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Challenges of *in vitro* conservation of *Citrus* germplasm resources

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The main problems of establishment a slow growth *in vitro* collection of citrus and other tree crops cultivars are high degree of fungal contamination of bud explants and low growth potential of shoots. In this regard, the aim of current research is to assess the efficiency of decontamination procedure and the possibility of tissue culture initiation and slow growth conservation of valuable lemon cultivars. The best results of surface sterilization were obtained using immersion solutions of 0.3 % Veltolen – 25 minutes or 10 % Domestos – 25–30 minutes. In these treatments, 27.7–33.0 % of aseptic explants were obtained, respectively. However, after the third subculture, the yield of aseptic viable explants decreased till 10 % as a result of secondary contamination by endophytic fungi. The addition of biocide ("Gavrish") in a nutrient medium at a concentration of 1 ml/l helped to increase the yield of aseptic viable explants till 50 %. However, after the third subculture the photosynthetic activity and the pigments content as well as growth rate decreased. Plants dropped yellowish leaves and eventually died. Thus, 37.35 % of plantlets survived after 8 months of conservation, and only 14.6 % survived after 10 months. Even after the third month of conservation significant decrease in the viability index and the coefficient of photosynthetic activity occurred in plants. Chlorophyll *a* in leaves decreased from 1.59 to 1.14 mg/g during 12 months *in vitro* conservation. The similar tendency observed on clorophyll *b* and carotenoids content. The experiments were carried out for 5 years using different lemon cultivars and other citrus varieties and cultivars. Thus, micropropagation and slow growth *in vitro* conservation of valuable lemon cultivars are still problematic and requires new technical solutions due to the low growth potential of plantlets raised from the mature buds that is consistent with the data of other researchers.
Key words: *Citrus*; nodal explant; contamination; micropropagation; slow growth conservation; culture media; true-to-type; endophytes.

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Проблемы сохранения *in vitro* гермоплазмы цитрусовых

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Главные проблемы создания депонированной коллекции сортов цитрусовых *in vitro*, как и некоторых других древесных растений, – высокая степень грибной контаминации вегетативных почек и последующее снижение ростового потенциала введенных в культуру *in vitro* микропобегов. В связи с этим цель нашей работы – выявить эффективность различных путей деконтаминации вегетативных эксплантов и оценить возможность введения в культуру *in vitro* и сохранения в медленнорастущей коллекции ценных сортов лимона. Наилучшие результаты поверхностной стерилизации были получены в вариантах с использованием растворов Велтолена 0.3 % – 25 минут и Доместос 10 % – 25–30 минут. В этих вариантах было получено 27.7–33.0 % стерильных жизнеспособных эксплантов соответственно. Однако после третьего пассажа выход стерильных эксплантов снизился до 10 % из-за вторичного инфицирования побегов эндофитными микроорганизмами. Использование биоцида («Гавриш») в питательной среде в концентрации 1 мл/л помогло повысить выход стерильных жизнеспособных эксплантов до 50 %. Несмотря на получение асептической культуры и начало морфогенеза, с течением времени происходило снижение фотосинтетической активности и содержания пигментов в листьях микропобегов. Ростовой потенциал микропобегов снижался с каждым последующим пассажем. После третьего пассажа микропобеги сбрасывали пожелтевшие листья, и рост их прекращался. Так, через 8 месяцев консервации выжило 37.35 % микропобегов на среде 1/2 Мурасиге–Скуга, их прирост составлял 0.33 см, а через 10 месяцев осталось лишь 14.6 % микропобегов. То есть спустя 10 месяцев консервации большая часть микропобегов погибла. Уже через три месяца консервации у растений наблюдалось существенное снижение индекса жизнеспособности и коэффициента фотосинтетической активности. Содержание хлорофилла *a* в листьях снижалось с 1.59 до 1.14 мг/г за 12 месяцев консервации *in vitro*. Аналогичная тенденция отмечена по содержанию хлорофилла *b* и каротиноидов. Эксперименты проводились в течение пяти лет с использованием разных сортов лимона и других цитрусовых. Таким образом, микроразмножение

и создание генбанка *in vitro* ценных сортов лимона все еще остается проблематичным и требует новых технических решений по причине низкого ростового потенциала эксплантов пазушных почек, что согласуется с данными других исследователей.
Ключевые слова: цитрусовые культуры; пазушные почки; контаминация; микроразмножение; *in vitro* консервация; питательная среда; генетическая однородность; эндофиты.

Introduction

Reliable true-to-type *in vitro* conservation of valuable cultivars of tree crops requires using vegetative buds and meristems as explants which are genetically identical to the maternal plant. However, tissue culture initiation of woody explants is associated with difficulties such as a high percentage of *in vitro* contamination, low bud growth potential, and occurring of secondarily contaminated plantlets (Niedz, Bausher, 2002; Çölgeçen et al., 2009; Kolomiets et al., 2014). The life of woody plants, in particular *Citrus* crops, goes in close cohabitation with fungal microorganisms (Kloepper et al., 2013; Kulyan, 2015; Ryndin, Kulyan, 2016). Surface sterilization of explants does not relieve tissue from internal infection, and on the nutrient medium the hyphae of the fungus proliferate through plant tissues, and prevents *in vitro* development of explants.

Several approaches are known in order to solve contamination problem: (1) fractional sterilization of explants, (2) combinations of sterilizing agents, (3) the creation of an isolated plant nursery – the source of explants, (4) pre-treatment of plants and shoots before tissue culture initiation and others (Gogoi, Borua, 2014; Kolomiets et al., 2014). However, heavy decontamination procedure can be a trigger of induction of genetic variability in tissue culture (Krishna et al., 2016), in this case true-to-type genotype conservation is again impossible. The aim of our work is to identify the efficiency of various decontamination procedures to establish slow growth collection and assess the possibility of *in vitro* conservation of valuable lemon cultivars.

Materials and methods

Plant material used in the experiments – young shoots, obtained from 5–8 year old plants grown in container culture under the greenhouse conditions from collection of the Russian Research Institute of Floriculture and Subtropical Crops (Sochi).

Nodal explants of *Citrus limon* (L.) Osbeck cultivars 'Novoafonskii', 'Beskoluchii', 'Jubilee', 'Villa Franka', 'Frost Eureka', and *Citrus* × *meyeri* Meyer were used for tissue culture initiation and conservation. The efficiency of shoots pre-treatment with the fungicide Fundazol was evaluated as follows: shoots without leaves with 5–7 buds were placed in aseptic wet vermiculite, treated with a solution of Fundazol prepared according to manufacturer's instructions and incubated for three days under the temperature of +25 °C.

The efficiency of eight surface sterilizing solutions in different concentrations was evaluated (0.2 % Mercuric chloride, 10–20 % Domestos, 0.2–0.3 % Veltolen, 0.2–0.3 % Novodez, 0.2 % Diacid, 15–20 % Hypochlorite Ca, 3–15 % Hydrogen peroxide), the incubation time was 10–30 min (Table 1). The effects of antibiotics (tetracycline hydrochloride of 200–500 mg/l) and the 0.5–1.0 ml/l Biocide ("Gavrish") added into the culture medium before autoclaving were assessed.

Culture medium 1/2 MS (Murashige, Scoog, 1962) supplemented with growth regulators BAP 1 mg/l, NAA 0.1 mg/l, casein hydrolyzate 500 mg/l, Phytagel 2.5 g/l, pH 5.7. Explants were cultured at standard lighting conditions 5 000 lux with periodicity 16/8 hours and temperature +22 °C. Plant growth observations were carried out every 2 weeks.

Physiological analysis were conducted on three lemon genotypes: 'Novoafonskii', 'Beskoluchii' and *Citrus* × *meyeri.* The coefficient of photosynthetic activity and the viability index were measured by the chlorophyll-fluorescent method (Budagovskaya et al., 2016). Chlorophyll and carotenoids contents were measured by taking 85 mg of leaf sample (mg/g fresh leaf weight). Pigments were extracted using acetone to a final volume of 50 ml. The absorbance of the extracts was measured spectrophotometrically at 662 and 644 nm for chlorophyll *a* (*ch a*) and chlorophyll *b* (*ch b*), respectively and at 440.5 nm for carotenoids. The concentration of chlorophyll *a*, *b* and total carotenoids were calculated according to Shlyk (1971).

The data were analyzed by one-way analysis of variance (ANOVA), and differences between treatments were considered as statistically significant at $p < 0.05$. The results were calculated as the mean value of at least 10 replicates in 3 repeats, mean ± SD were counted.

Results

The best results of surface sterilization were obtained using immersion in 0.3 % Veltolen for 25 minutes (variant IVb) and 10 % Domestos for 25–30 minutes (variants VIIa and VIIb); 27.7–33.0 % of aseptic viable explants were obtained, respectively, using these treatments (Fig. 1). However, subsequent subculturing, secondary shoots infection by endophytic microorganisms occurred, and resulted decrease in the yield of aseptic explants till 10 % after the third subculture.

Shoots pre-treatment with fungicide prior to tissue culture initiation have not affected significantly the yield of aseptic explants: during the shoots incubation with Fundazol fungal mycelium of endophytes released around the cut edges of shoot as heavy visible contamination on the surface of substrate. The addition of tetracycline to the nutrient medium at a concentration of 200–500 mg/l also have not affected the yield of aseptic viable explants. However, addition of 1 mg/l biocide in a culture medium showed positive effect and the yield of aseptic viable explants increased to 50 % (average data for cultivars), while the working concentration recommended by the manufacturer of 0.5 ml/l had no significant effect. The percentage of aseptic explants did not decrease during following subculture, this drug prevented the development of fungal infection on the surface and inside the nutrient medium, however, at the apical end of the explants, the output and proliferation of fungal endophytes were observed (Fig. 2, *a*).

The viability of lemon shoots was reduced during further *in vitro* conservation. After 4 months, 85.63 % of viable

Table 1. Variants surface sterilization of lemon shoot

Variant	Sterilizing solution	Concentration, %	Treatment time, min
I	Hypochlorite Ca	7	20
II	Mercuric chlorite	0.2	10
III	Dyacide	0.2	10
IVa	Veltolen™	0.2	25
IVb		0.3	25
IVc		0.5	25
Va	Novodez™	0.2	25
Vb		0.3	25
Vc		0.5	25
VIa	Domestos	10	15
VIb		20	15
VIIa		10	25
VIIb		10	30

Table 2. Effect of conservation period on the growth and viability of the lemon cv. 'Novoafonsky' *in vitro*

Conservation period, months	Shoot length gain, mm	Survival rate, %
2	6.35 ± 0.58	96.13 ± 2.23
4	4.65 ± 0.72	85.63 ± 2.60
6	1.93 ± 0.59	62.93 ± 2.92
8	0.33 ± 0.21	37.35 ± 3.44
10	0	14.60 ± 1.00

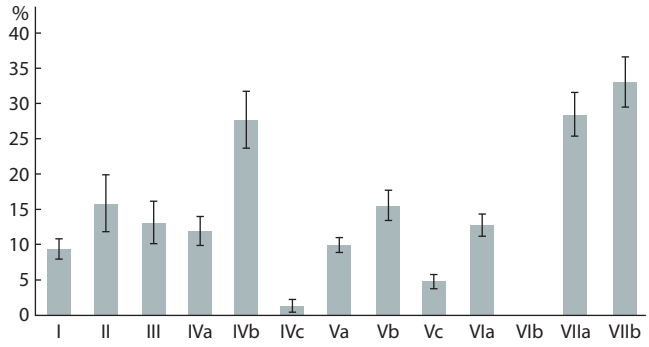

Fig. 1. Effect of sterilization treatments on sterile viable lemon buds, % (average for all cultivars).

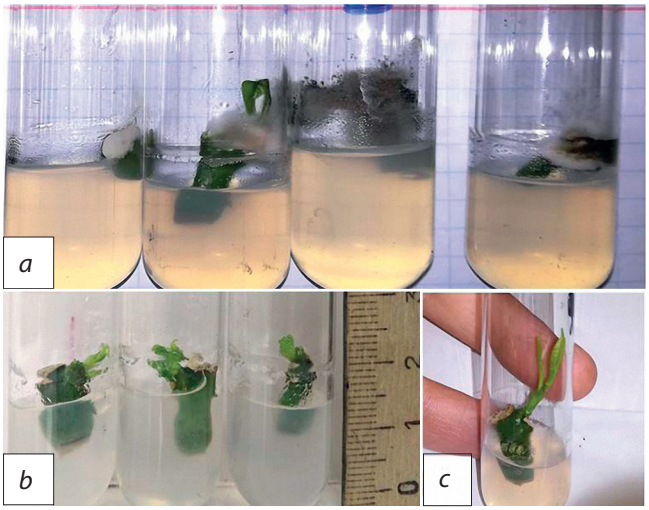

Fig. 2. Tissue culture initiation of lemon (cv. 'Frost Eureka'): *a* – growth of fungal endophytes in the medium with 0.5 ml of biocide (10 days in culture); *b* – aseptic explants in the medium with 1 ml/l biocide (20 days in culture); *c* – shoot growth initiation (35 days in culture).

plants were obtained (Table 2). The shoot length increased by 4.65 mm. During further conservation leaves of plants became yellowish and dropped. Thus, only 37.35 % of plants survived after 8 months of conservation, and their shoot length increased by 0.33 mm, and 14.6 % of plants survived after 10 months. Other lemon genotypes showed similar tendency: cv. 'Beskoluchii' and *Citrus* × *meyeri* survival rate was 10–15 % after 10 months of conservation. Three other cultivars ('Jubilee', 'Villa Franka', 'Frost Eureka') are not survived after the 10 months of conservation *in vitro*.

Significant effect of roots on the viability of plantlets during conservation was observed. The coefficients of photosynthetic activity and the viability index showed better rooted plant performance during the conservation of three lemon cultivars 'Novoafonskii', 'Beskoluchii' and 'Meyer' (Fig. 3). After 4 months of conservation, these physiological indicators were higher for rooted plants. The viability index (the ratio of the chlorophyll fluorescence maximum to the steady-state fluorescence level of the Kautsky curve) varied from 1.50 ('Meyer' lemon) to 1.94 ('Beskoluchii') in rooted plantlets (see Fig. 3). 'Novoafonskii' cultivar showed average viability index, and in rooted plantlets it was 1.75. In plantlets without a root system, viability index ranged from 1.26 ('Meyer' lemon) to 1.38 ('Beskoluchii'). The coefficient of photosynthetic activity which reflects the effectiveness of the use of light during photosynthesis, was 0.21–0.31 in rooted plantlets, and 0.12–0.16 in non-rooted plantlets. Positive correlation was observed between Viability index and Photosynthetic coefficient, and in rooted plantlets it was 0.99, in non-rooted plantlets was 0.98.

Chlorophyll *a* and *b* (*Ch a* and *Ch b*) in the leaves gradually decreased during *in vitro* conservation (Fig. 4). The content of *Ch a* decreased from 1.59 to 1.14 mg/g. A sharp decrease in *Ch b* was observed from the 1st to the 3rd months of plantlets conservation, when its content decreased by 0.16 mg, after which it did not change until the 6th month of conservation and amounted to 0.75 mg/g of fresh leaf weight. However, from the 6th to the 12th month of conservation, the content of this pigment again sharply decreased to 0.58 mg/g. During 12 months of conservation, the concentration of *Ch b* in leaves decreased by 0.33 mg. Gradual decrease in the level of carotenoids from 1.14 to 0.82 mg/g was also observed during conservation, so its concentration decreased by 0.32 mg in 12 months.

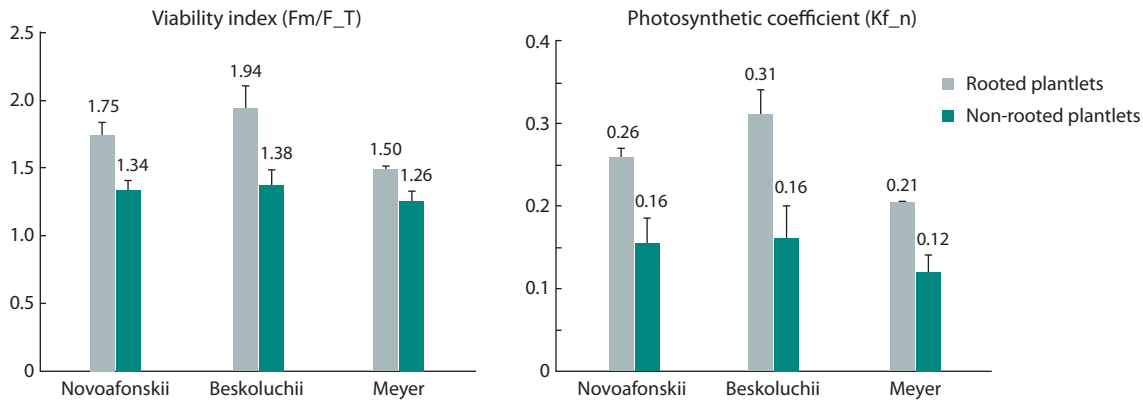

Fig. 3. Photosynthetic indices of rooted and non-rooted lemon plantlets after 4 months of conservation.

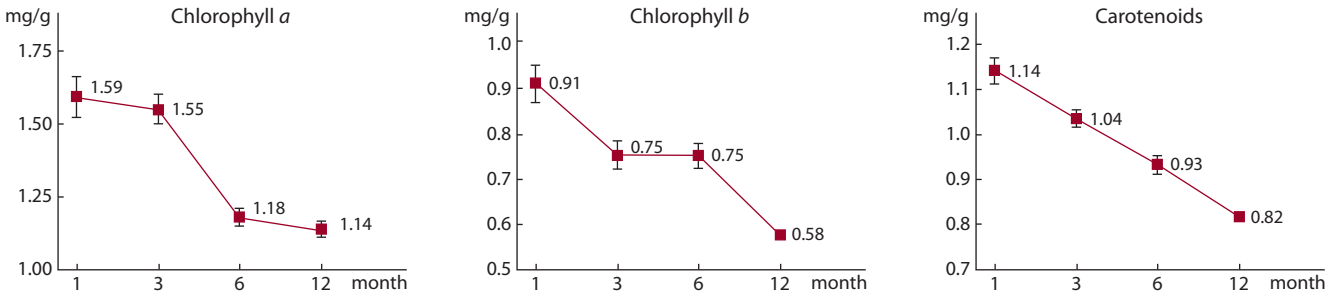

Fig. 4. Effect of conservation period on the content of chlorophylls *a*, *b* and carotenoids (mg/g of fresh leaf weight) in lemon 'Novoafonskii' cultivar *in vitro*.

Discussion

In the Black Sea coast of the Caucasus in conditions of humid subtropical climate, the endophytic fungal microorganisms inhabit woody plants abundantly (Samarina et al., 2012). During *Citrus* shoots decontamination surface of explants is cleaned of microorganisms, but inside the shoot, in the vascular tissue, the fungal mycelium is still alive. When the nodal explants or buds are placed on the nutrient medium, the hyphae of the fungus proliferates and prevents regeneration. In the most publications on the citrus *in vitro* culture, attention is not focused on the problem of contamination. Many of these articles describe simple standard decontamination protocols using 1–2 sterilizing agents (Rathore et al., 2007; Perez-Tornero et al., 2009; Samanhudi, Muji, 2010). In our work, the problem of contamination was acute, that is why we studied various ways of overcoming it. Addition of new generation of biocide, provided by the company "Gavrish", into a culture media showed a high efficiency of decontamination of explants of all lemon cultivars. However, further micropropagation and conservation *in vitro* of valuable cultivars were still problematic because of the low growth potential of regenerating plants from axillary buds.

Our results showed low values of photosynthetic coefficient of 0.2–0.3, in contrast normally in plants it has to be no lower than 0.6 units. Consequently, our plantlets showed low efficiency of using light in photosynthesis during conservation, which indicates a decrease in the growth potential. Reduction of chlorophylls and carotenoids also indicates a general low functional state of the photosynthetic apparatus, and the growth potential as a whole. The pigment content is an indicator that reflects the physiological adaptation of plants to various environmental factors (Belous et al., 2011). It can depend on many factors: humidity, temperature, lighting and others. In our work, we found a decrease in the pigment content in the leaves during slow growth conservation which may be a result of a decrease in nutrient concentrations in the medium and a change in the pH level. After 10 months of conservation only 14.6 % of lemon plantlets were survived *in vitro*. We tested different cultivars of lemon *C. limon* and other *Citrus* species such as *C. unshiu*, *C. sinensis*, *C. grandis*, *C. paradisi* etc., however the efficient micropropagation and conservation protocols for mature explants of valuable genotypes has not developed by us yet. Thus, despite the large body of successful protocols for slow growth conservation and micropropagation for many plant species, including elite genotypes (Pence, 2010), reliable micropropagation and *in vitro* conservation for citrus cultivars is still a problem.

Our conclusions are consistent with the findings of other researchers (Krueger, Navarro, 2007). They concluded that some species of plants easily growth *in vitro* using low temperatures and reducing the intensity of lighting and modification of the nutrient medium. In these protocols frequency of subculture occurs once a year. This procedure allows for the reliable conservation of many species germplasm *in vitro* (Engelmann, 1997). Attempts to replicate this approach on *Citrus* have not been successful in the world practice, since the protocols developed on juvenile material (*Citrus* seedlings), include a complex cycle of shoots from nodal segments, rooting and

recultivation (Marin, Duran-Vila, 1991), and these protocols are not applicable to adult plant explants (Krueger, Navarro, 2007). In addition, the citrus fruit has a low efficiency of rooting and micropropagation from bud explants, so the application of this procedure to adult genotypes is not effective (Krueger, Navarro, 2007). Thus, reliable *in vitro* conservation of valuable citrus genotypes is currently problematic and requires new technical solutions.

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