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Morphology and Chemistry of *Parmotrema tinctorum* (Parmeliaceae, Lichenized Ascomycota) Transplanted into Sites with Different Air Pollution Levels

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Abstract We performed an experiment involving transplantation of disks punched from *Parmotrema tinctorum* lobes at 3 sites with different air pollution levels in Shimizu Ward, Shizuoka City, Japan. Lobule formation was observed in the clean site, while disintegration of the cortex was observed in the polluted sites where NO_x, NO_2, SO_2, and O_3 levels were significantly higher. In addition, the amounts of atranorin were lower in the polluted sites than in the clean site, and they were lowest in the site where O_3 levels were the highest during summer. The difference between the amounts of lecanoric acid was not statistically significant among the test sites.

Key words: Atranorin, cortex disintegration, lichen, lobule formation, secondary substance, transplantation.

Introduction

Parmotrema tinctorum (Nyl.) Hale (Parmeliaceae, lichenized Ascomycota) is a bioindicator of air pollution in Japan. It is especially sensitive to sulphur dioxide (SO_2) and has been threatened or even extinct in urban areas where the annual mean concentration of SO_2 is more than 20 ppb (Sugiyama *et al.*, 1976). After an improvement in the pollution caused by SO_2, recolonization of *P. tinctorum* was observed in urban areas of Japan (Taoda, 1992; Ohmura *et al.*, 2006). However, *P. tinctorum* has been disappearing near arterial roads, and this may be related to changes in air quality caused by automobile exhausts (Ohmura *et al.*, 2008).

To confirm whether the disappearance of a particular species is truly due to deterioration of air quality, several experiments involving transplantation of various lichen species in polluted areas have been often carried out (*e.g.*, Brodo, 1966; LeBlanc and Rao, 1973; Søchting and Johnsen, 1978; Ferry and Coppins, 1979). Such experiments have revealed morphological degradation, discoloration or bleaching of the thallus, reduction of reproductive potential, and decline in various physiological activities of both the mycobiont and the photobiont of lichens in urban areas (Gries, 1996; Hamada and Miyawaki, 1998; Conti and Cecchetti, 2001; Białońska and Dayan, 2005).

In an experiment involving transplantation of *P. tinctorum* at a suburban site, after six months of exposure, lobule formation was observed at the margin of disks punched from the thallus, but no lobule formation was observed at an urban site (Kon and Ohmura, 2008). In addition to the morphological change, there was a decrease in the lecanoric acid levels in the transplanted thalli, probably due to acid rain (Bando and Sugino, 1997). Thus, we assume that the transplanted *P. tinctorum* thallus may show different morphological and chemical responses depending on the differences in air quality. However, this behaviour has not been well examined, and more experiments are needed in order to standardize the method for transplanting *P. tinctorum* for the evaluation of air quality.

The purpose of the present study is to examine whether morphological and physiological responses of the transplanted *P. tinctorum* thalli vary with differences in air pollution levels. We measured the levels of air pollutants such as nitrogen oxides (NO_x), nitrogen dioxide (NO_2), SO_2, and ozone (O_3) at the test sites by using simple passive samplers each month.

Materials and Methods

Test sites

An experiment involving transplantation of *P. tinctorum* was performed at three test sites in Shimizu Ward, Shizuoka City, Shizuoka Prefecture, Japan, from April 18 to December 25, 2006 (Fig. 1). In Shimizu Ward, the monthly values of mean temperature ranged from 6.0 to 26.7°C, precipitation varied from 97 to 311 mm, and the duration of sunshine varied from 56.2 to 175.6 h at the meteorological station (35°03′12″N, 138°31′18″E) (Japan Meteorological Agency, 2006). At the test site, the main pollutants currently affecting the distribution of *P. tinctorum* seem to be automobile exhausts resulting from the increase in traffic (Ohmura *et al.*, 2008). Therefore, we selected the following three test sites according to the distance from the national road "Route 1" where traffic jams occur often.

1. Umegaya-kita: This park (3,700 m^2) in a suburban area is located 2.2 km from Route 1 (35°02′38″N, 138°27′04″E), and the altitude is 25 m.
2. Sakuragaoka: This park (22,200 m^2) in an urban area is located from 1.0 km from Route 1 (35°00′36″N, 138°28′33″E), and the altitude is 5 m.
3. Shibukawa-naka: This park (3,900 m^2) in an urban area is located only 0.1 km from Route 1 (35°01′08″N, 1138°28′10″E), and the altitude is 5 m.

Measurement of air pollutants

We exposed simple passive samplers for NO_x,

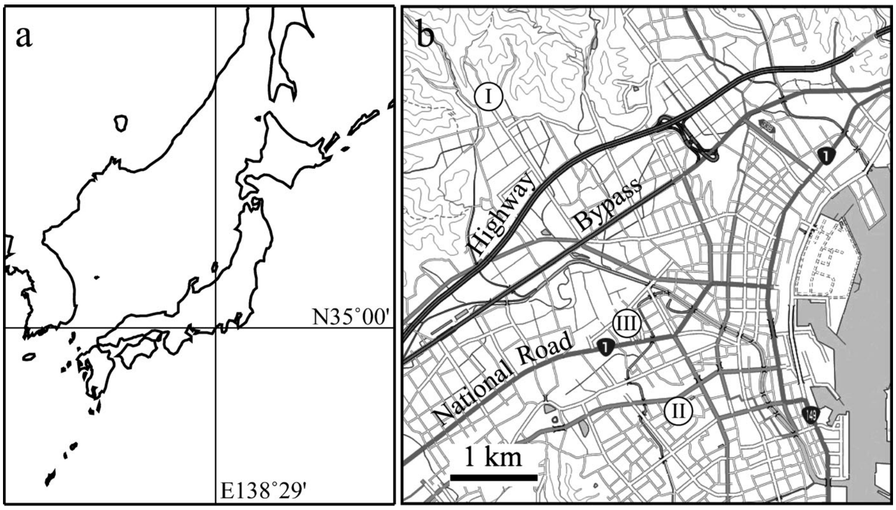

Fig. 1. Map of test sites. a. The geographical location of Shimizu Ward, Shizuoka City, Japan (cross point of the lines). b. The test sites of the transplantation experiment in Shimizu Ward: (I) Umegaya-kita, (II) Sakuragaoka, and (III) Shibukawa-naka.

NO_2, SO_2, and O_3 in ambient air (Nishikawa *et al.*, 2000) under a rain shield at a height of 2.0 to 2.5 m above the ground at each test site during the experimental period. The samplers were replaced each month, and the exudates were analysed by ion chromatography (DX-500, DIONEX, USA) with columns (IonPac AS12A/AG12A, DIONEX, USA) as reported by Nishikawa *et al.* (2000).

Lichen transplantation

Six thalli were collected from suburban areas of Shizuoka City on April 12, 2006 (Table 1). The voucher specimens have been deposited at the National Museum of Nature and Science, Tokyo (TNS). Round disks (5 mm in diameter) were punched from each thallus according to the procedure described by Kon and Ohmura (2008). The disks were punched from the flat parts of the lobes without rhizines, which comprise active growing zones. A set of 6 disks from different thalli was placed vertically at each test site. From the top, the disks were numbered I, II, III, IV, V, and VI (Table 1, Fig. 3). Kon and Ohmura (2008) fixed the disks directly to the bark by a nylon mesh; however, we attached the disks by using a double-sided adhesive tape (NW-15S, Nichiban, Japan) to the east side of the trunk of *Prunus*× *yedoensis* Matsum. at a height of 1.5 to 2.0 m above the ground in order to ensure uniform substrate condition. Before attaching the disks, the bark surface was cleaned to remove dust and lichens. The disks were attached onto a flat area

Table 1 Data for the disks in the transplantation experiment. All voucher specimens are deposited in the National Museum of Nature and Science, Tokyo (TNS)

Disk number	Locality in Shizuoka City, Japan	Specimen voucher (Y. Ohmura no.)
I	Tesshu-ji Temple (34°59′32″N, 138°28′58″E)	5887
II	Mt. Kuno-zan (34°57′47″N, 138°28′01″E)	5874
III	Gyuran-ji Temple (35°02′03″N, 138°26′53″E)	5892
IV	Gyuran-ji Temple	5888
V	Mt. Kuno-zan	5877
VI	Mt. Kuno-zan	5875

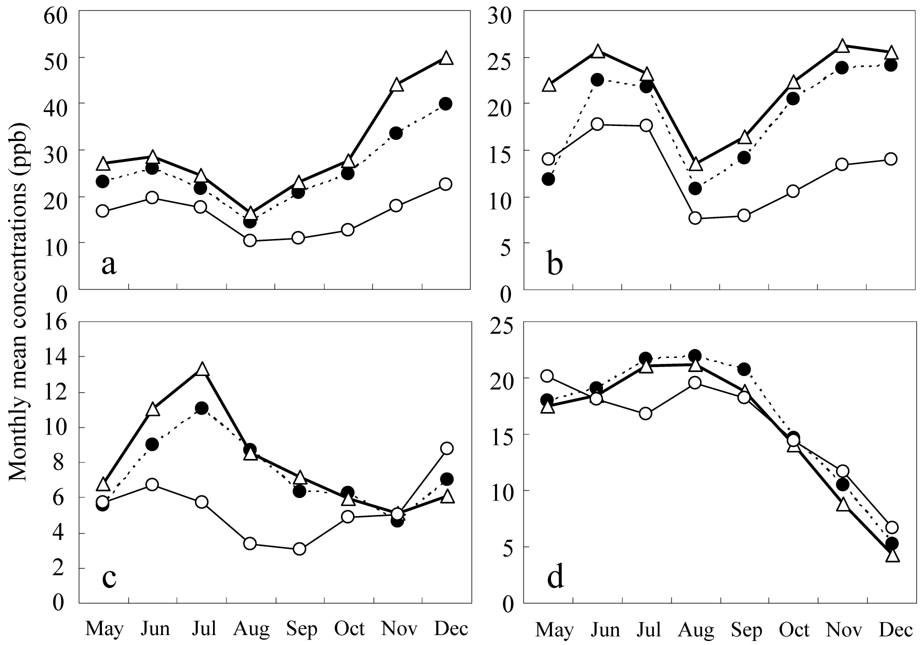

Fig. 2. Monthly mean concentrations (ppb) of various air pollutants measured during the experimental period. a. NO_x. b. NO_2. c. SO_2. d. O_3. —○— Umegaya-kita, ---●--- Sakuragaoka, and —△— Shibukawa-naka.

of the bark, thereby avoiding the rough surfaces on the bark, such as lenticels. The transplanted disks were then allowed to remain from April 18 to December 25, 2006 (ca. eight months). The disks were photographed each month. Distilled water (3–5 mL) was sprayed onto the set of disks once a month after capturing photographs. After the field experiment, the disks were retrieved from the barks and examined under a stereomicroscope.

Analysis of lichen substances in the thalli

After transplantation, a fragment of the thallus was excised from each disk and dried in a freeze dryer (Flexi-dry, FTS, USA) for 30 min. The dried thallus was weighed using an electronic microbalance (AX26, Mettler Toledo, Switzerland) and placed in 250 μL of 100% acetonitrile (high-performance liquid chromatography [HPLC] grade) overnight. Lichen secondary substances were separated with an analytical HPLC (LC-10, Shimadzu, Japan) with 6.0 i.d.×150 mm column (Unisil NQ C18, GL Sciences Inc., Japan) at 40°C. Ten microliter of the extract solution was injected onto the HPLC coupled with a photodiode-array detector (SPD-M10A, Shimadzu, Japan) set at a wavelength of 254 nm. The flow rate of the water–methanol–acetic acid (15:85:0.15) solvent was 1.0 mL/min, and the retention times (min) observed were 4.3 for lecanoric acid, 11.4 for atranorin, and 14.0 for chloroatranorin.

The content of these lichen secondary substances from each sample was calculated by comparing the chromatographic peak areas obtained with those of the authentic samples of lecanoric acid and atranorin, which were extracted by Miyawaki *et al.* (1998).

Statistical differences in the concentration of lichen secondary substances in the thalli that were transplanted to different sites were established using one-way analysis of variance (ANOVA), with $p<0.05$ as an indication of significant difference between groups.

Results

Air pollutant levels at the test sites

The monthly mean concentrations of NO_x, NO_2, SO_2, and O_3 at each site from May to December in 2006 are shown in Fig. 2. These values differ with seasonal changes. The concentration of NO_x was less than 17 ppb in August and reached 50 ppb in December. The NO_2 level was less than 14 ppb in August and reached 26 ppb in June and November. The level of SO_2 was less than 5 ppb in November and reached 13 ppb in July and that of O_3 was less than 7 ppb in December and reached 22 ppb in August. Despite these variations in concentrations, the pollution levels were almost stable at all the test sites. The ranking of the sites in decreasing order of NO_x and NO_2 concentration levels was Shibukawa-naka>Sakuragaoka>Umegaya-kita throughout the experiment, except for NO_2 in May (Figs. 2a, b). Higher levels of SO_2 (>10 ppb) were observed in June and July (Fig. 2c), during which the SO_2 levels were the same as the NO_x and NO_2 levels. Higher values of O_3 (>20 ppb) were observed from July to September (Fig. 2d), during which the ranking of the sites in decreasing order of O_3 pollution levels was Sakuragaoka>Shibukawa-naka>Umegaya-kita.

Morphological changes in the thalli

The morphological changes in the transplanted disks at the test sites are shown in Fig. 3. The disks punched from the thalli of *P. tinctorum* at all the test sites were round in shape (5 mm in diameter) with smooth edges around the disk observed at the beginning of the experiment. After three months of exposure, lobule primordia were found on the edge of disks I, IV, V, and VI at Umegaya-kita and disks I and VI at Sakuragaoka (Figs. 3a, c). Disintegration of the upper cortex was observed to a certain degree in all the disks transplanted at Sakuragaoka and Shibukawa-naka (Figs. 3c, e). After six months of exposure, lobules or lobule primordia were found on all disks at Umegaya-kita, and isidia-like protuberances were observed on the surface of disks III, IV, V,

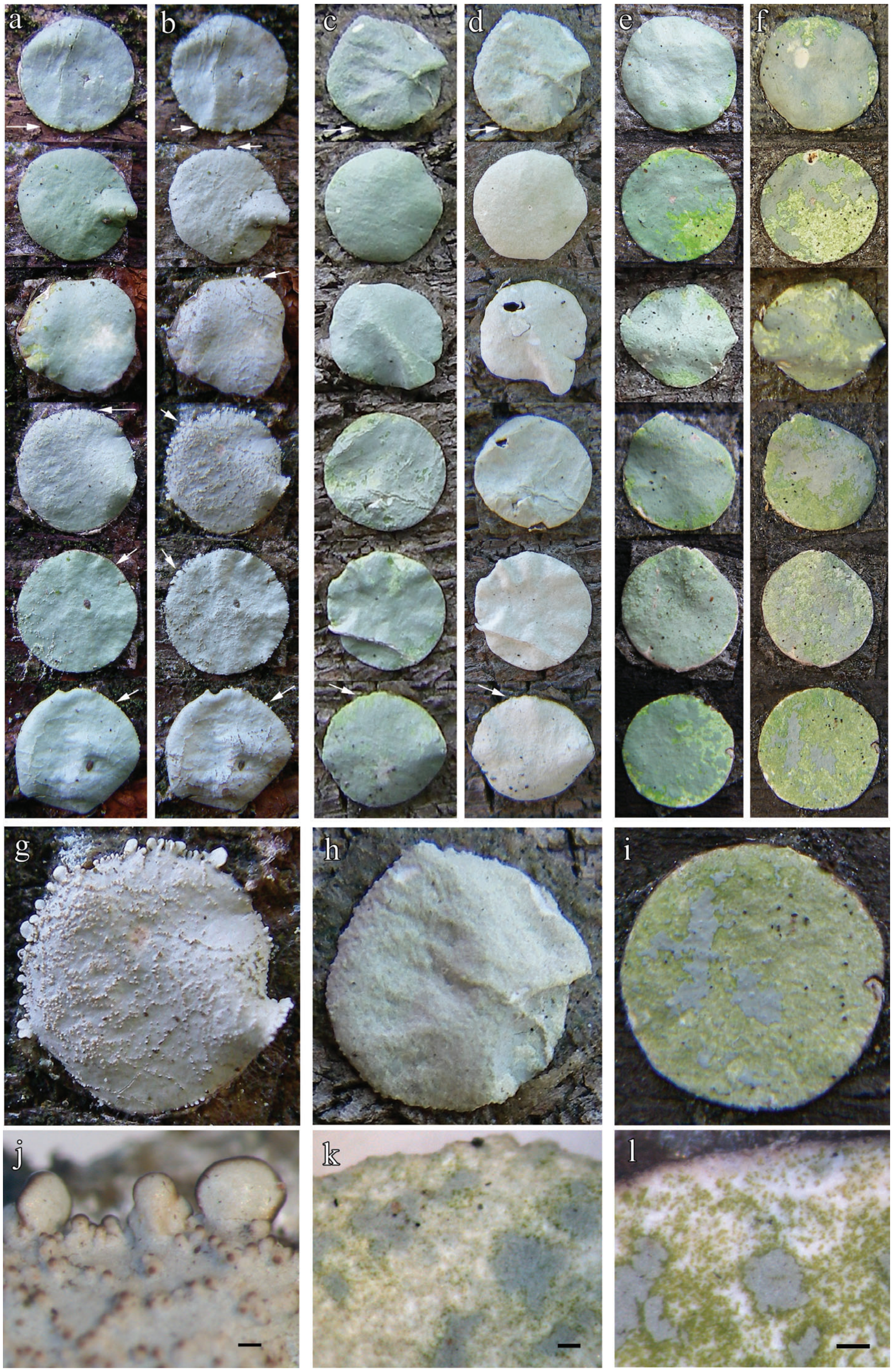

Fig. 3. Time-course changes in the morphology of the transplanted disks at the test sites. a, b, g, j. Umegaya-kita. c, d, h, k. Sakuragaoka. e, f, i, l. Shibukawa-naka. Disk numbers are I to VI from the top. a, c, e. Three-month exposure (Jul 31, 2007). b, d, f. Six-month exposure (Oct 30, 2007). g–l. Eight-month exposure (Dec 25, 2007) (g, j, disk IV; h, k, disk I; i, l, disk VI). a–d. Arrows indicate representative lobules or primordia of lobule. a–i. Diameter of disks is ca. 5 mm. j–l. Scale = 100 μm.

and VI at the site (Fig. 3b). However, the lobule primordia on disks I and VI at Sakuragaoka did not grow into lobules even after 8 months of exposure (Fig. 3d). The disintegration area of the upper cortex was expanded in all the disks at Sakuragaoka and Shibukawa-naka during the experimental period. All disks at Sakuragaoka showed damage to almost all the parts of their upper surface after four months of exposure, and most of their photobiont component was dead, except under the remaining cortex at the end of the experimental period (Fig. 3k). The disks transplanted at Shibukawa-naka were also heavily damaged at the cortex after 6 months of exposure, but much of the photobiont component on the exposed medulla of the disks seemed to be alive at the end of the experiment (Fig. 3l). Lobules, lobule primordia, or isidia-like protuberances were not found on the other disks at Sakuragaoka and Shibukawa-naka.

Lichen substances in transplanted thalli

Amounts (wt%) of atranorin and lecanoric acid in the dried transplanted disks are shown in Fig. 4. The values are those of each disk (I–VI) and the mean of them at the test sites. The amount of atranorin found in the disks ranged from 0.88% to 1.40% (1.11±0.19%; mean±SD) at Umegaya-kita, from 0.08% to 0.24% (0.16±0.06%) at Sakuragaoka, and from 0.21% to 0.61% (0.39±0.16%) at Shibukawa-naka. Differences between the mean values among the sites were statistically significant, as determined by ANOVA ($p<0.01$). All the disks at each site had significantly decreased amounts of atranorin, with the levels in Sakuragaoka being the highest, followed by those in Shibukawa-naka and Umegaya-kita.

The amount of lecanoric acid produced in the disks ranged from 33.4% to 35.8% (35.0±0.88%) at Umegaya-kita, 25.5% to 37.0% (31.5±4.91%) at Sakuragaoka, and 24.5% to 35.2% (31.5±4.47%) at Shibukawa-naka. The differences between the mean values of lecanoric acid among the sites were not statistically significant. Most of the transplanted disks in Sakuragaoka and Shibukawa-naka (7 out of 12) produced almost the same amount of lecanoric acid as that produced by the disks in Umegaya-kita (within±2.5%). However, the amount of lecanoric acid produced in disks II, III, and IV in Sakuragaoka and disks III and V in Shibukawa-naka were lower by more than 5.0% of that produced in Umegaya-kita.

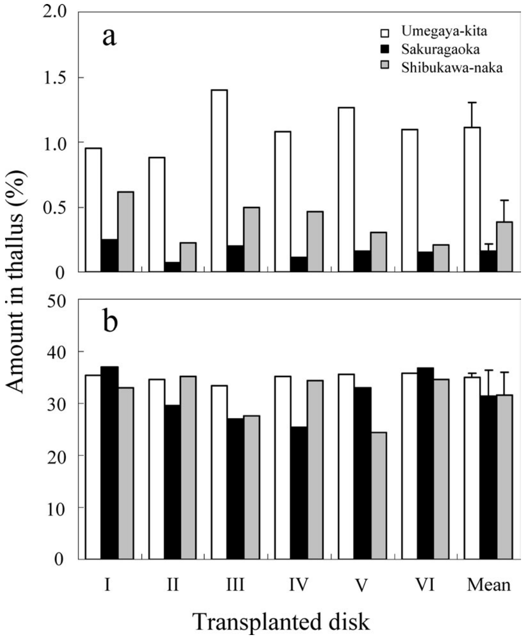

Fig. 4. Amounts (wt%) of lichen substances in the dried transplanted disks (I–VI) after 8-month exposure at the test sites with different pollution levels. a. Atranorin. b. Lecanoric acid. Mean±SD of the values for all disks at the site are also shown.

Discussion

All the disks of *P. tinctorum* that were transplanted in the clean site (Umegaya-kita) regenerated lobules, and the amounts of atranorin in the disks were significantly higher than those in the disks at the other sites after 8 months of transplantation. Although no previous studies have reported lobule formation within 6 months of exposure (Kon *et al.*, 2003; Kon and Ohmura, 2008), we observed the formation of small lobules three months after the exposure of the disks. The patterns of lobule formation were similar to those reported by Kon *et al.* (2003) and Kon and Ohmura (2008). The amounts of atranorin and lecanoric acid produced in the transplanted disks at the clean site were higher than those reported in previous studies (0.63% of a mixture of atranorin and chloroatranorin and 23.6–25.6% of lecanoric acid; Ahmann and Mathey, 1967).

All the disks in the polluted sites (Sakuragaoka and Shibukawa-naka) were damaged at the cortex, and the amounts of atranorin were significantly lower in these sites than in the clean site. We have reported these changes in transplanted *P. tinctorum* for the first time. Furthermore, the amounts of atranorin in the disks at Sakuragaoka were lower than those in the disks at Shibukawa-naka, and the photobiont component in the former was damaged to a greater extent than that in Shibukawa-naka. Since atranorin is deposited in the cortex of lichen thallus (Hale, 1983), the decrease in the amount of this substance in the disks at Sakuragaoka and Shibukawa-naka might be related with the disintegration of the cortex and death of the photobionts. Among all test sites, Sakuragaoka had the highest concentration of O_3 (22 ppb) in summer. Although the concentrations of NO_x, NO_2, and SO_2 were higher in Shibukawa-naka than in Sakuragaoka, the high levels of O_3 in Sakuragaoka may have resulted in more extensive damages to the cortex and photobiont at this site. O_3 has been thought to act synergistically in causing damage when combined with other pollutants such as NO_2 (Hale, 1983). One of the harmful effects of O_3 on lichens has been proven to be damage to the cell membrane (Conti and Cecchetti, 2001). However, the results of this study have not clarified the cause of cortex disintegration in *P. tinctorum*. Further experiments in controlled chambers are needed to confirm the harmful effects of O_3 on *P. tinctorum*. In addition, other pollutants such as hydroxyl radicals, heavy metals, and polycyclic aromatic hydrocarbons, which exert harmful effects on lichens (Hale, 1983; Modenesi, 1993; Kummerová *et al.*, 2005), also need to be investigated since they are generated in automobile exhaust. The amount of lichen substances, *e.g.*, atranorin and usnic acid, is known to vary in response to environmental stresses such as UV irradiation (BeGora and Fahselt, 2001). This study also suggests that the amount of atranorin in transplanted *P. tinctorum* may vary and indicate the influence of air pollution.

The differences among the sites with regard to the mean amount of lecanoric acid were not statistically significant. Decrease or absence of lecanoric acid in *P. tinctorum* has been observed in both urban and industrial areas (Nakagawa and Mitsugi, 1985; Nakagawa and Kobayashi, 1989; Hamada and Yamada, 1994; Bando and Sugino, 1997). However, this phenomenon has not been fully confirmed in this transplantation experiment. Lecanoric acid is deposited in the medulla of the lichen thallus (Ahmann and Mathey, 1967), while atranorin is deposited in the cortex. Since the cortex is directly exposed to air and attacked by various pollutants, the amounts of cortical substances may be more variable than those of medullary substances. In order to confirm the phenomenon of decrease in the amount of lecanoric acid in an air-polluted site, further experiments should be performed with longer exposure periods.

In summary, in our experiment, lobule formation was observed in a clean site and disintegration of cortex without lobule formation was observed in all disks at the polluted sites. In addition, the amounts of atranorin were lower in the polluted sites than in the clean site. These morphological and chemical differences appeared within a short period (6 to 8 months of exposures). Thus, this method of transplanting disks punched from the thallus of *P. tinctorum* can be useful for the evaluation of the morphological and chemical changes in this species with changes in air quality.

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