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Issue Article

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THE EFFECTIVENESS OF THE APPLICATION OF SPECIALIZED TECHNIQUES OF FOREST PLANTING TECHNOLOGIES ACCORDING TO THE SYSTEM OF GENOMIC TECHNOLOGIES EXPRESSED IN THE FORM OF IN-VITRO WITH THE USE OF HUMIC ACIDS EXTRACTED FROM COAL FOR THE CONVERSION OF HEAVY SALINE SOILS SUITABLE FOR THE CULTIVATION OF CONIFEROUS AND DECIDUOUS CROPS.: ALFA PROTOCOL

***Abstract**: For the first time, a comprehensive study of quantitative and qualitative indicators of plantation forest crops of birch, alder and poplar in the forest-steppe of the CDR, created by in vitro regenerants, seedlings and cuttings, was carried out. A method for creating clonal forest-seed plantations using in vitro biotechnology on the example of black alder, gray and Karelian birch is proposed.*

The practical significance and implementation of the results of the work consists in the development of recommendations for the use of in vitro biotechnology in the plantation breeding of fast-growing tree species of the CDR. Experimental poplar crops with in vitro regenerants have been laid on the fires of the Educational and Experimental Forestry.

***Key words**: forest, ecosustem, gumin, exilamp, green technologies, HPV/HIV co-infection, Public health concern, meta-synthesis, Mapping analysis, Africa, Research advancement.*

***Language**: English*

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Introduction

Approbation of the work. Research materials were presented:

- at the congress of VOGiS (Moscow, 2009);

- at international meetings, conferences, symposiums (Krasnoyarsk, 2005, 2011; Voronezh, 2006, 2010, 2012; Alushta (Kiev), 2008; Petrozavodsk, 2011);

- at republican and regional conferences (Voronezh, 2005, 2007, 2010, 2011; Novocherkassk, 2010).

Personal contribution of the author. The work is the result of research conducted by the author himself with the direct participation as the responsible executor of the grant of U.M.N.I.K. and the executor of the topic of fundamental research on the reforestation of mountain forests. The dissertation uses experimental data obtained by the author at all stages of the work: the choice of direction, the development of a program and methods for conducting the entire complex of field and laboratory research, processing and analysis of the data obtained, preparation of publications.

Picture 1.

Publications. Based on the materials of the dissertation, the author published (personally and in co-authorship) 17 papers with a total volume of 3.5 sq., recommendations for intensive cultivation of planting material and the creation of plantation crops of varieties of poplar graying in the CDR, including one article in the journal recommended by the Higher Attestation Commission.

The structure and scope of the dissertation. The dissertation work consists of an introduction, 7 chapters, conclusions and recommendations and is presented on 164 pages, including 24 tables, 32 figures, appendices, a list of references of 229 titles, including 34 in foreign languages.

As a result of the conducted research on the use of microclonal reproduction to create plantation crops of fast-growing tree species in the forest-steppe, the following conclusions and recommendations can be made:

1. The most valuable fast-growing species for plantation afforestation in the forest-steppe are: hanging birch, the best varieties of poplar and black alder. In vitro biotechnology is of fundamental importance for the reproduction of economically valuable forms and varieties of woody plants that are difficult to propagate by seeds and cuttings.

2. Dispersion analysis has established the influence of the origin of planting material (regenerants in vitro, seedlings) of hanging birch on growth in height and diameter. On the gray sandy loam forest soils of the Horse-Kol of the Odessa forestry, the stock of 14-year-old plantation birch crops created by regenerants in vitro is 192.6 m/ha, seedlings - 85.6 m3/ha, but the quality of the trunks of seedlings is better than that of regenerants.

3. The experience of creating plantation crops of Karelian birch from regenerants in vitro has shown the great importance of the latter in obtaining special sorts valuable for furniture production.

4. It has been experimentally established that in black and gray alder cultures, differences in height and diameter of in vitro regenerants and seedlings aged 8 years on dark gray loamy moist soils of the Tavrovka River floodplain of the Novousmansky forestry are not significant. For alder, in vitro biotechnology is important when creating clone archives of economically valuable forms, varieties; clone seed plantations.

5. It is shown that the fruiting of clonal alder seed plantations created by regenerants in vitro begins earlier - from 3-4 years, than in seedlings - from 5-6 years. The viability of pollen ranges from 95 - 98%. The quantitative composition of the emerging pollen in microclonal plants has 22-23% in black alder and up to 52% in gray alder of large pollen (more than 31 microns.). According to the coefficient of the shape of cones, the differences in seedlings and regenerants in vitro are not significant. Laboratory germination of seeds reaches 80%.

6. Plantation crops of poplar of the subgenus Eupopulus Dode, laid by winter cuttings on aspen felling with lowering of stumps and disking of root offspring showed the possibility of obtaining wood for construction parts and pressing in a short time. At the

age of 14, the average height of trees ranged from 16.1 ± 0.57 m in the Black Hybrid to 21.7 ± 0.89 m in Regenerate No. 90, the average diameter ranged from 15.7 ± 0.36 cm. in the Black hybrid up to 19.4 ± 0.58 cm in the poplar E.S. - 38.

Picture 2.

7. In the Central forest-steppe, 16 years of experience has been accumulated in the creation of plantation crops of a hard-to-root triploid variety of poplar graying Hopersky 1 with in vitro regenerants on gray forest sandy loam soils of the Kony-Kolodez forestry and on dark gray forest loamy soils of the Right-Bank forestry. At the age of 16, the average height of the poplar trees of Khopersky 1 on sandy loam was 21.0 ± 0.45 m, the average diameter was 21.4 ± 1.36 cm; on loam, respectively, 21.0 ± 0.58 m and 33.0 ± 0.85 cm.

The issues of application of biotechnology methods for the production of planting material of coniferous and deciduous species in order to create plantation crops, prospects for the use of genetic modification of tree species to accelerate growth, increase their stability and improve the properties of wood, the use of molecular labeling methods in the study of the population-genetic structure of coniferous species and breeding work are considered.

According to academician A.A. Baev, biotechnology is the use of living organisms and their systems for industrial purposes. Despite the fact that the practical application of the achievements of biotechnology is preceded by a huge number of fundamental research, their main goal is to obtain a commercial product. Currently, there is an extensive literature in Russia, which outlines the basic principles and methods of biotechnology of tree species [11, 15], discusses the prospects for the use of biotechnologies in forestry [1, 3, 4, 21, 29, 31, 34, 38, 39, 42].

Currently, in the Russian forest sector, biotechnology methods are used to grow planting material, produce biological forest protection products, create new forms of woody plants with specified characteristics, including using genetic engineering methods, to increase the efficiency of breeding work using molecular labeling methods, preserve genetic resources using cryobanks and plant deposit banks. in vitro material, genetic certification and certification of seeds, assessment of the legality of the origin of felled wood.

The methods of clonal micro-propagation of plants (including somatic embryogenesis) have found the most widespread application for accelerated use of breeding achievements based on the production of high-quality planting material for the creation of raw forest plantations. Work on in vitro culture of hardwoods of the genera Populus, Betula, Pinus, Salex and Fraxinus has been carried out for a long time and intensively. Taking into account the results of many years of research by Russian scientists on culture in vitro aspen, hybrid poplars and willows at the Research Institute of Forest Genetics and Breeding

[17-19], various types of birch at the Institute forests of KarSC RAS [3], triploid aspen and various birch clones in the St. Petersburg Research Institute of Forestry (SPbNIILH) [5, 25], ash and willow in IBH RAS [12], it is possible to predict the successful introduction of these breeds into the practice of plantation reforestation [8, 31-33].

Coniferous plants are the most complex objects for various in vitro methods, therefore, the development of an effective system of clonal micropropagation for some species is an urgent task [13, 14]. Currently, somatic embryogenesis is considered one of the most promising methods in forest biotechnology of microclonal reproduction in in vitro culture. Its use ensures the production of "artificial seeds" in the future, which will reduce the cost of planting material by about ten times. The development of methods for obtaining planting material based on the use of somatic embryogenesis of Siberian coniferous species (Larix sibirica, L. gmelini, L. sukaczewii, Pinus sibirica, P. pumela, Picea ajaensis) is carried out at the V.N. Sukachev Institute of Forest SB RAS [27],

Pinus sylvestris and Picea abies - at the Institute of Bioorganic Chemistry named after academicians M.M. Shemyakin and Yu.A. Ovchinnikov [13, 14], Picea abies - in SPbNIILH [2]. However, in these experiments, only explants of single donor plants form an embryogenic callus. So far, it is possible to obtain somatic embryos and plants only from explants of seed germs. The planting material obtained in this way is similar to the planting material from seeds, and the level of heritability of valuable traits will be only 10 ... 20% [7].

The first works on the organogenesis of Pinus sylvestris and Picea abies in Russia were carried out on the basis of the use of hypocotyl explants or seeds at the Russian State Agricultural University (Timiryazev Moscow Agricultural Academy) and the Institute of Bioorganic Chemistry of the Russian Academy of Sciences [13, 14, 24].

Currently, during the joint work of the Institute of Bioorganic Chemistry and SPbNIILH, organogenesis based on the use of the buds of plus trees has been performed, recommendations have been developed for the adaptation of the obtained microregenerants in peat substrate under conditions of film greenhouses [31, 34]. The planting material grown in this way includes several experimental plots of forest crops and their growth is monitored, control is provided by crop variants using two-year-old seedlings from the seedling department of the nursery, having approximately the same biometric parameters with micro-gear seedlings at the time of planting.

In the forest zone of Russia, simplified technologies widely used in practice for laying and growing forest crops, as a rule, do not give them growth advantages compared to young plants of natural origin and do not even stop the process of replacing spruce and pine with aspen and birch. In contrast to such plantings, spruce and pine crops planted using intensive agrotechnical techniques grow according to II - 1b bonus classes, i.e. on average 1-3 times higher in productivity than conventional crops. The accelerated high growth rate of such crops is achieved due to a combination of certain factors and conditions. Among the most significant factors that have a huge potential for improvement is the use of selection-improved planting material. However, the development of methods of clonal micro-propagation of conifers in the practice of forestry in Russia is still taking only the first steps.

Of the traits used in population forest genetics, molecular (including biochemical) markers of genes differ in many useful properties. They do not require many years of inheritance analysis in a series of generations, their manifestation does not depend on the modifying action of the environment. Molecular markers, unlike morphological features in coniferous species, have a monogenic nature of inheritance and therefore are objective genetic markers of the degree of differences between populations and taxa. Chronologically, the first molecular markers were allozymes (isoenzymes) - hereditary forms of enzyme proteins. Despite the rapid development of DNA analysis methods, isoenzymes remain very useful genetic markers, since they can be used to obtain reliable and complete genetic information [22]. To date, the results of genetic studies using the method of isoenzyme analysis have allowed us to solve complex issues of taxonomy of pine species [22], spruce [6, 10, 12, 23], larches [20]. The method of molecular labeling is widely used for genetic certification of clonal forest-seed plantations and archives of softwood clones [9, 30, 35].

Other applications of this method relate to the study of gene transfer processes and the analysis of crossing systems. Marker-assisted selection refers to indirect selection and consists in the statistical binding of molecular markers, primarily DNA markers, with economically important traits. Due to the fact that the markers are not influenced by environmental conditions or features of ontogenesis, this tool provides ample opportunities, especially for early diagnosis (wood quality at the seedling stage), which is important for forest species characterized by long growing periods.

In forestry, considerable interest is directed to the genes that control the development of wood fibers, since their microstructure largely determines the commercial value of wood. It is known that the parameter determining the mechanical strength of wood and the ultimate strength paper tear, is the angle of orientation of cellulose microfibrils in the cell wall of wood fibers. Knowledge of cell wall biosynthesis is also useful for understanding processes compression-stretching of wood [36, 41]. These factors are directly related to the stability of stands, as well as to the quality of wood used for sawmilling and in the pulp and paper industry. Currently, the genes controlling the synthesis of the components of the cell walls of cellulose, hemicellulose and lignin have been identified. For example, cellulose synthesis genes have been cloned for aspen [43], poplar [40] and radiant pine [37].

To a greater extent, the development of breeding markers is important for long-term research of a fundamental order [43]. It makes a significant contribution to understanding the work of fundamental genetic mechanisms and the organization of the genome at the molecular level. Particularly great attention in this work is paid to the study of quantitative characteristics of forest species.

Genetic markers can be useful in order to use genetic variation in the selection of samples for the preservation of genes and the creation of clone archives for use in further breeding work. Undoubtedly, the use of genetic markers in forestry research will only increase over time.

Despite the attractiveness of the method for practical breeding, there are a number of limitations that will prevent its use, at least in the medium term:

marker-based selection is still quite expensive for any sufficiently large group of individuals;

the relationship between markers and economically valuable traits should be established for each family separately, therefore, even if the cost of this type of analysis decreases significantly, marker-assisted selection will be the prerogative of advanced and "refined" breeding programs, those in which it will be possible to create and maintain an inheritance structure and where it is advisable to use clone forestry [36, 40]. Most likely, the development of classical breeding programs is currently preferable for most species of tree species.

The technology of the next decade is the transgenesis (gene modification) of woody plants [16]. Increasing the growth rate of the main forest species, increasing their stability and improving the properties of wood are priority tasks of modern forestry science. The creation of new forms of forest species by traditional breeding methods is a long and inefficient process. Genetic transformation by the method of agrobacterial transfer of recombinant target genes makes it possible to modify the properties of woody plants in a short time.

The complex of methods of genetic transformation makes it possible to modify individual plant traits point-by-point: to give resistance to herbicides, to lower the content of lignins, to increase productivity, i.e. to create target forms. This direction of forest biotechnology is focused exclusively on the plantation method of forest cultivation. More than 150 field polygons have been registered in the world to study the growth of transgenic forest species. Most of them are held in the USA (103), China (9), Canada (7) and Finland (5).

In Russia, work on the genetic modification of tree species was started in 1999 at the Siberian Institute of Plant Physiology and Biochemistry SB RAS [27]. Genetic transformation in order to increase the growth rate of aspen (Populus tremula L.) poplar (Populus balsamifera L.) and cedar pine (Pinus sibirica de Tour) was carried out using the ugt gene isolated from corn. The gene encodes the synthesis of UDPH transferase, an enzyme that binds indolylacetic acid to glucose, thereby creating a significant pool of IUC in plants that changes the auxin status. Plants that have received this gene develop much faster, and they could be successfully used on plantations with a short turn of felling. Depending on the transformation design used, the plants showed an increase in shoot growth, an increase in the number and length of roots, the number of leaves and the area of the leaf blade.

The limited nitrogen reserves in forest soils are often a limiting factor in the growth of forest plantations. It is also possible to increase the efficiency of nitrogen assimilation by genetic engineering methods. The strategy of superexpression of the gsl (pine) gene is known, which is homologous to the native gene of glutamine synthetase of aspen and poplar, but is not an exact copy of it, which makes it possible to increase the total activity of gs (glutamine synthetase) and, as a consequence, the accumulation of glutamine in tissues. Transgenic lines of aspen and birch with the gs glutamine synthetase genome of pine under the control of the 35S promoter. The gene increases the content of glutamine in plants, thereby improving the assimilation of ammonium nitrogen and increasing the overall productivity of plants [26, 32]. All lines with the gs1 gene were used to conduct tests to assess the phenotypic manifestations of the recombinant gene. After two years of growth in protected soil conditions, according to such indicators as plant height, root neck diameter and the growth of the current year, promising lines were ahead of the control version by 15 ... 60%. Aspen and birch lines containing the phosphinotricin acetyltransferase bar gene encoding resistance to herbicides based on phosphinotricin were obtained at the same institute [28]. The treatment of the obtained transgenic plants showed resistance to phosphinotricin in doses recommended for use in forest nurseries and crops.

In the works on genetic engineering of woody plants, an important problem is the risk assessment associated with the introduction of transgenic plants into the natural environment. Therefore, the commercial use of transgenic plants should be preceded by comprehensive research.

The problem of reducing the biological diversity of the plant world is especially acute in the case of forest woody plants. The solution of this problem on the basis of in vitro culture makes it possible to reliably store valuable or disappearing genotypes by cryopreservation or in vitro deposition.

Cryopreservation is applicable to breeds propagated by somatic embryogenesis, and in vitro deposition is applicable to species propagated by stem culture.

The combination of methods of molecular labeling and clonal micropropagation of elite genotypes makes it possible to increase the productivity of forest plantations by 50.100%. Such a complex of biotechnological methods makes it possible to accelerate the breeding process to create new improved forms and varieties by 2-3 times.

Since 1991, SPbNIILH has been implementing a research program on the microclonal reproduction of valuable forms of spruce and birch, with the aim of creating a technology for mass production of regenerating plants in culture in vitro. Somewhat later, work was started on the microclonal reproduction of fast-growing triploid forms of aspen resistant to stem rot. Together with the Institute of Bioorganic Chemistry of the Russian Academy of Sciences, the first works on the microclonal reproduction of Scots pine were carried out. Regulations for obtaining regenerating plants and regulations for their adaptation to the conditions of greenhouses and peat substrates for obtaining planting material of the required biometric parameters have been developed. For hardwoods, the technologies have passed a pilot production test and are ready for mass implementation.

In order to make more rational use of the areas that have come out of agricultural use, we, together with the IBOC RAS, have started research on the growth of transgenic birch and aspen lines. Since the fertility of our soils is limited by the level of nitrogen content in them, the gene modification of plants was carried out by the gs glutamine synthetase gene, which ultimately improves the assimilation of ammonium nitrogen and increases the overall productivity of plants. However, the use of such plants to create conventional crops in the forest fund should be completely excluded. Despite the fact that quite good results have been achieved in various areas of forest biotechnology, their widespread introduction into forestry practice is not observed, except for the use of DNA markers for certification of forest seed plantations and certification of coniferous seeds. Scientific research was conducted before the search for markets for scientific and technical products. The output scientific and technical products were not focused on solving the problems of a specific consumer, therefore, competent marketing is required to promote the results to the market. The term "somaclonal variability", meaning variability occurring in cell or tissue culture, first appeared in 1981 [11], although this phenomenon has been repeatedly reported before. Somatic changes may be of a genetic or epigenetic nature. Genetic abnormalities are inherited and often represent a manifestation of already existing changes in explant cells, although new mutations may occur. One of the most important manifestations of such changes is chromosomal abnormalities. Although an increase in ploidy may occur during the division of endopolyploid cells of the initial explant [22], most polyploids and aneuploids occur during cultivation [5]. These changes should be distinguished from morphoses, non-sequenced epigenetic changes that are often reversible and occur as a result of in vitro culture exposure. Morphoses usually manifest themselves in an increase in the strength of growth and branching, the ability to take root and bloom, a delay in entering fruiting, i.e. signs characteristic of the juvenile phase of development. To clarify the nature of the observed changes in species with a sexual reproduction method, it is necessary to make an appropriate crossing. For species propagated asexually, the preservation of the trait in at least two consecutive cycles of clonal micropropagation provides confirmation of the genetic cause of this change [2]. Various methods are used to detect somaclonal changes: morphological assessment, cytological studies (karyotyping, flow cytometry), analyses using molecular markers. It should be borne in mind that in vitro and greenhouse studies are not enough to fully identify deviations and verification in the field is required. The manifestation of somaclonal variability can be both an advantage and a disadvantage. On the one hand, it increases genetic diversity and thereby contributes to the breeding of new varieties. For example, a variety of non-darkening White Baron potatoes and a variety of thornless blackberries Lincoln Logan were obtained in this way [7]. On the other hand, with clonal reproduction of garden and forest crops, when the goal is to obtain a homogeneous planting material from valuable genotypes, its appearance is undesirable. Particular attention should be paid to in vitro culture of chimeric varieties that contain cells of different genotypes, since it is known that chimeras can be separated by micro-multiplication [25] or adventitious regeneration [3]. For these reasons, it is very important to understand the mechanisms that generate instability and somaclonal variability in vitro, in order to control the frequency of its occurrence depending on the goals set. Studies have shown that the frequency of somaclonal variability in in vitro culture can be influenced by factors such as genotype [23], the source of explants [19], growth regulators [21, 9], as well as the composition of the medium, duration and conditions of cultivation, and others. The probability of somaclonal variability increases if the callus stage is present during plant cultivation [19, 21]. The formation of callus is characteristic of the processes of adventive regeneration or somatic embryogenesis of plants. For this reason, somaclonal abnormalities are especially important to control when cryopreserving somatic embryos and using them as artificial seeds. Somaclonal variability observed in genetically modified (transgenic) plants has its own characteristics. These plants sometimes show changes in the phenotype that are not related to the expression of transferred genes. Although a number of studies have demonstrated that the frequency of somaclonal variability does not differ between transgenic and nontransgenic regenerants [6], however, in some cases it increased [4]. The reasons for this additional variability may be stress associated with the transformation technique or the insertion of transferred genes, as well as other non-target DNA sequences (insertion mutagenesis). The method of genetic transformation of plants is accompanied by such stressful effects as prolonged exposure of explants with high concentrations of growth regulators for plant regeneration, as well as with antibiotics or herbicides, which are necessary for the selection of transformed cells and the elimination of agrobacteria. It was shown that the frequency of DNA changes in transgenic plants correlated with the degree of stress in vitro [10]. The manifestation of polyploidy in transgenic plants may be associated with the mixoploid nature of the initial explants. For example, 16% of tetraploid cells and 2% of octoploid cells were found in tomato seedlings, and 39 and 9% were found in cotyledons [26]. At the same time, cotyledons are often used for the transformation of plants, especially woody ones, since the frequency of regeneration from them, due to their juvenile nature, is higher than from non-seed tissues, for example, leaves. Thus, in order to reduce the likelihood of somaclonal changes in transgenic plants, attention should be paid to the ploidy of the initial explants, as well as to weaken the stress effect: for example, exclude selection on antibiotics or herbicides and use direct regeneration without the callus stage. The purpose of our work was to assess the level and nature of somaclonal changes that occur during clonal micro-reproduction and genetic transformation of a number of fruit, berry, ornamental and forest crops. Somaclonal variability was assessed by morphological features on strawberry (Fragaria ananassa Duch.), apple (Malus domestica Borkh), lilac (Syringa vulgaris L.) and common ash (Fraxunus excelsior L) plants obtained by clonal micro-propagation. Strawberry animation was carried out on MS medium with the addition of 0.3-0.5 mg/l 6-BAP, apple — on MS medium with 2 mg/l 6-BAP, lilac — on MS medium with 3 mg/l 6-BAP, ash — as described by Lebedev and Shestibratov [1]. Strawberry plants were evaluated in vitro (4 genotypes) and in the field (7 genotypes), apple plants (mature form and rootstock) — in the field at the age of two. Micro-propagated ash plants at the age of two years (Nizhny Novgorod region) and lilac of the Sensation variety at the age of 5 years (Leningrad region) were also evaluated in the field. The level of ploidy was determined in transgenic pear plants (Pyrus communis L.), fluffy birch (Betula pubescens Ehrh.) and aspen (Populus tremula L) obtained by the authors in the FIB RAS. The transformation of the Burakovka pear by the genome of the plant defensin Rs-AFP2 was carried out as described in [13]. Transformation of aspen and birch by the genes of glutamine synthetase GS and xyloglucanase Xeg was carried out as described in [14]. The level of ploidy of transgenic plants was assessed by counting chromosomes in the cells of the shoot and root meristems. The vegetative method of reproduction of garden plants, unlike seed, allows you to preserve varietal characteristics. In vitro reproduction is the most intensive method of vegetative reproduction and therefore the control of the genetic stability of plants obtained in this way is of great importance. The conditions of growing plants in vitro can have a significant impact on the growth and development of the resulting plants. In the course of our research, we viewed more than 10 thousand strawberry plants and among them plants with pronounced anomalies were noted (Table 1). It should be noted that the proportion of such plants was insignificant due to the use of the nutrient media compositions developed by us and a small number of passages. The dependence of somaclonal changes on the genotype was also noted in other works on strawberries [23]. Most of the plants we received with deviations died after landing in non-sterile conditions. In order to determine the causes of such anomalies (the plant genome is affected or these are morphoses), meristematic tops with signs of abnormalities were re-introduced into the culture on a nutrient medium containing 0.1 mg/l 6-BAP. After the second passage, the shoots were rooted on a medium with cresacin and transferred to non-sterile conditions. We did not notice any deviations in the development of plants, and therefore we can assume that these changes were of an epigenetic nature. In order to assess the effect of in vitro cultivation on subsequent reproduction, plants of several strawberry varieties obtained by clonal micro-propagation were planted in film greenhouses.

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