THE INFLUENCE OF AIR POLLUTION ON CYTOGENETIC CHARACTERISTICS OF BIRCH SEED PROGENY

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ABSTRACT

Cytogenetic characteristics (mitotic index, level and spectrum of pathological mitosis, sizes of single and pair nucleoli, frequency of polynucleolar cells, nucleoli's types, frequency of persistent nucleoli in stages of metaphase – telophase of mitosis) of birch (*Betula pendula* Roth.) seed progeny were studied in some districts of Voronezh, which differed in degree of air pollution. The increase of spectrum and level of mitosis damages was revealed in experimental areas. The essential resistance of pathological mitosis level to different degrees of the environmental contamination has been shown. The influence of air pollution on mitotic activity was not observed. The increase of nucleoli's surface area and the number of nucleoli in cells, the appearance of persistent nucleoli at stages of metaphase – telophase and the rising of cell number with high-active nucleoli's types were marked on trial territories. Possible adaptive mechanisms to stress, which are connected with changes of nucleolar characteristics, are discussed. The correlation between cytogenetic characteristics (pathological mitosis level, surface area of single nucleoli in interphase cells) of birch seed progeny and frequency of micronuclei in children's buccal mucosa was revealed. We can conclude that cytogenetic characteristics of birch seed progeny are perspective for estimation of the environmental conditions for the humans.

Key words: adaptation, anthropogenic pollution, *Betula pendula* Roth., mitotic activity, nucleolar characteristics, pathological mitosis.

INTRODUCTION

The woody plants have a great application for landscaping of towns and making protective zones around industrial enterprises for pollutant retention. One of the recovery measures on the territories, which were contaminated by radioactive fallout after the Chernobyl Nuclear Power Plant accident, is the planting of woody plants delaying the spread of radionuclides (EVSTRATOV *et al.* 1993). For these aims it is important to use forms of plants resistant to pollution in order to raise the plantation stability to stress factors. However, the cytogenetic reactions and adaptive mechanisms of woody plants to pollution have not been studied very well. It has been shown for coniferous species that under conditions of contamination the increase of level and spectrum of pathological mitosis, the change of mitosis by amitosis, the appearance of micronuclei, the rise of number of nucleolar organizer regions (NOR) of chromosomes, the increase of metaphase chromosome size, the appearance of ring-like, dicentric and additional chromosomes have been marked (BUTORINA & EVSTRATOV 1996; DRUŠKOVIĆ 1997; KALASHNIK *ET AL.* 1997; MIČIETA & MURÍN 1997; ZOLDOS *et al.* 1997; ZUJEVA *et al.* 1997; KHAIDAROVA & KALASHNIK 1999; MURATOVA & SEDELNIKOVA 1999; SHAFIKOVA 1999; BUTORINA *et al.* 2000b; KALASHNIK & LYKHONOS 2000). Such publications for deciduous plants are known much less. In polluted areas, the delays of cells at stage of prophase or metaphase and the appearance of persistent nucleoli have been detected in oak (BUTORINA *et al.* 1997; KALAEV 2000). In other plants (birch, elm, willow, poplar) the rise of pathological mitosis level, the shift of picks of diurnal mitotic activity and micronuclei have been observed (NIKOLAEVSKIY 1998; VOSTRIKOVA 1999; BUTORINA *et al.* 2000b; GUSKOV *et al.* 2000). In this connection we have tried to study cytogenetic characteristics of birch (*Betula pendula* Roth.) seed progeny from some districts of Voronezh city, which differed in the degree of pollution. The choice of *Betula pendula* as the test-object is connected with its high frequency in protective plantations of the city. The study of cytogenetic reactions of woody plants to contamination will allow us to select the most acceptable criteria for cytogenetic monitoring of the environment. It is necessary to notice particularly that nucleolar characteristics of birch seed progeny were not studied earlier, but this criterion is the most sensitive to the stress (ARKHIPCHUK 1995).

MATERIALS AND METHODS

Voronezh is one of the largest industrial cities of Russia with the population of about 1 million people and various industrial, social and ecological structures. Our investigation of birch cytogenetic characteristics was carried out in 1999 in the following districts of Voronezh (MAMCHIK *et al.* 1997):

1. Left Bank Side District. The main sources of pollution on this area are the heating-power plant and enterprises of chemical industry producing rubber and tires. The atmosphere of this district is polluted by sulphur dioxide, nitrogen oxides, ashes and volatile organic compounds (xylene, toluene, butadiene, acetone, and gasoline).

2. Lenin District. The main pollutants are carbon oxide (CO), nitrogen oxides and formaldehyde. The sources of such substances are the mechanical plant, enterprises of chemical industry and automobiles.

3. Komintern District. The machine-building plants, building enterprises and the high traffic are the main sources of sulphur dioxide, nitrogen oxides, carbon oxide (CO) and benz(a)piren. There is the most unfavorable situation in air pollution by benz(a)piren, which is related to group of high-dangerous substances (TCHUBIRKO *et al.* 1997), in this district.

4. Soviet District. It is one of the so-called bedroom communities, where industries are absent. Recently, however, the area has been polluted with domestic wastes, and cars have been excessively accumulated in courtyards. That is why inorganic solid particles, carbon oxide (CO), nitrogen oxides, formaldehyde are present in atmosphere of this district and also benz(a)piren concentration exceeds the maximum allowable concentration (MAC).

For cytogenetic study of birch, we collected seeds in each district from 4 phenotypically normal trees without apparent damages by pests in September 1999. Seeds collected on ecologically "clean" territory (research station Venevitinovo of Voronezh State University) were taken as control. The seeds were germinated on wet filter paper in Petri dishes at 20 °C. Young plantlets with 0.5–1 cm root length were fixed in the 3:1 mixture of ethanol and glacial acetic acid at 9 a.m. Permanent preparations were made using the squash technique described earlier by BUTORINA *et al.* (2000a), according to which the stained material was put into a drop of Hoyer mixture. 15 microscopic preparations of plantlets originating from each experimental area and 5 preparations of plantlets originating from the control area were studied under the LABOVAL-4 microscope (Carl Zeiss, Jena) at magnification of 40 × 1.5 × 10 and 100 × 1.5 × 10. In each preparation we recorded the total number of cells (at least 500) and estimated mitotic activity by calculating the mitotic index (the ratio of quantity of dividing cells to the total quantity of analyzed cells). Quantity of pathological mitosis and persistent nucleoli we detected at the stages of metaphase, anaphase, telophase and calculated their percentage. The classification of pathological mitosis was made according to ALOV (1965). Frequency of various abnormalities was expressed as the percentage of their total number.

According to the data of some authors (ARKHIPCHUK 1995; BUTORINA & KALAEV 2000) the most sensitive criterion of cytogenetic monitoring is the nucleolar activity. It is the cytological manifestation of transcription activity of ribosomal genes and it is expressed by size of single or pair nucleoli and percentage of heteromorphic and homomorphic pair nucleoli. Pair nucleoli, surface area of which differs more than in 3 μm^2, are called "heteromorphic" (ARKHIPCHUK 1995). To study nucleolar characteristics we measured diameters of nucleoli using ocular micrometer (200 cells were analyzed in each preparation) and calculated the surface area of nucleoli; we counted the quantity of cells with various numbers of nucleoli, calculated their percentage and determined percentage of various nucleolus types according to the classification, given by TCHELIDZE and ZATSEPINA (1988).

The results were processed statistically using an IBM PC/AT with STADIA software package. The procedure of data grouping and treatment was described by KULAICHEV (1996). The comparison of birch cytogenetic characteristics were made in the following criteria: Van der Vaerden rank X-test for frequencies of persistant nucleoli and pathological mitosis, Student's *t*-test for mitotic indexes and nucleolar characteristics. The proportions of cells with homo- and heteromorphic nucleoli were compared using their angular transformation and Yates correction according to LAKIN (1990). To estimate the influence of birch growth-place factor on the surface area of single nucleoli we used Kruskal - Wallis one-way ANOVA. Correlations were estimated using the Spearman rank correlation coefficient. Cluster analysis was made using normalized Euclidean distances and complete linkage classification method.

RESULTS AND DISCUSSION

There are not revealed statistically verified differences between mitotic indexes in birch plantlet cells from experimental and control areas (Figure 1). Possibly, it is connected with high resistance of this criterion to pollutants, which was observed in our previous studies (BUTORINA & KALAEV 2000). The spectrum of pathological mitosis in experimental samples included

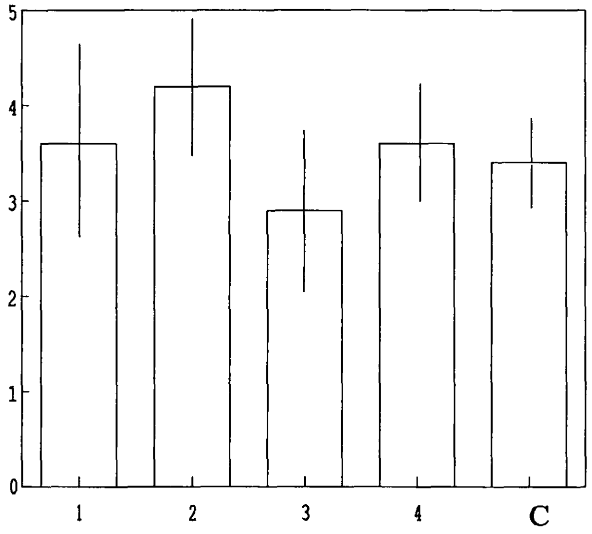

Figure 1. Mitotic indexes in apical root meristematic cells of birch from seeds collected in different districts of Voronezh. Ordinate - mitotic index, %. C - control, 1 - Lenin District, 2 - Left Bank Side District, 3 - Komintern District, 4 - Soviet District.

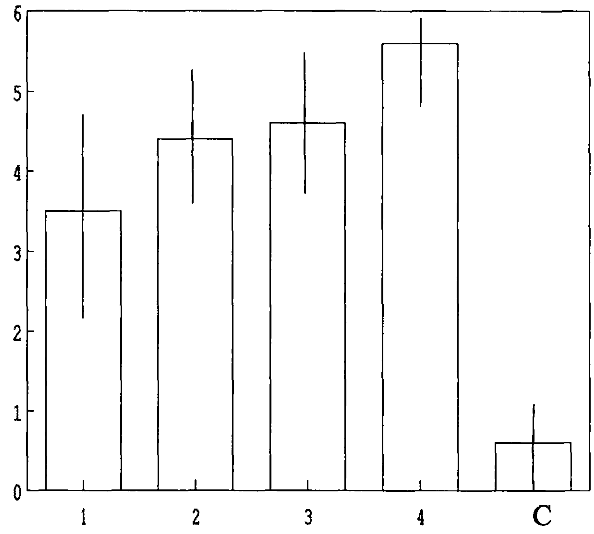

Figure 2. The frequency of pathological mitosis in apical root meristematic cells of birch from seeds collected in different districts of Voronezh. Ordinate - percentage of pathological mitosis. C - control, 1 - Lenin District, 2 - Left Bank Side District, 3 - Komintern District, 4 - Soviet District.

metakinetic chromosome lagging (34.3 %), anaphase chromosome lagging (7.1 %), bridges at the anaphase and telophase stages (48.5 %), chromosome agglutination in prophase (8.6 %) and asymmetric mitosis (1.4 %). In samples originating from the control area, bridges accounted for 100 % of mitotic pathology. The broader spectrum of mitotic abnormalities in the districts of Voronezh was apparently related to a high level of air pollution, compared to that in the control territory (BUTORINA & KALAEV 2000). Statistically significant differences between each of the experimental areas and control were found in parameters of pathological mitosis in birch root meristematic cells (Figure 2). This indicates that the air of Voronezh contains hazardous substances capable of disturbing cell division. The absence of differences in the pathological mitosis levels between the city districts might be explained by their pollution to approximately equal degree and possibly by low sensitivity of this parameter. This means that the level of pathological mitosis would be constant in large interval of pollution degree by changing other cytogenetic characteristics (nucleolar characteristics). For its statistically essential change, the difference in degree of pollution should be more considerable. The similar results have been obtained by GUSKOV *et al.* (2000) for four species of woody plants (*Populus deltoides* Marsh., *Ulmus pumila* L., *Salix babylonica* L., *S. triandra* L.) in Rostov on Don. It has been detected that in all the experimental areas in different districts of city the level of chromosomal aberrations was resembled, but it was authentically different from the control. The highest frequency of pathological mitosis was marked in Soviet District of Voronezh (5.7±2.1 %, in control - 0.6±0.6 %) what, probably, could be depended on the presence of high benz(a)piren concentration in atmosphere of this district.

Persistent nucleoli were observed in all stages of mitosis in root meristematic cells of birch plantlets in both experimental and control samples for the first time (Figure 3, a, b). Their frequencies are shown in Figure 4. In most cases we observed 1, rarely 2 or 3 persistent nucleoli in a cell. Persistent nucleoli were like small nodules (one or two) on one or both sides of metaphase plate (from lateral sight) or like oval bodies, connected with metaphase plate. by a thread. Rarely there was a persistent nucleolus, which was not connected with metaphase plate. In anaphase persistent nucleolus lays near the pole on one of the sister group of chromosomes. In telophase it could be observed like "sitting" on one of the cell poles. Earlier such phenomenon have been discovered in root meristematic cells of one-year-old seedling of oak (*Quercus robur* L.) from 30 km zone of Chernobyl nuclear power plant after accident in 1986 (BUTORINA & ISAKOV 1989; BUTORINA *et al.* 1997) and in normal (unstressful) conditions - in acorn plantlets of oak (predominance in mitotic activity peaks) (BUTORINA & KALAEV 1998). Other authors noted this phenomenon in plant and animal objects under the treatment by inhibitors of protein synthesis, under virus infection, under conditions of pathological

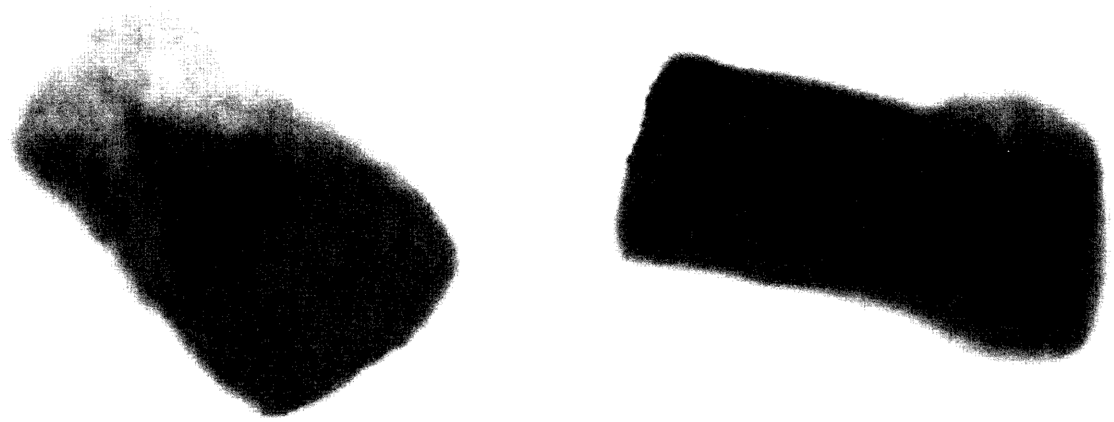

Figure 3. The persistent nucleolus at stage of metaphase (a) Figure 5. "Bark – core" nucleoli with vacuole (a) and without vacuole (b) in apical root meristematic cells of birch from seeds collected in different districts of Voronezh. 100 x 1.5 x 10.

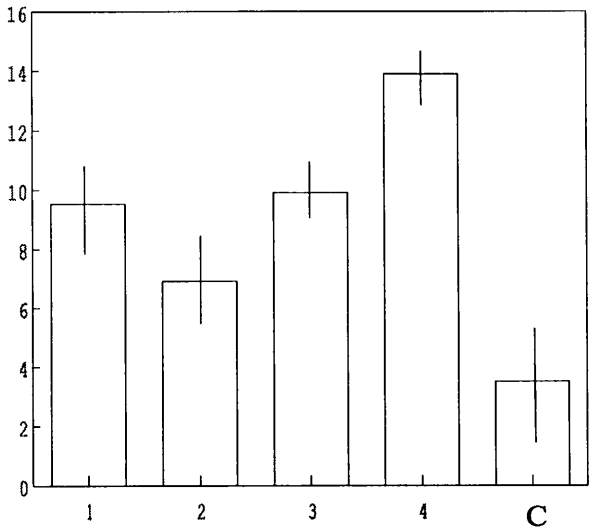

Figure 4. The frequency of persistent nucleoli at stage of metaphase – telophase of mitosis in apical root meristematic cells of birch from seeds collected in different districts of Voronezh. Ordinate – percentage of persistent nucleoli at stage of metaphase – telophase of mitosis. C – control, 1 – Lenin District, 2 – Left Bank Side District, 3 – Komintern District, 4 – Soviet District.

processes (SHELDON *et al.* 1961; HSU *et al.* 1964; HENEEN & NICKOLS 1966; NICKOLS 1970) and under introduction (NAZAROVA 2000). The appearance of persistent nucleoli in metaphase – telophase testifies to the puffing of chromosomes in loci of ribosomal genes and the increase of their number on experimental territories can be possibly regarded as response to stress induced by pollution of the environment. This fact could be construed as indemnification mechanism providing cells with normal amount of proteins under stressful conditions. In given parameter, Soviet District significantly differed from other experimental areas and control ($P < 0.05$), that also could be caused by the presence of high benz(a)piren concentrations exceeding MAC in the atmosphere of this district. It is interesting to notice the presence of positive correlation between frequency of persistent nucleoli and mitotic indexes (Spearman's rank correlation coefficient is $r_s = 0.482$, $P < 0.001$) and negative correlation between frequency of persistent nucleoli and level of pathological mitosis ($r_s = -0.234$, $P < 0.05$). As we suppose, this fact supports a conjecture about the compensation mechanism of persistent nucleoli origin. The high frequency of persistent nucleoli provides the high level of transcription activity of ribosomal genes and supports cell division. Possibly, this is the explanation of negative correlation with pathological mitosis (reparative proteins are synthesized actively).

The nucleolar characteristics of birch seed progeny are shown in the Table 1. The nonparametric ANOVA revealed significant effect of the birch location on surface area of single nucleoli in cells of its seed progeny ($P < 0.001$). This fact points out on dissimilar pollution of districts in Voronezh.

Single-nucleolar interphase cells of birch plantlet root meristem had the largest nucleolar surface area in Soviet and Komintern Districts (significantly different from the control). Perhaps, this is connected with the presence of benz(a)piren in air of these districts. The rising of nucleoli's size is the result of possible ribosomal gene amplification. Among two-nucleolar cells there were no distinctions in frequency of heteromor-

Table 1. The nucleolar characteristics in apical root meristematic cells of birch from seeds collected in different districts of Voronezh and on ecologically "clean" territory.

District	Surface area of single nucleoli, μkm²	Size of pair heteromorphic nucleoli, μkm²		Size of two homomorphic nucleoli, μkm²	Type of nucleoli, %			Frequency of cells with *n* nucleoli, %			Frequency of homo-rmorphic (1) and heteromorphic (2) nucleoli, %	
		"Large" nucleo-lus	"small" nucleo-lus		"Bark-core"	"bark-core" with vacuole	compact	2	3	4	1	2
Lenin	119.2 ±6.1	73.3 ±4.6	23.1 ±5.2[a]	99.5 ±12.2[a]	90.1 ±1.7[a]	9.6 ±1.6[a]	0.3 ±0.1	3.9 ±1.0[a]	0.1 ±0.0	0	20.9	79.1
Left Bank Side	129.2 ± 4.8	83.1 ±7.0	37.5 ±5.4[a]	105.9 ±19.0[a]	78.0 ±4.5	22.0 ±4.5	0	0.8 ±0.2	0.0 ±0.0	0	30.4	69.6
Komintern	184.7 ± 4.7[b]	116.9 ±6.0[b]	43.8 ±2.7[b]	143.8 ±13.1[b]	88.2 ±4.3[a]	11.5 ±4.4[a]	0.3 ±0.1	3.6 ±1.2[a]	0.6 ±0.2	0.0 ±0.0	17.4	82.6
Soviet	217.6 ± 5.3[a]	95.7 ±10.3	34.7 ±7.9	86.9 ±11.3[a]	84.2 ±2.8	15.4 ±2.8	0.6 ±0.3	1.7 ±0.3	0.0 ±0.0	0	32.7	67.3
Control	132.9 ± 6.9	71.2 ±9.7	22.0 ±4.3	56.6 ±5.1	79.1 ±1.9	20.9 ±1.9	0	0.9 ±0.4	0	0	18.2	81.8

[a] – significant differences with the control ($P < 0.05$); [b] – significant differences with the control ($P < 0.01$).

phic pair nucleoli between experimental and control samples, although, in most of trial territories its reduction was observed. These data indicate the large sensitivity of the single nucleoli's size parameter compared to the number of heteromorphic nucleoli.

The increase of the surface area of pair nucleoli was marked on trial territories. The sum of the areas of two heteromorphic nucleoli was less than the surface area of a single nucleolus, but it was more than the area of homomorphic nucleoli. All the districts of Voronezh differed from the control in the sum of surface area of heteromorphic nucleoli. Among homomorphic nucleoli the distinctions from the control were more essential. There was the interesting tendency: distinctions between "large" and "small' nucleoli in the cells with pair nucleoli were reduced in polluted areas. Perhaps, under conditions of contamination NOR, which is responsible for function of "small" nucleolus, intensifies its activity and "overtake" more active NOR forming "large" nucleolus. This hypothesis is confirmed by fact, that the surface area of "large" nucleoli did not change very essentially compared to the control. So, we can suppose that transcription activation of ribosomal genes does not occur by a steady activation of both NOR, but by an additional increase of functions of less active one.

We observed cells with 3 or 4 nucleoli; moreover, if the number of cells with 2 nucleoli increases, the number of cells with 3 nucleoli increases too (r_s = 0.491, $P < 0.01$). In opinion of a number of authors (SHERUDILO & SEMESHIN 1969; LAZAREVA 1999), the number of nucleoli in a cell indicates the quantity of active NOR. At norm, in the cells of Betula pendula one pair of chromosomes with NOR should be presented (MAKAROVA 1989). The increase of nucleoli's number is the original mechanism of indemnification, providing cell with normal amount of "total" production of nucleoli. This is confirmed by positive correlation between the percentage of two- and three-nucleolar cells and the sum of nucleolar surface area in two-nucleolar cells ($r_s = 0.191$ and $r_s = 0.245$, respectively, $P < 0.05$). The given parameter is maximal in Komintern District, what is possibly connected with the presence of substances in atmosphere of this district, which are capable to induce such effects. It may be benz(a)piren, for example. The increase of nucleoli's number and number of NOR in interphase cells and the decrease of nucleolus-nucleus ratio under stressful conditions of growth have been marked in coniferous plants by KHAIDAROVA and KALASHNIK (1999) and

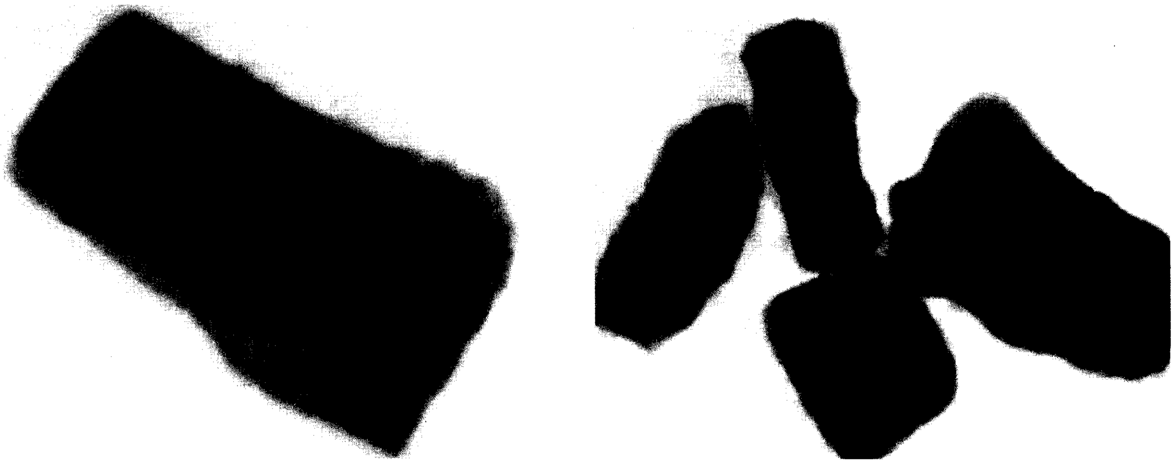

Figure 5. "Bark - core" nucleoli with vacuole (a) and without vacuole (b) in apical root meristematic cells of birch from seeds collected in different districts of Voronezh. 100 x 1.5 x 10.

MURATOVA and SEDELNIKOVA (1999). In opinion of these authors, this phenomenon is an adaptive sign of coniferous species to stressful conditions. We hypothesize, that the activation of latent NOR is also an adaptive mechanism to the conditions of anthropogenic pollution for *Betula pendula*. The types of nucleoli, which were found in birch root meristem, are shown in Figure 5 (a, b). It has been established, that "bark-core" nucleoli is the prevalence type of nucleoli in birch root meristematic cells: their main structures are RNP-fibrils in central part of nucleolus and RNP-granules forming continual layer in its periphery. These are high-active nucleoli, which are inherent for active meristematic tissue. "Bark-core" nucleoli with vacuole were marked also in both experimental and control samples. The appearance of vacuole in nucleolus testifies to the reduction of its functional activity (TCHELIDZE & ZATSEPINA 1988). In three out of four trial districts the decrease of cell number with vacuolate "bark-core" nucleoli and the rise of cell proportion with "bark-core" nucleoli without vacuole was observed. In Komintern, Soviet and Lenin Districts we found the presence of high-active compact nucleoli, which had the high degree of the development of granular component. This allows to assert about the rising of synthetic functions of nucleoli under conditions of anthropogenic pollution, especially in Soviet District. "Bark-core" nucleoli with vacuole had the largest surface area. There was marked the negative correlation between the percentage of vacuolate "bark-core" nucleoli and their surface area (r_s = -0.200, $P < 0.05$). Perhaps, it reflects the functional transitions between vacuolar and non-vacuolate nucleoli, assumed by TCHELIDZE and ZATSEPINA (1988). First, the rise of the size of vacuolate nucleoli takes place as a result of the intensive functional activity of cell. Then, a transition to new (more active) stages occurs. As we think, this opinion is supported by the positive correlation between the surface area of vacuolated "bark-core" nucleoli and percentage of both "bark-core" nucleoli without vacuole and compact nucleoli (r_s = 0.320, $P < 0.01$), that is if the sizes of vacuolar nucleoli increase, their number in cells decrease and the percentage of high-active nucleoli rise. We found the negative correlation between percentage of "bark-core" nucleoli and mitotic activity (r_s= -0.334, $P < 0.01$) and the positive correlation between percentage of these nucleoli and pathological mitosis (r_s = 0.188, $P < 0.05$). The opposite relationship was observed for vacuolar "bark-core" nucleoli (r_s= 0.246 and r_s = -0.235, respectively, $P < 0.05$). This points at the compensative character of the nucleolus type change in a high-active one, that could support a high level of protein biosynthesis under conditions of the functional repression of many unique genes by pollutants. The activation of latent NOR takes place by a transition to the more active nucleolus type. The positive correlation between the level of high-active nucleoli (compact, "bark-core" without vacuole) and the frequency of cells with 2, 3, 4 nucleoli (r_s = 0.371, $P < 0.01$) and the negative correlation between the level of high-active nucleoli and the number of cells with vacuolate nucleoli (r_s = -0.187; $P < 0.05$) allow to assert to favour of this phenomenon.

Thus, our experiment indicates, that in many cytogenetic characteristics the most adverse situation is in Soviet and Komintern Districts of Voronezh. Earlier we analyzed micronuclei in the cells of buccal mucosa of children living in the same districts of the city (BUTORINA *et. al.* 2000a). Comparison of micronucleus test's data with the data of cytological investigation of birch seed progeny should allow us to consider about environmental health. According to our data, the highest frequency of micronuclei was observed in children from Soviet District and it significantly differed from other districts. Komintern District was on the second place. This fact also testifies to high degree of air pollution by different compounds including benz(a)piren in these districts. We revealed the presence of correlation between following cytogenetic characteristics of human and birch: the frequency of micronuclei in epitheliocytes of children's mouth cavity, the surface area of single nucleoli and the level of pathological mitosis in apical root meristem of birch plantlets (the correlation coefficient in both cases is 1, $P < 0.05$). The data of cluster analysis of cytogenetic characteristics of birch seed progeny and frequency of micronuclei in buccal mucosa of children (Figure 6) allow to affirm about the closest cluster distances between the surface area of single nucleoli in birch seed progeny and the frequency of micronuclei in children. The above-mentioned data point out that the changes of these characteristics have similar causes. Therefore, the use of such characteristics as the level of pathological mitosis and the surface area of single nucleoli in roots of birch plantlets is perspective for cytogenetic monitoring of anthropogenic air pollution, because they will allow to make forecast of the genetic risk for the humans under unfavorable environmental conditions.

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