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Needle structure of mutational witches' brooms in *Pinus sibirica*

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Abstract: Mutational witches' brooms (WBs) in conifers are widely used in landscaping. However, there have been few WB morphology and anatomy investigations. The needle structure of witches' brooms and normal crown (NC) in *Pinus sibirica* clones was studied, using light microscopy technique. The aim was to compare the needle structure in WBs and NC and to reveal the effects of the mutation on the needle traits. Three WB and NC pairs of clones from the same trees were used in the study. WBs in the parental trees were characterized by different density in the affected part of the tree. In all pairs, WB needles were shorter and thinner than in NC. In contrast to NC needles, WB needle length was positively correlated with resin duct total area and width of endodermis cell. Thus, the affected needles had a changed structure, and the effect of the mutation was determined by mutation expression.

Keywords: needle anatomy, plant variation, dwarf conifer, clones, Siberian stone pine

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Introduction

A denser crown part is formed occasionally in the normal tree crown of various conifer species; this is called witches' broom (WB). Depending on their origin, two different types of WB can be distinguished. The first one is WB as a symptom of disease caused by some pathogens (Oner et al., 2009; Yun et al., 2009), while the second one is WB caused by somatic mutation in the bud in the apical meristem (Duffield & Wheat, 1963; Fordham, 1967; Waxman, 1975). Unlike the pathological WB, the mutational WB has a normal viability and cone bearing. The occurrence of mutational WB is about one in 5000 trees in conifer species (Yamburov & Goroshkevich, 2007). Mu-

tational WB has reduced shoots, increased branching and decreased apical dominance compared with the normal part of the crown (Yamburov & Goroshkevich, 2007; Zhuk et al., 2015). As a result of these morphological changes, the WB crown is more dense and compact.

The detailed anatomical traits of the needles in WB have not been studied until now. There are several investigations in which the needle length and width in WB in different pine species have been measured. Usually these traits were much less in WB than in the normal part of the tree crown (Khirova, 1973; Brown, 1994; Vrgoc, 2002), except in a few cases; for example, the only WB in *Pinus halepensis* had longer needles than the normal part of the crown

in the same tree (Vrgoc, 2002). A comparative analysis of the morphological and anatomical traits of WB needles and the normal part of crown has only been provided in *Abies sibirica* (Yamburov & Titova, 2013).

The needle anatomical traits are species-specific and can be used as taxonomic criteria (Engelmann, 1880; Mirov, 1967). The needle structure in *Pinus sibirica* has been well studied. The species leaves in cross section, like other five-needle pines, are triangular in shape. Mesophyll cells are unfolded or slightly folded, and the central cylinder has a single vascular bundle. There are three resin ducts in the mesophyll and they don't make contact with the hypodermis (Litvintseva, 1974; Nesterovich et al., 1986; Bender, 2003; Rudnik et al., 2007). There is a possibility that the mutation underlying the formation of WB can be expressed not only in the crown morphology, shoot and needle length, but also in the needle structure. The aim of this study was to compare the needle structure in WB and normal crown (NC) in *Pinus sibirica* and to reveal the effects of the mutation on the anatomical needle traits.

Material and methods

Plant material

Clones of both WB and NC were the studied objects. The use of clones in the same natural conditions enables the environment effect to be minimized and genetic-dependent anatomical signs revealed. The clones were made by grafting in 2007. The grafted plants were grown with 1 × 0.5 m spacing at the Kedr field station, which is managed by the Institute of Monitoring of Climatic and Ecological Systems SB RAS. The field station is situated 30 km south-east of Tomsk (56°13N 84°51E, 78 m above sea level, south-east of the West Siberian Plain, southern Taiga).

The grafts of each WB and NC pair were from the same trees, in total 3 trees with one WB in the crown. Two ortet trees (clones 040/041 and 036/037) orig-

inated from the mountain Siberian stone pine forests of Western Sayan, Khakassia. The third ortet tree (clones 08/09) originated from the flat Siberian stone pine forests, Tomskaya oblast (Table 1). The WB crown density was estimated by eye, and visually they were clearly distinguishable from each other. Crown density was ranked as low, medium and high based on the differences among studied WB only.

Clone characteristics

The current study is a continuation of the morphological investigation of the *P. sibirica* WB. The same clones that were studied in the work of Zhuk et al. (2015) were used. These WB clones were characterized by a more spherical crown, particularly 036. The ratio of the height of grafting to crown width in 036 was 0.98, in 08 it was 1.66 and in 040 it was 1.63, whereas NC clones had a ratio varying from 3.50 to 4.81. The shoot length in WB clones was significantly less than in NC clones. The shoot length was reduced by 1.5 times in 040, 2.1 times in 08 and 4.2 times in 036. Apical dominance of WB clones was decreased in the following order: 040, 08, 036. Another important characterized growth trait is the threshold of branching, i.e. the length of the shortest shoot with at least one branching structure, long shoot or latent bud. The WB threshold of branching was decreased by 3.0 times in 040, 3.7 times in 08 and 7.1 times in 036 (Zhuk et al., 2015).

Traits studied and statistical analyses

Current-year needles were collected at the end of the growth season in 2012. Thirty fascicles were selected from the leader shoot in each clone and then 30 needles from each fascicle were studied. The needles were fixed using 70% ethanol. Slices of about 30 μm thickness from the middle part of the needle were made by freezing microtome MZ-2 (Russia). Measuring was conducted by an Axiostar plus light microscope (Karl Zeiss, Germany) with a Watec LCL 217 HS video camera (Watec AC, USA) and SIAM-

Table 1. Origin and characteristics of the trees with witches' broom from which cuttings were collected for grafting

Characteristics	Clone number (witch's broom/normal crown)		
	040/041	08/09	036/037
Latitude, N	52°00'	56°10'	51°03'
Longitude, E	90°20'	84°00'	85°36'
Altitude above sea level, m	1350	140	1750
Tree age, year	190	170	170
Tree height, m	21	21	18
Diameter at breast height, cm	32	48	60
Witches' broom age, year	60	80	120
Witches' broom height, m	2,5	1,75	0,7
Witches' broom crown diameter, m	1,5	1,5	1,2
Witches' broom crown density	Low	Medium	High

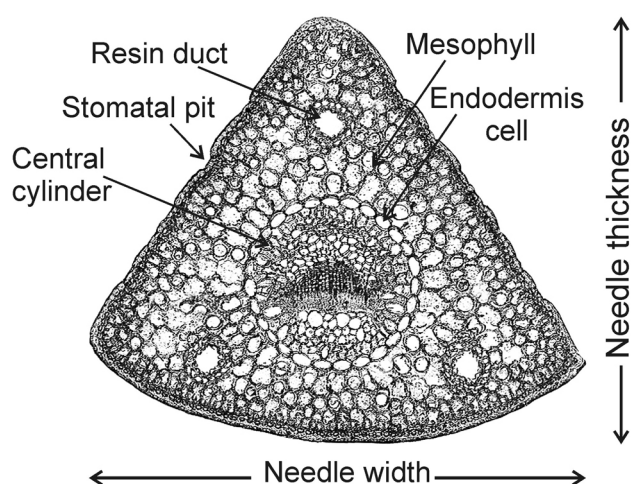


Fig. 1. Cross section of *P. sibirica* needle from normal crown

STM MesoPlant (SIAMS, Russia) software. A *P. sibirica* needle cross section is presented in Figure 1. The following traits were analysed:

- needle length, mm (NL)
- needle width, μm (NW)
- needle thickness, μm (NTh)
- ratio of needle width to needle thickness (NW/NTh)
- cross section area, μm^2 (Scs)
- central cylinder area, μm^2 (Scc)
- resin duct total area, μm^2 (Srd)
- mesophyll area, μm^2 (Smes=Scs-Scc-Srd)
- needle surface area, mm^2 (Ssurf= $P \times \text{NL}$, P – cross section perimeter)
- ratio of central cylinder area to cross section area (Scc/Scs)
- ratio of resin duct total area to cross section area (Srd/Scs)

- ratio of mesophyll area to cross section area (Smes/Scs)
- endodermis cell number surrounding the central cylinder (CN)
- width of endodermis cell, μm (WC)
- thickness of endodermis cell, μm (ThC)
- ratio of width of endodermis cell to thickness of endodermis cell (WC/ThC).

Pairwise comparisons of WB and NC clones originating from the same tree were performed by t-test. Discriminant analysis was conducted for main traits, namely needle length, width, thickness, mesophyll area, central cylinder area, resin duct total area, and width and thickness of endodermis cell. Correlation analysis of needle length and other studied needle traits for NC and WB was conducted; the adopted significance level was $p < 0.01$.

Results

Quantitative changes in WB needle structure

Comparative analysis of morphological and anatomical traits revealed considerable differences between WB and NC needles (Table 2). There are no significance differences for three traits only: the ratio of needle width to needle thickness in pair 036/037, the thickness of the endodermis cell in pairs 08/09 and 040/041, and the last pair didn't show any difference in the central cylinder area either. WB needles were usually characterized by smaller values of the measured traits, but WB with a low dense crown (040) exceeded the appropriate NC in some traits.

Table 2. Comparison of values (mean \pm standard variation) of studied needle characters from witches' broom (WB) and normal crown (NC) with results of t-test for each pair of them * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Characters	1		2		3	
	040 WB	041 NC	08 WB	09 NC	036 WB	037 NC
NL ¹	84 \pm 6	98 \pm 5***	59 \pm 3	104 \pm 5***	28 \pm 2	85 \pm 7***
NW	1301 \pm 80	1243 \pm 60**	1017 \pm 52	1278 \pm 55***	795 \pm 47	1181 \pm 76***
NTh	1034 \pm 69	1089 \pm 69**	797 \pm 46	1230 \pm 117***	644 \pm 38	956 \pm 52***
NW / NTh	1.26 \pm 0.11	1.14 \pm 0.06***	1.28 \pm 0.11	1.05 \pm 0.11***	1.24 \pm 0.09	1.24 \pm 0.08
Scs $\times 10^3$	794.7 \pm 67.7	832.3 \pm 76.5*	486.0 \pm 28.6	765.8 \pm 43.7***	321.1 \pm 29.6	685.8 \pm 60.6***
Scc $\times 10^3$	145.3 \pm 15.5	152.0 \pm 15.1	106.7 \pm 8.7	161.7 \pm 13.5***	61.3 \pm 7.8	131.5 \pm 15.5***
Srd $\times 10^3$	38.5 \pm 3.4	32.5 \pm 3.4***	28.1 \pm 1.9	38.8 \pm 4.0***	19.9 \pm 3.4	38.8 \pm 4.2***
Smes $\times 10^3$	611.0 \pm 51.9	647.8 \pm 63.4*	351.2 \pm 23.1	565.3 \pm 30.9***	240.0 \pm 21.0	515.5 \pm 44.1***
Ssurf	322.9 \pm 36.1	380.9 \pm 36.0***	177.4 \pm 11.9	390.9 \pm 25.7***	67.6 \pm 7.0	295.5 \pm 31.6***
Scc/Scs $\times 100$	18.3 \pm 0.9	18.3 \pm 1.2	21.9 \pm 1.3	21.1 \pm 1.0**	19.1 \pm 1.5	19.1 \pm 0.9
Srd/Scs $\times 100$	4.9 \pm 0.3	3.9 \pm 0.3***	5.8 \pm 0.4	5.1 \pm 0.4***	6.2 \pm 0.6	5.7 \pm 0.5**
Smes/Scs $\times 100$	76.9 \pm 1.0	77.8 \pm 1.2**	72.2 \pm 1.2	73.8 \pm 1.0***	74.8 \pm 1.6	75.2 \pm 0.8
CN	24.5 \pm 1.7	25.8 \pm 1.7**	24.9 \pm 1.8	27.1 \pm 1.7***	19.6 \pm 1.6	24.4 \pm 1.8***
WC	52.3 \pm 3.4	49.1 \pm 3.1***	43.9 \pm 2.2	49.5 \pm 3.2***	40.8 \pm 2.5	49.7 \pm 2.7***
ThC	26.8 \pm 1.6	27.3 \pm 1.7	28.9 \pm 1.6	29.2 \pm 1.6	29.1 \pm 1.9	31.2 \pm 1.7***
WC / ThC	1.95 \pm 0.10	1.80 \pm 0.09***	1.52 \pm 0.07	1.69 \pm 0.09***	1.41 \pm 0.10	1.60 \pm 0.08***

¹ – See Materials and Methods for the character codes.

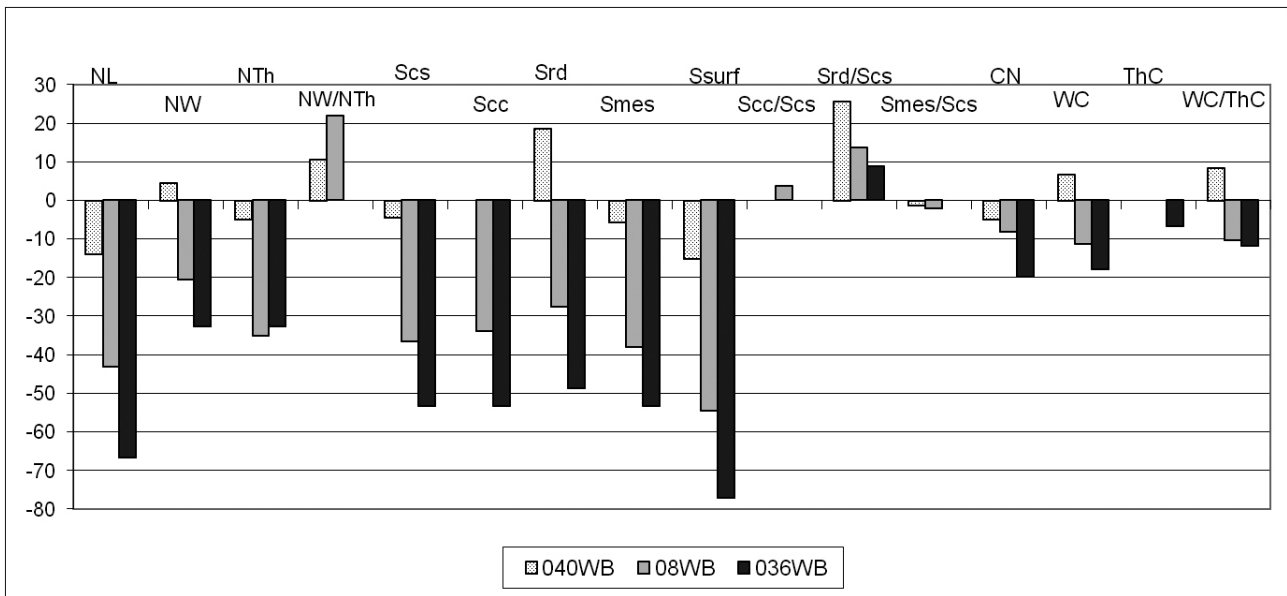


Fig. 2. Change of studied needle trait values in witches' broom (WB) relatively normal crown (%)

These traits were needle width, the ratio of needle width to needle thickness, resin duct total area, the width of the endodermis cell, and the ratio of the width of the endodermis cell to the thickness of the endodermis cell. In pair 08/09, WB had an increased value of the ratio of needle width to needle thickness compared to the corresponding NC, but not due to greater needle width as in the pair 040/041 but due to smaller needle thickness. The lowest values of the analysed traits, especially needle surface area, were observed in the WB with the densest crown (036). However, the largest reduction in both needle length and needle thickness was observed in WB with medium crown density (08) rather than in WB with the

densest crown (Fig. 2). As the figure shows, the needle structure traits in WB varied in different ways with respect to the appropriate NC. For example, the decrease of needle length by two-thirds in the 036 clone led to a decrease both of needle width and thickness by a third; the rest of the WB clones have another change pattern.

The complex of needle structure traits clearly differentiates WB and NC, and the greater the difference magnitude, the greater the WB crown density (Fig. 3). In discriminant analysis, the difference magnitude is expressed by squared Mahalanobis distance. The distance between WB and the appropriate NC increased with increasing WB crown density and

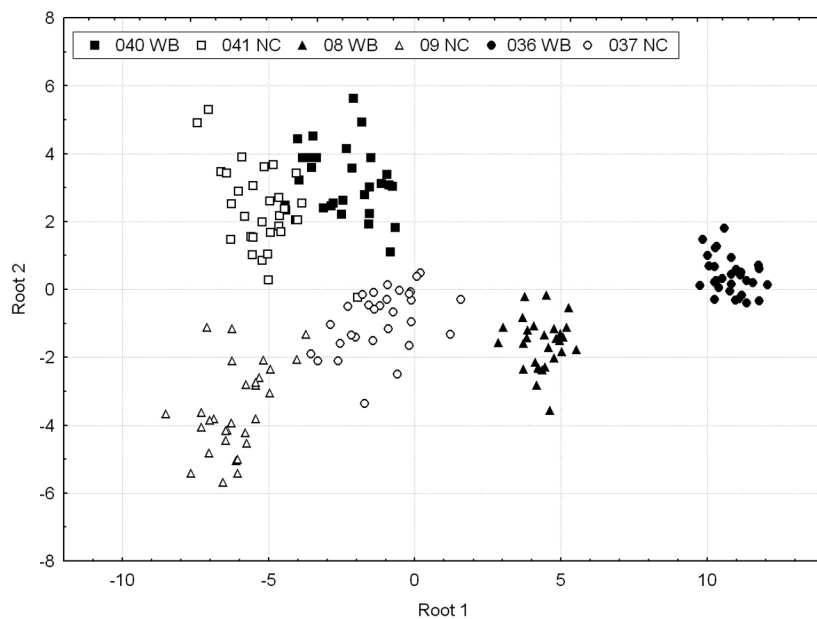


Fig. 3. Result of discriminant analysis based on measured needle structure traits from witches' broom (WB) and normal crown (NC) plotted along the first two canonical roots

decreasing WB needle length. The smallest distance was 29.1 for the 040/041 pair; it was longer (120.3) for the 08/09 pair and the 036/037 clones were the most distant (159.4). It should be noted that the maximum distance among NC clones was 65.0, whereas among WB clones it was 3 times more, at 198.2. Both discriminant functions (root 1 and root 2) accounted for 92.5% of total variation and 80.3% of it was attributed to root 1. The maximum correlation value was observed between root 1 and needle length (-0.908), and root 2 and mesophyll area (0.438).

Interrelations among morphological and anatomical traits

WB needle length reduced in the same order as the crown density – 040, 08, 036 – whereas NC needle length decreased in the order 09, 041, 037. The effect of the mutation on the needle structure can be determined by comparing the changes in the needle structure traits with a needle length reduction in the WB and NC. This approach enabled the identification of the three clear discordances between WB and NC needle. Firstly, the shape of the cross section expressed as the ratio of needle width to needle thickness was the same in the studied WB, and WB usually exceeded NC. This indicates that the WB cross section was flatter than the NC one irrespective of needle length. In NC, the longer needle led to a decrease in this ratio. Secondly, the WB needle length was positively related to resin duct total area. There was no relation in NC, and resin duct total area was nearly the same in the studied NC and didn't depend on the needle length. The third pronounced difference concerned the cell size. The width of the endodermis cell was nearly the same in the NC, whereas the WB needles were characterized by a positive correlation between the needle length and the width of the cell. The last observation indicates that a possible disturbance of cell elongation takes place in WB. This is supported by the fact that the WB endodermis cells became more rounded as needle length reduced.

Because of the interdependence of anatomical traits, we considered the ratio of different structures in cross section. Differences were observed in all WB/NC pairs. First of all, they concerned the relative resin duct area (Srd/Scs), which was higher in WB. Reduction of the relative mesophyll area ($Smes/Scs$) was observed in two pairs, but the WB with the shortest needles (036) had nearly the same relative mesophyll area as the appropriate NC. The relative central cylinder area (Scs/Scs) was the most invariable trait, with some difference only in the 08/09 pair. These observations suggest that the WB needles are not only shorter and characterized by lower values of

measured traits but they have disturbed anatomical trait relations in comparison with the NC needles.

Change of resin duct location in WB needles

As well as quantitative changes in WB needle structure, some qualitative changes were observed in the location of resin ducts. Siberian stone pine needles are characterized by three (in some cases more than three) resin ducts. Four resin ducts were observed only in one pair (040/041), two cases in NC and a single one in WB. An additional resin duct was located on the abaxial side and it was surrounded by mesophyll cells like main resin ducts. Sometimes one or two abaxial resin ducts are touched by the cells of dermal tissue. These external resin ducts were found in two WBs (08 and 036) and in one NC (037). Only two cases were found in 08. In pair 036/037, WB had external resin ducts in half cross sections which was twice more than NC had. It is possible that the location change is a direct consequence of too large a reduction of needle dimensions.

Discussion

There is no doubt that species-specific features of *P. sibirica* remain in WB needles. However, WB needle structure traits are characterized by lower values than those in NC. The reduction of WB needle dimensions is connected with the change of WB growth that is shown by the decrease of shoot length, apical dominance and branching intensification, which all together lead to different crown shape and density. The more pronounced changes in growth habit result in smaller needles. Needle length and other anatomical traits in different WBs do not change continuously, but stepwise. In needle structure traits, WB can be similar enough to the appropriate NC, like 040/041, and can significantly differ from the NC, like 036/037. The stepwise morphological differences were revealed earlier in the crown and shoot features of WB clones (Zhuk et al., 2015). This suggests that the mutation can be expressed in different degrees and probably the degree of the mutation is random like the mutation itself.

Changes of WB needle structure traits, being the consequence of the mutation, are determined by internal growth factors such as growth substance content and nutrient availability. WB grafts on normal tree root stock grow very well and restore normal growth habit to some degree in the first year; however, WB external appearance returns after some time (Duffield & Wheat, 1963; Khiron, 1973). Growth habit restoration is probably conditioned by the in-

flow of many substances that need normal growth including hormones from stock. But over time, WB requirements in different nutrients increase and the growing deficiency of certain substances results in the return of initial morphological features.

It is appropriate to draw an analogy with defoliation experiments. Growth rate decrease and annual shoot reduction were observed in the defoliation experiments in conifers (O'Neil, 1962; Kozłowski & Winget, 1964; Kulman, 1965; Britton, 1988). The WB needles with a small surface area like a partially or completely removed needle in the defoliation experiments cannot fully provide shoot growth by all necessary substances. Complete defoliation led to significantly reduced needle length the following year (Honkanen et al., 1999; Li et al., 2002). In addition, defoliation resulted in a decrease of apical dominance (Little, 1970). The decrease of apical dominance was observed in WB too (Yamburov & Goroshkevich, 2007; Zhuk et al., 2015). The growth deviation after defoliation was explained by a significant reduction of indoleacetic acid and gibberellin content in the shoot (Wang et al., 1997). The obtained results revealed that growth correlations of WB needles are abnormal and it is indirectly suggested that WB hormonal balance differs from that of NC.

Needles provide assimilation capability, serve as a main reserve of non-structural carbohydrates and are a source of many physiologically active substances. On the other hand, needle growth is determined by many factors and depends on the state and work of the whole organism. There is an acute shortage of investigations into mutant conifers. However, it has been shown recently that needle length correlates with flavonol content in dwarf trees in the genera *Taxus* and *Tsuga*. More flavonols and less cell nuclear activity were observed in the shorter needles (Feucht et al., 2014). It is known that flavonol effects on auxin transport and its distribution (Ringli et al., 2008; Kuhn et al., 2011) and the changes will have an impact on phenotype. Thus, WB is characterized by profound metabolism reorganization rather than simple changes of needle dimensions. The effects of the mutation on the WB physiological basis of growth and physiological functions such as photosynthesis, respiration and transpiration remain the subject of future investigations.

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