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Experiment of Intraspecific Hybridization of Siberian Stone Pine (*Pinus Sibirica Du Tour)* Clones in Middle Siberia

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Abstract

Experiments in intraspecific hybridization of Siberian stone pine, not just of this species but of different regional origins as well, have been carried out at a clone plantation in Middle Siberia (Krasnoyarsk forest- steppe). Crossings were realized using the principle of ecologo-geographical remoteness of populations. Siberian stone pine clones chosen for crossing had good growth and constant reproductive ability over many years. An analysis of the characteristics of hybrid female cones (weight, linear size, number of developed scales) and seeds (number, weight, seed fullness, viability) showed the positive influence of controlled pollination with combinations of different climatypes. The study of hybrid growing climatypes, resulting from crossing of the plain and mountain populations, also revealed signs of heterosis.

Key words: Intraspecific hybridization, Siberian stone pines, Climatypes, Clones, Clone plantation, Hybrids, Controlled pollination, Open pollination

Introduction

Hybridization of forest tree species, alongside with the selection and reproduction of the best plus trees, is one of the basic methods of forest selection to increase wood efficiency as well as improve their qualitative structure. Work in artificial inter- and intraspecific hybridization, both of deciduous and coniferous tree species, has made significant progress.

The control crossing of conifers, five-needle pines, has been realized in the USA, in Scandinavian countries, in Germany, Yugoslavia, Korea and other countries (Li Wen-Ying, 1964; Ahlgren C.E., 1972; Kriebel., 1972; Blada, 1994, 2000); Scots pine (Duffield, 1958; Wright and Gabriel, 1958; Saylor and Koenig, 1967; Hyun and Hong, 1969; Critchfield, 1967, 1968; Forbes, 1974; Karrfalt, 1975; Hyun, Ahn and Hong, 1972). Papers by N.T. Mirov (1967) and J.W. Wright (1978) fully describe interspecific hybridization of nuciferous pine. These papers report that in the following combination (Sugar pine x Korean pine) produced a large number of cones with empty seeds. A crossing of Swiss stone pine and Korean pine with the non- nuciferous tree species Macedonian pine and Eastern white pine also failed to produce hybrid seeds. According to N. Hagmann's data (1963) the crossings between these tree species show a high degree of incompatibility. Nevertheless, the perspectives of interspecific hybridization in forest science are big. Some combinations may be improved by repeated crossings of several selected trees of one species with the trees of another species (Wright, 1978). The following crossings Eastern white pine x Mexican white pine (Steinhoff, 1972), Western white pine x Limber pine (Mirov, 1967; Bingham, 1972), Western white pine x Southwestern white pine, Western white pine x Whiter bark pine (Bingham, 1972; Bingham *et al*., 1972) were successful.

The hybridization of the Pinus genus was started in Russia only in the 1970s (Makhalin, 1970; Prokazin, 1972; Ievlev, 1975; Belostotskaya, 1979; Patlai, Molotkov, 1981; Baumanis *et al*., 1987, etc.). Many experiments were carried out in the intraspecific crossing of pine trees, especially in crossing different climatypes (Mantsevich, 1975; Nenyukhin, 1983, 1988, etc.). The significance of the experiments undertaken in hybridization is not yet clear because not enough time has passed, therefore it is too early to speak about the potential of any crossing variants.

The inter- and intraspecific crossings of *Cembra* trees have been carried out. No positive results with the artificial interspecific crossings of Siberian stone pine with Korean pine (Demidenko, Urusov, Il'itchev, 1982) or with Japanese stone pine (Sokolov, 1972) have been achieved. Normally developed seeds were obtained by crossing the Siberian stone pine x Swiss stone pine. This hybrid grows fast, and produces seed early and abundantly (Titov, 2006). Crossing the Siberian stone pine x Macedonian pine, Siberian stone pine x Lacebark pine, Siberian stone pine x Chilgoza pine met with little success. Attempts to cross Siberian stone pine with Italian stone pine, a representative Pinus subgenus, failed (Titov, 2006).

The review of literature shows that crossings between species in the Pinus genus that belong to different sections but have an equal chromosome number are very difficult. Breeding of economically valuable hybrids depends, in many respects, on selecting the parent pairs (Wright, 1962). Provenance and native growing site of tree species have a specific impact on possible hybridization (Duffield, 1958;

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Critchfield, 1966; Mirov, 1967).

As a precondition for successful Siberian stone pine hybridization it is important to understand its high polymorphism, apparent in its geographic and especially in its intrapopulation variability (Pravdin, Iroshnikov, 1982; Krutovsky *et al*., 1988; Goncharenko *et al*., 1993; Iroshnikov, Politov, 2004).

The geographical variations of genotypical structure of natural populations of Siberian stone pine have not been studied sufficiently. Since the Siberian stone pine covers a vast area, there must be many different geographical forms. The following are some intraspecific ecological forms that have been identified (Iroshnikov, 1985): f. coronans Litw., f. turfosa Gorodk, f. depressa Kom., var. humistrata (Middend.).

The intrapopulation forms are the main source of the initial material for crossing and establishing Siberian stone pine sorts. In natural populations the Siberian stone pine trees differ: in development by sex type (male or female) (Nekrasova, 1972; Iroshnikov, 1974; Minina, 1979; Vorob'ev *et al*., 1989, Titov, 1991 etc.); also the start of the reproductive phase in ontogenesis (early or late ripening); with rapid development of cones; in the form of cones and apophysis; in the crown and bark form (Iroshnikov, 1974, Pravdin, Iroshnikov, 1982); gum productivity (Vorob'ev, 1983) and some other forms.

The theories of genetic balance and superdominance connect the appearance of hybrid vigor with inherited parental distinctions (Turbin, 1961). According to this theory, heterosis is one of the consequences of changed genetic balance in hybrids, which were obtained from crossing unrelated lines. The crossings with the most potential are the ones that result in hybrids with positive heterosis. They can be obtained under well-marked sex biopolarity, which means when the parent pairs are distinct from each other in pronounced sex features (Molotkovsky, 1966; Zhuchenko, Nesterov, Andryushenko *et al*., 1967; Minina, Larionova, 1979; Titov, 2006).

The successful hybridization of forest tree species relies on the principle of selecting pairs by the degree of ecologo-geographical remoteness of the parents. The obtained hybrids have accelerated growth and richer seed production (Yablokov, 1962; Steinhoff, 1972, Wright, 1978, Knyazeva, 1978; Shcherbakova, 1987 *et al*.; Nenyukhin, 1988 and others).

Developing methods for establishing a geographic forest seed grafting plantation of Siberian stone pine in the Siberian area was the main objective of foundation of clone Siberian stone pine plantation in 1965. Having a large collection of clones of different Siberian stone pine provenances at the graft plantation in Krasnoyarsk forest steppe allowed us to use the principle of selecting pairs according to their ecological- geographical remoteness.

The objective of this work is to obtain improved hybrids in the first generation (in growth and seed production) as a result of using geographically remote hybridization of Siberian stone pine grafts. Later the hybrids can be reproduced naturally so that we can develop recommendations for obtaining hybrid seeds at forest seed plantations.

Materials and Methods

The experiments in geographical hybridization of Siberian stone pine of different origin in Krasnoyarsk forest-steppe were carried out in 1986 at the Siberian stone pine clone plantation. Studying and selecting fast growing, vital and productive Siberian stone pine trees, and the realization of controlled crossings on established grafting plots to obtain improved hybrid seeds were the objectives.

Cuttings for grafting were taken from more than 40 growing sites of the Siberian stone pine areal. Naturally regenerated Scots pine trees growing on a logged area were used as stock. The parent trees of Siberian stone pine for the clone plantation were thoroughly screened and only those with a good cone crop over many years were selected. Five trees (ramets) of each clone of every climatype were pollinated. Not less than 25 macrostrobiles were pollinated in each variant. Since all the trees (ramets) of one clone inherit the features of the mother tree the remaining pollinated macrostrobiles of one clone were combined at the statistical treatment of cones and seeds. In the control open pollination not less than 25 macrostrobiles remained in each variant as well.

For the controlled pollination the pollen of the previous year was used. Its vitality was tested and pollen vitality was determined using a 15% sucrose solution in a moist sterile Petri dish at a constant temperature of 26 ^{o}C. After 3 days of growth the germinated pollen (from 150 to 250 pollen grains) was measured. Pollen vitality was determined by length of pollen tubes. The pollen grains were considered vital when pollen tube length was 1.5 – 2.0 times the length of the pollen grain body (Istratova, 1961). Pollen viability of the climatypes being tested was from 70% to 83%.

With controlled pollination the clones of geographically remote climatypes were used. They are shown in the Table 1. The following combinations were applied at controlled pollination:

- **1st variant**
  Yermakovsky climatype, 500 m a.s.l., (Krasnoyarsk krai) x Baikit climatype (Krasnoyarsk krai);
- **2nd variant**
  Verkh–Katunsky climatype, 1000 m a.s.l. (R. Altai) x Yermakovsky climatype, 1500 m a.s.l. (Krasnoyarsk krai**)**;
- **3rd variant**
  Kozylsky climatype, 200 m a.s.l. (Krasnoyarsk krai) x Yermakovsky climatype, 1500 m a.s.l.;
- **4th variant**
  Yermakovsky climatype, 500 m a.s.l. (Krasnoyarsk krai) x Angarsky climatype, 500 m a.s.l. (Irkutsk oblast).

Selection of these climatypes for study was determined by the assumption that heterosis posterity is possible owing to two facts: 1) the selected Siberian stone pine provenances are characterized by many years of good growth, constant reproductive ability and 2) the mother and father trees have remote geographic origins (provenances).

The semi-sibling posterity from the open pollination of mother trees in each variant was used as a control.

The data were analyzed by analysis of variance and significant means were separated according to Student's *t*-test (P=0.05).

Results and Discussion

Hybrid cones were produced from all the controlled pollination variants that were collected in August 1987. The cones of the same trees after open pollination were picked for the control. Survival of hybrid cones varied from 10% up to 54%. Analysis of the collected hybrid cones showed that all the crossing variants turned out to be heavier and larger (length, width) than the control cones from the parent trees.

The quality of seeds from the cones of different controlled pollination variants is shown in Table 2.

Table 1. List of Siberian pine clone provenances used for controlled hybridization.

Climatypes	Coordinates		Vegetation period characteristics (days with t > 5^oC)	
	NL	EL	Number of days	Sum of temperatures
Krasnoyarsk krai				
Kozylsky, 200, m, a.s.l.	$56^o38'$	$91^o30'$	140 - 153	1823 - 2050
Baikit. 500, m, a.s.l.	$61^o40'$	$96^o20'$	119 - 123	1475 - 1573
Yermakovsky, 500, m, a.s.l.	$53^o10'$	$95^o20'$	130 - 158	1501 - 2202
Yermakovsky, 1500, m, a.s.l.	$53^o20'$	$95^o20'$	111 - 112	1088 - 1103
Irkutsk oblast				
Angarsky, 500 m, a.s.l.	$55^o45'$	$103^o05'$	117	1377
Resbublica Altai				
Verkh-Katunsky, 1000, m, a.s.l.	$50^o43'$	$86^o06'$	129 - 150	1380 - 1920

Table 2. Characteristics of Siberian pine seeds of hybrid origin.

Crossing variants	Seed number in the cone (number seeds)			Weight of developed seeds in the cone, g	Weight of 1000 seeds, g	% of full seeds	% of empty seeds	% of seeds with polyembryons from full seeds	Viability, %
	Under - developed	Developed	Total						
No.1 variant									
Ermakovsky, 500 m a.s.l. x **Baikit (Evenkia)**, 500 m. a.s.l…	3.7±0.67	90.0±5.01	94.2±4.52	16.1±0.76	179	83	17	3	51
Ermakovsky, 500 m a.s.l. (open pollination)	5.3±1.26	71.5±3.53	76.1±3.28	13.4±0.49	187	84	16	2	50
No.2 variant									
Verkh- Katunsky, 1000m a.s.l… x **Yermakovsky**, 1500 m.a.s.l.	8.2±1.72	58.2±3.10	65.8±6.27	9.8±0.96	169	84	16	3	36
Verkh- Katunsky, 1000m a.s.l… (open pollination)	11.4±3.29	28.8±4.89	41.8±4.66	4.9±0.81	170	84	16	8	40
No.3 variant									
Kozulsky, 200 m.a.s.l. x **Yermakovsky**, 1500 m a.s.l...	2.5±0.50	21.0±6.80	23.5±6.50	5.6±1.55	267	95	5	37	53
Kozulsky, 200 m.a.s.l. (open pollination)	3.8±0.92	28.2±4.45	33.8±3.24	7.8±1.05	248	88	12	39	41
No.4 variant									
Yermakovsky, 500 m a.s.l… x **Angarsky**, 500 m a.s.l...	1.67	76.0±6.99	82.0±3.16	11.6±1.06	153	84	16	2	48
Yermakovsky, 500 m a.s.l... (open pollination)	8.6±1.36	80.7±3.44	89.7±4.25	10.8±1.26	134	61	39	2	29

Seed fullness in variant No.4 was much higher than in the control one (84% vs 61%). In the control, 39% of the seeds of variant No.4 were empty, possibly a result of insufficient pollination. Some research has shown that the high percent of empty seeds is connected with self-pollination (Hagman, Mikkola, 1963; Zemlyanoi, 1981). Some researchers believe the appearance of empty or underdeveloped seeds is a peculiar release of genetic load (Koski, 1973), or the result of unfavorable climate (Nekrasova, 1983; Tretiyakova, 1990; Shigapov, 1997 and others). The presence of empty seeds in hybrid seeds (from 5% to 17%) in our experiment is, likely, connected with the absence of insemination or with a disorder during embryo development (Kuznetsova, 1987; Romanovsky, 1989; Tretiyakova, 1990).

In variant No.3, a high percentage of seeds with polyembrions (experimental - 37% and control - 39%) was noted. It is believed that it is related to the influence of parent tree heredity (Table 2). The reasons for polyembriony in plants have not been well studied. Some researchers think that it appears owing to an increase in nourishment, others connect it with hybridization or hereditary factors (Zemlyanoi, 1973; Shimak, 1973; Iroshnikov, 1984 and others). On the clone plantation of Kozulsky and northern Baikitsky clones polyembryonal seeds have been constantly observed. This regularity becomes apparent over a period of many years which indicates the inherited character of this phenomenon (Kuznetsova, 2003). Low seed viability was observed in seeds of all the crossing combinations (from 36% up to 53%), and in the control, with open pollination, it was 29% - 50%. In variant No.2 with the crossing of the mountain populations seed viability was lower since the hybrid seeds had, mainly, a small embryo 0.1 – 0.2 of embryo canal length. In all the crossing combinations and in the control the embryo length of seeds was from 0.1 to 0.9 of embryo canal length. It should be noted that the underdeveloped endosperm is characteristic of all developed hybrid seeds. The underdeveloped endosperm in seeds is explained by the early collecting of the macrostrobiles. Weight of developed seeds in cones of variants No. 1, 2, and 4 exceeded that one of the control variant. However, the number of developed seeds in variants No. 3, and 4 was less. The weight of 1000 developed seeds was more in variants No. 3 and 4.

An analysis of cone characteristics (the weight, linear sizes, number of developed scales), as well as quality of seeds (the number, weight, seed fullness, viability) showed the positive influence of controlled pollination of Siberian stone pine climatypes in the crossing of variants (No. 3 and 4). The advantage was seen in the quality of the cone formation and in the increased number of full seeds.

Hybrid seeds, as well as seeds from open pollination, were sown in 1988 after the stratification. The percentage of germinated hybrid seedlings in all the controlled pollination variants was much higher (50% - 80%) than from open pollination (15% - 36%). The variability of cotyledon number in seedlings of all the

Table 3.Growth of hybrid Siberian pine seedlings in Krasnoyarsk forest- steppe (numerator - 9 years, denominator - 4 years). Significant differences (t-test; P=0. 05) between hybrids and seedling of open pollination are denoted by an asterisk.

Crossing variants	Height, cm	Cv, %	Increment, cm	Cv, %	Diameter, cm	Cv, %	Needle length, mm	Cv, %
No.1 variant								
Yermakovsky, 500 m a.s.l. x **Baikit (Evenkia)**, 500 m a.s.l...	54.2*/11.0	30/17	18.2*/4.5	18/75	2.5*/0.7	40/43	85.5/57.4	13/7
Yermakovsky, 500 m a.s.l. (open pollination)	46.0/9.1	36/8	10.5/3.0	54/47	1.8/0.5	33/40	93.6/44.8	13/22
No.2 variant								
Verkh- Katunsky, 1000m a.s.l. x **Yermakovsky**, 1500 m a.s.l...	48.8/9.3	29/19	12.3/2.6	41/46	1.6/0.8	44/25	86.1/50.4	10/17
Verkh- Katunsky, 1000m a.s.l. (open pollination)	68.2/10.7	10/11	22.0/1.5	10/11	2.0/0.6	13/50	99.7/46.5	17/9
No.3 variant								
Kozulsky, 200 m a.s.l… x **Yermakovsky**, 1500 m a.s.l.	71.2*/11.5	36/40	19.5*/3.2	22/19	2.3*/0.8	22/21	109.6*/20.8	9/19
Kozulsky, 200 m a.s.l... (open pollination)	34.0/8.1	36/22	9.3/1.3	40/22	1.6/0.4	56/25	100.0/53.8	15/22
No.4 variant								
Yermakovsky, 500 m a.s.l… x **Angarsky**, 500 m. a.s.l.	41.0*/8.6	37/29	12.0*/2.7	38/24	2.0/0.5	35/60	100.1*/46.4	8/19
Yermakovsky, 500 m a.s.l... (open pollination)	17.8/9.1	36/8	10.5/3.0	54/47	1.8/0.5	33/40	93.6/44.8	13/22

crossing variants was small. No large distinction in the cotyledon number in seedlings of all the crossing variants was seen.

The growth of hybrids, resulting from Siberian stone pine climatype controlled pollination, are shown in Table 3. We see from Table 3, that hybrid seedlings in the 4-year-old group exceeded the control ones (seedlings from open pollination) in all the parameters (growth, increment, diameter and needle length) in variant No. 1 and in variant No. 3 (excluding needle length). The 9-year-old seedlings exceeded the control ones in the same parameters as in variants No. 3, 4 and in variant No.1 (excluding needle length).

Table 3 shows that the hybrids from the controlled pollination of the plain and mountain populations of Siberian stone pine (variant No. 3) have the best growth of all the seedlings from open pollination, and were higher than those of other hybrid seedlings. The hybrids of combination No. 3 have visual signs of heterosis, they surpass the control ones by 50% in growth and diameter. Hybrid seedlings of variant 3 were two times taller (71.2 cm) than seedlings from the open pollination The reliability was confirmed by the Student's *t*-test (in height t=7 at P=0.05, in needle length t=3 at P=0.05). Seedlings of variant No. 1, the crossing of the southern and northern populations of Siberian stone pine, have good characteristics. The hybrids of variant No. 4 have less growth in comparison to variants No. 1 and No. 3, but nevertheless they exceed open pollination variants in all the characteristics. The reliability was confirmed by *t*-test (in height t=6.6 at P=0.05, in increment t=4.8 at P=0.05).

The above-mentioned data (Table 3) reflect the average distinctions in hybrid growth. However, if the maximum hybrid seedling growth of Siberian stone pine variant No. 3 is compared to the maximum seedling growth from open pollination, then the difference in growth is even more, variant No. 3 more than 50% larger.

Special attention should be paid to variant No. 2, the hybrids from the controlled pollination of mountain populations of Siberian stone pine. In this variant the seedlings from open pollination are distinguished by their growth (3). One would believe that no significant growth effect would noticeable at this stage in the crossed mountain populations. Our preliminary conclusion is that the large growth effect of hybrid posterity was not shown at crossing genotypes that differ in altitudinal belts (the high mountain Siberian stone pine forests). Controlled pollination of remote geographical genotypes is best when done as intraspecific combinations. We need to do further research on this point.

Conclusion

This study has shown that the controlled pollination of geographically remote Siberian stone pine climatypes can produce first generation hybrids with the somatic effect of heterosis in the first growth years. Hybrids from crossing systematically and geographically remote tree species have the greatest development potential as well as the ability to adapt most fully to environmental conditions (Yablokov, 1962). Further results of systematic observations of these hybrids will be of great interest. Heterosis is a complex biological phenomenon, the nature of which has not yet been completely determined. Genetic aspects are the essence of one of the heterosis problems, so genetic systems for cytoplasm control and the development of many economically valuable features in ontogenesis, and for the further study as potential "contributors" to the genotypical variability of flowering plants is needed (Wikie, 1964; Jinks, 1964). The heterosis effect is considered to be a particular form of stimulating a gene or complex of genes (Fedorov, 1968; Khotyleva, Tarutina, 1990; Tahir and Ketata, 1997). One of possible mechanisms of plant heterosis is the heterogeneity of the genome of organelles and high mitochondrial activity (Daniel, 1973; Berville, 1977; Srivastava, 1981) which is the biochemical base of heterosis (Zhuchenko, 1988; Filatov, 1988). Robert W. Allard (1999) presented a new conception of heterosis in plants. In his opinion, the heterosis effect is related, not with heterozygosity, but with homozygosity of genes in the genome. He has shown the similarity and distinctiveness of population-genetic processes in populations of crossed trees and tree self-pollinators and from that a new interpretation of heterosis appeared.

The duality and complexity of heterosis is still being discussed. There is also an opinion that the activity of the growth substances has an important general role in genetic factors. Related to this one more opinion is that the fast growth of successful crossings, and generally, the hybrid power is undoubtedly the result of deep and complex changes in metabolism of the whole system of growth regulators and enzymes (Ivannikov, 1980). Hence, the increment in trunk diameter and height is realized due to activity of substance exchange in structures of meristem cell using a genetic mechanism.

A further task is to observe the hybrid posterity during the complete ontogenesis to discover the best hybrid trees with accelerated growth, to identify the ones with increased environmental adaptation and to reproduce the best genotypes naturally. To further improve Siberian stone pine selection we will study the influence of parent components in hybrids obtained at the clone plantation using dispersion analysis, and by studying the influence of heterozygosity in separate genes on the effect of heterosis.

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