BIOTECHNOLOGICAL CONTRIBUTION FOR IMPROVING FRUIT TREES IN FRUITGROWING INSTITUTE, PLOVDIV, BULGARIA

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BIOTECHNOLOGICAL APPROACHES APPLIED IN FRUITGROWING INSTITUTE, PLOVDIV, BULGARIA

- Embryo Rescue for early ripening sweet cherry cultivars;
- Regeneration from somatic tissues;
- Somaclonal variation;
- Optimization of micropropagation protocols of woody fruit and ornamental plants;
- Physiology of *in vitro* cultivated plants;
- Virus elimination through tissue cultures;
Embryorescue for early ripening
sweet cherry cultivars

- In stone fruits and sweet cherries in particular, the use of \textit{in vitro} embryo culture in the earliest cultivars is difficult for the small embryo size.

- The use of \textit{in vitro} embryo culture, gave good results in embryos reaching 50-75\% of seed size at the time of isolation.

- Our previous studies showed that the type and the concentration of the cytokinins and carbohydrates used were of specific importance for the successful cultivation of smaller-size embryos.
Embryorescue for early ripening sweet cherry cultivars

- MS
- Wh
- DKW
- BAP
- TDZ
- SUCROSE
- SORBITOL
- LOW TEMPERATURE – 1-4 MONTHS
- SOMACLONS
EMBRYOCULTURE FROM EARLY RIPENING SWEET CHERRY CULTIVARS AT DIFFERENT STAGES - BAP

Initiation of embryoculture

Micropropagation of hybrid genotypes

Hybrid genotypes at acclimatization stage
Embryoculture from early ripening sweet cherry cultivars at different stages - TDZ
Elites developed at the Fruitgrowing Institute in Plovdiv via open pollination of the early cultivar Rivan, through embryo culture in vitro.

‘Kossara’ and ‘Rosita’ cultivars have very good compatibility with ‘GiselA-5’, *P. mahaleb* and *P. avium* rootstocks.
The studies are a part of the research program of the FGI aimed at the breeding of pome and stone fruit cultivars, resistant to main diseases and abiotic stress.

The program is based on methods of the classical breeding, plant biotechnology and immunology by means of plant regeneration and testing of somaclones *in vitro* and *in vivo* for disease resistance and abiotic stress.

The improvement of the shoot regeneration from somatic tissues is one of the key steps of the program.
Efficiency of somatic organogenesis depends to a great degree on the **physiological status** of the *in vitro* source plants. Important factors are the cultivation conditions – nutrient media, cultivation plates, gas-exchange possibilities, temperature and light regime.

Our studies in this field were focused on:

- **pear rootstock OHF 333** (*Pyrus communis* L.)
- **apple** (*Malus domestica* BORKH.) ‘Čadel’;
The apple cultivar ‘Čadel’® was obtained from a cross between ‘Golden Delicious’ × ‘Jonathan’, in 1984 in Serbia. A winter apple with high quality medium large to large fruits, suitable for transporting and successful cold storage until May, the cultivar ‘Čadel’® is moderate susceptible to *Venturia inaequalis* and *Podosphaera leucolricha*.

We investigated the effect of conditions for cultivating the source plants (cultivation plates and nutrient media) on the regeneration capacity of the apple cultivar ‘Čadel’ (*Malus domestica* BORKH.) leaf explants.

Pretreatment of the source plants in different cultural vessels - Glass jars (600 ml) or plastic vessels with gas-permeating cover (Combiness, Belgium, gas exchange – 10 GE/day).

The best results (over 80 % regeneration) are achieved when using explants of plants grown in plastic vessels in modified MS nutrient medium.

(Gercheva, P., Nacheva, L. and Dineva, V. 2009. The rate of shoot regeneration from apple (*Malus domestica* BORKH.) leaves depending on the in vitro culture conditions of the source plants. *Acta Hort.* (ISHS) 825:71-76.)

As a result of the research more than 1000 somaclones of the apple cultivar ‘Čadel’ are obtained and propagated.
We developed an efficient shoot regeneration system of pear rootstock OHF 333 (*Pyrus comunis* L.) leaves

Carbohydrate type and concentration in the cultural media significantly influence percentage of adventitious shoot formation. The best results are achieved on media 1/3 TIB, which is used as control in the next experiments.

The regeneration rate of leaf segments from OHxF333 improves with increase of the concentration of IAA.

The best efficiency of somatic organogenesis (over 80% regeneration and more than 4 regenerants from explant), high SFC and good development of regenerants on the elongation medium were achieved on two nutrient media – with 7.5 µM TDZ, 2.46 µM IBA and 20 µM IAA or with 9 µM TDZ, 2.46 µM IBA and 10 µM IAA.

Our results with plums (*Prunus domestica* L.) - local plum cultivar ‘Kyustendilska sinya’; ‘Improved French’ and ‘jo-jo’ are very promising.

Regeneration from leaf explants of plum (*Prunus domestica* L.) ‘Improved French’
Somaclonal variation is a potentially useful source of genetic variability for plant improvement and could result in new valuable genotypes. Several examples of somaclonal variation resulted in disease resistant forms have been reported in temperate fruit species.

All the in vitro obtained regenerants were cloned, propagated, rooted and adapted to ex vitro condition.

Apple somaclones after acclimatization.
Testing the resistance of in vitro obtained regenerants from apple cultivar ‘Čadel’® to the most widely distributed phytopathogens in apple – scab (Venturia inaequalis), powdery mildew (Podosphaera leucotricha) and fire blight (Erwinia amylovora).

<table>
<thead>
<tr>
<th>Accession number of somaclones</th>
<th>Degree of resistance after inoculation with Venturia inaequalis (scab)</th>
<th>Degree of resistance after inoculation with Podosphaera leucotricha (powdery mildew)</th>
<th>Degree of resistance after inoculation with Erwinia amylovora (fire blight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>115-6-A</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>188-1</td>
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<td>189-7 A</td>
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<tr>
<td>173-1</td>
<td>2</td>
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</tr>
<tr>
<td>199-1</td>
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<tr>
<td>3/1-B</td>
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</tr>
</tbody>
</table>

Ten out of the 187 in vitro obtained somaclones of apple cultivar ‘Čadel’® demonstrated good results after inoculation with scab (Venturia inaequalis), powdery mildew (Podosphaera leucotricha) and fire blight (Erwinia amylovora).

Three somaclones (199-1; 211-3 and 3/1-B) with complex resistance to scab and powdery mildew diseases and low susceptibility/tolerance to fire blight are selected for future field evaluation.
The aim of our study with pear rootstock ‘Old Home x Farmingdale’ (OHF 333) (*Pyrus communis* L.) was to obtain somaclones with resistance/tolerance to the fire blight. The artificial inoculation of this rootstock with local strain of *Erwinia amylovora* showed that 50% of examined plants demonstrate low degree of visible symptoms of disease and 10% - high degree of visible symptoms.

All obtained regenerants were cloned, propagated, rooted and acclimatized to greenhouse conditions. Somaclones were tested for resistance to fire blight (*Erwinia amylovora*).

It was found out that ten of the studied clones demonstrated a low susceptibility/tolerance to the fire blight. The field tests of the selected genotypes are in progress.
We investigated the effect of the type and the concentration of carbohydrates (sucrose and sorbitol) added to the nutrient medium with auxins (IBA, NAA and IAA) on the multiplication coefficient and the length of the newly formed shootlets in Gisela 5 Cherry Dwarf Rootstock.

The results of the investigations showed that the basic effect on both studied characteristics was exerted by the type and the concentration of the carbohydrates. The highest multiplication coefficient (number of shootlets per plant) was achieved when combining sucrose and sorbitol in a 2:1 ratio.

Sweet cherry rootstock Gisela 6 (*Prunus cerasus* x *Prunus canescens*).

Using a combination of sucrose and sorbitol in the nutrient medium had a positive effect on the multiplication rate of sweet cherry rootstock Gisela 6.

The hybrid Rutgers red leaf – open pollination (o.p) is obtained as a part of the selection program of Fruitgrowing Institute – Plovdiv.

It has from dark red to violet leaves, moderate growth and drought tolerance.

- are obtained for field tests as peach rootstocks and/or ornamental plants. The best multiplication rate with good quality of plantlets was achieved on media supplemented with sucrose (15 g/l) and sorbitol (15 g/l).
- The highest percentage of rooting was obtained on media with 0.3 mg/l IBA.
- After acclimatization a number of plants

In the summer of 2002 in the mountain of the Rhodopes (Bulgaria) a rare transsexual form of the normally dioecious type *Pistacia terebinthus* was found. Later the existence of a small isolated population of such trees was specified. The trees demonstrate exceptional drought resistance of the kind which allows cultivation in soils that are under no irrigation, small-productive, stony and sliding (pliant to erosion) soils.

The studies of the methods for *in vitro* propagation of the rare transsexual form of *P. terebinthus* will allow its preservation and use of the transsexual forms as rootstocks and eventually as a donor for monoeciousness in the Pistachio hybridization programs and further investigation.


As a part of hybridization program interspecific hybrids *P. terebinthus × P. vera* were obtained.

There were two problems:

- The germination capacity of the seeds of *Pistacia terebinthus* after conventional stratification is very low.
- The fruits and stones of *P. terebinthus* are small and the extraction of the embryos from the hard nut shell is extremely difficult

The aim of our research was to improve the germination efficiency of interspecific hybrids *P. terebinthus × P. vera*.

Interspecific hybrids *P. terebinthus × P. vera*.

**In Vitro Embryorescue of Mature Embryos**

*P. terebinthus* embryos were placed in culture at different stages - in the middle of August, at the beginning of September and at the end of September. The mesocarp was removed from the seeds, after which the embryos were extracted from the hard nut shell using a scalpel. After sterilization the embryos were cultivated in vitro on the nutrient media based on MS (Murashige and Skoog, 1962) with varying concentrations of carbohydrates and plant growth regulators.

**Stratification in Perlite**

Seeds from ripen fruits collected at the end of September with removed mesocarp were sterilized by the above described procedure. The sterilized pistachio seeds were put in the jars filled with sterile perlite and mixed. The jars were wrapped in polyethylene folio and stored in a refrigerator at 4°C for 3 months. After stratification in sterile perlite the *P. Terebinthus* seeds obtained by open pollination and by crossing with *P. vera* were taken out and seeded in a peat-perlite mixture at a temperature of 22°C and exposed to light of 16/8 h photoperiod (40 μmol m⁻² s⁻¹ PPFD).
Micropropagation of Walnut (*Juglans regia* L.)

**Step I – Initiation and establishment of in vitro culture**

**Step II – Multiplication**

**Step III – Rooting**

**Step IV – Acclimatization (Weaning)**
Preliminary studies on *in vitro* propagation of *Ginkgo biloba* L.

After registration of the *Ginkgo* leaf extract, EGb 761, for human use in 1974 in France, the use of *Ginkgo* has been growing at a very rapid rate worldwide and accordingly the pharmaceutical industry needs huge quantities of leaves.

Using vegetative explants of *Ginkgo* has not been widely studied as the production of whole plantlets *in vitro* has been limited to cultures of intact embryos.

We investigated the possibility of using shoot apices obtained from a single mature male *Ginkgo* tree as initial explants for establishing efficient mass micropropagation protocol.

Different explant types collected from a 17-year-old male *Ginkgo* tree were studied on various medium types.

Vegetative shoot apices of *Taxus*, commonly used as explants for *in vitro* shoot cultures, particularly mature ones, are under high risk of contamination. The routinely used protocols proved unsuccessful to eliminate this high rate of contamination.

WPM nutrient medium supplemented with 6.8 4-M zeatin exhibited the best initiation of shoot apices with high frequency of axillary bud induction averaged 2-3 buds/explant. WPM lacking growth regulators supported shoot elongation.


Physiology of in vitro cultivated plants

- *In vitro* cultivated apple plantlets can develop a positive carbon balance at suitable parameters of environmental factors (mainly light intensity, CO$_2$ concentration and sucrose in the nutrient medium).
- The sucrose concentration in the nutritional medium and the gas exchange between the cultural vessels and the environment had a significant impact on the growth of *in vitro* apple plantlets in the stage of elongation.
- The accelerated gas exchange between the cultural vessel and the environment lead to an increase in the chlorophyll content and actual quantum yield (Y*) of the apple plantlets.

CO$_2$-concentration curves (A) and light curves (B) of *in vitro* apple plants (MM106), cultivated at 2% sucrose in the nutrient medium in the vessels with different gas-exchange rate – T - 0.013 volumes/hour; G - 3 volumes/hour.
