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Physiological Characteristics of Pine Trees Growing in Automobile Exhaust Gas Pollution

By

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Summary

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The physiological characteristics of Japanese black pine (*Pinus thunbergii*) trees growing alongside roads with heavy traffic (polluted) were compared with those of trees growing away from the roads (control). The needles were directly exposed to the pollution, their surfaces were remarkably polluted and their stomata were severely clogged with particulate matter (PM) and fine dust. Polluted needles showed early defoliation two or three years after sprouting and oxidative responses in ascorbic acid and glutathione metabolism. But at the same time, the needles showed a strong reductive activity. Some characteristics relating to these phenomena were analysed.

Introduction

Japanese black pine (*Pinus thunbergii*) is widely distributed in Japan under various environmental conditions. This plant is frequently seen growing along the sides of highways or roads with heavy traffic. Under these environmental conditions, pine trees are exposed to automobile exhaust gas pollution, and show various physiological characteristics different from those of plants growing away from the traffic. Although Japanese black pine is highly tolerant to salt stress and desiccation, it seems to be sensitive to air pollution which results in a reduction in radial tree growth (HIRANO & MORIMOTO 1999). The effects of environmental pollution on pine trees have been widely investigated (HIRANO & MORIMOTO 1999, VENCLOVIENE & al. 2003, KUME & al. 2001, KAGAMIMORI & al. 1990), and many responses of pine trees to their environment have been reported. We have also in-

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investigated the effect of air pollution on *Pinus*. Our aim was to find an easy method to monitor the physiological status of pine trees. This report deals with some physiological characteristics of the needles of *Pinus thunbergii* growing along the sides of highways or roads with heavy traffic.

Material and Methods

Plant material

About 10 to 20-year-old, approximately 8 to 12 meter tall, Japanese black pine (*Pinus thunbergii*) trees were selected from natural areas and highway road sides around Numazu city, Japan. The road side trees were regarded as polluted, and the plants from natural areas were regarded as the control. Small trees (3 to 6 years old) cultivated in pots (40x40x30cm) were also used for artificial treatment with diesel exhaust gas.

Enzyme and chemical analyses

Crude enzymes were prepared from 3g of fresh pine needle tissue which was homogenized using a polytron homogeniser (Kinematica, Switzerland) with 10 ml of 0.1 M phosphate buffer. The crude extract was centrifuged at 4 °C for 5 min at 12,000 rpm and the supernatant was collected. One portion of the crude extract was fractionated by gel filtration using a Sephadex-G25 column (Sephadex PD-10, Pharmacia), and the high molecular weight fraction was used as the crude enzyme.

Peroxidase activity was measured using guaiacol as the substrate. The assay medium contained 10 ml of 0.1 M phosphate potassium buffer (pH 7.0), 50 µl of 0.2M guaiacol, 100 µl of 0.5 % H₂O₂ and 25 µl of crude enzyme solution. Activity was assayed spectrophotometrically at 470 nm, and a change in IAbs was calculated as 1 unit.

Peroxidase inhibiting activity was measured by the following equation.

$$A_{470} (0.1 \text{ U POD} + \text{Pinus extract}) / A_{470} (0.1 \text{ U POD, without Pinus extract}) \times 100 (\%)$$

Ascorbic acid was measured according to ARAKAWA & al. 1981. Pine needles were cut into 3mm sections, 0.5 g (FW) was homogenized with 5% trichloro acetic acid using a polytron homogeniser, centrifuged at 4 °C for 5 min at 12,000 rpm, and the supernatant was analysed.

Reduced ascorbic acid was assayed by mixing 1 ml of x10 diluted sample with 5% TCA, 1ml of 5% TCA, 1ml of ethyl alcohol, 0.5 ml of H₃PO₄-EtOH to make 5 ml, 1 ml of 0.5% 4,7-diphenyl-1,10-phenanthroline (Bathophenanthroline, BP) – EtOH, and 0.5 ml of 0.03% FeCl₂-EtOH. The activity was assayed spectrophotometrically at 534 nm after the assay solution was allowed to stand at 30°C for 90 min.

Total ascorbic acid (AsA plus DAsA) was basically determined using the above method but with the reduction of DAsA by 0.5 ml of 60 mg% DTT-EtOH and 0.5 ml of 0.2M Na₂HPO₄ – 1.2N-NaOH solution to adjust the solution's pH value to 7-8. Then the solution was allowed to stand at room temperature for 10 min for DAsA reduction to AsA. After reduction, an 0.5 ml of 240 mg% N-methylmaleimide-EtOH was added to block an excess of reducing reagent. Then, 0.5 ml of 20% TCA was added to adjust the final pH to 1-2 in order to develop a suitable colour formation. The following procedures for colour formation are the same as those for AsA determination.

Glutathione was measured according to Grace (GRACE & ROGAN 1996) by the cycling method (GRIFFITH 1980). Glutathione was extracted with 7% sulfosalicylic acid. Total glutathione was determined spectrophotometrically at 412 nm as described by Griffith (GRIFFITH 1980). A portion of the extract was neutralized by a 30-fold dilution in 0.5 M KH₂PO₄, 6.3 mM EDTA, pH 7.6 and subsequently assayed in the presence of 1.2 mM 5,5'-dithiobis-(2-nitrobenzoic acid) and 0.2 mM NADPH. The reaction was started by the addition of 0.2 unit of GR (from yeast, Boehringer Mannheim) in a total volume of 2 ml. All values are expressed as GSH equivalents, determined from a standard curve.

Fluorescence measurements. The needles were separated from the branch, immersed in 100ml of deionized water in 300ml flasks, and shaken for 1 hr, then fluorescence was measured using a fluorometer with excitation at 310 nm and emission at 425nm.

Results and Discussion

Characteristics of pine needles

The needle surfaces and stomata sampled from control trees were clean, but those from polluted trees were severely clogged with PM (particulate matter) and fine dust, especially one-year-old needles (Fig. 1). For two-year-old polluted needles, amounts of PM and fine dust were reduced because the needle surfaces were easily washed by rain due to epicuticular wax deterioration. The polluted needles contained higher concentrations of various organic compounds. Severe needle defoliation was observed in polluted trees. For the control trees, almost no defoliation was observed in one- and two-year-old needles, and 20% and 60% of the three- and four-year-old needles were defoliated, respectively. On the other hand, for the polluted trees, about 20% of the 2-year-old needles, and most of the three- and four-year-old needles were defoliated.

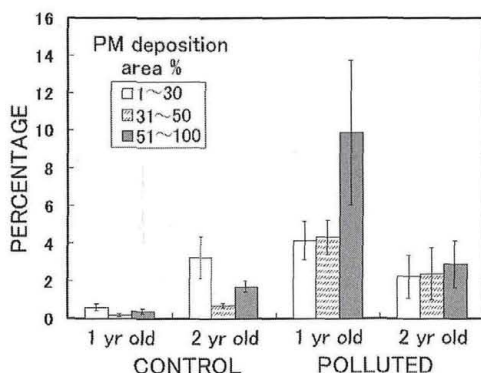


Fig. 1. Effect of particulate matter deposition on stomata. The percentages of the deposited area on the stomata were classified as: 1-30%, 31-50%, and 51-100%.

The physiological mechanism leading to defoliation is not fully understood because the internal response to air pollution appears after a long time. Some reports indicate a close relationship between needle longevity and the internal abnormality induced by pollution. For example, decreased needle longevity was shown to be closely related to increased heavy metal concentrations (LAMPPI & HUTTUNEN 2003). Environmental pollution seems to induce a specific physiological response, but the degree of the response is not clear when comparing to plants grown under normal conditions. KUME & al. 2001 reported that ethylene emissions did not increase evenly in polluted areas, but the frequency of trees emitting high

levels of ethylene increased (KUME & al. 2001). Such response variations make it difficult to study the effects of air pollution on plants growing under natural conditions.

Antioxidative stress relating metabolism

Higher ascorbic acid levels were detected in polluted needles compared to control needles (Fig. 2, left). Ascorbic acid in the control needles was mostly in the reduced form, but in the polluted needles, about 1/4 was in the oxidized form. The same tendency was observed for glutathione (Fig. 2, right). In the control needles, glutathione was mostly in the reduced form (GSH), but in the polluted needles, a large amount of the oxidized form (GSSG) was detected. Likewise, under UV-B radiation, the degradation of total glutathione, and a bigger proportion of oxidized glutathione, GSSG, was observed (LAAKSO 1999). The importance of the glutathione-ascorbate pathway in the defense mechanism (FOYER & al. 1994) is widely accepted, and this also acts as the defense mechanism in *Pinus thunbergii*.

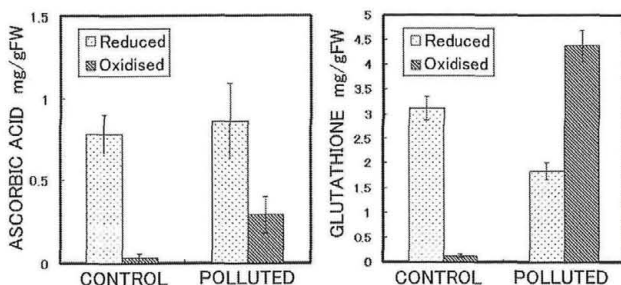


Fig. 2. Effects of air pollution on the composition of the antioxidative compound ascorbic acid and glutathione in the needles of black pine.

Peroxidase is also involved in the antioxidative pathway and detoxifies oxygen radicals. This enzyme was reported to show a positive response to environmental stress (SCALET & al. 1995). In the case of *Pinus thunbergii*, our results revealed that peroxidase activity was quite difficult to detect in polluted needles. In the case of control needles, peroxidase activity was easily detected, but in polluted needles, peroxidase activity was low or undetectable. The addition of hydrogen peroxide to the reaction solution enhanced the development of coloration in the guaiacol peroxidase reaction, but this faded with time. Our results indicate that the presence of a reductive compound induced the same results. Addition of the biologically active antioxidants, ascorbic acid and glutathione, to the assay solution induced the inhibition of peroxidase activity. This suggests that *Pinus thunbergii* has evolved excellent counter measures against environmental stress.

Pine needle extract was fractionated by gel filtration chromatography using gel packed PD-10 columns (Pharmacia), and peroxidase and the peroxidase inhibiting activity were measured. The results are shown in Fig. 3. In the control needles,

high molecular weight fractions equivalent to protein elutions revealed peroxidase activity, but in low molecular weight fractions, peroxidase activity was inhibited. In the case of polluted needles, both high and low molecular weight fractions showed inhibited peroxidase activity. The low molecular weight inhibitory activity may be due to antioxidative compounds including ascorbic acid and glutathione, but the compound which contributes to the inhibition in the high molecular weight fraction has not yet been purified.

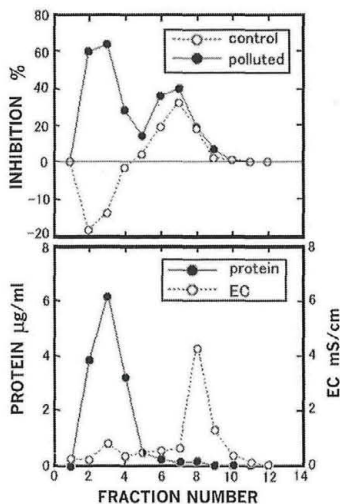


Fig. 3. Partial purification of the peroxidase inhibiting activity by a sephadex G-25 column (Sephadex PD-10 was used). The upper graph shows the peroxidase inhibiting activity of each fraction. The lower graph shows the elution patterns of protein and mineral salts.

Production of a hydrophilic fluorescent compound

Another important character of polluted pine trees is the stimulated production of a hydrophilic fluorescent compound. Diesel automobile exhaust gas was collected directly from the exhaust pipe into a polyethylene bag, and pine tree branches were directly exposed to this for 1, 2.5, 5, 7.5, 15, 30, 60 and 120 min. After 1 month the pines were collected, immersed in H_2O , and the fluorescence was measured. The fluorescence intensity increased as a result of diesel exhaust gas exposure for up to 60 min (Fig. 3). The treatment for 120 min caused the wilting of the branch, and caused a reduction in fluorescence intensity. This compound was difficult to separate, so its chemical structure and physiological characteristics have not yet been elucidated, but purification is in progress.

We have also detected such a highly hydrophilic fluorescent compound in other coniferous plant species (data not shown), especially in the needles of deteriorated or damaged trees (data not shown). It appears to be related to environ-

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mental conditions including air pollution and biotic/abiotic stresses. It is also related the internal physiological processes which respond to environmental conditions, and, after its elucidation, it may become a good indication of the physiological status of pine trees and also many coniferous trees.

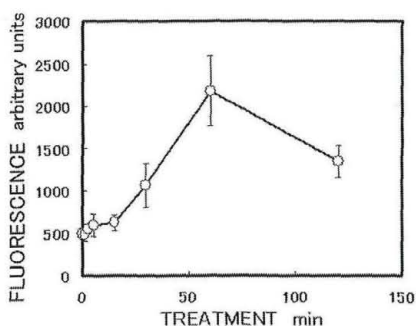


Fig. 4. Effect of diesel exhaust gas treatment on 6-year-old *Pinus thunbergii*.

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