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REGULAR PAPER

Effect of epicuticular wax crystals on the localization of artificially deposited sub-micron carbon-based aerosols on needles of *Cryptomeria japonica*

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Abstract Elucidation of the mechanism of adsorption of particles suspended in the gas-phase (aerosol) to the outer surfaces of leaves provides useful information for understanding the mechanisms of the effect of aerosol particles on the growth and physiological functions of trees. In the present study, we examined the localization of artificially deposited sub-micron-sized carbon-based particles on the surfaces of needles of *Cryptomeria japonica*, a typical Japanese coniferous tree species, by field-emission scanning electron microscopy. The clusters (aggregates) of carbon-based particles were deposited on the needle surface regions where epicuticular wax crystals were sparsely distributed. By contrast, no clusters of the particles were found on the needle surface regions with dense distribution of epicuticular wax crystals. Number of clusters of carbon-based particles per unit area showed statistically significant differences between regions with sparse epicuticular wax crystals and those with dense epicuticular wax crystals. These results suggest that epicuticular wax crystals affect distribution of carbon-based particles on needles. Therefore, densely distributed epicuticular wax crystals might prevent the deposition of sub-micron-sized carbon-based particles on the surfaces of needles of *Cryptomeria japonica* to retain the function of stomata.

Keywords Carbon-based particle · *Cryptomeria japonica* · Epicuticular wax crystal · Field-emission scanning electron microscopy (FE-SEM) · Sub-micron-sized particle

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Introduction

Anthropogenic air pollutants, such as aerosol particles generated by industrial activity, travel long distances from their sources, affecting neighboring countries as transboundary air pollution (Colvile 2002; Fowler 2002). The diameters of aerosol particles vary widely from a few nanometers to a few hundred micrometers (Chin et al. 2007) and sub-micron-sized particles are the major component of anthropogenic aerosols (Colvile 2002).

Deposited aerosol particles on the surfaces of leaves might influence surface conditions, growth and physiological functions such as photosynthesis of the leaves (Farmer 1993). Hygroscopic leaf surface particles affected gas exchange and the water relations of *Helianthus annuus* L., *Malus domestica* Borkh., *Sambucus nigra* L., *Solanum lycopersicum* L. and *Vicia faba* L. (Burkhardt et al. 2001, 2012; Pariyar et al. 2013). In addition, such aerosol particles induced the degradation of epicuticular waxes and the decrease in drought tolerance of *Pinus sylvestris* L. (Burkhardt and Pariyar 2014). Our previous study showed that artificially deposited ammonium sulfate particles affected net photosynthetic rate of *Cryptomeria japonica* D. Don (Yamaguchi et al. 2014). For understanding the mechanisms of the effects of aerosol particles on the growth and physiological functions of trees, it is necessary to elucidate the behavior of aerosol particles on the surfaces of leaves.

There are unique structures such as epicuticular wax crystals and trichomes on the surfaces of leaves of many species of trees (Neinhuis and Barthlott 1997a). The shapes and distribution of epicuticular wax crystals and trichomes differ among species of trees (Barthlott et al. 1998; Esau 1977). Micro-roughness derived from such surface structure affected deposition velocity and collection efficiency of particles under natural and experimental conditions (Freer-Smith et al. 2005; Hwang et al. 2011; Lin and Khlystov 2012; Neinhuis and Barthlott 1998; Räsänen et al. 2013; Reinap et al. 2009; Yunus et al. 1985). In addition, epicuticular wax crystals are related to water repellency of leaves (Holloway 1970). Such water repellency plays an important role in washing out deposited particles by rain, fog or dew (Neinhuis and Barthlott 1997a, b). Therefore, epicuticular wax crystal is a non-negligible surface structure to understand the mechanism of adsorption of aerosol particles to the outer surfaces of leaves.

Elucidation of the regions where particles deposit preferentially provides useful information for understanding the mechanism of effect of aerosol particles on the growth and physiological functions of trees. Nevertheless, microscopic information about the relationships between localization of sub-micron-sized particles and distribution of epicuticular wax crystals on leaves is limited. Burkhardt et al. (1995) reported that artificially deposited sub-micron-sized particles introduced via a wind tunnel preferentially deposited in stomatal regions where epicuticular wax crystals distribute densely. However, it is not clear whether epicuticular wax crystals affect the localization of particles in the same manner in the case of the particles with different size, chemical composition and driving force for deposition.

The black carbon (BC) is a kind of fine particulate matter. Combustion of fossil fuels and biomass burning are main sources of BC (WHO 2012). Anthropogenic sources of BC are most concentrated in developing nations in the tropics and East Asia (Ramanathan and Carmichael 2008). To evaluate the effect of BC on forest tree species, we have been used sub-micron-sized carbon-based particles as a model of BC particles (Yamaguchi et al. 2012; Yamane et al. 2012). In our previous studies, we established the method of exposure to sub-micron-sized carbon-based particles on the surface of leaves of tree seedlings and the visualization method of such particles with high-resolution scanning electron microscope (Yamaguchi et al. 2012; Yamane et al. 2012). In the present study, we applied these methods and examined the localization of artificially deposited sub-micron-sized carbon-based particles on the surfaces of needles of the conifer *Cryptomeria japonica*, a typical Japanese coniferous tree species to reveal the effect of epicuticular wax crystals on the deposition of carbon-based particles on the surfaces of needles. In *Cryptomeria japonica*, physiological dysfunction of the trees due to changes in physical and/or chemical condition, such as the amounts of epicuticular wax, of the surface of needles by deposition of air pollutants was reported (Sase et al. 1998; Takamatsu et al. 2001). Finally, we discussed about the function of epicuticular wax crystals on the surface of needles of *Cryptomeria japonica*.

Materials and methods

Plant and control materials

In July 2010, the current year's shoots were collected from three of two-year-old seedlings of *Cryptomeria japonica* growing in the campus of Tokyo University of Agriculture and Technology in Fuchu, Tokyo, Japan. We also used 0.04-mm-thick low-density polyethylene sheets (Unipack B-4; Seisannipponsha Ltd., Tokyo, Japan) as control substrate in exposure experiments. On the surface of polyethylene sheets, we could distinguish easily artificially deposited carbon-based particles because of their flatness and cleanness.

Exposure to carbon-based particles

The surfaces of needles were exposed to sub-micron-sized carbon-based particles as described by Yamane et al. (2012). The source of particles was a combustion-derived nanopowder (Tokai Carbon Co., Ltd., Tokyo, Japan), with primary diameters of approximately 30 nm, consisting mainly of elemental carbon (EC). These particles were solid at ambient temperatures and insoluble in water. The needles and the polyethylene sheets were exposed to carbon-based particles in aerosols generated either by electrostatic or ultrasonic force. Needles without exposure to aerosols were used as controls. For the aerosol generator that used electrostatic force (Fig. 1a), we used the electrostatic-spray (electrospray) system described by Lenggoro et al. (2006), with a stainless-steel capillary tube of 0.1 mm in inner diameter (Naim et al. 2010). The liquid flow rate and the applied voltage were set to 0.2 mL/h and between 2 and 3 kV, respectively. When we used the electrospray system, dry particles were deposited on the surfaces of substrates (Gen et al. 2014). For the aerosol generator that used ultrasonic force (Fig. 1b), we used an ultrasonic nebulizer

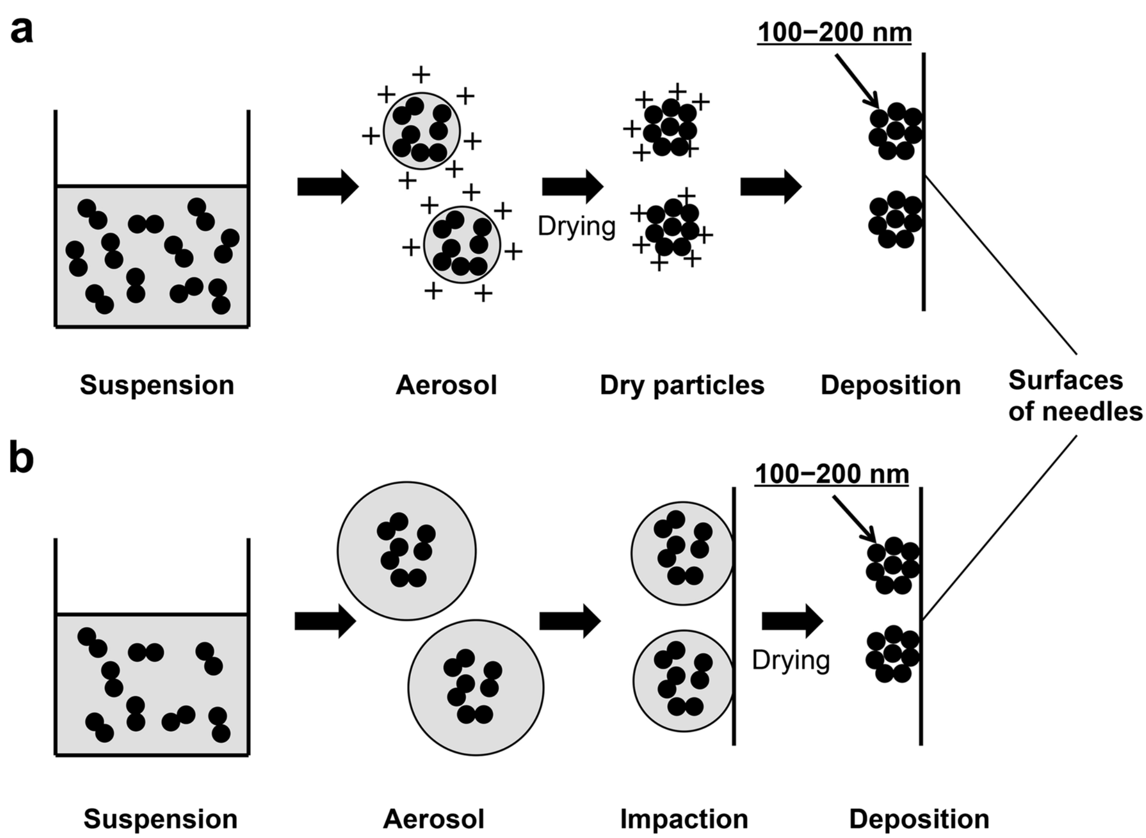

Fig. 1 Schematic diagrams of exposure experiment with aerosol generators that were used electrostatic force (**a**) and that were used ultrasonic force (**b**)

(NE-U17, Omron Co., Tokyo, Japan) operated at 1.7 MHz, which can generated droplet (as the carrier of carbon-based materials) in the size around 5 µm (Wang et al. 2008). Unlike electrostatic-spray, in the case of ultrasonic spray system, particles were carried and deposited on the substrates as "wet aerosols" (droplets) of suspension because we observed a visible white mist reaching plants during exposure experiments. Under natural conditions, we can assume that both dry and wet deposition of carbon-based particles occurs on needles. Therefore, we used the two exposure systems as models of "dry" and "wet" deposition. In both systems, the aerosols contained aggregates of carbon-based particles and the size distribution of the particles could be controlled via the size of droplets and the concentration of the suspension of particles. Concentrations of suspension for electrospray and ultrasonic spray were 0.01 and 0.001 wt%, respectively. In our experiments, the diameters of aggregated particles were approximately 100 nm in dry condition (i.e. the solid carbon-based particles) that were measured by a real-time technique based on a differential mobility analyzer (DMA) method (Lenggoro et al. 2002; Wang et al. 2008; Yamaguchi et al. 2012). The size range of the corresponding aerosols was also confirmed by a gas-phase measurement technique, namely an online (real-time) particle size/mobility analyzer system (SMPS. Model 3034, TSI, St. Paul, Gen et al. 2014). The durations of exposure were 120 min for the electrostatic aerosol generator and 70 min for the ultrasonic generator. The distance between the outlet nozzle of the aerosol generator and the test materials was 10 cm in all cases. Shoots were set at vertical orientation against outlet nozzle of the aerosol generator.

Field-emission scanning electron microscopy

After exposure to carbon-based particles, the polyethylene sheets were cut into squares (approximately 5 mm × 5 mm) and the needles were cut into lengths of approximately 5 mm with razor blades. The samples were then air-dried for 3 days in a desiccator. Specimens were mounted on specimen stubs for the FE-SEM and then coated with gold, platinum or platinum-palladium with a sputter coater (JFC1100 or JFC1500; JEOL, Tokyo, Japan) or a vacuum evaporator (JEE-4X; JEOL). The distribution of particles on needles and polyethylene sheets was observed under the FE-SEM (S-4800; Hitachi, JSM-6301F; JEOL or JSM-7100F; JEOL) at accelerating voltages from 0.5 to 10 kV (Sano and Jansen 2006; Sano et al. 2011;

Yamane et al. 2012). Individual 12 needles were examined in both exposure methods.

Measurements of the number of clusters of carbon-based particles and proportion of area covered by epicuticular wax crystals

The number of clusters of carbon-based particles was calculated on the surfaces of needles after exposure to carbon-based particles. Individual three needles were examined ($n = 3$) in each exposure method. Twenty SEM images (resolution of a micrograph, 1280 × 960 pixel; area, 12 × 9 μm; magnification, ×10,000) were obtained randomly from each needle. In each image, we counted the number of clusters of carbon-based particles, and calculated mean density of clusters of carbon-based particles (number μm^{-2}). In addition, proportions of area covered by epicuticular wax crystals in all of these SEM images were measured with the image-analysis software Image-J (National Institutes of Health, MD, USA).

Statistical analysis

The differences in density of clusters of carbon-based particles (number μm^{-2}) between regions with epicuticular wax crystals sparsely and densely were analyzed with an unpaired *t* test (SPSS Statistics 19; IBM, USA).

Results

Surface structures of needles of *Cryptomeria japonica*

Figure 2a–e shows FE-SEM images of the surface structure of needles of the conifer *Cryptomeria japonica*. There were no trichomes on the adaxial and abaxial surfaces of the needles (Fig. 2a), but stomata were distributed on both the adaxial and abaxial surfaces (Fig. 2a–c). There were copious epicuticular wax crystals on the surfaces of needles but the distribution and type of epicuticular wax crystals were not uniform. Densely distributed rod-like epicuticular wax crystals, which have smooth cross-sectional surface, were observed on all the stomata (Fig. 2d). Both rod-like and tube-like epicuticular wax crystals, which have a hole in their cross-sectional surface, were sparsely distributed in other regions (Fig. 2e). We classified surfaces of needles into two groups based on the density of epicuticular wax crystals: the region where smooth surface of needles could hardly be seen by densely distributed epicuticular wax crystals (Fig. 2d) and the region where smooth surface of needles could be seen due to sparsely distributed epicuticular wax crystals (Fig. 2e).

The distribution of particles on needles after exposure to sub-micron-sized carbon-based particles

Clusters of fine particles were deposited on the polyethylene sheets that were sprayed with suspensions of carbon nanopowder (arrowheads in Fig. 2f). We observed similar clusters on the surfaces of needles after exposure to carbon-based particles (arrows in Fig. 3). However, we did not find similar clusters on the needles that were not exposed to particles. Therefore, we concluded that the clusters of particles on the needles were derived from carbon-based particles that we generated. The aerosol generator by electrostatic force produced larger clusters of carbon-based particles than the aerosol generator by ultrasonic force. There were no clear differences between electrostatic and ultrasonic-based exposure methods in terms of patterns of distribution of particles on needles that were exposed to sub-micron-sized carbon-based particles.

On the surfaces of the needles of the conifer *Cryptomeria japonica*, there were many kinds of particles even in the control samples. Therefore, we had to distinguish artificially deposited carbon-based particles from other particles with diameter and shape under high magnification (e.g. ×40,000). In addition, we took into account for the possibility that carbon particles may be more easily found in the regions where epicuticular wax crystals were sparsely distributed than in the regions where epicuticular wax crystals were densely distributed. There were clusters of artificially deposited carbon-based particles in regions where epicuticular wax crystals were sparsely distributed (Fig. 3a, b, e, f). We did not find clusters of carbon-based particles that we generated in the regions where epicuticular wax crystals were densely distributed on stomata (Fig. 3c, d g, h). Density of clusters of carbon-based particles showed statistically significant differences between regions of sparse epicuticular wax crystals and those of dense epicuticular wax crystals (Fig. 4, $P < 0.05$). The mean percentage (±standard deviation, $n = 120$ SEM images) of the area covered by sparsely and densely distributed epicuticular wax crystals in the examined area were 23.1 ± 12.8 and 96.7 ± 1.8 %, respectively.

Discussion

In our experiments of artificial dispersion of sub-micron-sized carbon-based particles onto needle surfaces, we used two types of aerosol generator, one that used electrostatic force and one that used ultrasonic force. In this report, we discuss about the pattern of deposition of carbon-based particles.

After exposure to the particles, clusters of the particles were found on the surfaces of needles of *Cryptomeria*

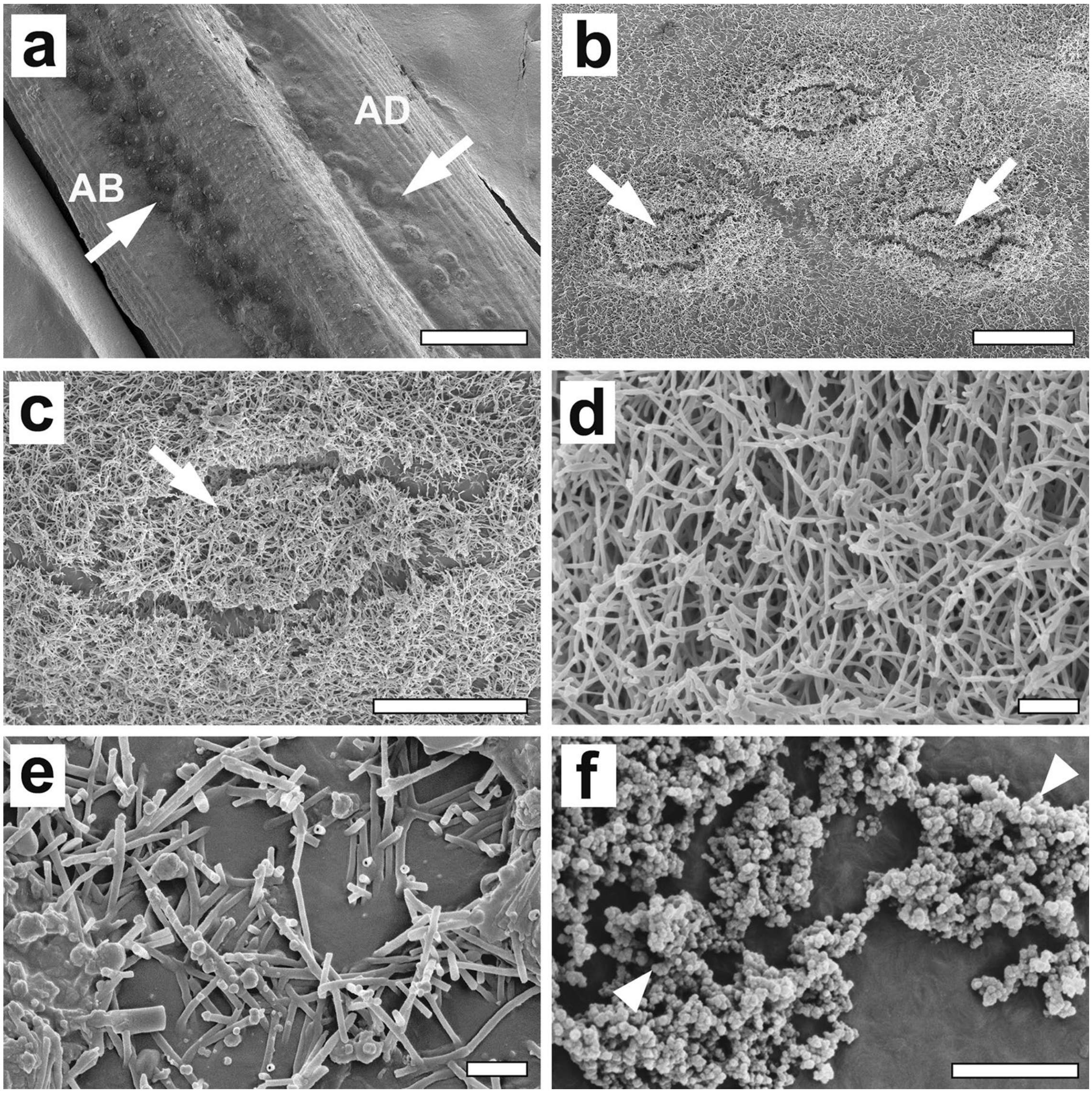

Fig. 2 FE-SEM images of the surface structure of needles of *Cryptomeria japonica* (**a**–**e**) and the polyethylene sheet (**f**). Lateral side of the surface of a needle (**a**). Areas where stomata are present beneath dense epicuticular wax crystals (**b**, **c**). Densely distributed epicuticular wax crystals on a stoma (**d**). Sparsely distributed epicuticular wax crystals (**e**). The carbon-based particles on a polyethylene sheet after exposure to carbon-based particles by electrospray (**f**). *Arrows* indicate epicuticular wax crystals covering stomata. *Arrowheads* indicate clusters of artificially dispersed carbon-based particles. *AD* adaxial surface, *AB* abaxial surface. The accelerating voltage was at 3 kV (**a**–**d**) and 2.5 kV (**e**, **f**). *Scale bars* **a** = 200 μm; **b** = 20 μm; **c** = 10 μm; **d**, **e** = 1 μm; **f** = 0.5 μm

japonica (Fig. 3). In the dry condition (i.e. the solid carbon-based particles), mean size of carbon-based particles was approximately 100 nm in both methods (Yamaguchi et al. 2012). However, on the surfaces of needles, size of many clusters of particles was larger than 100 nm (Fig. 3). Furthermore, the aerosol generator by electrostatic force produced larger clusters of carbon-based particles than the aerosol generator by ultrasonic force as mentioned by Yamane et al. (2012). In the case of dispersion of particles via electrostatic force, "dry" particles, which were strongly positively charged, were deposited on the surfaces of substrates (Naim et al. 2010). Artificially dispersed particles might form clusters on relatively lower-charged regions on the surfaces of needles. In addition, that electrostatic force might affect the formation of clusters of carbon-based particles as well as deposition. In the case of carbon-based particles via ultrasonic force, particles were deposited on the substrates as droplets of suspension by inertial force due to air flow from aerosol generator (Wang et al. 2008). These droplets of suspension might gather as a result of non-uniformity in hydrophilic properties of the surfaces. Then, large clusters of particles might be formed.

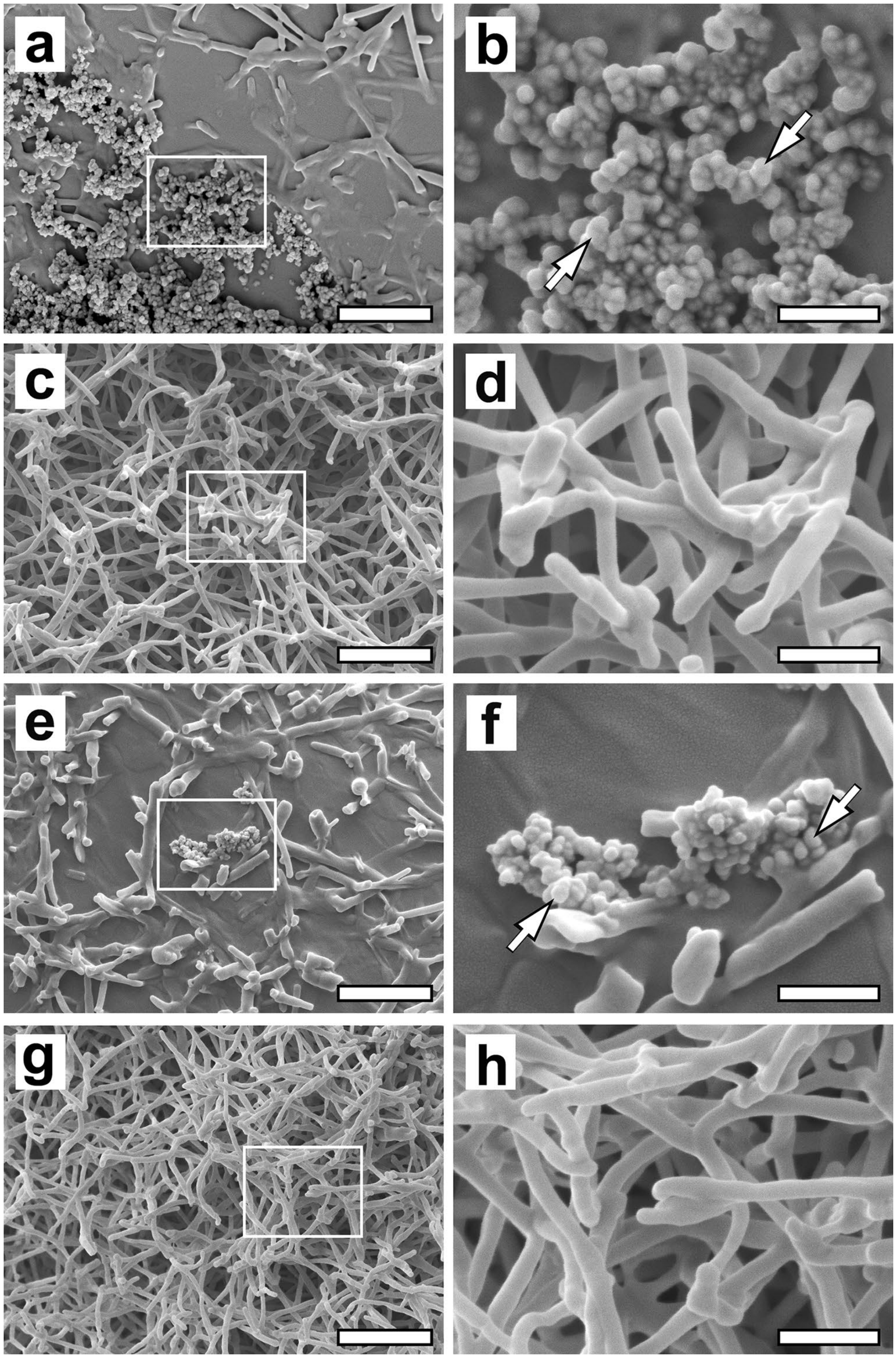

Fig. 3 FE-SEM images of the surfaces of needles of *Cryptomeria japonica* after exposure to carbon-based particles by electrospray (**a**–**d**) and ultrasonic spray (**e**–**h**). **a**, **e** Clusters of carbon-based particles on the region with sparse epicuticular wax crystals. **c**, **g** Cluster of carbon-based particles was not observed in the region with dense epicuticular wax crystals. **b**, **d**, **f**, **h** Higher-magnification images of the enclosed area in (**a**, **c**, **e**, **g**). The accelerating voltage was 2.5 kV. *Arrows* indicate clusters of artificially dispersed carbon-based particles. *Scale bars* (**a**, **c**, **e**, **g**) = 2 μm; (**b**, **d**, **f**, **h**) = 0.5 μm

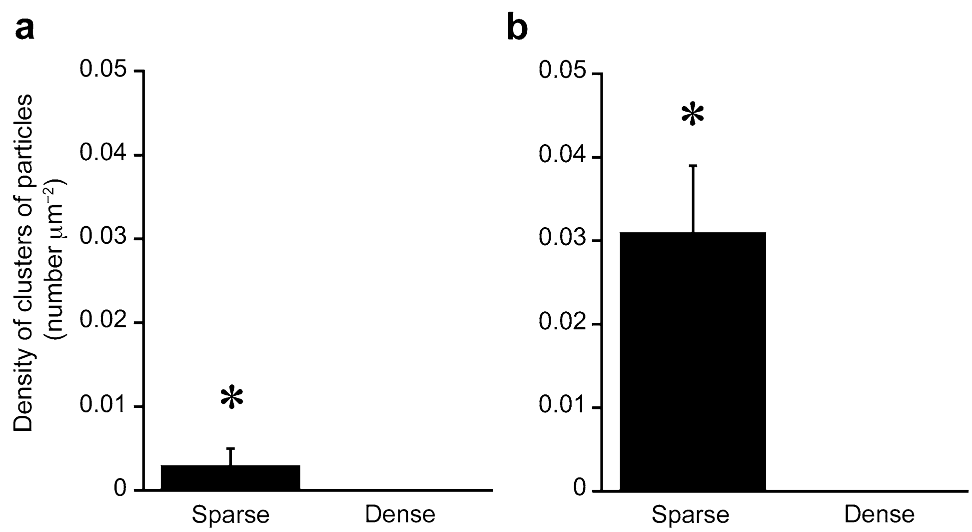

Fig. 4 Density of clusters of carbon-based particles artificially deposited by electrospray (**a**) and ultrasonic spray (**b**) on the surfaces of needles of *Cryptomeria japonica*. Each value shows the mean of three needles, and the standard deviation is given by *vertical bar*. *Asterisks* show statistically significant differences ($P < 0.05$, unpaired *t* test)

These patterns and differences in size of aggregation of the particles suggest that distribution and size of clusters of carbon-based particles might be affected by electric charge (surface potential) and hydrophilic properties of surfaces of needles.

In the present study, we focused on heterogeneously distributed epicuticular wax crystals on the surfaces of needles of *Cryptomeria japonica*. Rod-like epicuticular wax crystals were distributed densely on all of stomata (Fig. 2d). In other regions, sparsely distributed rod-like and tube-like epicuticular wax crystals were observed (Fig. 2e). Hereafter, we discuss about the differences in deposition of particles between these two regions on surfaces of needles of *Cryptomeria japonica*.

We found a clear relationship between the distribution of epicuticular wax crystals and the deposition of sub-micron-sized carbon-based particles. On the surfaces of needles of *Cryptomeria japonica*, clusters of the particles were deposited in regions where epicuticular wax crystals were sparsely distributed (Figs. 3a, b, e, f, 4). No clusters of the carbon-based particles were evident in regions where epicuticular wax crystals were densely distributed. These results suggest two possibilities: (1) carbon-based particles cannot deposit on the region where epicuticular wax crystals were densely distributed; or (2) carbon-based particles are only slightly attached to the region with densely distributed epicuticular wax crystals and fall off during the preparation procedure for scanning electron microscopy. In either case, the densely distributed epicuticular wax crystals might affect deposition of sub-micron-sized carbon-based particles. In our previous report, the exposure of living saplings to carbon-based particles with sub-micron size via similar methods for two growing seasons did not significantly affect the gas exchange rate of current year's needles of *Cryptomeria japonica* (Yamaguchi et al. 2012). This result implies that functions of stomata were not suppressed in the experiment of long-term exposure to sub-micron-sized carbon-based particles in *Cryptomeria japonica*. Therefore, it is speculated that epicuticular wax crystals might have a function preventing the deposition of sub-micron-sized carbon-based particles on the surfaces of needles of *Cryptomeria japonica*.

In contrast, Burkhardt et al. (1995) reported that artificially deposited sub-micron-sized aerosol particles of di-2-ethylhexyl-sebacate (DES) via wind tunnel were mainly distributed around the stomata where epicuticular wax crystals present densely in conifers, *Pinus sylvestris*, *Picea abies* (L.) H. Karst. and *Abies alba* Mill. In their experiment, the exposure method (driving force for deposition of particles) differed from our experiment. In addition, property of DES differed from that of carbon-based particles. For example, DES is liquid at ambient temperatures and insoluble in water. These contrary results suggest that property of particles and experimental condition affect deposition of particles on the surfaces of needles.

Epicuticular wax crystals play an important role in the water repellency of surfaces of leaves (Holloway 1970). Water repellency was related to the amounts of particles deposited on the surfaces of leaves (Neinhuis and Barthlott 1997a, 1998). Deposited particles on the surfaces of leaves with high water repellency were washed out by rain, fog or dew (Lotus-effect). Because the contact area between particles and leaf surface is reduced due to micro-roughness caused by epicuticular wax crystals, the contact area between particles and water droplets is consistent, and, thus, adhesion between particles and water droplets becomes relatively larger than that between particles and surfaces of leaves (Neinhuis and Barthlott 1997a, b). As a result, particles deposited leaf surfaces separate from the leaf surfaces when water droplets touch and move there. Therefore, epicuticular wax crystals are related to self-cleaning and adhesion with particles on the surfaces of leaves. In the present study, we demonstrated another possible function that densely distributed epicuticular wax crystals prevent the deposition of sub-micron-sized particles on the surfaces of needles of *Cryptomeria japonica*. This function of epicuticular wax crystals might be effective to retain the function of stomata. Our observations suggest that heterogeneity in distribution of epicuticular wax crystals within needle surfaces is an important factor for understanding of the mechanism of the effects of aerosol particles on the growth and physiological functions of trees.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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