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# Physiological Effects of Hydroxyl Radical (·OH) Generating Solution as Simulated Dew on the Needle Surfaces of Japanese Red Pine (*Pinus densiflora* Sieb. et Zucc.)

By

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Summary

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To investigate the effects of aqueous phase oxidants on the needle gas exchange of pine seedlings, simulated dew waters generating hydroxyl radicals (·OH) were sprayed onto the needle surfaces of pine seedlings. The nutrient contents and amounts of epicuticular wax of pine needles were also determined as a parameter with the effects of ·OH-generating solutions on gas exchange of pine needles. Two different sources and two different photoformation rates of ·OH-generating solutions, photo-Fenton reagents (100 and 200 μM HOOH- 1 μM Fe(III)- 5 μM oxalate) and 100 and 200 μM N(III), were sprayed three times a week in the early mornings for 3.5 months.

The results showed that maximum $CO_2$ assimilation rates ($A_{max}$) of pine needles treated with the two photo-Fenton reagents and 100 μM N (III) were smaller than those treated with a control solution after treatment; this did not occur with 200 μM N (III). Mg and Ca contents in the needles had positive correlations with $A_{max}$ among treatments. Amounts of epicuticular wax differed little among treatments. These results implied that needle nutrient status is one of the factors in reductions in $A_{max}$ of pine needles treated with ·OH-generating solutions.

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## Introduction

Oxidizing pollutant-induced oxidative stress in plants has been recognized as a main cause of forest decline (BYTNEROWICZ 1996, CAPE 1997, TAYLOR & al. 1994, WOHLGEMUTH & al. 2002). While the effects of gaseous oxidants such as ozone ($O_3$) on plant leaves have been investigated in many studies (IZUTA 1998, LONG & NAIDU 2002), the effects of aqueous phase oxidants have not been studied as extensively as gaseous oxidants. Oxidants in natural water have been investigated for hydrogen peroxide (HOOH) (DENG & ZUO 1999, SAKUGAWA & al. 1993), hydroxyl radicals (·OH) (ARAKAKI & al. 1998, FAUST & ALLEN 1993), singlet oxygen (HAAG & HOINÉ 1986), and so on. Among them, ·OH is the most potent (ZAFIRIOU & al. 1984) to react rapidly and unselectively with organic and inorganic matter (SEINFELD & PANDIS 1998, HAAG & YAO 1992).

In the forests of Japanese red pine on the Mt. Gokurakuji (Hiroshima Prefecture, wersten Japan), higher production of ·OH was found from the polluted dew water on pine needle surfaces in a pine stand near an urban area [eg. 3.36 (2.08-5.18) $\mu M\ h^{-1}$; mean and range on 5 days between Octorber and November 1999] compared to that on chemically inert surfaces (NAKATANI & al. 2001). In polluted dew water, dissolution of accumulated dry deposits on pine needles during non-precipitation periods enhanced concentrations of dissolved ions (CHIWA & al. 2003). The sources of ·OH production in the aqueous phase are photolysis of nitrate ($NO_3^-$), nitrite (N(III); $NO_2^-$ and $HNO_2$), and aqueous iron complexes (STUMM & MORGAN 1996). Thus, it is expected that ·OH production in natural water would be pronouced in smaller droplets at polluted sites, having adverse effects on plants due to its high oxidative reactivity. However, so far little attention has been given to the adverse effects of ·OH production in natural water on trees.

A few previous studies on the effects of aqueous phase ·OH production on the surfaces of pine needles demonstrated that the needle photosynthetic rates and stomatal conductance in Japanese red pine were significantly decreased when seedlings were exposed to mist solutions simulating the polluted dew water and containing sources of ·OH production (KOBAYASHI & al. 2002, KUME & al. 2001, NAKATANI 2004). However, little information is available about the chemical contents, including Mg contents, and amounts of epicuticular wax in the pine needles treated with ·OH generating solution. There are some studies on the impact of $O_3$ that have been concerned about needle nutrition status (EDWARDS & al. 1992, WALLIN & al. 2002) and wax amounts (BARNES & BROWN 1990, MAŇKOVSKÁ & al. 1999). Furtheremore, both of them are controlling factors of photosynthesis and stomatal aperture. This study determined them as a parameter with the effects of ·OH-generating solutions on gas exchange of pine needles.

## Material and Methods

### Growth chambers

Exposure experiments were conducted in growth chambers with open-air system, 3 m in diameter, 3 m high and with a capacity of ca. 20 $m^3$, established on the campus of Hiroshima Uni-

versity, western Japan (34°24'N, 132°44'E, 210 m asl). The octagonal framed chambers were wrapped by Ethylene-Tetrafluoroethylene copolymer (ETFE) film (F-CLEAN; Asahi Glass, Tokyo, Japan) sheets, which are transparent to both visible and UV light. Slanted conical roofs wrapped with F-CLEAN were placed above the each chamber to prevent ambient dew and rain from entering the chambers. A fan has been attached to each chamber to provide air circulation of 83 $m^3min^{-1}$ during day-time (08:00-20:00) and 40 $m^3min^{-1}$ during night-time (20:00-08:00), which means that air in the chambers changes ca. 4 and 2 times a minute during day-time and night-time, respectively. To reduce particulate matter and ozone in the ambient air, air was first filtered through a dust-filter then subsequently through a charcoal-filter before being drawn into the chamber. Air temperature and relative humidity in the chamber from 2 September to 11 December 2002 were 15.7 °C and 74.8 %, respectively. Even at the midday on a clear day of midsummer, air temperature in the chamber was only 2-3 °C greater than that in outside the chamber (KOBAYASHI & al. 2002). A detailed description of the growth chambers is given in KOBAYASHI & al. 2002.

Plant and soil materials

Two-year-old Japanese red pine (*Pinus densiflora* Sieb. et Zucc) seedlings were planted in pots (0.35 in diameter × 0.3 m deep, with a capacity of ca. 28 L) filled with sieved soil (20 mm) taken from brown forest soil at a depth of 0-0.5 m soil depth in a healthy pine woodland in the university campus (see Table 1 for chemical properties). They were then covered with organic materials taken from the soil surfaces in the pine woodland. Chemical properties of the collected soils were within the range of those of typical soils in western Japan. No nutrient solution was added to the pots during the growing periods. The pots were irrigated automatically with ca. 800 ml of water per pot every evening at 18:00 for 3 minutes so that the free water level was kept at above –0.1 MPa.

Table.1. Chemical properties of brown forest soil taken from a healthy pine woodland ($n$=8).

| | pH ($H_2O$) | $PO_4^{3-}$ | $NH_4^+$-N | Total-C | Total-N | Ex-Na | Ex-K | Ex-Mg | Ex-Ca |
|---|---|---|---|---|---|---|---|---|---|
| | | mg/dry-100g | | % | | cmol/dry-kg | | | |
| **Ave.** | 4.76 | 1.07 | 44.1 | 1.79 | 0.08 | 0.04 | 0.13 | 0.06 | 0.25 |
| **S.E**[a] | 0.00 | 0.38 | 8.08 | 0.13 | 0.01 | 0.00 | 0.01 | 0.01 | 0.00 |

[a] Standard error of mean of eight soil samples.

Mist treatments to pine foliage

Two types of ·OH-generating solutions (photo-Fenton reaction (F100 and F200); ZEPP & al. 1992, and photolysis of N (III) ($NO_2^-$ and $HNO_2$) (N100 and N200); ARAKAKI & al. 1999) added to control solution (C0) were applied to the pine foliage for a period of ca. 3.5 months from 23 August to 11 December 2002 (Table 2). As a control solution, concentrations of the major ions in dew water on chemically inert surfaces at Mt. Gokurakuji, Hiroshima prefecture, were prepared without adding N (III) or HOOH + Fe + oxalate; this solution produced less than 0.1 $\mu Mh^{-1}$ of ·OH (C0) according to KOBAYASHI & al. 2002. A 200-μM solution of HOOH added to a control solution (C0) was also prepared as a control for HOOH (C200).

Mist treatment with a volume of 50 ml was applied to the pine seedling in the growth chamber three times a week at one- or two-day intervals in the early morning (07:00 – 09:00) using an electric spray machine attached to a nozzle. During misting, waterproof sheets were placed over the surface soil to avoid direct changes in the chemical properties of the potted soil as a result of the mist solutions. After spraying, the surfaces of the pine needles were wetted for about 1 hour. During the exposure period of 3.5 months, the surfaces of the foliage of pine seedling were washed using ca. 500-ml control solution (C0) per one pine seedling at 10-day intervals in the early evening (17:00-19:00) in a same manner as mist treatments to remove gaseous and particulate matter on the surface of pine foliage. Also, pine seedings were rotated randomly among the six chambers at two-week

intervals to reduce any effects caused by differences among the chambers during the exposure period.

Table. 2. Additional components to a control solution (C0) and ·OH photoformation rates ($\mu Mh^{-1}$) of the six simulated mist treatments. For details on the determination of ·OH formation rates, refer to ARAKAKI & al. 1999.

| Treatment | Additional components | ·OH formation rates |
|---|---|---|
| F100 | HOOH 100 μM, Fe 1 μM, Oxalate 5μM | 7.7 |
| F200 | HOOH 200 μM, Fe 1 μM, Oxalate 5μM | 11.6 |
| N100 | N(III) 100 μM | 7.8 |
| N200 | N(III) 200 μM | 13.6 |
| C0 | — | < 0.1 |
| C200 | HOOH 200 μM | 2.6 |

Measurements of ecophysiological traits of pine needles

Current-year-old needles flushed in spring from seven pine seedlings treated with each mist solution were used for the ecophysiological trait measurements. Area-based maximum net $CO_2$ assimilation rates ($A_{max}$) were measured using a portable gas exchange measurement system (LI-6400; Li-Cor, Lincoln, NE, USA) at near-saturating irradiance (PPFD: 1500 μmol $m^{-2}$ $s^{-1}$) during the morning (07:00-11:30). The $CO_2$ concentration of the air entering the leaf chamber was kept at 360 μl $l^{-1}$ with a flow rate of 500 μmol $s^{-1}$. Area-based K, Mg, and Ca contents in the needles after 3.5-month mist treatments (112 days) were measured according to the method reported by NAKATANI & al. 2004, and area-based amounts of epicuticular wax on the needles after 3.5-month mist treatments (112 days) were measured based on the method of SASE & al. 1998. Needle area was determined according to the method reported by KUME & al. 2001.

## Results and Discussion

The $A_{max}$ of pine needles with all treatments, including the controls, decreased with time after the beginning of mist treatment (Fig. 1), mainly because of seasonal changes in the needle intrinsic processes during the exposure period of 3.5 months from 23 August to 11 December. Among treatments, the needles of pine seedlings treated with two photo-Fenton reagents and 100 μM N (III) tended to have smaller $A_{max}$ than those treated with control solutions. These reductions were consitant with previous exposure experiments involving application of ·OH-generating solutions to the surfaces of pine needles (KUME & al. 2001, KOBAYSHI & al. 2002, and NAKATANI 2004). On the other hand, needles of pine seedlings treated with 200 μM N (III) did not show a decrease in $A_{max}$ compared to those treated with photo-Fenton reagent and 100 μM N (III). Similar results were obtained by KOBAYASHI & al. 2002 who showed that 50 μM N (III) exposure to pine seedlings cause a decrease in $A_{max}$ of needles, but 100μM N (III) exposure did not. A probable reason for this is that N (III) solutions act not only as ·OH-generating solutions but also compensate for the adverce effects of ·OH production due to the nitrogen as a fertilizer (KOBAYAASHI & al. 2002) and the higher reaction rate constant of $NO_2^-$ with ·OH (NAKATANI 2004). In this study, however, 100 μM N (III)

exposure caused a decrease in $A_{max}$, while N (III) exposure of same concentration did not in the study reported by KOBAYAASHI & al. 2002. This is probably because the surfaces of the foliage of pine seedling were washed during the study periods at 10-day intervals in this study to remove gaseous and particulate matter on the surface of pine foliage, which could have resulted in relieving the effects of mist solution in this study.

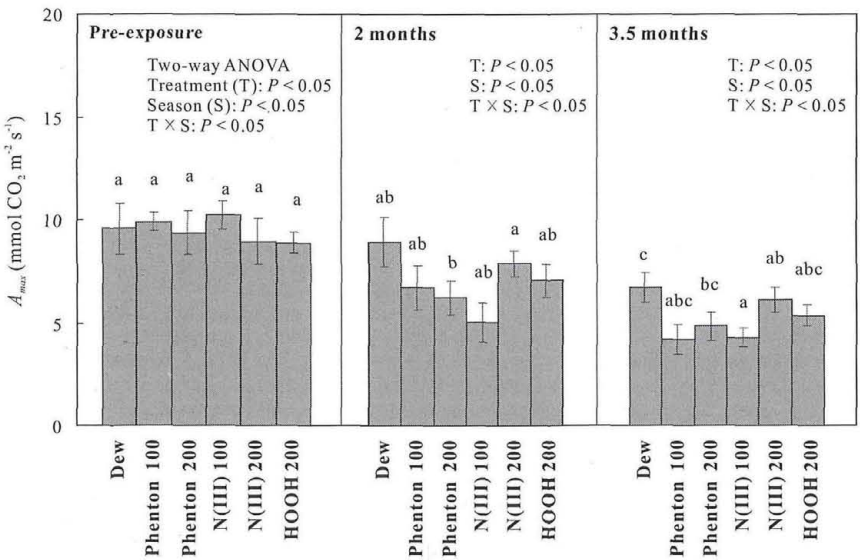


Fig. 1. $A_{max}$ (μmol $CO_2$ $m^{-2}$ $s^{-1}$) of current-year Japanese red pine (*Pinus densiflora*) needles subjected to six different simulated dew waters (see Table 2). Bars represent standard errors of 7 pine seedlings. Different letters indicate significant differences at $P$<0.05 (LSD) among treatment.

Area-based Mg and Ca contens in current-year needles were significantly correlated with $A_{max}$ among treatments (Fig. 2, Table 3). These relationships were also found for the mass-based Mg and Ca contents (data not shown). Between them, Mg content should have a direct influence on photosynthetic activity (LAING & al. 2000, NAKATANI & al. 2004), because of its constituent of chlorophyll molecules and effects some of the enzymatic reactions of photosynthesis (MARSCHNER 1995). These results implied that needle nutrient status, especially Mg, is one of the factors in reductions in $A_{max}$ of pine needles treated with ·OH-generating solutions.

There were small differences in the amounts of epicuticular wax among treatments (Table 4). This result was unexpected, because amounts of wax are reportedly reduced by air pollutants such as ozone (BARNES & BROWN 1990, MAŇKOVSKÁ & al. 1999). A plausible explanation for this is that pine seedlings treated with ·OH-generating solutions might maintain adequate amounts of wax due to the sufficient nutrients in the potted soils. Further invesitigations are re-

quired to elucidate the relationships between the effects of ·OH-generating solutions on plants and the nutrient status of soils.

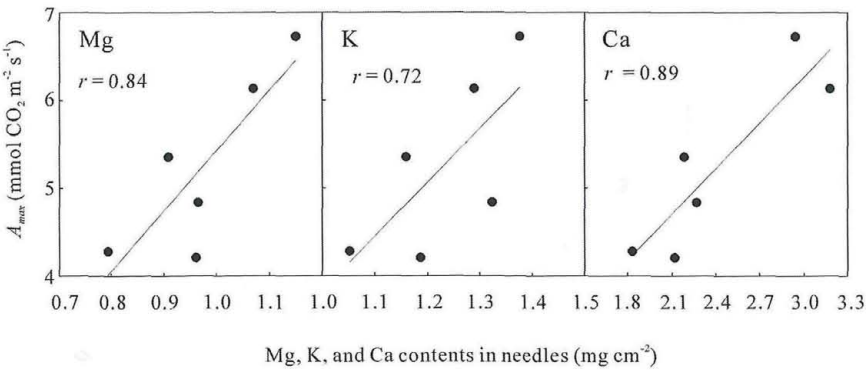


Fig. 2. Relationship between area-based $A_{max}$ and nutrient contens in current-year-old Japanese red pine (*Pinus densiflora*) needles subjected to six different simulated dew waters (see Table 2). Bars represent standard errors of 7 pine seedlings. Solid lines represent regression lines for simple linear function (Y=*a*X+*b*).

Table. 3. Pearson'scorrelation coecient (*r*) among the ecophysiological traits of the Japanese red pine (*Pinus densiflora*) needle subjected to six different simulated dew waters (see Table 2) (*n*=6).

| | $A_{max}$ | Mg needle | K needle | Ca needle | Wax amounts |
|---|---|---|---|---|---|
| $A_{max}$ | — | | | | |
| Mg needle | 0.836* | — | | | |
| K needle | 0.720 | 0.910** | — | | |
| Ca needle | 0.892* | 0.898* | 0.771 | — | |
| Wax amounts | -0.775 | -0.770 | -0.671 | -0.828* | — |

* $P$ <0.05
** $P$ < 0.01

Table. 4. Area-based epicuticular wax amounts (g $m^{-2}$) on current-year Japanese red pine (*Pinus densiflora*) needles subjected to six different simulated dew waters (see Table 2) ($n$ = 7).

| | F100 | F200 | N100 | N200 | C0 | C200 |
|---|---|---|---|---|---|---|
| **Ave.** | 1.78 | 1.75 | 1.75 | 1.67 | 1.64 | 1.81 |
| **S.E**[a] | 0.09 | 0.08 | 0.12 | 0.11 | 0.08 | 0.17 |

One-Way ANOVA, $P$ = 0.900

[a]Standard error of mean seven samples.

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