

**Studies on needle browning and death of evergreen conifers
distributed in the alpine region of Mt. Norikura, Japan
- From a viewpoint of cuticle property -**

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2013**

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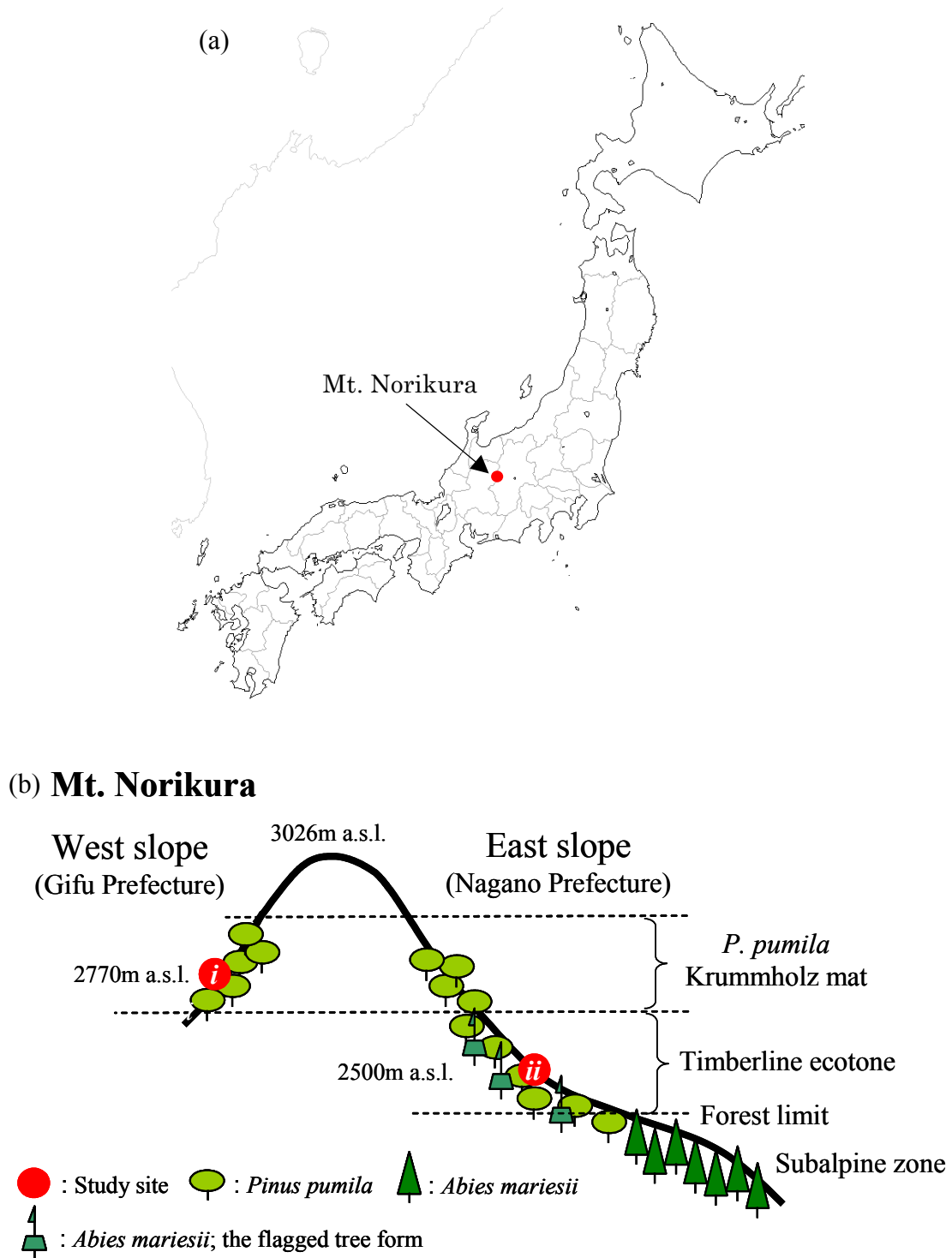
Chapter 1. General Introduction

High mountainous habitat is one of the most severe habitats for plants (Körner 2003). In Europe and North America, the timberline of the uppermost subalpine zone transits the alpine zone via the Krummholz zone, which is the timberline ecotone (Tranquillini 1979; Körner 2003). The vegetation of the Krummholz zone is composed of dwarf scrubs of the same species as in the subalpine zone. While in the Krummholz zone of the alpine region of Mt. Norikura, one of the high mountains in Japan located in the northern Japanese Alps, at its upper part the dwarf scrub *Pinus pumila* (Pallas) Regel. forms the community, and at its lower part *Abies mariesii* Mast. scatters within a *P. pumila* community (Fig. 1).

Trees in alpine regions tend to suffer from severe stresses caused by environmental conditions such as a short growth period, strong wind, low temperature, and soil freeze during winter, as well as shallow root systems (Tranquillini 1979; Körner 2003; Smith et al. 2003). Therefore, needle browning and death in some evergreen coniferous trees in the high mountains of Europe, North America, and Japan often occur in late winter and early spring and seems to result from winter desiccation (Tranquillini 1979; Herrick and Friedland 1991; Körner 2003).

Because there is a cessation of water absorption from frozen soil (Tranquillini 1979; Wieser and Tausz 2007), a remarkable decrease in root permeability, and an increase in water viscosity (Kaufmann 1975; Running and Reid 1980; Lopushinsky and Kaufmann 1984; Cochard et al. 2000), the evergreen conifers in the subalpine zone close their stomata and needle cuticle thickness increases. The cuticle and epicuticular waxes increase resistance to water loss through the epidermis, and the occlusion of stomatal pores with wax greatly reduces water loss (Pallardy 2008). It is thought that these adjustments prevent the needles' desiccation, which would otherwise arise from excess winter transpiration. Needles of *P. pumila* and *A. mariesii* in a alpine region of Mt. Norikura, often turn brown and die in March, which is earlier than the usual thaw that occurs from May to June. Browning of *P. pumila* occurs mainly in 1-year old needles of shoots protruding from the snowpack (Fig. 2). *A. mariesii* at the timberline

ecotone shows the flagged crown above snowpack and the cushion-shaped crown below snowpack in winter (Fig. 3). Needles in the flagged crown of *A. mariesii* often become brown and die in early spring, but remain green and intact in the cushion-shaped crown (Fig. 4).



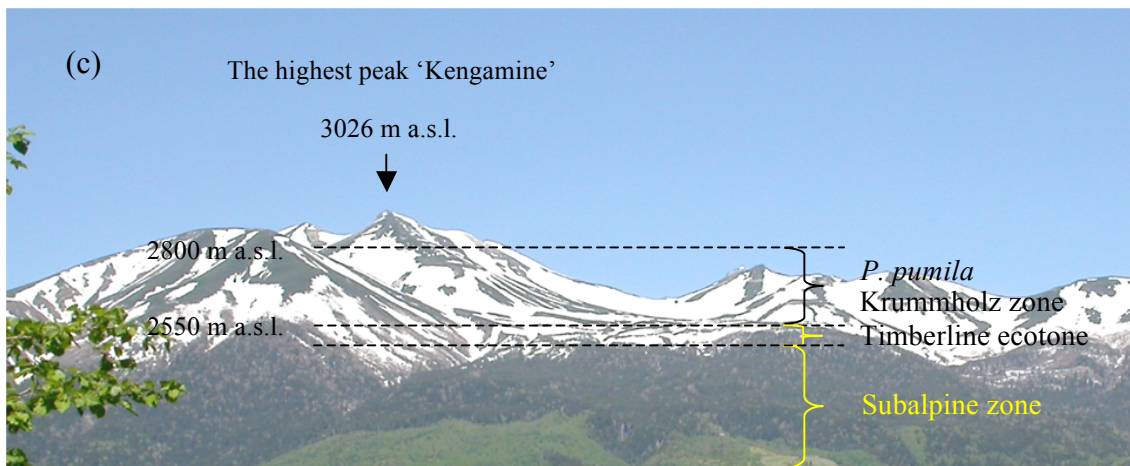


Fig. 1. Outline of study area. (a) Location of Mt. Norikura in Chubu Mountain National Park, Nagano, Japan (36°61'N, 137°33'E, 3,026 m a.s.l.) at the southernmost tip of the northern Japanese Alps in the central mountainous area of the Japanese Archipelago. (b) Vertical distribution of vegetation. *i* is *P. pumila* Krummholz mat. *ii* is timberline ecotone which *A. mariesii* is scatters with in a *P. pumila* community. (c) Distant view of vegetation.



Fig. 2. *P. pumila* shoots protruded above snowpack in June 2008. Needle browning was observed mainly in 1-year-old needles.

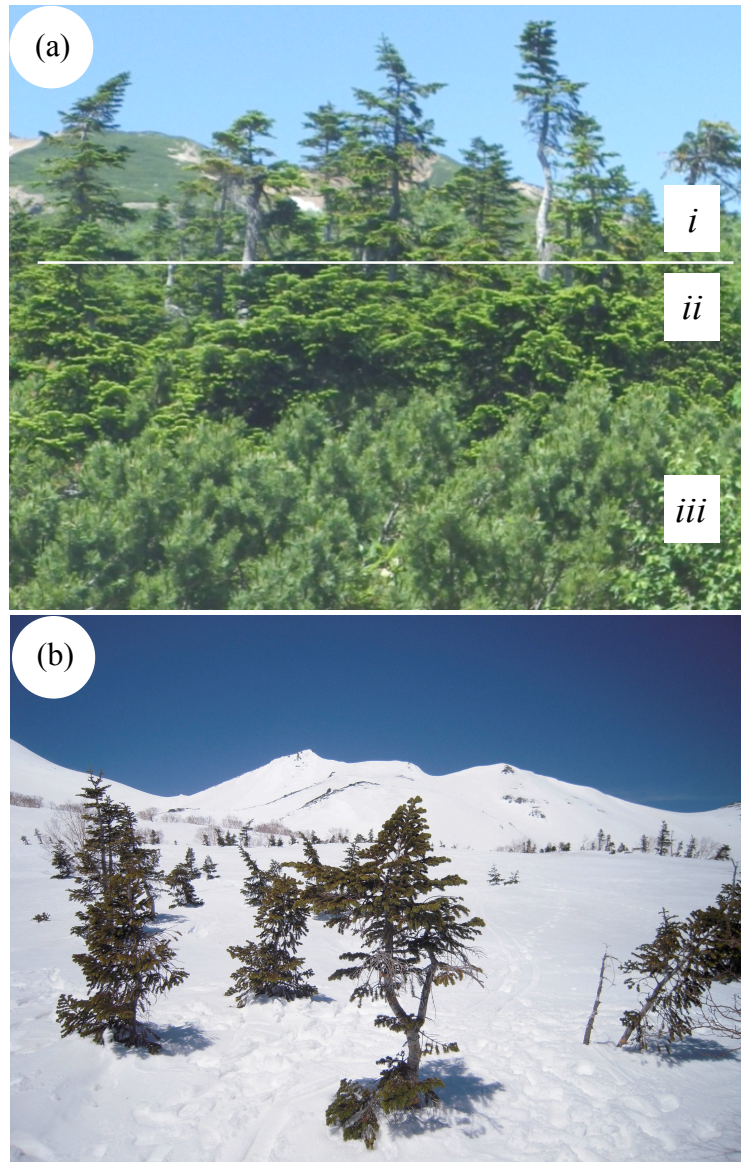


Fig. 3. Timerline ecotone of Mt. Norikura, Japan, in summer (a) and in winter (b). *Abies mariesii* distributed on a shallow slope (2,500 m above sea level) showing the flagged crown (i) and the cushion-shaped crown (ii). The dominant vegetation is a dwarf scrub *Pinus pumila* community (iii). White bar indicates the approximate snow level during winter.

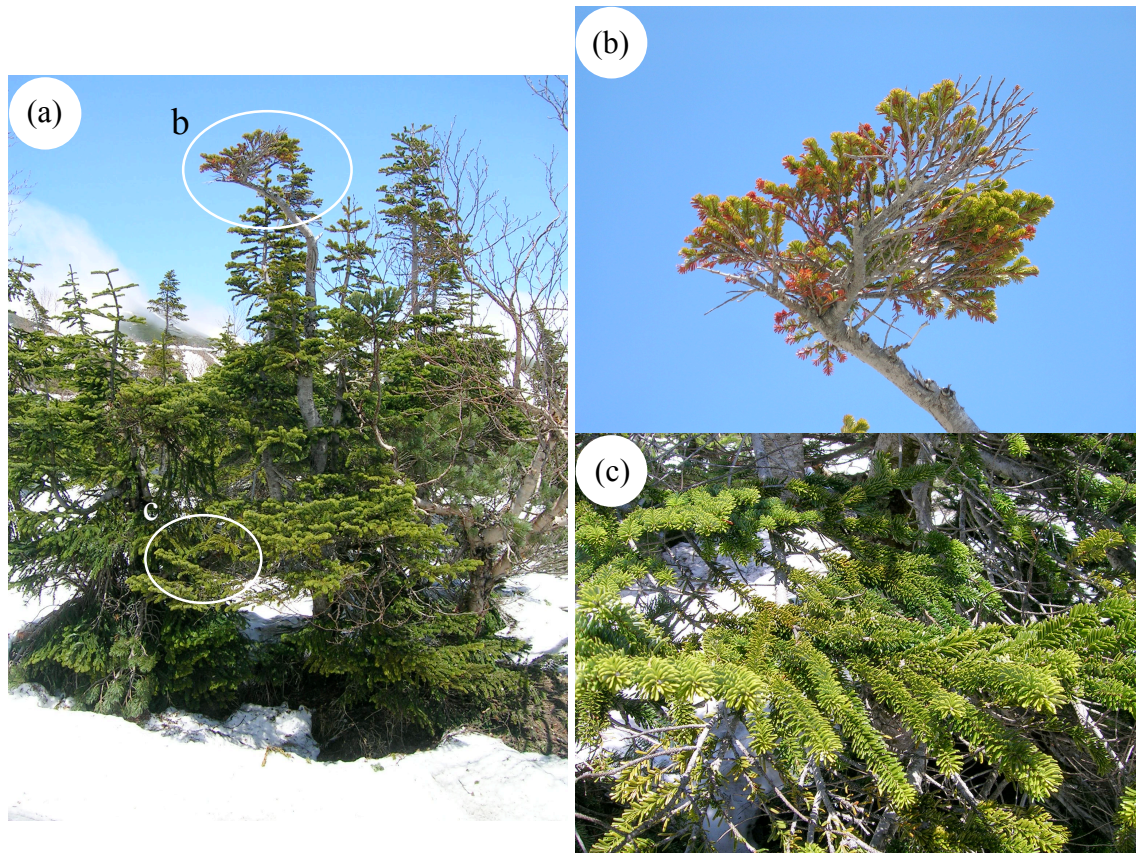


Fig. 4. *Abies mariesii* showing the flagged tree form (a). (b) Closeup of b shown in (a), the abaxial side of shoots in the flagged crown: needle color was brown and most of needles already died and dropped in June. (c) Closeup of c shown in (a), the adaxial side of shoots in the cushion-shaped crown: needle color was green. Needle color of the abaxial side of shoots was also green (see Chapter 2).

Snow cover of the crown prevents needle browning and death during late winter and early spring, because the snow protects the shoot from strong winds and low temperatures (Hadley and Smith 1986, 1987). The protruded parts might be abraded by snow and ice particles during winter (Hadley and Smith 1986, 1987). Therefore, increasing cuticular transpiration of damaged needles seems to be one of the possible causes of needle browning and death. This has been thought to determine the location of the timberline in high mountains (Tranquillini 1976, 1979, 1982; Sowell et al. 1982;

Hadley and Smith 1983, 1990; DeLucia and Berlyn 1984; Herrick and Friedland 1991; Wieser and Tausz 2007).

Because the needle cuticle of trees in high mountains do not fully mature before winter due to the short growing period (Tranquillini 1979), cuticular resistance is low and cuticular transpiration is high. This has been thought to induce winter desiccation of foliage (Wardle 1971; Sowell et al. 1982; Hadley and Smith 1983; DeLucia and Berlyn 1984; Herrick and Friedland 1991). Mechanical damage to foliage caused by wind abrasion during winter has already been reported (Wilson 1980, 1984). Hadley and Smith (1986, 1987) showed that damage during winter in *Picea engelmannii* and *Abies lasiocarpa* distributed in the upper part of the subalpine zone of the Rocky Mountains was due to a decrease in needle water content at the end of winter. This decrease occurred because wounded cuticles were polished by snow and ice particles, which increased cuticular transpiration. In contrast, conifer needle browning and death at the timberline and in the subalpine zone occurred from late winter to early spring in many mountains, but their needle water content did not necessarily decrease (Marchand and Chabot 1978; Kincaid and Lyons 1981; Hadley and Amundson 1992). The relationship between the structural integrity of the cuticle and needle browning and death is not fully understood.

Snow cover in the mountains has decreased in most regions of the world, especially in spring and summer (Intergovernmental Panel on Climate Change 2008). It may be that early thaws in the mountains facilitate the increase in browning and death of needles. Cuticular transpiration through the foliage cuticle cannot be prevented, even when stomata are completely closed (Riederer and Schreiber 2001). In general, cuticular transpiration is very small in comparison with stomatal transpiration (Pallardy 2008). Because severe physical changes are caused by mechanical damage from snow and ice particles during winter and (or) by chemical changes induced by air pollutants, which can cause excess water loss through the cuticle, cuticular transpiration might often determine the water status in the needles of evergreen conifers (Anfodillo et al. 2002). For trees living under severe environmental conditions, the role of the cuticle seems to be very important for their survival.

In this study, we focused on the cuticle of the needles of *P. pumila* and *A. mariesii* in the alpine region of Japan and studied the relationship between needle browning and death and needle cuticle integrity such as cuticular resistance, cuticle thickness and cuticle components in alpine regions.

Chapter 2. Needle browning and death in *Pinus pumila* in the alpine region of central Japan were not related to mechanical damage of cuticle and cuticle thickness

Introduction

Pinus pumila (Pallas) Regel. is the dwarf scrub of evergreen conifer characterizing the vegetation in the alpine region of Japan (Maruta 2000). Needles of *P. pumila* on Mt. Norikura, one of the high mountains in Japan located in the northern Japanese Alps, often turn brown and die in March. Browning of *P. pumila* occurs mainly in 1-year old needles of shoots protruding from the snowpack.

In this study, we focused on the cuticle of the needles of *P. pumila* in the alpine region of Japan and studied the relationship between needle browning and death and needle cuticle integrity in alpine regions. We assessed mechanical damage of the cuticle and studied the influence of artificial cuticle abrasion on needle browning and death. Finally, we evaluated the effect of cuticle thickness on cuticular resistance.

Materials and methods

Study site and species

The study was conducted in a *P. pumila* community distributed on a flat terrain (2770 m above sea level (a.s.l.)) on the northern slope of Mt. Norikura in Chubu Mountain National Park, Gifu, Japan (36.61°N, 137.33°E, 3026 m a.s.l.), which is located at the southernmost tip of the northern Japanese Alps in the central mountainous area of the Japanese Archipelago and a heavy-snow region. The minimum, mean, and maximum air temperatures were -22.7, -5.6, and 17.4 °C, respectively, from 2004 to 2008. The mean air temperature of the growing period was 0.2 °C in June, 5.2 °C in July, 8.6 °C in August, and 6.1 °C in September. The first snow and last snow at the study area occurred in early October and early June, respectively.

Pinus pumila is a haploxylon species with five needles. As shoots flushed in late July, the growth period in a year was only about two months. Tree height, the degree of needle browning, and snow depth differed greatly as a result of a slight difference in topography. Two sites of the study area were selected: (i) site P (wind-protected); and (ii) site E (wind-exposed). At site P, mean tree height of the community was 1.1 ± 0.1 m. There was a lot of snow accumulation and the *P. pumila* community was almost entirely covered with snow during the winter. Needle browning occurred rarely (Fig. 1a). At site E, mean tree height of the community was 0.6 ± 0.1 m. Snow accumulation was minimal, and the community was not entirely covered with snow. Needle browning was observed frequently (Fig. 1b).

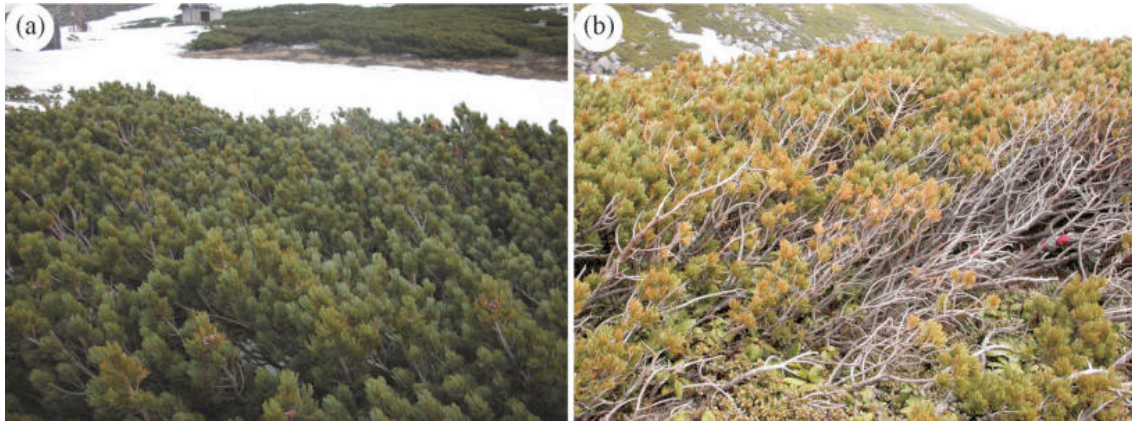


Fig. 1. *Pinus pumila* community at (a) site P (wind-protected) and (b) site E (wind-exposed) on Mt. Norikura, Japan, distributed on flat terrain (2770 m above sea level). Shoots at site P were almost completely snow covered during winter, whereas those at site E were not entirely covered with snow during winter. (a) *P. pumila* community at site P in early June 2008; needle browning occurred rarely. (b) *P. pumila* community at site E in early June 2008; needle browning was observed frequently.

Preparation of specimens for microscopy

There were two manners of preparation of specimens for observation by scanning electron microscopy and light microscopy. One involved the use of a chemical fixative and the other did not. Chemical fixation of tissues often induces structural artifacts, structure or organelle dissolution, and artifactual synthesis (Ruzin 1999); therefore, we

needed to confirm whether chemical fixation was appropriate or not for cuticle observation of *P. pumila* needles and to establish the plant microtechnique in a suitable manner. The first approach was as follows: browning and healthy green needles collected in the field were immediately fixed in cold 3 % glutaraldehyde and stored in a cool box. Fixed samples were brought to the laboratory and kept in a refrigerator. The second approach did not involve a chemical fixative. Browning and healthy green needles collected in the field were immediately placed in a plastic bag, stored in a cool box, and brought back to the laboratory and kept in a refrigerator. Browning needles and healthy green needles with or without a chemical fixative were treated and observed as described below.

For scanning electron microscopy, needles treated with a chemical fixative were dehydrated in an EtOH series and substituted in a *t*-butyl alcohol series. Samples were dried using freeze dehydration equipment (JFD-310, JEOL Datum Ltd., Tokyo, Japan). Fresh needles without a chemical fixative were kept in their natural state. Both prepared samples were observed using a scanning electron microscope (JSM-5510LV, JEOL Datum Ltd., Tokyo, Japan) after coating with Au in an ion-sputter coater (IB-3, Eiko Engineering Co., Ltd., Tokai, Ibaraki, Japan).

For light microscopy, fixed needles were embedded in epoxy resin, and ultrathin sections 2 μm thick were prepared using a rotary microtome (EM Super Nova, JEOL Datum Ltd., Tokyo, Japan) and stained with safranin (0.5 %). Fresh needles were fitted into pith sticks, and sections 25 μm thick were prepared using a sliding microtome (Yamato Koki, Asaka, Saitama, Japan) and stained with Sudan III (0.5 %). Both stained sections were observed by a light microscope (Olympus BX51TF, Tokyo, Japan) and a digital camera (Camedia C-5060, Olympus Corp., Tokyo, Japan). Then cuticle thickness was measured using Photoshop CS2 (Adobe Systems, San Jose, California, USA).

Scanning electron microscopy and light microscopy showed that the features of the needle surface differed depending on whether or not a chemical fixative was used (Fig. 2). For the surface of epidermal cells in needles prepared without a chemical fixative, the epicuticular waxes comprised the outer part of the cuticle, occluded the openings of stomatal antechamber (Figs. 2a, 2b), and plugged up the stomatal

antechamber (Fig. 2b). In contrast, for those prepared with a chemical fixative, the epicuticular waxes mostly disappeared, the openings of the stomatal antechamber were clearly seen (Figs. 2c, 2d), and the epicuticular waxes in the stomatal antechamber disappeared (Fig. 2d). This difference in preparation, i.e., use of a chemical fixative, caused the dissolution of epicuticular wax. Cuticle thickness of the adaxial surface prepared with a chemical fixative was also significantly thinner than that prepared without a chemical fixative in both browning needles ($P < 0.01$) and healthy green needles ($P < 0.01$) (Table 1). These results showed that not only the epicuticular wax, but also the cuticle itself, were dissolved with a chemical fixative. As a result, fresh samples prepared without a chemical fixative just after collection were considered appropriate for observing the needle surface and its cross section in a natural state.

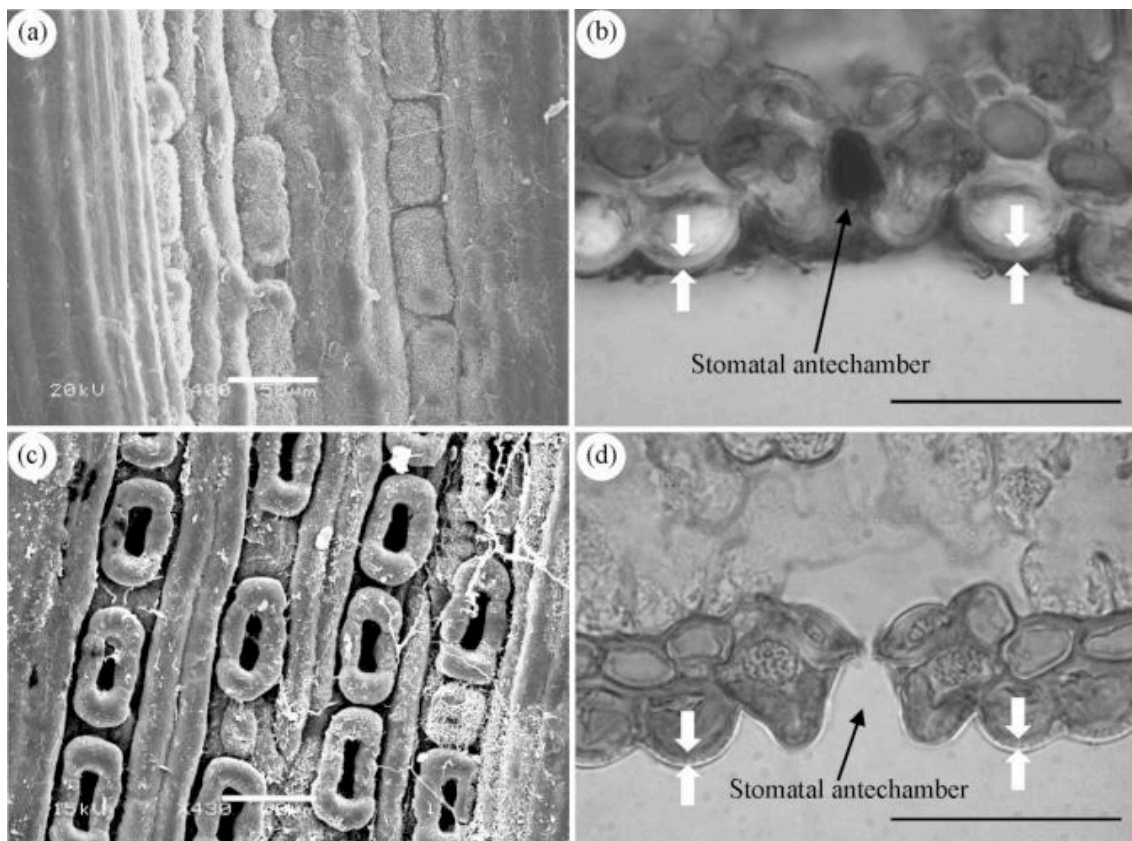


Fig. 2. Features of the needle surface in *P. pumila*: abaxial surface and abaxial cross section of needles (a and b) without a chemical fixative and (c and d) with a chemical fixative. (a and c) Observed by scanning electron microscopy; (b and d) observed by light microscopy; cuticle thickness is that part sandwiched between the two arrows. Scale bars = 50 μm .

Table 1. Cuticle thickness of *P. pumila* needles prepared with or without a chemical fixative.

	Cuticle thickness ($\mu\text{m} \pm \text{SE}$)		<i>P</i>
	With a chemical fixative	Without a chemical fixative	
Browning needle	2.32 ± 0.06	3.01 ± 0.08	< 0.01
Healthy green needle	2.24 ± 0.05	3.17 ± 0.03	< 0.01

Sampling procedures

To confirm the presence or absence of cuticle mechanical damage, the influence of artificial abrasion of the cuticle on needle browning and death, and the effect of cuticle thickness to cuticle resistance, we collected leafy shoots at sites P and E in June, early August, and late September 2008. Shoots collected in June did not have current-year needles because the forest floor was covered with snow and current shoots had not yet flushed. In early August, each bunch of current year needles had just flushed but had not yet unfolded. Late September is just prior to the first snow. Shoots collected in the field were immediately placed in plastic bags, stored in a cool box, and brought back to the laboratory where they were kept in a refrigerator. All collected shoots and needles were treated without a chemical fixative as described above. Shoots at site P in June were collected from two parts of the tree: above snowpack and below snowpack. Artificial abrasion of cuticles was done in August and September 2008. Treated samples were collected in September 2009.

Observation of mechanical damage of cuticle

To confirm whether mechanical damage of the cuticle existed or not, adaxial and abaxial needle surfaces were observed according to the microtechnique for scanning electron microscopy described above.

Artificial abrasion test of cuticle

To confirm whether mechanical damage of the cuticle causes needle browning, an artificial abrasion test of the cuticle was performed in the field as follows. Cuticles of the adaxial surface of approximately 1 cm of the needle tip on current-year and 1- and

2-year-old needles were filed away using waterproof abrasive paper (#2000 type DCC, Sankyo Rikagaku Co., Ltd., Okegawa, Saitama, Japan) in August and September 2008 (Fig. 3). This test was carried out on needles of different shoots. The filing frequency was 15 times per test because filing of 20 times and more per test wounded the needle epidermis. Shoots with filed needles were collected in September 2009, and the following items were investigated: (i) survival rates of needles, i.e., the ratio of needles

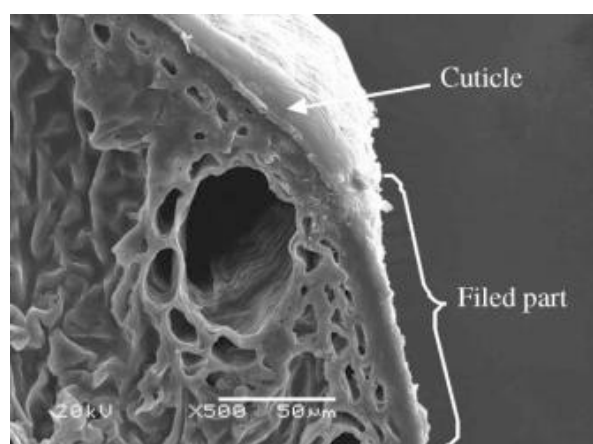


Fig. 3. Features of artificially abraded *P. pumila* needle observing by scanning electron microscopy. Cross section of needle indicating where the cuticle had been filed away. Scale bars = 50 μm .

survived in September 2009 to needles with cuticle filed away; (ii) comparison of needle color between naturally damaged needles in June 2008 and tested needles with cuticles filed away; color was determined according to the Guide to Color Standard (Japan Color Research Institute, Tokyo, Japan); and (iii) histological observation of the artificially abraded part of needle according to the microtechnique for light microscopy described above.

Cuticular resistance

To confirm whether excessive transpiration causes needle browning and death, cuticular resistance was measured as follows. The cut ends of the shoots stored in a refrigerator were refreshed under water and were watered sufficiently in the laboratory.

Shoots collected in the field were kept and watered additionally for more than 12 h in a dark room. They were cut into internodes for each year, and the cut ends were sealed with nail polish to prevent evaporation. For assessment of cuticular resistance to water loss, r_c (ks m^{-1}), eq. 1 was used:

$$E = (e_l^0 - e_a)/r_c \quad (1)$$

where E ($\text{g s}^{-1} \text{m}^{-2}$) is the amount of water vapor loss from needles; e_l^0 (g m^{-3}) is the saturated water vapor concentration inside a needle (at needle temperature); and e_a (g m^{-3}) is the water vapor concentration in air. The influence of boundary layer resistance of the needle was reduced by circulating air at $1.2\text{-}2.3 \text{ m s}^{-1}$ in a dark room. Samples of internodes of each year were weighed, maintained at room temperature (approximately $20 \text{ }^\circ\text{C}$) for 30 min, and reweighed. Measurements were repeated until E became constant. Leaf area was measured by using an image analysis software (Scion Image, Beta 4.0.3, Frederick, Maryland, USA). Air temperature and humidity were monitored during measurement (Thermo Recorder RS-10, ESPEC MIC Corp., Osaka, Japan). Needle temperature was measured using a fine-wire copper-constantan thermocouple (0.1 mm diameter).

Cuticle thickness

To confirm whether cuticle thickness relates to cuticular resistance, 10 needles were collected in each needle age, and the cuticle thickness was measured at two locations on each needle, i.e., at the tip and in the middle, according to the microtechnique for light microscopy described above. The mean value of the two measurements was adopted as the cuticle thickness of each needle. The *P. pumila* needle has one adaxial side and two abaxial sides as the cross section of needle is fan-shaped. Cuticle thickness was measured at three points on the adaxial side and at one point on each abaxial side for a total of five measurements per needle.

Data analysis

Statistically significant differences between sites P and E were determined using Student's *t* test. To assess the difference in measurement months and needle age, one-way analysis of variance (ANOVA), the Kruskal-Wallis test, and Scheffé's test were performed. Either linear or nonlinear regression analysis was used to characterize a relationship between cuticular resistance and cuticle thickness.

Results

Mechanical damage of cuticle

Histological observations by scanning electron and light microscopy of all needles did not find any mechanical damage to the needle cuticle in *P. pumila* growing in a natural environment (Figs. 2a, 2b, 6a, 6b). The epicuticular waxes comprise the outer part of the cuticle, occlude the openings of the stomatal antechamber (Figs. 2a, 2b), and plug up the stomatal antechamber (Fig. 2b).

The color of artificially abraded needle tips of all trees tested in August 2008 had changed from green to brown at sites P and E one month (September 2008) (Fig. 4) and one year (September 2009) after the filing treatment. Browning occurred not only at the filed part, but also in an area around the filed part. In contrast, colors of untreated parts of filed needles and untreated needles in a natural environment did not change. These results indicated that only the color of that part of needle with the abraded cuticle changed to brown.



Fig. 4. Color of artificially abraded needle tips in August 2008 that changed to brown in September 2008.

At site P, survival rates of 1- and 2-year-old needles filed in September 2008 were significantly lower than those of needles filed in August 2008 and those of needles growing in a natural environment ($P < 0.01$ and $P < 0.01$, respectively) (Fig. 5a). This showed that damage to the cuticle in late autumn and early winter made defoliation of 1- and 2-year-old needles faster than damage to the cuticle in summer. Meanwhile, at site E, an artificial abrasion test of the cuticle did not influence survival rates of needles (Fig. 5b).

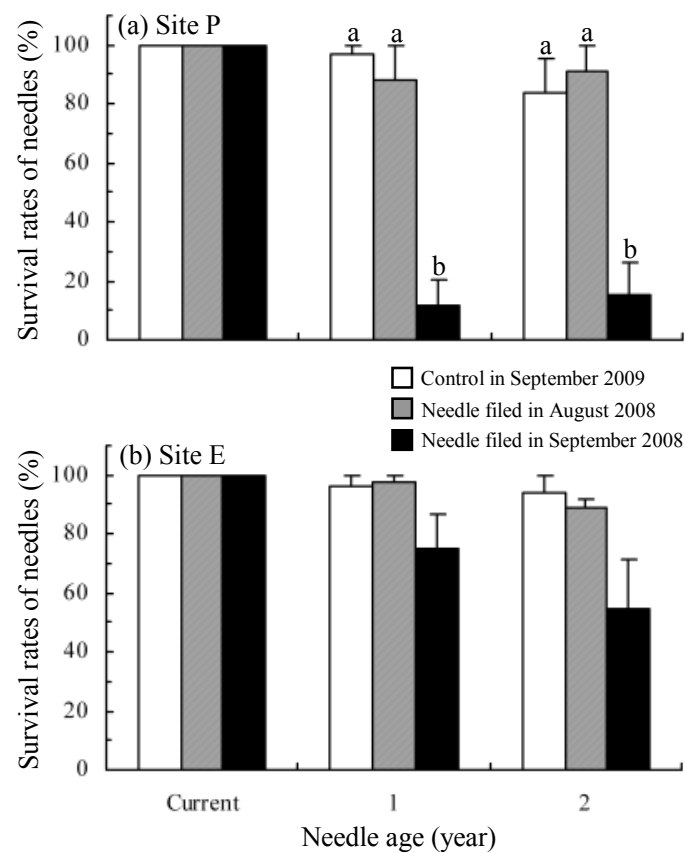


Fig. 5. Survival rates of current-year and 1- and 2-year-old needles in September 2009 ($n =$ six shoots) after an artificial abrasion test of the cuticle in August ($n =$ four shoots) and September ($n =$ four shoots) 2008. (a) At site P, an artificial abrasion of the cuticle in September made defoliation of 1- and 2-year-old needles faster than damage to the cuticle in August. (b) At site E, an artificial abrasion test of the cuticle did not influence the survival rates of needles. Different letters indicate significant difference ($P < 0.01$) within a needle age. Bars = SE.

Colors of the artificially abraded needle tips and of needles that turned brown naturally were compared using Munsell notation and color names according to the Guide to Color Standard (Japan Color Research Institute, Tokyo, Japan). For the needles filed in August 2008, three colors were common at both sites P and E one month after the filing treatment in September 2008: 3rO 15/3 and grayish red brown, 4O 14/4 and brown, and 4O 13/4 and chestnut brown. For the needles filed in August and September 2008, four colors were common at both sites P and E in September 2009: N 8/0 and grayish white, 7.5YR 8/1 and light gray, 2.5Y 7/4 and light yellow, and 4O 13/4 and chestnut brown. For the needles that turned brown naturally without any mechanical damage, colors in June 2008 at both sites were 3rO 15/3 and grayish red brown and 4O 14/4 and brown. Colors 3rO 15/3 and grayish red brown and 4O 14/4 and brown were common in all tested needles; however, the needles that turned brown naturally did not show the colors N 8/0 and grayish white, 7.5YR 8/1 and light gray, and 2.5Y 7/4 and light yellow. This indicated that needle browning by abrasion of the cuticle was different from discoloration in a natural environment.

For the part of needle just after filing treatment, only the cuticle became thinner (Fig. 6c); epidermal cells and mesophyll cells did not show distinct changes (Fig. 6d) in comparison with healthy green needles (Figs. 6a, 6b). The needle cuticle with filing treatment in August 2008 remained thin and did not regenerate in September 2008 (Fig. 6e), and the epidermal and mesophyll cells changed to a brown color and degenerated (Fig. 6f). The cross-sectional shape of the damaged part was shrunken (Fig. 6f). One year after the filing treatment, the cuticle did not regenerate (Fig. 6g), and the epidermal and mesophyll cells collapsed (Fig. 6h). On the other hand, that part of needle without filing treatment was similar to the needle without any filing treatment as healthy green needles (Figs. 6a, 6b). It was found that artificial abrasion of the cuticle was a mortal wound not only for cells just beneath a filed cuticle, but also for all cells on a vertical section of a filed part. This induced needle browning and death.

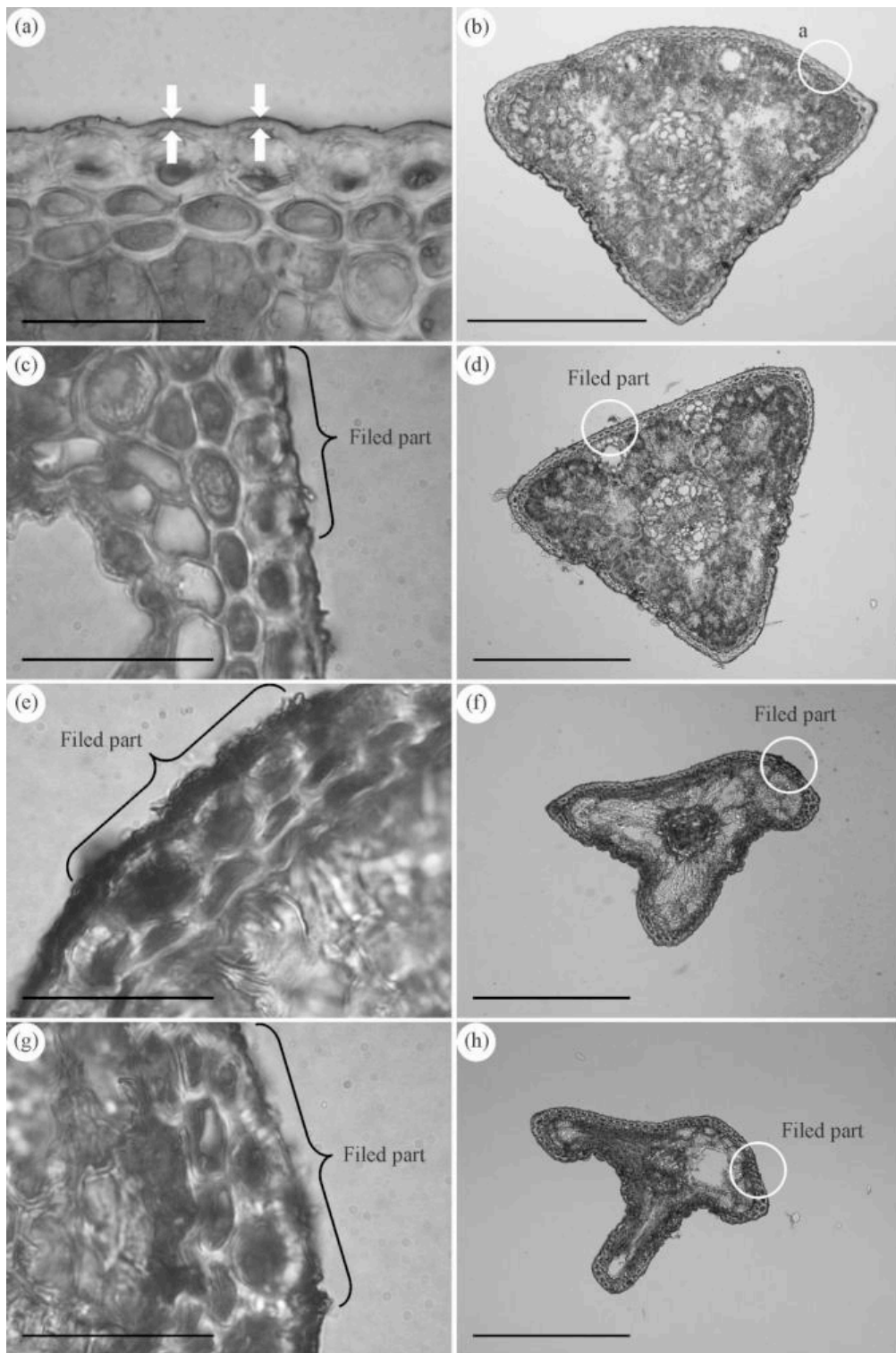


Fig. 6. Cross section of *P. pumila* needle observing by light microscopy. The cuticle was artificially abraded in August 2008. (a) Closeup of needle treated without a chemical fixative shown in (b). (b) Needle treated without a chemical fixative. (c) Closeup of needle filed part

shown in (d). (d) Filed needle in August 2008. (e) Closeup of needle filed part shown in (f). (f) Filed needle in September 2008. (g) Closeup of needle filed part shown in (h). (h) Filed needle in September 2009. The cuticle of the needle with filing treatment remained thin and did not regenerate. The colour of epidermal and mesophyll cells changed from green to brown and the cells degenerated. In (a), cuticle thickness is that part sandwiched between the two arrows. In (a), (c), (e), and (g), scale bars = 50 μm ; in (b), (d), (f), and (h), scale bars = 0.5 mm.

Cuticular resistance

Cuticular resistance of each needle age at sites P and E was compared among measurement months (Fig. 7). At site P, cuticular resistance of the current-year needle was $0.65 \pm 0.03 \text{ ks m}^{-1}$ (\pm SE) in August and $0.82 \pm 0.04 \text{ ks m}^{-1}$ in September. It significantly increased from August to September ($P < 0.01$) (Fig. 7a). Cuticular resistance of the 1-year-old needle was $4.97 \pm 0.46 \text{ ks m}^{-1}$ in June, $4.47 \pm 0.46 \text{ ks m}^{-1}$ in August, and $0.84 \pm 0.05 \text{ ks m}^{-1}$ in September, and that of the 2-year-old needle was $3.68 \pm 0.60 \text{ ks m}^{-1}$ in June, $5.23 \pm 0.47 \text{ ks m}^{-1}$ in August, and $0.74 \pm 0.04 \text{ ks m}^{-1}$ in September. Cuticular resistance in 1- and 2-year-old needles did not differ between June and August and significantly decreased in September ($P < 0.01$) and increased in June of the following year ($P < 0.01$). At site E, cuticular resistance of the current-year needle was $0.75 \pm 0.05 \text{ ks m}^{-1}$ in August and $1.91 \pm 0.26 \text{ ks m}^{-1}$ in September. It significantly increased from August to September ($P < 0.01$) (Fig. 7b). There was no significant difference in cuticular resistance of the 1-year-old needle. Cuticular resistance of the 2-year-old needle was $3.77 \pm 1.41 \text{ ks m}^{-1}$ in June, $3.30 \pm 0.49 \text{ ks m}^{-1}$ in August, and $1.80 \pm 0.31 \text{ ks m}^{-1}$ in September. It significantly decreased in September ($P < 0.05$). Cuticular resistance of each needle age in September at site P was lower than that at site E (Fig. 7).

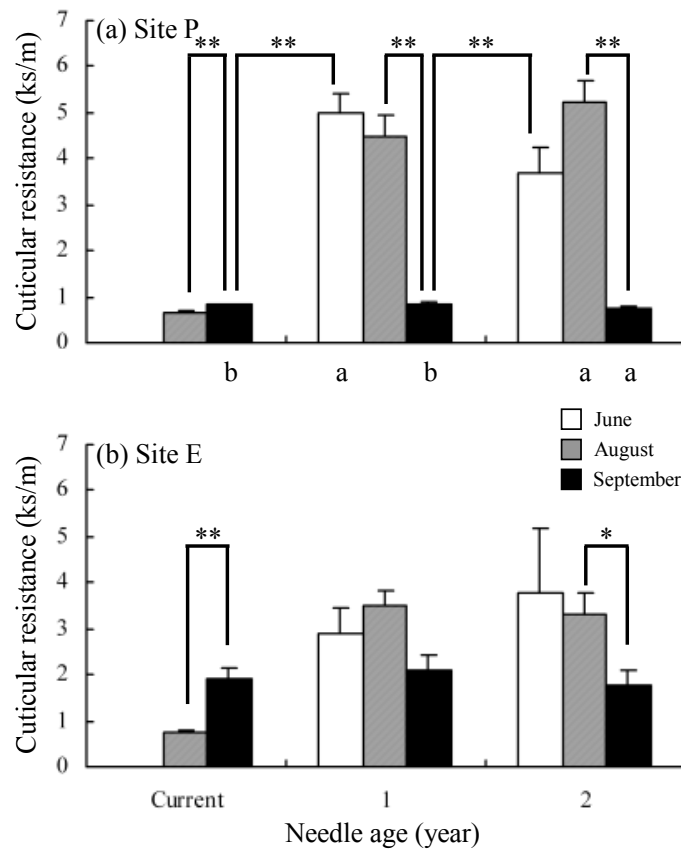


Fig. 7. Cuticular resistance in current-year and 1- and 2-year-old needles in June ($n = 40$ shoots), August ($n = 60$ shoots), and September ($n = 60$ shoots) 2008. Asterisks indicate significant differences between months: *, $P < 0.05$; **, $P < 0.01$. Letters indicate significant differences between site P and site E: a = $P < 0.05$; b = $P < 0.01$. Bars = SE.

Cuticle thickness

Cuticle thickness of each needle age at sites P and E was compared among measurement months (Fig. 8). At site P, cuticle thickness in the current-year needle was $2.58 \pm 0.09 \mu\text{m}$ (\pm SE) in August and $2.84 \pm 0.07 \mu\text{m}$ in September. It was significantly thicker in September than in August ($P < 0.05$) (Fig. 8a). There was no significant difference in cuticle thickness of 1- and 2-year-old needles among measurement months. At site E, cuticle thickness in the current-year needle was $2.84 \pm 0.08 \mu\text{m}$ in August and $3.05 \pm 0.07 \mu\text{m}$ in September. It was significantly thicker in September than in August ($P < 0.05$) (Fig. 8b). Cuticle thickness tended to become thick with needle development

from August to September at both sites. Cuticle thickness of the 1-year-old needle at site E was $2.86 \pm 0.10 \mu\text{m}$ in June, $3.10 \pm 0.09 \mu\text{m}$ in August, and $3.38 \pm 0.09 \mu\text{m}$ in September, and that of the 2-year-old needle was $2.88 \pm 0.09 \mu\text{m}$ in June, $3.12 \pm 0.07 \mu\text{m}$ in August, and $3.43 \pm 0.07 \mu\text{m}$ in September. It was significantly thicker in September

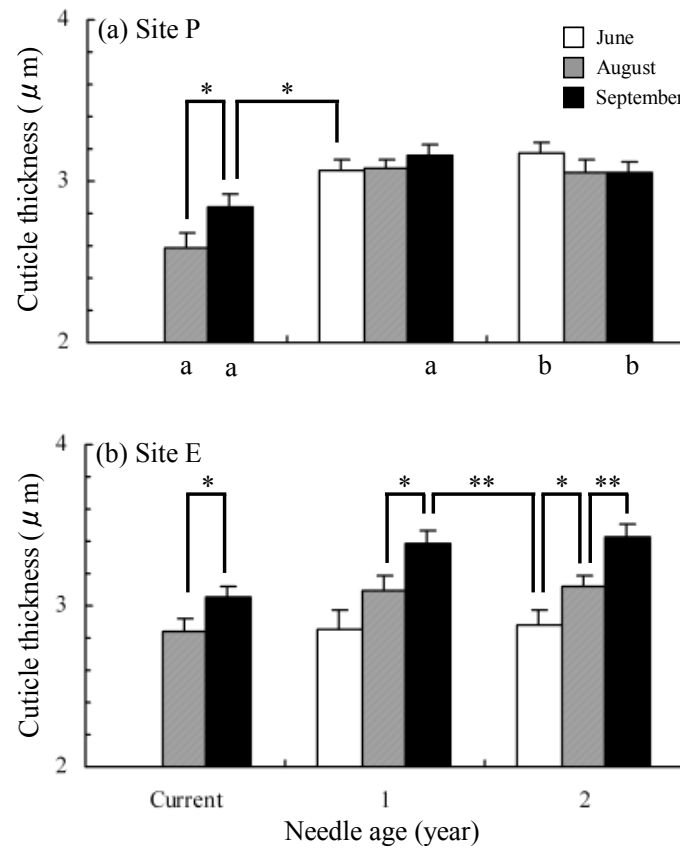


Fig. 8. Cuticle thickness in current-year and 1- and 2-year-old needles in June ($n = 40$ shoots), August ($n = 60$ shoots), and September ($n = 60$ shoots) 2008. Asterisks indicate significant differences between months: *, $P < 0.05$; **, $P < 0.01$. Letters indicate significant differences between site P and site E: a = $P < 0.05$; b = $P < 0.01$. Bars = SE.

than in August for the 1-year-old ($P < 0.05$) and 2-year-old ($P < 0.01$) needles (Fig. 8b). Cuticle thickness of the 1-year-old needle in September significantly decreased in June of the following year ($P < 0.01$). Cuticle thickness of the current-year needle at site E was thicker than that at site P (Fig. 8).

At site P in June, cuticle thickness and cuticular resistance of needles above snowpack (shoots protruding from snowpack) were compared with those of needles below snowpack (shoots buried in snowpack). Although there was no significant difference in cuticle thickness between above and below snowpack in each needle age (Fig. 9a), cuticle resistance of the 1-year-old needle above snowpack was lower than that below snowpack ($P < 0.05$) (Fig. 9b).

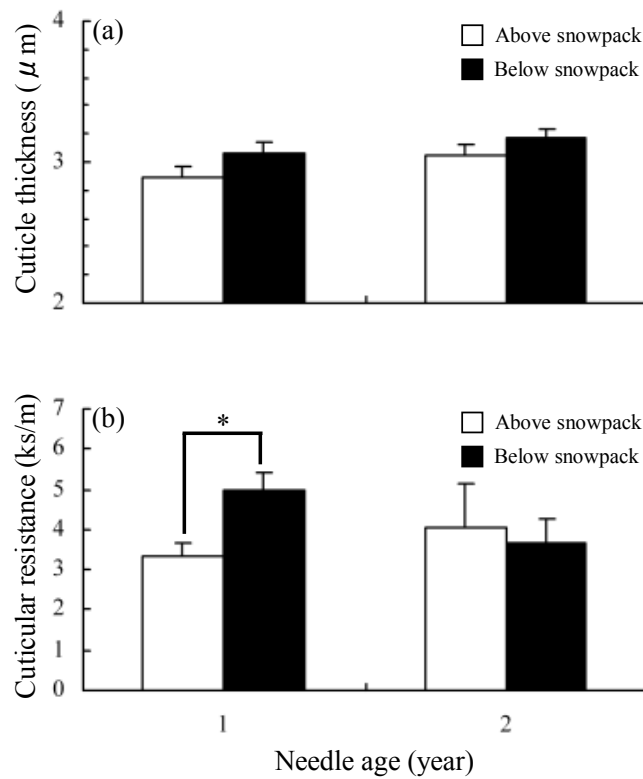


Fig. 9. (a) Cuticle thickness ($n = 40$ needles) and (b) cuticular resistance ($n = 40$ shoots) of needles above snowpack were compared with those below snowpack at site P in June 2008. Although there was no significant difference in cuticle thickness of 1-year-old needles between above and below snowpack in each needle age, cuticular resistance of 1-year-old needles above snowpack was significantly lower than that below snowpack. Asterisk indicates significant differences between above and below snowpack: *, $P < 0.05$. Bars = SE.

Relationship between cuticular resistance and cuticle thickness

There was no significant relationship between cuticular resistance and cuticle thickness in total (Fig. 10a), which was reconstructed by integrating the data of site P (Fig. 10b) and site E (Fig. 10c), as well as for each site.

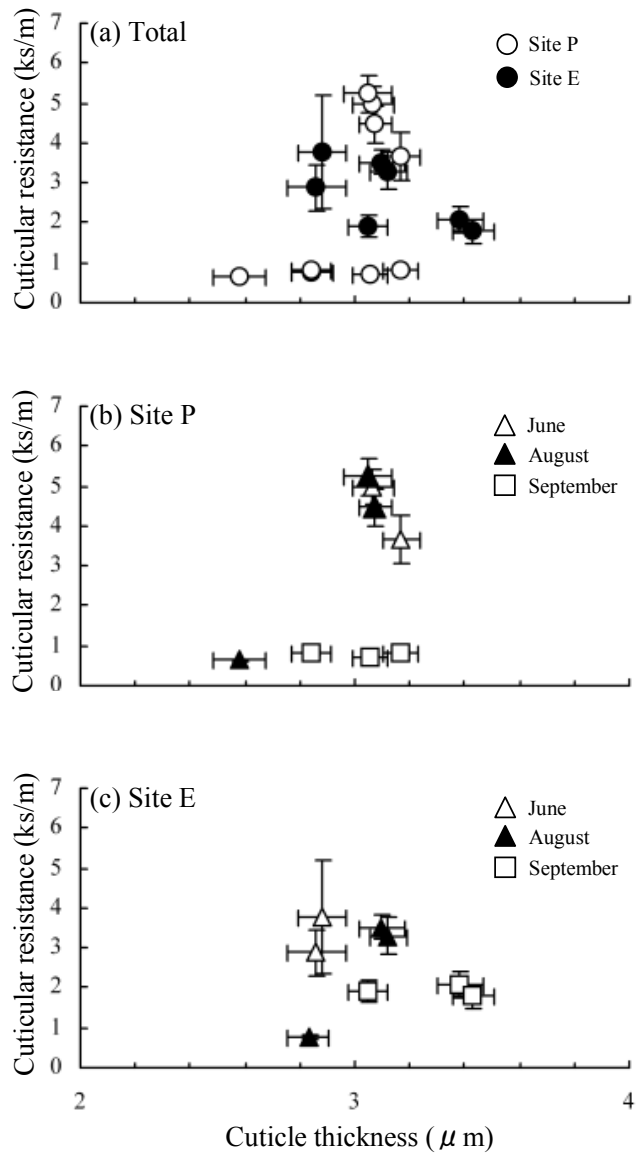


Fig. 10. Relationship between cuticular resistance ($n = 160$ shoots) and cuticle thickness ($n = 160$ needles). (a) Total was reconstructed by integrating the data of (b) site P and (c) site E. Bars = SE.

Discussion

That increased transpiration in conifer needles with damaged cuticles in high mountains induces decreased water content of needles to a lethal level causing needle death has been considered (Baig and Tranquillini 1976; Hadley and Smith 1983, 1986). To confirm whether this concept applies to needle browning and death of *P. pumila* in the alpine region of Mt. Norikura, Japan, we discussed following issues: (i) does mechanical damage of the cuticle cause needle browning and death? and (ii) is there any relationship between cuticular resistance and cuticle thickness?

Features of intact needle and artificially abraded needle cuticle

Whether browning of needles exposed above the winter snowpack in high mountains was caused by abrasion of the wax and cuticle by impacts of wind-borne snow and ice particles is discussed (Marchand and Chabot 1978; Tranquillini 1979; Hadley and Smith 1983, 1986, 1987). It was reported that foliage of woody plants (Wilson 1980, 1984; van Gardingen et al. 1991) and herbaceous plants (Thompson 1974; MacKerron 1976; Pitcairn et al. 1986) were mechanically damaged from being rubbed against each other by strong wind. In *Picea engelmannii*, the surface wax of needles above the winter snow level was 60 % less than that of needles below the winter snow level (Hadley and Smith 1987). Excessive transpiration was induced when strong wind blew damaged needles (Lindsay 1971; Tranquillini 1979; Hadley and Smith 1987; Grace 1990; Herrick and Friedland 1991) and low soil temperature prevented water uptake (Lindsay 1971; Larcher 1995). This caused needle water loss and death. However, the microscopic study of *P. cembra* did not show any damage of needle cuticles abraded by snow and ice particles (Tranquillini 1979). We also showed in this study that in *P. pumila* growing in a natural environment on Mt. Norikura, mechanical damage of the cuticle of all needle ages was not observed, and the epicuticular waxes covered the needle surface and plugged up the stomatal antechamber. Even if neither wax nor cuticle was abraded, needle color of a few tree species changed to brown.

Moreover, to confirm whether damage of the cuticle causes needle browning, an artificial abrasion test to the needle cuticle was performed in August 2008. The color of only that part of the needle where the cuticle was artificially abraded changed to brown one month after the filing treatment. The artificial abrasion test in September 2008 showed needle browning one year after the filing treatment. The artificial abrasion test of the cuticle also influenced survival rates of needles. At site P covered with snow in winter, most of needles abraded in September 2008 defoliated one year after filing treatment on 1- and 2-year-old needles (Fig. 5a), and the damaged cuticle did not regenerate. Needle parenchyma cells degenerated, needle morphology greatly changed, and the needle died. On the other hand, at site E not covered with snow in winter, needles did not fall (Fig. 5b), even though needle color changed to brown. The difference in survival rates of needles between site P and site E was thought to be that the shoot not protected with snow at site E is more tolerant to severe environment in winter than the shoot protected with snow at site P.

It was clear that artificial abrasion of *P. pumila* cuticle led to browning of needle color. However, the cuticle of *P. pumila* needles that browned in a natural environment had not been damaged, and the color of these browned needles obviously differed from that of needles subjected to the filing treatment and untreated parts of needles subjected to the filing treatment. From these results, the cause of needle browning of *P. pumila* could not be explained by the idea of Tranquillini (1979) and Hadley and Smith (1983, 1986, 1987) that cuticle damaged needle wilts result from decreases in needle water content to lethal levels due to excess transpiration.

Relationship between cuticular resistance and cuticle thickness

Cuticular transpiration is thought to be another factor to bring a desiccation stress to foliage besides cuticle abrasion. It has reported that the growing period of foliage in high mountains was short and that the cuticle of foliage could not mature to reach its final thickness during the current growing period (Tranquillini 1976, 1982). Therefore current-year needles could not completely fulfill the function of controlling water loss

from foliage. This induced the increase in transpiration during winter and led to needle browning (Baig and Tranquillini 1976, 1980; Sowell et al. 1982; Tranquillini 1982).

Cuticular resistance decreased with the altitude and differed between windward side and leeward side, needle ages, and seasons (Tranquillini 1976; Sowell et al. 1982; Herrick and Friedland 1991). Transpiration rate of *Picea abies* of the European Central Alps in December was $14 \text{ mg (g shoot dry mass)}^{-1} \text{ h}^{-1}$ in the krummholz (2140 m) and seven times higher than that in the valley (1000 m). Cuticular resistance of *Pinus cembra* was lower in the krummholz (2140 m) than in the valley (1000 m) (Tranquillini 1976). Cuticular resistance of windward needles of *Picea engelmannii* in the Medicine Bow Mountains of southeastern Wyoming was lower than that of leeward needles (Hadley and Smith 1983). Further, in the Rocky Mountain, US, cuticular resistance of wind-exposed needles of *P. engelmannii* in the lower timberline ecotone (3200 m) in southeastern Wyoming declined from $100\text{--}250 \text{ ks m}^{-1}$ in autumn to less than 30 ks m^{-1} in midwinter (Hadley and Smith 1986). Cuticular resistance of *P. pumila* in Mt. Norikura, Japan, differed between sites and seasons. Site P corresponds to a leeward side and site E corresponds to a windward side. At site P, cuticular resistance in September significantly increased in June of the following year (Fig. 7a), but at site E, there was no difference in cuticular resistance between September and June of the following year (Fig. 7b). Then cuticle thickness was measured to clarify whether the difference in cuticular resistance was related to the difference in cuticle thickness.

It was reported that cuticle thickness in some species differs with altitude. The cuticle of 1-year-old needles in *Picea abies* in the krummholz limit on Patscherkofel Mountain near Innsbruck, Austria, was thinner by 84 % than that of the treeline, and this caused the increase in transpiration (Baig and Tranquillini 1976; Tranquillini 1976). Mean cuticle thickness of the adaxial surface of 1-year-old *Abies balsamea* needles in a subalpine forest on Mt. Moosilauke, New Hampshire, US, decreased with an increase in altitude from $3.01 \text{ }\mu\text{m}$ at the base to $2.21 \text{ }\mu\text{m}$ at the treeline (DeLucia and Berlyn 1984). Meanwhile, the cuticles of *Picea abies* and *Pinus cembra* in the Dolomites (northeastern Italian Alps) were thicker in the high altitude area than in the low altitude area (Anfodillo et al. 2002). The tendency of increases and decreases in cuticle thickness

according to altitude was different between study sites and tree species. Moreover, it was shown that cuticle thickness differs between needle ages. The cuticle of 1-year-old needles of *Picea abies* in the krummholz limit on Patscherkofel Mountain near Innsbruck, Austria, was thinner by 73 % than that of 2-year-old needles, and the difference in cuticle thickness between 1-year-old and 2-year-old needles was attributed to the growing period (Baig and Tranquillini 1976; Tranquillini 1976). Cuticle thickness of 1-year-old needles on *P. pumila* in Mt. Norikura, Japan, was approximately 2.9 μm and thinner than that of 2-year-old needles, which was approximately 3.3 μm . Cuticle thickness was equal to approximately 3.3 μm in *Pinus cembra* (Baig and Tranquillini 1976) in the same genus, *Pinus*.

In *P. pumila* in Mt. Norikura, the cuticle thickness differed between seasons. At site P, after needle emergence in early August, the cuticle of current-year needles became thickened as needles matured and was thickest in June of the following year (Fig. 8a). Therefore, it was thought that it took about one year for the needle cuticle of *P. pumila* in high altitudes to reach final thickness. At site E, cuticle thickness of 1- and 2-year-old needles remained constant. Cuticle thickness of current-year needles grew thick as needles matured (Fig. 8b). Cuticles of 1- and 2-year-old needles were thinnest after overwinter and became thicker during the following months. This was different from that at site P. Cuticles of current-year and 1- and 2-year-old needles just before winter were thicker at site E than at site P. This showed that needles at site E might have to endure a more severe environment than those at site P.

Despite that cuticle thickness was approximately 3 μm and constant (Fig. 8a), cuticular resistance repeatedly increased and decreased (Fig. 7a). Therefore, this means that cuticular resistance does not reflect cuticle thickness and that needles being covered with snow during the winter at site P need not increase cuticular resistance in autumn. On the other hand, the increase in cuticular resistance after passing the winter would protect shoots above snowpack during thaw. At site E, there was no difference in cuticular resistance of 1-year-old needles (Fig. 7b), but cuticles were thinnest after winter and then became thicker again (Fig. 8b). This also means that cuticular resistance

does not reflect cuticle thickness at site P. These results indicate that there is no remarkable relationship between cuticular resistance and cuticle thickness.

Meanwhile, there was no difference in cuticle thickness in each needle age between needles that overwintered above the snowpack and those that overwintered below the snowpack (Fig. 9a). However, cuticular resistance of 1-year-old needles above the snowpack was lower than those below snowpack (Fig. 9b). This also indicated that cuticular resistance did not reflect cuticle thickness.

In general, it has been considered that a thicker cuticle is more effective in decreasing water loss (Tranquillini 1976, 1982; Hadley and Smith 1983; Edwards et al. 1996). However, although cuticles of *P. abies* and *P. cembra* in the Dolomites (northeastern Italian Alps) were thicker in high altitudes than in low altitudes, the correlation between cuticle thickness and cuticular resistance was not seen (Anfodillo et al. 2002). The correlation between cuticular resistance and cuticle thickness of *P. pumila* in Mt. Norikura, Japan, was also not seen. Therefore, the difference in cuticular resistance of *P. pumila* does not reflect the difference in cuticle thickness (Fig. 10a).

The cuticle became thick in high altitudes because desiccation stimulates the development of a thick cuticle (Hull et al. 1975). Cuticles also provide defence against ultraviolet rays (Day 1993; Riederer 2006). Thick cuticles have been thought to be more effective than thin cuticles in reducing water loss from foliage (Tranquillini 1976, 1982; Hadley and Smith 1983; Edwards et al. 1996). On the other hand, there are a few studies in which thin cuticles are more effective as a water barrier than thick cuticles (Becker et al. 1986; Schreiber and Riederer 1996) and cuticle water permeability does not correlate with cuticle thickness, weight, and wax coverage (Schönherr 1982; Riederer and Schreiber 2001). Moreover, it was reported that damage to leaves by water loss was attributed to a stomatal dysfunction caused by mechanical damage and by direct damage to the leaf cuticle rather than to a thinner or less developed cuticle (Grace 1990). As mentioned above, various discussions have been developed.

The cuticle is a thin (0.1–10 μm thick) continuous membrane consisting of a polymer matrix (cutin), polysaccharides, and associated solvent-soluble lipids (cuticular

waxes) (Jeffree 1996; Riederer and Schreiber 2001; Jeffree 2006). It was reported that the amount of water taken up by an isolated cuticle was closely correlated with cuticular polysaccharide content in some species (Kerstiens and Lenzian 1989; Chamel et al. 1991). Needle browning of *P. pumila* on Mt. Norikura, Japan, was not caused by mechanical damage to the cuticle, a thinner cuticle, or the disappearance of epicuticular wax. We then considered that the difference in cuticular resistance was attributed to the difference in water permeability through a cuticle, which was induced by a change and deterioration of the cuticle quality and structure in *P. pumila*. This is different from the idea by Tranquillini (1979) and Hadley and Smith (1983, 1986, 1987) that damaged cuticles of needles cause excess transpiration resulting in needle death due to water content decreasing to lethal levels. To estimate desiccation stress in relation to a cuticle, we need to elucidate not only cuticular resistance and cuticle thickness, but also cuticle quality and structure and other stresses.

Conclusion

We found that needle browning and death of *P. pumila* on Mt. Norikura, the alpine region of Japan, was not caused by mechanical damage of the cuticle or thinner cuticle. More research is needed to better understand not only cuticular resistance and cuticle thickness, but also cuticle quality and structure and other stresses.

Chapter 3. Needle browning and death in the flagged crown of *Abies mariesii* in the timberline ecotone of the alpine region in central Japan

Introduction

The timberline ecotone of alpine regions is the transitional zone between the forest and the Krummholz limit. Here, evergreen conifers frequently produce the flagged crown which is an asymmetric growth form associated with severe stresses during winter (Tranquillini 1979; Raven et al. 1999; Körner 2003; Smith et al. 2003). Above snowpack, the flagged crown is characterized by a wind-deformed leader above a section free of branches and needles. Below the snowpack, the crown is cushion-shaped (Fig. 1; Tranquillini 1979; Pereg and Payette 1998). Tree form largely depends on the winter snow cover, since shoots projecting above the snow are severely damaged regularly (Tranquillini 1979; Körner 2003). The flagged crown has been thought to develop primarily due to winter desiccation of windward shoots and secondarily due to damage by mechanical action of the wind (Tranquillini 1979; Holtmeier 2003). Needle browning and death in the flagged crown often occur in late winter and early spring followed by needle drop.

A timberline ecotone in the high mountains of Japan is characterized by scattered *Abies mariesii* within a *Pinus pumila* community. *A. mariesii* at the timberline ecotone shows the flagged crown above snowpack and the cushion-shaped crown below snowpack in winter (Fig. 1). Needles in the flagged crown of *A. mariesii* often become brown and die in early spring, but remain green and intact in the cushion-shaped crown. In this study, I assessed mechanical damage of the needle cuticle of *A. mariesii* in a timberline ecotone and evaluated the effect of cuticle thickness on cuticular resistance.



Fig. 1. *Abies mariesii* showing the flagged crown (a) and the cushion-shaped crown (b) distributed on a shallow slope (2,500 m above sea level) of Mt. Norikura, Japan, in June 2008. White bar indicates the approximate snow level during winter.

Materials and Methods

Study area and tested trees

The study was conducted in a timberline ecotone distributed on the eastern gentle slope [5°; 2,500 m above sea level (a.s.l.)] of Mt. Norikura in Chubu Mountain National Park, Nagano, Japan (36°61'N, 137°33'E, 3,026 m a.s.l.) located at the southernmost tip of the northern Japanese Alps in the central mountainous area of the Japan Archipelago. In this area, snow depth during the winter reaches approximately 2.5 m. Air temperature was continuously sub-zero °C just above snowpack from late November to late April and minimum temperature has reached below -20 °C in late February (Yamazaki et al. 2003). Thus, -20 °C might be also reached before and after late February in other years. The dominant vegetation is a dwarf scrub *Pinus pumila* community, including *A. mariesii* trees. *A. mariesii* is a dominant species of subalpine coniferous forest where its tree form is the conical crown of the excurrent tree. However, *A. mariesii* in a timberline ecotone shows the flagged tree form (Fig. 1). Needle color of the adaxial and

abaxial sides in the cushion-shaped crown was green in June (Figs. 2a, 2b) and needle browning hardly occurs. In contrast, needle color of the abaxial side in the flagged crown becomes brown frequently in early spring and most of the browning needles have already dropped (Fig. 2c). New shoots flushed in late July, making the growing season only about 2 months long. We studied mechanical damage to needle cuticles and the effect of cuticle thickness on cuticular resistance of *A. mariesii* in a timberline ecotone.

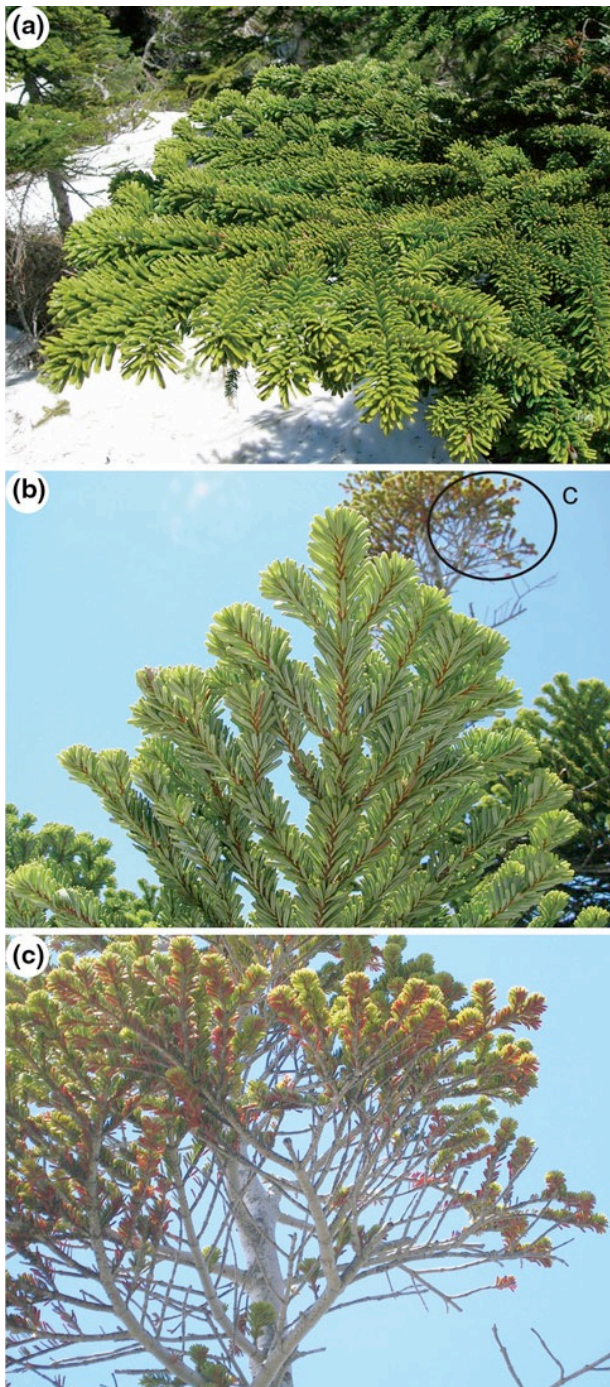


Fig. 2. Needle color of *A. mariesii* shoots in June 2008. (a) The adaxial side of shoots in the cushion-shaped crown: needle color was green. (b) The abaxial side of shoots in the cushion-shaped crown: needle color was green. (c) Closeup of c shown in (b), the abaxial side of shoots in the flagged crown: needle color was brown and most of needles already died and dropped in June.

Microscopy for needle surface and cuticle thickness

Fresh samples for microscopy are often fixed with chemicals after collection. Chemical fixation of tissues often induces structural artifacts, and structure or organelle dissolution (Ruzin 1999). In the needle cuticle of *P. pumila*, not only the epicuticular wax but also the cuticle itself was dissolved with a chemical fixative (see Chapter 2). Therefore, to make sure whether chemical fixation is appropriate or not for cuticle observation of *A. mariesii* needles, we compared needles with and without chemical fixative. In the former, shoots collected in the field were immediately fixed in cold 3 % glutaraldehyde and kept in a refrigerator. In the latter, shoots collected in the field were immediately put into a plastic bag without a chemical fixative and kept in a refrigerator. Browning and healthy green needles with or without a chemical fixative were treated and observed as described below ($n = 10$ needles).

For scanning electron microscopy, needles treated with a chemical fixative were dehydrated in an EtOH series, substituted in a *tert*-butyl alcohol series and dried using freeze dehydration equipment (JFD-310, JEOL Datum Ltd., Tokyo, Japan). Fresh needles without chemical fixative were kept in their natural state. Both prepared samples were observed using a scanning electron microscope (JSM-5510LV, JEOL Datum Ltd., Tokyo, Japan) after coating with Au in an ion-sputter coater (IB-3, Eiko Engineering Co., Ltd., Tokai, Ibaraki, Japan).

For light microscopy, fixed needles were embedded in epoxy resin, and ultrathin Sects. 2 μm thick were prepared using a rotary microtome (EM Super Nova, JEOL Datum Ltd., Tokyo, Japan) and stained with safranin (0.5 %). Fresh needles were fitted into pith sticks, and Sects. 25 μm thick were prepared using a sliding microtome (Yamato Koki, Saitama, Japan) and stained with Sudan III (0.5 %). Both stained sections were observed by a light microscope (Olympus BX51TF, Tokyo, Japan) and a digital camera (Camedia C-5060, Olympus Corp., Tokyo, Japan). Then adaxial and abaxial cuticle thicknesses were measured using Photoshop CS2 (Adobe Systems, San Jose, CA, USA).

Sampling

Branches collected from flagged crown and cushion-shaped crown of different 4 trees in the field in June, early August and late September 2008 were immediately put into a plastic bag, stored in a cool box, and brought back to the laboratory and kept in a refrigerator. The tree heights and diameters at breast height of tested trees ($n = 4$) were 4.85 ± 0.33 m and 19.75 ± 3.93 cm, respectively. Ten shoots in branches of flagged crown and cushion-shaped crown of four trees (two or three shoots in each crown per tree, respectively) were used for measurements of cuticle thickness and cuticular resistance.

Measurement of cuticle thickness

One needle in each age internode of a single shoot was used for measurement. It made a total of 10 needles in each flagged crown and cushion-shaped crown in all. Cuticle thickness was measured at five points of the middle of an adaxial surface and one point each of two white stomata belts on the abaxial surface according to the light microscopy as mentioned above, respectively. It made a total of seven points in each needle in all.

Measurement of cuticular resistance

The shoots were refreshed under water and watered additionally for more than 12 h in a dark room. They were cut into internodes bearing needles for each year, and the cut ends were sealed with nail polish to prevent evaporation. Cuticular resistance of each internode was measured separately. For assessment of cuticular resistance to water loss, r_c (ks m^{-1}), Eq. 1 was used:

$$E = (e_1^0 - e_a)/r_c \quad (1)$$

where E ($\text{g s}^{-1} \text{m}^{-2}$) is the rate of water vapor loss from needles; e_1^0 (g m^{-3}) is the saturated water vapor concentration inside a needle (at needle temperature); and e_a (g m^{-3}) is the water vapor concentration in the air. The influence of boundary layer

resistance of the needle was reduced by circulating air at 1.2-2.3 m s⁻¹ in a dark room. Samples of internodes for each year were weighed, maintained at room temperature and humidity (approximately 20 °C and 52 %, respectively) for 30 min, and reweighed. Measurements were repeated until *E* became constant. Leaf area was measured by using an image analysis software (Scion Image, Beta 4.0.3, Frederick, Maryland, USA). Air temperature and humidity were monitored during measurement (Thermo recorder RS-10, ESPEC MIC Corp., Osaka, Japan). Needle temperature of each internode was measured before reweighing using a fine-wire copper-constantan thermocouple (0.1 mm diameter).

Data analysis

Statistically significant differences between with and without a chemical fixative were determined using Student's *t* test. To assess the differences in measurement months and needle ages, one-way analysis of variance (ANOVA), Tukey test and Kruskal–Wallis test were performed. Nonlinear regression analysis fitted by an exponential function was used to characterize a relationship between cuticular resistance and cuticle thickness.

Results

Effect of chemical fixative to needle cuticle thickness

Scanning electron microscopy and light microscopy showed that the features of the needle surface differed depending on whether or not a chemical fixative was used (Fig. 3). For the surface of epidermal cells in needles prepared without a chemical fixative, cuticle abrasion and wounding were not observed on both adaxial (Fig. 3a) and abaxial sides (Fig. 3b). On the abaxial surface, the epicuticular waxes comprise the outer part of the cuticle and occlude the openings of stomatal antechambers (Figs. 3b, 3c). In contrast, for those prepared with a chemical fixative, the cuticle appeared abraded and wounded on both adaxial (Fig. 3d) and abaxial sides (Fig. 3e), and stomatal pores were clearly observed (Fig. 3f). The epicuticular waxes mostly disappeared and

the openings and the interior of the stomatal antechamber were clearly seen (Figs. 3e, 3f). This difference implies the dissolution of epicuticular wax with a chemical fixative. Cuticle thickness of the adaxial surface prepared with a chemical fixative was also significantly thinner than that prepared without a chemical fixative in both browning needles ($P < 0.01$) and healthy green needles ($P < 0.01$) (Table 1). These results showed that not only the epicuticular wax but also the cuticle itself were dissolved by a chemical fixative. As a result, fresh samples prepared without a chemical fixative after collection were considered to be appropriate for observing the needle surface and its cross section in a natural state.

Table 1. Cuticle thickness of *Abies mariesii* needles prepared with or without a chemical fixative.

	Cuticle thickness ($\mu\text{m} \pm \text{SE}$)		<i>P</i>
	With a chemical fixative	Without a chemical fixative	
Browning needle	5.64 ± 0.17	7.77 ± 0.25	< 0.01
Healthy green needle	4.82 ± 0.10	7.36 ± 0.05	< 0.01

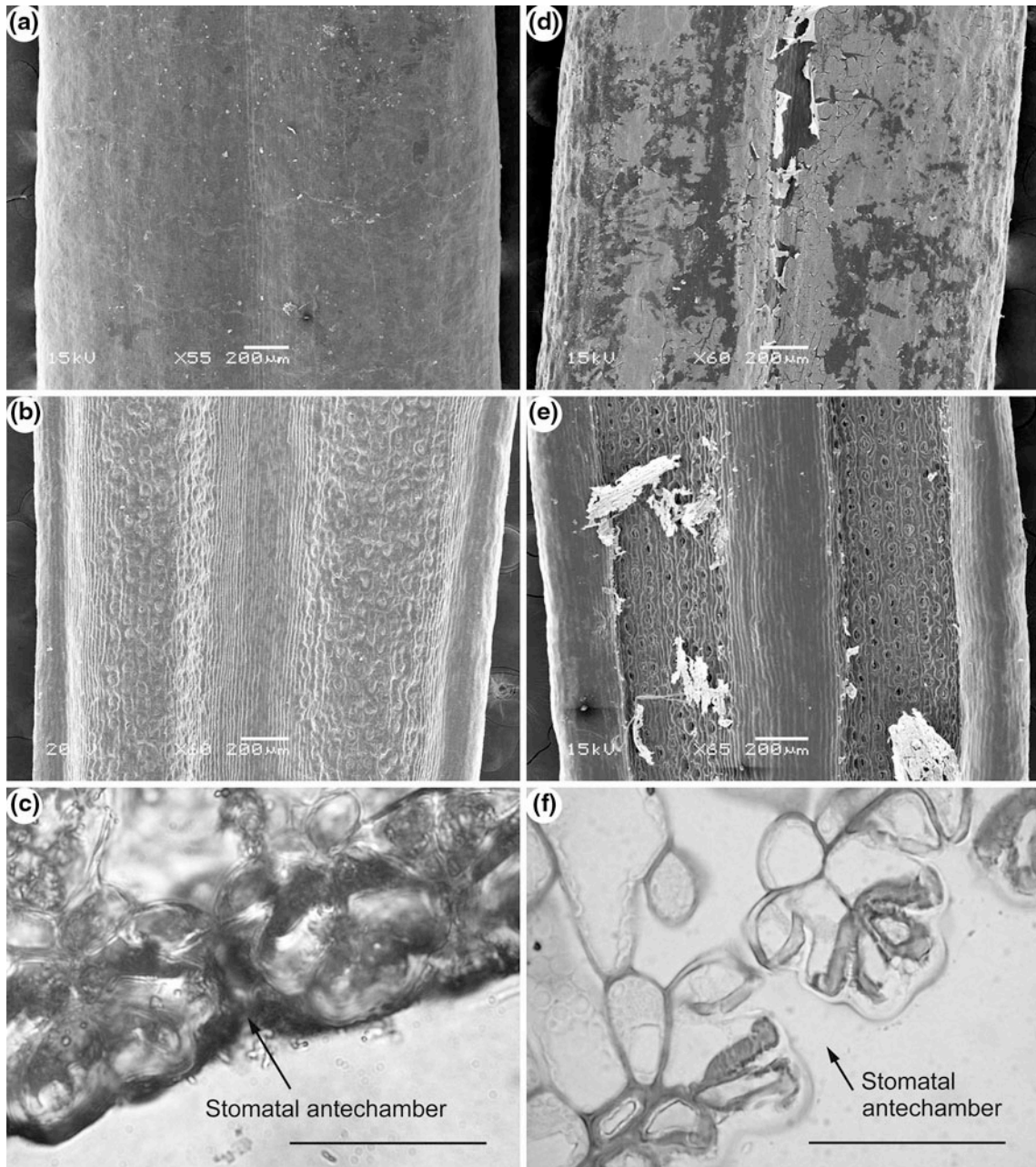


Fig. 3. Features of the needle surface in *A. mariesii*: adaxial surface, abaxial surface and abaxial cross section of needles. (a)–(c) Without a chemical fixative and (d)–(f) with a chemical fixative. (a), (b), (d) and (e) observed by scanning electron microscopy; (c) and (f) observed by light microscopy. In (a), (b), (d) and (e), scale bars = 200 μm; in (c), (f), scale bars = 50 μm.

Mechanical damage of cuticle

Histological observations of all needles by a scanning electron microscope and a light microscope did not show any mechanical damages to the needle cuticle in *A. mariesii* (Figs. 3a, 3b, 3c). In the 2-year-old needles, the cuticle features of the abaxial side were different between flagged and cushion-shaped crowns (Fig. 4). In the flagged crown, the abaxial surface of needles appeared smooth (Fig. 4a) and epicuticular wax was not seen (Fig. 4b). In contrast, in the cushion-shaped crown, the epicuticular wax comprised the outer part of the cuticle (Fig. 4c), occluded the openings of stomatal antechamber and plugged up the stomatal antechamber (Fig. 4d).

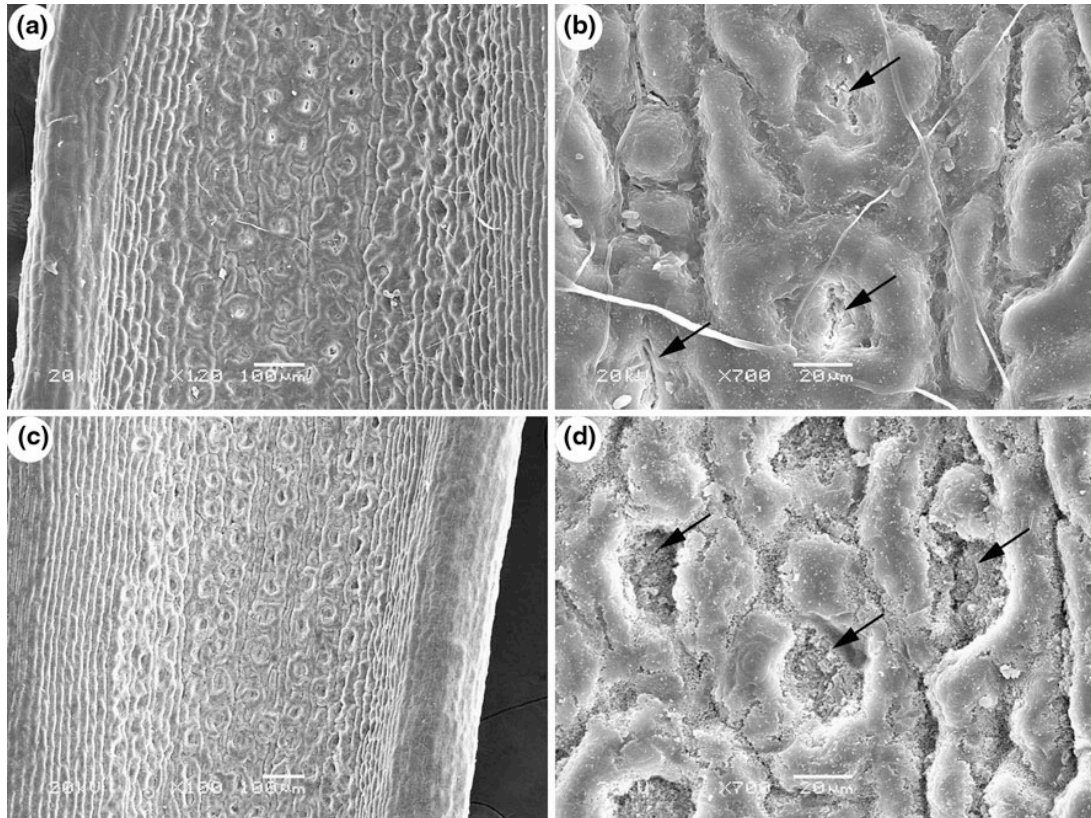


Fig. 4. Features of the abaxial surface of the 2-year-old needles in *A. mariesii* observed by scanning electron microscopy: needle in the flagged crown (a, b) and the cushion-shaped crown (c, d). (b) Closeup of a, the openings of stomatal antechambers in the flagged crown; arrows show that epicuticular wax mostly disappeared. (d) Closeup of c, the openings of stomatal antechamber in the cushion-shaped crown; arrows show that epicuticular wax plugged the openings of stomatal antechamber. In (a), (c), scale bars = 100 μm ; in (b), (d), scale bars = 20 μm .

Cuticular resistance

First, we compare seasonal changes in cuticular resistances within each crown type and age group (Fig. 5). In both flagged and cushion-shaped crowns, cuticular resistance significantly increased from June to August ($P < 0.05$) except for the 1- and 2-year-old needles in the cushion-shaped crown and then decreased from August to September ($P < 0.05$) (Fig. 5). In other words, cuticular resistance increased at the peak of the growing season and decreased just before winter. Data of cuticular resistance in current-year needles in June were not available because shoots flushed in late July.

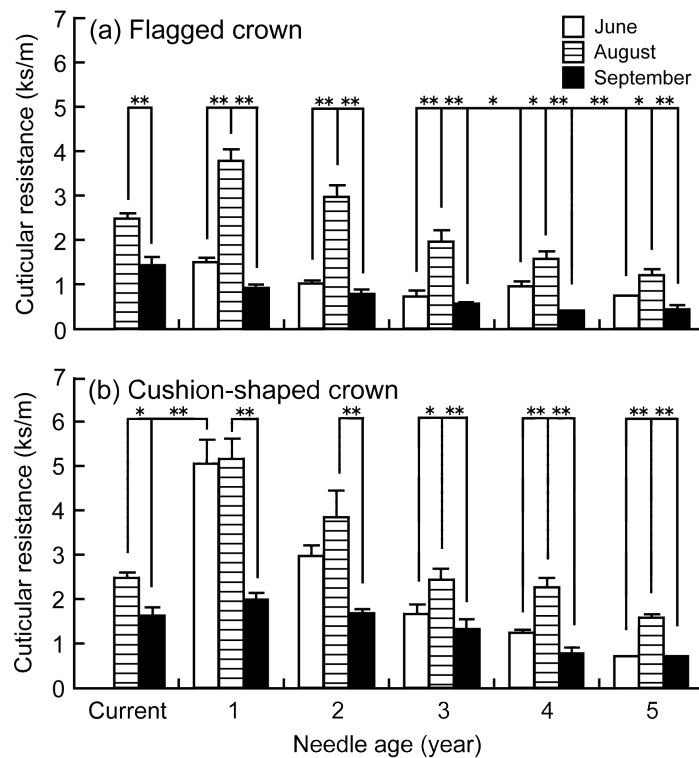


Fig. 5. Comparison of seasonal changes in cuticular resistances from current- to 5-year-old needles in June ($n = 10$ shoots), early August ($n = 10$ shoots), and late September ($n = 10$ shoots), 2008 within each crown type and age group. The adjacent values were compared. Asterisks indicate significant differences between months: *, $P < 0.05$; **, $P < 0.01$. Bars = SE.

Between September, before winter, and June, after winter, cuticular resistances in the flagged crown significantly increased from 3- to 4-year-old needles ($P < 0.05$) and from 4- to 5-year-old needles ($P < 0.01$) (Fig. 5a). This shows that cuticular resistance

increased after winter in older needles. It was noteworthy that cuticular resistance in the cushion-shaped crown significantly rose about threefold from September (current-year) to June (1-year-old) ($P < 0.01$) (Fig. 5b). This was not shown in the flagged crown.

Next, we compare age-related changes in cuticular resistances within each season and crown type. In September, before winter, there was no significant difference in cuticular resistance between current- and 1-year-old needles and between 1- and 2-year-old needles in either crown type. But cuticular resistance significantly decreased from 2- to 3-year-old needles ($P < 0.05$) in the flagged crown and from 3- to 4-year-old needles ($P < 0.01$) in both crowns (Table 2). In June, after winter, cuticular resistance significantly decreased from 1- to 2-year-old needles ($P < 0.05$) and from 2- to 3-year-old needles ($P < 0.05$) in the flagged crowns (Table 2). In August, cuticular resistance significantly increased from current- to 1-year-old needles ($P < 0.01$) in both crowns and then it significantly decreased from 2- to 3-year-old needles ($P < 0.05$) in the flagged crowns (Table 2). The function of the cuticle to reduce water loss through the needle might complete in August of the following year.

Table 2. Comparison of age-related changes in cuticular resistances or cuticle thicknesses within each season and crown type.

	Needle age (year-old)				
	Current - 1	1 - 2	2 - 3	3 - 4	4 - 5
Cuticle resistance (ks/m \pm SE)					
Flagged crown					
September	1.43 \pm 0.22 - 0.93 \pm 0.11	0.93 \pm 0.11 - 0.77 \pm 0.08	0.77 \pm 0.08 - 0.56 \pm 0.03*	0.56 \pm 0.03 - 0.44 \pm 0.01**	0.44 \pm 0.01 - 0.45 \pm 0.03
June	-	1.53 \pm 0.17 - 1.04 \pm 0.10*	1.04 \pm 0.10 - 0.70 \pm 0.07*	0.70 \pm 0.07 - 1.01 \pm 0.15	1.01 \pm 0.15 - 0.84 \pm 0.11
August	2.42 \pm 0.16 - 3.77 \pm 0.27**	3.77 \pm 0.27 - 3.01 \pm 0.29	3.01 \pm 0.29 - 2.01 \pm 0.26*	2.01 \pm 0.26 - 1.59 \pm 0.17	1.59 \pm 0.17 - 1.26 \pm 0.14
Cushion-shaped crown					
September	1.65 \pm 0.24 - 1.97 \pm 0.23	1.97 \pm 0.23 - 1.65 \pm 0.11	1.65 \pm 0.11 - 1.37 \pm 0.13	1.37 \pm 0.13 - 0.84 \pm 0.09**	0.84 \pm 0.09 - 0.79 \pm 0.02
June	-	5.02 \pm 1.09 - 3.09 \pm 0.55	3.09 \pm 0.55 - 1.69 \pm 0.20	1.69 \pm 0.20 - 1.23 \pm 0.14	1.23 \pm 0.14 - 0.79 \pm 0.08*
August	2.46 \pm 0.12 - 5.10 \pm 0.51**	5.10 \pm 0.51 - 3.88 \pm 0.62	3.88 \pm 0.62 - 2.46 \pm 0.20	2.46 \pm 0.20 - 2.24 \pm 0.20	2.24 \pm 0.20 - 1.60 \pm 0.08**
Cuticle thickness (μ m \pm SE)					
Flagged crown					
September	8.94 \pm 0.20 - 8.82 \pm 0.17	8.82 \pm 0.17 - 8.62 \pm 0.16	8.62 \pm 0.16 - 8.68 \pm 0.20	8.68 \pm 0.20 - 8.87 \pm 0.19	8.87 \pm 0.19 - 8.73 \pm 0.12
June	-	7.53 \pm 0.20 - 7.96 \pm 0.21	7.96 \pm 0.21 - 8.59 \pm 0.22*	8.59 \pm 0.22 - 8.37 \pm 0.24	8.37 \pm 0.24 - 7.13 \pm 0.16**
August	5.55 \pm 0.21 - 6.43 \pm 0.21**	6.43 \pm 0.21 - 6.44 \pm 0.25	6.44 \pm 0.25 - 6.83 \pm 0.18	6.83 \pm 0.18 - 6.80 \pm 0.16	6.80 \pm 0.16 - 7.09 \pm 0.13
Cushion-shaped crown					
September	7.38 \pm 0.18 - 7.38 \pm 0.17	7.38 \pm 0.17 - 7.83 \pm 0.17	7.83 \pm 0.17 - 7.84 \pm 0.13	7.84 \pm 0.13 - 7.99 \pm 0.22	7.99 \pm 0.22 - 7.94 \pm 0.13
June	-	6.73 \pm 0.21 - 6.97 \pm 0.15	6.97 \pm 0.15 - 7.92 \pm 0.12**	7.92 \pm 0.12 - 7.77 \pm 0.17	7.77 \pm 0.17 - 7.45 \pm 0.17
August	5.42 \pm 0.15 - 6.73 \pm 0.14**	6.73 \pm 0.14 - 6.86 \pm 0.10	6.86 \pm 0.10 - 7.26 \pm 0.24	7.26 \pm 0.24 - 7.55 \pm 0.22	7.55 \pm 0.22 - 7.67 \pm 0.16

Asterisks indicate significant differences in cuticular resistance or cuticle thickness between needle ages: *, $P < 0.05$; **, $P < 0.01$.

Table 3. Comparison of cuticular resistances or cuticle thicknesses between crown types within each season and age group.

Needle age (year-old)		1		2		3		4		5	
Current											
Flagged crown : Cushion-shaped crown											
Cuticular resistance (ks/m ± SE):											
September	1.43 ± 0.22	1.65 ± 0.24	0.93 ± 0.11	1.97 ± 0.23**	0.77 ± 0.08	1.65 ± 0.11**	0.56 ± 0.03	1.37 ± 0.13**	0.44 ± 0.01	0.84 ± 0.09**	0.45 ± 0.03
June	-	-	1.53 ± 0.17	5.02 ± 1.09**	1.04 ± 0.10	3.09 ± 0.55**	0.70 ± 0.07	1.69 ± 0.20**	1.01 ± 0.15	1.23 ± 0.14	0.84 ± 0.11
August	2.42 ± 0.16	2.46 ± 0.12	3.77 ± 0.27	5.10 ± 0.51*	3.01 ± 0.29	3.88 ± 0.62	2.01 ± 0.26	2.46 ± 0.20	1.59 ± 0.17	2.24 ± 0.20*	1.26 ± 0.14
Cuticle thickness (µm ± SE):											
September	8.94 ± 0.20	7.38 ± 0.18**	8.82 ± 0.17	7.38 ± 0.17**	8.62 ± 0.16	7.83 ± 0.17**	8.68 ± 0.20	7.84 ± 0.13**	8.87 ± 0.19	7.99 ± 0.22**	8.73 ± 0.12
June	-	-	7.53 ± 0.20	6.73 ± 0.21**	7.96 ± 0.21	6.97 ± 0.15**	8.59 ± 0.22	7.92 ± 0.12**	8.37 ± 0.24	7.77 ± 0.17	7.13 ± 0.16
August	5.55 ± 0.21	5.42 ± 0.15	6.43 ± 0.21	6.73 ± 0.14	6.44 ± 0.25	6.86 ± 0.10	6.83 ± 0.18	7.26 ± 0.24	6.80 ± 0.16	7.55 ± 0.22*	7.09 ± 0.13

Asterisks indicate significant differences in cuticular resistance or cuticle thickness between the flagged crown and the cushion-shaped crown. The left number the right number in a column show the value of the flagged crown and that of the cushion-shaped crown, respectively: *, $P < 0.05$; **, $P < 0.01$.

Finally, we compared cuticular resistances between crown types within each season and age group. In September, cuticular resistance of needles in the flagged crown was significantly lower than that in the cushion-shaped crown ($P < 0.01$) except for the current-year-old needles (Table 3). In June, cuticular resistance of 1-, 2- and 3-year-old needles in the flagged crown was significantly lower than that in the cushion-shaped crown ($P < 0.01$) (Table 3). In August, cuticular resistance of 1- and 4-year-old needles in the flagged crown was significantly lower than that in the cushion-shaped crown ($P < 0.05$) (Table 3).

Cuticle thickness

In the flagged crown, cuticle thicknesses significantly decreased from June to August ($P < 0.01$) except for the 5-year-old needles and increased from August to September in any age needles ($P < 0.01$) (Fig. 6a). In other words, cuticle thickness in the flagged crown decreased at the peak of the growing season and increased just before winter. Comparing cuticle thicknesses between September and June, they significantly decreased from current- to 1-year-old needles ($P < 0.01$), from 1- to 2-year-old needles ($P < 0.01$) and from 4- to 5-year-old needles ($P < 0.01$) (Fig. 6a). Cuticle thickness decreased after winter in both younger and older needles. In the cushion-shaped crown, cuticle thickness significantly decreased from June to August only in the 3-year-old needles ($P < 0.05$) (Fig. 6b). Cuticle thickness in current-, 1-, 2- and 3-year-old needles significantly increased from August to September ($P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.05$, respectively), but did not differ in older needles (Fig. 6b). Cuticle thickness only decreased significantly from current-year needles in September ($P < 0.05$) to 1-year needles in June (Fig. 6b). To summarize, cuticle thickness did not change below snowpack before and after winter except for younger needles.

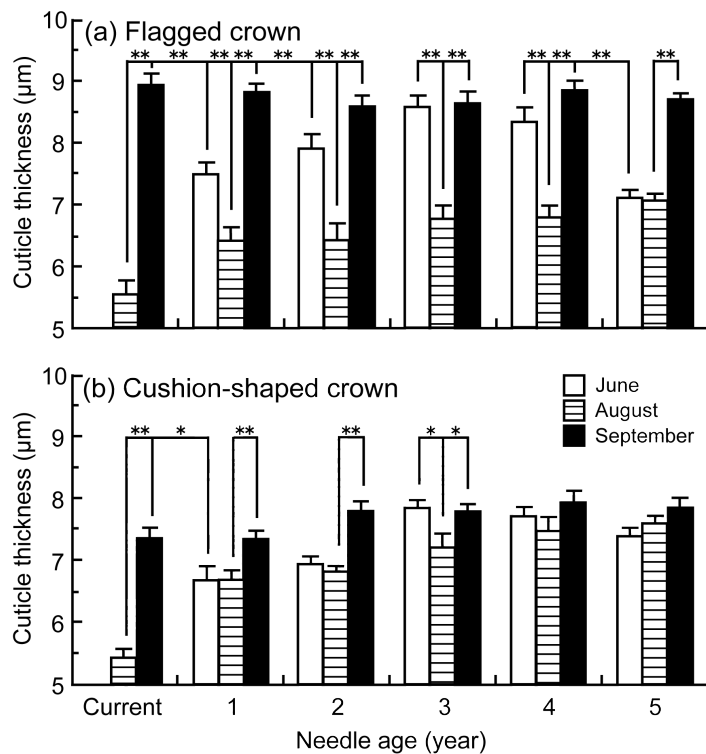


Fig. 6. Comparison of seasonal changes in cuticle thicknesses from current- to 5-year-old needles in June ($n = 10$ needles), and early August ($n = 10$ needles), and late September ($n = 10$ needles), 2008 within each crown type and age group. The adjacent values were compared. Asterisks indicate significant differences between months: *, $P < 0.05$; **, $P < 0.01$. Bars = SE.

Next, cuticle thickness in each season and crown type was compared among needle ages. In both crown types in September, there were no significant differences in cuticle thickness between any needle ages. In June, cuticle thicknesses significantly increased from 2- to 3-year-old needles ($P < 0.05$) in both crowns, but it significantly decreased from 4- to 5-year-old needles ($P < 0.01$) in the flagged crown (Table 2). In August, cuticle thickness significantly increased from current- to 1-year-old needles ($P < 0.01$) followed by no significant changes in either crown (Table 2).

Finally, cuticle thicknesses were compared between crown types in each season and each needle age. In September, cuticle thicknesses of all age needles in the flagged crown were significantly greater than that in the cushion-shaped crown ($P < 0.01$) (Table 3). In June, cuticles of 1-, 2- and 3-year-old needles in the flagged crown were significantly thicker than those in the cushion-shaped crown ($P < 0.01$), but there were no significant differences in older needles (Table 3). In August, cuticles of 4- and 5-year-old needles in the flagged crown were significantly thinner than that in the cushion-shaped crown ($P < 0.05$, $P < 0.01$, respectively), but there were no significant differences in younger needles (Table 3).

Relationship between cuticular resistance and cuticle thickness

The relationship between cuticular resistance and cuticle thickness in both crowns was compared in every season (Fig. 7). Data of current-year needles in August were eliminated because the shoot just flushed in late July and needles were not mature. In the flagged crown, there was no correlation between cuticular resistance and cuticle thickness in September and June, but there was a significant negative correlation in August ($r = -0.93$, $P < 0.05$) (Fig. 7a). In other words, cuticular resistance did not relate to cuticle thickness before and after winter, but it decreased when cuticle thickness increased at the peak of the growing season. In the cushion-shaped crown, there was a significant negative correlation between cuticular resistance and cuticle thickness in September ($r = -0.82$, $P < 0.05$) and August ($r = -0.96$, $P < 0.01$), but there was no correlation in June (Fig. 7b). That is, cuticular resistance decreased with increasing cuticle thickness before winter and during growing season, but it was not related to cuticle thickness after winter.

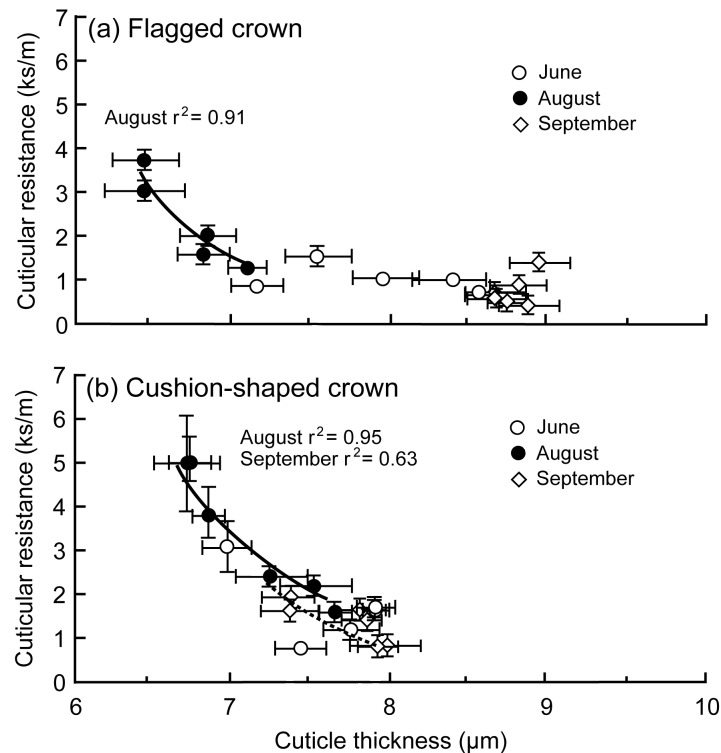


Fig. 7. Comparison of the relationship between cuticular resistance ($n = 10$ shoots) and cuticle thickness ($n = 10$ needles) in both crowns in every season. (a) In the flagged crown. (b) In the

cushion-shaped crown. Curves were fitted by an exponential function and indicated a significant negative correlation in August (solid lines; both crown forms) and September (broken line; cushion-shaped only). Bars = SE.

Discussion

Increasing transpiration of conifer needles with damaged cuticles in high mountains decreases needle water content to a lethal level and then the needles die (Baig and Tranquillini 1976; Hadley and Smith 1983, 1986). Moreover, a thick cuticle has been thought to be more effective at reducing water loss than a thin cuticle (Tranquillini 1976, 1982; Hadley and Smith 1983; Edwards et al. 1996). To confirm whether the needle browning and death in a flagged crown of *A. mariesii* in the alpine region of Mt. Norikura, Japan, conform to these concepts, we asked the following questions: (1) Does mechanical damage to the cuticle cause needle browning and death? (2) Is there any relationship between cuticular resistance and cuticle thickness?

Effect of mechanical damage of cuticle to needle browning

It has been reported that in high mountains the cuticle and wax of needles exposed above snowpack during winter were abraded by wind-born snow and ice particles (Marchand and Chabot 1978; Tranquillini 1979; Hadley and Smith 1983, 1986, 1987; van Gardingen et al. 1991). Many trees transpire excessively through abraded parts of their needles. However, the microscopic study on *Pinus cembra* (Tranquillini 1979) and *P. pumila* (see Chapter 2) did not show any damage of needle cuticles due to snow and ice particle abrasion. Our study also showed no signs of mechanical damage to the cuticle of *A. mariesii* in Mt. Norikura, Japan (Figs. 3a, 3b). The cause of the needle browning and death of *A. mariesii* could not be explained by the idea that cuticle damage leads to excessive transpiration that lowers needle water content to lethal level (Tranquillini 1979; Hadley and Smith 1983, 1986, 1987).

Epicuticular waxes which comprise the outer part of the cuticle are physiologically important because they restrict transpirational water loss (Pallardy 2008).

The permeability coefficient for diffusion of water vapor through the cuticle has been shown to increase by 300–500 times following removal of the cuticular wax (Schönherr 1976). Leaf waxes play an important role in desiccation avoidance by plants (Pallardy 2008). In *A. mariesii* on Mt. Norikura, the cuticle of the abaxial side of needle was different between the flagged crown and the cushion-shaped crown (Fig. 4). In the flagged crown the abaxial surface was smooth because the epicuticular wax was mostly gone (Figs. 4a, 4b), but in the cushion-shaped crown the rough epicuticular wax was present (Figs. 4c, 4d). In the cushion-shaped crown the openings of stomatal antechambers of the needle with sunken stomata were plugged up with epicuticular waxes (Fig. 4d), but stomata in the flagged crown were not plugged entirely (Fig. 4b). These results show that epicuticular waxes in the flagged crown might be abraded away due to strong wind. The waxes in the stomatal antechambers of the conifers with sunken stomata may be more important in controlling water loss than the waxes on the epidermal surface (Pallardy 2008). Wax that occluded the stomatal pores of Sitka spruce was estimated to decrease transpiration by nearly two-thirds when stomata were fully open (Jeffree et al. 1971). Disappearance of epicuticular waxes in the flagged crown might be one of reasons for greater transpirational water loss.

Relationship between cuticular resistance and cuticle thickness

One of the main functions ascribed to the cuticle is to minimize water loss from plants when stomata are closed (Chamel et al. 1991; Burghardt and Riederer 2006). Cuticles are very efficient barriers to water loss, but the growing period of foliage in high mountains is so short that the cuticle of foliage cannot mature to reach its final thickness during the current growing period (Tranquillini 1976, 1982). Cuticle thicknesses of 1- and 2-year-old needles of *Picea abies* in the Krummholz limit on Patscherkofel Mountain, Austria, were 3.45 and 4.7 μm , respectively, and the growing period was related to this difference (Baig and Tranquillini 1976; Tranquillini 1976). In *A. mariesii* on Mt. Norikura, cuticular resistance of the 1-year-old needle in the flagged crown and the cushion-shaped crown showed the maximum in all seasons (Fig. 5; Table 2). After the first year, cuticular resistance tended to decrease with age (Fig. 5;

Table 2). Maximum cuticular resistance of *P. cembra* at a treeline site (2,170 m a.s.l.) in the Dolomites was lower in 1-year-old needles than that in the current-year needle (Anfodillo et al. 2002). These results show that the cuticle's ability to restrict water loss decreases with age. Therefore, water loss through the cuticle might occur even if needle cuticles are not mechanically damaged.

Cuticular resistance of foliage has been shown to decrease with increasing altitude in high mountains (Tranquillini 1976; Baig and Tranquillini 1980; Sowell et al. 1982; Herrick and Friedland 1991), to differ between windward and leeward side (Hadley and Smith 1983), and to fluctuate seasonally (Sowell et al. 1982; Hadley and Smith 1986; see Chapter 2). These studies showed that severe environment in high mountains induced low cuticular resistance. Winter desiccation seems to have occurred when cuticular resistance decreased and water loss by transpiration increased (Tranquillini 1979; Baig and Tranquillini 1980). In *A. mariesii* on Mt. Norikura, cuticular resistances in September, before winter, and June, after winter, were lower than that in August of the growing period in the flagged crown and the cushion-shaped crown (Fig. 5). From this result, cuticular resistance was thought to be low during winter. In accordance, maximum cuticular resistance of *Picea abies* in current-year needles at a treeline site (2,170 m a.s.l.) was lower in February than in May, August and December (Anfodillo et al. 2002). Cuticular resistance of *P. abies* was also low in winter, and it was the same result in *A. mariesii* on Mt. Norikura. Moreover, higher cuticular resistance of *A. mariesii* in August seems to contribute to reduced water deficit of needles in the growing period.

Cuticular resistance in the flagged crown was lower than that in the cushion-shaped crown (Table 3). Flagged crowns are located above snowpack during the winter and therefore subject to severe stresses and winter desiccation. In contrast, cushion-shaped crowns are located below snowpack during winter, which offers protection from severe stresses and winter desiccation. Consequently, the needle browning and death in the flagged crown may occur even if needle cuticles are not mechanically damaged. In the cushion-shaped crown, cuticular resistance increased from September in the current-year needles to June in the 1-year-old needles, but cuticle

thickness at this point decreased (Figs. 5, 6). It can be presumed that quality and structure of needle cuticles below snowpack varied.

Becker et al. (1986) and Schreiber and Riederer (1996) showed that plant species with thin cuticles have lower cuticular conductance than species with thick cuticles, refuting the premise that a thick cuticle is more effective in reducing water loss. However, there is no significant relationship between maximum cuticular resistance and cuticle thickness in *Picea abies* and *P. cembra* (Anfodillo et al. 2002). Our study showed that there was a significant negative correlation between cuticular resistance and cuticle thickness or there was no significant difference (Fig. 7). Therefore, cuticle thickness did not necessarily increase in order to raise cuticular resistance. The cuticle must have become thick for other reasons.

The cuticle, often together with the outer epidermal cell wall and the vacuoles of the epidermis, can contribute to an effective screening of ultraviolet radiation (Day 1993; Riederer 2006). In *A. mariesii*, cuticle thickness of the flagged crown was thicker than that in the cushion-shaped crown (Table 3). It is inferred that the development of a thicker cuticle boosts the function to defend needles from UV rays. Moreover, it was reported that water permeability of the cuticle does not correlate with cuticle thickness and weight and wax coverage (Schönherr 1982; Riederer and Schreiber 2001). The amount of water taken up by isolated cuticle was closely correlated with cuticular polysaccharide content in some species (Kerstiens and Lenzian 1989; Chamel et al. 1991). We then considered that the difference in cuticular resistance was attributed to the difference in water permeability through the cuticle, which was induced by change and deterioration of the cuticle quality and structure in *A. mariesii*. To estimate desiccation stress in relation to the cuticle, we need to elucidate not only cuticular resistance and cuticle thickness, but also cuticle quality and structure.

Conclusion

We found that the needle browning and death of *A. mariesii* on Mt. Norikura in the alpine region of Japan was not caused by mechanical damage to the cuticle or a thinner

cuticle. Cuticles became thick for other reasons except to prevent the excess water loss through needle cuticles. More research is needed to better understand not only cuticular resistance and cuticle thickness, but also cuticle quality and structure.

Chapter 4. Relationship between cuticular resistance or cuticle thickness and isolated needle cuticle components in the flagged crown of *Abies mariesii* in the timberline ecotone of the alpine region in central Japan

Introduction

To estimate desiccation stress in relation to the cuticle, we need to elucidate not only cuticular resistance and cuticle thickness, but also cuticle quality. The main function ascribed to the cuticle is the protection and waterproofing of the plant surface (Jeffree 1996), and to minimize water loss from plants when stomata are closed (Tranquillini 1982, Chamel et al. 1991). Evergreen conifers in the subalpine zone are thought to prevent winter desiccation of needles by closing stomata and increasing cuticle thickness (Tranquillini 1979, Wieser and Tausz 2007). In fact, the cuticle became thick in the high altitude because desiccation induces the development of thick cuticle (Hull et al. 1975), and the thicker cuticle is thought to be more effective in decreasing water loss (Tranquillini 1976, 1982, Hadley and Smith 1983, Edwards et al. 1996). Epicuticular waxes increase resistance to water loss through the epidermis (Pallardy 2008). In some species, however, thin cuticles are more effective as a water barrier than thick cuticles (Becker et al. 1986, Schreiber and Riederer 1996). Cuticular permeability of water did not correlate with cuticle thickness and weight and wax coverage (Schönherr 1982, Kerstiens 1994, Riederer and Schreiber 2001). The mechanism of water barrier in foliage cuticles is not fully solved.

The cuticle is made up of cutin, main structural component of the cuticle, an insoluble polymer composed of cross-linked hydroxy-fatty acids and waxes constituting soluble cuticular lipids which are embedded with in the cutin and which cover, in many diverse forms, the outer surface of the cuticle (Chamel et al. 1991, Jeffree 1996, 2006). Waxes are the most water-repellent of the lipids (Raven et al. 1999), and its predominating role for the transport-limiting barrier of cuticular membranes is evident because cuticular water permeability increases by one to two orders of magnitude after

complete removal of waxes (Burghardt and Riederer 2006). Cuticles also contain non-lipid components such as polysaccharides (cellulose, pectins) and phenolics (Chamel et al. 1991, Jeffree 1996, 2006). In accordance with the heterogeneous compositions of the cuticle two pathways for the permeation of water have been discussed: a lipophilic route through the cutin and amorphous wax fraction and a hydrophilic pathway across polar pores of molecular dimensions (Burghardt and Riederer 2006). It has been thought that the hydrophilic pathway probably consist of reticulum polysaccharide microfibrils ramifying and stretching through the cuticular membrane (Jeffree 1996, Burghardt and Riederer 2006). Mean water uptake was closely correlated with mean polysaccharide content in some species (Kerstiens and Lenzian 1989, Chamel et al. 1991), but not in *Abies alba* Mill. (Chamel et al. 1992). Correlation between water permeability or water uptake through the cuticle and cuticle components is not fully understood. We confirmed whether the cuticular resistance and cuticle thickness of *A. mariesii* needles are relate to needle cuticle components.

Materials and methods

Study area and tested trees

The study was conducted in a timberline ecotone distributed on the eastern gentle slope (5°; 2500 m above sea level (a.s.l.)) of Mt. Norikura in Chubu Mountain National Park, Nagano, Japan (36° 61'N, 137° 33'E, 3026 m a.s.l.) located at the southernmost tip of the northern Japanese Alps in the central mountainous area of the Japan Archipelago. In this area, snow depth during the winter reaches approximately 2.5 m. Air temperature was continuously sub-zero °C just above snowpack from late November to late April and minimum temperature has reached below -20 °C late February (Yamazaki et al. 2003). Thus, -20 °C might be also reached before and after late February in other years. The dominant vegetation is a dwarf scrub *Pinus pumila* community, including *Abies mariesii* trees. *A. mariesii* is a dominant species of subalpine coniferous forest where its tree form is the conical crown of the excurrent tree. However, *A. mariesii* in a timberline ecotone shows the flagged tree form. Needle color

of the adaxial and abaxial sides in the cushion-shaped crown was green in June and needle browning hardly occurs. In contrast, needle color of the abaxial side in the flagged crown becomes brown frequently in early spring and most of the browning needles have already dropped in June. New shoots flushed in late July, making the growing season only about 2 months long.

Sampling

Branches collected from flagged and cushion-shaped crown of different 4 trees in the field in late September 2009, before winter, and June 2010, after winter, were immediately put into a plastic bag, stored in a cool box, and brought back to the laboratory and kept in a refrigerator. The tree heights and diameters at breast height of tested trees ($n = 4$) were 4.88 ± 0.34 m and 19.75 ± 3.93 cm, respectively. Ten shoots in branches of flagged crown and cushion-shaped crown of 4 trees (2 or 3 shoots in each crown per tree, respectively) were used for measurements of cuticular resistance. And then 3 to 5 shoots were selected from shoots measured cuticular resistance to measure the cuticle thickness and content of isolated needle cuticle components.

Measurement of cuticular resistance

The shoots were refreshed under water and watered additionally for more than 12 h in a dark room. They were cut into internodes bearing needles for each year, and the cut ends were sealed with nail polish to prevent evaporation. Cuticular resistance of each internode was measured separately. For assessment of cuticular resistance to water loss, r_c (ks m^{-1}), eq. 1 was used:

$$E = (e_1^0 - e_a) / r_c \quad (1)$$

where E ($\text{g s}^{-1} \text{m}^{-2}$) is the rate of water vapor loss from needles; e_1^0 (g m^{-3}) is the saturated water vapor concentration inside a needle (at needle temperature); and e_a (g m^{-3}) is the water vapor concentration in the air. The influence of boundary layer resistance of the needle was reduced by circulating air at $1.2\text{-}2.3 \text{ m s}^{-1}$ in a dark room.

Samples of internodes for each year were weighed, maintained at room temperature and humidity (approximately 20 °C and 52 %, respectively) for 30 min, and reweighed. Measurements were repeated until *E* became constant. Leaf area was measured by using an image analysis software (Scion Image, Beta 4.0.3, Frederick, Maryland, USA). Air temperature and humidity were monitored during measurement (Thermo recorder RS-10, ESPEC MIC Corp., Osaka, Japan). Needle temperature of each internode was measured before reweighing using a fine-wire copper-constantan thermocouple (0.1 mm diameter).

Measurement of cuticle thickness

Three needles in each age internode of a single shoot were used for measurement. It made a total of 12 needles in the flagged crown in each year of 2009 and 2010 in all, and a total of 9 and 15 needles in the cushion-shaped crown in each year of 2009 and 2010 in all. Fresh needles were fitted into pith sticks, and Sections 25 µm thick were prepared using a sliding microtome (Yamato Koki, Saitama, Japan) and stained with Sudan III (0.5 %). Stained sections were observed by a light microscope (Olympus BX51TF, Tokyo, Japan) and a digital camera (Camedia C-5060, Olympus Corp., Tokyo, Japan). Then adaxial and abaxial cuticle thicknesses were measured using Photoshop CS2 (Adobe Systems, San Jose CA, USA). Cuticle thickness was measured at five points of the middle of an adaxial surface and one point each of two white stomata belts on the abaxial surface. It made a total of seven points in each needle in all.

Isolation of needle cuticles

Cuticles were enzymatically isolated using an aqueous mixture of 20 % (w/v) pectinase (Pectinase G “Amano”, Amano Enzyme Inc., Nagoya, Japan) and 4 % (w/v) cellulose from *Aspergillus niger* (22178 EC 3.2.1.4., SIGMA, Tokyo, Japan) buffered at pH 3.8 with sodium acetate and acetic acid according to Lenzian et al. (1986) and Chamel et al. (1992). The needles of approximately 1 g fresh weight and/or 70 needles per each age internode, freshly collected in late September 2009 and June 2010, were superficially sterilized using an aqueous solution of K₂CO₃ (1 %), and then extensively

washed in deionized water. Each needle except for 1 mm of a tip and base was cut longitudinally with a razor blade. Two half needles were obtained, one corresponding to the adaxial surface and the other to the abaxial surface of needles. Samples of half-needles were vacuum-infiltrated separately with the enzymatic solution (20 ml solutions) and incubated for 5-7 days at 30 °C on a shaker in an incubator. And then the adaxial and abaxial cuticles were recovered, washed in an ultrasonic bath and carefully rinsed with distilled water to remove remnants of cell walls. The isolated cuticles were air-dried, kept in a desiccator and weighed on the electric microbalance (Sartorius model CP225D, Göttingen, Germany).

Determination of isolated needle cuticle components

Soluble cuticular waxes were extracted by immersing isolated cuticles in chloroform (3 ml solvent) for 24 h at 25 °C according to Chamel et al. (1992). Hydrolysable components, including polysaccharides, were removed from samples of dewaxed cuticles by acid-hydrolysis using 6 N HCl at 110 °C for 14 h. The solid residue recovered after this treatment was carefully washed with distilled water, air-dried, kept in a desiccator and weighed. This fraction is referred to as cutin, the insoluble polymer composed of cross-linked hydroxyl-fatty acids (Riederer 1989).

Data analysis

Linear regression analysis was used to characterize a relationship between cuticular resistance or cuticle thickness and content of isolated needle cuticle components such as cutin, wax, and hydrolysable components. To assess the difference in needles ages, measurement months and crowns, one-way analysis of variance (ANOVA), Scheffé's test and Kruskal-Wallis test were performed.

Results

Relationship between cuticular resistance and isolated needle cuticle components

The relationship between cuticular resistance and content of isolated needle cuticle

components in both crowns were compared before and after winter (Fig. 1). In the flagged crown in 2009, there was a significant positive correlation between cuticular resistance and content of hydrolysable components of isolated needle cuticles ($P < 0.05$) (Fig. 1a). In the flagged crown in 2010, there were a significant positive correlation between cuticular resistance and content of hydrolysable components of isolated needle cuticles ($P < 0.01$) and a significant negative correlation between cuticular resistance and content of cutin of isolated needle cuticles ($P < 0.01$) (Fig. 1b). In contrast, in the cushion-shaped crown both in 2009 and 2010, there were no significant correlations between cuticular resistance and content of all components of isolated needle cuticles (Figs. 1c, 1d). In other words, in the flagged crown needles with higher cuticular resistance have a higher content of hydrolysable components of isolated needle cuticles (Figs. 1a, 1b), but in the cushion-shaped crown it did not relate to all components of isolated needle cuticles (Figs. 1c, 1d).

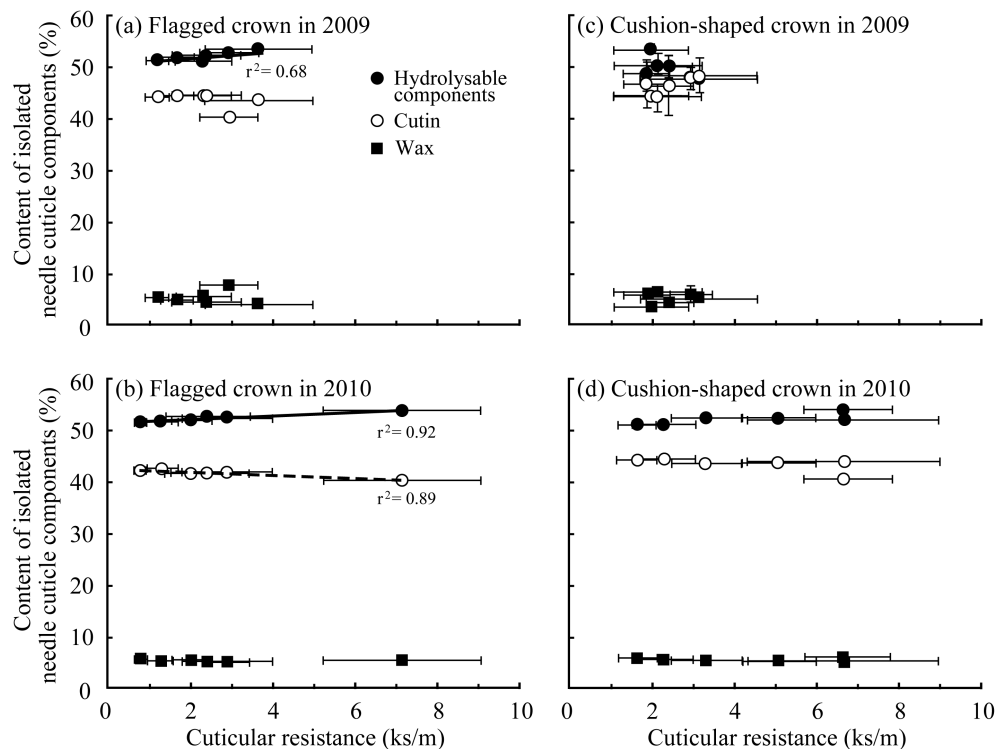


Fig. 1. Comparison of the relationship between cuticular resistance and content of isolated needle cuticle components in both crowns before and after winter. (a) Flagged crown in 2009 ($n = 4$ shoots). (b) Flagged crown in 2010 ($n = 4$ shoots). (c) Cushion-shaped crown in 2009

($n = 3$ shoots). (d) Cushions-shaped crown in 2010 ($n = 5$ shoots). Curves were fitted by a linear function and indicated a significant positive correlation in hydrolysable components (solid lines; flagged only) and cutin (dashed lines; flagged in 2010 only). Bars = SE.

Relationship between cuticle thickness and isolated needle cuticle components

The relationship between cuticle thickness and content of isolated needle cuticle components in both crowns were compared before and after winter (Fig. 2). In the flagged crown in late September 2009, there was a significant positive correlation between cuticle thickness and content of cutin of isolated needle cuticles ($P < 0.01$) (Fig. 2a), and a significant negative correlation between cuticle thickness and content of wax of isolated needle cuticles ($P < 0.05$) (Fig. 2a). These indicate that the thicker cuticles contained higher content of cutin and lower content of wax of isolated needle cuticles than the thinner cuticles. In contrast, in the flagged crown in June 2010 and the cushion-shaped crown in September 2009 and June 2010 there were no significant correlations between cuticle thickness and content of all components of isolated needle cuticles (Figs. 2b, 2c, 2d).

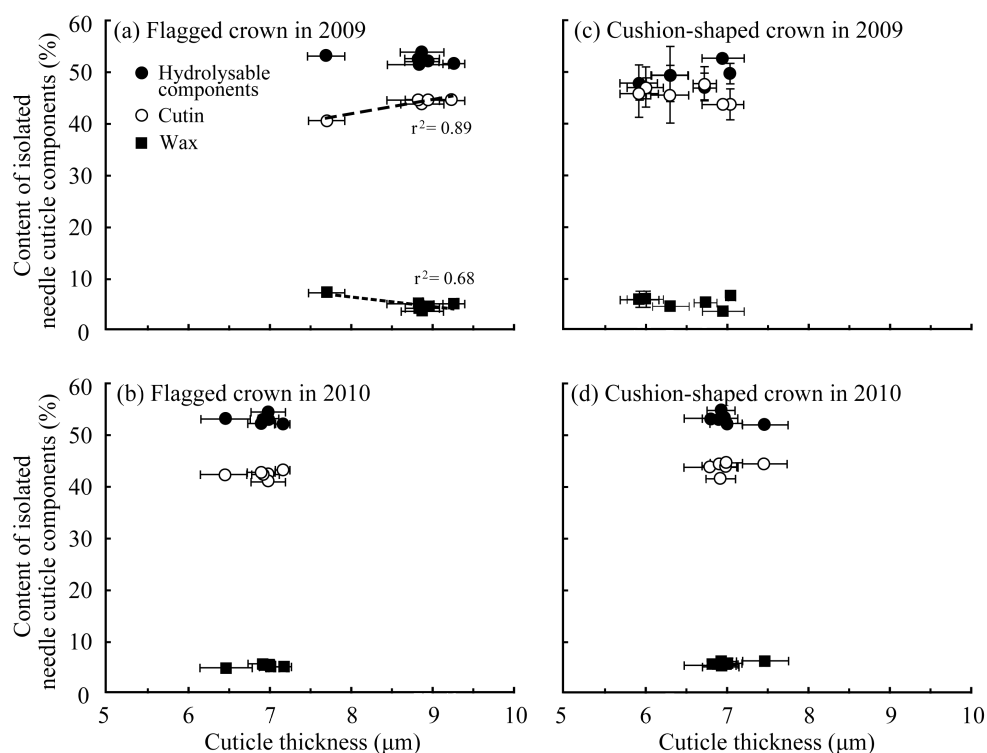


Fig. 2. Comparison of the relationship between cuticle thickness and content of isolated needle

cuticle components in both crowns before and after winter. (a) Flagged crown in 2009 ($n = 12$ needles). (b) Flagged crown in 2010 ($n = 12$ needles). (c) Cushion-shaped crown in 2009 ($n = 9$ needles). (d) Cushions-shaped crown in 2010 ($n = 15$ needles). Curves were fitted by a linear function and indicated a significant positive correlation in cutin (dashed line; flagged in 2009 only) and wax (broken line; flagged in 2010 only). Bars = SE.

Comparison of age-related changes in cuticular resistance, cuticle thickness and content of isolated needle cuticle components

Firstly, in both crowns, there were no significant difference in cuticular resistance between late September 2009 and June 2010 except between current- and 1-year-old needles in the cushion-shaped crown (Table 1). In the flagged crown, cuticle thickness in late September 2009 was significantly greater than that in June 2010 ($P < 0.01$) except between current- and 1-year-old needles (Table 1). In the cushion-shaped crown, cuticle thickness significantly increased from current-year needles in late September 2009 to 1-year needles in June 2010 ($P < 0.05$) and from 3-year needles in late September 2009 to 4-year needles in June 2010 ($P < 0.01$) (Table 1).

Table 1. Comparison of age-related changes between late September 2009 and June 2010 in cuticular resistance, cuticle thickness and content of isolated needle cuticle components with in each crown type.

Variable	Needle age (year-old)		2009-2010			
	Current - 1	1 - 2	2 - 3	3 - 4	4 - 5	5 - 6
Flagged crown:						
Cuticular resistance (ks/m)	2.86 ± 0.71 - 7.13 ± 1.91	2.25 ± 0.68 - 2.88 ± 1.09	3.58 ± 1.30 - 2.38 ± 1.02	2.32 ± 0.84 - 2.00 ± 0.50	1.62 ± 0.41 - 1.29 ± 0.38	1.13 ± 0.28 - 0.76 ± 0.14
Cuticle thickness (µm)	7.70 ± 0.22 - 7.03 ± 0.22	8.84 ± 0.39 - 7.03 ± 0.13**	8.87 ± 0.27 - 6.48 ± 0.32**	8.83 ± 0.17 - 6.96 ± 0.14**	8.95 ± 0.13 - 7.21 ± 0.9**	9.25 ± 0.13 - 6.94 ± 0.18**
Hydrolysable components (%)	52.6 - 54.1	50.8 - 52.9	53.3 - 53.0	51.9 - 52.5	51.5 - 52.0	51.2 - 51.9
Cutin (%)	40.1 - 40.7	44.0 - 42.3*	43.2 - 42.2	44.1 - 42.2	44.1 - 43.0	44.0 - 42.5
Wax (%)	7.3 - 5.2*	5.2 - 4.8	3.5 - 4.8*	4.0 - 5.3	4.4 - 5.0	4.8 - 5.6
Cushion-shaped crown:						
Cuticular resistance (ks/m)	2.85 ± 0.50 - 6.73 ± 1.10*	2.36 ± 0.55 - 6.75 ± 2.27	3.06 ± 1.37 - 5.08 ± 0.86	1.83 ± 0.54 - 3.30 ± 0.94	2.10 ± 1.02 - 2.25 ± 0.71	1.93 ± 0.88 - 1.61 ± 0.47
Cuticle thickness (µm)	5.99 ± 0.23 - 6.86 ± 0.18*	6.30 ± 0.23 - 6.85 ± 0.21	6.74 ± 0.14 - 6.74 ± 0.32	5.91 ± 0.24 - 6.92 ± 0.13**	7.06 ± 0.04 - 6.93 ± 0.20	6.97 ± 0.26 - 7.38 ± 0.27
Hydrolysable components (%)	47.2 - 53.6	49.7 - 51.9	47.1 - 52.1	48.1 - 52.0	49.9 - 51.0	52.7 - 50.9
Cutin (%)	47.0 - 40.6*	45.8 - 43.2	47.8 - 42.8	46.2 - 42.8	43.7 - 43.7	43.8 - 43.4
Wax (%)	5.8 - 5.8	4.5 - 4.9	5.1 - 5.1	5.7 - 5.1	6.4 - 5.3	3.4 - 5.7*

Asterisks indicate significant differences in cuticular resistance, cuticle thickness or content of isolated needle cuticle components between needle ages: *, $P < 0.05$; **, $P < 0.01$.

Next, in both crowns, there was no significant difference in content of hydrolysable components of isolated needle cuticles between late September 2009 and June 2010 in any age needles (Table 1). In the flagged crown, content of cutin of isolated needle cuticles in 1-year-old needles in 2009 was significantly higher than that in 2-year-old needles in 2010 ($P < 0.05$), but there were no significant differences in other age needles (Table 1). Content of wax of isolated needle cuticles in current-year-old needles in late September 2009 was significantly higher than that in 1-year-old needles in June

2010 ($P < 0.05$) (Table 1). In contrast, content of wax of isolated needle cuticles in 2-year-old needles in late September 2009 was significantly lower than that in 3-year-old needles in June 2010 ($P < 0.05$) (Table 1). In the cushion-shaped crown, content of cutin of isolated needle cuticles in current-year-old needles in late September 2009 was significantly higher than that in 1-year-old needles in June 2010 ($P < 0.05$) (Table 1). Content of wax of isolated needle cuticles in 5-year-old needles in late September 2009 was significantly lower than that in 6-year-old needles in June 2010 ($P < 0.05$), but there were no significant differences in other age needles (Table 1).

Next, both in 2009 and 2010, there were no significant differences in any age needles of cuticular resistance between flagged and cushion-shaped crowns (Table 2). In 2009, cuticles in the flagged crown were significantly thicker than that in the cushion-shaped crown in any age needles ($P < 0.01$) (Table 2). In contrast, in 2010, there were no significant differences in any age needles of cuticle thickness between flagged and cushion-shaped crowns (Table 2).

Finally, in late September 2009, content of hydrolysable components of isolated needle cuticles of the 2-year-old needles in the flagged crown was significantly higher than that in the cushion-shaped crown ($P < 0.05$) (Table 2). Content of cutin of isolated needle cuticles of the current-year-old needles in the flagged crown was significantly lower than that in the cushion-shaped crown ($P < 0.05$) (Table 2). Content of wax of isolated needle cuticles of the 4-year-old needles in the flagged crown was significantly lower than that in the cushion-shaped crown ($P < 0.05$) (Table 2). In June 2010, content of hydrolysable components of isolate needle cuticles of the 5- and 6-year-old needles in the flagged crown were significantly higher than that in the cushion-shaped crown ($P < 0.05$) (Table 2). In contrast, there were no significant differences in content of cutin and wax of isolated needle cuticles between flagged and cushion-shaped crowns in any age needles (Table 2).

Table 2. Comparison of cuticular resistance, cuticle thickness and content of isolated needle cuticle components between crown types before and after winter in each age group.

Variable	Needle age (year-old) Flagged crown : Cushion-shaped crown					
	Current	1	2	3	4	5
2009						
Cuticular resistance (ks/m)	2.86 ± 0.71 : 2.85 ± 0.50	2.25 ± 0.68 : 2.36 ± 0.55	3.58 ± 1.30 : 3.06 ± 1.37	2.32 ± 0.84 : 1.83 ± 0.54	1.62 ± 0.41 : 2.10 ± 1.02	1.13 ± 0.28 : 1.93 ± 0.88
Cuticle thickness (µm)	7.70 ± 0.22 : 5.99 ± 0.23**	8.84 ± 0.39 : 6.30 ± 0.23**	8.87 ± 0.27 : 6.74 ± 0.14**	8.83 ± 0.17 : 5.91 ± 0.24**	8.95 ± 0.13 : 7.06 ± 0.04**	9.25 ± 0.13 : 6.97 ± 0.26**
Hydrolysable components (%)	52.6 : 47.2	50.8 : 49.7	53.3 : 47.1*	51.9 : 48.1	51.5 : 49.9	51.2 : 52.7
Cutin (%)	40.1 : 47.0*	44.0 : 45.8	43.2 : 47.8	44.1 : 46.2	44.1 : 43.7	44.0 : 43.8
Wax (%)	7.3 : 5.8	5.2 : 4.5	3.5 : 5.1	4.0 : 5.7	4.4 : 6.4*	4.8 : 3.4
2010						
Cuticular resistance (ks/m)	7.13 ± 1.91 : 6.73 ± 1.10	2.88 ± 1.09 : 6.75 ± 2.27	2.38 ± 1.02 : 5.08 ± 0.86	2.00 ± 0.50 : 3.30 ± 0.94	1.29 ± 0.38 : 2.25 ± 0.71	0.76 ± 0.14 : 1.61 ± 0.47
Cuticle thickness (µm)	7.03 ± 0.22 : 6.86 ± 0.18	7.03 ± 0.13 : 6.85 ± 0.21	6.48 ± 0.32 : 6.74 ± 0.32	6.96 ± 0.14 : 6.92 ± 0.13	7.21 ± 0.09 : 6.93 ± 0.20	6.94 ± 0.18 : 7.38 ± 0.27
Hydrolysable components (%)	54.1 : 53.6	52.9 : 51.9	53.0 : 52.1	52.5 : 52.0	52.0 : 51.0*	51.9 : 50.9*
Cutin (%)	40.7 : 40.6	42.3 : 43.2	42.2 : 42.8	42.2 : 42.8	43.0 : 43.7	42.5 : 43.4
Wax (%)	5.2 : 5.8	4.8 : 4.9	4.8 : 5.1	5.3 : 5.1	5.0 : 5.3	5.6 : 5.7

Asterisks indicate significant differences in cuticular resistance, cuticle thickness or content of isolated needle cuticle components between the flagged and the cushion-shaped crowns. The left number and the right number in a column show the value of the flagged crown and that of the cushion-shaped crown, respectively: *, $P < 0.05$; **, $P < 0.01$.

Discussion

In general it has been considered that a thicker cuticle of foliage inhibits more excessive transpiration from the foliage (Hull et al. 1975; Tranquillini 1976, 1979, 1982; Hadley and Smith 1983; Edwards et al. 1996; Wieser and Tausz 2007). However, some studies did not necessarily adhere to these concepts (Schönherr 1982; Becker et al. 1986; Kerstiens 1994; Schreiber and Riederer 1996; Riederer and Schreiber 2001; Anfodillo et al. 2002; see Chapter 2 and 3). Namely the water permeability of cuticle is not only directly proportional to the cuticle thickness.

Kerstiens and Lenzian (1989) and Chamel et al. (1992) showed the relationship between the water permeability and the polysaccharide in isolated cuticle of tomato fruit (*Lycopersicon esculentum* Mill.) and white fir needle (*Abies alba* Mill.). In the flagged crown of *A. mariesii*, needles with a higher cuticle resistance contained a higher hydrolysable component, containing polysaccharide (Figs. 1a, 1b). While in the cushion-shaped crown, there was no correlation between cuticular resistance and cuticle components (Figs. 1c, 1d). These results suggested that the relationship between cuticular resistance and needle cuticle components in *A. mariesii* differs from crown forms, and water permeability of cuticles relates to the content of polysaccharide of needle cuticles only in the flagged crown.

Cuticle thickness was different between study sites and tree species (Baig and Tranquillini 1976, Tranquillini 1976, DeLucia and Berlyn 1984, Anfodillo et al. 2002), needle ages (Baig and Tranquillini 1976, Tranquillini 1976, Hadley and Smith 1994) and seasons (see Chapter 2 and 3). Before winter in the flagged crown of *A. mariesii*, needles with a thicker cuticle contained higher content of cutin and lower wax (Fig. 2a). On the other hand, there was no correlation between cuticle thickness and content of all components of isolated needle cuticles in the flagged crown after winter and in the cushion-shaped crown (Figs. 2b, 2c, 2d). This suggested that the relationship between cuticle thickness and content of needle cuticle components differs from the crown forms and seasons. There were, however, no remarkable tendencies and differences in each content of needle cuticle components between before and after winter, and between the

flagged and the cushion-shaped crown in each age needles (Tables 1, 2). These suggested that the change in cuticular resistance or cuticle thickness in *A. mariesii* needles did not necessarily relate to the change in content of needle cuticle components, and respond to not only desiccation but also other stresses.

Focusing on abiological factors relating to water permeability of cuticle, previous studies showed that temperature (Baur et al. 1999, Schreiber 2001, Buchholz 2006) and humidity of air affected to cuticle permeance to water (Meidner 1986, Chamel et al. 1992, Schreiber et al. 2001, Schönherr 2006). Water permeance of cuticle in 12 plant species almost doubled with increase in air temperature from 15 to 35 °C (Riederer and Schreiber 2001). Increase in air humidity from 2 to 100 % raised a cuticular permeability of water by factors of 2 to 3 (Schreiber et al. 2001). In the alpine region of Mt. Norikura, Japan annual range of air temperature is about 40 °C (see Chapter 2). Therefore it was considered that a cuticular permeability of *A. mariesii* was changing throughout the year. Besides solar ultraviolet radiation is likely to be an important constraint for growth and development of alpine plants, and UV-B radiation caused reductions in needle elongation, leaf area, and biomass (Turunen and Huttunen 1991, Fischbach et al. 1999). It was reported that the cuticle, often together with the outer epidermal cell wall and the vacuoles of the epidermis, can contribute to an effective screening of ultraviolet radiation (Day 1993; Reiderer 2006). Before winter cuticles in the flagged crown of *A. mariesii* were thicker than that in the cushion-shaped crown (Table 2). This presumed that the development of a thicker cuticle of needle in the flagged crown protruding from the snowpack during winter to spring enhanced the function to defend needles from UV rays. Moreover, it was suggested that needle browning of trees in high mountains relates to strong sunlight reflection from the snow surface, low temperature (Smith et al. 2003, Yamazaki et al. 2003), and photoinhibition (Schulze et al. 2005). Both photoinhibition and winter desiccation are closely linked and occur together under field conditions in evergreen trees (Neuner et al. 1999). In addition, air pollutants and acid rain can destroy epicuticular waxes (Turunen and Huttunen 1990, Huttunen 1994, Kerstiens 1995), and it predisposes trees to winter desiccation in high mountains (Barnes and Davison 1988). To estimate the desiccation stresses of foliage in

relation to cuticles, we need to elucidate the fine structure of cuticles for predicting the route which water penetrates, the harmful effects of ultraviolet ray, and air pollutants that affect the structure on foliage surface.

Conclusion

We found that only in the flagged crown of *A. mariesii* on Mt. Norikura in the alpine region of Japan, needles with a higher cuticular resistance contained higher hydrolysable components including polysaccharide. It was suggested that the correlation between cuticular resistance and content of needle cuticle components differs from the crown forms, and water permeability of cuticles relates to the content of polysaccharide of needle cuticles only in the flagged crown. Only in the flagged crown before winter needle with a thicker cuticle contained higher content of cutin and lower wax. This result suggested that the change in cuticle thickness may be respond to other stresses because there was no remarkable correlation between cuticle thickness and content of needle cuticle components. More research is needed to better understand; we need to elucidate the fine structure of cuticles for predicting the route which water penetrates the influence of ultraviolet rays which may have harmful effects, and air pollutants that affect the structure on foliage surface.

Chapter 5. General Conclusion

Trees in alpine regions tend to suffer from severe stresses caused by environmental conditions, therefore, needle browning and death in some evergreen coniferous trees in the high mountains of Europe, North America, and Japan often occur in late winter and early spring and seems to result from winter desiccation (Tranquillini 1979; Herrick and Friedland 1991; Körner 2003). Needles of *Pinus pumila* and *Abies mariesii* in an alpine region of Mt. Norikura, Japan, often turn brown and die in March, which is earlier than the usual thaw that occurs from May to June. For trees living under severe environmental conditions, the role of the cuticle seems to be very important for their survival, but the relationship between the structural integrity of the cuticle and needle browning and death is not fully understood. In this study, we focused on the cuticle of the needles of *P. pumila* and *A. mariesii* in the alpine region of Japan and studied the relationship between needle browning and death and needle cuticle integrity such as cuticular resistance, and cuticle thickness and cuticle components.

Assessment of needle browning and death in *P. pumila* in relation to the mechanical damage to the cuticle, cuticular resistance, and cuticle thickness was shown in Chapter 2. Mechanical damage was not observed on needle cuticles of *P. pumila* browning in a natural environment. The color of needles with artificially abraded cuticles changed from green to brown in the abraded part. However, the brown color at the abraded part differed from the brown of a browning needle in its natural environment. There was no correlation between cuticular resistance and cuticle thickness. Needle browning and death in *P. pumila* were not related to mechanical damage of the cuticle or to cuticle thickness but might be due to changes in the quality and structure of the cuticle and other stresses.

Assessment of mechanical damage of *A. mariesii* needle cuticle in a timberline ecotone and evaluation of the cuticle thickness on cuticular resistance were shown in Chapter 3. Mechanical damage on needle cuticles of *A. mariesii* was not observed. In the cushion-shaped crown, epicuticular wax covered the cuticle and plugged stomatal antechambers. In contrast, in the flagged crown, epicuticular wax was mostly absent.

Cuticular resistance in the flagged crown was lower than that in the cushion-shaped crown. However, the cuticle in the flagged crown was thicker than that in the cushion-shaped crown. The needle browning and death in the flagged crown of *A. mariesii* occurred even though needle cuticles were not mechanically damaged. The thicker cuticle of the flagged crown may play a role in other stresses. To estimate desiccation stress in relation to the cuticle, it was concluded that we need to elucidate not only cuticular resistance and cuticle thickness, but also cuticle quality and structure.

From the result in Chapter 4, we confirmed whether the cuticular resistance and cuticle thickness of *A. mariesii* needles are related to needle cuticle components. Only in the flagged crown, needles with a higher cuticular resistance contained higher hydrolysable components including polysaccharide, and needle with a thicker cuticle contained higher cutin and lower wax. However, in the cushion-shaped crown, there were no differences between cuticular resistance or cuticle thickness and content of isolated needle cuticle components. Moreover, there were no remarkable differences in content of isolated needle cuticle components in any age needles between flagged and cushion-shaped crown, and between before and after winter. It was suggested that the change in cuticular resistance or cuticle thickness did not necessarily relate to the change in content of needle cuticle components, and respond to not only desiccation but also other stresses.

Consequently, it is considered that needle browning and death of *P. pumila* and *A. mariesii* did not relate to water loss from needle induced excessive transpiration caused by cuticle abrasion. This result showed clearly that it differs from the idea by previous studies, e.g. Tranquillini (1979), Hadley and Smith (1983, 1986, 1987), that damaged cuticles of needles cause excess transpiration resulting in needle death due to water content decreasing to lethal level. Moreover, we showed that thicker cuticles did not necessarily relate to control of water transpiration from needles. Furthermore, in *A. mariesii*, there were no remarkable differences between content of isolated needle cuticle components and cuticular resistance or cuticle thickness, and the change in content of needle cuticle components did not necessarily induce the change in the amount of water which transpires from cuticle. Especially, the change in cuticle

thickness was considered to the response to other stresses. It was suggested that epicuticular waxes which comprise the outer part of the cuticle relates water permeability in a cuticle. Current studies address to the elucidation of the mechanism of water permeability in a cuticle used by fresh foliage or isolated from foliage. The elucidation of the mechanism of desiccation stresses which vary by species is expected by the multiple comparisons including abiological factors, such as temperature, humidity, light, and air pollutants.

Summary

The browning and death of needles of evergreen conifers in alpine regions occur mainly in early spring at the point where the shoot protrudes from the snowpack. They are thought to be caused by excessive transpiration due to mechanical damage to the cuticle or to a thinner cuticle. However, there are a few studies that do not necessarily agree with this idea. We assessed needle browning and death in *Pinus pumila* (Pallas) Regel. in the alpine region of Japan in relation to mechanical damage to the cuticle, cuticular resistance, and cuticle thickness in Chapter 2. Mechanical damage was not observed on needle cuticles of *P. pumila* browning in a natural environment. The color of needles with artificially abraded cuticles changed from green to brown in the abraded part. However, the brown color at the abraded part differed from the brown of a browning needle in its natural environment. There was no correlation between cuticular resistance and cuticle thickness. Needle browning and death in *P. pumila* were not related to mechanical damage of the cuticle or to cuticle thickness but might be due to changes in the quality and structure of the cuticle and other stresses.

In the flagged crown, which is asymmetric growth formed by severe stresses during winter in alpine regions, needles of evergreen conifers often became brown and died in early spring, but did not in a cushion-shaped crown. Needle browning and death is thought to occur by increasing transpiration due to a thinner cuticle or mechanical damage to the cuticle by wind-born snow and ice particles. To confirm whether the needle browning and death in the flagged crown of *Abies mariesii* Mast. in the alpine region of Japan conform with this concept, we assessed mechanical damage of the needle cuticle in a timberline ecotone and evaluated the effect of cuticle thickness on cuticular resistance in Chapter 3. Mechanical damage on needle cuticles of *A. mariesii* was not observed. In the cushion-shaped crown, epicuticular wax covered the cuticle and plugged stomatal antechambers. In the flagged crown, epicuticular wax was mostly absent. Cuticular resistance in the flagged crown was lower than that in the cushion-shaped crown. However, the cuticle in the flagged crown was thicker than that in the cushion-shaped crown. The needle browning and death in the flagged crown of

A. mariesii occurred even though needle cuticles were not mechanically damaged. The thicker cuticle of the flagged crown may play a role in other stresses. To estimate desiccation stress in relation to the cuticle, we need to elucidate not only cuticular resistance and cuticle thickness, but also cuticle quality and structure.

The flagged crown of evergreen conifers grown under severe winter stress in alpine regions developed due to winter desiccation of shoots on the windward side of crowns. Evergreen conifers in alpine regions are thought to prevent winter desiccation of needles by increasing cuticle thickness. Furthermore, cuticular water permeability is thought to associate with cuticular polysaccharide. We confirmed whether the cuticular resistance and cuticle thickness of *A. mariesii* Mast. needles in the alpine region are relate to needle cuticle components in Chapter 4. Only in the flagged crown, needles with a higher cuticular resistance contained higher hydrolysable components including polysaccharide, and needle with a thicker cuticle contained higher cutin and lower wax. However, in the cushion-shaped crown, there were no differences between cuticular resistance or cuticle thickness and content of isolated needle cuticle components. Moreover, there were no remarkable differences in content of isolated needle cuticle components in any age needles between flagged and cushion-shaped crown, and between before and after winter. It was suggested that the cuticular resistance or cuticle thickness did not necessarily relate to content of needle cuticle components, and multiple correspondence against other stresses might be relate to them.

Acknowledgement

I would like to express the deepest appreciation to my supervisor, Professor Takefumi Ikeda, Kyoto Prefectural University for his considerable encouragements, kind and carefully instruction, meaningful discussions, suggestions, and leading to development of this doctoral research.

I would like to express my gratitude to Professor Emiko Maruta, Toho University for excellent suggestions, Professor Satoshi Terabayashi, Kyoto Prefectural University and Professor Keiji Takabe, Kyoto University for teaching the microtechnique, Emeritus Professor Kazuya Minato, Kyoto Prefectural University for teaching the technique of isolation of needle cuticles and determination of isolated needle cuticle components, Dr. Duncan Smith, University of Utah and Dr. Uwe G. Hacke, University of Alberta for editing a part of the manuscript and the staff of the Norikura Solar Observatory, University of Tokyo for helpful assistance.

I would like to express appreciation to Dr. Masafumi Ueda, Dr. Kimiko Hirayama, Professor Kazuhiro Tanaka, Professor Hikaru Takahara, Professor Makoto Ohkoshi, Professor Kazuki Matsumura, Emeritus Professor Yutaka Yoshiyasu, Emeritus Professor Yutaka Ishimaru, Dr. Yuzo Furuta, Dr. Hisashi Miyafuji, Dr. Masami Lintuluoto, Mr. Nobuhiko Kasuya, Dr. Yasushi Minowa, and Dr. Andre Freire Cruz, Kyoto Prefectural University for their helpful and constructive advice and considerable encouragement.

I would like to thank members in the laboratory and my all close friends for having a meaningful and delightful time, especially, I thank Ms. Saori Mizuno, Ms. Sanae Yamamoto, Ms. Yuko Sakai, Dr. Haruka Suzuki and Mr. Atsushi Shibahara.

Special thanks to Ms. Natsuyo Kishimoto, who is living in heaven. Although we are in the different world, I can feel your smile. You encouraged me sincerely all of the time. I feel gratitude for you, *Mimura-san*, I miss you.

Finally, I am deeply grateful to my parents, Mr. Masao Nakamoto and Ms. Taeko Nakamoto, and sister Ms. Akane Nakamoto, and my husband, Dr. Takeshi Nakatani for their endless love, understanding, tremendous support and sincere encouragement.

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