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Original Study Article

Senescence metabolomics of *Nicotiana tabacum* L. VBI-0 heterotrophic suspension cultures

Roman K. Puzanskiy [1, 2], Anastasiia A. Kirpichnikova [2], Alexey L. Shavarda [1, 2], Vladislav V. Yemelyanov [2], Maria F. Shishova [2]

[1] Komarov Botanical Institute of the Russian Academy of Sciences, Saint Petersburg, Russia;
[2] Saint Petersburg State University, Saint Petersburg, Russia

ABSTRACT

BACKGROUND: Heterotrophic cell cultures are widely used as a model in plant biology. During a culture cycle the composition of the medium changes: the sucrose and other substrates are depleted, metabolism products are accumulated and the density increases. Finally, arrest of a growth is followed by cell death in a short time. These processes are accompanied with physiological alterations, corresponding to senescence.

AIM: To resolve metabolic features of tobacco cells in growing and stationary senescent suspension cultures VBI-0.

MATERIALS AND METHODS: *Nicotiana tabacum* VBI-0 cells were cultured in suspension MS medium supplied with 3% sucrose. Cells were sampled at 7th day, during intensive growth, and at 28th day, when the culture was in the stationary phase. The GC-MS method was used to profile the metabolites.

RESULTS: Sucrose depletion in media caused starvation of heterotrophic tobacco cell culture and was associated with a decrease in the accumulation of free amino acids. At the same time, the level of pentoses and complex sugars, including sucrose, increased, while the levels of glucose and fructose were not changed significantly and levels of hexose phosphates decreased. During culture senescence cells showed higher levels of accumulation of malate, pyruvate and some other carboxylates.

CONCLUSIONS: The metabolomic data indicate that culture senescence was associated with a drop in amino acids metabolism, a decrease in the activity of the upper part of glycolysis, and the accumulation of complex sugars, pentoses and carboxylates.

Keywords: *Nicotiana tabacum* VBI-0; metabolomics; plant cell culture; senescence; carbon starvation.

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Оригинальное исследование

Метаболомика старения гетеротрофных суспензионных культур *Nicotiana tabacum* L. VBI-0

Р.К. Пузанский [1, 2], А.А. Кирпичникова [2], А.Л. Шаварда [1, 2], В.В. Емельянов [2], М.Ф. Шишова [2]

[1] Ботанический институт им. В.Л. Комарова Российской академии наук, Санкт-Петербург, Россия;
[2] Санкт-Петербургский государственный университет, Санкт-Петербург, Россия

АННОТАЦИЯ

Гетеротрофные клеточные культуры широко используют в качестве модельных объектов в биологии растений. В процессе развития культуры меняется состав среды: истощается субстрат, накапливаются продукты метаболизма, растет плотность клеток. В финальной фазе рост останавливается и через непродолжительное время культура погибает. Эти процессы сопровождаются физиологическими изменениями клеток, которые можно назвать старением культуры. Методом газовой хроматографии, сопряженной с масс-спектрометрией, было проведено профилирование метаболитов гетеротрофных клеток *Nicotiana tabacum* VBI-0, поддерживаемых в суспензионной культуре. Сравнивали клетки культур возрастом 7 сут, во время интенсивного роста биомассы, и 28 сут, когда культура находилась в стационарной фазе. Было установлено, что старение сопряжено с падением накопления аминокислот. В то же время возрастал уровень пентоз и сложных сахаров, включая сахарозу, тогда как уровень глюкозы, фруктозы и сахарофосфатов снижался. Для стареющих культур характерен больший уровень накопления малата, пирувата и некоторых других карбоксилатов. Таким образом, полученные метаболомные данные свидетельствуют, что старение сопряжено с изменением обмена аминокислот, снижением активности начального этапа гликолиза, накоплением сложных сахаров, пентоз и карбоксилатов.

Ключевые слова: *Nicotiana tabacum* VBI-0; метаболомика; культура растительных клеток; старение; суспензионная культура.

Как цитировать

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BACKGROUND

Cell cultures of higher plants, including transformed ones, are widely used as model systems for studying numerous processes, including cell division, embryogenesis, differentiation, various stages of primary and secondary metabolism, etc. [1, 2]. The study of cultures enables us to understand the cellular mechanisms underlying the adaptation of plants to biotic [3] and abiotic stressors [4] better. Additionally, plant cell cultures are in wide demand in modern biotechnology for the production of biologically active compounds, heterologous proteins, etc., [5]. The application of aseptic, strictly controlled conditions enables the achievement of high reproducibility of results, cell cycle synchronization, and enhanced growth rates [2, 6]. Plant cell cultures can be either photosynthetic [7] or heterotrophic [1]. Heterotrophic cultures are maintained in the dark, and their only sources of carbon and energy are the organic compounds included in the medium. Plant cells are capable of consuming exogenous organic compounds, especially sucrose [8] which is the main form of transported carbon in higher plants [9].

Sucrose metabolism begins with cleavage, which is catalyzed by two types of enzymes, invertases and sucrose synthases. Invertases hydrolyze sucrose into glucose and fructose. Acidic invertases are localized to the vacuole, and neutral/alkaline invertases to the cytoplasm and apoplast [10]. Sucrose synthases cleave sucrose molecules into fructose and uridine diphosphoglucose, which is a precursor for the synthesis of many metabolites. Several sucrose metabolism pathways can probably operate in parallel, and thus, the question arises about their relationship under normal and stressful conditions. The products of sucrose hydrolysis can be catabolized through glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate pathway to produce energy. Conversely, exogenous carbon can be involved in the accumulation of biomass, directed into the synthesis of amino and fatty acids or other compounds. In addition, it can be stored in the form of starch or lipids. The activity of the listed metabolic processes depends on the species' characteristics, the physiological and biochemical activity of its constituent cells, and developmental alterations.

During cultures growth, the substrate is exhausted and starvation occurs, the first consequence of which is a decline in physiological activity. Removal of sucrose rapidly decreases respiration levels [11, 12]. The expression of genes encoding the TCA cycle and oxidative phosphorylation enzymes is suppressed [13]. The glycolysis level also decreases, due to a reduction in the contents of sugar phosphates [14, 15] and the expression of the corresponding genes [11, 13]. In addition, when cells are starved, reserves are mobilized. Vacuolar pools of sucrose and malate serve as a rapid but short-term carbon source [14]. An enhancement in the expression of certain genes associated with starch hydrolysis was noted [13]. Lipid mobilization occurs, which is ensured by the induction of genes encoding lipases and fatty acid oxidation-related enzymes [11, 13]. Simultaneously, membrane degradation may occur [16]. The presentation is complemented by a decrease in the expression of genes for enzymes involved in fatty acid biosynthesis [11]. Starved cells are characterized by high proteolytic activity since amino acids can also be a carbon source during starvation. This is indicated by an increase in the expression levels of genes encoding enzymes associated with amino acid catabolism [17], especially the branched-chain amino acids [18]. This process entails a restructuring of nitrogen metabolism [19]. However, in heterotrophic cultures, starvation can only last for a highly limited period. The cell viability of Arabidopsis suspension cultures begins to decline rapidly, after 24 h of starvation; after 48 h, the culture loses the ability to recover after passaging, which indicates the impossibility of restoring a new development cycle that begins with proliferation [11].

The depletion of nutrients and other environmental changes induces several physiological processes that end with the death of the cultured cells; this stage can be called senescence. Growth stops, the level of respiration and synthetic processes decreases [20, 21]; the composition of fatty acids changes [22]; and the morphology and number of organelles alter [23–25]. The comparative analysis of the processes occurring in plant cell cultures during starvation and at late stages of development, with those during ontogenetic or induced senescence of cells in native organs, is of particular interest [19, 26]. It was established that old starving heterotrophic Arabidopsis cultures differed markedly from ontogenetically aging organs at the transcriptional level; of the genes whose expression increased in senescent cell culture, only about 40% were enhanced in leaves during dark-induced senescence [27]. A further study of the features of ontogenetic senescence, carbon starvation and senescence of cell culture is necessary to examine the development and trophic levels of plant adaptability.

It can be concluded that substrate uptake and the metabolic response to its deficiency are primarily associated with the central or primary metabolism. It is a collective concept of a set of metabolic processes that provide the cell with carbon, energy, and metabolites necessary to maintain life, as well as precursors for the synthesis of secondary compounds [28, 29]. The set of primary metabolites, mostly represented by small molecules such as C_2–C_5 carboxylic acids, amino acids, monosaccharides, fatty acids, etc., constitute a specific metabolic profile that characterizes the state of a biological object. Gas chromatography coupled with mass spectrometry (GC-MS), one of the fundamental techniques in metabolomics, is a promising method for metabolic profiling [30].

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The present study compares the metabolomic profiles of the heterotrophic cell suspension culture VBI-0 obtained from the stem parenchyma of *Nicotiana tabacum* L. cv. Virginia Bright Italia 0 [31]. VBI-0, like the culture By-2 (*N. tabacum* L. cv. Bright Yellow 2), is easily synchronized and has a specific filamentous phenotype [32]. Cells at different stages of development are well distinguished morphologically; they are small, forming chains during the proliferation period of the first week of growth, but single, large, and elongated after two weeks in the stationary phase [33]. The cells from these two stages, contrasting in their physiological state, were used in this study. Metabolite profiling was performed during the period of biomass growth (day 7) and at the stage of senescence, on the eve of death (day 28). The work aimed to identify metabolic variations between cells from the growing and senescent stationary suspension cultures of VBI-0.

MATERIALS AND METHODS

Plant material

The etiolated *Nicotiana tabacum* suspension cell culture (VBI-0) was maintained in the dark, at 26 °C and with constant stirring on a rotary shaker (120 rpm). Samples were collected at 7 days (biomass growth) and 28 days (senescence) by filtration using a water jet vacuum pump. For analysis, 200 mg of crude biomass was used.

Sample preparation

Samples were flash-frozen in liquid nitrogen. The cells were disrupted in a TissueLyser LT bead mill (QIAGEN, Germany) and extracted with cooled methanol–chloroform–water at 5 : 2 : 2. The extract was cleared from debris by centrifugation for 10 min, 15,000 g at 4°C. Then the extract was evaporated in a vacuum evaporator. The dried material was dissolved in a mixture of pyridine and silylating agent BSFA–TMCS (99 : 1), and added with an internal standard (tricosane, normal hydrocarbon C_{23}). The material was derivatized by incubating the samples at 90°C for 20 min.

Metabolite profiling

For the GC-MS analysis, Agilent 5860 gas chromatograph coupled to Agilent 5975C mass spectrometer (Agilent Technologies, USA) was used. Agilent 7693A autosampler was utilized for automatic sample injection. Separation was performed using a DB5-HT capillary column (Agilent). The carrier gas used was helium, with a constant flow of 1 ml/min. The evaporator temperature was 250 °C, applied in a splitless mode. The initial column thermostat temperature was 70°C, followed by a linear increase at a rate of 4°C/min up to 320°C.

The PARADISe software [34] in combination with NIST MS Search (National Institute of Standards and Technology, NIST, USA) was utilized to process the chromatograms. Additionally, AMDIS (Automated Mass Spectral Deconvolution and Identification System, NIST, USA) was applied for the deconvolution and identification of metabolites. Compounds were annotated by matching the obtained mass spectra (MF > 800) and Kovacs retention indices with library records in NIST2020 (USA), Golm Metabolome Database (GMD, Germany) [35], and the homelibrary of the Laboratory of Analytical Phytochemistry of the Botanical Institute of the Russian Academy of Sciences (St. Petersburg, Russia).

Statistical analysis and visualization

Data analysis was performed in the R4.3.1 “Beagle Scouts” environment. Data were normalized to the observation median, logarithmized, and standardized. If a compound was absent in a sample but present in the remaining replicates, this was considered a technical error and imputation was performed using the KNN (k-nearest neighbors) method utilizing the impute package [36]. Principal component analysis (PCA) was performed using pcaMethods [37]. Orthogonal projections in latent structure-discriminant analysis (OPLS-DA) were performed utilizing the ropls package [38]. The fgsea package was applied for metabolite set enrichment analysis (MSEA) [39]. Metabolite sets for identifying the biochemical pathways for MSEA and reaction pairs for metabolic map construction were downloaded from the KEGG database [40] using the KEGGREST package [41]. The list of metabolites belonging to different biochemical pathways was manually adjusted as required pathways were added for certain metabolites. Compounds for which a class was annotated were placed in the appropriate paths. The metabolic map was constructed on the Cytoscape platform [42].

RESULTS

On day 7, the cultures were characterized by an intensive increase in biomass density. On day 28, the raw mass density did not change for >7 days, which indicates complete exhaustion of resources and accomplishment of culture development (Fig. 1).

The metabolite profiles were obtained using GC-MS, which included ~300 compounds; 84 of them were identified by matching the mass spectra and retention indices with those from the library, and a chemical class was annotated for another 44. The remaining spectra were unidentified but still analyzed. The most abundant profiles obtained were sugars and their derivatives (60 in total), sugar alcohols, sugar acids, phosphosugars, pentoses (including ribose), hexoses (including glucose and fructose), and oligosaccharides such as sucrose. Several complex molecules containing sugar residues were annotated, apparently including numerous di- and

trisaccharides, as well as secondary compounds such as glycosides. The profiles also included 24 amino acids, consisting of 17 standard ones, and 20 carboxylic acids, which included the intermediates of energy metabolism. Free fatty acids and sterols were detected in small quantities.

PCA was performed to determine the similarity of metabolite profiles of the cultures in the growth and senescence phases. The profiles were scattered in the count space of the first two principal components (PCs) (Fig. 2). The observations were separated according to age along PC1, explaining 56.8% of the variance. Individual differences among the cultures of the same age were associated with PC2 explaining 23%.

Since metabolite profiles were grouped based on the culture development stages, OPLS-DA was performed next to identify metabolites that accumulated differentially depending on the development stage. We selected senescence-associated metabolites based on the VIP (variable importance in projection) values [38]. The results are presented on a simplified map of central metabolism (Fig. 3), plotted based on the main reaction pairs (substrate–product) accessed from the KEGG database [40].

Senescent cultures differed from growing ones by enhanced levels of complex sugars, including sucrose, while those of glucose and fructose did not vary remarkably. Simultaneously, the volumes of hexose phosphate pools either did not change or declined. Senescence was also associated with the accumulation of pentoses and, to a limited extent, hexoses. The level of the final product of glycolysis, pyruvate, as well as malate, was higher in stationary cultures. However, those of other detected TCA cycle intermediates did not alter. Interestingly, those of many other carboxylic acids not directly involved in energy metabolism also increased. The most striking difference in the metabolite profiles of senescent cultures appears to be the reduced accumulation of most free amino acids. These include the nitrogen metabolism intermediates, namely glutamate, glutamine, and ornithine. Interestingly, in contrast, a significant accumulation of urea was noted. Notably, aminocyclopropanecarboxylic (ACC) acid, a precursor of ethylene, was detected only at the growth stage. Almost no alterations were registered in the sterol and free fatty acid levels. The exception was a marked decrease in one unidentified sterol and an increase in one very long chain (lignoceric) fatty acid (C_{24}) (Fig. 3).

Enrichment analysis was performed to determine the relationship between these changes and the biochemical pathways activated. It enables the assessment of the strength and probability of directed coordinated changes in the accumulation of a particular set of metabolites [39]. We used lists of metabolites from biochemical pathways downloaded from the KEGG database [40]. The results indicate that the reduced accumulation of most free amino acids the reduced accumulation of most free amino acids senescent cultures differed from growing ones in the greater accumulation of sugars involved in sucrose, galactose, and ascorbate metabolism (Fig. 4). Parallelly, the pools of amino acids decreased, which may be due to the repression of protein synthesis and, possibly, nitrogen-containing secondary compounds, being their precursors. Intermediates of the TCA cycle, sterol, and fatty acid synthesis pathways did not show any obvious unidirectional changes.

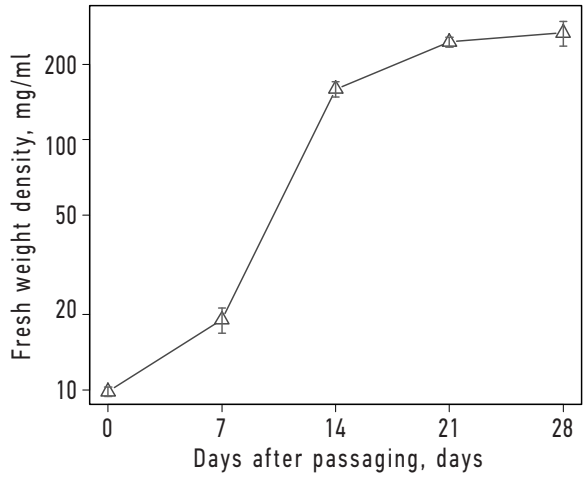

Fig. 1. Growth of heterotrophic suspension cell culture *N. tabacum* VBI-0: fresh weight density mg per ml
Рис. 1. Рост гетеротрофной суспензионной культуры *N. tabacum* VBI-0

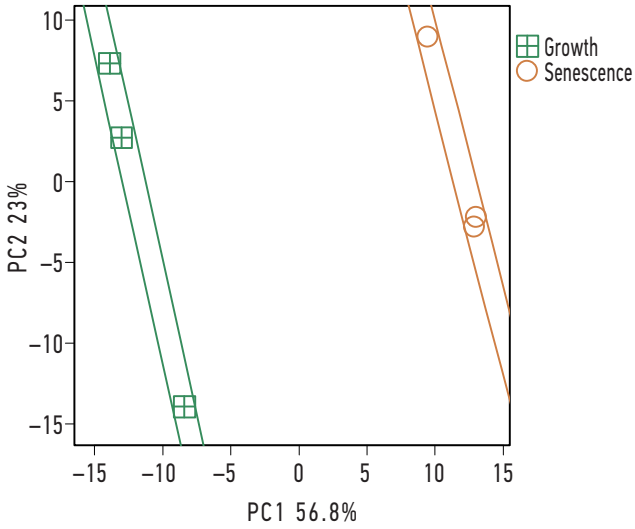

Fig. 2. Score plot from PCA of metabolite profiles extracted from heterotrophic suspension cell culture *N. tabacum* VBI-0 at growth and senescence. Eclipses — are the 95% confidence intervals, % — percent of variation
Рис. 2. Рассеяние профилей метаболитов в пространстве счетов главных компонент (ГК), полученных при анализе суспензионных культур клеток *N. tabacum* VBI-0 на стадиях роста и старения. Эллипсы — 95 % доверительные интервалы, % — доля дисперсии, связанная с главной компонентой

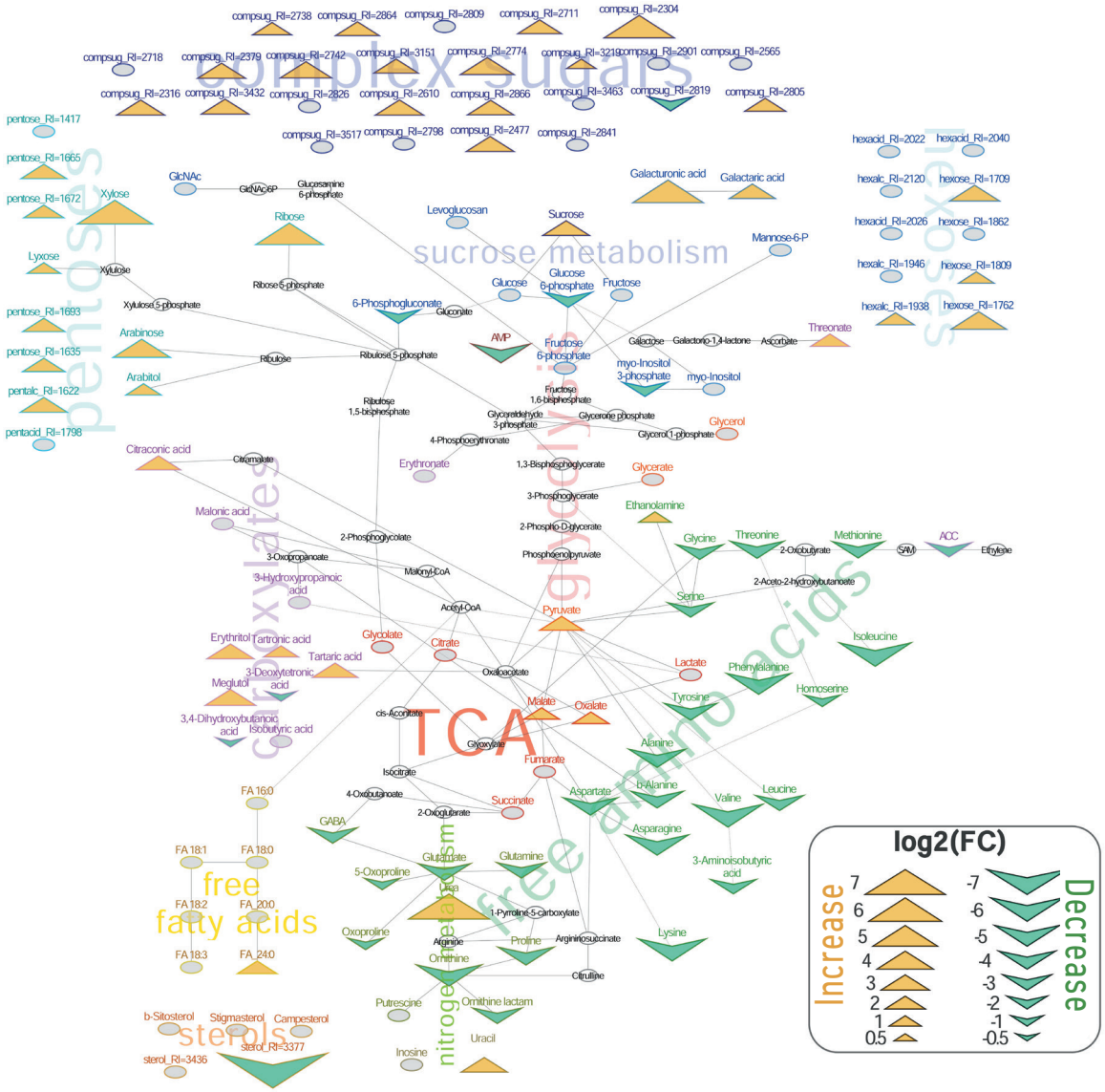

Fig. 3. Visualization of differentially accumulated metabolites (DAMs) in suspension cell culture *N. tabacum* VBI-0 at growth and senescence stages. DAMs were selected by rule: VIP > 1. Increase refers to higher level at senescence. FC — fold changes

Рис. 3. Визуализация метаболитов, дифференциально накапливающихся (ДНМ), в растущих и стареющих культурах клеток *N. tabacum* VBI-0. Выбор ДНМ осуществлен по VIP > 1. FC — кратность различий средних значений (fold changes). Увеличение соответствует более высокому накоплению на стадии старения

DISCUSSION

The composition of the metabolite profiles of VBI-0 cell cultures was similar to those of native tobacco seedlings [43]. While the spectra were obtained and annotated using similar methods, the number of compounds in the culture profiles was significantly smaller. In both seedlings and cultures, carbohydrates were the most widely represented group. However, in cultures, the number of compounds annotated as carbohydrates was 1.5-fold less than in seedlings. Mainly, fewer complex carbohydrates were registered in cultures. This may be due to the differentiation of organs, tissues, and cells in a multicellular organism, which can also be traced at the biochemical level [44]. Conversely, seedlings have fully functional plastids, which upon illumination, photosynthesize, presumably expanding the metabolic network and making the metabolite profile more diverse. Sugar residues are part of many secondary compounds [45], and the synthesis of specialized molecules is usually triggered in response to unfavorable influences [3, 46]. Since the experimental suspension cultures were maintained under stable and favorable conditions, the mechanisms for the biosynthesis of many secondary compounds could remain inactive. This finding was consistent with the observation that the cell cultures analyzed were characterized by a limited number of secondary compounds identified. For example, nicotine, a characteristic of *N. tabacum*, was not detected in tobacco cultures. We emphasized earlier that these compounds were identified in seedlings [43].

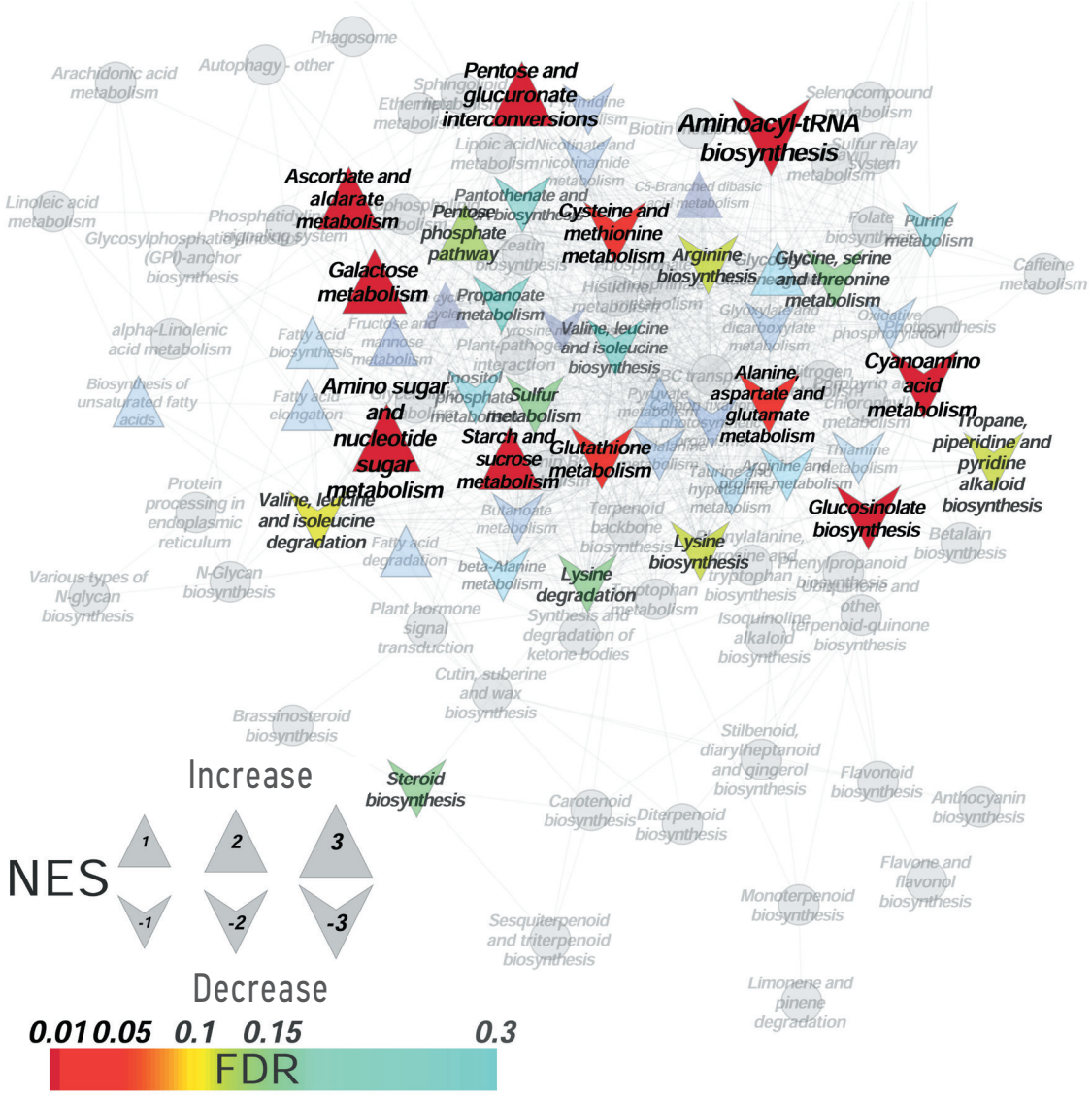

Fig. 4. Metabolite sets enrichment analysis based on loadings from OPLS-DA classification. Nodes of graph are KEGG pathways for *N. tabacum*, edges, contracting nodes, are presence of common metabolites in profiles. Size — strength of influence (NES, normalized enrichment score), color — FDR (false discovery rate), up triangles refer to accumulation of pathway intermediates at senescence

Рис. 4. Анализ обогащения, полученный на основе нагрузок предиктивной компоненты ОПЛС-ДА. Сеть метаболических путей, реализуемых в клетках *N. tabacum*. Узлы — метаболические пути из базы данных KEGG. Если они имеют общие метаболиты в профиле, то они соединены ребрами, которые их стягивают. Размер — NES, нормализованная оценка обогащения (normalized enrichment score), цвет — FDR, уровень ложноположительных результатов (false discovery rate). Треугольники с вершиной, направленной вверх соответствуют более высокому накоплению интермедиатов пути при старении

Finally, changes in cell wall composition may be an important factor in carbohydrate profile formation. The appearance of a large number of oligosaccharides in the profile of the cultures studied may result from the partial degradation of cell wall polysaccharides, which was previously noted during the development of tobacco suspension cultures.

It is well known that plant organs and tissues undergo physiological changes with age. This observation is also reflected in the fact that the metabolite profiles of plants at different ages [47, 48] or organs during development [49] vary significantly. As a rule, in the early stages of the development of plants, organs, and cell cultures, there is an increase in coupling with high biosynthetic activity. For example, actively dividing tomato cells in suspension cultures are characterized by a high level of carbon flux into protein synthesis [21]. High protein synthesis activity is associated with a high content of free amino acids, which is noted at the early stages of plant development [50].

Biosynthetic activity must be supported by appropriate resources. Since sugars are the source of carbon and energy for a heterotrophic culture, their metabolic pathways play a crucial role in providing the cell with energy. Modeling of flows in heterotrophic Arabidopsis cells revealed that glycolysis and TCA cycle play a major

role in glucose utilization [51]. The combination of a high level of phosphorylated hexoses and a low ATP/ADP (adenosine triphosphate/adenosine diphosphate) ratio can impart glycolysis with high importance. This presentation is registered, for example, during the period of proliferation of pericarp cells of tomato fruits [52]. The same was also true in the case of our study, as in suspension cultures, the contents of all sugar phosphates were high during the growth period. A reduction in their level during senescence indicates a decline in the activity levels of the initial reactions of glycolysis. The end of the proliferation of heterotrophic tomato cells in culture coincided with a decrease in flows through glycolysis and the TCA cycle [21]. As heterotrophic tobacco cell cultures age, the aerobic respiration activity decreases [20]. Presumably, this is the result of carbon starvation. A rapid decrease in respiration levels associated with a reduction in the phosphorylated sugar contents was previously noted in starved cultures after removing the substrate from the medium [14, 15]. Parallel, as the main osmotic agent, the high sucrose levels in the cells of older cultures can be due to its accumulation in the central vacuole, the volume of which increases during cell growth [53]. The accumulation of free sugars is detected at the later stages of plant and organ development [50, 54]. Vacuole growth is also probably associated with an enhancement in the malate pool, which usually accumulates in apparent quantities in this organelle [55, 56]. The accumulation of malate and other carboxylates may be mediated by the partial oxidation of sugars for energy supply [57], as can an increase in the level of pyruvate, which is the end product of glycolysis.

Based on the results of the enrichment analysis, it can be suggested that ascorbate metabolism is activated during senescence, as a result of enhanced oxidative stress in senescent cultures. High culture density and depletion of nutrients in the medium can be stress factors. A decrease in respiration levels in the late stages of development can be considered as a mechanism for reducing oxidative stress and slowing down the senescence process.

CONCLUSION

To summarize, it should be noted that cells of senescent heterotrophic cultures experience stress associated with carbon starvation and changes in the culture medium composition. The metabolomic data obtained indicated that senescence is associated with a decrease in the intensity of the biosynthetic processes, a reduction in the activity of the initial steps of glycolysis, and the accumulation of complex sugars, pentoses, and carboxylates.

ADDITIONAL INFORMATION

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Authors' contribution. All authors have made a significant contribution to the development of the concept, research, and preparation of the article, as well as read and approved the final version before its publication. Personal contribution of the authors: R.K. Puzanskiy — experimental design, collecting and preparation of samples, GC-MS, data analysis, writing the main part of the text, making final edits, funding acquisition; A.A. Kirpichnikova — maintaining cell cultures, collecting and preparation of samples; A.L. Shavarda — GC-MS, data analysis; V.V. Yemelyanov — collecting and preparation of samples, making final edits; M.F. Shishova — experimental design, data analysis, writing the main part of the text, making final edits.

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Competing interests. The authors declare that they have no competing interests.

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AUTHORS' INFO

Roman K. Puzanskiy, Cand. Sci. (Biology);
ORCID: 0000-0002-5862-2676; eLibrary SPIN: 6399-2016;
e-mail: puzansky@yandex.ru

ОБ АВТОРАХ

Роман Константинович Пузанский, канд. биол. наук;
ORCID: 0000-0002-5862-2676; eLibrary SPIN: 6399-2016;
e-mail: puzansky@yandex.ru

AUTHORS' INFO

Anastasiia A. Kirpichnikova;
ORCID: 0000-0001-5133-5175; eLibrary SPIN: 9960-9527;
e-mail: nastin1972@mail.ru

Alexey L. Shavarda, Cand. Sci. (Bioligy);
ORCID: 0000-0003-1778-2814; eLibrary SPIN: 5637-5122;
e-mail: stachyopsis@gmail.com

Vladislav V. Yemelyanov, Cand. Sci. (Bioligy), Associate Professor;
ORCID: 0000-0003-2323-5235; eLibrary SPIN: 9460-1278;
e-mail: bootika@mail.ru

***Maria F. Shishova,** Dr. Sci. (Biology), Professor;
address; 7/9 Universitetskaya emb., Saint Petersburg, 199034, Russia; ORCID: 0000-0003-3657-2986; eLibrary SPIN: 7842-7611;
e-mail: mshishova@mail.ru

ОБ АВТОРАХ

Анастасия Алексеевна Кирпичникова;
ORCID: 0000-0001-5133-5175; eLibrary SPIN: 9960-9527;
e-mail: nastin1972@mail.ru

Алексей Леонидович Шаварда, канд. биол. наук;
ORCID: 0000-0003-1778-2814; eLibrary SPIN: 5637-5122;
e-mail: stachyopsis@gmail.com

Владислав Владимирович Емельянов, канд. биол. наук, доцент; ORCID: 0000-0003-2323-5235; eLibrary SPIN: 9460-1278;
e-mail: bootika@mail.ru

***Мария Федоровна Шишова,** д-р биол. наук, профессор;
адрес: Россия, 199034, Санкт-Петербург, Университетская наб., д. 7/9; ORCID: 0000-0003-3657-2986; eLibrary SPIN: 7842-7611;
e-mail: mshishova@mail.ru

* Corresponding author / Автор, ответственный за переписку