STUDIES ON THE MAJOR FACTORS AFFECTING *IN VITRO* MICROPROPAGATION OF TWO INTERGENERIC HYBRIDS *FRAGARIA* × *POTENTILLA*

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Abstract
In order to establish the major factors affecting in vitro micropropagation of intergeneric hybrids Fragaria × Potentilla, respectively 'Pink Panda' and 'Serenata', basic culture media Murashige-Skoog (MS), Lee-Fossard (LF) and Knop, were supplemented with 6-benzylaminopurine (BAP), kinetine (Kin), indole-3-acetic acid (AIA), indole-3-butyric acid (AIB) and gibberellic acid (GA_3), in different combination and concentration. In ornamental strawberry 'Serenata', which showed a genetic potential of shoot regeneration significantly higher compared with 'Pink Panda', a high multiplication rate associated with a high vigor of shoots was obtained on MS medium supplemented with 0.5 mg/l BAP + 0.1 mg/l AIB + 0.1 mg/l GA_3. The same combination of growth regulators, added in MS medium in higher concentrations, namely 1.0 mg/l BAP + 0.2 mg/l AIB + 0.1 mg/l GA_3 led to the highest rate of multiplication in 'Pink Panda' intergeneric hybrid of Fragaria × Potentilla.

Keywords: *Fragaria* × *Potentilla*, micropropagation, genetic potential, basic culture media, growth regulators.

1. INTRODUCTION

Intergeneric hybrids *Fragaria* × *Potentilla* known as ornamental strawberry, such as 'Pink Panda', 'Serenata', 'Lipstick', 'Red Ruby', 'Rosalyne', 'Rosseberry', 'Gerald Straley', 'Tristan', 'Roman', 'Pikan', 'Pretty in Pink' and 'Whiting', are distinguished by pink - red flowers, while producing edible fruit.
The conventional propagation of these varieties does not allow the obtention of high number of stolons of guaranteed authenticity and biological value in a very short time. Therefore, taking into consideration that they resemble octoploid cultivated strawberries, the *in vitro* micropropagation is the first choice.
Knowing the fact that the efficiency of micropropagation through axillary shooting depends to a great extent of the genetic potential of regeneration, the culture media composition, the type of hormones and their concentrations used for the initiation of shoot cultures and maintenance of subcultures, we initiated a study aiming at the elaboration of an reliable protocol for the high rate *in vitro* propagation of the ornamental strawberry.

2. MATERIAL AND METHODS

Two varieties of ornamental strawberry (*Fragaria x Potentilla*), named 'Pink Panda' and 'Serenata', respectively, were established *in vitro* culture starting from meristems and then subcultured succesively on either Murashige-Skoog (1962), Lee-Fossard (1977) or Knop (1965) media, supplemented with various combinations of growth regulators (Table 1).
For the initiation of shoot cultures, meristems with 2-3 leaf primordia, of 0.1- 0.3 mm in size, excised from runners formed by field grown stock plants were immersed successively in 94˚etilic alcohol for 7 minutes and 6% calcium hypoclorite for 14 minutes, with the aim of disinfection. Runner tips were subsequently rinsed with sterile distilled water. Meristems with 2-3 leaf primordia, of 0.1-0.3 mm in size were cultured on LF basal medium, supplemented with MS vitamins, dextrose at 40 g l^{-1} and agar at 7.0 g l^{-1} concentration. The pH was adjusted to 5.8.
Six treatments with different combinations and concentration of BAP, Kin, IAA, IBA, GA_3, added to MS, LF and Knop basal culture media (Table 1), were used in order to find an adequate medium for obtaining a high rate of micropropagation while maintaining a good vigor of micropropagated

shoots. The concentration of cytokinins in the experimental treatments covered the range currently used with commercial strawberry, thus allowing the establishment of that inducing the best morphogenetic response. To avoid major statistical errors, at least 6 culture flasks with 5 shoots per flask were used as repetitions in each of the experimental treatment investigated.

Table 1. The combinations and concentration of growth regulators added to MS and LF media respectively, tested in order to establish an efficient protocol for the micropropagation of Fragaria x Potentilla varieties.

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

The cultures have been incubated in a growth chamber at the temperature of 22-24°C, with a photoperiod of 16 hours light/8 hours darkness, and a light intensity of about 40 µmol m^{-2} s^{-1}.
The observations were carried out at every 4 weeks, respectively at the moment of subculturing the micropropagated shoots. The micropropagation rate was calculated as the average number of shoots regenerated on each primary explant cultured *in vitro* on each of the media tested.
Statistical analysis of the data obtained with ”Pink Panda” and ”Serenata” varieties respectively on the MS, LF and Knop media supplemented with various combinations of growth regulators were performed using Windows SPSS 16.0 program (SPSS, 2007) at $p < 0.05$.

3. RESULTS AND DISCUSSION

Regardless of the genotype, all apical meristems produced shoots and the appearance of the first shoots was noted in approximately 16 days after initiation of culture. However, the analysis of variance test revealed that ‘Serenata’ and ‘Pink Panda’ genotypes, basal culture media and plant growth regulators interacted significantly, with respect to the mean number of shoots formed per explant.
Effect of genotype. Significant differences on shoot regeneration and multiplication was observed between the two genotypes of ornamental strawberry. Regardless of the basic culture medium or plant growth regulators combination and concentration, the multiplication rate was found to be higher in ‘Serenata’ than in ‘Pink Panda’ ornamental strawberry. In this context, it is significant that the average number of 16.4 shoots formed per primary explant calculated after the first subculture for ‘Serenata’, was approximately two times higher than the multiplication rate calculated for ‘Pink Panda’, equivalent to a 8.46 shoots formed per primary explant.
By transffering the shoots to fresh multiplication media, in the second subculture, genotype ‘Serenata’ also showed the same significantly higher genetic potential for regeneration, leading to a multiplication rate of 17.66 shoots formed per primary explants, compared with intergeneric hybrid ‘Pink Panda’, for which was calculated an average of only 6.6 shoots formed per primary explants.
The correspondence between the genotype and *in vitro* multiplication capacity followed the same pattern after the third and fourth subculture, ‘Serenata’ intergeneric hybrid maintaining the advantage of genotype with high *in vitro* proliferation capacity. The results are consistent with data from literature, genotype influence on the ability of *in vitro* multiplication of strawberry being often reported (Simpson and Bell, 1989; Landi and Mezzetti, 2006; Kikas *et al*., 2006; Debnath and Teixeira da Silva, 2007).

As compared to the 'Pink Panda' (which was calculated an average of 7.49 shoots formed per primary explant), observations on the number of shoots regenerated after four successive subcultures, revealed a potential of 1.96 times higher for intergeneric hybrid 'Serenata' (for which was calculated an average of 14.69 shoots formed per primary explant), as evidenced in Figure 1.

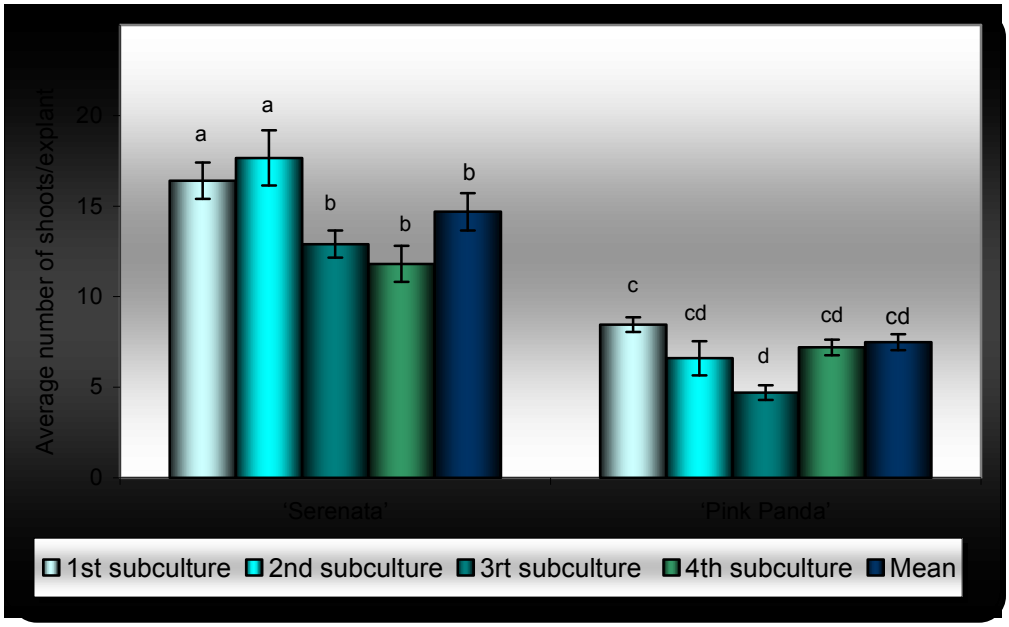

Figure 1. The influence of genotype on the in vitro multiplication capacity through axillary shoot formation in the Fragaria x Potentilla intergeneric hybrids (bars represents the standard deviation; a, b, c, d: assessment of the significance of differences, by Duncan's Multiple Range Test, p<0.05).

Effect of basic culture medium. In order to develop an effective protocol for the *in vitro* micropropagation of intergeneric hybrids *Fragaria* × *Potentilla*, three basal culture media were initially tested, respectivelly MS, LF and Knop, supplemented with the same combination and concentration of growth regulators (Table 1).

The regeneration capacity of intergeneric hybrids 'Pink Panda' and 'Serenata' was negatively influenced by the basal culture medium Knop, regardless of the combination and concentration of growth regulators. Thus, low multiplication rate (2-5 shoots formed per primary explant in 'Pink Panda' and 4-8 shoots formed per primary explant in 'Serenata', respectively), was associated in each subculture with a reduced vigor of shoots and a premature drying of a significant percentage of shoots for 'Pink Panda' hybrid, which therefore could not be subjected to further studies (Fig. 2). Based on these findings, in the next studies were used only MS and LF basal media to determine the ability of multiplication of shoots.

The statistical analysis have not revealed significant differences between the mean of micropropagation rate obtained on MS medium and LF medium, respectively (Fig. 3). Thus, in 'Serenata' intergeneric hybrid, the mean number of 15.74 of shoots formed per primary explant calculated for micropropagation experiments on MS medium was close to the mean number of 13.64 of shoots formed per primary explant calculated for experiments on LF medium, regardless of the combination and concentration of growth regulators. As compared to the 'Serenata', in 'Pink Panda' intergeneric hybrid, the difference between the average rate of micropropagation obtained on MS medium (7.36 shoots formed per primary explant) and LF medium (6.6 shoots formed per primary explant), was only 1.11 shoots formed through axillary shoot proliferation, suggesting the decisive influence of genotype × basic culture medium interaction on multiplication (Fig. 3).

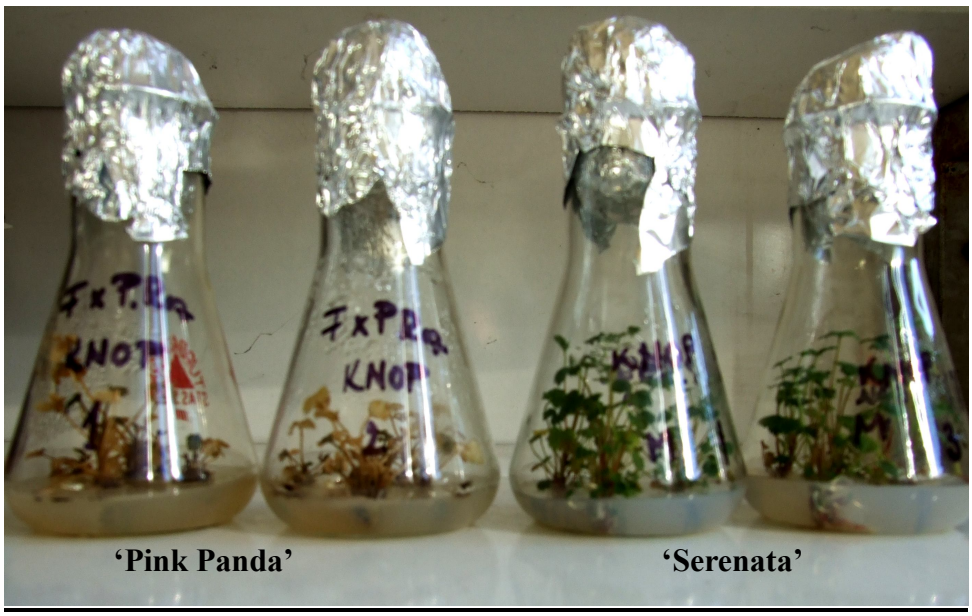

Figure 2. The influence of the basic culture medium Knop on in vitro multiplication capacity through axillary shoot formation in the Fragaria x Potentilla intergeneric hybrids (the second subculture).

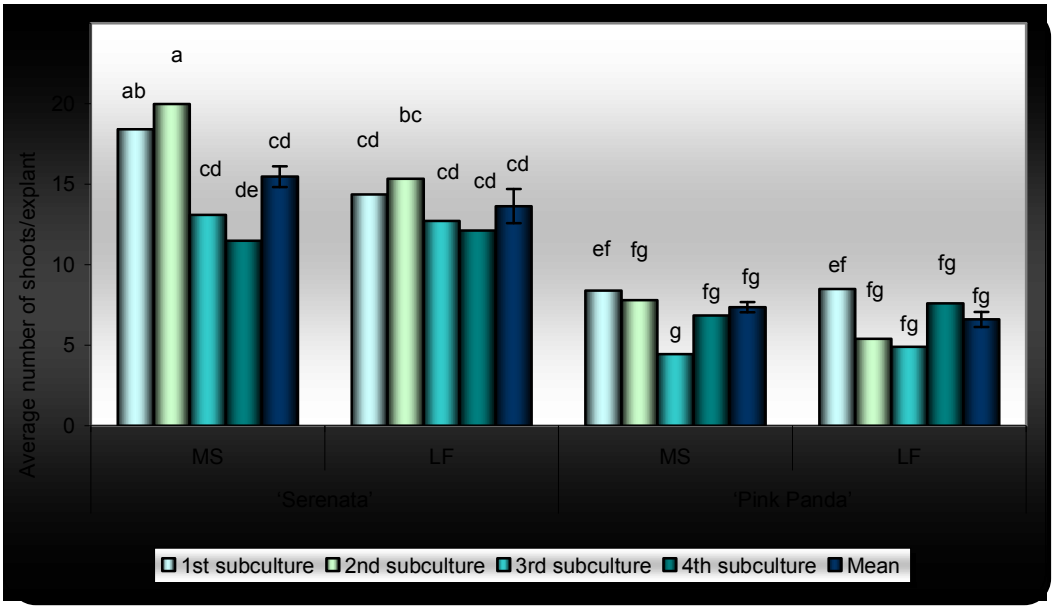

Figure 3. The influence of basic culture medium on the in vitro multiplication capacity through axillary shoot formation in the Fragaria x Potentilla intergeneric hybrids (bars represents the standard deviation; a, b, c, d, e, f, g: assessment of the significance of differences, by Duncan's Multiple Range Test, $p<0.05$).

In the absence of significant differences in the values of the multiplication rate, which could be attributed to the influence of the basic culture medium, higher vigor of shoots regenerated on the experimental variants carried out by using MS medium, indicates that the MS basal culture medium had a favorable mineral composition for the *in vitro* micropropagation of intergeneric hybrids *Fragaria* × *Potentilla*.

This finding was also supported by the observation that in both genotypes, the highest values of the multiplication rate was attributable to MS basal medium (Fig. 2). It was noted that, although the two intergeneric hybrids have different origin, the basic interaction with the environment was similar.

Effect of combination and concentration of growth regulators. Separation of shoots from clusters and their transfer onto fresh culture medium, maintaining the correspondence of experimental variants, showed that specific combination of growth regulators must be designed for each genotype. Although during the four successive subcultures of axillary shoots, a wide variability in the reactivity of the intergeneric hybrids according to the composition of the basic culture medium was observed, statistical analysis revealed significant differences between the average of micropropagation rate corresponding for each experimental variant. Thus, in 'Serenata' intergeneric hybrid, the average number of 15.93 shoots formed per primary explant in MM1 experimental variant, significantly different of all the values calculated for the other variants, clearly showed that the combination 0.5 mg/l BAP + 0.1 mg/l AIB + 0.1 mg/l AG_3 has a favorable influence on shoot proliferation and multiplication (Fig. 4). Also, the lower *in vitro* regeneration potential of 'Pink Panda' intergeneric hybrid is underscored by the higher values obtained in experimental variants with higher concentration of growth regulators, respectively MM2 (5.88 shoots formed per primary explant), MM4 (5.72 shoots formed per primary explant) and MM6 (5.73 shoots formed per primary explant).

Similarly, several other authors suggested 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} IAA + 0.05 mg l^{-1} GA_3; 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} IBA + 0.1 mg l^{-1} GA_3 (Boxus, 1999; Litwińczuk, 2004); 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} IBA (Bozena, 2001) and 0.2 mg l^{-1}BAP +0.01 mg l^{-1} IBA + 0.1 mg l^{-1} GA_3 (Li *et al*., 2009) combinations of growth regulators added to the basal culture medium as most appropriate for strawberry micropropagation.

4. CONCLUSIONS

1. Regardless of the basal culture medium or combination of growth regulators the micropropagation rate of intergeneric hybrid 'Serenata' was significantly higher, compared to 'Pink Panda', reflecting its superior genetic potential of multiplication *in vitro*.

2. The basal culture medium has a strong influence on the number of shoots obtained from micropropagation through axillary branching. In both 'Pink Panda' and 'Serenata' intergeneric hybrids *Fragaria* × *Potentilla*, the average number of shoots formed per primary explant was generally higher when the explants were subcultivated on the MS medium, rather than on LF medium (currently used for the micropropagation of the octoploid cultivated strawberry), indicating a more adequate composition of nutrients to the *in vitro* growth requirements of these intergeneric hybrids.

3. The combination and concentration of growth regulators has a marked influence on the ability of shoot regeneration. In ornamental strawberry 'Serenata', supplementing culture medium with 0.5 mg/l BAP + 0.1 mg/l AIB + 0.1 mg/l GA_3 resulted in the highest multiplication rate (15.93 shoots formed per primary explant). The same combination of growth regulators, but in higher concentrations, namely 1.0 mg/l BAP + 0.2 mg/l AIB + 0.1 mg/l GA_3 led to the highest rate of multiplication in ornamental strawberry 'Pink Panda'.

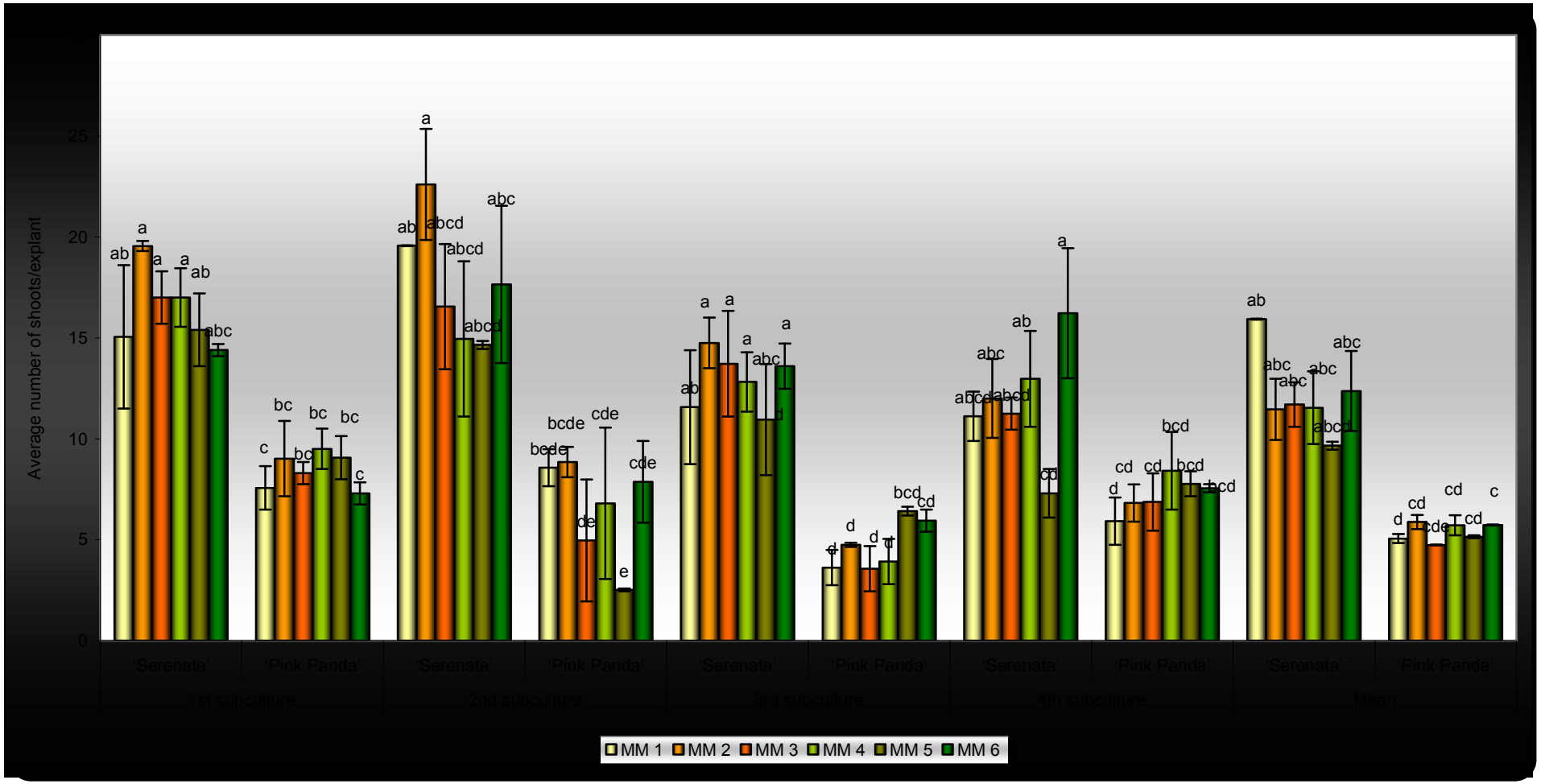

Figure 4. The influence of combination and concentration of growth regulators on the in vitro multiplication capacity through axillary shoot formation in the Fragaria x Potentilla intergeneric hybrids (bars represents the standard deviation; a, b, c, d, e: assessment of the significance of differences, by Duncan's Multiple Range Test, p<0.05).

P<0,05).

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