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Effects of copper ions on the growth and photosynthetic activity of *Scopelophila cataractae*

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Scopelophila cataractae is a rare species that grows in environments with high concentration of copper. We analyzed the relationship between plant growth and copper ion concentration in the rainwater in a field study and the relationship between photosynthetic activity and copper ion concentration in a laboratory. We surveyed six sites with *S. cataractae*; these sites had rainwater copper ion concentrations ranging from 16.8 to 29.2 ppm. We found that the growth area, gametophore size, and leaf size of *S. cataractae* decreased as copper ion concentration increased. We observed reduced photosynthetic activity at copper ion concentrations of 20.0 ppm or higher. We discovered that the growth of *S. cataractae* gametophores was adversely affected by copper ions at concentrations commonly observed under copper roofs. Thus, *S. cataractae* may inhabit areas with high concentrations of copper ion (~30 ppm) partly because of a selective advantage when interspecific competition is high. We recommend that copper roofs should be renewed partly rather than all at once, as aged copper roofs create environments with copper ion concentrations suitable for *S. cataractae*.

Keywords: copper moss, copper ion concentration, growth inhibition, delayed fluorescence, habitat conservation

Scopelophila cataractae (Mitt.) Broth. is regarded as a rare species because it is found in only a small number of unique habitats. In *S. cataractae*, sexual reproduction is very rare, yet the species maintains relatively high levels of genetic divergence among populations, despite its worldwide distribution, being found in Asia, North and Latin America and Europe (Rabinowitz 1981, Shaw 1995, Longton and Hederson 1999, Cleavitt 2005).

The habitats of the moss *S. cataractae* are environments in which copper is present at high concentrations, such as areas containing copper ore (Shaw 1987, Guerra et al. 2002, Thouvenot and Aubert 2016). In Japan, copper has been traditionally used in the construction of the elegant and beautiful patina roofs of shrines and temples (Suzuki and Koizumi 1994). *Scopelophila cataractae* is often observed growing under copper roofs in shrines and temples (Kitagawa 1987, Yamaoka 1993, 1995, 1996a, b, Deguchi and Yamaguchi 2010, Satake 2014, Yamaura and Higuchi 2017).

Scopelophila cataractae is a type of bryophyte known as a ‘copper moss’, so named as they commonly accumulate

high concentrations of copper within their bodies (Shaw 1987, 1995, Satake 2014). Although it is generally known that high concentrations of copper ions are toxic to plants (Yruela 2005), it is thought that *S. cataractae* is adapted to higher concentrations of copper ions than other plants (Satake 2014). Because copper is selectively accumulated in the cell walls of *S. cataractae*, it is sometimes thought that *S. cataractae* is copper-resistant, a trait made possible by its ability to isolate harmful copper ions (Satake 2014). However, it is unclear at what concentration copper ions begin to have adverse effects on the growth of gametophores within this species.

Therefore, the goals of this research are to clarify the effects of different concentrations of copper ions on the growth and photosynthetic activity of *S. cataractae* and to use this information to make suggestions to aid in the conservation of *S. cataractae* in shrines and temples.

Material and methods

Study sites

We identified nine shrines and temples located near Yoshida Shrine and Munetada Shrine in Kyoto City, Japan, where *Scopelophila cataractae* populations have previously been

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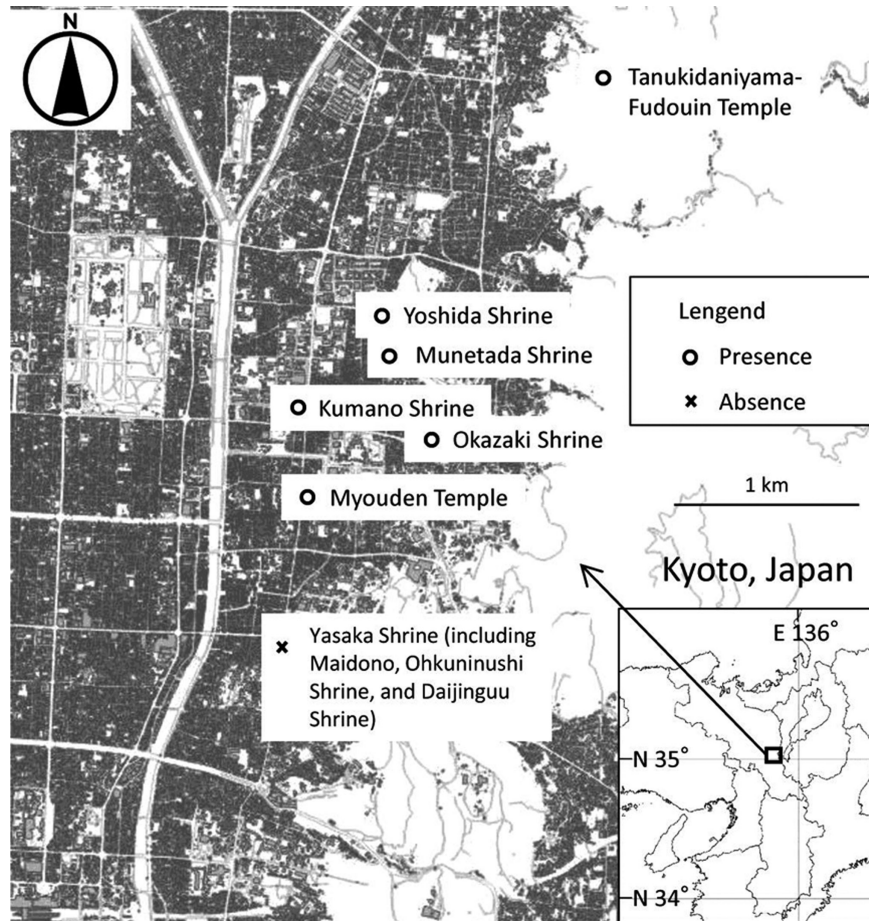


Figure 1. The location of study sites including six sites where *Sceloporphila cataractae* was present and the three sites where it was absent.

recorded (Kitagawa 1987) (Fig. 1). Three shrines where *S. cataractae* did not grow were also included as study sites to investigate whether copper ion concentrations in rainwater relate to the presence or absence of *S. cataractae*.

Sceloporphila cataractae inhabited where rainwater was supplied through copper roofs. At the five sites where *S. cataractae* was present, except for Kumano shrine, it grew on ditches or walls made of concrete or stones. At Kumano shrine, it grew on the soil. At the three sites in Yasaka shrine, where *S. cataractae* was absent, rainwater through copper roofs was similarly supplied to concrete or stone ditches or the soil.

Analysis of rainwater

We analyzed copper ion concentrations in rainwater because growth foundations of *S. cataractae* were mainly concrete and stones in our study sites, and also because bryophytes have not evolved roots and an efficient conducting system unlike vascular plants (Proctor 1982) and absorb water over their whole surface (Jones and Dolan 2012).

Rainwater was collected from copper roofs at Yoshida Shrine, Munetada Shrine, Okazaki Shrine, Kumano Shrine, Myouden Temple, Yasaka Shrine Maidono, Ohkuninushi Shrine and Daijinguu Shrine on 15 October 2017 and at Tanukidaniyama-Fudouin Temple on 14 November 2017.

The daily mean temperature and daily precipitation at Kyoto Local Meteorological Observatory in Kyoto City were 12.0°C and 18.0 mm day⁻¹ on 15 October 2017 and 17.3°C and 12.5 mm day⁻¹ on 14 November 2017. We collected rainwater using a disposable plastic container directly from the roof or from drains if rain gutters were present. Only water running directly over copper roofs was collected.

Immediately after collection, ten 20 µl droplets of the rainwater was accurately dropped onto a filter paper and dried at 50°C for 24 h or longer in a temperature-controlled oven, forming one sample. Two of these samples were prepared for each study site. The peak Cu element intensity contained in each sample was measured using an energy dispersive X-ray fluorescence spectrometer. Then, CuSO₄ (0.100 mg Cu ml⁻¹), a standard solution used in atomic absorption spectrometry, was diluted with deionized water to prepare an aqueous solution with copper ion concentrations of 10.0, 20.0 and 40.0 ppm. Peak intensity was measured to allow the preparation of a calibration curve, which was used to determine the copper ion concentration of each sample.

After taking mean ion concentration values for the two samples for each study site, Welch's t-test was applied to test for differences in the mean copper ion concentrations between the six sites where *S. cataractae* was present and the three sites where it was absent.

Analysis of the relationship between growing status and copper ion concentration

On 22 and 23 December 2017, the growth area of individuals of *S. cataractae* was measured and gametophores were collected to allow the measurement of gametophore size ($n=6$) and leaf length ($n=3$) at each study site using a stereo microscope. We defined the gametophore size as the whole length of a sample including rhizoids because rhizoids could not be distinguished clearly from the other body parts. Regarding the leaf length, a larger leaf near the top of a gametophore was measured. A simple linear regression analysis was applied to test if copper ion concentration in rainwater significantly explains the growing status of *S. cataractae*, i.e. whether it affects growth area, gametophore size, or leaf length. Growth area in sites where *S. cataractae* was absent were input as 0.0 (m^2) for data of the absent sites.

Analysis of the relationship between the condition of copper roofs and copper ion concentration

Because *S. cataractae* is often observed growing under copper roofs in shrines and temples (Kitagawa 1987, Yamaoka 1993, 1995, 1996a, b, Deguchi and Yamaguchi 2010, Satake 2014, Yamaura and Higuchi 2017), it is important to know the relationship between the condition of the copper roofs and copper ion concentration for the habitat conservation of this rare species. We hypothesized that the surface area of a copper roof (S), the length from the ridge to the eaves (L) and the roof age (the time since construction or replacement) affect copper ion concentration of rainwater. The structure of each copper roof was visually checked at each site. S and L , on which the collected rainwater was assumed to flow, were determined with the aid of Google Earth and QGIS ver. 2.14 Essen. For the Okazaki Shrine and Kumano Shrine sites, the collected rainwater seemed to have passed along multiple roofs. In these cases, measurements from the longest of these roofs was adopted as L . The roof age was obtained by interviewing persons associated with each site.

A multiple linear regression was applied to test which factors of the copper roofs significantly explained variation in copper ion concentrations in rainwater. S , L , and the roof age were used as explanatory variables and were selected by stepwise method based on F -values (criteria: inclusion if probability of F -value ≤ 0.05 , removal if probability of F -value ≥ 0.10).

Analysis of the relationship between photosynthetic activity and copper ion concentration

Delayed fluorescence (DF) is light that is emitted from green plants, algae and photosynthesizing bacteria after they have been exposed to light (Goltsev et al. 2009). DF is a common indicator used to evaluate the photosynthetic activity of plants, and is a more sensitive indicator of plant stress than chlorophyll fluorescence because the mechanism by which the excited singlet state of the photochemical system II (PS II) chlorophyll antenna pigment is achieved is different (Goltsev et al. 2009). In the case of chlorophyll fluorescence, the excited state of the antenna chlorophyll is achieved

by the direct absorption of light or by fast energy transfer from other chlorophylls. However, in the case of DF, it is achieved via back electron transfer and charge recombination in the reaction center of PS II. Thus, DF is more closely related to the entire photochemical processes. In addition, it has been demonstrated that DF could be an effective index when investigating the influence of heavy metals including copper on duckweeds, *Lemna minor* (Drinovec et al. 2004) and mercury on blue-green alga *Spirulina platensis* (Katsumata et al. 2005), and when conducting rapid ecotoxicological bioassay with green alga *Pseudokirchneriella subcapitata* (Katsumata et al. 2006). Therefore, we decided to evaluate the photosynthetic activity of *S. cataractae* under different copper ion concentrations using DF.

Fifty-four healthy gametophores of *S. cataractae* were collected in the field and stored in deionized water for 12 h in dark conditions. DF was measured with a 0.1 s interval using a photon detection unit TYPE-6100A (Hamamatsu Photonics K.K.) after excitation by red light having peak wavelength of 680 nm (corresponding to, or close to, the absorption bands of chlorophyll a and b) was applied for 1.0 s with the intensity of $16.7 \mu\text{mol m}^{-2} \text{s}^{-1}$ (maximum irradiation of the measurement apparatus, but not strong level that induces photoinhibition). The time length and the intensity of the irradiation were optimized by changing settings of the measurement apparatus in prior with a few samples. The integrated value of DF from 1.0 to 60.0 s (DFI) was recorded as the initial value, DFI₀. Subsequently, about half of the height of each specimen kept soak in CuSO_4 aqueous solution with copper ion concentrations of 0.0, 1.0, 10.0, 15.0, 20.0, 30.0, 50.0, 70.0 and 100.0 ppm ($n=6$ for each copper ion concentration level) for 24 h in a growth chamber (Drinovec et al. 2004). The environment of the chamber was set at room temperature ($21.0 \pm 0.2^\circ\text{C}$), relative humidity = $41.0 \pm 2.1\%$, and photosynthetically active radiation = $27.5 \mu\text{mol m}^{-2} \text{s}^{-1}$. A 12-h light and 12-h dark cycle was also implemented. After the treatment, DFI was taken, here referred to as DFI₁.

The DFI change rate, ΔDFI (DFI₁/DFI₀) was then calculated (Drinovec et al. 2004). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to detect significant differences between ΔDFI values in plants subjected to different copper ion concentration levels.

All the above statistical tests were conducted using SPSS Statistics ver. 23.0 (IBM Corp.) with a significance level of 5%.

Results

Copper ion concentration in rainwater

The copper ion concentrations in rainwater samples collected under copper roofs were significantly higher at the three sites where *Scopelophila cataractae* was absent when compared to the six sites where *S. cataractae* was present ($p=0.009$; Fig. 2). Mean copper ion concentrations ranged from 16.8 to 29.2 ppm in sites where *S. cataractae* was present and from 29.4 to 35.0 ppm in sites where *S. cataractae* was absent.

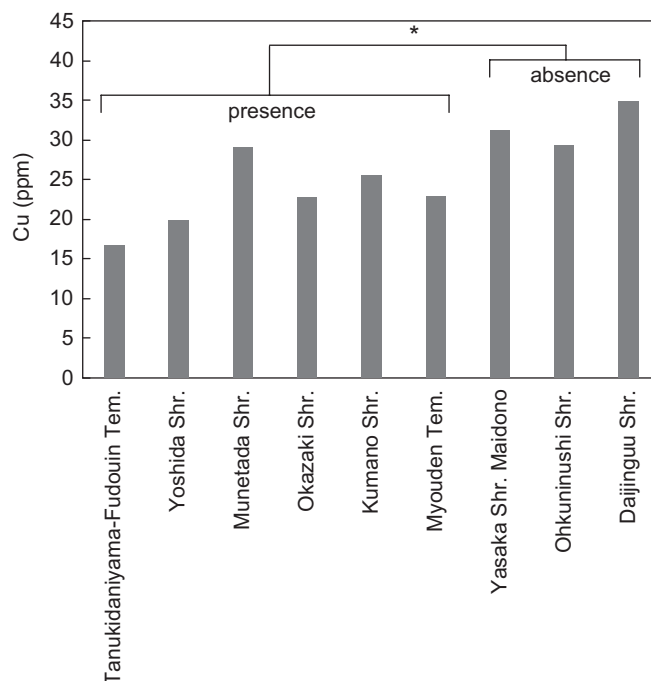


Figure 2. Copper ion concentrations in rainwater determined by the energy dispersive X-ray fluorescence spectrometry.

Relationship between growing status and copper ion concentration

Based on simple linear regression analyses, the copper ion concentration of rainwater was a statistically significant predictor of growth area ($p=0.004$, $R^2=0.71$), gametophore size ($p<0.001$, $R^2=0.46$), and leaf length ($p<0.001$, $R^2=0.60$). The growth area, gametophore size and leaf length decreased as copper ion concentration in rainwater increased (Fig. 3–5).

Relationship between condition of copper roofs and copper ion concentration

The multiple linear regression analysis using the stepwise method identified only roof age as a significant variable in explaining variation in rainwater copper ion concentration ($p=0.008$, $R^2=0.65$); S and L were not selected. Copper ion concentrations in rainwater sampled from older roofs were lower when compared to ion concentrations in water samples taken from newer roofs (Fig. 6).

Relationship between photosynthetic activity and copper ion concentration

Δ DFI decreased in an S-shape manner as copper ion concentration increased (Fig. 7). Δ DFI of the control group (copper ion concentration = 0 ppm) was 1.20 ± 0.46 (mean \pm SD). The effect of copper ion concentration level was statistically significant in ANOVA ($p<0.001$). DFI values from the treatment groups exposed to copper ion concentrations of 1.0, 10.0, 15.0, 20.0, 30.0, 50.0, 70.0 or 100.0 ppm for 24 h were all significantly lower than the control group ($p<0.03$). When compared to 1.0 ppm, the Δ DFI of plants exposed to concentrations of 20.0 ppm or higher was significantly lower ($p<0.04$). The DFI value halved at a concentration

between 10.0 and 15.0 ppm. The Δ DFI at concentrations of 50.0 ppm or higher were less than 0.01, and gametophores in these treatment groups turned visibly brown in color.

Discussion

Scopelophila cataractae is largely restricted to soils or other man-made substrata with high levels of heavy metal concentrations (Shaw 1987, Guerra et al. 2002, Thouvenot and Aubert 2016). In Japan, it is always discovered on sites where copper ions are eluted such as under a copper roof, under a bronze statue or at an ore deposit containing copper (Satake 2014). In the present study, *S. cataractae* was observed at

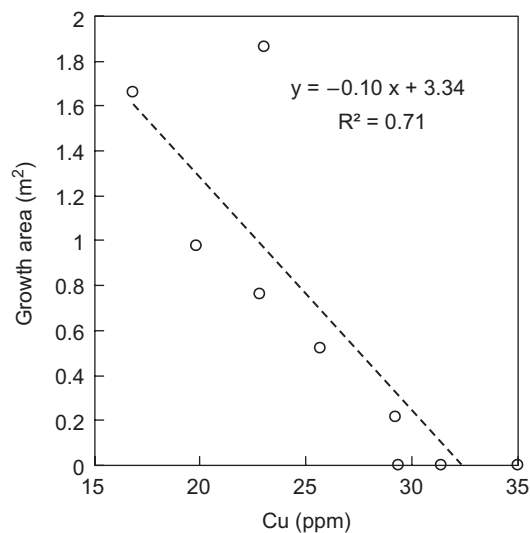


Figure 3. Relationship between copper ion concentration in rainwater and growth area of *Scopelophila cataractae*.

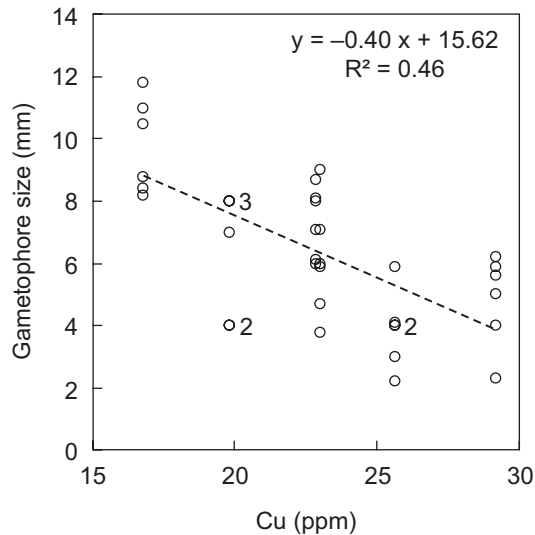


Figure 4. Relationship between copper ion concentration in rainwater and gametophore size of *Scopelophila cataractae*. The number beside the data point shows number of samples overlaid at the same data point.

the six sites where rainwater contained copper ion concentrations ranging from 16.8 to 29.2 ppm. In these sites, the growth area of *S. cataractae* decreased (Fig. 3) and the gametophores and leaves of *S. cataractae* became increasingly smaller (Fig. 4, 5) as the copper ion concentration in rainwater runoff from copper roofs increased. These results suggest that copper ions at concentrations from 16.8 to 29.2 ppm have adverse effects on the growth of this species.

This was supported by the results of the experiment in which *S. cataractae* individuals were exposed to different copper ion concentrations. Photosynthetic activity as indicated by DF was significantly reduced at copper concentrations of 1.0 ppm or higher when compared to the control (0.0 ppm), and DF values of plants exposed to copper concentrations

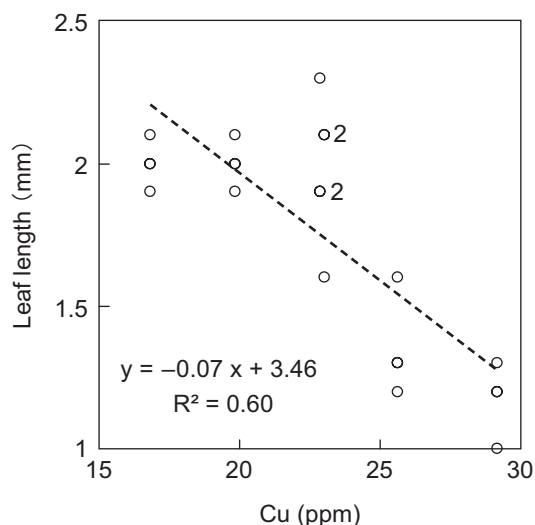


Figure 5. Relationship between copper ion concentration in rainwater and leaf length of *Scopelophila cataractae*. The number beside the data point shows number of samples overlaid at the same data point.

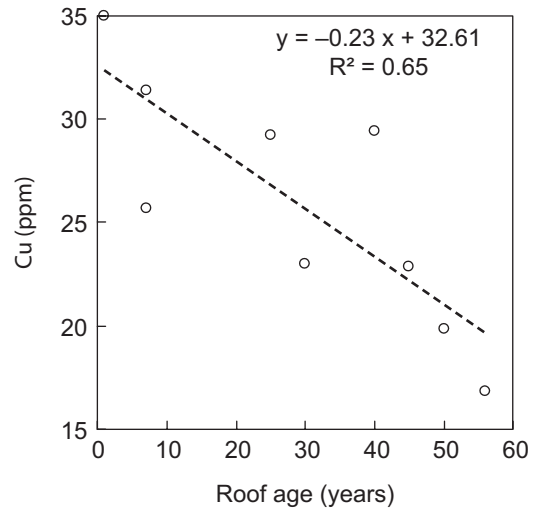


Figure 6. Relationship between number of years after construction or replacement of a copper roof and copper ion concentration in rainwater.

of 20.0 ppm or higher were significantly lower than those of plants exposed to copper ion concentrations of 1.0 ppm.

As the copper ion concentration was often higher than 20.0 ppm under copper roofs (Fig. 2), it is likely that photosynthetic activity in this species is frequently inhibited in these habitats. Nomura and Hasegawa (2011) argued importance of copper-sensitive asexual reproduction in the unique life strategy of *S. cataractae* because the moss with high concentrations of copper suppressed gemma formation but promoted protonemal growth. However, our results suggest that the gametophore of *S. cataractae* is not necessarily copper-preferring in terms of photosynthetic activity. DFI values in exposed *S. cataractae* halved between

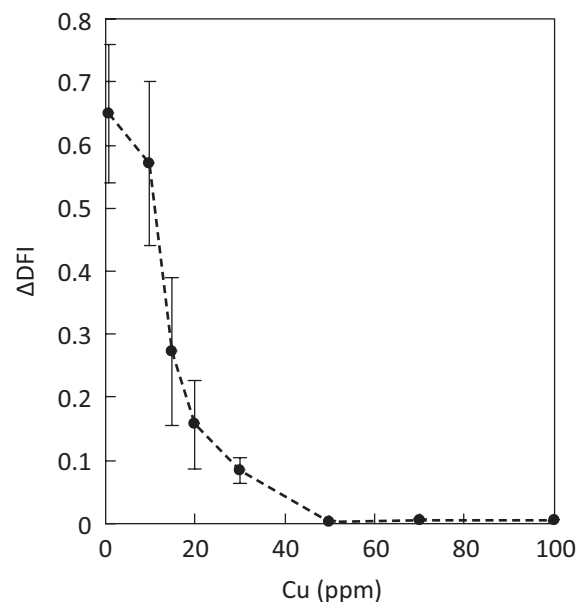


Figure 7. Change rate of integrated value of delayed fluorescence from 1.0 to 60.0 s (Δ DFI) after samples were exposed to different copper ion concentrations via CuSO_4 aqueous solution for 24 h ($n=6$ for each copper ion concentration level, mean \pm SD, the results for 0.0 ppm shown in text).

copper ion concentrations of 10.0–15.0 ppm (Fig. 7), whereas DFI of *L. minor* was halved at concentrations as low as 2.0 ± 0.2 ppm (Drinovec et al. 2004). This indicates that *S. cataractae* has a higher copper ion tolerance compared to *L. minor*. Based on this, we suggest that *S. cataractae* grows under relatively high copper ion conditions because it is advantageous when interspecific competition is high.

However, because *S. cataractae* was absent at the three sites where rainwater contains high concentrations of copper ions (from 29.4 to 35.0 ppm), when copper ion concentrations are too high (likely over 30 ppm), it becomes difficult for populations of *S. cataractae* to survive. The concentration of copper ions in rainwater runoff from copper roofs decreased as roof age increased (Fig. 6). As a result of aging, copper roofs produce a patina and the number of eluted copper ions decreases (Krätschmer et al. 2002). Thus, the aged copper roofs provide a more suitable habitat for *S. cataractae*. From the viewpoint of conserving the habitat of *S. cataractae*, it is recommended that roofs should not be completely replaced to prevent sharp rises in copper ion concentrations. The formation of a natural patina takes about ten years (Takeuchi et al. 2004), and so a long-term plan for renewing copper roofs is needed. Furthermore, copper roofs are frequently replaced with corrosion-resistant titanium roofs to protect against acid rain (Kimura 2000). The replacement of copper roofs in this way should be carefully monitored when attempting to conserve this species.

Conclusion

This is the first example of research which has investigated the effects of copper ions on the growth and photosynthetic activity of *S. cataractae* gametophores. We analyzed the relationship between growing status and copper ion concentration in rainwater in the field, whilst the relationship between photosynthetic activity and copper ion concentration was analyzed by laboratory experiment. Although this study has limitation in sample size and rainwater collection period, we obtained consistent results between the field observation and the laboratory experiment. At the six sites where *S. cataractae* was present and rainwater contained copper ion concentrations between 16.8 and 29.2 ppm, the growth area, gametophore size and leaf size of *S. cataractae* decreased as copper ion concentration increased. Photosynthetic activity as indicated by DF was significantly reduced at ion concentrations of 20.0 ppm or higher when compared to plants exposed to 1.0 ppm ion concentrations. *S. cataractae* was absent at the three sites where rainwater contained copper ions at high concentrations (29.4–35.0 ppm), and so it is likely that copper ion concentrations upwards of 30 ppm are too high to be tolerated by *S. cataractae*.

We found that *S. cataractae* is copper-resistant in terms of photosynthetic activity of gametophores, and can grow under relatively high copper ion conditions up to about 30 ppm although this threshold is required to be confirmed by further researches. Growing in areas where copper ion concentration is high likely pertains some advantage when interspecific competition is high. For the conservation of this species, we recommend that copper roofs are not replaced all at once to limit copper ion concentrations and that the replacement of copper roofs with titanium roofs is avoided.

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