THE EFFECT OF THE *IN VITRO* ENVIRONMENTAL CONDITION ON ORGANOGENETIC POTENTIAL OF TWO INTERGENERIC HYBRIDS *Fragaria X Potentilla*

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Abstract. Using liquid culture medium provided with filter-paper bridges, a simple plant regeneration system via organogenesis from leaf and petiole explants of two intergeneric hybrids *Fragaria x Potentilla*, named 'Pink Panda' and 'Serenata', has been developed. The regeneration capacity of the explants was influenced by the light condition and plant growth regulators concentration. Dark incubation during the first 6 weeks, followed by the transfer of the cultures under a photoperiod of 16 hours light/8 hours darkness, at a relatively low intensity of light was the most effective condition to successfully induce shoots regeneration in both intergeneric hybrids. The pretreatment darkness of 21 days, followed by the cultures transferring under a photoperiod of 16 hours light/8 hours darkness and a light intensity of about 40 µmol m^{-2} s^{-1} has been associated with an incapacity of calluses to form adventitious buds, regardless of the explant type or hormonal balance. Optimal shoot regeneration was obtained with BAP in a concentration of 3.0 mg/l, in combination with 1.0 mg/l IBA, added to modified Murashige-Skoog medium. Shoot regeneration frequency were as high as 54.66% in 'Serenata' genotype and 43.33% in 'Pink Panda' genotype.

Keywords: ornamental strawberry, liquid culture, callus, organogenesis, shoot regeneration

INTRODUCTION

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 [13], 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 [14], 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 [11], 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 [28]. 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 [26], 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.

Although there have been numerous reports on adventitious bud and shoot regeneration from somatic explants [6 - 8, 16, 21, 27, 29, 30, 32] cultured on agar-gelled media, liquid culture has been recently considered as an alternative approach to strawberry micropropagation. Some modern techniques including shaken of liquid culture and bioreactor [10, 15, 34, 35], or liquid culture system combined with some kinds of substrate made of simple material [9] have been successfully applied for strawberry micropropagation. In shake culture and bioreactor where the problem of explant asphyxiation exists, it can be avoided by taking the advantage of the surface tension and floating explants [8] or supporting of explants on different kinds of absorbent substrate. The use of filter-paper bridges for liquid culture media have been proved to be an extremely efficient method for *in vitro* culture of different kind of explants from different plant species [4, 5].

In this study, the reactivity of leaf and petiole explants of 'Pink Panda' and 'Serenata' genotypes supported on the surface of the liquid culture through filter paper bridges was investigated, as method which can reduce strawberry micropropagation cost.

MATERIAL AND METHODS

Plant material. 'Pink Panda' and 'Serenata' genotypes from the *Fragaria* Germplasm Collection of the Institute for Fruit Growing, Pitesti, Mărăcineni, Romania were cultured *in vitro* starting from meristems on Lee & Fossard [20] basal medium, supplemented with 1.0 mg/l kinetin (Kin) and 2.70 mg/l indolyl-acetic acid (AIA). Leaf and petiole explants collected from six weeks old *in vitro* plantlets, precultured for two weeks on LF basal medium containing 0.5 mg/l BAP, 0.5 mg/l IBA and 0.2 mg/l GA_3 [32], were used as explants.

Callus induction experiments. Callus induction media consisted of Murashige & Skoog [24] basal medium modified ($CaCl_2$ being introduced at a concentration of 330.0 mg/l, instead of 440.0 mg/l as in the original recipe) and supplemented with three different combinations of plant growth regulators, as following: 0,5 mg/l AIB + 3,0 mg/l BAP as MS1 variant, 1,0 mg/l AIB + 3,0 mg/l BAP as MS2 variant and 1,0 mg/l AIB + 5,0 mg/l BAP as MS3 variant. Dextrose, at a concentration of 40 g/l, was used as carbon source in all culture media.

Both the leaf explants (0.3-0.5 mm diameter) and petiole segments (0.3-0.5 mm) were supported on the surface of the liquid medium on filter paper bridges. Modelling of the filter paper bridges and applying this method have been completed according to the methodology proposed by Blidar [3]. The liquid culture medium was added to 25 cc Pyrex tubes after introduction of filter paper bridges, such containers being sterilized by autoclaving. Into the each tube on filter paper bridge was placed one somatic explant. The leaf explants were placed with their abaxial surface in contact with the filter paper bridges and petiole segments were placed horizontally on the filter paper bridges and nutrition of the somatic explants was done through a wick system.

Thus, using liquid culture medium, provided with filter paper bridge, we initiated a comparative study, as follows:

- on the one hand, after 21 days of incubation in the dark, a number of experimental variants were maintained under a photoperiod of 16 hours light/8 hours darkness and a light intensity of about 40 µmol m^{-2} s^{-1};
- on the other hand, after 42 days of incubation in the dark, a similar number of experimental variants were maintained under a photoperiod of 16 hours light/8 hours darkness and at a relatively low intensity of light (starting from a light intensity of about 40 µmol m^{-2} s^{-1}), obtained by covering the culture flasks with large sheets of white paper.

In both experiments, the cultures were incubated in the growth chamber at the temperature of 22-24°C. The number of explants forming callus was scored after 21 days and 42 days of culture for both varieties.

Shoot organogenesis. Callus cultures initiated from the leaf- and petiole-derived calli were maintained on the same media without refreshing them, until the shoots induction. The number of shoots formed per callus was determined after 70 days in culture.

Experimental design and statistical analysis. The rate of callogenesis (%) was determined as the ratio of the number of explants that developed callus to the total number of explants. Similarly, the frequency of shoots regeneration was determined as the ratio of the number of calli that regenerated shoots. To avoid major statistical errors, all of the experimental treatments were performed with twenty-five replicates of one somatic explant. Statistical analysis of the data obtained with 'Pink Panda' and 'Serenata' varieties respectively, on basal media containing different concentrations of auxins for callus induction and shoot regeneration, were performed using Duncan's Multiple Range test, at $p < 0.05$, working with Statistical Package for the Social Science (SPSS) statistical software (version 16.0).

RESULTS

The callogenic response was induced after 12 days of *in vitro* culture, in response to IBA and BAP added in the modified MS basic medium. These calli were formed all over the surface of the somatic explants and were predominantly initiated at the wound sites of the petiole segments and leaf fragments. In this respect, it is known that plant hormones are present in higher quantities after wounding and are involved in cell proliferation at the wound site [18].

After transferring cultures, maintained for 21 days in the dark at a light intensity of about 40 µmol m^{-2} s^{-1}, under a photoperiod of 16 hours light/8 hours darkness, callus proliferation was stagnant, regardless of genotype, explant type or liquid culture media

composition, necrosis processes becoming extensive in a shorter time period of 10 days.

The influence of pretreatment darkness and effect of the culture medium composition in callogenesis. For both intergeneric hybrids, increasing the time period of dark pretreatment had a favorable effect on cell proliferation, number of explants which formed callus after 42 days of incubation in the dark, being significantly higher than that recorded after only 21 days, regardless of the explant type or hormonal balance. Also, after 42 days of incubation in the dark, between the two genotypes of ornamental strawberry there were no significant differences on average percentage of somatic explants which formed callus. The highest rate of callus induction was 100% from leaf explants and petiole explants, in both intergeneric hybrids (Fig. 1), in the modified MS basal medium [24], containing 0.5 mg/l AIB + 3.0 mg/l BAP, indicating a more adequate composition of nutrients to the *in vitro* regeneration requirements of these intergeneric hybrids.

On the other hand, an inhibition of callogenesis occurred for leaf explants on media with an increased BAP concentration (from 3.0 mg/l to 5.0 mg/l BAP). Thus, the rate of callogenesis was 40% for leaf explants, in 'Pink Panda' intergeneric hybrid, and 38.8% in 'Serenata', respectively. The same concentration of BAP promoted callus formation from the petiole explants on MS medium, with a frequency of 64.2% in 'Pink Panda' and 54.4% in 'Serenata', respectively (Fig. 1).

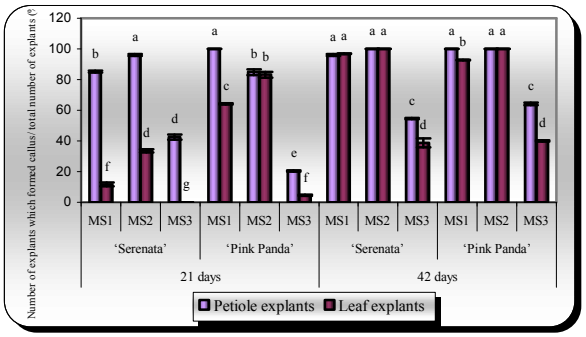

Figure 1. The effects of the pretreatment of darkness on the callogenesis ability of leaf and petiole explants cultured on liquid culture media provided with filter paper bridges (bars represent standard deviation; letters above the columns indicate percentages significantly different from each other, $p<0.05$).

The influence of pretreatment darkness in shoot organogenesis. For both intergeneric hybrids, 'Serenata' and 'Pink Panda', respectively, the pretreatment darkness of 21 days, followed by the cultures transferring under a photoperiod of 16 hours light/8 hours darkness and a light intensity of about 40 $\mu mol\ m^{-2}\ s^{-1}$ has been associated with an incapacity of calluses to form adventitious buds, regardless of the explant type or hormonal balance.

In contrast, in the ornamental strawberry cv. 'Serenata', maintaining cultures of somatic tissues in darkness for a period of 42 days, allowed the occurrence of adventitious buds, first observed after 27 days from petiole explants inoculation and after 30 days from leaf explants inoculation, respectively. In ornamental strawberry 'Pink Panda', adventitious buds were first formed from leaf explants, in the absence of light, after 38 days from initiation of cultures and after 50 days from petiole explants inoculation. In fact, these were the conditions which allowed adventitious shoot regeneration from petiole explants in the presumably recalcitrant 'Pink Panda' genotype [33], in a relatively short period, without dividing the calli and transferring them on fresh culture media (Fig. 2).

Frequency regeneration of shoots increased gradually, so that after 42 days in the dark, process of *in vitro* morphogenesis was reported in 30.64% of calli derived from leaf explants in 'Serenata', and only 4.12% in 'Pink Panda'. Also, formation of adventitious buds was observed only in 14.85% of calli derived from petiole explants in 'Serenata', and in 'Pink Panda' has not been observed formation of adventitious buds and shoots (Table 1).

At the same time, the favourable effect of a relatively lower intensity of light, as a result of covering the culture flasks with large sheets of white paper, has been confirmed after seven days only, by increasing cell proliferation and forming of morphogenetic callus from apparently necrotic callus (Fig. 3). In this context, it is important to note that proliferative processes of parenchimatous cells and adventitious buds formation occurred mainly at the contact surface of the explants with filter paper bridges.

The transfer of the *in vitro* cultures of somatic tissue explants to low intensity light was associated with the stimulation of anthocyanins secretion in the parenchimatous cells from the surface of the callus, regardless of its origin in petiole segments, or leaf

Table 1. Regeneration frequency of shoots via callus after 42 days of dark incubation

Basal medium	Hormonal balances (mg/l)	Regeneration frequency of shoots (%)			
		'Serenata'		'Pink Panda'	
		Leaf explants	Petiole explants	Leaf explants	Petiole explants
Murashige-Skoog [24]	0.5 mg/l AIB + 3.0 mg/l BAP (MS1)	12.2 ± 0,62	6.17 ± 0,09	4.12 ± 0.6	0
	1.0 mg/l AIB + 3.0 mg/l BAP (MS2)	18.44 ± 0,11	8.68 ± 0,4	0	0
	1.0 mg/l AIB + 5.0 mg/l BAP (MS3)	0	0	0	0

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

fragments. Moreover, the red pigmentation, characteristic for anthocyanins, was observed preponderently in the parenchimatous cells from the calli with high regeneration capacity (Fig. 4).

Effect of the culture medium composition in shoot organogenesis. After 70 days from the initiation of *in vitro* cultures on liquid nutritive media, provided with filter paper bridges, the frequency of caullogenesis became stable in those experimental variants characterized by incubation of cultures for 42 days in the dark, followed by the transfer of the culture under a photoperiod of 16 hours light/8 hours darkness and at a relatively low intensity of light.

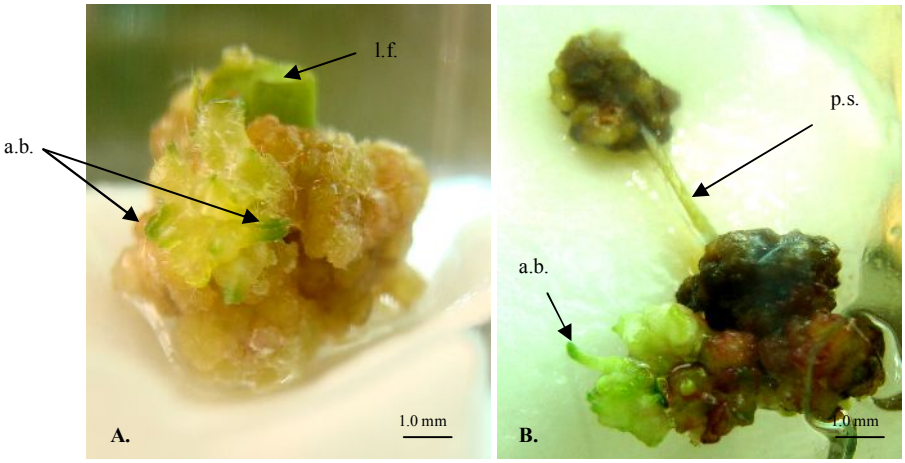

Figure 2. Organogenesis in the callus obtained by the *in vitro* culture of somatic tissue explants on liquid culture media, provided with filter paper bridges, in the 'Pink Panda' genotype. A – shoot regeneration from leaf-derived callus; B – shoot regeneration from petiole-derived callus (l.f. – leaf fragment; p.s. - petiole segment; a.b. – adventitious buds)

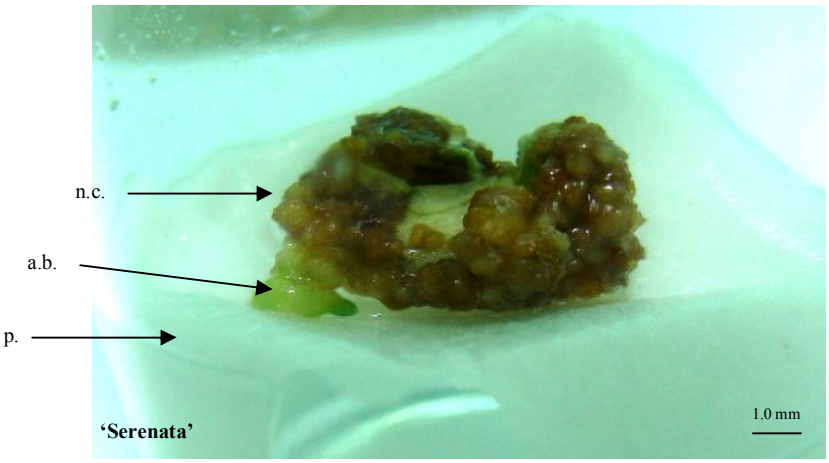

Figure 3. Formation of morphogenetic callus from apparently necrotic callus, derived from leaf explant (n.c. – necrotic callus; a.b.- adventitious buds; p. – paper filter bridge)

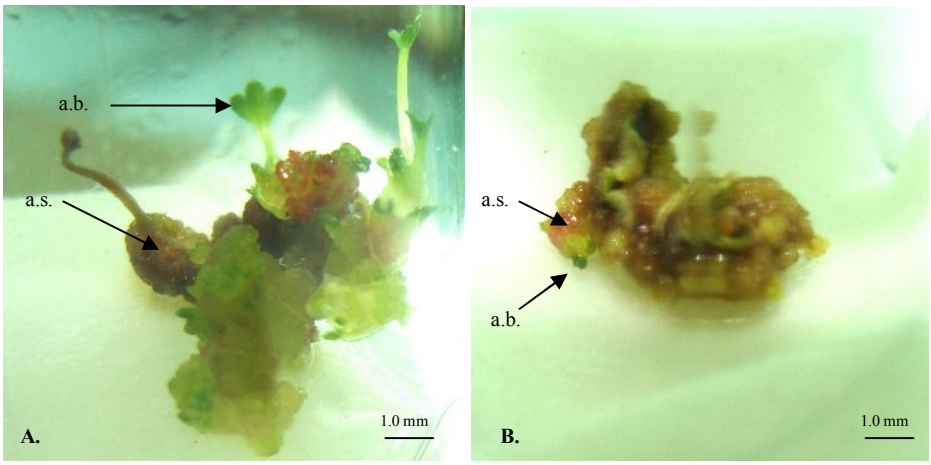

Figure 4. Anthocyanins secretion in the parenchimatous cells from the surface of the callus, derived from petiole explant (A) and leaf explant (B) cultivated on liquid culture media provided with filter paper bridges, in the 'Serenata' genotype (a.b. – adventitious bud; a.s. – anthocyanins secretion)

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

Suplementation of the culture medium with BAP in a concentration of 3.0 mg/l, in combination with 1.0 mg/l IBA (MS2) allowed obtention of highest percentage of calli which regenerated shoots in 'Serenata' genotype, the average rate of adventitious shoots regeneration being as high as 54.66 % calli which regenerated shoots. The same combination of growth regulators, led to the highest rate of shoot regeneration in 'Pink Panda' genotype, equal to 43.33% calli which regenerated adventitious shoots.

As compared with frequency of organogenesis obtained in MS2 variant of culture medium, the average percentage of explants from which were regenerated adventitious buds and shoots in MS3 variant of culture medium was of only 4.05% in 'Serenata' genotype and 2.98% in 'Pink Panda' genotype.

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.

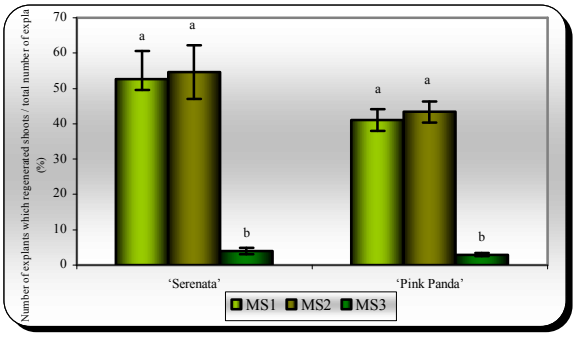

Figure 5. Influence of combination and concentration of growth regulators on the frequency of *de novo* plantlets from calli maintained on liquid culture media provided with filter paper bridges (the bars represents standard deviation; a, b, c, d: significance of differences, as revealed by Duncan test, $p<0.05$).

DISCUSSION

Considering the results of these experiments, it is obviously that dark pretreatment of the somatic tissue explants and their maintenance under conditions of low light intensity represents critical factors for inducing shoot regeneration in 'Pink Panda' and 'Serenata' genotypes and in the same time for obtaining a higher frequency of adventitious shoot regeneration. Although the positive influence of the dark pretreatment on the expression of callus regenerative potential has been demonstrated in a large number of species, the results reported for strawberry genotypes were contradictory, which often led to assert that such pretreatment is not absolutely necessary.

However, callus formation and plantlets regeneration is a process that is carried out differently in diferent species and cultivars as working protocol for regeneration differs from species to species and cultivar to cultivar [2, 12]. In previous studies on regeneration potential of strawberry a initial dark pretreatment for four weeks has been followed by the transfer of the explants to light, on fresh culture media [2, 27]. According to Nehra (1989) a dark pretreatment immediately following the explant culturing is necessary, because light degrades critical media components wich are necessary for plantlet development.

Moreover, Barceló *et al.* [2], reported no significantly differences between the treatment groups receiving various light irradiation level, but the cultures receiving no light had significantly fewer regenerated plantlets. We found that, for intergeneric hybrids 'Pink Panda' and 'Serenata', light intensity of about 40 µmol m^{-2} s^{-1} had unfavorable influence on the regeneration process and a lower intensity of light being necessary for shoots regeneration. Similar results have been obtained in other species, such as *Spinacea oleracea* L. [1], *Capsicum annum* L., *Cucurbita pepo* L., *Cucumis melo* L., *Gardenia jasminoides* L., *Rosa hybrida* L. [19], *Saccharum officinarium* [31], etc.

In terms of culture medium composition, the precise concentration of growth regulators is critical in producing the desired response. Hormonal balance consisting of 1.0 mg/l IBA + 3.0 mg/l BAP (MS2) 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 in strawberry.

Hormonal balance consisting of 1.0 mg/l IBA + 5.0 mg/l BAP (MS3) had an inhibitory action on the regenerative processe in ornamental strawberry cvs. 'Pink Panda' and 'Serenata', respectively. Adventitious shoots regeneration frequency significantly lower for this variant, compared to the other two variants of culture medium (MS1 and MS2, respectively), can be attributed to higher concentration of cytokinin in the culture medium.

IBA is an auxin of high efficiency in inducing the proliferation process and callus formation from somatic tissue explants in 'Pink Panda' genotype. The importance of the auxin type used for *in vitro* regeneration of adventitious shoots was reported by Nehra et al. (1989) [25] and Barceló et al. (1998) [2], which suggested that in the regeneration of adventitious shoots regeneration in different cultivars of strawberry 2,4-dichlorophenoxyacetic acid (2,4-D) and benzo-selenyl acetic acid (BSAA) are highly dependent of the genotype in promoting regeneration of shoots, especially under a photoperiod of 16 hours light/8 hours darkness.

Based on these results it can be concluded that in both the investigated ornamental strawberry genotypes 'Pink Panda' and 'Serenata', respectively, dark pretreatment of the somatic tissue explants and their maintenance under conditions of low light intensity are mandatory. Supplementation of the liquid culture medium with 1.0 mg/l IBA + 3.0 mg/l BAP, led to the highest rates of callusogenesis, respectively to the highest frequencies of adventitious shoot regeneration.

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