ENVIRONMENTAL PROTECTION THROUGH BIOTECHNOLOGY USING *IN VITRO* PROPAGATION OF SOME FRUIT SPECIES

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Abstract
This paper presents research results for development of biotechnology using in vitro propagation of dwarf nectarine and peach dwarf species as an alternative to conventional technology obtaining propagating material from these species. It was studied the behavior of dwarf peach varieties Paul and Puiu, and dwarf nectarine variety Valerica of in vitro propagation technology.
Research has considered the establishment of biotic and abiotic factors influence in the initiation phase of in vitro culture, the necessary conditions to in vitro propagation explants process, determines the influence of auxins, cytokinins and photoperiodism related to propagation processes and rhizogenesis of explants and finally plant acclimatization to septic conditions emissions.
The best results obtained in the phase of initiation of culture were 78-97% explants, in response to the nutrient medium, variety and time of sampling.
Propagation rate was between 6,4-14,2 microshoots / explant, the percentage of rooting in vitro was best 64,3% - 87,0% depending on the nutritional medium, variety and level photoperiodism.
Based on the results obtained was calculate the volume of pollutants using in vitro propagation compared with the conventional technology propagations. The level of environmental pollution is insignificant in case of propagation using in vitro technology compared with conventional technology, which requires the entire stream, namely: the establishment and maintenance of seed plantation, plantation graft branches, of juvenile school, nursery field I and II, over 12 tones of diesel, over 6 tones of chemical fertilizers and plant protection treatments over 60 year.

Keywords: in vitro, biotechnology multiplication, dwarf, acclimatization, environment, pollution, nursery.

1. INTRODUCTION

Production of propagation material by biotechnological methods has become a current technique for many species (Barbe J.P [1]), (Fiorino P [4])], (Rosati P. [6]), (Sansavini S. [7]). Annual production of plants obtained by *in vitro* propagation is over 500 million, which is obtained in approx. 600 laboratories (Teodorescu A. [11]).

In horticulture the impact of cell cultures and plant tissues has been felt mainly in plant propagation by in vitro techniques, those biotechnology used on an industrial scale in many laboratories and an increasing number of species and varieties (Barbe J.P. [1]), (Buvat R. [2]), (Navatel J.C. [5]), (Pierik R.L.M. [9]), (Stănică F. [10]).

In vitro plants propagation achieves to the major objective of ecological agriculture, which refers to the avoidance of forms of environmental pollution, specific to conventional technologies for the production of planting material. This is one reason for the development of research in developing and promoting biotechnology techniques as an alternative to classical technologies for the propagation of fruit trees and bushes. There are many species of trees, shrubs and ornamental trees in which propagation by in vitro techniques became the current production (Damiano C. [3]), (Boxus Ph. [8]), (Zuccherelli G. [12]).

Dwarf genotypes of peaches and nectarines are susceptible to more intensive culture systems, with over 2000 trees per hectare to about 800 trees per hectare to standard varieties. These genotypes are also susceptible to culture containers and gardens, as they have also a great decorative value (Dumitru, Liana [13]), (Teodorescu, R. [14]), (Teodorescu, R.[15]).

Limiting factor for extending the growing dwarf varieties of peaches and nectarines is the difficulty of multiplying these varieties through conventional technology.

In the present study are the results obtained on the behavior of dwarf genotypes of peaches and nectarines using in vitro propagation

techniques in order to develop the biotechnology for propagation of these species.

Based on results it has made a comparative study on the impact of pollution in case of using in vitro propagation techniques versus conventional propagation technology of peach and nectarine dwarf.

2. MATERIAL AND METHOD OF WORK

Biological material consists of two dwarf peach varieties and a dwarf nectarine variety, obtained at SCDP Constanta and recommended for fruit production and as ornamentals.

Variety Paul**,** peach dwarf with height 1.0-1.2 m. globular crown, lush foliage and abundant flowering (Fig. 1).



Fig. 1. Variety Paul

Variety Puiu dwarf peach height of 0.8-1.2 m, has large and highly colored leaves, branches bearing very short internods present, abundant flowering (Fig. 2).


Fig. 2. Variety Puiu

Variety Valerica, nectarines with a height of 1.5 m., the branches of fruit have a short internods and a denity of floral bud (Fig. 3).


Fig. 3. Variety Valerica

Working method followed specific protocol for *in vitro culture.* For the initiation phase of *in vitro* culture were used explants taken from shoots in active growth phase and branches in the dormant phase. Disinfection of biological material was made in 94% alcohol for 4 and 10 minutes and calcium hypochlorite for 10 or 20 minutes.

Sampling of explants was done under the magnifying binocular, inside the hood with sterile laminar airflow.

Nutrient medium for culture initiation phase

There were established six variants of different nutrient medium containing mineral salts, vitamins and phytohormones (Table 1).

Table 1. Nutrient medium used in the growth phase of explants

Components (mg/l)	Multiple factors					
	A.1	*A.2.*	*A.3.*	*A.4.*	*A.5*	*A.6*
Macroelements						
Potassium nitrate	1.900	950	950	190	1.800	1.800
Ammonium nitrate	1.650	825	825	165	400	400
Calcium chloride	440	220	220	44	-	-
Magnesium sulphate	370	185	185	37	360	360
Phosphate Monopotassium	170	85	85	17	270	270
Calcium nitrate	-	-	-	-	1.200	1.200
Microelements						
Manganese Sulfate	22,3	11,55	11,55	2,23	0,75	0,75
Zinc sulphate	8,6	4,3	4,3	0,86	8,6	8,6
Boric acid	6,2	3,1	3,1	0,62	12,0	12,0
Copper sulphate	0,025	0,0125	0,0125	0,0025	0,025	0,025
Sodium molybdate	0,25	0,125	0,125	0,025	0,25	0,25
Cobalt chloride	0,025	0,0125	0,0125	0,0025	0,025	0,025
Potassium iodide	0,83	0,415	0,415	0,083	0,08	0,08

NaFeEDTA	32,0	32,0	32,0	32,0	32,0	32,0
Vitamins						
Nicotinic acid	0,5	0,5	0,5	0,05	-	2,462
Pyridoxine	0,5	0,5	0,5	0,05	-	0,616
Thiamin	0,1	0,1	0,1	0,01	0,4	0,674
Clicină	2,0	2,0	2,0	0,2	-	0,375
Inositol	100,0	100,0	100,0	10,0	100,0	54,048
Biotin	-	-	-	-	-	0,048
Calcium pantothenate	-	-	-	-	-	0,476
Riboflavin	-	-	-	-	-	0,376
Ascorbic acid	-	-	-	-	-	0,176
Choline	-	-	-	-	-	0,104
Cysteine	-	-	-	-	-	7,269
Phytohormones						
Giberelic acid	0,1	-	-	-	0,1	0,1
Naftilacetic acid	0,004	0,004	0,004	-	-	-
Benzilaminopurină	2,0	1,0	2,0	2,0	1,0	1,0
Other components						
Glucose	40.000	40.000	40.000	40.000	40.000	40.000
Agar	7.000	7.000	7.000	7.000	7.000	7.000

Nutrient medium for *in vitro* multiplication phase

In order to determine the propagation capacity of genotypes considered for the study, explants growth in the initiation phase were cultivated on four nutrient medium (Table 2).

Table 2. Nutrient medium used in the multiplication phase

Component (mg/l)	Tipes of nutrient medium			
	A.1.	A.2.	A.3.	A.4.
Macroelements	M&S	M&S	M&S	M&S
Microelements	M&S	M&S	M&S	M&S
Vitamins	M	M	M	M
NaFeEDTA	32	32	32	32
Dextrose	40.000	40.000	40.000	40.000
Agar	7.000	7.000	7.000	7.000
Naftilacetic acid	0,1	0,1	0,1	0,1
Benzilaminopurină	1,0	2,0	-	-
Kinetine	-	-	4,0	-
2iP	-	-	-	5,0

Legend: M & S = Murashige - Skoog (1962), M Miller (1982) A.

Macroelements M&S (mg/l)		**Vitamins M (mg/l)**	
Potassium nitrate	1.900	Thiamin	2
Ammonium nitrate	1.400	Nicotinamide	4
Calcium chloride	474	Pyridoxine	4
Magnesium sulphate	370	Calcium pantothenate	2
Phosphate Monopotassium	170	Biotin	2
		Folic acid	1
Microelements M&S (mg/l)		Choline	2
Manganese Sulfate	22,3	P. aminobenzoic acid	1
Zinc sulphate	8,6	Riboflavin	1
Boric acid	6,2	Ascorbic acid	50
Copper sulphate	0,025	Vitamin B 12	3
Sodium molybdate	0,25		
Cobalt chloride	0,025		
Potassium iodide	0,83		

Nutrient medium for *in vitro* rooting phase

For in *vitro* rooting of micro cuttings obtained from micro shots obtained in the multiplication phase, there were used four nutrient medium (Table 3).

Table 3. Nutrient medium used in the rooting phase

Component (mg/l)	Multiple factors			
	A.1.	A.2.	A.3.	A.4.
Macroelements	M&S 1/2	M&S ½	M&S ½	M&S 1/2
Microelements	M&S 1/2	M&S ½	M&S ½	M&S1 /2
Vitamins	LS	LS	LS	LS
NaFeEDTA	38,0	38,0	38,0	38,0
Dextrose	30.000	30.000	30.000	30.000
Agar	7.000	7.000	7.000	7.000
Giberelic acid	0,1	0,1	0,1	0,1
Indolilbutiric acid	0,2	0,4	0,6	0,8

Key: LS = Linsmaier - Skoog (1965) LS Vitamins (mg / l): Thiamine - 0.4, inositol - 100.0

For acclimatization of vitro plants were used six nutrient medium (Table 4).

Table 4. Nutrient media used for vitro plants acclimatization

Alternating factor Composition of substrate	**The proportion of components**	**pH**
A.1. Red + black peat + clayey sandy earth	4:3:3	6,5
A.2. Red + black peat + clayey sandy earth + perlite	4:3:3:1	6,5
A.3. clayey sandy earth	-	6,3
A.4. clayey sandy earth + perlite	3:1	6,3
A.5. clayey sandy earth + manure + perlite	3:2:1	7,0
A.6. manure + perlite	3:1	7,7

Growth phases of explants, multiplication and rooting *in vitro* were conducted in specially equipped premises at 22-24^0 C, light period 12, 14, 16 hours and light intensity of 3000 lux.

3. RESULTS AND DISCUSSION

The influence of nutrient medium and genotype on explants growth

Statistically analyzing the variation number of explants (%) according to the nutrient medium for the varieties studied are found that at the average effect the highest percentage values of growth explants were obtained when used A.6 nutrient medium by 87.3% differences over all

other nutrients are provided statistical average (Duncan test for $P \leq 0.05$).

Percentage immediately below were obtained on nutrient media A.3 and A.5 (78.6 and 77.1% respectively), their differences were not statistically assured. A.2 nutrient medium determined a lower rate of growth explants namely increased 70.4%, differences being statistically assured to the other graduations of A factor.

The lowest percentages of growth explants were obtained using nutrient medium A.1 (54.3%), the value is significantly lower than all other nutrient medium. As the nutritional environment A.4 explants did not survive, this nutrient medium was excluded from the statistical interpretation (Fig. 4).

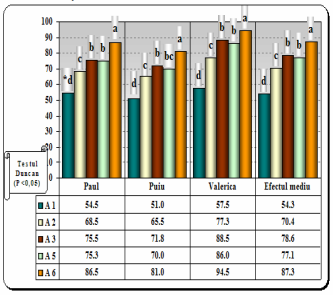

Fig. 4. Explants number variation (%) growth depending on nutrient medium and variety

Explants growth phase lasted 32 days, after this phase explants had 4 - 7 leaflet (Fig.5).

Fig. 5 Explants growth *in vitro*

The influence of nutrient medium and genotype on the rate of multiplication

The statistical interpretation of the results shows that at the average effect the higher value of the multiplication rate was 10,7 micro shoots / explants obtained on nutrient medium A.2 Differences over all nutrient medium are statistical assured (Fig. 6).

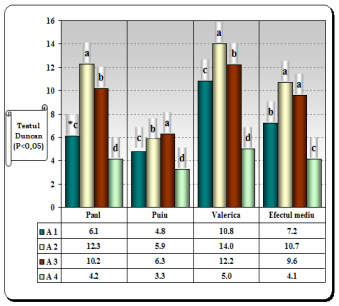

Fig. 6. Changes in the rate of multiplication in response to the nutrient medium and variety

A close value of 9.6 of multiplication rate is recorded on the nutrient medium A.3, where average differences over all other nutrient medium are also statistical assured.

Formations resulting in the multiplication process are very compact, with adventitious shoots very close and ranging in size from 4 - 10 mm. (Fig. 7).

Fig. 7. Multiplication phase aspect

Expression of rhizogenic potential response to the nutrient medium and variety

Statistical interpretation of the research results *of in vitro* rooting process caused by a nutrient medium in interaction with average effect of varieties shows that the highest percentage of rooted plants obtained using the nutrient medium A.2 is 75.8%.

Analyzing the interaction between nutrient medium and each variety it can be seen that the two varieties (Puiu and Valeria) keep the same trend as the average effect namely the highest values are determined by the nutrient medium A.2, while for the variety Paul the highest percentage of rooted plants is determined by the nutrient medium A.1.

Best results are determined by the nutrient medium A.2 of 84.9% rooted plants for variety Valerica. Differences over other nutrient medium are statistical assured.

The lowest percentages of rooting were obtained on nutrient medium A.3 and A.4 of 65.0% and 52.0%. (Fig. 8)

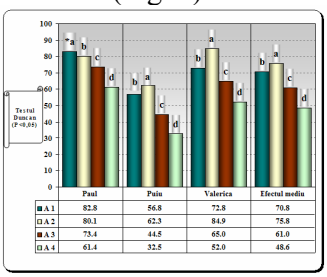

Fig. 8 Changes in the percentage of rooting *in vitro* in response to the nutrient medium and variety

Rooting period was 30 days. The vitro plants obtained formed a number of 2 - 4 malt, with a length of between 9 - 43 mm. (Fig. 9).

Fig. 9. Rooted plants

Results obtained on acclimatization of vitro plants

Interpreting the obtained results shows that the highest percentage of acclimatized plants, of 60.0 to 70.2 was obtained using the nutrient mixture A.1 (Fig. 10).

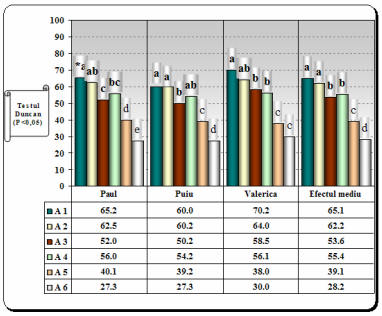

Fig. 10. The influence of nutrient mixture in the aclimatization phase

Acclimatization duration was 17 days. The end of acclimatization phase was marked by the onset of active growth of plants by the appearance of the first leaflet (Fig. 11).

Fig. 11 Acclimatized plants

The comparative study of the pollutants used for propagation through conventional and biotechnological methods

Specific pollution factor using biotechnology techniques for propagation by in vitro culture is considered nutrient medium through which it contains ingredients while the conventional technology the pollution factor consist in pesticides, chemical fertilizers and diesel.

Calculation of pollutants has been done for a total of 32,000 plants, representing the average of trees per hectare which is obtained in field II by conventional technology.

To obtain a total of 32,000 plants through *in vitro* propagation consumes 276 liters of nutrient medium containing approx. 1 kg macro elements, 29 g microelements, 26 g vitamins, 0.2 g phytohormones, aprox. 8.9 kg. glucose or dextrose and about 1.9 kg agar (Table 5).

Table 5. Nutrients used to obtain 32,000 plants through biotechnological methods

Specification	Initiation phase of culture	Phase multiplier	Rooting phase	Total
Nutrient medium (l)	1	55	220	276
Of which: Macroelemente(g) Macroelements(g)	4,03	211,2	844,8	1049,03
Micronutrients (g)	0.05	4,0	24,4	28,45
Vitamins	0,07	3,85	22,0	25,92
Phytohormones (g)	0,001	0,1	0,11	0,21
Glucose or dextrose (g)	40,00	2200,00	6600,00	8840,00
Agar)g)	7,00	385,00	1540,00	1932,00
TOTAL (g)	51,15	2793,15	9031,31	11875,61

Analyzing the data presented shows that for a production of 32,000 plants propagated *in vitro* is necessary to use less than 12 kg nutrients, which are necessary for optimum growth, multiplication and roots of vitro plants. One can appreciate that after the completion of phases the nutrient medium are completely

depleted and the waste resulted are practically non-polluting.

For growing 32,000 trees are needed by classical technology only for field I and II 15 treatment plant nursery 863 kg chemical fertilizer and 3643 liters of diesel.

For all sectors which contribute to the production plant are required 61 treatments, 6000 kg chemical fertilizer and 12691 liters diesel (Table 6).

Table 6. Pollutants using conventional technology

Nursery Division	Number of phytosanitary treatments performed annually per hectare	Chemical fertilizers Kg/Ha	Diesel l/ha.
Planting stock slips branches: the establishment and maintenance	11	1000NPK	2916
Planting stock slips industries: mining	11	400 Ammonium nitrate	587
Plantation seeds: the establishment and maintenance	7	1000 NPK	2916
Plantation seedlings: mining	9	300 Ammonium nitrate	336
School of juvenile	8	1000 NPK	2293
Field I	7	1500 NPK	2293
Field II	8	800 Ammonium nitrate	1350
TOTAL	61	6000	12691

4. CONCLUSIONS

Bases on the results obtained can be developed the biotechnology of propagation by in vitro techniques of dwarf peaches and nectarines, as an alternative to the conventional technology of propagation of these genotypes.

Pollutants in the case of biotechnology of propagation by in vitro techniques of dwarf peaches and nectarines are almost reduced to zero, because *in vitro culture* media components are calculated at level of biological need for vitro plants so that the end stages of culture media nutrients are fully exhausted.

Conventional technology requires a major consumer of pesticides, chemical fertilizers and diesel, which pollutants is very large impact on the environment.

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