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Improvement of the efficiency of sanitation and primary propagation technology of garden strawberry in *in vitro* culture

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Abstract

The relevance of the research is conditional upon the great economic value of strawberries in the horticulture of our country and abroad. The purpose of the work is to propose various methods and evaluate their effectiveness in improving the technology of recovery and primary *in vitro* propagation of strawberries. The leading method of the research was the method of clonal micropropagation of plants. In the course of work the influence of preliminary *in vitro* cultivation of microcuttings of garden strawberries (sources of expelants) on the effectiveness of meristems' introduction *in vitro* and the rate of their regeneration was evaluated. The impact of L-aspartic acid and D-desthiobiotin on the callus formation process and the growth of microplants were assessed. In the process of clonal micropropagation, the survival of explants, the degree of callus formation, the size and overall condition of the microrosettes were taken into account. As a result of the researches it has been established that preliminary *in vitro* cultivation of strawberry apexes increases the survival rate of meristem isolated from them and being 0.1-0.3 mm in size, on average, 2 times and their growth rate - 1.6 times. When adding L-aspartic acid in the nutrient medium at concentration of 1 and 6 mg/l or D-destiobiotin at concentration of 0.1 and 0.5 mg/l, the degree of callus formation decreases and, at the same time, the overall state of the strawberry microrosettes *in vitro* improves. The materials of the article can be useful for researchers in the field of plant biotechnology.

Keywords: garden strawberry, micropropagation, *in vitro* culture, regeneration, meristem, explant, callus formation.

INTRODUCTION

For centuries, people plasticized vegetative reproduction of plants; however, only modern methods of tissue culture (*in vitro* clonal micropropagation) significantly expanded the area of this reproduction method use. Tissue culture is a powerful tool for expanding the potential of regeneration of cells and tissues of different plant species. Due to the fact that when cultivating meristematic tissues *in vitro* the vectors of systemic diseases are largely eliminated, these methods are very convenient for the recovery, rapid propagation and storage of useful plants, and garden strawberry in particular.

The purpose of the work is to propose different methods and evaluate their effectiveness in improving the technology of recovery and primary *in vitro* propagation of strawberries.

The relevance of the research is conditional upon the great economic value of strawberries in the horticulture of our country and abroad.

The novelty of the research is the use of new growth regulators and new technological techniques in the technology of sanitation and primary propagation of garden strawberries in the garden *in vitro* culture.

ANALYTICAL REVIEW

The world literature has accumulated a great amount of information on the *in vitro* cultivation of fruit and berry plants. At present time clonal micropropagation is widely used, including for the rapid production of a large amount of virus-free material in plants, the reproduction of which by traditional methods does not present difficulties, but that are prone to viral diseases, such as, for example, garden strawberry.

For vegetative propagation, various mechanisms are used for the formation and reproduction of meristematic tissues, which give rise to *in vitro* microshoots. One of the most common approaches now is the propagation by axillary shoots. Depending on the type of branching characteristic of roses, only a limited number of axillary meristematic tissues develop. Their development is inhibited by the presence of apical dominance. Addition of cytokinins to the medium causes active development and growth of axillary shoots. Garden strawberry [1], apple and its rootstocks [2, 3] can be given as examples.

The reasons why the method of clonal micropropagation is increasingly included in the technology of production of healthy planting material of fruit and berry crops are as follows [4-7]:

1. A sufficiently high multiplication factor - 1:500 - 1: 1,000 pieces per year.
2. The use of the cytokinin nature substances practically does not affect the frequency of spontaneous mutations; therefore, it is possible to obtain a homogeneous vegetative geniture.
3. The cultivation conditions make it possible to reject plants affected by pathogens.
4. This method allows producing plants throughout the year, regardless of climatic conditions.

This method becomes especially valuable in propagating healthy (selected) single virus-free plants.

However, it has a number of shortcomings, which determine the high final cost of plants, in particular:

1. Low level of survival ability of meristems when introducing explants into *in vitro* culture.
2. Fallouts and growth and development retardation of cloned microshoots due to callus overgrowth.

To reduce the cost of cultivated plants (mericlones), a search was made to improve the efficiency of introducing explants into the *in vitro* culture, to control callusogenesis, and to improve the quality of the regenerated microshoots.

MATERIALS AND METHODS

Strawberry apexes from 1 to 3 mm in size (microcrops) and meristems (0.1-0.3 mm) isolated from the rapidly growing tendrils of the first-order strawberry (from the mother bush) of the Marmolada and Elsanta varieties served as the material.

Material preparation and sterilization. Apex washing in running water for 1 -1.5 hours.

Sterilization in two stages:

1. Benlat (2%) - 45 seconds;
2. Sulema (0.1%) - 30 seconds or calcium hypochloride (3%) - 15 minutes.

Washing after the first stage - 5 minutes, after the second - 20 minutes in sterile distilled water.

All in vitro cultivation work has relied on or taken into account the methods of P. Boxus [8], O.O. Beloshapkina [9], K. Moradi et. al. [10], etc.

The medium included macro- and microsalts according to Murashige and Skoog [11], 0.8 mg/l of 6-BAP, 0.08 mg/l of indolylacetic acid, ascorbic acid – 1 mg/l, B1, B6 and PP vitamins 0.5 mg/l each, mesoinositol - 100 mg/l, sucrose - 24 g and agar 0.7%.

When studying the growth and development of explants, the following records were made:

- The percentage of the explants' survival was calculated from the total intact during planting (after 5 days) and uninfected (30 days) material.
- The growth of microrosettes regenerated by meristems was taken into account in millimeters with the help of a graduated scale of a binocular microscope.
- The degree of callus formation was measured in points on a 5-point scale (1 - callus is not formed, 5 - callus completely closes the cut, grows in a large colony, 2-4 - intermediate state).
- The condition of the microrosettes was assessed in points on a 5-point scale, the size, leaf coverage, saturation of the color of the ground tissues were taken into account (1 - the smallest size from the estimated sample, rudimentary leaves, chlorotic color of the ground tissues, 5 – the maximum size in the estimated sample, developed leaflets, bright green color of the covers).

The point-based system is developed from the minimum to the maximum and can be common for the crop when comparing different methods or varieties or for each variety or series of experiments separately, based on the available minimum and maximum indicators.

The results obtained are processed by means of an analysis of variance. All necessary calculations were performed using MS Office (Excel) and Stat Soft STATISTICA 5.5 [12].

RESULTS AND DISCUSSION

As a rule, the introduction of strawberry meristems of 0.1-0.3 mm in size in in vitro culture shows a low survival rate (30-40%), often the explants begin to regenerate callus instead of differentiated organs, the surviving meristems lack or have low growth momentum. In order to increase the survival rate of strawberries' meristem, prevent callus formation, and accelerate the growth and development, the following experiments were carried out:

- Preliminary cultivation of microcuttings, sources of the *in vitro* explants,
- Addition of L-aspartic acid and desthiobitin to the medium containing auxins and cytokinins.

The results on the introduction of apexes (microcuttings) of 3 mm in size into the culture, followed by the isolation of meristems of 0.1-0.3 mm in size (preliminary cultivation of microcuttings, explant sources) are presented in Tables 1, 2.

It can be seen from the Table 1 that the survival of meristems 0.1-0.3 mm in size, isolated from *in vitro* cultivated microcuts, in comparison with meristems isolated from growing strawberry tendrils almost doubled (from 39% to 67%).

The analysis of variance of the results of the introduction of the strawberry meristems into *in vitro* culture in two ways showed that the method of preparation of explants (isolation of meristems directly from growing strawberry tendrils and isolation of meristems from *in vitro*pre-cultivated microcuts) significantly affected the efficiency of meristems survival rate on nutrient medium (Fact. > F tab.). Factor influence share was 87.5 (Table 2).

Table 1 - The influence of preliminary cultivation of explant sources on survival of strawberries' meristem

Year	Variety	Meristems 0.1-0.3 mm, isolated from growing tendrils			Meristems 0.1-0.3 mm, isolated from the *in vitro* cultivated microcuts		
		Amount		% of survivability	Amount		% of survivability
		Planted	Survived		Planted	Survived	
2015	Marmolada	45	18	40	45	33	73
	Elsanta	45	16	36	45	29	64
2016	Marmolada	45	20	44	45	31	69
	Elsanta	45	17	38	45	28	62
2017	Marmolada	45	17	38	45	32	71
	Elsanta	45	18	40	45	27	60
			Medium	39		Medium	67

Table 2 - Analysis of variance of the results of direct introduction of meristems and with the preliminary cultivation of the source of strawberry (explant) meristems (factor 1 - year, factor 2 - variety, factor 3 - technology option)

Variability	Degree of freedom	Average square	F-ratio	Variance	Share of influence
Between years	2	0.194	0.206	0.00	0.0
Between varieties	**1**	**6.250**	**6.618**	**0.29**	**2.9**
Between technology options	**1**	**156.250**	**165.441**	**8.63**	**87.5**
Year x variety	2	0.083	0.088	0.00	0.0
Year x option	2	1.083	1.147	0.00	0.0
Variety x option	1	1.361	1.441	0.00	0.0
Interactions: Year x variety x option	2	0.861	0.912	0.00	0.0
Residual	24	0.944	-	0.944	9.6

Table 3 - Dimensions of microrosettes regenerated with meristems isolated from growing strawberry tendrils and from the *in vitro* cultivated microcuts, mm

Variety	Meristems 0.1-0.3 mm, isolated from growing strawberry tendrils			Meristems 0.1-0.3 mm, isolated from *in vitro* cultivated plants		
	10 days	15 days	20 days	10 days	15 days	20 days
Marmolada	0.53	1.18	2.21	0.79	1.75	3.64
Elsanta	0.71	1.62	2.76	0.88	2.18	4.03
Average by varieties	0.62	1.4	2.49	0.84	1.97	3.84

Table 4 - Analysis of variance of the sizes of microrosettes regenerated by meristems isolated from growing strawberry tendrils and from the in vitro cultivated microcuts (factor 1-variety, factor 2-technology option)

Variability	Degree of freedom	Average square	F-ratio	Variance	Share of influence
Explant size					
Between varieties	1	3.528	8.810	0.1	7.4
Between options	**1**	**27.015**	**67.454**	**0.86**	**63.2**
Variety x option	1	0.0725	0.181	0.00	0.0
Residual	58	0.40	-	0.40	29.4

The effectiveness of meristems' introduction into the *in vitro* culture differed in experimental varieties: in the Marmolada variety, the survival rate of meristems 0.1-0.3 mm in size, isolated from *in vitro* microcuts, was 71% (73%, 69%, 71%, Table 1). In the Elsanta variety, the same indicator was 62% (64%, 62%, 60%, Tab. 1). According to the results of the variance analysis, the differences were reliable (Fact. > F tab.). Factor influence share was 2.9 (Table 2).

Varietal (i.e. genetically mediated) differences exist also in the survival rate of meristems with direct isolation from growing strawberry tendrils. In the Marmolada variety, the survival rate of meristems 0.1-0.3 mm in size, isolated from tendrils, averaged 41% (40%, 44%, 38%, Tab. 1). In the Elsanta variety, the same indicator was 38 % (36 %, 38 %, 40 %, Tab. 1). According to the results of the variance analysis, the differences were reliable (Fact. > F tab.). Factor influence share was 2.9 (Table 2).

The survived meristems began to regenerate the shortened microshoots, characteristic for garden strawberry - microrosette. The results of measuring the size of regenerated microrosettes after 10, 15 and 20 days from the beginning of explant growth are shown in Table 3.

It has been established that the preliminary cultivation of the explant source increased the rate of microrosettes' regeneration by strawberry meristems in comparison with meristems isolated directly from strawberry tendrils: on day 10, the excess was 0.3 mm (0.55 mm on average - meristems from tendrils, 0.85 mm in average - meristems from the *in vitro* cultivated microcuts), on day 15 the excess was 0.73 mm (respectively, 1.31 mm and 1.84 mm), and on day 20 - 1.22 mm (respectively, 2.44 mm and 3.68 mm, Table 3).

The results of the measurements of the size of microrosettes' regenerated with meristems isolated from growing strawberry tendrils and from the *in vitro* cultivated microcuts were subject to a dispersion analysis (Fig. 4).

The analysis showed that the method of explant preparation (isolation of meristems directly from the growing strawberry tendrils and isolation of meristems from the preliminary *in vitro* cultivated microcuts) significantly influenced the size of microrosettes regenerated by meristems of experimental varieties (Fact. > F tab.). Factor influence share was 63.2 (Table 4).

The growth rate of microrosettes *in vitro* regenerated by meristems differed in experimental varieties: in the Marmolada variety, the size of the microrosettes in the option with the *in vitro* cultivation of microcuts was 3.64 mm on the 20th day (Table 3). In the Elsanta variety, the same indicator was 4.03 mm (Table 3). According to the results of the variance analysis, the differences were reliable (Fact. > F tab.). However, the factor influence share of 7.4 was low (Table 4).

Varietal differences exist also in the size of microrosettes regenerated by meristems from direct isolation from growing strawberry tendrils. In the Marmolada variety, the size of the microrosettes was 2.21 mm on the 20th day (Table 3). In the Elsanta variety, the same indicator was 2.76 mm (Table 3). According to the results of the variance analysis, the differences were reliable (Fact. > F tab.). The factor influence share of 7.4 was low (Table 4).

Thus, for 20 days of cultivation, the size of the regenerated microrosettes for the Marmolada variety was 1.4 times more in the experimental option than in the traditional technology, for the Elsanta variety - 1.8 times, and on average, 1.6 times.

The possible causes of an increase in the survival and growth rates of explants in the option with the preliminary *in vitro* cultivation of microcuts (a source of meristems) are the following: the adaptation of explants to cultivation, the reduction of the phenol level, and the absence of phytotoxic effect of the sterilizer just before meristem division.

It has been concluded that the method of introducing strawberry meristem into the *in vitro* culture allows to increase the efficiency of the in *vitro* introduction of meristems and to increase the rate of their regeneration.

Meristems introduced into the culture develop into microplants at the following stage of the sanitation and clonal micropropagation technology of garden strawberry. Callusogenesis is one of the effects, often accompanying the growth and development of explants in the *in vitro* culture. [13]. The development of callus tissue is usually promoted by the excessive content of auxins in the nutrient medium, the ontogenesis stage of the stool (donor plant), the varietal predisposition to explant callusogenesis, the stresses withstood by the stools or by the explants themselves, infection, the reaction of explants to sterilizers and cultivation modes, etc. For the clonal micropropagation technology, the development of callus on explants has a completely negative significance - under its influence the tissue differentiation and organogenesis of microplants are damped, and then completely stop (Fig. 1).

There exist various approaches to limiting the *in vitro* callusogenesis of explants. We have tested the ways of limiting the induction of callus by explants in vitro by adding growth regulators such as aspartic acid and desthiobiotin to the nutrient medium.

Aspartic acid (amino succinic acid), one of the dicarboxylic amino acids, has slightly acidic properties, it is a precursor for all other amino acids, and takes part in nitrogen metabolism and protein synthesis [14]. The L-isomer of aspartic acid was used in the experiment. The acid was added to the nutrient medium at concentration of 1, 6, 12 mg/l. A nutrient medium without

the addition of L-aspartic acid served as a control. Cultivation lasted 30 days, and after this period, the degree of callus formation and the overall state of the microrosettes were evaluated. The results of the measurements are shown in Table 5.

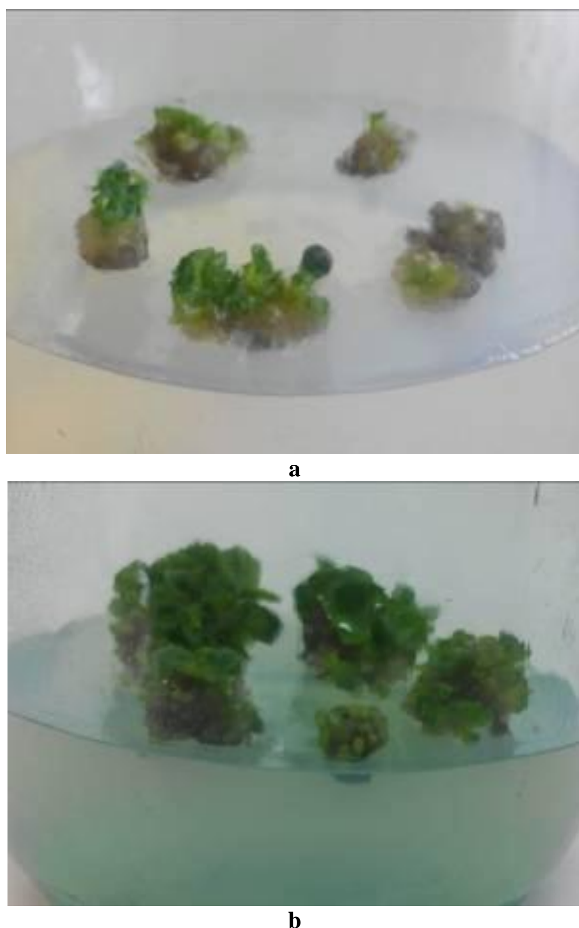

Figure 1 - "Occlusion" of garden strawberry regenerated microshoots with callus: a - Marmolada variety, b - Elsanta variety

From the table we see that in the control, i.e. on a medium containing auxins and cytokinins without the addition of L-aspartic acid, the state of the cultivated microrosettes is satisfactory (3.3-3.5 points), the proportion of microrosettes with callus and the degree of callus formation are the highest in the experiment: in the Marmolada variety, 43% and 3.2 points, respectively, in the Elsanta variety - 40% of microrosettes have occluded with callus, the degree of callus formation - 3.0 points (Table 5).

Adding L-aspartic acid at concentration of 1 and 6 mg/l to the nutrient medium decreases the proportion of microrosettes with callus (17-23%) and the degree of callus formation (1-1.5 points), and simultaneously improves the condition of microrosettes (3.7-4, 1 point).

The increase in L-aspartic acid in the nutrient medium to 12 mg/l reduces the share of explants with callus (3-7% in experimental varieties), reduces the degree of callus formation in the option with the Elsanta variety (0.5 points), but retains it at a level above the average in Marmolada (2.0 points), and sharply worsens the general condition of microrosettes (1.6 points for Elsanta, 1.8 for Marmolada; 5).

In the experiment on the study of the effect of L-aspartic acid on callus formation in microrosettes of Marmolada and Elsanta varieties *in vitro* cultivated for 30 days, the results of accounting the number of microrosettes in which callus developed or did not develop were subjected to the analysis of variance (Table 6).

The analysis of variance showed that the addition of L-aspartic acid to nutrient media significantly influenced the regeneration of callus by microrosettes of the *in vitro* cultivated experimental varieties (Fact. > F tab.). Factor influence share was 86.2 (Table 6).

D-desthiobiotin (D-desthiobiotin or 5-methyl-2-oxo-4-imidazolidinehexanoic acid, microbial growth stimulant). Desthiobiotin is a precursor of biotin, its action is not inhibited by other growth regulators. In plant cells it turns into biotin, but the transformation is slow and continuous, providing a growing body with biotin for a long time, protecting it in unfavorable conditions, positively influencing the growth of plants and, especially, the formation of a strong root system.

According to the developers of the preparation, during the growth of meristem cultures there is no antagonism of desthiobiotin and auxins, as in the example with pure biotin. Desthiobiotin causes strong *in vitro* growth of the root system, accelerating the process of obtaining planting material, especially at concentrations of 0.01-0.1 mg/l of the medium. At higher concentrations, no harmful effect is observed [15].

D-desthiobiotin was added to the nutrient medium at concentration of 0.1; 0.5 and 1 mg/l. A nutrient medium without the addition of D-desthiobiotin served as a control. Cultivation had lasted 30 days, and after this period, the degree of callus formation and the overall state of the microrosettes were evaluated. The results of the measurements are shown in Table 7.

Table 5 - The influence of L-aspartic acid on callus formation in microrosettes of Marmolada and Elsanta varieties *in vitro* cultivated for 30 days

The concentration of L-aspartic acid in mg/l	Number of cultivated microrosettes	Callus forming microrosettes		The degree of callus formation in points, on average	The state of microrosettes, in points, on average
		pcs.	%		
Marmolada					
0	30	13	43	3.2	3.5
1	30	6	20	1.5	3.7
6	30	5	17	1.5	4.1
12	30	2	7	2.0	1.8
Elsanta					
0	30	12	40	3.0	3.3
1	30	7	23	1.5	3.9
6	30	5	17	1.0	3.9
12	30	1	3	0.5	1.6

Table 6 - Analysis of variance of the results of the effect of L-aspartic acid on callus formation in microrosettes of Marmolada and Elsanta varieties *in vitro* cultivated for 30 days (factor 1- variety, factor 2 - technology option)

Variability	Degree of freedom	Average square	F-ratio	Variance	Share of influence
Callus formation					
Between varieties	1	0.062	0.0769	0.00	0.0
Between options	**3**	**21.063**	**25.9231**	**5.06**	**86.2**
Variety x option	3	0.229	0.2821	0.00	0.0
Residual	8	0.81	-	0.81	13.8

Table 7 - The effect of D-destiobiotin on the growth and callus formation of strawberry apexes

D-desthiobiotin concentration, mg/l	Number of cultivated microrosettes	Callus forming microrosettes		The degree of callus formation in points, on average	The state of microrosettes, in points, on average
		pcs.	%		
Marmolada					
0	30	12	40	2.8	3.3
0.1	30	9	30	1.2	3.7
0.5	30	9	30	1.5	4.0
1.0	30	15	50	3.5	3.2
Elsanta					
0	30	11	37	2.5	3.1
0.1	30	8	27	1.7	3.6
0.5	30	7	23	1.3	4.3
1.0	30	13	43	3.2	3.0

Table 8 - Analysis of variance of the results of the effect of D-destiobiotin on callus formation in microrosettes of Marmolada and Elsanta varieties *in vitro* cultivated for 30 days (factor 1- variety, factor 2 - technology option)

Variability	Degree of freedom	Average square	F-ratio	Variance	Share of influence
Callus formation					
Between varieties	1	2.250	2.5714	0.00	0.0
Between options	**3**	**7.833**	**8.9524**	**1.74**	**66.7**
Variety x option	3	0.083	0.0952	0.00	0.0
Residual	8	0.87	-	0.87	33.3

a

b

Figure 2 - Microrosettes of garden strawberry: a - Marmolada, b - Elsanta

According to the data in Table 7, the state of the cultivated microrosettes is satisfactory (3.1-3.3 points), the share of explants with callus (37-40%) and the degree of callus formation (2.5- 2.8 points) are quite high without the addition of D-desthiobiotin (control).

Adding D-desthiobiotin at concentration of 0.1 and 0.5 mg/l to the nutrient medium decreases the proportion of explants with callus (23-30 %) and the degree of callus formation (1.2-1.7 points), and simultaneously improves the condition of microrosettes (3.6-4,3 points). An increase in the content of D-destiobiotin in the nutrient medium to 1.0 mg/l increases the share of explants with callus (43-50%), increases the degree of callus formation to the maximum values (3.2-3.5 points), and sharply worsens the overall condition of the microrosettes (3.0-3.1 - minimal in the experiment; Table 7).

In the experiment on the study of the effect of D-destiobiotin on callus formation in microrosettes of Marmolada and Elsanta varieties *in vitro* cultivated for 30 days, the results of accounting the number of microrosettes in which callus developed or did not develop were subjected to the analysis of variance (Table 8).

The analysis of variance showed that the addition of D-destiobiotin to nutrient media significantly influenced the regeneration of callus by microrosettes of the *in vitro* cultivated experimental varieties (Fact. > F tab.). Factor influence share was 66.7 (Table 8).

As a result, the introduction of the L-aspartic acid and D-desthiobiotin growth regulators into nutrient media allowed to *in vitro* reduce the level of callus formation by explants and to obtain qualitative viable microrosettes of garden strawberry for further propagation (Fig. 2).

CONCLUSION

As a result of the studies conducted, in order to increase the effectiveness of the technology of sanitation and primary reproduction of garden strawberry, the method of preliminary *in vitro* cultivation of apexes, future meristem donors, was tested, and the growth activity of L-aspartic acid and D-desthiobiotin growth regulators introduced into the nutrient medium was studied.

It has been established that preliminary *in vitro* cultivation of strawberry apexes increases the survival rate of meristem isolated from them and being 0.1-0.3 mm in size, on average, 2 times and their growth rate - 1.6 times.

When adding L-aspartic acid in the nutrient medium at concentration of 1 and 6 mg/l or D-desthiobiotin at concentration of 0.1 and 0.5 mg/l, the degree of callus formation decreases and, at the same time, the overall state of the strawberry microrosettes *in vitro* improves.

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