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Mannitol can mitigate negative effects of simulated acid mist and fluoranthene in juvenile Japanese red pine (*P. densiflora* Sieb. et Zucc.)

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ABSTRACT

The negative health effects of simulated acid mists and fluoranthene on juvenile Japanese red pine were investigated, and the methods of protection from these pollutants were examined. The needle gas exchange, chlorophyll fluorescence, chemical contents and visual damage to needles caused by acid mist applied alone or its conjunction with fluoranthene were investigated over 60 d and 20 d, respectively. Acid mist at pH 2 and 3 caused physiological and visual damage, which was enhanced by the addition of fluoranthene to the mist. However, fluoranthene and acid mist at pH 4 and 5 showed only minor effects. These findings indicate that acid mist may be more harmful to pine trees if it occurs in conjunction with polycyclic aromatic hydrocarbons. Moreover, suppression of the singular and additive effects of these compounds was achieved using mannitol, which may be widely applicable to suppression of reactive oxygen species-mediated plant damage.

1. Introduction

Acid deposition may be in the form of acid rain, snow, fog, humidity, and acid gas and dust (acidity < pH 5.6). Because human–industrial activity produces additional acid-forming compounds in far greater quantities than natural sources of acidity, wet deposition with pH values of 3.0 or lower have been reported. Highly acidic deposition can cause many problems, ranging from killing freshwater fish and damage to crops, to erosion of buildings and monuments (Casiday and Frey, 1998). Additionally, acid deposition alters soil chemistry, nutrient availability, and plant growth; thus, its effects on terrestrial ecosystems may be chronic and accumulative (Shan, 1998), leading to trees and shrubs being weakened and becoming more vulnerable to insects, diseases, and fungal infestations (Eamus and Fowler, 1990; USEPA, 2010).

Automobile exhaust is a large source of atmospheric PAHs. Fluoranthene is a typical polycyclic aromatic hydrocarbon (PAH) emitted in automobile exhaust and one of most abundant PAHs in the atmosphere of Japan and other countries (Poor et al., 2004; Gocht et al., 2007; Tham et al., 2007). Many of our previous studies have clearly indicated significant physiological effects of PAHs to plant. Fluoranthene applied in mist to the surfaces of needles and leaves of some higher plants (including Japanese red pine) produced negative effects after short- and long-term periods (Oguntimehin and Sakugawa, 2008, 2009; Oguntimehin et al., 2010a). Some other studies have also examined the effects of PAHs on the foliar (above-ground) regions of other terrestrial plants with similar results (Edwards, 1983; Huang et al., 1996).

Japanese red pine is one of the most widespread and typical species of vegetation in Japan, and red pine forests are the predominant ecosystems in low lying areas of Hiroshima prefecture and other Chugoku district prefectures. It is well known that Japanese red pine forests in prefectures of the Chugoku District have been declining during the last few decades (Kume et al., 2000). Acid deposition and air pollution may be responsible for the decline of these red pine forests (Naemura et al., 1996; Nakane et al., 2000). Our previous studies have established the significances of aqueous phase (rain, fog and dew) chemistry on the surfaces of plant leaves, where reactive oxygen species (ROS) would be generated and reduce photosynthetic activities (Kume et al., 2001; Kobayashi et al., 2002; Chiwa et al., 2003; Nakatani et al., 2007; Sakugawa et al., 2011).

In the present study, we evaluated the short-term (1–2 months) effects of acid mists and fluoranthene deposition on Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) using the physiological, ecological and visible foliar assessments of the plant in chamber conditions. Especially, we attempted to elucidate the combined ecophysiological effects of PAHs and acid mist on Japanese red pine seedlings. This study was divided into two stages. In the first stage, the effects of acid mists on Japanese red pine were investigated. In

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the second phase, the effects of fluoranthene applied in conjunction with acid on the conifer were evaluated. Specifically, the combined treatments were investigated to determine if they exerted additive or synergistic effects, or alleviated the effects of the acid precipitation. Additionally, the effects of mannitol on the aforementioned treatments were also investigated. Oguntimehin and Sakugawa (2008) reported that damage to Japanese red pine conifers caused by fluoranthene could be mitigated by spraying the plants with mannitol. Therefore, the mitigation effects of mannitol on acid mist and fluoranthene treatments were our special concerns to be investigated in this study.

2. Materials and methods

2.1. Plant, soil and growth conditions

Two-year-old Japanese red pine seedlings grown in a nursery at Fukuoka prefecture were purchased and transplanted into 0.35 m by 0.3 m deep pots (one seedling per pot) in the spring of 2007 and 2008. The pots were filled with 21 L of a mixture of yellow sandy soil (weathered granite '*Masado*'), perlite (white loam 4–20 mm, Toho-Leo Co.), isolite (CG2, Isolite Insulating Products Co.) and humus (Midori-Sangyo Inc.) at a volumetric ratio of 11:2:2:4. Tree leaf litter composed of healthy pine woodland collected from the university campus was spread over the soil surface of each pot (approximately 50 g per pot). The pots were then watered with de-ionized water once a day by 07:00 using an auto-irrigation system. The soil water potential was monitored using a soil tension meter (DM-8, Takemura Denki Seisakusho, Tokyo) installed inside one pot in each chamber. Three months after transplanting the pine seedlings into pots, nutrient solution (N/P/K = 6:10:5; Hyponex, Murakami Bussan, Tokyo, Japan) was added monthly at 1 mL of concentrated nutrient solution per 500 mL of Milli-Q water per pot. Pine seedlings were rotated bi-monthly in the 2007 experiment and weekly in the 2008 experiment within a greenhouse during the exposures to nullify any bias due to the relative position of the greenhouse.

The greenhouse (3 × 16 × 3 m height) used to cultivate the plants has been described elsewhere (Oguntimehin and Sakugawa, 2008). The upper halves of the framed house (excluding the two ends) were covered with transparent ethylene–tetra fluoro ethylene copolymer film (ETFE) (F-CLEANs, Asahi Glass Green-Tech Co. Ltd., Japan), which allows maximum ultraviolet light transmission (over 95% sunshine transparency). The differences between the meteorological conditions inside and outside of the greenhouse were negligible during plant growth and exposure. The mean photosynthetic photon flux density (PPFD) measured using an LI-190SA Quantum Sensor (Licor, USA) revealed that at noon on a typical sunny day of measurement during the study period, the PPFD incident on the foliage of the pine seedlings ranged between 1103 and 1227 μmol m^{-2} s^{-1}. The mean air temperature and relative air humidity logged by a Thermo recorder TR-72S (T&D Corp., Japan) in the greenhouse from 1 June to 31 July 2007 were 28.5 °C and 78.5%, respectively (corresponding to a vapor pressure deficit – VPD of 5.64), which were similar to the values obtained from 25 April to 20 June 2008, when the mean air temperature inside the chambers was 26.7 °C and the mean relative air humidity was 73.1% (corresponding to a VPD of 5.68).

2.2. Solution preparations and exposures

Fluoranthene was purchased from Sigma–Aldrich (USA) and used without further purification. A stock solution of 1 mM fluoranthene was prepared in 50% aqueous acetone solution (Wako Pure Chemical Industries, Japan).

For spraying applications, the stock solution was diluted to a final concentration of 10 μM fluoranthene with Milli-Q water (hereafter referred to as F). This final concentration of fluoranthene is comparable to the minimal concentration used elsewhere for exposure experiments to plants (Huang et al., 1996; Kummerova and Kmentova, 2004; Oguntimehin et al., 2008). The final concentration of acetone in the solution was about 0.5%, which was assumed to be harmless to the normal health status of the plant (Oguntimehin et al., 2008). The concentration of fluoranthene in the final solutions was determined based on the method described by the USEPA (1990) using HPLC with a fluorescence detector.

A stock solution of 0.1 M H_2SO_4 (Nacalai Tesque Inc., Kyoto Japan) was prepared by adding 1.53 mL of the acid into 150 mL of Milli-Q water and then diluting the solution to 200 mL in standard flasks. Aliquots of the stocks of the acid were diluted in Milli-Q water to the target pHs of 2, 3, 4 and 5 (20 °C) inside a 2 L beaker.

The simulated acids and fluoranthene solutions were applied to the foliage of pine seedlings using safe environmental practices through an electronic spray machine with a nozzle (BS-4000, Fujiwara Sangyo, Miki, Japan) in the early morning (06:00–07:00) 3 days a week. Since the exposures targeted the above ground portions of the plants, soil surfaces were covered with corrugated aluminum water proof sheets during application of the treatments to prevent excess liquids from entering the root portions. Where more than one solution was sprayed on the plant, the treatments were applied individually and allowed to dry before the next application.

Each treatment group was made of five healthy pine seedlings that were carefully selected. Two exposure experiments were conducted in this study to examine the effects of acid mist and fluoranthene on pine needles. The first experiment was conducted during August–October 2007 (60 days). The treatments in the first experiment consisted of the following: Control 'C' (Milli-Q water only) and "S" (H_2SO_4 at pH 2 and 3) with and without mannitol treatment 'M'. Overall, there were five treatments, C, pH2S, pH2S + M, pH3S and pH3S + M. During treatment, mannitol solution (1 mM) was pre-applied to the pine needles and allowed to dry naturally for ~30 min.

The second experiment was carried out during May–June 2008 (20 d). In the second experiment, the following treatments were applied: 'C' (Milli-Q water only) and "S", (H_2SO_4 at pH 2, 3, 4 and 5) with and without F and M. Overall, there were 12 treatments, C, pH2S, pH2S + F, pH2S + F + M, pH3S, pH3S + F, pH3S + F + M, pH4S, pH4S + F, pH4S + F + M, pH5S + F and pH5S + F + M. Mannitol and fluoranthene were pre-applied separately as described above. A pH5S treatment was not applied in this study because a preliminary study revealed almost no difference between pH5S and C treated plants (data not shown). Neither F nor M were applied alone in this study. In our previous studies these treatments have been examined for the red pine seedlings (Oguntimehin et al., 2008; Oguntimehin and Sakugawa, 2008, 2009). In these studies, decreased gas exchange rate, chlorophyll and rubisco contents and visible injury of pine needles were observed in the F alone treatment, but almost no difference between the M alone and C treated plants was observed.

2.3. Gas exchange measurement, chlorophyll fluorescence analysis and needle chemical content

Carbon dioxide and water vapor exchange measurements were conducted on the 1-yr-old needles. Each seedling was assessed between 06:00 and 09:30. The photosynthesis rate at saturated irradiance (A_{max}), stomata conductance (g_s) and intercellular/interstitial CO_2 concentration (C_i) were determined for three pairs of intact 1-yr-old needles of each seedling per treatment. Measurement procedures for the A_{max}, g_s and C_i were reported in our previous papers (Kume et al., 2001; Kobayashi et al., 2002; Nakatani et al., 2007). Briefly, A_{max}, g_s and C_i were measured at a saturating irradiance of 1500 μmol m^{-2} s^{-1} PPFD and a needle temperature of 24 ± 2 °C. The leaf to air vapor pressure deficit was maintained between 0.80 and 1.32 kPa. The reference CO_2 concentration of the air entering the leaf chamber was fixed at 370 μmol CO_2 $mol^{-1}$and a flow rate of 500 μmol s^{-1} using an open-flow infra-red gas analyzer with light and temperature control systems (LI-6400, Li-cor Inc., Lincoln, NE, USA). After each measurement, the pine needles used in the measurements were harvested, and their width and length were measured using a digital vernier caliper (CD-15, Mitutoyo Co., Kanagawa, Japan). The cross-section of the needle was approximated as a semi-circle, with a diameter equal to the measured width. In addition, half of the leaf surface area of the needle was used to calculate the effective leaf area, which was used to determine the A_{max}, g_s and needle dry mass per unit area (NMA) (Oguntimehin et al., 2008). Chlorophyll fluorescence was measured at night (19:00–20:00) with a portable chlorophyll fluorometer (MINI-PAM, Heinz Walz GmbH, Effeltrich, Germany) equipped with a leaf-clip holder 2030B (Heinz Walz GmbH, Germany). Needles were arranged compactly in a parallel array and clamped with the holder to measure the initial chlorophyll fluorescence (F_0), maximum chlorophyll fluorescence (F_m), and photochemical efficiency of PS II in the dark (F_v/F_m, where F_v represents the variable fluorescence).

The concentrations of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and chlorophylls *a* + *b* (Chla + *b*) were determined by N,N-dimethyl formamide extraction of 100 mg of needles that were collected from close to those used for the gas exchange measurements. Absorption of the extract was measured at 663.8 and 646.8 nm and the concentrations of the chemical contents were calculated using equations described elsewhere (Porra et al., 1989).

2.4. Calculating the crown surface area of Japanese red pine seedlings

The crown surface area of two-year-old Japanese red pine seedlings was estimated from the crown diameter and length using the method described by Brack and Wood (1998). If we assume that the crown shape as a solid can be appropriated to a Japanese red pine seedling crown, a conic shaped crown surface area can be determined using the following equation:

$$C_A = \mathrm{pi} * d_b L/2 \quad (1)$$

where,

C_A = the crown surface area (m^2);
d_b = the diameter at the base of the crown (m);
L = sloping length from the apex to the base of the crown (m).

Using the mean ± standard deviation obtained from measurement of 24 *P. densiflora* seedlings in August, 2007 gave values of 0.5 ± 0.1, 0.5 ± 0.15 and 0.4 ± 0.2 for d_b, L and C_A, respectively. The width or diameter at the base of the crown