Chemotypic structure of \textit{Pinus sylvestris} population in Usmansky and Khrenovskoy pine forests

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\textbf{Abstract.} The main target of the paper is to study structure of main pine forest types in Usmansky and Khrenovskoy pine forests, their chemical composition of essential oil (EO) monoterpene fraction. This paper is on problems of Scots Pine (\textit{Pinus sylvestris}) breeding and seed-growing in the forest-steppe European part. It is done for forest culture improvement, based on genetics and breeding. The characteristic of main components of pine-needles essential oil in free and controlled cross-breeding was investigated. The established peculiarities of monoterpene biosynthesis in pine-needless essential oil are genetically motivated. The long-term opportunity for essential oil composition usage as characteristics of \textit{Pinus sylvestris} breeding on resistibility to diseases and pests was defined. The structures of main forest types in Usmansky and Khrenovskoy pine forests according to basic monometric and biochemical indices were studied. The data on composition, changeability and inheritance regularity of monoterpenes may be used for recommendations on creating of \textit{Pinus sylvestris} seed base on genetic and breeding bases.

1. \textbf{Introduction}

Studying of \textit{Pinus sylvestris} essential oil is a part of work efficiency increase in breeding of main forest forming species. Changeability of coniferous essential oil is not studied enough, as well as connection levels and directions between all the components of monoterpene fraction composition. Another current problem is the investigation of natural pine stands population structure [1, 2] and artificially created forest cultures according to essential oil composition. The question of essential oil significance in pine seed-growing is still of vital importance.

The exceptional genetic variety and presence of specific features of the genetic structures of the \textit{Pinus sylvestris} populations in the forests of the Central Forest-Steppe make it necessary to study and develop a strategy for conservation of the remains of the natural gene resource of \textit{Pinus sylvestris}. Natural old-growth areas of the forest are the only living labs in which people can learn to create sustainable forests. Studying and preserving the sites of ancient forests is an issue of paramount importance, because they have a genetic code and all the features that are crucial for a healthy and flexible forest.

The main aim of this paper is to approach to recommendations on the forest cultures creating in Central forest-steppe of Russian European part by means of planting material with a definite composition of coniferous essential oil. The total number of forest cultures should correlate with the structure of forest types of natural plantation in present forest vegetation conditions, taking into consideration definite pine chemotypes interchange, resistant or unstable to diseases and pest damage.
2. Materials and methods

Natural stands, forest cultures, seed plantations, crops and *Pinus sylvestris* plantations in Central Chernozem region were taken as the objects for studying.

Chemical composition and phenotypic features of monoterpenes biosynthesis in essential oil of *Pinus sylvestris* pine-needles were studied in natural plantations of experimental silviculture enterprise of Voronezh State University of Forestry and Technologies (VSUFE), Somovo silviculture enterprise, Voronezh State Reserve and Davidovsky silviculture enterprise (the Voronezh region).

Four years old (1+3) seedlings of Davidovsky silviculture enterprise forest area and two years old seedling of VSUFE experimental silviculture enterprise forest nursery were investigated for analysis of essential oil composition of *Pinus sylvestris* seedlings after reciprocal cross-breeding.

Population structures of *Pinus sylvestris* according to monoterpenes biosynthesis type were tested on four forest types of Usman pine forest, three forest types of Khrenovskoy pine forest and 120 and 50 years old cultures of Khrenovskoy Forestry Colledge (the Voronezh region).

Cones monoterpenes fraction and pine-needles monoterpenes individual changeability in connection with pine damaging by cone pests were investigated on forest seed plantation clones in Somovo silviculture enterprises (the Voronezh region).

The experiments of pine breeding on resistance to pine fungus were carried out in Levoberezhny and Zhivotinovo forestry plantations of VSUFE experimental silviculture enterprises, Khrenovskoy and Braginsky forestries, Khrenovskoy Forestry College and Semeno-Alexandrovsky forestry of Bobrov forest complex.

For precise analysis of essential oil it is necessary to obtain biologically homogeneous material and for problems, connected with forestry and biological importance studying, it is essential to investigate biotypes individual changeability of compositions of *Pinus sylvestris*.

Using the correlation of essential oil components output, the principal changeability of extraction method application by diethyl ester was found as well as changeability of essential oil monoterpenes fraction of *Pinus sylvestris*.

EO monoterpenes fraction composition was adopted by the method of gas-liquid chromatography on the chromatograph “Chrom-5”. Helium was used as a carrier gas at the flow rate of 1ml/min, while HP-5MS (30m, 0.25mm, 0.25um) capillary column was used. The initial temperature was programmed at 50-100 °C at the rate of 5 °C/min and then 100-250 °C at the rate of 3°C/min followed by a constant temperature at 250°C for a period of 25 minutes. Sample (2 µl) was injected to the column programmed at 200 °C and resolution of components was attained. Identification of individual components was carried out by comparison of their relative retention time with those of authentic samples by co-elution and MS analysis. For the components like terpenes and aliphatic compounds, the reference samples were not available. It is determined that stable composition of EO main components (α-pinene and Δ3-karen) is set under correlation of pine-needles mass and solvent volume 1:2. Two grams are a minimal weight of pine-needles sample, recommended for extraction, in which EO monoterpenes fraction composition sets the most precisely.

3. Results and discussion

Analysis of monoterpenes fraction components composition of pine-needles EO in seedling after reciprocal crossing, as well as their class distribution, show that quality of seedlings with monoterpenes homogeneous content and quantity of seedlings, that inherits EO monoterpenes composition from parent clones, do not exceed 40% of the whole quantity of plants under analysis with connection group coefficient 0.96±0.85 and space arcCos equal to 0.15±0.52.

According to the discovered peculiarities of monoterpenes fraction composition inheritance of essential oil in *Pinus sylvestris*, under reciprocal clones crossing with definite terpenes biosynthesis type, the production of definite seedlings number with required quality of α-pinene and Δ3-karen may be planned. To fulfill this it is necessary to set biosynthesis type of monoterpenes fraction EO in crossed clones.
Up to 35-40% of pinen and intermediate types of plants with monoterpane fraction EO composition, appropriate to parent clone composition (±5%), are defined under controlled crossing of intermediate type clone (α-pinen, 36 ±5%; Δ³-karen - 36±5%) or pinen type clone (α-pinen 43 ±5%; Δ³-karen - 25 ±5%).

After controlled crossing, 30-35% of pinen type seedlings were obtained. The controlled crossing was used for pure pinen type clone (α-pinen - 54% and more; Δ³-karen - 12% and less) with pinen type clone (α-pinen 42 ±5%; Δ³-karen - 18 ±5%).

To obtain 30-35% of pinen type plants (α-pinen 42 ±5%; Δ³-karen - 18 ±5%) the controlled crossing should be carried out: karen type clone (α-pinen 18 ±5%; Δ³-karen - 36 ±5%) with pure pinen type clone (α-pinen 54% and more; Δ³-karen - 12% and less) or pure pinen type clone (α-pinen 54% and more; Δ³-karen - 12% and less) with karen type clone (α-pinen 24±5%; Δ³-karen - 36 ±5%).

Table 1. Characteristics of monoterpenes biosynthesis types according to α-pinen and Δ³-karen content in pine-needles essential oil.

| Content components | Biosynthesis types | I | II | III | IV | V | VI | VII | VIII | IX |
|--------------------|-------------------|---|----|-----|----|---|----|-----|------|----|
| Observed phenotypes |               | 54<&> | 54-42 | 41-31 | 30-18 | 41-31 | 48-42 | 24-12 | 30-18 | 12<&> |
| Hypothetical genotypes |             | Aabb | AABb | Aabb | aabb | AaBb | AABB | aaBb | AaBB | aaBB |
| α-pinen to Δ³-karen correlation |           | 4.5:1 | 3:1 | 2:1 | 1:1 | 1.5:1.5 | 2:2 | 1:2 | 1:3 | 1:4.5 |

An investigation of monoterpane fraction composition of essential oil of about 2000 Pinus sylvestris trees has been carried out. Nine groups, united into three biosynthesis types of essential oil monoterpenes were distinguished (table 1).

Differences authenticity between average values of selected groups is proved in 5% significance level (F_r = 49.4; F_05=2.03) under HCP=4.8- X_r and under 1% level with confidence interval = (X±6.45X).

Group number and percent modulus of components content, appropriate to them, are defined in extracts classification for proximity measures determination of multidimensional objects and boundaries determination between homogeneous groups.

α-pinen and Δ³-karen are taken as the tests for biosynthesis types characteristics, because they form the main part of essential oil monoterpane fraction. The connection between α-pinen and Δ³-karen is proved, it is and high (Z=0.83-0.91).

Differences in indices of correlative ratio connection inadequacy (η=0.91) and correlation coefficient proved to be unessential (t=1.55 < 3), that is why correlation between α-pinen and Δ³-karen is linear and high. Regression coefficient calculation (R_α/Δ³=-0.79; R_α/Δ³=-0.87) (the authenticity of which is demonstrated under high level of significance) distinguishes, that 1% α-pinen increase causes decrease of Δ³-karen up to 0.87% and 1% Δ³-karen increase causes α-pinen decrease up to 0.79%. Connection between main components has linear character and is expressed through regression equation

\[ (X_\alpha/\Delta^3=60.2 - 0.79y) \text{ and } (Y_{\Delta^3}/\alpha=58.5-0.87x). \]

Using method, applied in works [2,3], calculation for phenotypic boundaries determination of presumed genotypes and cluster analysis for proximity measures determination of multidimensional

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objects, the hypothetical genotypical characteristics of selected biosynthesis types of main monoterpenes was obtained. This characteristic was used in hybridization analysis of progeny from reciprocal (controlled) four clones crossing (3, 4, 22, 27) in Somovo forest seed plantation (the Voronezh region). During the results analysis, the hypothesis of monogenic and digenic splitting was tested.

In calculations of theoretically expected frequency of progeny genotypes output and correlation with experimentally observed genotypes frequency of sibs and semi-sibs, based on the law of independent gene combination under their simple cooperation [4] the result was the following: for semi-sibs of clone 27 criterion \( \chi^2 = 4.0 \) for clone 22 it is 0.81 and for semi-sibs of clone 4 criterion is 4.9. For sibs of family 27x22 criterion \( \chi^2 = 10.05 \), for family 22x27 it is 9.06 and for families 4x22 and 22x4 it is 10.91 and 1.72, when \( \chi^2_{0.05} = 11.07 \). Consequently, according to the results, it may be considered, that differences between theoretically calculated and experimental genotypes output frequency support null hypothesis \( X_{0.05}^2 > X_{\text{fact}}^2 \).

Revealed regularities of conducted study of pine-needles essential oil monoterpene fraction and reciprocal crossing analysis prove that \( \Delta^2 \)-karen synthesis is under isolated gene control. We can affirm that \( \alpha \)-pinen synthesis also has monogenic base. Allelic pairs of these components have definite levels and bound directions.

Consequently, three biosynthesis types of essential oil, characterized by content monoterpene fraction main components, have genetic base and genotypical forms characteristics.

It was found that clone 3 of pure pinen form I, genotype (AAbb) in Somovo seed plantation gives up to 50-60% of hollow seeds. It is reflected in peculiar distribution of genotypes output factual frequency in sibs and semi-sibs analysis of clone.

Analysis showed that three main biosynthesis types of monoterpenes, typical for Pinus sylvestris on the territory of Usman pine forest, are the following: karen, intermediate and pinen.

While comparing general groups frequencies of biosynthesis types the index of population similarity (R) [5], correspondence criterion \( \chi^2 \) and student-t-test calculation \( t \), it was found, that presumed equal distribution of trees according to biosynthesis types is typical for lichen and green-moos pine forests. Grass-moor pine forest vitally differs from the previous two types in trees distribution.

These results to some extend prove the data [6] that soil conditions influence quantity content of monoterpenes composition in resin terpene oil of Pinus sylvestris population in the Perm region and the Mari El Republic.

For practical output the fact is noteworthy, that under conditions of pine habitat with limited moisture degree and inorganic nutrition, the part of karen type trees is 23% and vice versa in damp and overdamp conditions 67% of pinen trees are dominant, but the representatives of karen type biosynthesis are not distinguished [7-9].

Khrenovskoy pine forest (Voronezh region) is located in the very South boundary of Pinus sylvestris natural area and it is the representative of insular forest tracts. Obviously, for a long period of time it is to certain extend isolated from gene influx of other populations.

Correspondence criterion estimated values \( \chi^2 \) (K. Pirson) of trees occurrence frequency of Pinus sylvestris with definite biosynthesis type of coniferous essential oil as well as with definite resin composition for Khrenovskoy pine forest plants under studying show, that null hypothesis about compared frequency correspondence is rejected for selections of all investigated types of forest, excluding cultures of N.D. Sukhodskoy and industrial ones.

Null hypothesis is not rejected for them in case of 1% significance level. This correlation between selections is distinguished both under frequency comparison according to biosynthesis types of pine-needles essential oil and under resin turpentine composition. The fact, obtained for Usman pine forest is assumed: there observed the connection between occurrence frequency of karen and pinen types trees of pine-needless essential oil biosynthesis in dependence on habitat conditions and place. So, in Khrenovskoy pine forest lichen forest has up to 36% of karen type specimen and only about 20% of pinen, and moline pine forest - two times less: 18% of karen type trees, 48% of pinen type
biosynthesis. Pine forest, that grows near-by steppe area, has 28% of karen and 41% of pinen type trees, so it takes intermediate position.

Thus, the investigation of main forest types structure of Pinus sylvestris in Khrenovskoy pine forest shows, that tree distribution both according to types of coniferous essential oil biosynthesis and resin composition differs from natural plantations, growing in different forest vegetation conditions [8,9].

The results of cones monoterpenes composition study during summer period show its significant change in quantity in comparison to winter period fraction composition. It is true both for cones themselves and for coniferous essential oil, mainly α-pinene content decrease for 1.5-2.0 times (from 44.8 to 17.9%), Δ3-karen increase for 1.0-1.5 times (20.6 to 38.9%) and 1.8 - cineole increase up to 10% (from 3.6 to 12.1%). Their stabilization in relative rest period allows to make a supposition about peculiarities in biochemical processes of terpenes transformation in cones.

4. Conclusion

1. As a result of experiments in Somov grafting forest seed plantation (the Voronezh region) it was found, that cone pests significantly damage Pinus sylvestris trees with content of α-pinene and Δ3-karen main components in coniferous essential oil in ratio (5 (4.5) : 1), less significantly in ratio (2 : 1) and far not significantly with ratio (1.5 : 1).
2. Trees with main components ratio (1:1) are damaged at different degree, depending on their position to other clones. Trees with ratio (1:2) are considered to be more resistant in comparison with all other trees.
3. In two clones with the same essential oil type, the clone, presented by more homogeneous graftings (according to monoterpenes composition), has low degree. Cones monoterpenes fraction composition may serve as addition in determining one or another way of possible damaging degree, but not as the resistance index.
4. In pine-needles essential oil of trees, not affected by pine fungus, the increased proportion of Δ3-karen and terpinene was discovered. These substances are known to suppress growth of mycelium fungus - the disease agent.
5. Essential oil Δ3-karen content of sound trees in pine fungus centers fluctuates from 20.2 to 60.1%, while adjacent value is 37.0%.
6. In N.D. Sukhodsky cultures, not significantly damaged by pine fungus, Δ3-karen content fluctuates from 5 to 68.4%, while adjacent value is 35.1%. Terpinolene content in essential oil of compared trees groups is 2.7 and 3.5% accordingly.

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