Clonal micropropagation of essential oil rose cultivars and breeding samples at long-term subcultivation *in vitro*

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Abstract. The development of explants of 12 cultivars and breeding samples of essential oil rose (obtained with the participation of *Rosa damascena* Mill., *R. gallica* L., *R. alba* L.) during long-term micropropagation was investigated. At the second multiplication stage 5 subcultures were carried out. Increase of the studied morphometric parameters of explants to 3-4th subcultures was established. In some genotypes, the maximum multiplication index was in the third subculture (cultivars ‘Raduga’, ‘Zolushka’, ‘Lada’, ‘Krymskaya Krasnaya’, ‘Lany’, ‘Vesna’ and samples N37-24, M215), while in others (cultivar ‘Kazanlykskaya’ and samples N138, G2168, N37-2) – in the fourth. The largest increase of the multiplication index in 3-4th subcultures compared to the first (3.0-4.8 times) was found in the cultivars ‘Lada’, ‘Lany’, ‘Raduga’, ‘Krymskaya Krasnaya’ and sample N37-24. In the fifth subculture the multiplication index decreased. However, in most cultivars and breeding samples it was higher than in the first subculture. The best morphogenetic potential was noted for cultivar ‘Raduga’ and samples G2168, N37-2, in which the multiplication index reached 14.2, 14.4 and 11.8, respectively. The minimal ability to propagation *in vitro* was in samples M215 and N138 – their multiplication index did not exceed 1.1-4.0.

1 Introduction

Essential oil rose is one of the best known aromatic plants worldwide. It is mainly cultivated in the Mediterranean and the Middle East countries – Bulgaria, Turkey, France, Morocco, Iran, India, etc. The genus *Rosa* (family Rosaceae Juss.) includes hundreds of species and thousands of cultivars. But only a few species of this genus are used to produce essential oils – *R. damascena* Mill., *R. gallica* Linn., *R. moschata* Herrm., *R. centifolia* Linn., *R. bourboniana* Desportes., *R. chinensis* Jacq., *R. alba* Linn. [1]. One of the most important essential oil *Rosa* species is *R. damascena*, which belongs to the Damask group of roses and is known for its strong fragrance [2].

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Interest in this plant is associated with a wide range of its use. There are four main products which are obtained from essential oil rose – essential oil, concrete, absolute and rose water. Rose oil is widely used as a base component of many high-quality perfumes and cosmetics – perfumes, eau de toilette, shampoos, creams and other skin care products. Rosa has many culinary uses as a flavor additive or as an antioxidant and antibacterial agent in food industry. The modern investigations on *R. damascena* have confirmed the antibacterial, antiviral, anti-inflammatory, anticancer, antidepressant, analgesic and anticonvulsant activities [3]. In medicine rose is applied for the treatment of diseases of mouth and throat, cardiovascular system, cholecystitis, gastritis, ulcers and many others.

In Russia, essential oil rose is currently grown in the southern regions, mainly in the Crimea. Most of the high-yielding and adapted to the conditions of this region cultivars were obtained by hybridization with the participation of species of *R. damascena*, *R. gallica*, *R. alba* [4]. To improve the breeding of roses, it is necessary to develop biotechnological methods, in particular, clonal micropropagation, which has many advantages compared to traditional methods. Propagation *in vitro* allows accelerating the reproduction of valuable breeding samples, hybrids, new cultivars, as well as receiving virus-free planting material. This method is also the basis for creating collections *in vitro*.

There are far less works devoted to research on clonal micropropagation for essential oil roses than for ornamental ones. These publications mostly covered issues of *R. damascena* propagation *in vitro* [2, 5-8]. As explants when introduced into an aseptic culture, axillary or apical buds and meristems [5, 8-10], stem segments with a node [7, 9, 11-12] were usually used. There is evidence of the use of direct shoot regeneration from leaf, stem and petiole for *R. damascena* micropropagation [1]. Protocol for *in vitro* propagation using induction of shoot buds from leaf explants of *in vitro*-raised shoots of *R. damascena* cultivar 'Jwala' was developed [2]. A. Ginova et al. reported about induction of indirect organogenesis from rose callus [2]. However, to obtain a genetically stable planting material, meristems or buds are most frequently used as explants, from which the probability of obtaining somaclonal variants is less.

The information on micropropagation of essential oil rose presented in various literature sources almost always focuses on the optimization of the culture medium composition for the different stages of propagation *in vitro*. The compositions of culture media recommended by different authors for the multiplication stage are very diverse [1-2, 9, 11, 13]. As cytokinin, many authors recommend the introduction of 6-benzylaminopurine (BAP) into the culture medium composition [6, 9-11, 14]. Thus, some studies have shown the efficiency of relatively high concentrations of this growth regulator – 3.0 mg/l [11-12], 4.0 mg /l [9], 6.0 mg/l [7]. Often, for better shoot proliferation, BAP was used in combination with indole-3-butyric acid (IBA) [15] or α -naphthaleneacetic acid (NAA) [16]. Different studies have shown the efficiency of thidiazuron [1, 17] or the combined use of cytokinin, gibberellic acid (GA₃) and auxin (α -naphthaleneacetic acid, indole-3-acetic acid) [1-2, 6]. B. Allahverdi Mamaghani et al. [5] recorded the highest level of shoot multiplication rate at a combination of 5 mg/l BAP and 0.1 mg/l thidiazuron. For three genotypes (Kashan damask rose, Yasooj aromatic rose and their hybrid), the best shoot formation from shoot tip explants was achieved on MS medium with BAP (0.5 mg/l), N⁶-2-isopentenyl adenine (0.5 mg/l) and IBA (0.1 mg/l) [8]. At the same time, M. Salekjalali [13] for *R. damascena* revealed the effectiveness of introducing into the medium not only BAP and NAA, but also phloroglucinol, which enhanced the multiplication rates *in vitro*. H. M. Noodezh et al. found that an increase in the content of NH₄NO₃, CaCl₂ and FeSO₄ in the culture medium contributed to a 5-10-fold increase in the number of shoots per explant in *R. damascena* [9].

In addition to the influence of the culture medium composition, in some studies the effect of other exogenous or endogenous factors on the development of rose explants during

micropropagation was analyzed. An important role in this process of the genotype [5-6, 8, 15], explant type [1-2], explant size [7], explant isolation season [5, 10] was noted for scented roses. So, in the work of Allahverdi Mamaghani et al. it was noted that appropriate seasons for introduction *in vitro* were summer and autumn [5]. For different regions of the Crimea, the best time for introducing explants into the culture was February-March [10] or August [18]. Some papers have noted the influence of light source on rose micropropagation [1]. A. Kumar and L.M.S. Palni [3] showed enhanced growth and better shoot proliferation in shoot cultures grown under photosynthetically active radiation as compared with cultures maintained under cool fluorescent lights.

To increase the efficiency of fragrant rose propagation or to overcome the problems that arise during this process, some authors used different conditions of cultivation. There is evidence of the successful use of the liquid medium for micropropagation [1, 6, 9]. In order to regenerate *R. damascena* on a large scale, an efficient propagation system has been established using transverse thin cell layer culture [16]. A. Ginova and V. Kondakova [14] for *R. damascena* Mill. f. *trigintipetala* Dieck. propagation used a temporary immersion bioreactor system to overcome the problem with top necrosis of microshoots. A. Bosh et al. [7] also showed that a temporary immersion system was significantly better than solid ore liquid media for shoot multiplication.

However, the data presented in the literature are sometimes ambiguous and many questions of the clonal micropropagation of roses have not been studied enough. So, in several works the influence of the genotype is noted, however, only 2-3 cultivars or samples were studied [5-6, 8, 15]. In other articles, there is no exact indication of the studied sample or cultivar [9, 13-14]. Many studies do not specify the number of subcultures carried out at the micropropagation stage. Several publications present rather contradictory data on the effect of the number of subcultures on the development of explants and the formation of shoots of essential oil roses [1, 11, 17]. However, this is an important question, since the effectiveness of *in vitro* micropropagation and storage techniques depends on this, as well as the ability to obtain the required number of propagated plants.

The aim of the work was to study the influence of long-term subcultivation on explants development *in vitro* and micropropagation of essential oil rose cultivars and breeding samples.

2 Materials and methods

The objects of our investigations were essential oil rose cultivars ('Raduga', 'Vesna', 'Krymskaya Krasnaya', 'Zolushka', 'Lany', 'Lada', 'Kazanlykskaya') and breeding samples (N138, G2168, M215, N37-2, N37-24), obtained with participation of species *Rosa damascena* Mill., *R. gallica* L. and *R. alba* L. [4]. Plants were grown in the collection plot of Research Institute of Agriculture of Crimea. To introduce *in vitro*, meristems with two leaf primordia (0.3-0.5 mm) were used as primary explants. They were excised from the vegetative buds under a binocular microscope MSP-1 (LOMO, Russia). Sterilization of plant material was carried out using 70° ethanol (1 min), and then 50% "Bradofen" solution (Florin, Hungary) for 10 minutes. For micropropagation, stem segments with one node isolated after microcutting from test tube shoots were used as explants.

The explants were cultured on modified Murashige and Skoog (MS) culture medium with 1.0 mg/l BAP and 0.1 mg/l GA₃ (Sigma, USA), previously optimized for clonal micropropagation of essential oil rose [18]. Culture medium was solidified with agar 7 g/L. The cultivation of meristems was carried out in test tubes with 10 ml of medium. On multiplication stage, the cultures were grown in 200-ml glass jars containing 40 ml of medium with five explants per jar. Jars were closed with aluminium foil. Explants were

kept under standard culture conditions at 24-26°C, 70% of relative air humidity, under 16-h photoperiod with light intensity 2000-3000 lux.

The explants were cultivated for 30 days, after which analysis of morphometric parameters of rose explants was performed. Then microcuttings of the shoots were carried out. For morphometric analysis, length and number of shoots (per explant), number of leaves (per explant), nodes number (per shoot), frequency of multiple shoot formation (%) were determined. The multiplication index was calculated as the number of microcuttings that can be obtained during one subculture. For this, the number of shoots formed on an explant was multiplied by the number of nodes on a shoot.

Experiments were carried out in three replications; 15 explants were analyzed at each variant. The data were processed using the Microsoft Office software package (Excel 2010). Significance of differences was assessed by the Student criterion at $P \leq 0.05$. The tables and the graphs show average values and their standard errors.

3 Results

In an aseptic culture *in vitro* on the MS medium with 1.0 mg/l BAP and 0.1 mg/l GA₃ meristems of 7 cultivars and 5 breeding samples were introduced. The analysis of cultures revealed a relatively slow development of meristems for all genotypes. An increase in the size of explants and the formation of the first leaf blade were observed two weeks later. The number of viable developing explants was minimal in N138 (78.6%), and in the remaining genotypes it was 92.7-100.0%. After one month of cultivation, the length of the shoots reached 6.6–8.5 mm (Table 1.). At the same time, small rosettes of leaves were formed representing microshoots with shortened internodes. The number of leaves was maximum for G2168 and N37-2 (2.0 and 2.2 pcs per explant). The studied genotypes (except N138) developed several small shoots and buds. Frequency of multiple shoot formation varied depending on the cultivar and breeding sample. The smallest ability to form several shoots was typical for cultivar ‘Vesna’ (18.8%); the maximum frequency of multiple shoot formation was observed in N37-2 (72.4%). The average number of shoots per explant ranged from 1.0 (N138) to 2.5 (N37-2) pcs.

Table 1. Development of meristems of different essential oil rose cultivars and breeding samples under introduction *in vitro*.

Cultivar, breeding sample	Frequency of multiple shoot formation (%)	Shoots number per explant (pcs)	Shoot length (mm)	Leaves number per explant (pcs)
‘Raduga’	68.9±5.8	2.0±0.1	7.9±0.3	1.9±0.2
‘Vesna’	18.8±2.2	1.2±0.1	7.7±0.5	1.5±0.1
‘Krymskaya Krasnaya’	56.5±4.0	1.7±0,1	6.6±0.4	1.4±0.1
‘Zolushka’	42.9±4.5	1.5±0,1	8.5±0.7	1.6±0.1
‘Lany’	34.6±2.7	1.2±0.1	7.6±0.2	1.5±0.1
‘Lada’	39.5±2.9	1.2±0.1	7.3±0.3	1.4±0.1
‘Kazanlykskaya’	43.8±3.1	1.5±0.1	7.0±0.3	1.5±0.1
N138	0	1.0	7.7±0.5	1.4±0.1
G2168	64.3±5.4	2.1±0.1	6.8±0.4	2.0±0.2
M215	30.0±2.5	1.4±0.1	7.2±0.3	1.2±0.2
N37-2	72.4±6.5	2.5±0.2	7.8±0.4	2.2±0.1
N37-24	46.3±4.0	1.2±0.1	7.2±0.3	1.2±0.2

For further propagation at the second multiplication stage, stem segments with a node were used as explants. They were transplanted every month to MS culture medium with 1.0 mg/l BAP and 0.1 mg/l GA₃. The analysis of morphometric parameters of 12 rose genotypes was carried out during 5 subcultures. The results of a study of explants development in the first and 3-5th subcultures are presented in Table 2. When explants were cultured for 1 month, 1-2 microshoots were developed from axillary buds. Several adventitious buds or shoots were also formed frequently. Since the developing adventitious shoots were small, the average shoot length was relatively small, too. Many microshoots, especially in the first subculture, had shortened internodes and micro rosettes of leaves. In the first subculture shoot length vary from 6.6 ('Kazanlykskaya') to 9.8 mm (N37-2). Further subculture led to formation longer shoots with normal internodes. The average microshoot length in 3-5 subcultures varied from 8.5 to 16.0 mm depending on the genotype and the number of subcultures. The smallest shoots were developed in sample M215 (8.5-8.8 mm); the maximum average shoot sizes (up to 14.1-16.0 mm) were noted in cultivars 'Lada' and 'Raduga' and samples N37-2, N37-24, G2168. The results in Table 2 show that most of the studied genotypes demonstrated high potential for multiplication. The percentages of the multiplied explants vary from 11.8 to 91.5% in the first subculture and from 48.5 to 100% in the third. In the first subculture active formation of additional microshoots was observed. On average, from 1.1 to 4.2 shoots per explant (depending on genotype) were formed. By the third passage, the number of shoots increased (1.5-5.8 pcs per explant), and in the fifth – decreased to 1.3-4.0 pcs per explant. The minimum frequency of multiple shoot formation was observed in the samples M215 and N138 – in the first subculture did not exceed 29.3%, and in a fifth – 41.7%. These samples developed, on average, no more than 1.5-1.8 shoots per explant. The biggest average number of shoots from explant was induced in the first subculture in the cultivar 'Raduga' (4.0 pcs) and sample N37-2 (4.2 pcs). By the third passage, the number of shoots increased in the cultivar 'Raduga' up to 5.8 pcs per explants and in the sample N37-2 up to 5.0 pcs per explants. In 4-5 subcultures this parameter decreased in all studied cultivars and breeding samples.

Table 2. Influence of subcultures number and genotype on the development of explants during the second stage of essential oil rose micropropagation *in vitro*.

Number of subcultures	Cultivar, breeding sample	Shoots number per explant (pcs)	Shoot length (mm)	Nodes number per shoot (pcs)	Frequency of multiple shoot formation (%)
1	'Raduga'	4.0±0.4	8.0±0.3	1.1±0.1	85.7±7.5
	'Vesna'	2.8±0.2	9.6±0.4	1.3±0.1	72.4±6.2
	'Krymskaya Krasnaya'	1.9±0.2	7.2±0.4	1.1±0.1	60.0±5.2
	'Zolushka'	2.0±0.2	9.6±0.5	1.2±0.1	76.3±6.5
	'Lany'	1.6±0.2	9.1±0.4	1.2±0.1	37.3±2.2
	'Lada'	1.8±0.2	8.5±0.4	1.2±0.1	38.9±2.5
	'Kazanlykskaya'	2.3±0.2	6.6±0.3	1.1±0.1	68.2±5.4
	N138	1.4±0.1	8.0±0.4	1.0	29.3±2.8
	G2168	2.7±0.3	8.5±0.4	1.2±0.1	72.7±6.1
	M215	1.1±0.1	6.8±0.4	1.0	11.8±0.8
	N37-2	4.2±0.3	9.8±0.5	1.3±0.1	91.5±8.2
3	N37-24	3.2±0.3	8.9±0.4	1.2±0.1	70.4±6.2
	'Raduga'	5.8±0.5	15.8±0.5	2.4±0.1	92.8±7.8
	'Vesna'	3.4±0.3	12.9±1.0	2.0±0.2	72.4±7.5
	'Krymskaya Krasnaya'	3.2±0.3	12.5±0.7	2.0±0.2	91.7±7.8
	'Zolushka'	3.2±0.3	12.6±0.6	2.1±0.1	81.8±7.2
	'Lany'	3.1±0.5	13.7±0.6	2.1±0.1	82.8±6.8
	'Lada'	4.2±1.2	16.0±1.2	2.5±0.2	70.8±5.4
	'Kazanlykskaya'	3.1±0.3	10.6±0.5	1.5±0.1	90.5±8.5
	N138	1.8±0.2	11.1±0.6	1.8±0.2	66.2±4.2
	G2168	2.1±0.2	13.2±1.1	2.0±0.2	53.8±4.3
	M215	1.5±0.1	8.5±0.4	1.2±0.1	48.5±3.5
4	N37-2	5.0±0.4	13.2±0.7	2.0±0.2	100.0
	N37-24	5.3±0.4	14.1±0.8	2.2±0.2	85.8±7.6
	'Raduga'	3.4±0.3	13.1±0.7	2.6±0.2	94.2±7.1
	'Vesna'	3.2±0.3	12.8±0.9	2.1±0.2	78.5±7.1
	'Krymskaya Krasnaya'	3.0±0.3	12.2±1.0	2.0±0.2	91.7±7.5
	'Zolushka'	2.4±0.2	10.6±0.7	1.3±0.1	68.5±6.2
	'Lany'	3.0±0.3	12.6±0.4	2.0±0.1	79.8±5.1
	'Lada'	3.1±0.3	12.4±0.5	2.5±0.2	90.0±8.4
	'Kazanlykskaya'	3.2±0.3	10.3±0.3	1.6±0.1	83.3±7.1
	N138	1.9±0.2	11.7±0.7	2.1±0.2	54.5±4.9
	G2168	2.6±0.3	11.3±0.6	1.8±0.1	66.7±5.3
5	M215	1.3±0.1	8.8±0.4	1.1±0.1	28.2±2.1
	N37-2	5.3±0.4	14.1±0.9	2.7±0.2	95.3±9.2
	N37-24	3.3±0.3	13.5±0.8	2.6±0.2	77.2±7.0
	'Raduga'	2.3±0.2	14.5±0.8	2.9±0.3	76.5±5.6
	'Vesna'	2.0±0.3	11.3±0.7	2.1±0.2	60.5±5.6
	'Krymskaya Krasnaya'	2.6±0.3	11.4±0.7	1.9±0.2	78.6±7.2
	'Zolushka'	2.5±0.2	10.9±0.7	1.6±0.1	67.5±5.9
	'Lany'	2.4±0.3	12.0±0.6	2.2±0.2	75.0±5.6
	'Lada'	2.1±0.2	12.2±0.8	2.2±0.2	60.9±5.5
	'Kazanlykskaya'	2.9±0.3	11.9±0.5	1.7±0.1	79.3±6.6
	N138	1.7±0.2	12.3±0.8	1.8±0.2	41.7±3.1
	G2168	1.8±0.2	14.3±0.8	2.5±0.2	59.5±4.3
	M215	1.3±0.1	8.9±0.4	1.1±0.1	19.5±1.4
	N37-2	4.0±0.4	12.9±0.7	2.5±0.2	82.1±7.7
	N37-24	2.8±0.3	11.2±0.5	2.0±0.2	78.4±8.1

Figure presents data on the change in the multiplication index in 12 genotypes of essential oil rose for 5 consecutive subcultures. The results show that this parameter, as the most important for assessing the propagation efficiency, changed depending on subcultures number and genotype. In the first passage, the multiplication index ranged from 1.1 to 5.6 and was maximum at N37-2. Then a gradual increase of this parameter to 3-4th subcultures was observed. In the third subculture multiplication index ranged from 1.8 to 14.2, and in the fourth – from 1.4 to 14.4. In the fifth passage in most cultivars and breeding samples multiplication index decreased. In some genotypes ('Vesna', M215, G2168, N37-24), the multiplication index in the fifth subculture did not significantly differ from that in the first. However, for other genotypes the multiplication index in the fifth subculture was significantly higher than in the first. For example, in the sample N37-2, multiplication index in the first subculture was 4.0 and in the fifth – 10.0.

Fig. 1. Influence of subcultures number and genotype on the multiplication index during essential oil rose micropropagation *in vitro*.

4 Discussion

Development of *in vitro* propagation methods for rapid multiplication of rose cultivars and valuable genotypes (hybrids, mutants, collection samples, obtained in tissue culture regenerants, etc.) is of great importance. A major objective of the present investigation was to study the influence of long-term subcultivation on the micropropagation of essential oil rose cultivars and breeding samples. This question, despite the available publications on the development of essential oil rose *in vitro* propagation methods, is very poorly studied. However, analysis of the explants development over several subcultures is very important for the creation of effective biotechnologies, since it allows one to find out how long plants can be propagated *in vitro* without losing their quality. For the practical use of these methods in breeding programs, it is necessary to conduct such studies for a wide range of genotypes grown in a certain region. In our work rose cultivars ('Raduga', 'Vesna', 'Krymskaya Krasnaya', 'Zolushka', 'Lany', 'Lada', 'Kazanlykskaya') and samples (N138, G2168, M215, N37-2, N37-24), used in the breeding process, were studied. At the first stage of initiation of culture, a significant influence of the genotype on the ability to propagation was noted. Frequency of multiple shoot formation and shoots number were maximum for breeding sample N37-2 and 'Raduga' cultivar, which formed 2.5 and 2.0 shoots per explant, respectively. During sample N138 cultivation, very weak development

of meristems and the absence of additional buds were noted. The remaining morphometric parameters varied slightly in the studied genotypes.

The formation of several shoots at the stage of explant introduction *in vitro* was noted in many works devoted to the micropropagation of essential oil roses [2, 8-10, 12]. However, some authors noted that explants exude dark colored compounds into the culture medium (phenols, pigments) that are released from the cut ends of the explants [14]. This can cause browning of tissue and medium, which is often connected with poor culture establishment and reduced regeneration ability. The media enriched with activated charcoal, various antioxidants – ascorbic or citric acid, polyvinylpyrrolidone or other substances are often employed to nullify this effect [2]. Frequent subculture, incubation in shaking liquid culture, reduced culture temperature have also been used to deal with this problem [2]. For the genotypes of essential oil roses that we studied, there was no significant negative effect of phenolic compounds. Darkening of explants was observed only in isolated cases. The amount of viable developing meristems was usually over 90-95%.

At the second stage of micropropagation (multiplication stage) not only the forming of small shoots were used for rose propagation, but also microcutting of the shoots with normal internodes was carried out. Some shoots (especially in the 3-4th subcultures) reached 30-40 mm and had 3-5 nodes, so it was advisable to carry out their cuttings. Such single-node stem segments were transferred to a fresh medium. This allowed us to get more explants for propagation and increase the multiplication index. For all cultivars and breeding samples, multiple shoot formation was detected. In the first subculture, the average number of shoots ranged from 1.1 to 4.2 pcs per explant, and to the third – increased up 1.5-5.8 pcs per explant. In most genotypes the average number of shoots in 3-4 subcultures was more than 3.0 pcs per explant, and the ‘Raduga’, N37-2 and N37-24 had more than 5.0 pcs per explant.

Our data on the number of proliferating shoots during micropropagation of rose varieties and samples are comparable with the results of other researchers. When studying *in vitro* propagation of three cultivars of *R. damascena* and *R. gallica*, formation from 2.5 to 4.2 shoots per explant was reported [15]. S. Mirzaei et al. at micropropagation of Kashan damask rose, Yasooj aromatic rose and their hybrid received up to 3.4-3.9 shoots per explant depending on genotype [8]. During multiplication *in vitro* of different *R. damascena* cultivars and samples it was reported obtaining 4.4-6.0 [13], 3.7-4.0 [11], 2.9-3.8 [12], 5.9 [5] shoots per explant. Z. Jabbarzadeh and M. Khosh-Khui [11] revealed the development of 1.6-7.5 shoots depending on the number of subcultures and the composition of the culture medium. The largest shoots number per explant was obtained from a temporary immersion system (12.3 pcs) [7], or using transverse thin cell layer culture (22.3 pcs) [16].

In our study, we found that in almost all genotypes, the studied morphometric parameters gradually increased to 3-4th subcultures and then decreased with prolonged micropropagation. So, in cultivars ‘Lany’, ‘Lada’, ‘Raduga’, ‘Zolushka’, ‘Krymskaya Krasnaya’ and sample N37-24 the number of shoots in the third passage increased by 1.5-2.3 times compared with the first. During 2-4th subcultures, a slight increase in shoot length and, accordingly, the number of nodes was also observed. This made it possible to obtain more explants during microcutting for further propagation. All this led to an increase of multiplication index in almost all genotypes in 3-4th subcultures, which was significantly higher than in the first one. In the third subculture, the maximal multiplication index was in cultivars ‘Raduga’, ‘Zolushka’, ‘Lada’, ‘Krymskaya Krasnaya’, ‘Lany’, ‘Vesna’ and samples N37-24, M215. At the same time, in cultivar ‘Kazanlykskaya’ and samples N138, G2168, N37-2 the maximal multiplication index was noted in the fourth subculture. The largest increase of the multiplication index in 3-4th subcultures compared to the first subculture was found in the cultivars ‘Lada’ (4.8 times), ‘Lany’ (3.4 times), ‘Raduga’ (3.2

times), 'Krymskaya Krasnaya' (3.1 times) and sample N37-24 (3.0 times). For several samples (G2168 and M215), this increase was small (1.4-1.6 times). During subsequent cultivation (in the fifth passage) the multiplication index decreased. However, in most cultivars and breeding samples it was higher than in the first subculture. This was mainly due to the longer shoot length and the number of nodes, which made it possible to increase the efficiency of microcutting.

There are only a few reports on the effect of the number of subcultures on the micropropagation of essential oil roses. Z. Jabbarzadeh and M. Khosh-Khui [11] subcultured shoots of *R. damascena* four times on MS medium containing 2.5-3.0 mg/l BAP and 0.1 mg/l IBA. Among the multiplication stages, the first subculture resulted in the highest proliferation rate. In the first subculture 7.46 proliferated shoots was detected, while in the fourth it was only 1.63. In *R. moschata* the highest proliferation was obtained in the first subculture, but in the second one there were fewer shoots [1]. At the same time, A. Kumar et al. [17] showed the possibility of successful propagation of *R. damascena* during 8-12 subcultures on a medium containing BAP, after which the shoots attained the capacity to grow and proliferate on a medium free from plant growth regulators. For decorative garden rose (*Rosa hybrida* cvs. 'Cri Cri', 'Pariser Charme', 'First Red'), the frequency of shoot multiplication was the best up to the 6th-7th subculture and thereafter it declined [19].

We revealed an increase of multiplication index to 3-5th subcultures during *in vitro* propagation for some other essential oil plants. The experiments with micropropagation of lavender in 9 subcultures showed that the number of shoots increased in all five studied cultivars up to the third subculture and decreased thereafter [20]. During the micropropagation of five mint cultivars and breeding samples during six subcultures, it was found that the multiplication index increased in 3-5th passages, reaching 12.1-48.7 depending on the genotype and duration of cultivation [21]. When studying the effect of cultivation duration on propagation of *Origanum vulgare in vitro*, 13 passages were conducted. The maximum number of shoots (54.8 pcs per explant) and the multiplication index (74.1) were obtained in the fifth subculture. With further subcultures, the multiplication index decreased [22]. For three cultivars of *Melissa officinalis*, the maximum value of the studied morphometric parameters and the multiplication index (up to 12.0-17.6) also were noted in the 3-5th subcultures [22].

Other works also show an increase in the proliferative ability of *in vitro* cultures to a specific passage. At a continuous multiplication of *Gerbera jamesonii*, the multiplication rate of most of the 17 cultivars began to increase starting from the 2-3rd subcultures (3.8-4.0); reached its maximum in the 4th to 6th (9.8-10.5) subcultures; thereafter started to decline [23]. In the same work, it was shown that in *Philodendron tuxlanum* the maximum number of shoots per explant was in 4-5th subcultures, 6th and 7th subcultures led to a decrease in the multiplication rate. Multiple shoot formation of *Glycyrrhiza glabra* was increased from the first (2.2 shoots per explant) to the fourth subculture (6.6 shoots per explant) [24]. G.R.A. Mohamed et al. when developing a protocol for micropropagation of *Vaccinium corymbosum* showed that the fourth subculture gave the best results for multiplication [25]. However, after the fifth passage, hyperhydrated shoots appeared.

Such an increase of proliferative ability to 3-4th subculture revealed in our study for essential oil roses (and also shown for a number of other plant species) may be due to the optimal combination of the level of endogenous and exogenous (in the nutrient medium) plant growth regulators during this period. A further decrease in the multiplication index, apparently, is associated with a change in the hormonal state of cultured tissues, leading to gradual physiological aging of cultures. Therefore, in the future, it is promising to conduct a comparative analysis of the content of endogenous phytohormones in cultivated rose microshoots of different subcultures.

However, for some plant species, such an increase of multiplication index to a specific subculture was not found. Repeated subculture of the nodes of *Mahonia leschenaultii* through at least 10 passages enabled consistent production of 6-7 shoots per explant without loss of vigour, growth and morphological abnormalities [26]. *In vitro* shoots of vegetative rootstocks cherry, plum and pear were subcultured for 10 passages. Shoot formation capacity decreased over repeated subculturing in all genotypes [27]. Such a decrease may, however, be due to the lack of optimal cultivation conditions or culture medium.

In our study, when culturing rose explants during 5 passages, green microshoots and leaves without morphological anomalies were developed. Only a few cases of the appearance of hyper-hydrated shoots or yellowing of leaves were noted. At the same time, A. Ginova and V. Kondakova [14] reported that during subcultures chlorosis and then necrosis of leaves and apex were appeared. The appearance of yellow leaves, shoot tip necrosis, or abnormal shoots during the rose micropropagation *in vitro* was noted by a number of authors [2, 5-6].

It should be noted that in our study a significant influence of the genotype on the efficiency of micropropagation of essential oil rose was revealed. The maximum proliferative ability of explants at the stage of initiation of culture was noted for cultivar 'Raduga' and samples G2168, N37-2, and the minimum – for M215. With prolonged cultivation (on the multiplication stage), the best morphogenetic potential was shown by cultivar 'Raduga' and samples N37-2, N37-24. By the third passage, they developed more than 5.0 shoots per explant, and the multiplication index reached 14.2, 14.4, and 11.8, respectively. Most of the studied rose genotypes were propagated quite effectively under the conditions used by us. In 1-2nd passages, a low multiplication index was noted only in sample N138 and cultivars 'Lany', 'Krymskaya Krasnaya'. However, with further subcultures, their multiplication index increased significantly, which indicated the need for such samples to carry out several microcuttings for obtaining the required number of microplants. Minimum capacity for propagation showed sample M215; adventitious shoots almost did not develop from explants. Its multiplication index was 1.1-1.8, depending on the number of subcultures. Such different cultivars and samples *in vitro* reaction may be due to not only a low genetically determined ability to reproduce but also to an unsuitable culture medium. Therefore, for the successful propagation of these samples, it is, probably, necessary to carry out further optimization of the medium composition. In some studies of the species, cultivars and samples of essential oil rose, a significant influence of the genotype on the efficiency of micropropagation *in vitro* was also established [1, 5-6, 10, 15].

5 Conclusions

Thus, the peculiarities of explants development of 7 cultivars and 5 breeding samples of essential oil rose at the first and second stages of micropropagation *in vitro* were investigated. In the meristem culture of the studied genotypes (except sample N138), multiple shoot formation was observed with a frequency of 18.8-72.4%. At the second multiplication stage 5 subcultures were carried out, during which an average of 1.1-5.8 shoots per explant were formed, depending on the genotype and passage. Increase of the studied morphometric parameters of explants to 3-4th subcultures was established. At the same time, in some genotypes the maximum multiplication index was in the third subculture (cultivars 'Raduga', 'Zolushka', 'Lada', 'Krymskaya Krasnaya', 'Lany', 'Vesna' and samples N37-24, M215), while in others (cultivar 'Kazanlykskaya' and samples N138, G2168, N37-2) – in the fourth. The largest increase of the multiplication index in 3-4th subcultures compared to the first one was found in the cultivars 'Lada' (4.8

times), 'Lany' (3.4 times), 'Raduga' (3.2 times), 'Krymskaya Krasnaya' (3.1 times) and sample N37-24 (3.0 times). For several samples (G2168 and M215), this increase was small (1.4-1.6 times). In the fifth subculture the multiplication index decreased, but in most cultivars and samples it was higher than in the first subculture. The influence of the genotype on the efficiency of micropropagation of essential oil rose was revealed. At long-term subcultivation, the best morphogenetic potential was noted for cultivar 'Raduga' and samples G2168, N37-2, in which the multiplication index reached 14.2, 14.4 and 11.8, respectively. The minimal ability to propagation *in vitro* was in samples M215 and N138; their multiplication index did not exceed 1.8 and 4.0, respectively. The results indicate the effectiveness of long-term micropropagation of cultivars and breeding samples of essential oil rose (for at least 5 subcultures) without loss of microshoots quality.

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