

Effect of witches' broom mutation on growth of *Pinus sibirica* seedlings

Galina V. Vasilyeva^{1*}, Evgeniya A. Zhuk¹, Sergey N. Goroshkevich¹

¹Institute of Monitoring of Climatic and Ecological Systems, Siberian Branch, Russian Academy of Sciences, 10/3, Akademicheskoy Ave., Tomsk, 634055, Russia

*Corresponding author: Galina V. Vasilyeva, e-mail: galina_biology@mail.ru

Abstract

Mutational witches' brooms (WB) spontaneously arise in the tree crown. There are no male cones in *Pinus sibirica* WB and pollination always occurs with normal pollen. We studied 2-year-old seed progeny obtained from open-pollinated cones of WB and normal crown (NC) pines. There were significant morphological differences in two pairs of WB and NC families, while the third family pair studied showed barely pronounced differences. Segregation analysis of WB seed progeny based on needle length (growth trait) and total bud number (branching trait) revealed that about half of seedlings had a normal phenotype, while mutants were from 15.6 to 35.7 %. The rest seedlings could not be unambiguously identified, because they have not yet fully demonstrated the phenotype. Looking normal seedlings from WB families differed not only from mutants but also from NC progeny. Therefore, the mutation had some effect on both mutant seedlings and seedlings with a normal phenotype. Moreover, the denser was maternal WB the more differences were observed between WB and NC progeny.

Keywords: *conifers, witches' broom, seed progeny, morphology, branching*

Introduction

Witches' broom (WB) is a peculiar formation in a tree crown that constitutes a local shoot system with abundant branching and slow growth. Plenty of pathogens cause WB formation in trees; these may be fungi (Van Sickle, 1977; Gardner, 1997) or bacteria (Al-Ghaithi et al., 2017). Pathological WB has focal

distribution, sickly appearance and can result in plant death (Agrios, 2005).

In addition to pathological WB is mutational WB resulting from somatic mutation in an apical meristem (Duffield and Wheat, 1963; Fordham, 1967). Based on whole-genome sequence methods, it has been shown that the genome of an adult tree can accumulate several tens of different somatic mutations (Schmid-Siegert et al., 2017; Hanlon et al., 2019), and some of them are inherited by the seed progeny (Plomion et al., 2018). However, mutational WB in conifers occurs rarely in wild populations, and estimates of WB occurrence in scientific literature are rarer. In *Pinus sibirica* Du Tour, the estimate is 1 WB per 5,000 trees in a wild stand (Yamburov and Goroshkevich, 2007). WB occurrence can be substantially higher in some species and regions. So, 1 WB per 152 trees has occurred in *P. sylvestris* stands in Altai Krai, presumably due to proximity to the Semipalatinsk nuclear test site (Zhuk and Vasilyeva, 2016).

Mutational WB is a goldmine for ornamental cultivar breeding in conifers (Auders and Spicer, 2012). However, for some reason, it attracts much fewer researchers than breeders. Meanwhile, control of the growth and shape of a tree to optimize economically valuable species cultivation is the most important task facing breeders and plant scientists. A study of WB as a mutation resulting in abnormal morphogenesis and growth allows the elucidation of the mechanisms of their regulation and solving tree shape related problems.

Today, WB morphology is well studied. WB in conifers is characterized by reduced apical dominance as well as shorter shoots and needles (Duffield and Wheat, 1963; Brown et al., 1994; Zhuk et al., 2015). The mutation affects not only needle dimensions but also some of the structural traits (Vasilyeva and Zhuk, 2016). Moreover, WB has smaller cones and seeds compared to that of the normal crown (NC) (Polyakova et al., 2019).

Mutation-caused WB can be expressed in different degrees, i.e., WB can have weak, medium, or strong differences from NC (Zhuk et al., 2015).

WB seed progeny segregates into two discrete groups, namely mutants and normal plants. The ratio of the groups is considered 1:1 (Johnson et al., 1968; Waxman, 1975). However, there is a large variation, and the proportion of the mutants has varied from 12 to 65 % (Noskov and Negrutskiy, 1956; Fordham, 1967; Khiron, 1973). Growth features of the mutant seedlings compared to normal ones are little-studied and only in a few conifer species. No studies have described seedling morphology in detail. The study aimed to reveal the WB mutation effect on the seed progeny growth in *P. sibirica*.

Materials and Methods

The study was carried out at the “Kedr” field station, managed by the Institute of Monitoring of Climatic and Ecological Systems situated 30 km from Tomsk, Russia (56°13' N 84°51' E, 78 m. a. s. l., south-east of the West Siberian Plain, south taiga). The initial graft material was three *P. sibirica* trees with mutational WB in the crown (Table 1). The main trait that distinguished initial WB is the crown density. The higher WB crown density, the more morphological differences are observed between WB and NC (Zhuk et al., 2015). Scions were cut from WB and NC, and then they were grafted onto local 5-year-old *P. sibirica* rootstock.

Table 1
Characteristics of the trees with witches' broom in the crown

Characteristics	Clone number (witches' broom/normal crown)		
	08/09	054/055	038/039
Geographic coordinates and altitude above sea level, m	56°10' N 84°00' E, 140 m	56°10' N 84°00' E, 140 m	52°04' N 89°45' E 1500 m
Tree age, year	170	180	200
Tree height, m	21	21	25
Diameter at breast height, cm	48	53	42
Witches' broom age, year	80	90	60
Witches' broom height, m	1.75	3.8	3.0
Witches' broom crown diameter, m	1.5	5.0	1.5
Witches' broom crown density/Normal crown density	3	2	1.5
Comparative witches' broom crown density	Maximal	Middle	Minimal
WB shape	Globose	Globose	Conical

To obtain the seed progenies, cones were collected separately from WB and NC clones when at an age of 5 years. Obtained seeds were stratified under the same conditions (from 0 to +5°C over 6 months). Drill sowing was carried out; the distance between drills was 12–15 cm, and the distance between seeds

within drills was about 3 cm. Watering and weeding were performed as required.

There are no microstrobiles in *P. sibirica* WB (Yamburov and Goroshkevich, 2007; Zhuk et al., 2015), and so all-female cones are pollinated by pollen from normal trees. Morphological analysis was carried out in the 2-year-old seed progenies. Seedling height, including hypocotyl length and juvenile shoot length formed in the first vegetation season, the first mature shoot length that grows in the second vegetation season, and terminal bud length were measured. Moreover, hypocotyl diameters, cotyledons, and the needle lengths of the first mature shoots were measured, as well as the lateral bud numbers of the juvenile and the mature shoots, which were counted separately. Raw data were processed by statistical methods; the T-test and ANOVA were used for normally distributed data, and the Kolmogorov-Smirnov test and Spearman correlation were performed for sampling differing from normal distribution.

Results

The greatest differences were in the two pair families (08-09 and 054-055); particularly seedlings from the NC family were higher, about 10 % (Table 2). Weak differences were observed in the 038-039 pair, the heights of the seedlings from the WB and NC families were similar. Hypocotyl length and juvenile shoot length were on average significantly lower in the 08 and 054 families than that in 09 and 055 families, respectively. The length of the first mature shoot was different only in the 054-055 pair. The terminal bud length in the WB families was less than that in the NC families. Cotyledon length was usually less in WB progeny compared to NC one, but 038-039 family pair. Needle length was lower by about 20–30 % in the WB progeny compared to the NC progeny.

The total bud number was significantly higher in the WB progeny than in the NC progeny, except for the 038-039 pair. There were more buds in the first mature shoots than in the juvenile shoots. Half of the 1-year-old seedlings in the 054 progeny and two thirds in the 08 and 038 progeny did not have lateral buds; however, in the second year of life, the number of the buds usually increased significantly. Therefore, among the 08 progeny, there were 1-year-old seedlings without lateral buds, which then had 1–5 buds in the second growing season.

The distribution pattern of the bud number was different in the 08 and 09 families; seedlings having up to 13 lateral buds occurred in the WB progeny, while seedlings from the NC progeny had up to four buds (Fig. 1). Hence, skewness of the bud number distribution in the WB progeny was 2 times more than that in the NC progeny, 1.6 vs. 0.8. The distribution pattern of the bud number was more similar in the rest two pairs of the families. The excess of the bud number distribution was 2.7 and 0.4 for the 08 and 09 progeny, respectively. The excess was -0.01 and 1.9 for 054 and 055 progenies, respectively, and 4.6 and 3.4 for 038 and 039 progenies, respectively.

Table 2
Morphological traits (mean $\pm$ standard deviation) of the 2-year-old seed progeny of witches' brooms and normal crowns

Traits	Seed progeny of the clones					
	08	09	054	055	038	039
N	129	88	132	107	96	106
Hypocotyl length, cm	5.5 $\pm$ 0.9	6.4 $\pm$ 0.9 ***	5.5 $\pm$ 1.0	5.9 $\pm$ 1.3 *	5.8 $\pm$ 1.0	5.8 $\pm$ 1.0
Cotyledon length, cm	2.9 $\pm$ 0.4	3.5 $\pm$ 0.4 ***	3.2 $\pm$ 0.4	3.4 $\pm$ 0.1 **	3.2 $\pm$ 0.5	2.8 $\pm$ 0.4 ***
Juvenile shoot length, mm	2.2 $\pm$ 0.6	2.6 $\pm$ 0.8 ***	3.5 $\pm$ 1.4	2.7 $\pm$ 1.1 ***	2.5 $\pm$ 0.8	2.4 $\pm$ 1.0
Bud number on juvenile shoot	0.6 $\pm$ 1.3	0.3 $\pm$ 0.2 *	0.9 $\pm$ 1.3	0.3 $\pm$ 0.7 **	0.5 $\pm$ 1.0	0.4 $\pm$ 0.8
Length of the first mature shoot, cm	2.0 $\pm$ 0.6	2.1 $\pm$ 0.5	1.3 $\pm$ 0.4	1.8 $\pm$ 0.5 ***	1.3 $\pm$ 0.5	1.3 $\pm$ 0.4
Needle length, cm	2.6 $\pm$ 0.9	4.0 $\pm$ 0.8 ***	3.9 $\pm$ 1.4	4.8 $\pm$ 1.2 ***	2.8 $\pm$ 1.0	3.0 $\pm$ 0.9
Bud number on the mature shoot	1.5 $\pm$ 1.7	1.0 $\pm$ 1.0	1.1 $\pm$ 1.0	0.8 $\pm$ 0.8	0.7 $\pm$ 1.0	0.6 $\pm$ 0.9
Terminal bud length, mm	6.0 $\pm$ 2.5	8.1 $\pm$ 2.7 ***	4.8 $\pm$ 2.4	5.6 $\pm$ 1.7 **	6.7 $\pm$ 3.7	8.0 $\pm$ 4.1 *
Seedling height, cm	8.3 $\pm$ 1.2	9.5 $\pm$ 1.1 ***	7.7 $\pm$ 1.2	8.5 $\pm$ 1.2 ***	8.1 $\pm$ 1.3	8.2 $\pm$ 0.9
Hypocotyl diameter, mm	1.8 $\pm$ 0.2	2.1 $\pm$ 0.3 ***	2.0 $\pm$ 0.3	1.9 $\pm$ 0.3 ***	2.0 $\pm$ 0.5	2.1 $\pm$ 0.4
Total bud number	2.1 $\pm$ 2.6	1.0 $\pm$ 0.9 **	2.0 $\pm$ 1.8	1.2 $\pm$ 1.1 **	1.2 $\pm$ 1.8	0.9 $\pm$ 1.3

Asterisks show significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) according to T-test or Kolmogorov-Smirnov test in case of a bud number.

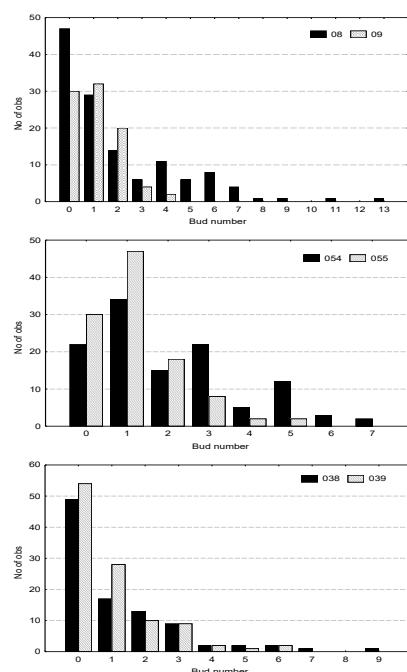


Figure 1
Distribution of the seed progeny of witches' broom and normal crown by the total bud number

In addition, differences between WB and NC progenies were observed for needle length distribution patterns (Fig. 2). The differences were expressed in the significantly lower mode and median in the WB progeny compared to the NC progeny, even in the 038-039 pair where the difference did not reach the level of statistical significance. The skewness of the distribution

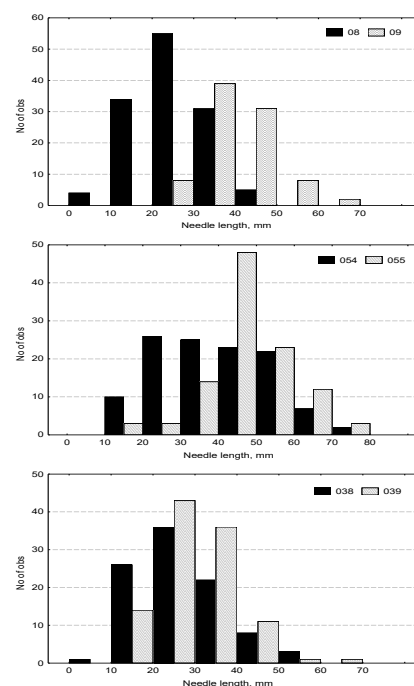


Figure 2
Distribution of the seed progeny of witches' broom and normal crown by the needle length

varied from -0.03 to 0.73 in WB progeny and from 0.08 to 0.82 in NC progeny. The excess of the distribution varied from -0.71 to 0.30 in the WB progeny and from 0.65 to 1.0 in the NC progeny.

The morphological differences between WB and NC progeny were mainly caused by the heterogeneity of the WB progeny, i.e., the presence of both mutant seedlings and seedlings with a normal phenotype. The differences were often observed in the 1-year-old seedlings because of different bud numbers (Fig. 3a, 3b). The second differentiating trait, namely the needle length of the first mature shoot, was observed in 2-year-old seedlings (Fig. 3c, 3d).

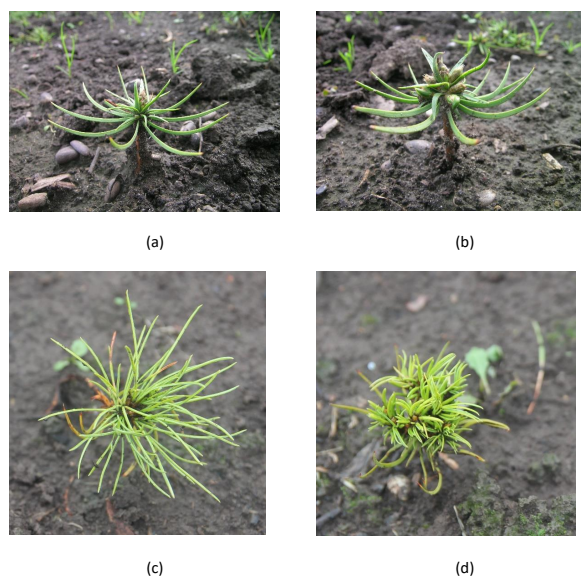


Figure 3
One-year-old (a, b) and 2-year-old (c, d) *P. sibirica* seedlings with normal (a, c) and mutant phenotypes (b, d).

Needle length characterizes the growth, and the total bud number defines the branching. These two traits were chosen to separate WB progeny into two groups. Needle length in the 08 progeny was 65 % of that in the 09 progeny, while total bud number was 190 % of that in the 09 progeny. For the 054-055 pair, these values were 81 and 167 %, respectively. For the 038-039 pair, these values were 93 and 133 %, respectively. Correlation analysis showed that in the WB progeny in contrast to the NC progeny, the Spearman correlation was negative and significant, -0.597 in the 08 progeny and -0.381 in the 054 progeny (in both cases $p < 0.001$). Also in the 038 progeny, the correlation was negative but it did not reach the level of statistical significance.

To distinguish seedlings with normal and mutant phenotypes in WB progeny, a certain value of the trait or a range of the values was assigned a score from 0 to 10 (Table 3). Then the points on both traits were summarized. The maximum score (20) corresponded to a combination of traits: short needles and plenty of buds, which characterized the mutant phenotype. The minimum score (0) corresponded to a combination of traits: long needles and few buds, which characterized the phenotype of a normal plant. Seedlings scored from 0 to 8 points were defined as normal-looking plants and plants that

scored from 12 to 20 points were defined as mutants. The third group was composed of seedlings with 9–11 points that were difficult to unambiguously identify in the group of normal or mutant plants, and these remained unidentified.

Table 3
Correspondence between score and trait values in studied witches' broom families

Trait	Total bud number					
Value	<=1	2	3	4	>4	–
Score	0	5	5	8	10	–
Trait	Needle length (mm) for 08 progeny					
Value	<20	20-30	30-40	40-50	>50	–
Score	10	8	5	3	0	–
Trait	Needle length (mm) for 054 progeny					
Value	<20	20-30	30-40	40-50	50-60	60-70
Score	10	9	8	5	3	1
Trait	Needle length (mm) for 038 progeny					
Value	<19	19-28	28-37	37-46	46-55	>55
Score	10	8	6	4	2	0

Performing this approach, we obtained the segregation of seedlings in the WB progeny (Table. 4). This segregation was not always close to 1:1, although seedlings with a normal phenotype were about half of the total.

Table 4
Segregation of witches' broom seed progeny based on needle length and total bud number

WB family	Mutants	Looking normal	Unidentified
08	46 (35.7%)	70 (54.3%)	13 (10.0%)
054	40 (30.3%)	64 (48.5%)	28 (21.2%)
038	15 (15.6%)	53 (55.2%)	28 (29.2%)

For further analysis, we selected two WB families (08 and 054) that were most different from the NC progeny. We had two tasks. The first one was to determine traits susceptible to changes due to mutation in the two separated groups of half-sibs: normal looking and mutants. The second task was to determine how large the differences were between normal-looking seedlings from WB and NC progeny.

The analysis of variance showed that the three seedling groups (mutants and normal-looking seedlings from the WB family and normal seedlings from the NC family) were significantly different for measured traits (Table 5). Both the genotypes of the maternal tree and mutation availability had a significant effect on morphological traits, but hypocotyl length in the 054 and 055 pair was influenced only by the genotype of the maternal tree.

Table 5
Analysis of variance (degrees of freedom (df), total sum of squares (SS), and F value) of morphological traits in two pairs of WB and NC families

Source	08 and 09			054 and 055		
	df	SS	F value	df	SS	F value
<i>Hypocotyl length</i>						
Family	1	528823.4	6309.4***	1	519468.3	4106.4***
Group	2	2661.7	15.9***	2	379.2	1.5
Error	168	14080.9		165	20872.7	
Total	170	16742.6		167	21251.9	
<i>Cotyledon length</i>						
Family	1	152468.7	9078.2***	1	176073.6	14042.5***
Group	2	1264.9	37.7***	2	91.0	3.6*
Error	168	2821.6		165	2068.9	
Total	170	4086.5		167	2159.9	
<i>Juvenile shoot length</i>						
Family	1	889.1	1978.2***	1	1717.5	1074.2***
Group	2	4.5	5.0**	2	30.2	9.4***
Error	168	75.5		165	263.8	
Total	170	80.0		167	294.0	
<i>Length of the first mature shoot</i>						
Family	1	62959.6	2416.3***	1	35610.3	2258.3***
Group	2	224.4	4.3*	2	650.0	20.6***
Error	168	4377.4		165	2601.8	
Total	170	4601.8		167	3251.8	
<i>Needle length</i>						
Family	1	147127.5	2929.1***	1	312645.9	2662.4***
Group	2	13477.9	134.2***	2	15303.2	65.2***
Error	168	8438.5		165	19376.1	
Total	170	21916.3		167	34679.3	
<i>Terminal bud length</i>						
Family	1	6696.6	1208.0***	1	4270.335	1048.7***
Group	2	437.7	39.5***	2	120.484	14.8***
Error	168	931.3		165	671.891	
Total	170	1369.0		167	792.375	
<i>Seedling height</i>						
Family	1	1187600	10622.0***	1	1032743	8595.4***
Group	2	7001	31.3***	2	2658	11.1***
Error	168	18783		165	19825	
Total	170	25784		167	22483	
<i>Hypocotyl diameter</i>						
Family	1	558.8	8131.1***	1	674.4	8135.0***
Group	2	5.7	41.1***	2	0.78	4.7*
Error	168	11.5		165	13.7	
Total	170	17.2		167	14.5	

Asterisks indicate significant differences, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

In the 08 progeny, mutant seedlings were significantly shorter compared to looking normal half-sibs due to lower both length of the first mature shoot and terminal bud length (Fig. 5). However, there were no differences by traits that characterized the first year of seedling life, hypocotyl length, juvenile shoot length, cotyledon length, and hypocotyl diameter. It is important to note that normal-looking plants from the 08 progeny differed from the 09 progeny in all the traits of the 1-year-old seedlings, but the lengths of both the first mature shoots and terminal buds were nearly the same. Needle length and seedling height were strictly intermediate in normal looking seedlings compared to mutants and NC progeny. These observations indicate the factor effected on growth apart from

mutation. In addition, this factor was observed in the 054-055 pair, and its effect was less pronounced; normal looking seedlings from the 054 progeny were shorter than the 055 progeny and they had shorter mature shoots as well.

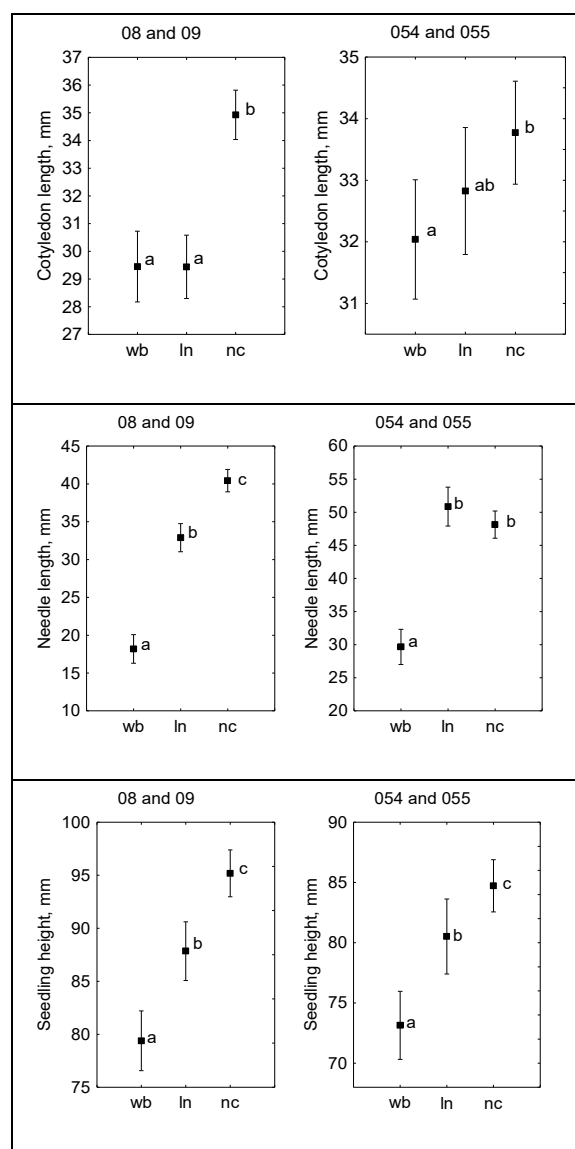


Figure 5
Comparison of the seed progeny of witches' broom clones (08 and 054) and normal crown clones (09 and 055) in cotyledon length, needle length, and seedling height. WB—mutant seedlings from witches' broom progeny, ln—normal looking seedlings from witches' broom progeny, and NC—normal seedlings from normal crown progeny. Vertical bars denote 0.95 confidence intervals. Different letters show a significant difference between means according to the Newman-Keuls test, $p < 0.05$.

Total bud number was lower in normal looking seedlings from the 08 progeny compared to the 09 progeny (0.4 vs 1.0, Kolmogorov-Smirnov test, $p < 0.001$). In addition, the bud number was lower in normal looking seedlings from the 054 progeny compared to the 055 progeny (0.7 vs 1.2); however, the difference did not reach the level of statistical significance.

As the density of maternal WB increased, the needle length in the seed progeny decreased, and the Spearman correlation was -0.524 ($p < 0.01$). Hence, the more the WB morphogenesis was changed in comparison with NC, the more these changes affected their seed progeny.

Discussion

There are a few observations of the WB seed progeny growth. A.J. Fordham (1967) noted that WB progeny of several *Pinus* species had globe-shaped crown and lateral growth often exceeded height growth, which resulted in plants that were more broad than tall. The author obtained interesting results for *P. virginiana* and *P. rigida*. Up to 4 inches in height, growth of the mutant seedlings was ordinary, and then it became horizontal and shoots turned pendulous, which resulted in a prostrate growth form. Growth analysis was carried out in some conifer species (Table 6). These studies indicated that height and needle length in normal seedlings were 2–3 times more than that in mutants. The slow growth of WB progeny is related to mitotic activity disturbance and reduced cell number rather than cell length (Molotkov et al., 1989; Brown et al., 1994). In our study, the difference between WB and NC progeny was much smaller, and in the 038-039 pair, the differences were barely pronounced. We attribute this to the fact that the maternal WB that gave rise to clone 038, had a minimal difference from NC. The difference between mutants and normal looking seedlings from the WB family was even less. This is mainly because *P. sibirica* in the first years of life has an extremely slow growth, the intensity of which begins to increase only after 3–5 years under favorable conditions (Nikolaeva et al., 2011). The maximum seedling height from NC progeny reached only 12 cm and was determined by the hypocotyl length of about two thirds.

Table 6
Growth traits of witches' broom seed progeny in different conifer species

Species	Age	Plant height, cm		Needle length, cm		Reference
		Normal plants	Mutants	Normal plants	Mutants	
<i>Pseudotsuga taxifolia</i>	3	80	36	–	–	Duffield and Wheat, 1963
<i>Pinus sylvestris</i>	5	80,5	32,1	5,1	3,2	Noskov and Negrutskiy, 1956
<i>P. sylvestris</i>	5	48,7; 59,6	18,7; 27,5	6,3; 5,6	2,9; 3,3	Shulga, 1979
<i>P. sylvestris</i>	8	151,7	24,2	6,4	2,7	Shulga, 1979
<i>P. halepensis</i>	2	42,4; 49,6	12,5; 24,3	3,5; 5,6	0,9; 2,2	Vgroc, 2002

In those few articles devoted to the WB progeny, the differences between normal looking seedlings from WB progeny and NC progeny are not discussed. We showed that the former was inferior to the later in many growth traits. Most probably, it is related to seed dimensions. It is well known that seed mass depends on maternal tree genotypes (Castro, 1999), and seedling mass is mainly determined by seed mass (Castro et al., 2008). At germination time, lipids that are abundant in the megagametophyte of the *Pinus* species seeds are transformed to sucrose and are transported in the seedling, supplying the energy for growth (Murphy and Hammer, 1994; Jordy and Favre, 2003). In *P. sibirica*, WB has smaller seeds than NC (Polyakova et al., 2019). Therefore, WB seeds contain less nutrition required for the early development of a germinant and seedling growth. The growth of the normal seedlings from WB and NC progeny may be equal at the third year of life because they have the same terminal bud size. However, needles in normal seedlings from the WB progeny are often shorter than those of the NC progeny were, and needles, as donors of nutrients, primarily carbohydrates, can determine the reduced growth of the following shoots in normal seedlings from WB progeny. Hence, equalizing of the growth curves can occur later.

We showed that mutant and normal phenotypes in 2-year-old *P. sibirica* WB progeny could be distinguished based on the total bud number and needle length. There is no clear boundary between the two seedling groups; nevertheless, these traits allow us to determine the portion of the mutants, which varied from 15.6 to 35.7 %, while the portion of normal seedlings was about 50 %. Sometimes the portion of the mutants was substantially decreased, even to 12 % (Fordham, 1967). Several factors can affect the portion of the mutants in WB progeny; first, there is a low germination capacity of the WB seeds and increased loss of mutant seedlings at the early developmental stages because of reduced vitality and low competitive ability. Observed unidentified seedlings will show their phenotypes and may refill the mutant seedling group. The fact that 1-year-old seedlings from WB progeny without lateral buds can grow several buds at the 2-year age indicates the age factor of the mutational phenotype appearance. Thus, the mutational appearance in tree ontogeny can be delayed for one year and, possibly, for some years.

Earlier, it was shown that the expression of the mutation is different in crown, shoot, and cone morphology (Zhuk et al., 2015; Polyakova et al., 2019). We also observed this phenomenon. The extent of the differences was varied in the studied family pairs, and the maximum difference was in the progeny of the 08 and 09 clones and the minimum difference was in the 038-039 pair. We believe that features of the maternal WB, particularly the crown density, determine the growth traits of WB progeny, i.e., the higher WB crown density the more differences there are between WB and NC families.

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