BABEŞ-BOLYAI UNIVERSITY

FACULTY OF BIOLOGY AND GEOLOGY

RESEARCHES ON *IN VITRO* MICROPROPAGATION CAPACITY AND GENETIC STABILITY OF SOME INTERGENERIC HYBRIDS *FRAGARIA X POTENTILLA*

SUMMARY OF THE DOCTORAL THESIS

SCIENTIFIC COORDINATOR:

Professor GABRIEL CORNEANU

PhD STUDENT:

NICOLETA ANCA ȘUȚAN

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CONTENT

Key words: intergeneric hybrids *Fragaria x Potentilla*, *in vitro*, axillary shoot formation, direct organogenesis, indirect organogenesis, genetic stability.

Introduction

Retrospectively looking back, all the benefits oferred by the plants to the humankind represented the basis of the plant breeding activities, which begins about 10 000 years ago and culminate in our days with the use and intensive exploitation of biotechnology for increasing the amount of planting material and improvement of its quality. Additionally, the isolation, cloning and sequencing of genomic DNA, in order to identify genes and find their role, or to determine and evaluate the genetic polymorphism, have became routine activities, more accesibile and less expensive.

The strawberry is among the few species characterized by a high rate of replacement of varieties and assortments, closely related to the consummer demands, the extensive breeding activity offering the possibility of growing strawberry from the temperate areas to the subtropical regions and cold regions in the northern hemisphere. Generally, the breeding objectives for the cultivated strawberry are the identification of highly valuable phenotypes, their hybridization and selection of progenies which can be either released as new varieties or used as genitors in the next breeding generation.

However, as a consequence of the limited genetic diversity observed within the *Fragaria x ananassa* germplasm (Graham et al., 1996), it is considered that the obtention of some favorable combinations of traits into the new strawberry varieties will be possible only by the increase of the number of genitors belonging to the exotic germplasm (Hancock et al., 2002), or tho the related genera.

If for long time the intergeneric hybridization was considered impossible to use in the case of species with different ploidy level (Evans, 1974), the advances from the last two decades in manipulating the ploidy and the optimization of techniques for hybridization and rescue of zygotic embryos resulted from distant hybridizations, have determined an almost a radical reconsideration of the practical use of this method for the genetic improvement of cultivated strawberry.

The intergeneric hybrids *Fragaria x Potentilla*, known as ornamental strawberies, are harmoniously combined with the exigences of the present, and have an increasing commercial value. Thus, the production in a short time of the required amount of planting material, guaranteed for authenticity and biological value, is essential.

Moreover, the hybridization of ornamental species is constantly seeking for new technologies, which could help substantially the reduction of production costs, enhancement of the quality of resulted product, as well as the diversification of varieties and assortments. In this way, the increase of productivity in the new obtained variety is achieved by improving the desired traits, such as size, color and flavor of fruits, together a prolonged period of flowering and fruiting. Thus, micropropagation represent a system generally accepted for the large scale propagation of the strawberry varieties, under conditions of the use with maximum attention of an well established procedure. Incontestably, there is a fact that *Fragaria x ananassa* was one of the pioneer species in applying the *in vitro* culture techniques on large scale (Jungnickel, 1988; Popescu, 1998). In strawberry, meristem culture, used as a method for micropropagation, has already a history of four decades. Concerning the induction of adventitious shoot formation, from the first attempt to induce callus formation from meristems and regenerate plants (Nishi and Oosawa, 1973), the range of explant types tested has considerably enlarged, including leaf, petiole, root, immature embryos, cotyledons, anthers, receptacle, petal, etc. Efficient protocols for adventitious shoot regeneration from calli cultivated *in vitro* have been established only in the last two decades. The experimental results have shown that, in general, the capacity to form callus and the regenerative response are influenced by the explant type, source of explants, type and concentration of growth regulators, culture conditions, and genotype. Very often, the conditions standardized for a variety are not optimal for others. This suggests possibly the interaction between the levels of endogenous and exogenous hormones in the expression of regenerative response. As the existence of a strong interaction between the type of explant, growth regulators and culture conditions became obvious, all these variables must be taken into consideration simultaneously in elaboration of a regeneration system for a commercial strawberry variety.

On the other hand, it is already recognized for many years that the strawberry plants regenerated from *in vitro* cell or tissue culture are not always identical. Therefore, the confirmation of genetic stability of the regenerants by using molecular markers is absolutely necessary when establishing a protocol for the *in vitro* multiplication.

Based on this, the researches carried out by us within the doctoral thesis have as main objectives the evaluation of *in vitro* multiplication capacity of two genotypes of ornamental strawberry, which are intergeneric hybrids *Fragaria x Potentilla* at their origin, by using various techniques for micropropagation, as well as the evaluation of genetic stability of the regenerants, by using molecular techniques.

1. RESEARCH OBJECTIVES, MATERIALS AND METHODS USED WITHIN THE EXPERIMENTS

1.1. RESEARCH OBJECTIVES

Having as main objective the establishment of an optimized protocol, allowing high efficiency of micropropagation in genotypes of ornamental strawberry by promoting either the axillary shoot formation or shoot regeneration directly from somatic tissues or via callus formation, and maintaining the genetic identity of the *in vitro* micropropagated plants, the researches carried out by us were primarily divided into a complex of experiments designed to meet the following secondary objectives:

- Finding the most appropriate culture medium and type of explant for obtaining a high rate of micropropagation, associated with the clonal uniformity of the regenerants.
- Establishing the composition of the culture medium, and the type and concentration of growth regulators, which promotes *de novo* formation of shoots, their growth and proliferation *in vitro.*
- Evaluation of the genetic potential of the investigated *Fragaria* x *Potentilla* intergeneric hybrids for *in vitro* multiplication.
- Establishment of culture medium composition, type of growth regulators and their concentrations, which promotes *in vitro* rooting of the multiplicated shoots at a high rate.
- Evaluation otf the acclimatization capacity of vitroplants, after the *in vitro* rooting.
- Identification of primers allowing to detect the existence of polymorphism at the molecular level.
- Confirmation of the genetic identity of plants multiplicated *in vitro* by using the RAPD markers
- Detecting the eventual genetic differences occurred between the plantlets regenerated *de novo* from somatic explants via calus and the plant from which they have originated, by RAPD analysis.

The researches initiated by us represents the first attempts of micropropagation and evaluation of genetic stability by using the RAPD technique, in the ornamental strawberry genotypes ‘Pink Panda’ and ‘Serenata’.

1.2. BIOLOGICAL MATERIAL

The biological material investigated was represented by two genotypes of ornamental strawberry, namely ‘Pink Panda’ and ‘Serenata’, from the National Collection of *Fragaria*, at the Research and Development Institute for Fruit Growing, Piteşti - Mărăcineni.

‘Pink Panda’, is an intergeneric hybrid *Fragaria x Potentilla*, released as ornamental strawberry variety, with pink flowers, and characterized by a prolonged flowering.

‘Serenata’ is another intergeneric hybrid *Fragaria x Potentilla*, released as a distinct variety of ornamental strawberry, with flowers of intense, dark pink.

Both of these intergeneric hybrids have the ability to form edible fruits, with acceptable size and flavor.

1.2.1. Biological material used in the *in vitro* micropropagation experiements of *Fragaria* × *Potentilla* intergeneric hybrids

The biological material used for initiation of the *in vitro* cultures was represented by caulinar apexes, for the both ornamental strawberry genotypes, ‘Pink Panda’ and ‘Serenata’, respectively.

The microshoots (originating from apexes) obtained after 4 succesive subcultures, reprezented the biological material used for studying of the *in vitro* rooting capacity of the two genotypes of ornamental strawberry. The rooted plantlets were transferred for acclimatization in the greenhouse.

In the studies on calli induction and shoot regeneration via calus or by direct organogenesis, somatic tissues were used, respectively petiole segments and leaf fragments, collected from the *in vitro* micropropagated plantlets.

1.3. CULTURE MEDIA USED IN EXPERIMENTS

1.3.1. Basal media used in experiments

1. For **initiation of the *in vitro* culture of caulinar apexes** have been used the Lee – Fossard (1977) basal medium, modified by us, in that the calcium chloride ($CaCl_2$) was added to the medium in a concentration of 330.0 mg/l. The medium was solidified by adding of 7.0 g/l agar-agar.

2. For **multiplication of the microshoots regenerated from caulinar apexes,** there have been used Murashige – Skoog (1962) and Lee – Fossard (1977) basal media, modified by us as described above. In a first stage of evaluation of the micropropagation capacity of ‘Pink Panda’ and ‘Serenata’ genotypes of ornamental strawberry, Knop (1965) basal medium was also tested.

3. For **initiation of the *in vitro* culture using leaf and petiole explants** there have been used the same basal media (MS and LF), modified as mentioned for the previous stages.

The culture media were used either in liquid state, with filter paper bridges, or solidified with agar-agar. In all the experiments, the culture media lacking growth regulators represented the control treatment.

1.3.2. Growth regulators used in experiments

The experimental variants have been organized depending on the combination and concentration of growth regulators introduced into the culture media, as well as depending on the objective, as follows:

1. For **initiation of the *in vitro* culture by using caulinar apexes** the LF basal medium was supplemented with 3.2 mg/l Kin and 2.7 mg/l IAA.

2. For **multiplication of shoots regenerated from caulinar apexes**, the MS and LF basal media were supplemented each with IAA, IBA, BAP, GA_3 and Kin, in six experimental variants, as shown in Table 1.

3. For **induction of callus formation from somatic tissues and subsequent shoot regeneration**, the leaf fragments and petiole segments have been cultivated on the MS and LF basal media, each of them supplemented with varios combinations and concentrations of 2.4-D, IBA and BAP (Table 2).

4. For **regeneration of shoots from the somatic tissue explants by direct organogenesis**, the MS basal medium was supplemented with 2.4-D or IAA, together with TDZ, in six different combinations, as shown in Table 3.

5. ***In vitro*** **rooting of the obtained shoots** was promoted by supplementing the solidified basal medium, containing ½ MS macroelements, ½ LF microelements and MS vitamins, with various concentrations of the auxins IBA and IAA, together with 0.1 mg/l GA_3 (Table 4).

Table 1.

Combinations and concentrations of growth regulators used in the culture media for multiplication of shoots regenerated from caulinar apexes.

Codes of the experimental variants	Basal medium	Growth regulators used and their concentration in the culture medium (mg/l)				
		BAP	IBA	IAA	GA_3	Kin
MM1	MS, LF	0.5	0.1	-	0.1	-
MM2	MS, LF	1.0	0.2	-	0.1	-
MM3	MS, LF	0.5	-	0.5	0.1	-
MM4	MS, LF	1.0	-	1.0	0.1	-
MM5	MS, LF	2.0	-	1.0	-	-
MM6	MS, LF	1.0	-	-	2.0	0.5

Table 2.

Combinations and concentrations of growth regulators in the culture media used for promoting the cell proliferation and shoot regeneration by indirect organogenesis.

Codes of the experimental variants	Basal medium	Growth regulators used and their concentration in the culture medium (mg/l)		
		2.4-D	IBA	BAP
CIM 1	MS, LF	0,5	-	3,0
CIM 2	MS, LF	1,0	-	3,0
CIM 3	MS, LF	1,0	-	5,0
CIM 4	MS, LF	-	0,5	3,0
CIM 5	MS, LF	-	1,0	3,0
CIM 6	MS, LF	-	1,0	5,0

Table 3.

Combinations and concentrations of the growth regulators in the culture media used for shoot regeneration by direct organogenesis.

Codes of the experimental variants	Basal medium	Growth regulators used and their concentration in the culture medium (mg/l)		
		2,4-D	IBA	TDZ
DO 1	MS	0.5	-	0.5
DO 2	MS	1.0	-	1.0
DO 3	MS	1.0	-	1.5
DO 4	MS	-	0.5	0.5
DO 5	MS	-	1.0	1.0
DO 6	MS	-	1.0	1.5

Table 4.

Composition of the culture medium used for *in vitro* rooting of shoots multiplicated by axillary shoot formation

Codes of the experimental variants	Basal medium	Growth regulators used and their concentration in the culture medium (mg/l)		
		IBA	IAA	GA_3
RM1	Macroelemente MS ½ n, Microelemente LF ½ n, Vitamine MS n	0.25	-	0.1
RM2	Macroelemente MS ½ n, Microelemente LF ½ n, Vitamine MS n	0.5	-	0.1
RM3	Macroelemente MS ½ n, Microelemente LF ½ n, Vitamine MS n	-	0.5	0.1

1.4. BIOLOGICAL MATERIAL USED IN THE EXPERIENTS OF GENETIC ANALYSIS

The donor plants, from which the caulinar apexes were excised for initiating the *in vitro*, cultures, together with the shoots obtained after several succesive subcultures on multiplication media, as well as the somaclones regenerated from leaf and petiole explants, have served as biological material for studying the genetic stability in the *in vitro* cultures of 'Pink Panda' and 'Serenata' genotypes of ornamental strawberry. The conditions in which the microshoots and somaclones were obtained, composition of culture media, and the age of *in vitro* cultures are presented in Table 5. A code was attributed to each somaclones, respectively to each shoot, which can be find in the images with amplification products.

Table 5.

The control plants, microplantlets and somaclones investigated for evaluating the genetic stability of micropropagated plants.

Genotype	**Microshoots and somaclones**	***In vitro* culture conditions**	**Method of micropropagation/ type of explant**	**Age of the *in vitro* culture**
'Serenata'	S1	**Germplasm collection**		
	S2	MS (agarized medium) - 1.0mg/l BAP, - 1.0 mg/l IAA, - 0.1 mg/l GA_3	Axillary shoot formation / caulinar apex	120 days
	S3	LF (agarized medium) - 1.0mg/l BAP, - 1.0 mg/l IAA, - 0.1 mg/l GA_3	Axillary shoot formation / caulinar apex	120 days
	S4	LF (agarized medium) - 0.5 mg/l IBA, - 3.0 mg/l BAP	Indirect organogenesis / leaf fragments	90 days
	S5	LF (agarized medium) - 0.5 mg/l IBA, - 3.0 mg/l BAP	Indirect organogenesis / petiole segments	90 days
	S6	MS (agarized medium) - 1.0 mg/l IBA, - 3.0 mg/l BAP	Indirect organogenesis / leaf fragments	90 days
	S7	MS (liquid medim) - 1.0 mg/l IBA, - 3.0 mg/l BAP	Indirect organogenesis / leaf fragments	70 days
	S8	MS (liquid medim) - 1.0 mg/l IBA, - 3.0 mg/l BAP	Indirect organogenesis / petiole segments	70 days

'Pink Panda'	PP1	**Germplasm collection**		
	PP2	MS (agarized medium) - 1.0mg/l BAP, - 1.0 mg/l IAA, - 0.1 mg/l GA_3	Axillary shoot formation / caulinar apex	90 days
	PP3	LF (agarized medium) - 1.0mg/l BAP, - 1.0 mg/l IAA, - 0.1 mg/l GA_3	Axillary shoot formation / caulinar apex	90 days
	PP4	MS (liquid medim) - 1.0 mg/l IBA, - 3.0 mg/l BAP	Indirect organogenesis / leaf fragments	70 days
	PP5	MS (liquid medim) - 1.0 mg/l IBA, - 3.0 mg/l BAP	Indirect organogenesis / petiole segments	70 days

1.5. INITIATION OF THE *IN VITRO* CULTURE

In the stage of in vitro culture initiation, caulinar apexes with 2-3 leaf primordia and size ranging between 0.1 and 0.3 mm were used. These were inoculated onto the solidified culture medium with inclined plane. The asepsization of the biological material was realized in two stages: (1) the immersion of runners in 94° ethanol , for 1 - 2 minutes; (2) the immersion of runners in calcium 6% hipochloride, for 14 minutes, followed by three rinses in sterile distilled water.

1.6. Experiments carried out for evaluation of the capacity of multiplication by axillary shoot formation

The microshoots obtained at the end of the phase of *in vitro* culture initiation were separated and transferred on the multiplication media, in six experimental variants with different combinations and concentrations of growth regulators (Table 1).

1.7. Experiments carried out for evaluation of the capacity of multiplication by direct and indirect organogenesis

Within the experiments designed to induce callus formation and shoot regeneration via calus in 'Serenata' and 'Pink Panda' genotypes of ornamental strawberry, the cultures of calli were initiated from both petiole segments (0.3 – 0.5 cm in length), and leaf fragments (0.3 – 0.5 cm in diameter), excised from plants grown *in vitro.* Thus, microshoots regenerated from

meristems have been transferred onto LF basal medium supplemented with 0.5 mg/l BAP, 0.5 mg/l IBA and 0.2 mg/l GA_3, as recommended by Sorvari et al. (1993). This pretreatment was aiming at increasing the uptake of growth regulators by the microshoots, allowing both their vigorous growing and excision of explants having a high potential for direct or indirect shoot regeneration. The regenerative potential of somatic tissue explants was assayed for both the cultures on agarized media (as in the most of current applications), and liquid media with filter paper bridges (as recommended by Blidar, 2004).

1.8. Experiments carried out for assessing the influence of pretreatment on the regenerative potential of somatic tissue explants

By using exclusively liquid culture media, with filter paper bridges, we have initiated a comparative study, as follows:

- in a series of experimental variants, duration of the darkness pretreatment was of 21 days, after this the cultures being transferred under a photoperiod of 16 hours light / 8 hours darkness, with a light intensity of about 40 µmol m^{-2} s^{-1};
- in a similar series of experimental variants, the darkness pretreatment with a duration of 42 days, was followed by the transfer of vitrocultures under a photoperiod of 16 hours light / 8 hours darkness, but with a relatively lower intensity of light, as a result of covering the culture flasks with large sheets of white paper.

1.9. Experiments carried out for studying the genetic stability of plants propagated clonally *in vitro*

1.9.1. Selection of primers used in the study of genetic stability

Polymorphism generation, clarity, luminosity and the high number of bands formed by the amplification products represented criteria for selection of the ten decanucleotide primers (produced by Mycrosinth), which have been used for assaying the genetic stability of plants regenerated *in vitro* (Table 6).

1.9.2. DNA extraction for RAPD analysis

For all the somaclones, extraction of genomic DNA was performed by using DNEasy Plant Mini Kit (Qiagen method), and following the protocol recommended by the producer.

Table 6.

Primers used for DNA amplification

No.	Primer	Sequence (5' - 3')
1	OPA02	TGCCGAGCTG
2	OPA07	GAAACGGGTG
3	OPA20	GTTGCGATCC
4	OPB05	TGCGCCCTT
5	OPB10	CTGCTGGGAC
6	OPB17	AGGGAACGA
7	OPC05	GATGACCGCC
8	OPC06	GAACGGACTC
9	OPC08	TGGACCGGTG
10	OPC010	TGTCTGGGT

1.9.3. RAPD amplification

The reaction of amplification was performed in the TC-512 Gradient Thermocycler (Bibby Scientific Ltd), programmed 2 minutes at 95ºC for the preliminary denaturation of DNA, followed by 45 cycles with the following temperature profile: 30 seconds at 92ºC – denaturation; 25 seconds at 36ºC – primers annealing; 74 seconds at 72ºC – extension; 7 minutes at 72ºC – final extension.

1.9.4. Electrophoresis in agarose gel

Electrophoretic migration was performed in the Tris-borat 0.5 x (TBE) buffer, at 21℃ and 150 v constant voltage for 35 minutes, until the bands migrated to the lower edge of the gel. The PCR producst were stained with ethydium bromide (10.0 mg/ml), added to the gel.

1.9.5. Image capture

Visualization of the amplification products was performed in UV light, and the gel images were captured with a Gene Flash Syngene Bio Imaging.

1.9.6. Image analysis

The bands were detected by using LabImage software, which gives also the size of amplified DNA fragments, by their comparison with a standard DNA (Ladder ADN 100 pb), consisting of 14 fragments with sizes ranging from 100 to 3000 pb. Also, it was calculated the

genetic distance between ‘Pink Panda’ and ‘Serenata’ genotypes, by using the Jaccard index of similarity with the FreeTree software, and the UPGMA (Unweighted Pair Group Method based on Arithmetic mean) method.

1.10. STATISTICAL METHODS FOR ANALYSIS AND INTERPRETATION OF RESULTS

Using the SPSS for Windows (Statistical Package for the Social Science), version 16.0 (2007), for comparing three or more variants, the ONE-WAY ANOVA was applied. The significance of differences between the effects of experimental factors or that of their interaction, for which the calculated F had significant values at a confidence level of 95%, was noted with small letter. For multidimensional series of data, whose statistical dependence is of probabilistic nature, the simple correlation coefficient was calculated, and the regression of data was established.

2. RESULTS AND DISCUSSIONS

2.1. CAPACITY OF MICROPROPAGATION THROUGH AXILLARY SHOOT FORMATION IN SOME INTERGENERIC HYBRIDS *FRAGARIA* × *POTENTILLA*

2.1.1. Capacity of shoot regeneration from caulinar apexes

Starting from the premise that the plants regenerated by axillary shoot formation are characterized, generally, by a higher genetic uniformity, and the rate of multiplication can be maintained to a high level over many subcultures, in a first stage of our researches, we have choosed for a micropropagation system of the intergeneric hybrids *Fragaria* × *Potentilla*, based on the multiplication of microplantlets regenerated from caulinar apexes.

For both of the investigated genotypes, the frequency ofshoot regeneration from caulinar apexes was 100%, and the occurrence of first shoots was noted at about 16 days after the culture initiation. In Figure 1 are presented microshoots of ornamental strawberry regenerated from caulinar apexes excised from runners collected from mature plants in the field.

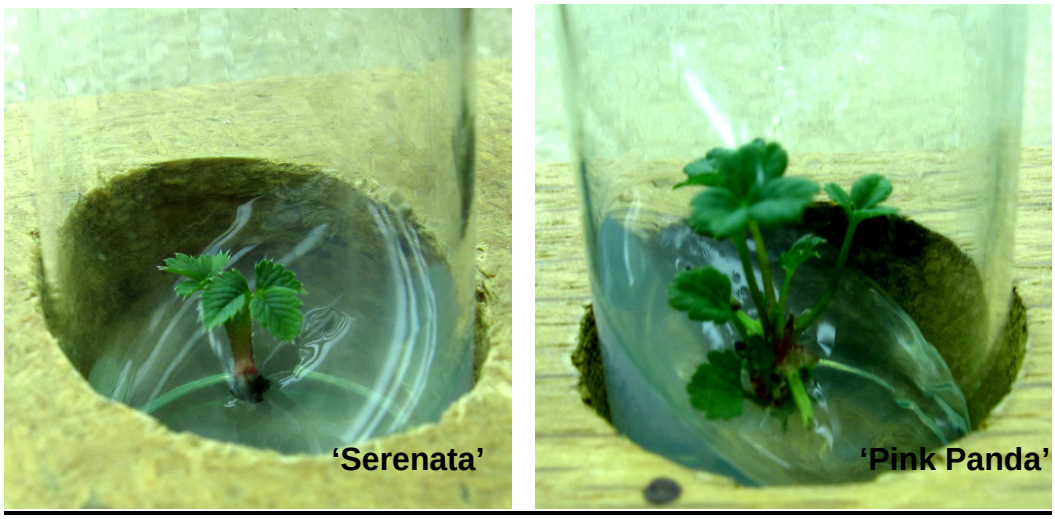


Figure 1. Plantlets regenerated from caulinar apexes in ‘Serenata’ and ‘Pink Panda’ ornamental strawberry varieties.

2.2. Capacity of multiplication *in vitro*

2.2.1. Influence of genotype (A) on the rate of multiplication *in vitro*

From the multitude of factors involved in the regeneration process, genotype is the most important, a relevant proof being the fact that the ‘Serenata’ intergeneric hybrid maintained its advantage of genotype with a superior capacity of multiplication *in vitro*, during all the four subcultures.

The data presented in Figure 2, shows that the assessment of average number of shoots regenerated after the four succesive subcultures revealed a 1.96 times higher potential in the ‘Serenata’ genotype (for which an average number of 14.69 shoots formed per initial explant was determined), as compared with the ‘Pink Panda’ genotype (for which an average number of 7.49 shoots formed per initial explant was calculated).

Due to the problems associated with the genetic stability of plants clonally multiplicated by the proliferation of axillary buds in succesive subcultures (Rosati, 1993), within the researches initiated by us, the transfer of shoots on fresh medium for multiplication was limited to four subcultures.

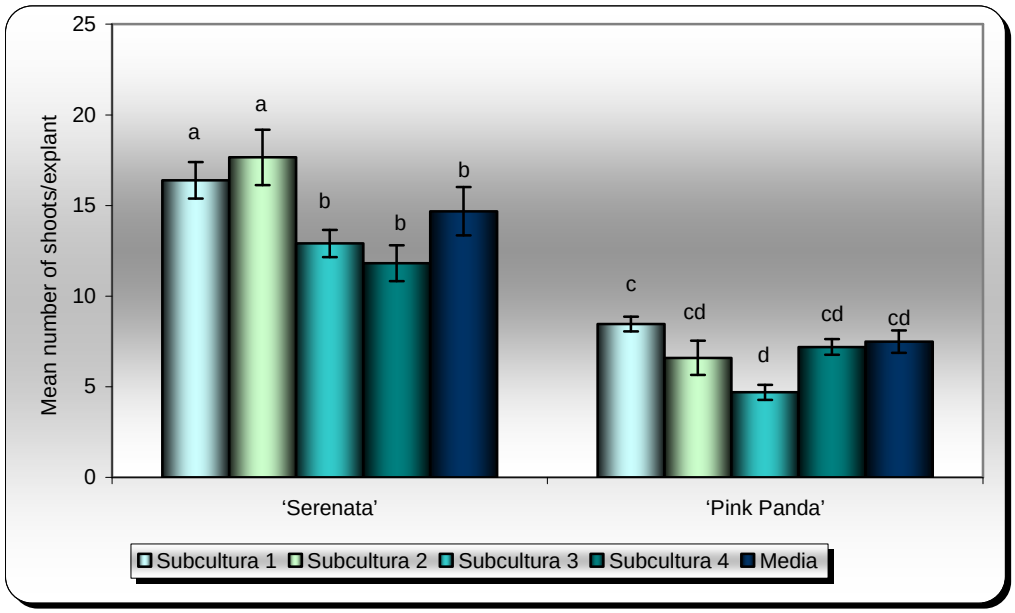


Figure 2. Influence of genotype (A) on the rate of multiplication *in vitro*, in 'Pink Panda' and 'Serenata' genotypes (the bars represents standard deviation; a, b, c, d: significance of differences, as revealed by Duncan test, $p<0.05$).

2.2.2. Influence of basal mediun (B) on the rate of multiplication *in vitro*

The results obtained in experiments designed for shoot regeneration in the intergeneric hybrids *Fragaria* × *Potentilla*, clearly reflects the important role of culture medium composition among the overall factors influencing the expression of regeneration potentrial, the different effect of the same combinations of growth regulators in basal media with a sensibly different composition in salts being a conclusive proof.

Regardless of the combination of growth regulators, composition of the Knop basal medium has negativelly influenced the *in vitro* regeneration capacity of intergeneric hybrids *Fragaria* × *Potentilla.* In this respect, must be mentioned the fact that, the reduced rate of multiplication (2 - 5 shoots formed per initial explant in 'Pink Panda' genotype, respectively 4 - 8 shoots per initial explant in 'Serenata' genotype) was associated, in each subculture, by a reduced vigor of the shoots and premature withering in a high percentage of the shoots in 'Pink genotype Panda' (Figure 3). Therefore, these shoots couldn't be subjected to the subsequent studies, concerning the capacity of rooting *in vitro* and acclimatization in greenhouse conditions. Similarly, the rate of multiplication was low in the case of control variants, the lack of growth regulators in culture media resulting in regeneration incapacity for a great number of explants, starting with the second transfer of shoots on fresh nutritive media.

Starting from these findings in the studies aiming at the assessment of multiplication ability of the shoots regenerated from caulinar apexes, exclusively MS and LF basal media have been used.

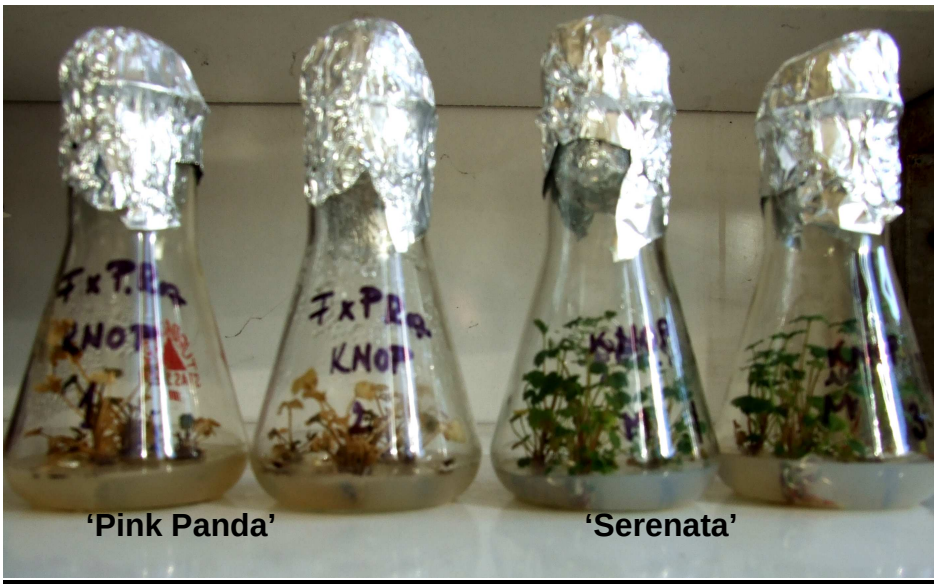


Figure 3. Influence of the Knop basal medium on the rate of multiplication *in vitro* in 'Pink Panda' and 'Serenata' genotypes (the second subculture).

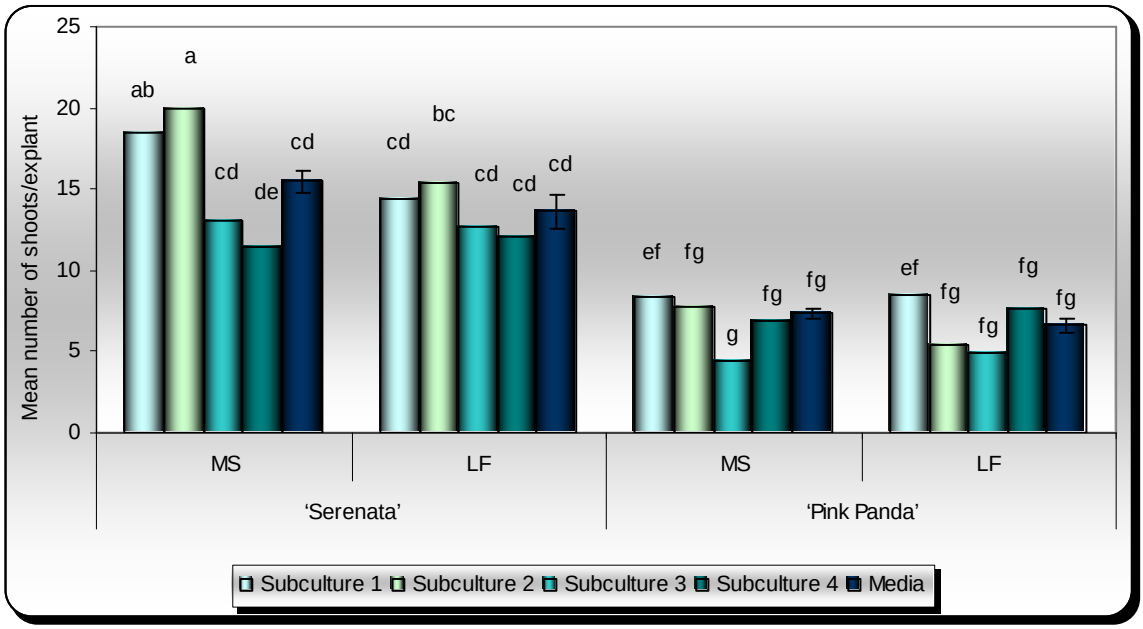


Figure 4. Influence of basal medium (B) on the *in vitro* rate of multiplication, in 'Pink Panda' and 'Serenata' genotypes (the bars represents standard deviation; a, b, c, d: significance of differences, as revealed by Duncan test, $p<0.05$).

In Figure 4 there are presented the overall results concerning the influence of basal medium on the number of shoots obtained by *in vitro* multiplication, in this respect being relevant the difference of 2.1 shoots formed per initial explant in 'Serenata' genotype, calculated between experimental variants based on MS medium and LF medium, respectively. The difference of only 1.11 shoots formed per initial explant in 'Pink Panda' genotype, found between the two basal media, shows the decisive role of genotype × basal medium interaction on this process.

2.2.3. Influence of combination and concentration of growth regulators (C) on the rate of multiplication *in vitro*

Owing to the fact that separation and transfer of the shoots on fresh culture media, maintaining the corespondence of experimental variants, revealed the favourable interaction between the investigated genotypes of ornamental strawberry and specific combinations and concentrations of the growth regulators, we choose for graphic representation of the results obtained after each subculture of shoots, and finally of the averages calculated after the four succesive subcultures (Fig. 5).

Supplementation of the culture medium with BAP, in a concentration of 0.5 mg/l, in combination with 0.1 mg/l IBA and 0.1 mg/l GA_3 allowed obtention of better results in 'Serenata' genotype, the average rate of multiplication being as high as 15.93 shoots formed per initial explant and per subculture. The same combination of growth regulators, but in higher concentrations, respectively 1.0 mg/l BAP, in combination with 0.2 mg/l IBA and 0.1 mg/l GA_3, led to the highest rate of multiplication in 'Pink Panda' genotype, equal to 5.88 shoots formed per initial explant and per subculture.

Although the average number of shoots formed per initial explant was high also in the experimental variant containing 2.0 mg/l BAP + 0.5 mg/l Kin + 2.0 mg/l GA_3, this phytohormone balance is not recommended due to the vitrification of shoots. Also, the phytohormone combination of 1.0 mg/l BAP + 1.0 mg/l IAA + 0.1 mg/l GA_3, characterized by a higher concentration of the auxin, is not recommended for the *in vitro* clonal multiplication in *Fragaria x Potentilla* hybrids, as it was shown to induce callus formation, which may result in somaclonal variation.

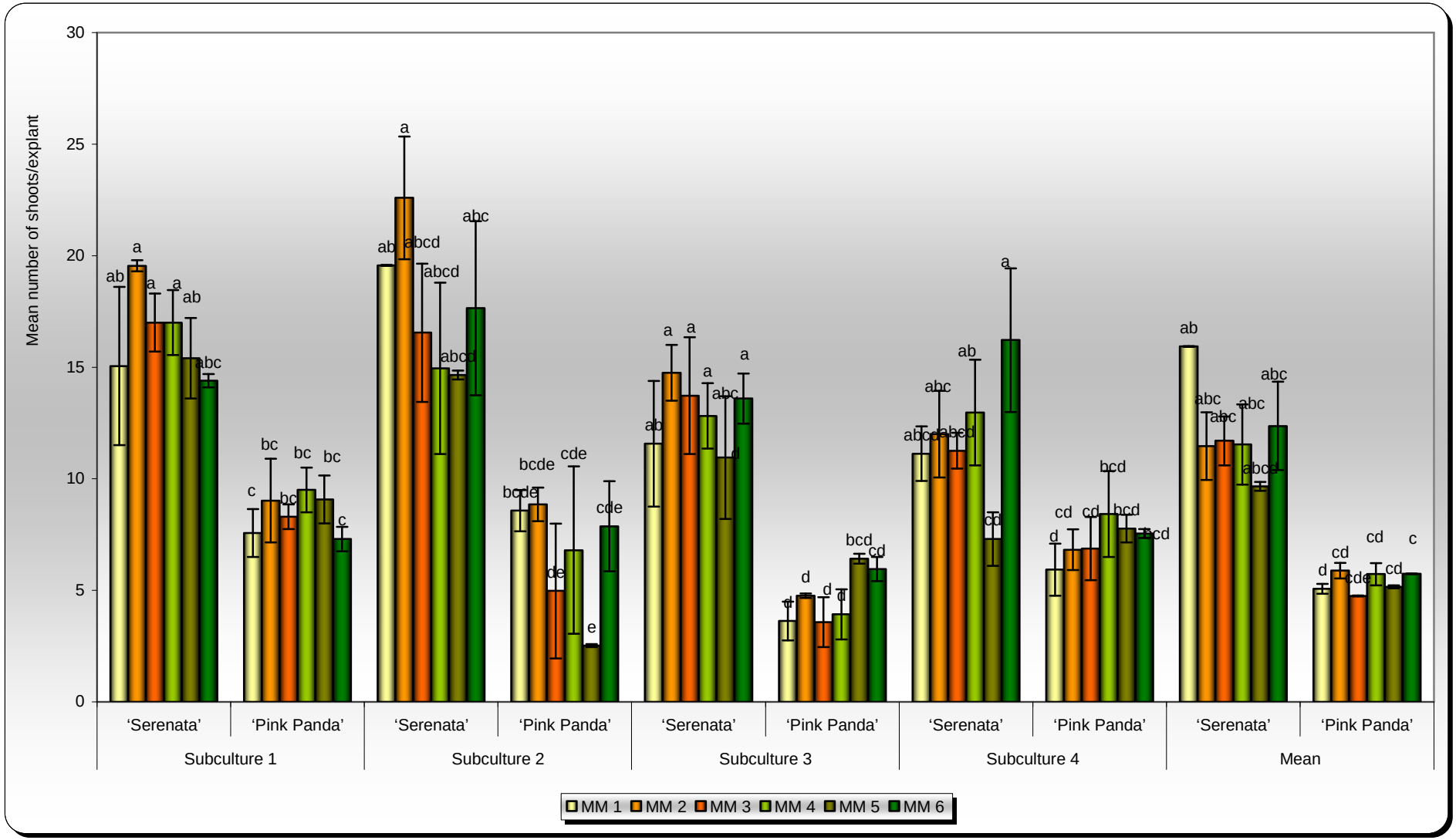


Figure 5. Influence of combination and concentration of growth regulators (C) on the rate of multiplication *in vitro*, in 'Pink Panda' and 'Serenata' genotypes (the bars represents standard deviation; a, b, c, d: significance of differences, as revealed by Duncan test, $p<0.05$).

2.2. CAPACITY OF SHOOT REGENERATION BY INDIRECT ORGANOGENESIS USING AGARIZED CULTURE MEDIA

2.2.1. Capacity of callus formation from leaf and petiole explants cultivated on agarized culture media

In order to prevent the negative effect of a development stage “ante” or “post” to those proved to be optimum, the plantlets (originating from caulinar apexes), used as explant source, have been subjected to the pretreatment recommended by Sorvari et al. (1993), which aimed at enhancing the level of endogenous phytohormones, prior to excision of the somatic tissue explants. In this context, is important to mention that, in the preliminary studies which we have carried out for inducing organogenesis in the intergeneric hybrids *Fragaria* × *Potentilla*, in the absence of pretreatment for improving the physiological state of plants used as source of explants, the processes of callus formation and morphogenesis have failed, as all the explants being affected by necrosis in 7 - 10 days from the initiation of *in vitro* cultures.

Also, the absence of growth regulators in the culture media, in the control variant, had visible effects, the first signs of necrosis occurring in less than 14 days in the area of wounding. In the following three weeks, the necrosis became total in both the leaf and petiole explants.

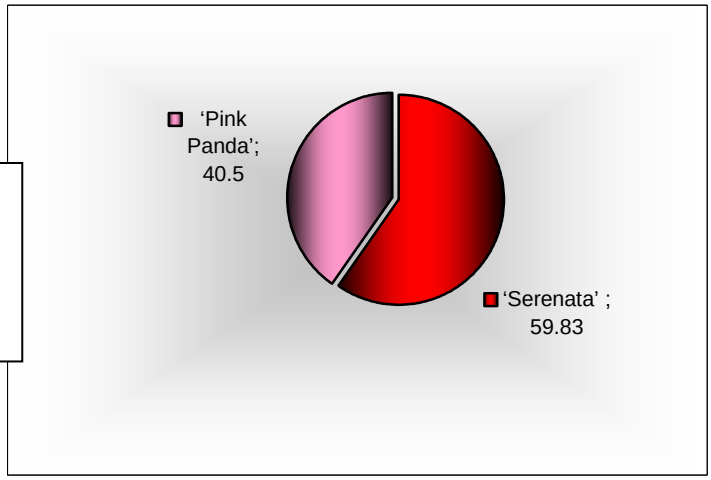
A friable callus, with morfogenetic potential, have been developed after 40 - 45 days from the *in vitro* inoculation of the somatic tissue explants.

2.2.1.1. Influence of genotype (A) on the capacity of callus formation in leaf and petiole explants cultivated on agarized culture media

Based on the overall results obtained in experiments for determining the potential for callus formation, was calculated an average percentage of 59.83% of somatic tissue explants (leaf fragments and petiole segments) which formed callus in ‘Serenata’ genotype, and of only 40.5% in ‘Pink Panda’ genotype (Fig. 6), the difference being significant for $p<0.05$, according to the Independent T-test. This result represent a confirmation of the different micropropagation potential in the two genotypes, as was shown in the previous subchapters.

Although the recalcitrance *in vitro* of the ‘Pink Panda’ genotype maintained during the experiments designed for induction of callus formation, it was observed that once the process of callus formation was initiated, the cell proliferation was more intense, resulting in bigger calli, as compared to those formed in ‘Serenata’ genotype on culture medium with the same composition.

Figure 6. Influence of genotype (A) on the frequency of callus formation, induced on agarized culture medium



2.2.1.2. Influence of combination and concentration of growth regulators (C) on the capacity of callus formation in leaf and petiole explants cultivated on agarized culture media.

As shown by the data presented in Figure 7, the culture medium supplemented with 2.4-D and BAP in a ratio equal to 0.16 (CIM1 variant) had a favorable influence on the callus formation in 'Serenata' genotype. This balanced composition of the growth regulators resulted in an average percentage of leaf explants forming calli of 94%, the same percentage being calculated also for the petiole segments.

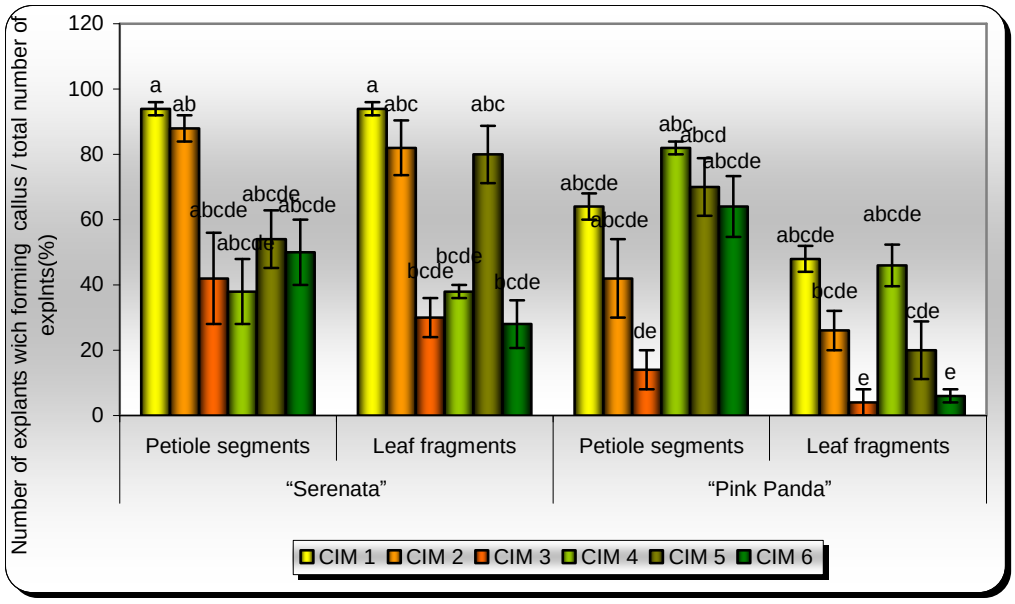


Figure 7. Influence of combination and concentration of growth regulators (C) on the capacity of callus formation in leaf and petiole explants cultivated on agarized culture media (the bars represents standard deviation; a, b, c, d: significance of differences, as revealed by Duncan test, $p<0.05$).

On the other hand, IBA is an auxin of high efficiency in inducing the proliferation process and callus formation from somatic tissue explants in 'Pink Panda' genotype. Thus, 82% from the petiole segments, respectively 46% from the leaf fragments placed on culture media supplemented with 0.5 mg/l IBA + 3.0 mg/l BAP (CIM4 variant) formed callus. In comparison, in 'Serenata' genotype, good results were obtained with a concentration of 1.0 mg/l IBA, added to the culture medium together with 3.0 mg/l BAP (CIM5 variant), which induced callus formation in 80% from the leaf explants and 54% from the petiole segments, respectively.

Also, in 'Serenata' genotype was noticed formation of secondary green callus from the apparently entire brown callus, originated from leaf fragments, exclusively on the media variants containing 2.4-D (Fig. 8).

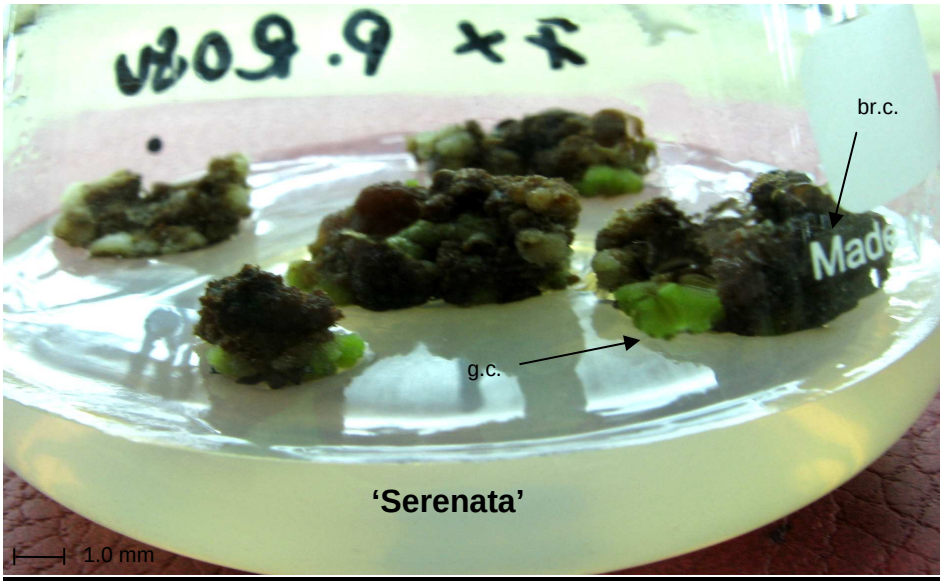


Figure 8. Formation of green callus from brown callus, resulted by culturing the leaf explants on the medium variant CIM2, containing 1.0 mg/l 2.4-D + 3.0 mg/l BAP (g.c. – green callus; br.c. – brown callus).

2.2.2. Regeneration potential of the calli maintained on agarized culture media

2.2.2.1. Influence of genotype (A) on the frequency of shoot regeneration from calli maintained on agarized culture media

Counting of the number of calli which regenerated shoots, as well as counting of the number of plantlets formed *de novo* after five months in culture, when visible signs of calli ageing were noticed and the regeneration process ceased, revealed the extremely important influence of the genotype.

As in previous experiments, ‘Serenata’ genotype of ornamental strawberry proved to possess a potential for *in vitro* regeneration by indirect organogenesis superior to that of ‘Pink Panda’ genotype.

As compared with induction and proliferation of the callus, organogenesis had a relatively low frequency in ‘Serenata’ genotype, the average percentage of explants from which were regenerated adventitious buds and shoots being of only 11.33%. Moreover, in ‘Pink Panda’ genotype, shoot regeneration did not occur, regardless of the type of explant and culture medium composition. Thus, in ‘Serenata’ genotype, shoot regeneration was obtained only in the case of explants which formed callus in high amounts. The highest frequency of regeneration (36%) was calculated for the calli derived from leaf explants cultured on the medium variant CIM4. The regeneration ability of the calli derived from petiole explants was even lower, as in the same culture conditions shoot formation occurred on only 10% of them (Table 7).

It is also important to mention that the secretion of antocianines, observed in the cells of the leaf and petiole derived calli in ‘Serenata’ genotype, did not have a negative influence on the process of shoot regeneration (Fig. 9).

Table 7.

Frequency of shoot regeneration from the leaf- and petiole-derived calli in ‘Serenata’ genotype of ornamental strawberry.

intergeneric hybrid ‘Serenata’	**Basal medium**	**Growth regulators**	**Frequency of shoot regeneration (%)**		**Number of regenerated plantlets**	
			Leaf fragments	Petiole segments	Leaf fragments	Petiole segments
	LF	0.5 mg/l AIB + 3.0 mg/l BAP (CIM4)	36.0 ± 2.3 a	10.0 ± 1.1 a	6.8 ± 0.9 a	3.4 ± 0.32c
	MS	1.0 mg/l AIB + 3.0 mg/l BAP (CIM5)	22.0 ± 1.6 b	-	9.6 ± 0.06 b	

* Each value represents the mean ± SE. In each column, differences between any of two variants, followed by at least a common letter, are not significant for $p<0.05$, according to the Duncan test.

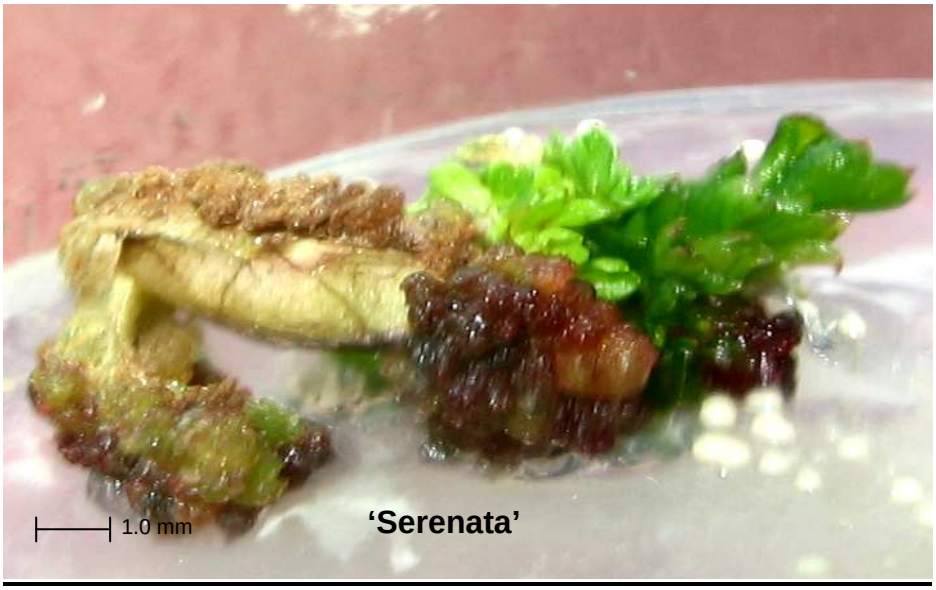


Figure 9. Shoot regeneration via callus from an immature leaf fragment placed on agarized culture medium.

2.2.2.2. Influence of combination and concentration of growth regulators (C) on the frequency of shoot regeneration from calli maintained on agarized culture media

For each genotype and for each stage of micropropagation it is necessary an adequate formula of the growth regulators which can be added to the agarized culture media. In this respect, a conclusive proof is that the 2.4-D auxin in a concentration of 0.5 mg/l was efficient in promoting and maintain the process of callus formation in ‘Serenata’ genotype, but shoot regeneration was stimulated by IBA in a concentration of 0.5 mg/l, which resulted in a frequency of adventitious shoot regeneration of 36%. In case of ‘Pink Panda’ genotype, the IBA auxin proved to be more efficient in stimulating the cell proliferation, but it couldn’t sustain the regeneration process.

2.2.2.3. Influence of explant type (E) on the frequency of shoot regeneration from calli maintained on agarized culture media

In ‘Serenata’ genotype, the capacity of callus formation from the leaf explants (57.66%) inoculated on agarized culture media was inferior to that of petiole segments (62%), but the potential of shoot regeneration from leaf explants (58%) was significantly higher to that exhibited by the petiole explants (10%), suggesting that it isn’t a close correlation between the competence for cell proliferation of the somatic tissue explants, and that for shoot regeneration.

In this context, in Figure 10 it is ilustrated the shoot regeneration from leaf-derived calli in ‘Serenata’ genotype of ornamental strawberry, these presenting a lower rate of callus formation.

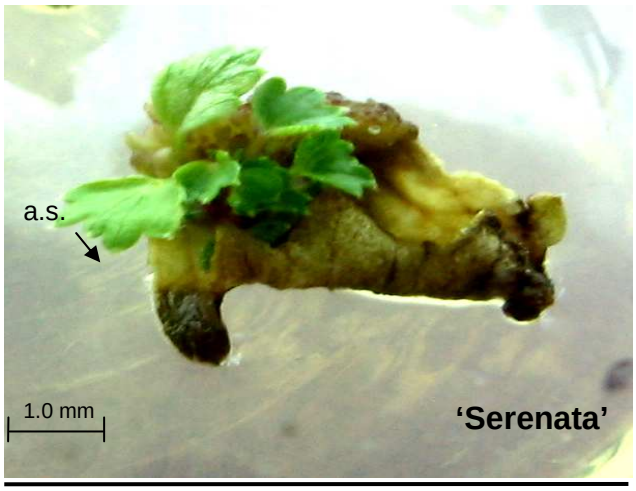


Figure 10. Shoot regeneration from leaf-derived callus (a.s. – adventitious shoot).

2.3. CAPACITY OF SHOOT REGENERATION BY DIRECT ORGANOGENESIS USING AGARIZED CULTURE MEDIA

In the attempts to overtake the recalcitrance to organogenesis of ‘Pink Panda’ genotype, as well as to obtain a higher frequncy of shoot regeneration in the studied intergeneric hybrids, we choosed for supplementation of the agarized culture media with tidiazuron (TDZ), in combination with IBA or 2.4-D. Replacement of BAP with TDZ led to obtention of some surprising results, shoot regeneration being obtained in less than 14 days directly from the somatic tissues, without callus formation, in both the investigated ornamental strawberry genotypes. The intense organogenetic activity in these genotypes is obviously trigerred by the TDZ, which was demonstrated to induce a diversity of morphogenic responses, from the cell proliferaration to formation of adventitious shoots or somatic embryos.

The frequency of shoot regeneration by direct organogenesis from somatic tissue explants was calculată at the end of the two weeks of incubation in darkness. In this context, is important to mention that, for the first time from the initiation of experiments which aimed at the micropropagation of *Fragaria* × *Potentilla* intergeneric hybrids by organogenesis, became evident the inhibitory influence of 16 hours of light / 8 hours of darkness photoperiod, as the transfer of in vitro cultures to light conditions triggered the suspension of regeneration process in all the somatic tissue explants, for both the investigated ornamental strawberry varieties.

2.3.1. Influence of genotype (A) on the frequency of shoot regeneration by direct organogenesis

Influence of genotype has once more revealed, the average percentage of explants regenerating shoots being of 7.75% in 'Serenata' genotype, and only 5.22% in 'Pink Panda' genotype (Fig. 11). Although the 'Serenata' genotype have maintained its advantage of having a superior potential for regeneration *in vitro*, the frequency of shoot regeneration by direct organogenesis was extremely low, even when compared to the results obtained in experiments for shoot regeneration by indirect organogenesis.

Otherwise, TDZ is is recognized as an cytokinin-like compound extremely efficient in inducing the proliferative processes, but the growth regulators mode of action, implicitly that of TDZ, is highly dependent on specific genetic factors (Passey et al., 2003).

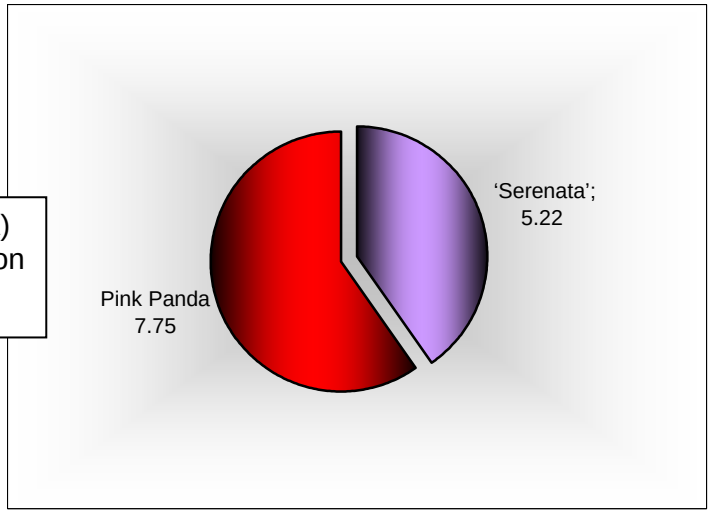


Figure 11. Influence of genotype (A) on the frequency of shoot regeneration by direct organogenesis

2.3.2. Influence of the interaction between basal medium (B) and combination and concentration of growth regulators (C) on the frequency of shoot regeneration by direct organogenesis

By analysing the data presented in Figure 12 concerning the influence of interaction between basal medium and growth regulators formula on the capacity of shoot regeneration by direct organogenesis, we found that supplementation of basal medium with moderate to high concentrations of TDZ, had a positive influence on the frequency of shoot regeneration from petiole explants cultivated on LF basal medium, without callus formation.

2.3.3. Influence of explant type (D) on the frequency of shoot regeneration by direct organogenesis

In the experiments designed with the objective to stimulate shoot regeneration by organogenesis, from the two types of somatic tissue explants tested, only the petiole explants expressed their potential for shoot regeneration, without callus formation (Fig. 13). In this respect, is important to notice that Foucault (1990) reported that, in case of using TDZ, the percentages of leaf explants showing regeneration were similar, or even lower than those induced by BAP.

The points of organogenesis initiation were always at the ends of petiole segments, probably as a consequence of accumulation of endogenous phytohormones at the level of explant sectioning, as well as a consequence of uptaking from the culture medium, through the wounding areas, of high amounts of phytohormones required for both initiating and maintaining the regeneration process.

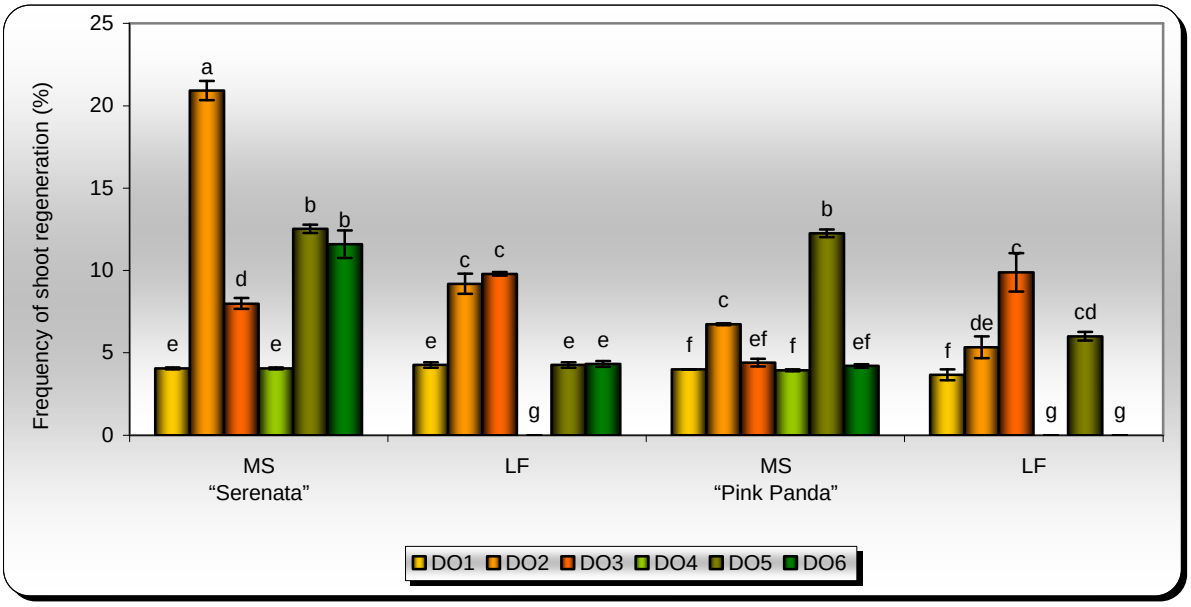


Figure 12. Influence of the interaction between basal medium (B) and combination and concentration of growth regulators (C) on the frequency of shoot regeneration by direct organogenesis (the bars represents standard deviation; a, b, c, d: significance of differences, as revealed by Duncan test, $p<0.05$).

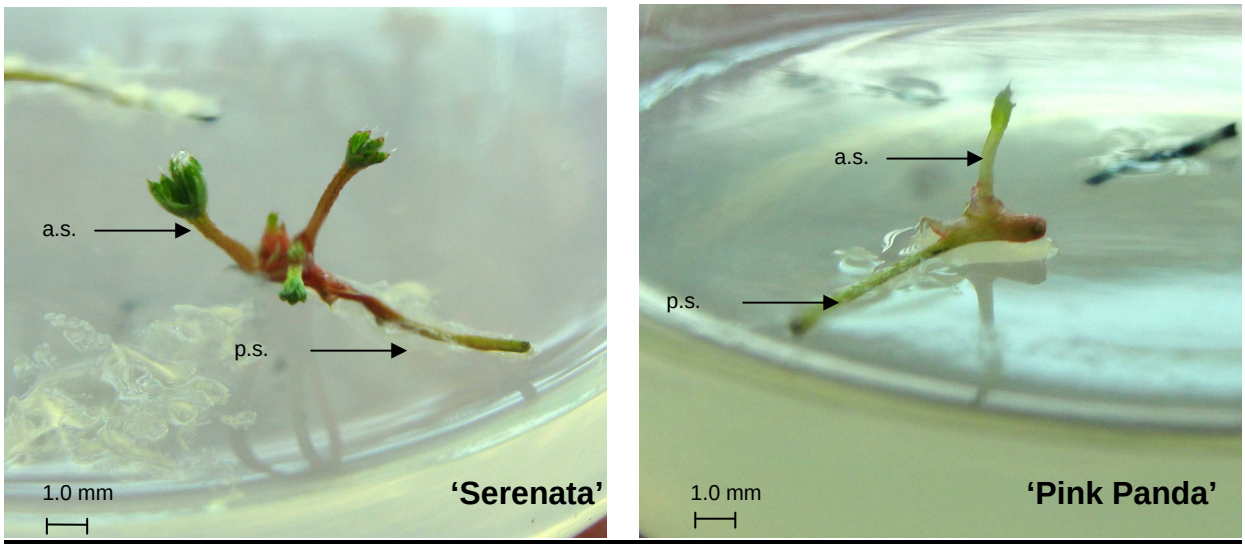


Figure 13. Shoot regeneration by direct organogenesis from petiole explants inoculated on MS basal medium, supplemented with 1.0 mg/l IBA + 1.0 mg/l TDZ (a.s. – adventitious shoot; p.s. – petiole segment).

2.4. CAPACITY OF SHOOT REGENERATION BY INDIRECT ORGANOGENESIS USING LIQUID CULTURE MEDIA PROVIDED WITH FILTER PAPER BRIDGES

2.4.1. Influence of dark pretreatment (F) on the capacity of callus formation in leaf and petiole explants cultivated on liquid culture media provided with filter paper bridges

After the transfer of cultures maintained for 21 days in darkness, to a light intensity of about 40 µmol m^{-2} s^{-1} and under a photoperiod of 16 hours light / 8 hours darkness, the proliferation of calli comes to a standstill, regardless of the genotype, type of explant, or composition of the liquid culture media. The processes of necrosis developed rapidly in a period shorter than 10 days. Once again, the duration of pretreatment to darkness represented a critical factor for the processes of callus proliferation and shoot regeneration via calus, even in the absence of TDZ, known as having a favorable influence on the *in vitro* morphogenesis, especially, in the conditions of maintaining the somatic tissue explants in darkness (Landi and Mezzetti, 2006). However, it must be stated precisely that at the end of pretreatment to darkness, with a duration of 42 days, no significant differences have been found between the two ornamental strawberry genotypes in the average percentages of somatic tissue explants which formed callus (Fig. 14).

Thus, in the case of experimental variant CIM5, characterized by the presence of 1.0 mg/l IBA + 3.0 mg/l BAP, the percentage of somatic tissue explants which formed callus was maximum (100%) for both ornamental strawberry genotypes investigated. On the other hand, the lowest frequency of callus formation was recorded in the case of leaf fragments, in experimental variant CIM6, as the cell proliferation was noticed in only 40% of the leaf explants in the 'Pink Panda' genotype, and 38.8 % in the 'Serenata' genotype, respectively.

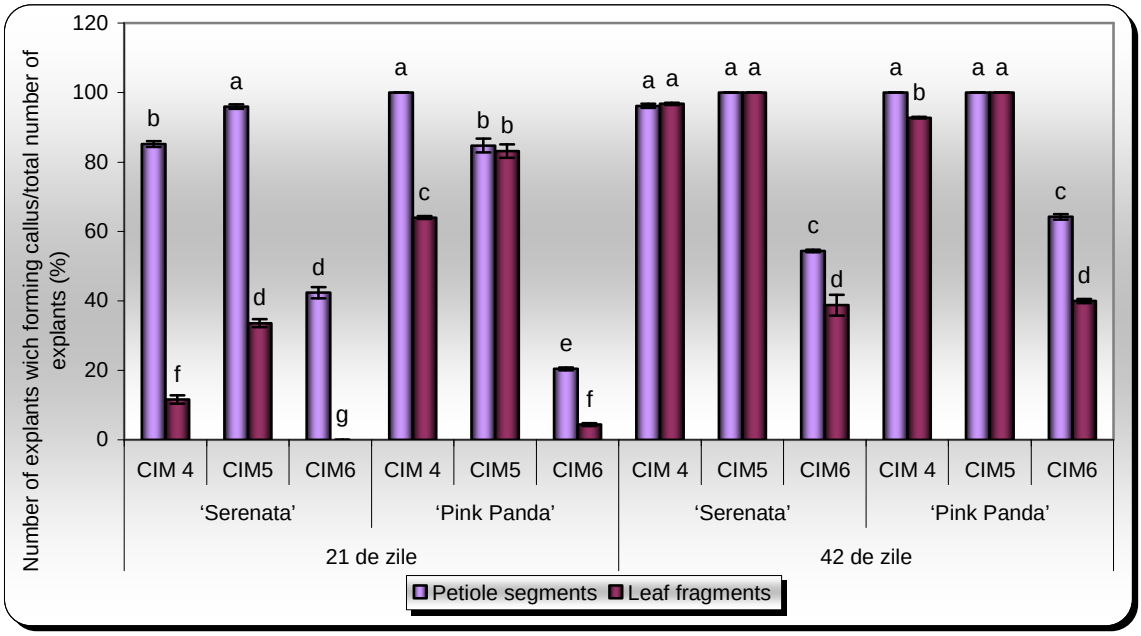


Figure 14. Influence of the darkness pretreatment (E) on the capacity of callus formation from somatic tissue explants cultivated on liquid nutritive media, provided with filter paper bridges (the bars represents standard deviation; a, b, c, d: significance of differences, as revealed by Duncan test, $p<0.05$).

2.4.2. Regeneration potential of the calli maintained on liquid culture media provided with filter paper bridges

2.4.2.1. Influence of genotype (A) on the frequency of shoot regeneration from calli maintained on liquid culture media provided with filter paper bridges

The maintenance of somatic tisue cultures in darkness for a period as long as 42 days, in the growth room, led to occurrence of the first regeneration events, observed in 'Serenata' genotype after about 27 days from the inoculation of petiole explants, and 30 days from the inoculation of leaf explants, respectively. In 'Pink Panda' genotype, the first adventitious buds have occurred from leaf fragments, in the absence of light, after about 38 days from the initiation of *in vitro* cultures (Fig. 15).

The transfer of the in vitro cultures of somatic tissue explants to low intensity light was associated with the stimulation of antocianines secretion in the parenchimatous cells from the surface of the callus, regardless of its origin in petiole segments, or leaf fragments. Moreover, the red pigmentation, characteristic for antocianines, was observed preponderently in the parenchimatous cells from the calli with high regeneration capacity.

The favorable influence of a relatively low light intensity was confirmed also by the increase of the frequency of shoot regeneration in both ‘Serenata’ and ‘Pink Panda’ genotypes. In fact, these were the conditions which allowed adventitious shoot regeneration from petiole explants in the presumably recalcitrant ‘Pink Panda’ genotype, in a relatively short period, of about 50 days, without dividing the calli and transferring them on fresh culture media (Fig. 15).

After 70 days from the initiation of *in vitro* cultures on liquid nutritive media, provided with filter paper bridges, the frequency of callus formation became stable. An average percentage of 37.12% calli from which shoots were regenerated was calculated for the ‘Serenata’ genotype, while in ‘Pink Panda’ genotype the frequency of calli regenerating shoots was of only de 28.77%. According to the independent T-test, these values are significantly different for $p<0.05$ (Fig. 16).

Concerning the number of shoots formed from the same callus, there were no significant differences between the two genotypes. Thus, the average number of shoots formed *de novo* per callus was 8.9 in ‘Serenata’ genotype, and 8.64 in ‘Pink Panda’ genotype of ornamental strawberry, respectively.

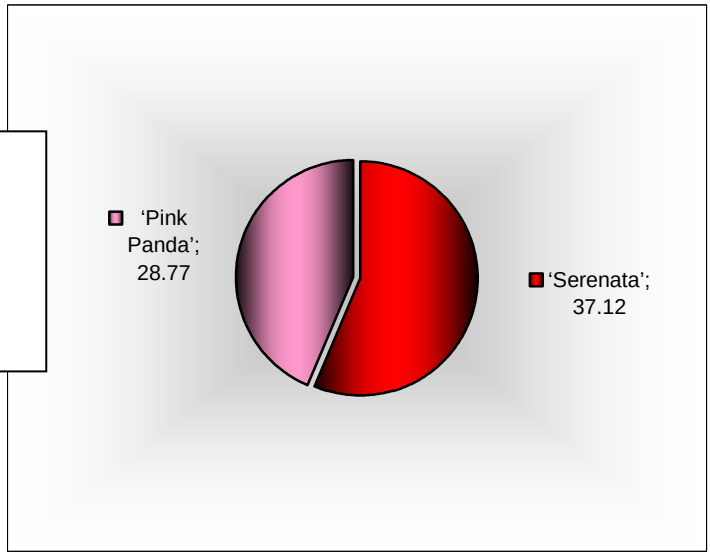


Figure 16. Influence of genotype on the frequency of shoot regeneration via tissue-derived callus from the explants cultivated on liquid nutritive medium provided with filter paper bridge.

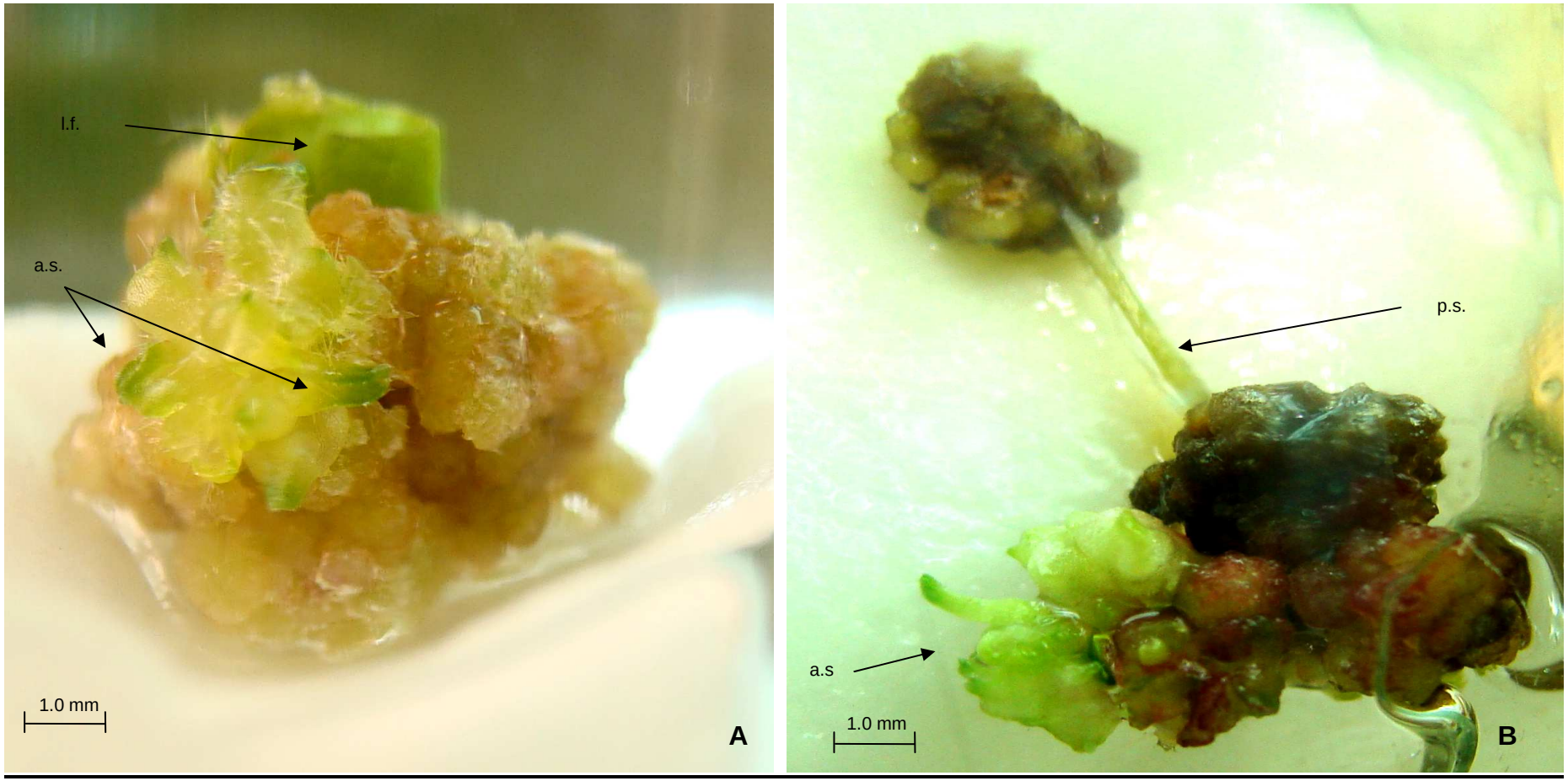


Figure 15. Organogenesis in the callus obtained by the *in vitro* culture of somatic tissue explants on liquid culture media, provided with filter paper bridges, in the 'Pink Panda' genotype. A – shoot regeneration from leaf-derived callus; B – shoot regeneration from petiole-derived callus (l.f. – leaf fragment; p.s. - petiole segment; a.s. – adventitious shoot).

2.4.2.2. Influence of combination and concentration of growth regulators (C) on the frequency of shoot regeneration from calli maintained on liquid culture media provided with filter paper bridges

By analysing the overall results presented in Figure 17, can be observed that the hormonal balance consisting of 1.0 mg/l IBA + 3.0 mg/l BAP induced cell proliferation in the highest number of somatic tissue explants and stimulated the shoot regeneration in the highest percentage of calli, which suggests the necessity of presence in the culture medium of a higher concentration of the auxin with the best potential of promoting the organogenic potential, as compared to the agarized culture media.

Supplementation of the liquid culture media with only 3.0 mg/l BAP, regardless of the concentration of IBA (0.5 mg/l or 1.0 mg/l), induced adventitious shoot formation in 52.66% and respectively 54.66% from the explants of 'Serenata' genotype, these percentages being not significantly different from those calculated for 'Pink Panda' genotype (41%, and respectively 42.33%, on the CIM5 culture medium variant).

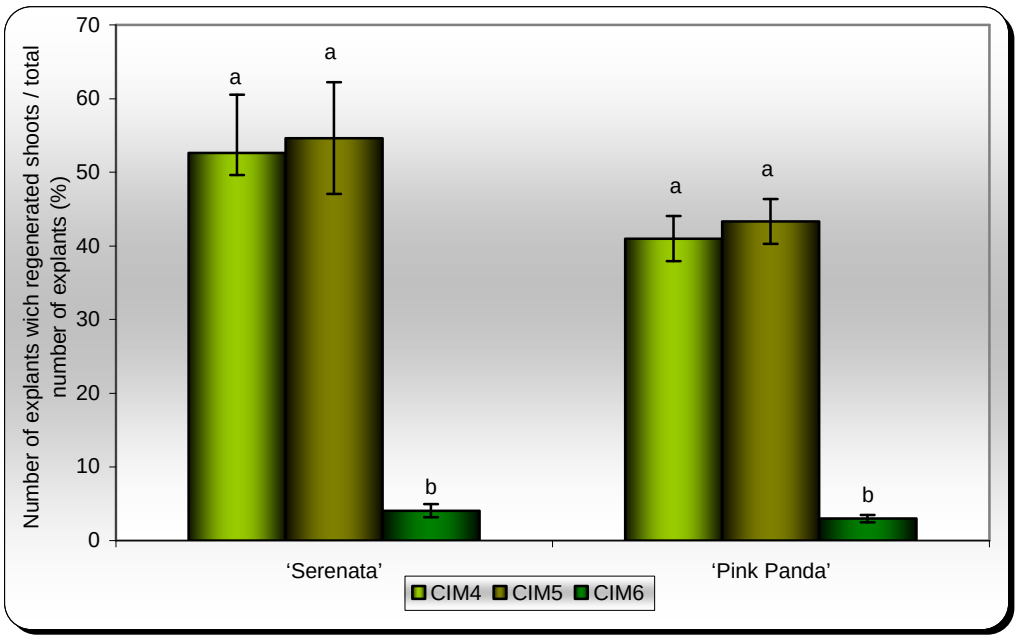


Figure 17. Influence of combination and concentration of growth regulators (C) on the frequency of de novo plantlets from calli maintained on liquid culture media provided with filter paper bridges (the bars represents standard deviation; a, b, c, d: significance of differences, as revealed by Duncan test, $p<0.05$).

2.5. THE *IN VITRO* ROOTING CAPACITY OF SOME INTERGENERIC HYBRIDS *FRAGARIA* × *POTENTILLA*

2.5.1. Factors with major influence on the expression level of *in vitro* rooting capacity of the shoots originating from meristems

Starting from the hypothesis that the culture medium used for the multiplication of meristem-derived shoots can have a significant influence on the rhyzogenesis process *in vitro*, experiments designed for the assessment of rooting ability were organized separately for each multiplication medium. Thus, the rooting rate of the shoots (ratio between the number of rooted shoots and the total number of shoots transferred on the rooting medium, as percentage), and the number and length of roots formed by each shoot, have been determined separately, the same three variants of rooting medium being used for the assessment of rooting ability of shoots regenerated in each variant of medium for multiplication.

2.5.1.1. Influence of genotype (A) on the capacity of rooting *in vitro*

If in the experiments designed for the stimulation of axillary shoot formation and organogenesis, the ‘Serenata’ ornamental strawberry distinguished by a superior capacity of shoot regeneration and proliferation, the ‘Pink Panda’ genotype distinguished by a high ability for *in vitro* rooting of the shoots.

Thus, while in ‘Pink Panda’ intergeneric hybrid the rooting of shoots started after 10 days from the intiation of culture and continued with an average rate of 81.43%, in ‘Serenata’ genotype, the rhyzogenesis process was initiated after 16 days from the inoculation of shoots, and the average rate of rooting was 36.17%.

By analysing the data presented in Figure 18 we can observe that in ‘Pink Panda’ genotype there are positive correlations between the three characteristics investigated (rooting rate, average number of roots per rooted shoot, average length of the roots), which were proven to be statistically significant for $p<0.01$. In comparison, in ‘Serenata’ genotype (Fig. 19), a positive correlation, statistically significant for $p<0.01$, was established only between the rooting rate of the multiplicated shoots and the average length of roots.

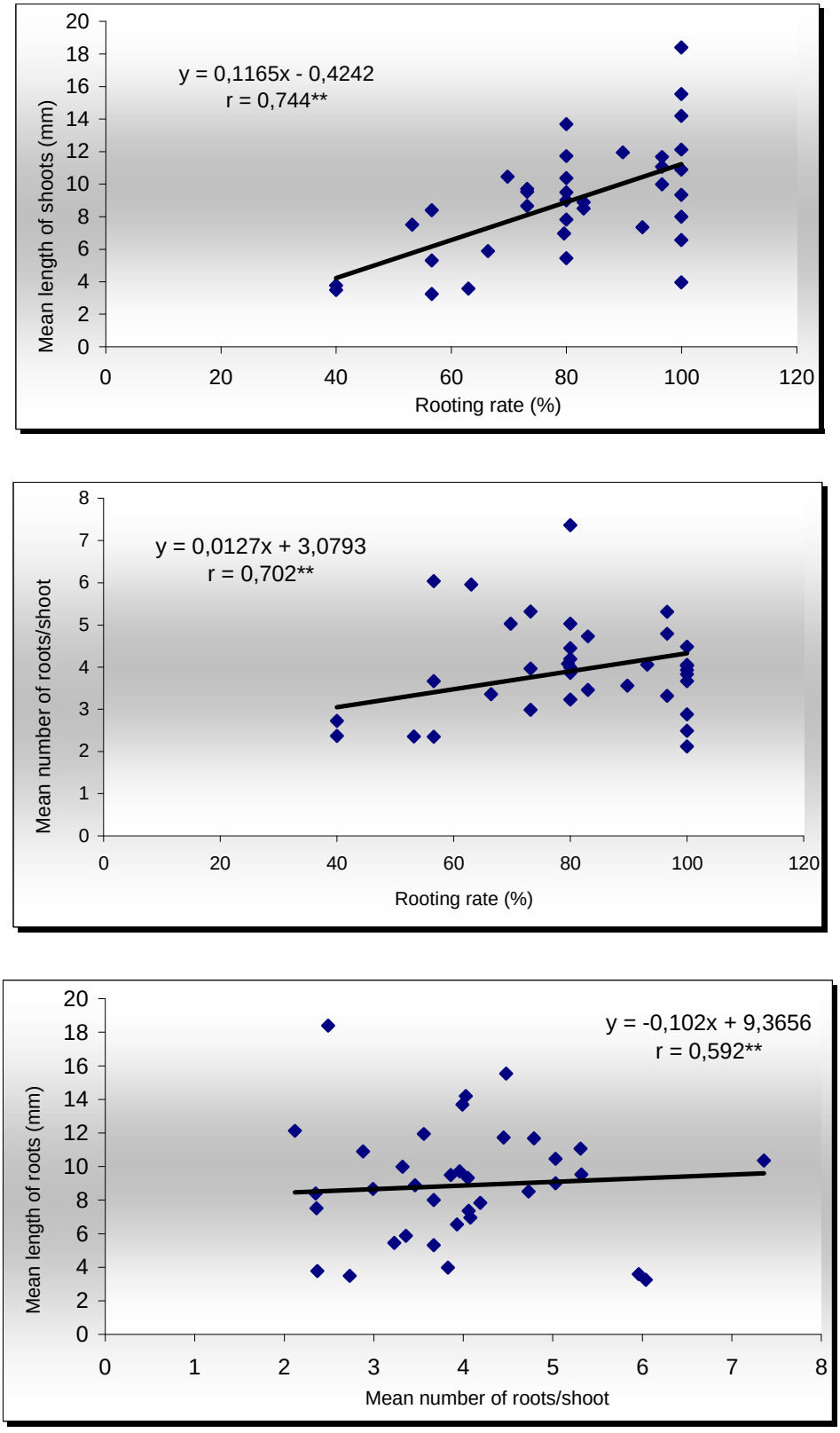
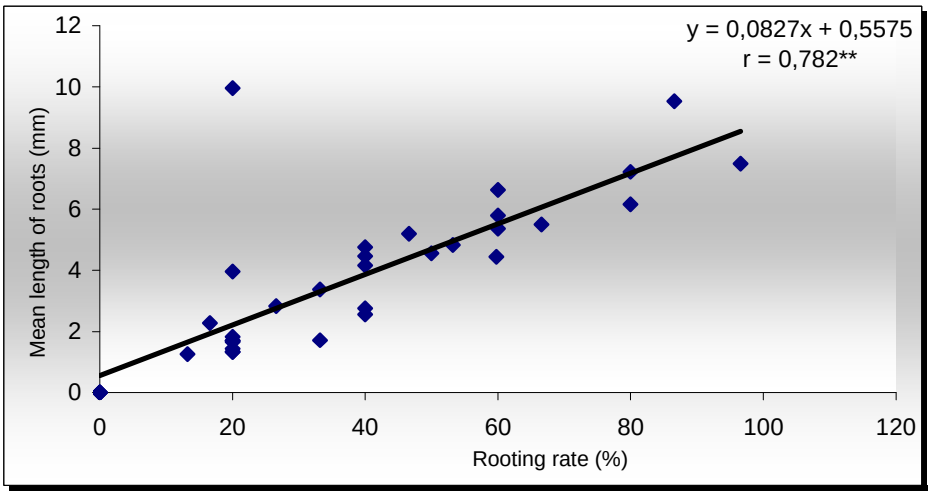
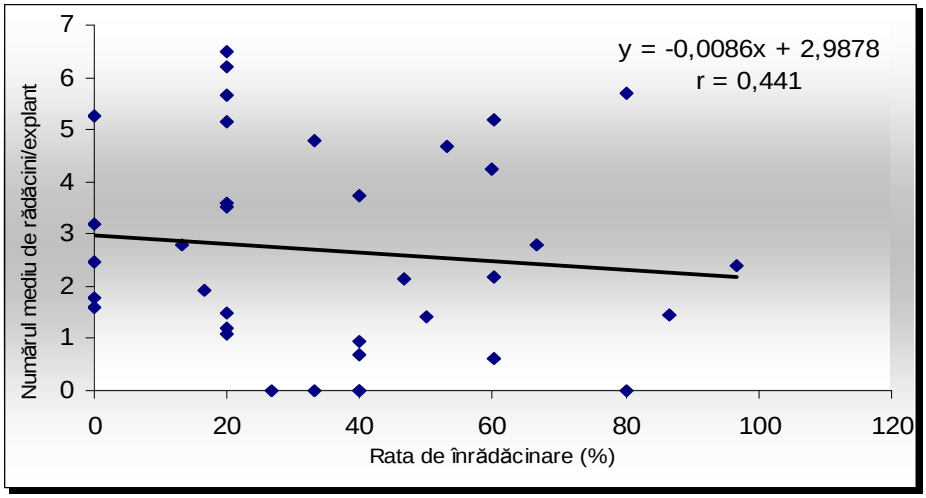


Figure 18. Correlation between the average length of roots and the rooting rate, between the average number of roots formed per explant and the rooting rate, and between the average number of roots per explant and the average length of roots, in 'Pink Panda' genotype. (** The correlation is statistically significant for $p<0.01$).





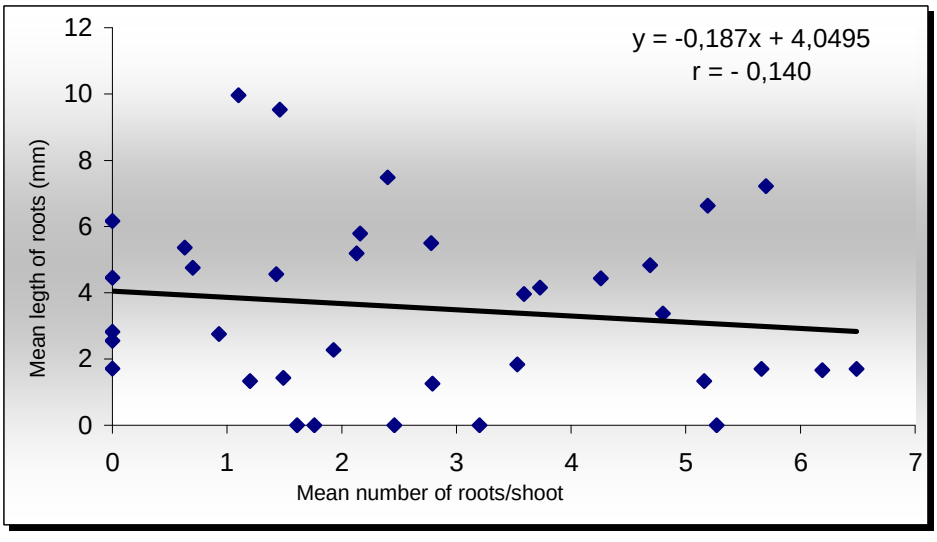


Figure 19. Correlation between the average length of roots and the rooting rate, between the average number of roots formed per explant and the rooting rate, and between the average number of roots per explant and the average length of roots, in 'Serenata' genotype. (** The correlation is statistically significant for $p<0.01$).

2.5.1.2. Rooting capacity of the microshoots regenerated on culture media with various composition

The effect of endogenous phytohormones content and composition of culture mediumis cumulative and significant for the stimulation of rhyzogenesis. There have been ascertained two ways of interaction: on the one hand, LF basal medium supplemented with low concentrations of growth regulators × rooting medium supplemented with 0.25 mg/l IBA + 0.1 mg/l GA_3 and, on the other hand, MS basal medium supplemented with higher concentrations of growth regulators × rooting medium supplemented with 0.5 mg/l IBA or IAA + 0.1 mg/l GA_3, which intensified the rooting capacity of shoots multiplicated *in vitro*.

In 'Pink Panda' genotype, the analysis of overall results highlighted a positive correlation between mineral composition of the MS basal medium used for shoot multiplication and their rooting capacity, the rooting rate ranging from 53.2% to 100%. Comparatively, the percentages of shoots multiplicated on LF basal medium which responded by root formation on the rooting medium ranged from 40% to 100% (Fig. 20).

The analysis of data presented in Figure 21, concerning to the influence of culture medium on the rooting rate in 'Serenata' genotype, revealed the fact that, similarly to the results presented previously, LF medium used for shoot multiplication, regardless of the combination and concentration of growth regulators, had a positive influence on the rooting capacity of shoots inoculated inoculated on RM1 medium variant, thus confirming the favorable interaction between LF basal medium composition and low concentration of IBA. A deviation from this finding was observed in the case of plantlets regenerated on MM3 and MM4 media variants, for which the increase of IBA concentration from 0.25 mg/l to 0.5 mg/l determined a rooting rate of 80% and 86.6%, respectively.

2.6. ACCLIMATIZATION CAPACITY OF THE MICROPLANTLETS ROOTED *IN VITRO*

In the acclimatization stage, the two genotypes showed different capacity of adaptation, which resulted in a different survival rate. Thus, the percentage of plants which survived over the acclimatization stage was 86.8% in 'Pink Panda' in genotype, slightly lower than in 'Serenata' genotype (92.4%). After acclimatization, all the plants were transferred individually in pots, and subsequently in the field.

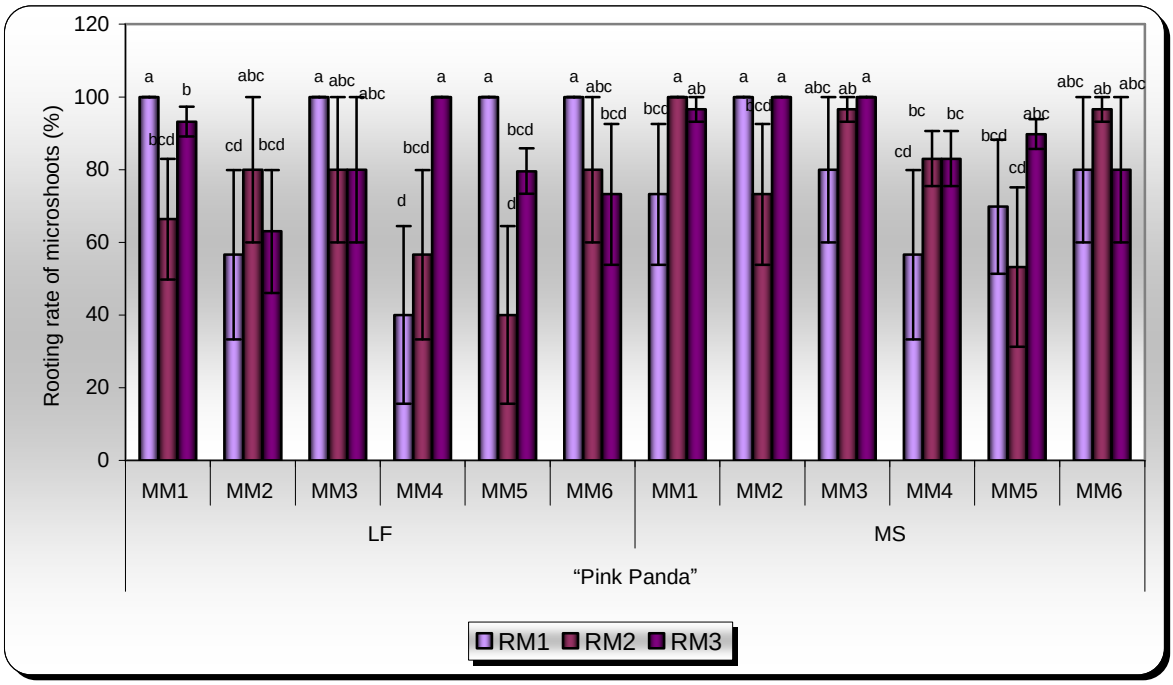


Figure 20. The rooting rate of microshoots in ‘Pink Panda’ genotype (the bars represents standard deviation; a, b, c, d: significance of differences, as revealed by Duncan test, p<0.05).

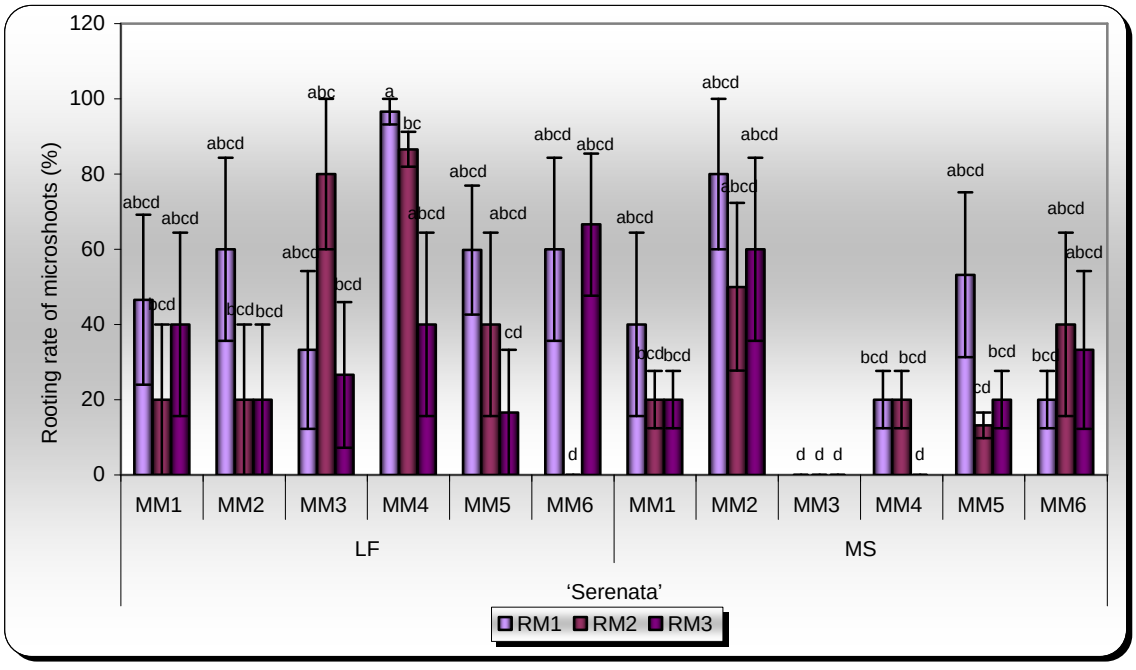


Figure 21. The rooting rate of microshoots in ‘Serenata’ genotype (the bars represents standard deviation; a, b, c, d: significance of differences, as revealed by Duncan test, p<0.05).

2.7. THE GENETIC STABILITY OF PLANTS CLONALLY MULTIPLICATED *IN VITRO*, REVEALED BY RAPD ANALYSIS

In the case of 'Serenata' genotype, the selected RAPD primers generated a total number of 59 bands, which were monomorphic for all the analyzed plants, including the control plant. The number of bands per primer ranged between 3 (OPC05) and 10 (OPC08), with an average value of 5.9 bands per primer. The size of DNA amplified fragments varied between 2665 pb and 287 pb.

For 'Pink Panda' genotype, the selected primers generated a total number of 54 bands, also monomorphic, and the number of bands per primer varied between 2 (OPB17) and 8 (OPA20 and OPC08), with an average value of 5.4 bands per primer. The size of DNA amplified fragments varied in the same interval as in case of 'Serenata' genotype.

Analysis of the gels from experiments organized for assessing the genetic stability of plants multiplicated in succesive subcultures, revealed identical patterns of bands between the control plant and shoots regenerated after the fourth subculture, the percentage of monomorphism per genotype being of 100%. As shown in Figure 22, the highest number of bands was obtained with primers OPA08 (10 bands), OPA20 and OPB05 (8 bands), the other primers being less efficient in generating succesive PCR products.

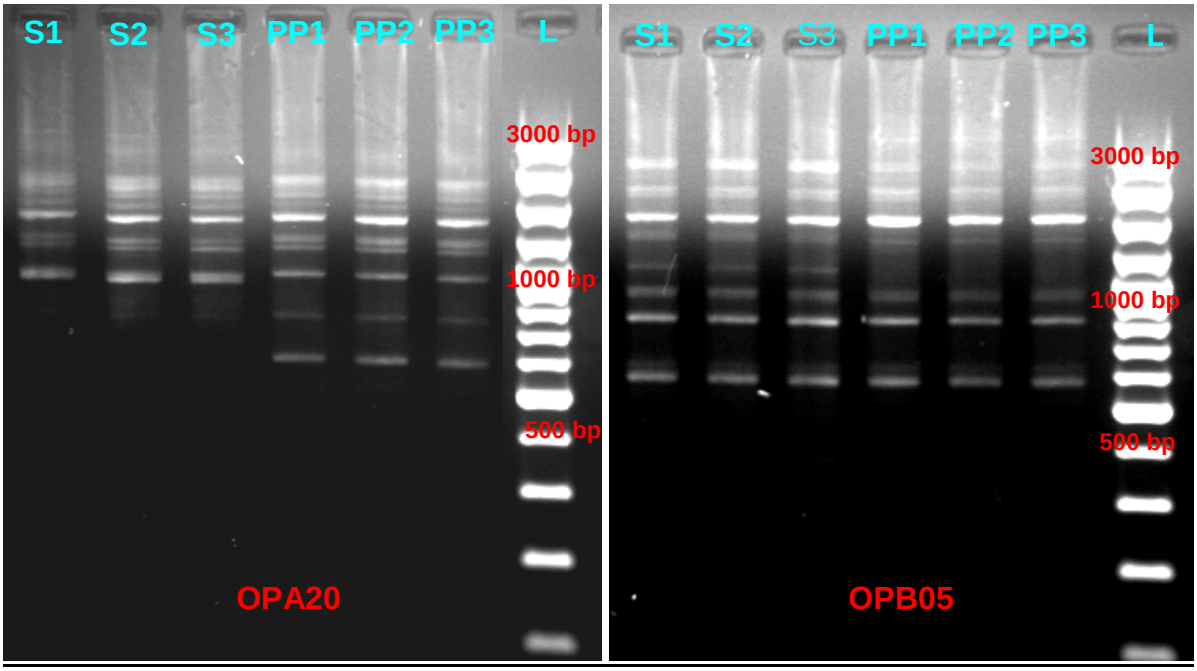


Figure 22. RAPD profile of 'Serenata' and 'Pink Panda' genotypes (control plants and microshoots obtained after the fourth subculture) with the primers OPA20 and OPB05; L= marker DNA, 100 pb.

Taking into consideration that, the most genetic variation induced by *in vitro* cell and tissue culture occurs during the callus phase, it was necessary to assess, at the molecular level, the genetic stability of plantlets regenerated *de novo* from somatic explants, inoculated on either agarized or liquid culture media. The analysis of RAPD profiles, obtained by amplification of the DNA probes with the same 10 primere revealed the absence of any genetic differences between the control plants and the corresponding regenerants. The amplification products obtained with primers OPC05 and OPC08 in the somaclones regenerated via calus, on liquid culture media provided with filter paper bridges, are presented in Figure 23.

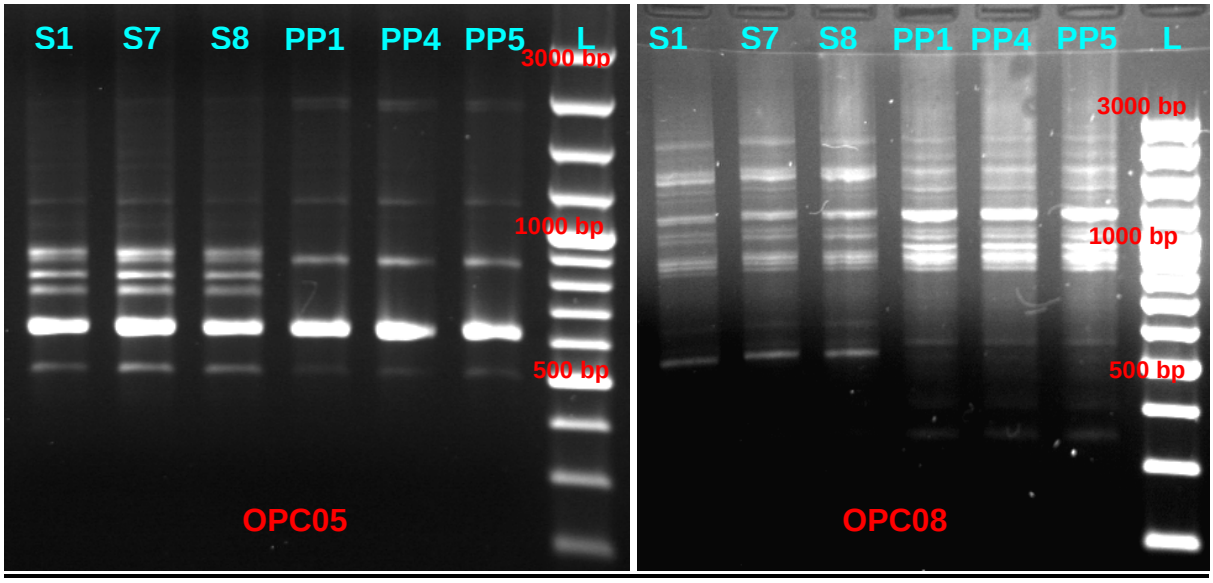


Figure 23. RAPD profile of 'Serenata' and 'Pink Panda' genotypes (control plant and somaclones regenerated from tissue explants cultivated on liquid media) with primers OPC05 and OPC08; L= marker DNA, 100 pb.

Concerning the genetic polymorphism between the two *Fragaria x Potentilla* intergeneric hybrids, from the 69 bands generated by the selected primers, 25 bands were polymorphic. The number of polymorphic bands varied between 1 (OPC05) and 4 (OPC06 and OPC08), with an average value of 2.5 bands per primer (Table 8). The value of Jaccard similarity coefficient (Jaccard index) was 0.63, indicating the fact that, although they have similar phenotypic characteristics, the 'Pink Panda' and 'Serenata' intergeneric hybrids are genetically distant related.

Taking into consideration that in generation of somaclonal variation have been frequently noticed the influence of genotype, based on the demonstrated genetic stability of plantlets regenerated by axillary shoot formation, and especially of those regenerated *de novo* from leaf and petiole explants, we can consider that the 'Serenata' and 'Pink Panda' genotypes of ornamental strawberry are stable under conditions of *in vitro* culture.

Table 8.

The level of genetic polymorphism between the ‘Pink Panda’ and ‘Serenata’ ornamental strawberry varieties

Primer	Sequence (5’ – 3’)	Total number of bands	Number of polymorphic bands	Percentage of polymorphic bands (%)
OPA02	TGCCGAGCTG	6	2	33.3
OPA07	GAAACGGGTG	7	3	42.8
OPA20	GTTGCGATCC	8	2	25
OPB05	TGCGCCCTT	8	2	25
OPB10	CTGCTGGGAC	7	2	28.5
OPB17	AGGGAACGA	4	2	50
OPC05	GATGACCGCC	4	1	25
OPC06	GAACGGACTC	7	4	57.1
OPC08	TGGACCGGTG	11	4	36.3
OPC10	TGTCTGGGT	7	3	42.8

3. GENERAL CONCLUSIONS

The researches carried out of us within the Laboratory for Tissue Culture, at the Research and Development Institute for Fruit Growing Pitesti, the Laboratory for Molecular Biology, at the National Research and Development Institute for Biotechnology in Horticulture, and Laboratory of Genetics, at the Faculty of Science, University of Pitesti, aiming at the assessment of *in vitro* multiplication capacity of two *Fragaria* × *Potentilla* intergeneric hybrids, and respectively the assessment of genetic stability of the micropropagated plants, led to the following conclusions:

1. The capacity of *in vitro* micropropagation of *Fragaria* × *Potentilla* intergeneric hybrids is strongly dependent by genotype. Concerning the multiplication by axillary shoot formation, the 'Serenata' genotype showed a regeneration potential 2.17 times higher in comparison to the 'Pink Panda' genotype of ornamental strawberry. The genotype with best response to the in vitro culture of somatic tissue explants was also 'Serenata', whose potential for shoot regeneration from somatic tissue-derived calli was on average 3.65 higher as compared with 'Pink Panda'. On the other hand, in 'Pink Panda' variety, the rate of *in vitro* rooting of the microshoots was 2.22 times higher as compared to 'Serenata'. The superior rhyzogenesis potential of 'Pink Panda' genotype was clearly shown by the positive correlation, statistically significant, found between the rooting rate, average number of roots and their average length.

2. Supplementation of the culture medium with IBA and BAP, as well as with GA_3 - in the case of micropropagation by axillary shoot formation, led always during the experiments to the highest rates of multiplication, respectively to the highest frequencies of adventitious shoot regeneration. The use of liquid culture media provided with filter paper bridges allowed the establishment of fact that the different genotypes of ornamental strawberry have a different response to the *in vitro* culture of somatic tissue explants, and also indicated that the IBA auxin must be present in a high concentration in the culture medium.

3. The results of researches carried out by us have conclusively revealed that by changing the *in vitro* culture conditions can be controlled the pattern of shoot regeneration from the somatic tissue explants. Thus, by replacing the BAP cytokinin with the TDZ cytokinin-like compound, are avoided conditions which favorize callus formation, thus enabling shoot regeneration directly from explant and assuring the way of micropropagating plants which should be genetically identical and, implicitly, phenotypically uniform.

4. Dark pretreatment of the somatic tissue explants and their maintenance under conditions of low light intensity represents a critical factor for inducing shoot regeneration in ‘Pink Panda’ genotype and in the same time for obtaining a higher frequency of adventitious shoot regeneration, in both the investigated ornamental strawberry genotypes.

5. Genetic analyses aided by RAPD markers, revealed the genetic uniformity of the plantlets micropropagated clonally by axillary shoot formation, and also of the shoots regenerated from somatic tissue-derived calus, either onto agarized culture media, or onto filter paper bridges at the surface of liquid culture media.

6. Based on the overall results obtained following the researches which represented the scientific support of this PhD thesis, have been elaborated two working protocols for the *in vitro* micropropagation of ‘Serenata’ and ‘Pink Panda’ genotypes of ornamental strawberry.

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