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The micropropagation of the strawberry cultivar 'Idea'

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Abstract

This work presents the production technology of the strawberry cultivar 'Idea' by micropropagation. Meristems of 0.5mm size were isolated in June from the stolons of examined strawberry sorts. They were raised on Murashige and Skoog's (Murashige and Skoog, 1962) medium in presence of 6-Benzylaminopurine (BAP), Indole-3-butyric acid (IBA) and gibberellic acid (GA_3). Fifty days later meristems were organized into a foliate rosette. Multiplication was achieved on Murashige and Skoog's substratum with BAP and IBA. The plants which came to the size of about 10mm were shifted to the substratum for tree rooting. Tree rooting was achieved on Murashige and Skoog's substratum with IBA. Sixty days later the plants with strongly developed roots were transplanted into the peaty briquettes. One month later the plants were transplanted into flowerpots and kept in a greenhouse at a temperature of 10-15^{0}C.

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Introduction

The district of Jablanica is known for the volume production of the berry fruits. The most represented berry fruits are strawberry, raspberry and blackberry.

If during the growing of commercial strawberry plants, the runner plants from the production plantings are used, diseases and pets are easily transmitted, contributing mostly to the low yield and poor quality fruit. Therefore, the raise of modern plantation of strawberries gives the emphasis on the use of varietal pure, quality, healthy and virus-free planting material.

Since the various fungal, bacterial, virus and other diseases are transmitted using conventional methods of multiplication, and this leads to a yield reduction, the production of healthy planting material is imposed as a special problem.

The modern production of strawberry runner plants consists of the production of selected and virus-free strawberry runner plants. Strawberries can be virus-free *in vitro* growth conditions by using micropropagation method. There are three basic methods of plant regeneration *in vitro*: micropropagation through axillary bud formation, organogenesis through the formation of adventitious buds and somatic embryogenesis through the formation of somatic embryos (Murashige, 1974). Micropropagation methods are based on the ability of plants to regenerate from single cells, tissues or organs into a new plant. This multiplication is provided only under laboratory conditions.

Micropropagation is performed on a special medium, which contains a larger number of nutrients, especially minerals, sucrose and growth stimulators. The influence of hormones on the plant growth and cell division, and their development into an organized structure, cannot be inferred on the basis of pedigree. Thus, for example, a certain hormonal treatment for the induction, division and the growth of plant cells applied to one sort of strawberries did not show itself as efficient when compared to another sort. A lot of unsolved issues mainly regard the specific features of a certain sort. Experiments with this promising method of reproduction are still ongoing around the world and are already used in practice.

Micropropagation methods can be applied for obtaining healthy planting materials of strawberry. The aim of this research was to determine the production technology of the strawberry cultivar 'Idea' using the method of micropropagation.

Material and methods

Isolation of initial explants

During our three-year research the starting material for examination of the sort 'Idea' was taken from the mother plantation of strawberries in the social company ''Porečje Vučje''. Preparation of the starting material and Murashige and Skoog's medium was performed in the biological laboratory ''Zdravlje - Actavis'' Leskovac, whereas the isolation of plant material was performed in the laminar microbiological laboratory of the mentioned company. The impacts of different phytohormones *in vitro* growth conditions were also carried out in these laboratories.

Sterilization of nutrient medium

The nutrient medium is prepared with double-distilled water (ddH_2O) and after that it is placed into an Erlenmeyer flask or a test tube with cotton wool. Thus prepared medium is sterilized in autoclave at 120^{0}C for 20 minutes.

Conditions of growing the cultures

The explants were kept at ''Zdravlje - Actavis'' Leskovac in controlled conditions and at a temperature 20 -25 ^{0}C, with light intensity of 2000 – 2500 lx and the length of brightness of 16 hours per day and 8 hours per night. Adjustment to environmental conditions was carried out in the greenhouses of the company under the ''mist'' system. After adjustment, the plants were planted on the plots of ''Porečje Vučje'' and one private producer in the village of Strojkovce for further research.

Results and discussion

Factors that determine induction and organogenesis

There are direct and indirect ways of organogenesis. When the callus formation is omitted on the primary explants, it is a direct way of organogenesis. And contrary, when callus is primarily formed on the primary explants, it is an indirect way of organogenesis. The explants multiplicated *in vitro* do not form callus, if they originate from the apical meristem. It means that this method of multiplication of strawberries manifests direct organogenesis. However, when the concentration of IBA hormones is increased above 1.5 mg/l then it comes to the callus formation, i.e. it comes to the multiplication of strawberries by indirect way of organogenesis (Nikolić, 1996). Based on these data, it is evident that the regeneration of strawberries can be carried out by direct organogenesis.

Stolons of the runner plants of the strawberry sort 'Idea' were used for the isolation of meristems. Plant material was collected by the end of May and the beginning of June from the stolons that were in the air. For the first stage, the initiation of culture, the sizes of the explants, time of obtaining the explants and nutrient medium have an important role. The largest number of explants can be developed in a short period of time when used with the apical buds size of 1-2 mm. In addition, an important role is played by the time of taking the explants, as well as the age of the plants from which explants are taken. Regarding the strawberries, the largest number of meristems develops when the explants are taken during the period from the second half of May and June (Nikolić, 2006). At the apical meristem culture, better results are obtained if the explants are taken from young plants (Ružić, 2004).

The composition of the nutrient medium plays an important role for the organogenesis and regeneration. Among the known nutrient medium Murashige and Skoog's medium is most frequently used. The isolated strawberry meristems were cultured on Murashige and Skoog's mineral solution with GA_3, BAP and IBA at various concentrations (Table 1). In order to identify significant differences, LSD test was performed.

Table 1. The influence of hormone concentrations of initiation of culture of the strawberry cultivar 'Idea'

Hormones mg/l	Number of isolated meristems	Uninfected cultures		Initiated cultures		Success rate
		No.	%	No.	%	%
0.0	30	18	60.00	-	-	-
0.1BAP+0.1IBA+0.1GA_3	30	23	76.67	7	30.43	23.33
0.1BAP	30	20	66.67	2	10.00	6.67
0.5BAP+0.1IBA+0.1GA_3	30	25	83.33	12	48.00	40.00
0.5BAP	30	21	70.00	3	14.29	10.00
1BAP+0.1IBA+0.1GA_3	30	26	86.67	17	65.38	56.67
1BAP	30	22	73.33	4	18.18	13.33

Using the applied treatments optimal hormone balance for the culture of primary explants of strawberry cultivar 'Idea' is 1.00 mg/l BAP, 0.1 mg/l IBA and 0.1 mg/l GA_3 (Table 2). Changes in explants were already observed after five or six days, and after twenty days formed callus and growth are noticed. After 40 days the average number of surviving plants was 36.73% at the optimal concentration of hormones 56.67%. Milosavljević et al. (1999) achieved lower percentage of initiating the cultures of strawberry cultivars 'Marmolada' (20%) and 'Cortina' (50%). The highest results that they recorded were by the cultivar 'Selena' (70%) and the lowest by the cultivar 'Sena' (10%). High concentration of cytokinins, in this case 1 mg/l BAP, has a positive effect on the initiation of culture. The percentage of initiation at this concentration of cytokinins for the cultivar 'Senga Sengana' was 81.25% (Petrović and Jaćimović-

Plavšić, 1990), for the cultivar 'Selena' it was 76.67%, and for the ever-bearing strawberry cultivar 'Hummel' was 63.33% (Nikolić et al., 2004). At the optimal combination of hormones survival rate for the cultivar 'Senga Sengana' was 73.33%, 'Marmolada' - 40% and 'Cortina' - 60% (Nikolić, 2006).

Table 2. The least significant difference of the influence of hormones concentration for the initiation of culture of the strawberry cultivar 'Idea'

Hormone concentration mg/l	x	x-2	x-3	x-4	x-7	x-12
1BAP+0.1IBA+0.1GA_3	17	15**	14**	13**	10**	5**
0.5BAP+0.1IBA+0.1GA_3	12	10**	9**	8**	5**	
0.1BAP+0.1IBA+0.1GA_3	7	5**	4*	4*		
1BAP	4	2	1			
0.5BAP	3	1				
0.1BAP	2					

$LSD_{(0.05)}$ =3.41; $LSD_{(0.01)}$ =4.78; *significant difference at the level 0.05; **significant difference at the level 0.01

Multiplication and elongation of scions

The main aim of this stage was to produce a maximum number of units for reproduction. At this stage, the apical meristem is organized into the leaf rosette. The most abundant and genetically stable method is multiplication by axillary buds (Boxus, 1974)

After 40 days of cultivation in a culture, formed scions size 10-20mm were transferred to the medium of reproduction. Multiplication of isolated buds was achieved at Murashige and Skoog's medium with phytohormones BAP and IBA (Table 3). The highest multiplication index is achieved by using hormones at concentrations of 1 mg/l BAP and 1 mg/l IBA. Without the presence of BAP hormones in growth medium multiplication did not exist, and the omission of hormones IBA has led to a reduction in the multiplication index.

Table 3. The influence of different concentrations of BAP hormones on multiplication of the strawberry cultivar 'Idea'

Hormone combination mg/l	Number of plants/cultures	Number of produced plants/passage	Multiplication index
0.0 BAP+1.0 IBA	20	-	-
0.1 BAP+1.0 IBA	20	31	1.55
0.5 BAP+1.0 IBA	20	48	2.40
1.0 BAP+1.0 IBA	17	96	5.65
1.5 BAP+1.0 IBA	20	105	5.25
2.0 BAP+1.0 IBA	20	101	5.05
1.0 BAP+0.0 IBA	20	98	4.90

Cytokinins are used at the stage of multiplication to overcome the dominance of scions and to increase branching of the lateral buds from the leaf axils. In their researches, Nikolić et al. (2004) reached the lowest index of multiplication with the cultivar 'Marmolada' (5.42), and the highest index was with the cultivar 'Senga Sengana' (8.77). Petrović and Jaćimović-Plavšić (1990) reached the multiplication index 8.67 with the cultivar 'Senga Sengana'. During the examination of multiplication of some strawberry cultivars by the method of meristem culture, Milosavljević et al. (1999) achieved the multiplication index 4.0 for the cultivar 'Sena' and 6.0 for the cultivar 'Careca'.

If the hormone concentration is increased above 1 mg/l BAP, there is a higher percentage of callus formation of 30% (15% for combinations of 1mg/l

BAP, 45% of 1.5 mg/ BAP). In order to obtain plants without mutations, it is recommended to remove this callus. The combination of 1 mg/l BAP is favored, due to the fact that it provides a lower percentage of calluses (Nikolić, 1996).

The significance of the influence of BAP hormones on multiplication was determined by LSD test (Table 4). Between the optimal concentration of hormones and other hormones combinations, there are significant differences at 0.05 and 0.01 levels.

Table 4. The least significant difference of the influence of different concentrations of BAP hormones on multiplication of the strawberry cultivar 'Idea'

Hormone combination mg/l	x	x-1.55	x-2.40	x-5.05	x-5.05	x-5.25
1.0 BAP+1.0 IBA	5.65	4.10 **	3.25**	0.75*	0.60*	0.40*
1.5 BAP+1.0 IBA	5.25	3.70 **	2.85**	0.35	0.20	
2.0 BAP+1.0 IBA	5.05	3.50**	2.65**	0.15		
1.0 BAP+0.0 IBA	4.90	3.35**	1.55**			
0.5 BAP+1.0 IBA	2.40	0.85*				
0.1 BAP+1.0 IBA	1.55					

$LSD_{(0.05)}$ =0.659; $LSD_{(0.01)}$ =0.959; *significant difference at the level 0.05; **significant difference at the level 0.01

The presence of BAP hormones in nutrient medium effectively prevented rooting of strawberries. At the optimal concentration of hormones, multiplication index was higher than 1:5 and it was approximately 1:5-7 (in some passages even 8). Omission of IBA hormones leads to the reduction the multiplication, but not to the omission of multiplication. The biological potential of starting material is not significantly reduced by the three-year cultivation, and it ranges within the boundaries of the calculated multiplication index value. High value of the multiplication factor indicates that the use of micropropagation methods can achieve rapid and mass production of strawberries.

Rooting

Strawberry scions obtained in the phase of multiplication and elongation, are distinguished by the absence of rootlets. Therefore, when the plantlets reached a height of 10-20 mm, they were transferred at the rooting medium. The composition of this medium includes macro and micro salts, sucrose, and agar inositol, and regarding the hormones, hormone IBA at different concentrations (Table 5).

Table 5. The influence of IBA hormone concentration on rooting strawberry cultivar 'Idea' after 30 days

Hormone combination IBA mg/l	Number of plants in a culture	Number of rooted plants	Percentage of rooted plants	The average number of roots
0.0	30	13	43.33	2.84
0.1	30	19	63.33	4.79
0.5	30	22	73.33	5.82
1.0	30	16	53.33	4.07

The optimal concentration of IBA hormones for the rooting of the cultivar 'Idea' is 0.5 mg/l. At a higher concentration of IBA hormones the larger mass of callus was formed at the basal part of the scion, but at a lower concentration of hormones, the number of rooted scions was lower.

A high percentage of rooting was achieved with the strawberry cultivar 'Senga Sengana' - 80% (Petrović and Jaćimović-Plavšić, 1990); cultivar 'Cortina' - 90% cultivar 'Marmolada' - 83.33% (Nikolić, 2006). In the experiments with a large number of strawberry cultivars, the highest rooting percentage is achieved with the cultivar 'Cortina' (85%), while other cultivars ('Sena',

'Selena') have a lower percentage and it ranges from 18.87% to 29.63% (Milosavljević et al., 1999).

The significance of the influence of BAP hormones on rooting was determined by LSD test (Table 6).

Table 6. The least significant difference of the influence of different concentrations of IBA hormone on rooting strawberry cultivar 'Idea' after 30 days

Hormone combination IBA mg/l	x	x-13	x-16	x-19
0,5	22	9**	6**	3
0,1	19	6**	3	
1,0	16	3		
0,0	13			

$LSD_{(0.05)}$ =3.98; $LSD_{(0.01)}$ =5.58; *significant difference at the level 0.05; **significant difference at the level 0.01

Transferring to non-sterile conditions

After rooting, in vitro regenerated plants are transferred to non-sterile conditions. The success of transplantation and survival of plantlets greatly depends on the quality of root (Pons et al., 1983).

The most convenient moment for transplanting the obtained plantlets after rooting is 60 days after incubation of scions on agrarian 1/2 Murashige and Skoog's medium, when the rootlets have reached the length of 30-50 mm. Then the roots of young plants were carefully separated from medium, followed by rinsing with water to remove the agar, in order to avoid harmful settled microflora. Thus washed, young plants were placed into sterile plastic dishes with a mixture of vermiculite, peat, and sand 1:1:1. 1/2 Murashige and Skoog's mineral solution was added to this mixture. The courts were placed in plastic tubs with water (2-3 cm) and covered with jar. After that, the plants were kept for a month under plastic sheets or uncovered, and then transplanted into pots with soil and taken out. By the end of the critical period of 30 days, the plants were transplanted to the open field. Percentage of adapted plants for this strawberry cultivar is 72.50%.

Conclusion

Considering the conducted analyses, we can conclude that multiplication of the strawberry sort 'Idea' is possible using the micropropagation method.

For the strawberry cultivar 'Idea', good organogenesis and regeneration *in vitro* is achieved when apical buds are used as initial explants. The best results are achieved when the explants are taken in May or June from stolons that are in the air.

The optimal balance of the hormones concentration for the initiation of cultures is: 1 mg/l BAP, 0.1 mg/l IBA and 0.1 mg/l GA_3. The best multiplication of the strawberry scions is achieved by a combination of phytohormones: 1.0 mg / l BAP and 1.0 mg / l IBA. On the medium without BAP hormones, despite the presence of IBA hormones, multiplication does not occur. Phytohormone BAP improves reproduction and reduces apical dominance strawberries. The optimal concentration of IBA hormones for the rooting of the cultivar 'Idea' is 0.5 mg/l. During the adaptation to external conditions the number of adapted plants for the cultivar 'Idea' is 72.50%.

The results presented in this study indicate that micropropagation can significantly speed up the process of obtaining high-quality and healthy plant material.

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