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PROCESSES OF ESSENTIAL OIL ACCUMULATION IN PETALS OF *ROSA* (ROSACEAE) AND MYCELIUM *EREMOTHECIUM* (EREMOTHECIACEAE)

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Introduction

Essential oil-bearing crops unite rather big group of plants, which have such a distinctive feature as capacity to have biosynthesis process and accumulate essential oils. One of these plants that has a high-valuable oil is *Rosa* L. In essential oil industry the following sorts are of great demand: *R. damascena* Mill., *R. gallica* L., *R. alba* L., *R. centifolia* L. as well as hybrids bred on their basis [9,13].

But nowadays due to obstacles, caused by huge effect of ecological factors and labor intensity, plantation cultivation can`t meet such a great demand for natural fragrant substances in the field of food production, perfume and cosmetic industry, chemical and pharmaceutical industry, what drives at development of their alternative sources. It was found out that an amount of synthesized oil in the cell culture of rose is much lower than in petals of intact plant. In this way composition of extracted oils differs from traditional rose oil. In 90s a new method of fragrant product extract was discovered, which is based on stock homotallic ascomycetes of *Eremothecium ashbyi* Guilliermond and *E. gossypii* Kurtzman, close to rose essential oil extracted out of fresh flowers [2, 3, 10, 13].

Nevertheless in the first works, points, related to accumulation of essential oil at the cellular level of study cases, weren`t discussed in comparison aspects and now provoke a great interest [10]. The study purpose was comparison analysis of structural peculiarities of accumulation process in essential oil of *Rose* and *Eremothecium,* composition and quality of synthesized substances on different development stages.

Objects and methods of the research

The study objects were Rosa cultivars: *R. alba* L., *R. centifolia* L., *R. gallica* L., *R. damascena* Mill., *R. rugosa* Thunb., *R. canina* L., *R. cinnamomea* L., *R. odessiana* hort., *R. lutea* Mill., stocks *E. ashbyi* Guilliermond: BKMF-124, BKMF-3009, BKMF-4565D, BKMF-4566D, ВКПМF-36, ВКПМF-340 and *E. gossypii* Kurtzman: BKMF-2627, BKMF-3276, that differ by level of synthesis and monoterpenic accumulation, as well as fragrant alcohols as principal components of rose essential oils.

Researches were carried out with plants, cultivated on collection areas (village Krymskaya Roza – Belogorsk region, the Republic of Crimea), located in north piedmont part of the Crimean peninsula, as well as under conditions of Botanical garden named after I.I. Sprygin (the city of Penza). Plant material was registered on the late phase of onthogenesis (generative period, budding and blooming phase) in acetalcohol (1:3) and 6% in formalin; cross sections were prepared by a razor or applying freezing microtome (agreeable method) [6].

Micromycetes were supported on skew sow and sucrose, potato and glucose, glucose and peptonic with yeasty extract, Sabouraud and Chapeka mediums, wort and maltha agars.

Stock cultivation was conducted in liquid and nutrient mediums under conditions that correspond to those published before [10].

Microscopy of native and colored preparations with methylene blue, iodine, sudan III, black ink was implemented using microscopes MIKMED-1, BIOMED-6 (magnification degree 4, 10, 40, 100). Cameras Nikon Coolpix 2500, Nkon Coolpix 6300, Panasonic DMC-FX100 were applied to make photographs of micro- and macro-objects. Micropreparatons were described according to modern methodical and reference data [6].

Patterns for electronic microscopy (24,36, 48, 56, 64 hours of micromycetes cultivation) were prepared applying 5% solution of glutaraldehyde in phosphate buffer as a fixative (pH 5,8-7,0 depending upon pH of cultural liquid on a certain stage of study stock development). Fixation was keeping on for 24 hours, after that material was washed with phosphate buffer for 10 minute and fixed again for 1,0-1,5 hours with 1% water solution O_sO_4.

Later the patterns were extra stained for 12 h by 5% uranyl acetate prepared in 70% ethanol. The ultrathin sections were made by an ultramicrotome and imbued for 5 min by Reynolds' lead citrate. Then they were investigated via a microscope JEM-100 C (magnification 15000-41000).

For quantification of the essential oil, the method described in the Russian State Pharmacopoeia (XI ed.) [4], or the gravimetry after triple extraction by an organic solvent were used. The solvent was removed by a rotor vacuum evaporator. The oil component composition was tested by gas-liquid chromatography (polar column, flame-ionization detector).

Experimental data was processed statistically in accordance with G.F. Lakin [5], significance level p=0,95.

Results and discussion

The results of the anatomical investigation of rose (dog roses) petals have shown that adaxial epidermal cells are polygonal, adjoin closely to each other, stretch to cone-shaped nipples, and are covered by a highly rugous cuticle. The abaxial cells are elongated, with slightly anfractuose or straight walls, have the cuticle (Fig. 1). The separate groups of the papilate adaxial (in case of *R. alba, R. gallica, R. damascena* also abaxial) epidermal cells are able to synthesize and secrete the essential oil which accumulates in small drops under the cuticle and causes its detachment and spot formation. The content of the cells in an epidermis is colored because of pigments dissolved in vacuoles. It adds different roses (*R. damascena, R. cinnamomea, R. canina, R. odessiana*), red (*R. gallica, R. centifolia, R. rugosa*) shades to the petals during the budding and blossoming periods. Small stomata are anomocytic, mostly located on the inferior petal side with low frequency of occurrence.

The parenchymal tissue consists of layers of non-colored rounded or irregular cells with thin walls which form a big intercellular space (*R. canina* has considerably less of it). The number of the parenchymal layers ranges from 4-6 (*R. lutea, R. centifolia, R. cinnamomea*) to 10-12 (*R. gallica, R. alba, R. rugosa*). The strengthening tissue was not found. The vascular tissue is presented by spiral tracheides surrounded by small parenchymal cells adjoined closely to each other. The tracheides are located in groups of 8-10 or 3-4 (*R. gallica, R. rugosa*).

By rose flower tripping the endogenous secretory structures – essential oil lysigenous oval conceptacles are deeply located in the parenchymal tissue and contain drops of the essential oil (Fig. 1, Table 1).

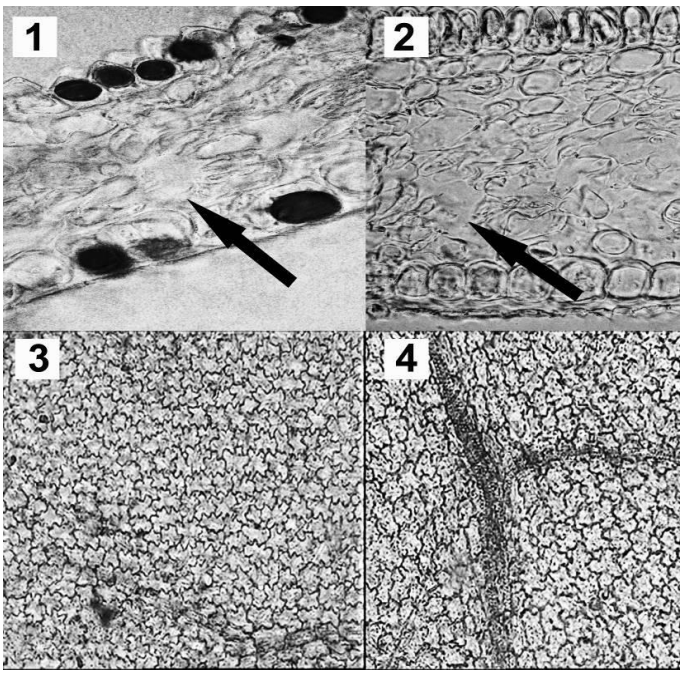

Fig. 1. The anatomical structure of the rose petals: 1 (*R. rugosa*), 2 (*R. gallica*) – transverse sections (lysigenous essential oil conceptacles are pointed by arrows; magnification x40); the surface: 3 – adaxial *R. cinnamomea*; 4 – abaxial *R. canina* epidermal cells (magnification x10)

Table 1

Anatomical-morphological features of secretory structures of different oil-bearing rose species

Cultivar	Glandular epidermal cells		Endogenous structures (secretory conceptacles)			
	availability	localization	localization	frequency, lim pcs/mm^2	sizes, μm	sizes interrelation of lysigenicous conceptacles and parenchymal cells
R. cinnamomea	–	–	close to a surface (2-3 cell layers)	1,29-2,08	89,6±10,2 × 86,1±9,8	0,95
R. canina	+	adaxially	in a depth of parenchyma (4 cell layers)	0,62-1,65	64,3±5,2 × 62,6±4,5	0,63
R. odessiana	+	adaxially	in a depth of parenchyma (3-6 cell layers), uniformly	0,55-1,32	83,0±8,2 × 82,1±8,3	0,88
R. rugosa	–	–	in a depth of parenchyma (4-6 cell layers)	1,58-2,69	88,7±9,2 × 85,4±8,8	0,94
R. alba	+	adaxially and abaxially	close to a surface (3-5 cell layers)	0,52-1,15	106,6±14,2 × 96,5±9,1	0,95
R. lutea	–	–	close to an abaxial epidermis (4 cell	4,98-6,01	84,9±11,1 × 80,7±10,8	1,01

			layers)			
R. gallica	+	adaxially and abaxially	in a depth of parenchyma (4-6 cell layers), uniformly	3,83-4,68	75,5±8,4 × 61,2±7,0	0,83
R. centifolia	+	adaxially	in 2-3 parenchymal layers	2,83-3,68	81,2±9,4 × 66,7±7,6	0,84
R. damascena	+	adaxially and abaxially	close to a surface (2-3 cell layers)	4,83-5,68	87,4±10,2 × 66,7±×6,8	1,13

Note: «+» – structures were found out; «–» – structures were not found out.

Their sizes, localization and frequency ranges. For instance, the conceptacles of *R. canina, R. gallica* are small and evenly distributed in the parenchyma deep. The ones of *R. alba, R. cinnamomea, R. rugosa* are large, rare and located in different way: close to the petals surface (*R. alba*); by groups adjoining to the epidermis (*R. cinnamomea*); in the parenchyma deep (*R. rugosa*).

It should be noticed that cell conversion to the essential oil biosynthesis occurs during the late ontogenetic phases (generative period) and concurs with the budding and blossoming. The interconnection of localization of the endogenous secretory structures and the mass concentration of the volatile fragrant substances per petal raw mass was revealed during the qualitative and quantitative analysis of the essential oil extracted from plant raw materials. It is shown, the maximum levels of the rose oil content is typical for *R. damascena*, *R. lutea* which have the frequent volatile-oil-bearing conceptacles which are closely located to the adaxial and abaxial epidermis and have bigger sizes than the adjoined parenchymal cells (Table 1, 2). Thus, the mass concentration of the essential oil and, conformably, the level of the synthesis and the accumulation of its components in the rose petals are lower when the secretory structures are deeply located in the parenchymal tissue, scarce, and small-sized.

Table 2

Comparative characteristics of the studied rose species

Name	Petals color	Flower type [11]	Essential oil content (EOC), %
R. canina	pale pink	simple	0,0435
R. odessiana	pale with a pink bottom	simple	0,0487
R. alba	white	semidouble	0,0518
R. cinnamomea	pink	simple	0,0702
R. rugosa	red	simple	0,0767
R. gallica	ruddy	double	0,0865
R. centifolia	dark red	thickly double	0,1148
R. lutea	yellow	semidouble	0,1413
R. damascena	pale pink	semidouble	0,1528

As the rose and eremothecium oils have similar qualitative and quantitative component composition, it is supposed that ways of the essential oil synthesis, its intracellar transport, and mechanisms of its excretion in micromycetes could be like in the oil-bearing rose.

Findings are evidence that there are electron light lipid bodies in hyphae of the one-day submerged culture. They are located in the intermembranous space of an agranular endoplasmic reticulum. As a rule these lipid bodies appear in 36 h when the spores germinate and the mycelium starts to form. The quantity of spherosomes changes synchronously in

accordance with the level of the essential oil accumulation in the cultural liquid. In 36-48 h of cultivation the frank vacuolization of the mycelium was noticed. There were the osmiumphilic lipid bodies in the formed vacuoles (Fig. 2, 3). More intensive vacuolization, presented by the numerous small vacuoles, was characteristic for the strains with the high intensity of the essential oil synthesis (for instance, VKPM F-340). Moreover, high level of vacuolization was noticed from the earlier to the later ontogenetic stages.

During all development phases there were the lipid bodies of another type which were electron-dense (osmiumphilic), rounded. The small ones merged to the bigger formation of irregular shape (flows). It was typical for all study strains that in the beginning of the stationary phase, increase of efficiency of the essential oil synthesis was accompanied with the rise of spherosome quantity and sizes. This causes the intensification of affinity for osmium (Table 3).

Table 3

Comparative characteristics of results of essential oil synthesized by *Eremothecium* and *Rosa* species

Strain-producer, species of oil-bearing rose	Ratio			Efficiency of production process within essential oil, mg per g of biomass per h
	2PE/ MTA	geraniol/ citronellol	geraniol/ nerol	
E. ashbyi				
VKMF-4566D	0,08-0,31	0,98-6,90	2,22-10,17	0,825-1,237
VKMF-4565D	0,01-0,37	4,66-12,21	15,64-48,86	1,032-1,682
VKPM F-36 (NRRLY-1363)	0,22-0,39	2,51-7,04	3,65-68,20	0,930-1,358
VKPM F-340	0,11-0,24	37,62-51,94	– **	0,976-1,240
VKM F-3009	0,02-0,12	9,12-15,30	13,6-24,65	0,813-1,298
VKMF-124	0,11-0,28	12,43-16,65	6,96-12,21	0,158-0,239
E. gossypii				
VKMF-3276	0,79-1,29	7,76-13,21	4,79-26,42	0,627-2,198
VKMF-2627	1,12-1,27	13,92-77,33	6,96-34,72	1,514-1,915
R. alba	0,05-0,13	0,26-1,20	1,43-3,20	0,002-0,003
R. gallica	2,33-3,00	2,00-2,40	1,00-4,20	0,004-0,006
R. damascena	0,04-0,08	0,13-1,12	0,55-7,67	0,001-0,005
R. rugosa	0,01-0,14	0,14-0,53	3,07-4,21	0,002-0,006
R. canina	2,02-3,23	– *	– **	0,001-0,003
R. centifolia	0,99-2,14	1,17-35,00	1,05-2,10	0,004-0,006
R. lutea	– ***	– *	– **	0,004-0,007

Note: 2PE – 2-phenylethanol; MTA – monoterpene alcohols; –* – citronellol was not identified; –** – nerol was not identified; –*** – cyclohexanone was identified.

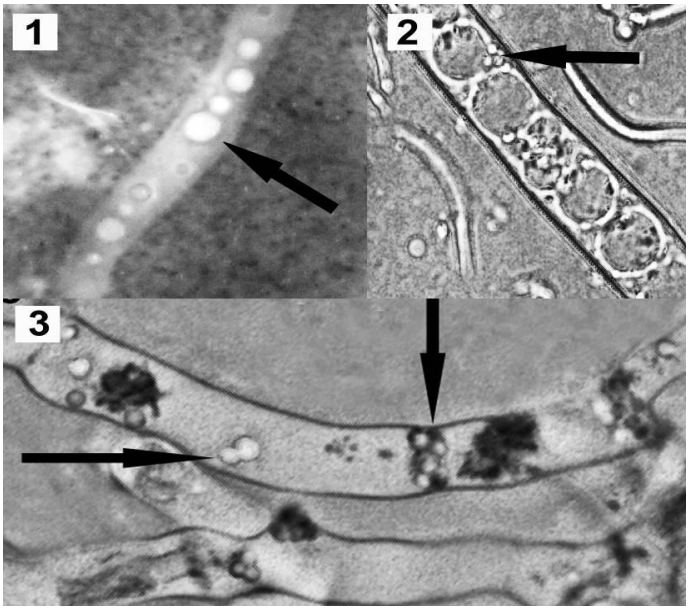

Fig. 2. The anatomical structure of *Eremothecium* oil synthetizing hyphae (lipid bodies are pointed by arrows; magnification x100) stained by: 1 – ink; 2 – iodine; 3 – Sudan III

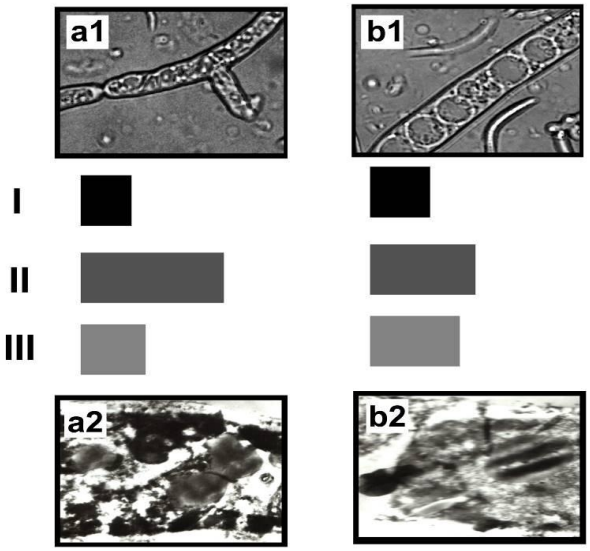

Fig. 3. The cytomorphological features of the *Eremothecium* mycelium and the level changes in the accumulation of the main biologically active substances: I – riboflavin, II – essential oil, III – geraniol; a_1, a_2 – 36 h, b_1, b_2 – 48 h of cultivation in a liquid nutrient medium (magnification: a_1, b_1,– x100; a_2 – x18500; b_2 – x20000)

The interconnection between increase of the total protoplasm affinity to osmium and rise of the total biosynthetical activity of every strain was revealed. At the same time the rounded lipid bodies started to locate near the main cell membranes (plasmalemma, tonoplast). The maximum of the essential oil productivity of the study taxons concurred with the massive flows of the electron-dense substance to the cell membrane. The affinity to osmium for all strains significantly decreased after lipids excretion into the environment. The lipid bodies practically disappeared. The electron-dense content was not found in the vacuoles. Besides the lipid bodies might get into the environment because of the cell lysis as a result of aging.

Analyzing structural organization of the essential oil synthesis and its accumulation among representatives of the *Eremothecium* genus in comparison with *Rosa,* it should be

taken into account, that the study objects have the significant micromorphological differences caused by their taxonomic positions. The fungal cells form hyphae which make mycelium (Fig. 2). And no further tissue differentiation like in the oil-bearing rose occurs. So the described earlier secretory structures are not typical for the *Eremothecium* species. Moreover, the differences, like in the rose cells, in the productional ability of the separate mycromycete cells were not revealed. Concerning the synthesis of other secondary metabolites, that is riboflavin and its forms, it is known that only certain cells of the mycelium (~60%) are able to synthesize and accumulate them, and the rest (~40%) do not have this ability [10]. This assumption is based on the results of the microscope investigation which has shown that vitamin crystalline inclusions were not found in each cell. Moreover, there is no reliable data about permeases, participating in the active transport of the synthesized riboflavin to the environment and genes coding them.

The findings of the histological investigation of the rose species is coordinated with the data described previously for kinds Krymskaya Krasnaya [3] and Anna [1] belonging to *R. gallica* and *R. hybrida,* and *R. rugosa* [14]. Every petal tissue is characterized to synthesize the certain component of the essential oil. For instance, in the petals of the Michurinka kind the synthesis of the main monoterpene alcohols (MTA) took place mostly in the adaxial and abaxial layers of the epidermis, but 2-phenylethanol is mainly produced in the parenchyma. This might be connected with the difference in the influence of endogenous and exogenous factors on the genes participating in the synthesis of the certain substances in the differently located cells. From our point of view, the MTA biosynthesis is associated with the presence (level) of active oxygen, and the synthesis of an aromatic alcohol is intensified in the anaerobic conditions that might be observed in the rose petals tissue.

Due to the fungi organization the plastids, particularly the leucoplasts, are absent in their cells. So the synthesis of the main MTA precursor, isopentenyldiphosphate, from 2-C-methyl-D-erythrol-4-phosphate (Fig. 4, 5) [12] cannot be realized.

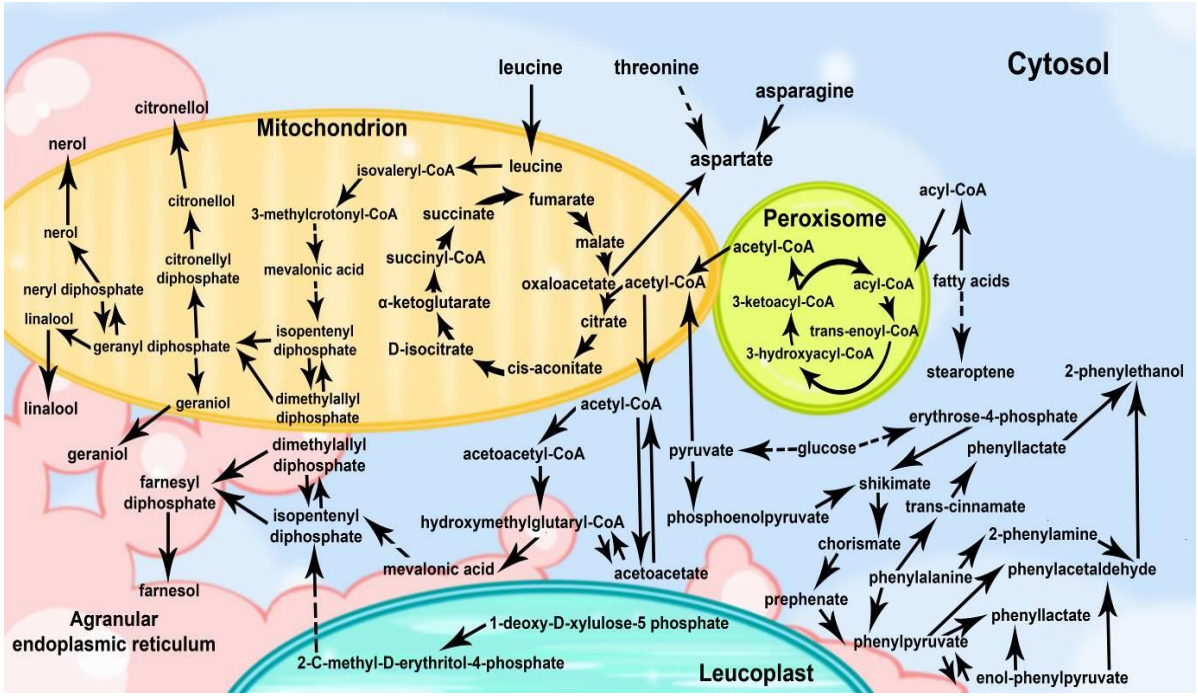

Fig. 4. The hypothetic metabolic model of the biosynthesis of the main essential oil components in the petals of the oil-bearing rose

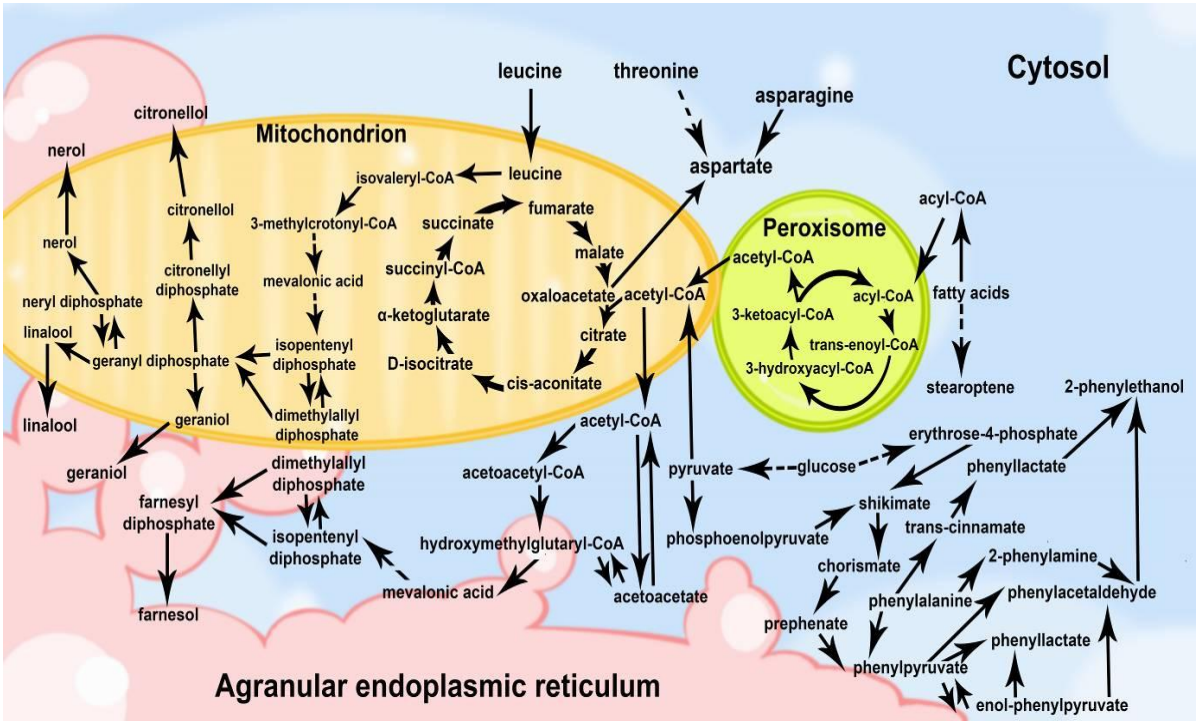

Fig. 5. The hypothetic metabolic model of the biosynthesis of the main essential oil components in the *Eremothecium* mycelium

Perhaps for *E. ashbyi* and *E. gossypii* the synthesis of this precursor can be accomplished only due to mevalonic acid which is produced in a cytosol and mitochondria from hydroxymethylglutaryl-CoA. Isopenthyldiphosphate isomerizes into dimethylallyldiphosphate, and then after condensation of these two compounds farnesyldiphosphate or geranyldiphhosphate might be formed depending on the precursors localization. Such MTA as linalool, nerol, and citronellol come from geranyldiphosphate due to the specific reactions. The formation of the oxidated terpene compounds is connected with the agranular endoplasmic reticulum. Moreover, the production of other lipophilic compounds (stearoptenes, triacylglycerols, fatty acids, etc.) is common for both *Rosa* and *Eremothecium*. It is possible that the structural organization of the plant cells and the hyphae is caused by the intensity of the processes of the secretion synthesis and its transport. The morphological parameters of the vacuoles and the spherosomes (quantity, sizes, localization) characterize the mycelium functional activity like in the rose cells [1]. The increase of the vacuolization is observed from early to late growth stages that correspond to the data related to the oil-bearing rose [15]. Besides it was found that the rounded lipid bodies are located at the same places like in the rose petals: near the plasmalemma and the tonoplast. The maximum of the essential oil productivity of the *Eremothecium* strains concurred with the massive flows of the electron-dense substance to the cell membrane. The same phenomenon was noticed in the rose petal cells [3, 15].

Conclusions

The results of the carried out research permit to make the following conclusions:

1. The rose secretory structures are presented as the glandular epidermis and the endogenous oval conceptacles which are located in the deep of the parenchymal tissue and contain the drops of the essential oil.

2. The small volatile-oil-bearing conceptacles in the parenchyma deep are characteristic for collection samples with the low essential oil content.

3. The over-synthesis of the fragrant substances among species of the *Eremothecium* genus is closely connected with the high cell functional activity which becomes apparent as

the increase of the spherosomes and the filling of the vacuoles by the osmiumphilic substance like in the case of the rose.

4. The fungal secretion of the fragrant substances to the environment is supposed to be one of the regulatory functions of their synthesis ("mechanism of overfill").

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Semyonova Ye.F., Shpichka A.I., Presnyakova Ye.V., Mezhennaya N.A. Processes of essential oil accumulation in petals of *Rosa* (Rosaceae) and Mycelium *Eremothecium* (Eremotheciaceae). // Bull. of the State Nikit. Botan. Gard. – 2016. – № 118. – P. 25-33.

The plantation cultivation of an oil-bearing rose is not able to cover the increasing demand of the industry. Therefore, the interest to fungi strains *Eremothecium ashbyi* Guilliermond and *E. gossypii* Kurtzman, is rising. The features of secretory structures of the *Rosa* and *Eremothecium* species were found out. The investigation of biosynthesis, accumulation, and secretion of essential oils with a rose scent is crucial either for development of new ways to produce them or for rating the biological role of *Rosa* and *Eremothecium* secondary metabolites.

Key words: *essential oil; oil accumulation; spherosomes; secretory structures; Rosa; Eremothecium*

PLANT BIOCHEMISTRY

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BIOLOGICALLY ACTIVE SUBSTANCES OF NEPETA CATARIA L.

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Introduction

Nepeta Cataria L. is a perennial plant that belongs to Lamiaceae family. Overground mass of the cultivar possesses pretty lemon fragrance, pungent taste and takes a great interest in the field of food, perfume and soil boiling industries.

Various preparations that include *Nepeta Cataria* are used in folk medicine as spasmolytic, carminative, tonic and stimulative remedy. Besides, extracts of its overground part can be used to treat gastrointestinal and respiratory diseases, stagnations of gallbladder and bile passages, histerical and depressive attacks [6, 12].

Complex of biologically active substances, such as volatile compounds, phenolic substances and vitamins in plant material of *Nepeta cataria* specifies its medicinal properties [5, 8, 14].

Essential oil (EO) has got a fine herbaceous and citrus fragrance and presents high antimicrobial action. Content and component composition of *Nepeta cataria* EO ranges a lot what depends upon ecological and genetical factors. Essential oil includes geranial, geraniol, camphora, carvacrol, caryophyllene, nepetalactone, nerol, citral, citronellal, citronellol and eugenol [9, 15]. Phenolic compounds of *Nepeta cataria* are presented by hydroxicoric acids – rosemary, caffeic, n-cumarin, ferulic acids; and flavonoids – flavones (apigenin, lutheoline) and flavonols (quercetin, kaempferol, myricetin) [2, 7, 8].