Phytotoxicities of fluoranthene and phenanthrene deposited on needle surfaces of the evergreen conifer, Japanese red pine (*Pinus densiflora* Sieb. et. Zucc).

Fluoranthene and phenanthrene caused negative effects on the needles of Japanese red pine.

Ilemobayo Oguntimehin, Nobutake Nakatani, Hiroshi Sakugawa*

Department of Environmental Dynamics and Management,
Graduate School of Biosphere Science,
Hiroshima University,
1-7-1 Kagamiyama, Higashi-Hiroshima, 739-8521, Japan.

*Corresponding author

Address: 1-7-1 Kagamiyama, Higashi-Hiroshima,

739-8521, Japan.

Tel & Fax: +81-82-424-6504.

Email: hsakuga@hiroshima-u.ac.jp

Abstract

Polycyclic aromatic hydrocarbons (PAHs) have been widely studied with respect to their carcinogenic and mutagenic effects on animals and human cells. Phenanthrene (PHE) and fluoranthene (FLU) effects on the needle photosynthetic traits of two-year-old Japanese red pine (*Pinus densiflora* Sieb. et. Zucc) seedlings were investigated. Three months after fumigation of foliage with solutions containing these PAHs (10 μM each), FLU had negative effects on net photosynthesis at near-saturating irradiance, stomatal conductance, initial chlorophyll fluorescence, and the contents of total chlorophyll, magnesium, and ribulose 1,5-bisphosphate carboxylase (rubisco) of current-year needles. PHE had similar negative effects to FLU but in lesser magnitude. The effects of the PAHs were mitigated by the addition of an OH-radical scavenger (mannitol) into the PAHs solutions. PAHs deposited on the surface of pine needles may induce the generation of reactive oxygen species in the photosynthetic apparatus, a manner closely resembling the action of the herbicide paraquat.

Capsule:

Fluoranthene and phenanthrene caused negative effects on the needles of Japanese red pine.

Key words: PAHs fumigation, Fluoranthene, Phenanthrene, Mannitol, Pine needles.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants found in all environmental compartments. Sources vary widely from natural to anthropogenic (Harvey, 1997). PAHs enter plants either directly via stomata or indirectly through the root system (Kuhn et al., 2004; Rohacek and Bartak, 1999; Samsoe-Peterson et al., 2002). They may be transferred to soil by litter fall, which indicates their adsorption on leaf and needle surfaces (Matzner, 1984), but atmospheric deposition on leaves often greatly exceeds uptake from soil by roots as a route of PAHs accumulation (Vaughan, 1984). Pine needles were used as passive samplers in assessing ambient atmospheric concentrations of persistent organic contaminants, such as PAHs and dichlorobenzene-*p*-dioxins on regional and global scales (Dmuchowski and Bytnerowicz, 1995; Safe et al., 1992; Tremolada et al., 1996). Gaseous diffusion from the air to the waxy layer of plant leaves has been shown to be a major uptake process for these lipophilic organic contaminants (Jensen at al., 1992; Keymeulen et al., 2001; Kylin and Sjodin, 2003; Wild and Jones, 1991; Wild et al., 2005, 2006). Though many limitations may exist, including kinetic constraints over the pollutants uptake and analytical problems relating to matrix complexity (waxy, lipid rich plant tissue), this approach has provided a time integral of the analytes' airborne concentrations. Past studies involving PAHs effects on plants have mostly used root exposure methods (Kummerova et al., 2006a 2006b; Wild and Jones, 1992). Among the few that examined the effects of PAHs on the foliar (above-ground) regions of terrestrial plants, for example, Edwards (1983) and Huang et al. (1996), none considered PAHs effects on evergreen Japanese red pine. Wang et al. (2005) in their work asserted that PAHs on surfaces of pine needles (*Pinus thunbergii*) applied by dry or wet deposition would be stable and would accumulate to reach a certain concentration. Compared with PAHs dissolved in water, it was found that PAHs sorbed on pine needles had low photolysis rates, thus suggesting that the waxes of the pine needles can stabilize PAHs photolysis (Miller and Olejnik, 2001; Wang et al., 2005). Phenanthrene (PHE) and fluoranthene (FLU) are among U.S Environmental Protection Agency (USEPA's) priority PAHs, and they are examples of low and high molecular weight PAHs respectively (ASTDR, 1995). These two PAHs are widespread in the environment. Plants are very sensitive and respond rapidly to their presence (Awata et al., 1998; Huang et al., 1996; Kummerova et al., 2006a 2006b) thus justifying their choice as model compounds. These volatile PAH compounds with a vapor phase component in the air are subject to an air-leaf exchange process moving towards equilibrium over time (Tremolada et al., 1996; Wild et al., 2004). They can therefore enter plant tissues primarily by gaseous diffusion via open stomata. The main advantage of using Japanese red pine as markers of exposure to PAHs is that vegetation acts as a natural "air sampler", with accumulation of PAHs occurring overtime. In this study, we fumigated the PAHs directly onto the needle surfaces of the evergreen conifer with the aim of investigating the impacts of the PAHs on the Japanese red pine. Japanese red pine seedlings treated with fluoranthene and phenanthrene are expected to internalize these model PAHs, and exhibit altered physiological, morphological, and possibly growth changes. More so, because of the eco-

toxicological effects that this might present to the plant, stress responses are expected to provide insights into plants' PAHs-perception and stress-signaling mechanisms.

2. Materials and Methods.

2.1. Growth chambers

The growth chambers used in this work were designed as described previously by Kobayashi et al. (2002) and Nakatani et al. (2007). The chambers were covered with transparent ethylene–tetrafluoroethylene copolymer film (ETFE) made by F-CLEAN®, Asahi Glass Green-Tech Co. Ltd., Japan. F-CLEAN® has high sunshine transparency of over 95% and allows maximum ultraviolet light transmission. The maximum photosynthetic photon flux density (PPFD), (LI-190SA Quantum Sensor, Licor, USA*)* incident on the foliage of the pine seedlings was approximately 1450 μmol $m^{-2}s^{-1}$ at noon on a summer day (August 2006). A charcoal filter removed excess O_3 and SO_2 and maintained their concentration below 10 and 4 ppb, respectively (as measured on 18 July 2006). The mean air temperature and relative air humidity in the chamber from 1 August to 15 November, 2006 were 25.6 °C and 79.5% respectively. Even at midday on a clear day in mid-summer, air temperature in the chamber was only 2 to 3 °C greater than ambient.

2.2. Plant and soil materials

Two-year-old Japanese red pine (*P. densiflora*) seedlings grown in Fukuoka prefecture were purchased and transplanted into 0.35 m × 0.3 m deep pots (1 seedling/pot) on 6 March 2006. They were left in the open for one month before being transferred into the growth chambers. Pots were filled with 21 L of a soil mixture consisting of yellow sandy soil (weathered granite), perlite (white loam 4-20 mm, Toho-Leo Co.), isolite (CG2, Isolite Insulating Products Co.) and humus soil (Midori-Sangyo Inc.) at 11:2:2:4 volumetric ratios, respectively. Neat litter (mainly pine needles) of healthy pine woodland from the university campus was collected and spread over the soil surface in the pots (50 g/pot). The soil contained free water (above -0.01 MPa), as it was supplied with water at least once daily by an automatic irrigation system (Kobayashi et al., 2001). 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 the transplant of pine seedlings into pots, nutrient solution (N:P:K = 6:10:5; Hyponex, Murakami Bussan, Tokyo, Japan) was added monthly at the rate of 1 mL of concentrated nutrient solution in 500 mL of MilliQ water per pot. Pine seedlings were rotated bi-monthly within chambers to nullify any bias due to the relative position of the chambers to one another. New needles were fully expanded by the end of July. On July 8, 2006 (almost one month before fumigation), the seedlings were 609 ± 78 mm in height and had stem basal diameters of 11.8 ± 1.7 mm. On August 1, 2006 (a day before fumigation), the seedlings were 634 ± 80mm in height and 12.3 ±1.9 mm in stem basal diameter (mean ± standard deviation, *n* = 48) at heights of approximately 20 cm from the chamber floor. Stock solutions (1 mM) of fluoranthene (FLU) (Sigma-Aldrich, USA) and phenanthrene (PHE) (Nacalai, Kyoto, Japan) were

prepared in 50% acetone (Wako Pure Chem. Ind., Japan) and MilliQ water (Millipore Co., Japan), respectively. Each stock solution was diluted to a final concentration of 10 μM with MilliQ water bringing the final concentration of acetone in the solution to 0.5%. This final concentration of FLU is comparable to the minimal concentration used elsewhere (Huang et al, 1996, Kummerova and Kmentova, 2004). When this concentration of acetone was used in a preliminary study, there was no effect on any of the eco-physiological parameters considered in the present study. Mannitol (MANN) (Nacalai, Kyoto, Japan) (1 mM) was used as an OH radical scavenger because reactive oxygen species are thought to play an active role in the photolysis of the PAHs adsorped on the surface of pine needles (Wang et al., 2005). Mannitol is produced in some plants and has long been recognized as a potent ROS quencher, it was used to scavenge hydroxyl radicals (OH) generated by cell-free oxidant systems (Upham and Jahnke, 1986), mannitol (1mM) could not cause osmotic stress to the Japanese red pine. Past studies that reported osmotic stress in plant used mannitol in the concentration range above 90 mM. For example, 92-276 mM of mannitol solution was used by Lin and Kao (2002) and a 750 mM mannitol was used by Gill et al. (2002). The five types of solutions used as treatments were; control (MANN), FLU, FLU + MANN, PHE and PHE + MANN. The solutions were applied to the foliage of pine seedlings using an electronic spray machine with a nozzle (BS-4000, Fujiwara Sangyo, Miki, Japan) in the early morning (6:00-8:00 AM), four alternate days weekly for three and half months. On the average each plant received 50 mL of the fumigant per one spraying period. The soil surface was covered with a waterproof sheet during application to prevent solutions from entering the roots of the seedlings.

2.3. Photosynthesis measurement and fluorescence analysis

Physiological measurements were conducted on current needles from July to November; the first was the pre-fumigation measurements. In each pot of the six chambers and between the hours of 6:00 AM and 10:00 AM except for November; from 9:00 AM to 1:00 PM, net photosynthesis at near-saturating irradiance (A_{max}), stomata conductance (g_s) and intercellular CO_2 concentration (C_i) were measured for six intact current-year needles (three pairs of leaves) of each seedling. The A_{max}, g_s, and C_i were measured at near-saturating irradiance of 1500 μmol m^{-2} s^{-1} PPFD and at a needle temperature of 27 ± 2 °C except in November (23 ± 2 °C). Leaf to air vapour pressure deficit (Vpdl) was maintained between 0.8 and 1.3 kPa. CO_2 concentration of the air entering the leaf chamber was kept 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 for measurement were harvested and their width and length measured with a digital caliper (CD-15, Mitutoyo Co., Kanagawa, Japan). The cross-section of the needle was approximated as a semicircle having a diameter equal to the measured width; half of the leaf surface area of the needle was used as the effective leaf area for A_{max} and g_s determination (Kume et al., 2001). The area-based chlorophyll content is a measure of the ability of light capture in the needles (Evans, 1998); therefore photosynthetic capacity should be directly influenced by area-based chlorophyll content in the needles (Nakatani et al., 2004). All data of

needle eco-physiological traits are expressed based on the effective needle area. Chlorophyll fluorescence was measured at night (8:00-9:30 PM) with a portable chlorophyll fluorometer (MINI-PAM, Heinz Walz GmbH, Effeltrich, Germany) with leaf-clip holder 2030B (Heinz Walz GmbH, Germany), needles were arranged compactly in a parallel array and clamped with the holder. Initial chlorophyll fluorescence (F_o) and the photochemical efficiency of PS II in the dark (F_v/F_m; F_v is the variable fluorescence and F_m the maximum fluorescence) were measured.

2.4. Needle Chemical Content

For rubisco analysis, total protein was extracted as described by Makino et al. (1986); Nakaji and Izuta (2001). Fresh needles (100 mg) were frozen in liquid nitrogen and then homogenized in 1 mL extraction buffer containing 100 mM HEPES (pH 7.5), 5 mM EDTA, 2% PVPP (w/v), 0.7% polyethylene glycol 20000 (w/v) and 24 mM 2-mercaptoethanol. This procedure was carried out at 4 °C. The homogenate was centrifugated at 9000 g for 30 s, and the supernatant was used in the rubisco assay. Precisely, 20 µL of the prepared samples was loaded onto a slab gel (13.7 x 12.7 x 0.1 cm) containing a 12.5% (w/v) acrylamide in the resolving gel and 3.5% (w/v) acrylamide in the stacking gel. The Laemmli buffer system was used. Electrophoresis was carried out at 30 mA per gel. Gels were stained in 0.25% (w/v) CBB-R250 and eluted in 1.0 mL formamide. The absorbance of the resultant solution was read at 595 nm with a 2400-UV/VIS spectrophotometer (Shimadzu Co., Japan). The concentrations of Chlorophyll a (Chl a), Chlorophyll b (Chl b), and Chlorophylls a+b (Chl $_{(a+b)}$) were determined by extraction of 100 mg needles (collected close to those used for A_{max} and g_s) with N, N-dimethylformamide (DMF). Absorption of the extract was measured at 663.8 and 646.8 nm and concentrations of Chl a, Chl b and Chl $_{(a+b)}$, were calculated with equations used by Porra et al. (1989). Dried current-year needles collected in November were ground into powder using a mortar and pestle. K, Na, Ca and Mg concentrations were determined using inductively coupled plasma atomic emission spectrometry (Perkin-Elmer, Optima 3000) following sample digestion by conc. HNO_3 using the microwave oven system (O.I Analytical. model 9175) as described by Nakatani et al. (2004).

2.5. Statistical analysis

For statistical evaluation of the results, the program SPSS 12 (SPSS, USA) was used. The results are averages of values from five pine seedlings in each treatment group. The significance of the differences of the average values among the treatments was evaluated by analysis of variance of simple classification after preceding verification of normality and homogeneity of the variance (one-way ANOVA $p < 0.05$). The comparison of means was based on the method of Tukey contrast test, except for November (Fisher's least significant difference 'LSD' test). Pearson's correlation coefficient (r, $p < 0.05$) was used to test the correlation among the needle eco-physiological traits.

3. Results

The analytical values of current-year pine needles after the three months fumigation are presented in Table 1. Relating the eco-physiological parameters in this study with A_{max}, our results indicated that FLU had negative effects on the photosynthetic pigments of the pine needles. September is the likely peak of photosynthesis in pine seedlings. A_{max} values for the month of October (90 days fumigation) until the end of fumigation in November (105 days) showed consistently decreased values (Fig. 1). FLU treatment greatly diminished A_{max} values in seedlings in October and November, and a similar pattern was observed in PHE-treated seedlings. Stomatal conductance to water vapour appeared to vary with treatment types. A regular pattern of decrease in stomatal conductance, similar to A_{max} values of the FLU and PHE treatments, was obtained at the end of the exposure experiment. These values were significantly different to those of the control treatment. C_i showed no clear decrease pattern in all treatments. Treatments containing equal concentrations of PAHs in addition to mannitol did not show any significant decrease in A_{max} and g_s values, and were not significantly different from the control treatment. Generally, F_o of pine needles showed no significant difference within the treatments until September (Fig. 1). However, for FLU treatments an increment in the F_o values started at the end of the 30-day measurement and increased constantly till the 90^{th} day (Fig. 1). Even though the increment in October was not significantly different ($p < 0.05$), after a fumigation period of 105 days the F_o of the FLU treatment was significantly higher than that of the control treatment (Table 1). However, the F_o of the FLU + MANN treatment was lower than that of the FLU treatment. This implies that F_o value decreased in the presence of mannitol. Photochemical efficiency of PSII in the dark (F_v/F_m) indicated a decreasing trend for FLU and PHE treatments as shown in Fig. 1. After the 105-day measurement, F_v/F_m values were not significantly different from other treatments or from the control. A positive correlation coefficient ($r = 0.61$, $p < 0.01$; Table 2) was found between A_{max} and photochemical efficiency of PS II (F_v/F_m) even though F_o is significantly negatively correlated with A_{max}. Chlorophyll contents of *P. densiflora* current-year needles at the end of 105 days fumigation are shown in Table 1. There was a significant decrease in chlorophyll contents of seedlings in FLU and PHE treatments. However, Chl a/Chl b ratios were unaffected by treatments with the PAHs, as their values were not significantly different compared with the control treatments. Fig. 2a shows the relationship between A_{max} and chlorophyll content of pine needles ($r = 0.76$, $p < 0.01$). Plate 1 shows the photos of potted pine seedlings for control treatments and treated pine seedlings before and after fumigation exposure. FLU-treated plants had the lowest amount of rubisco as shown in Table 1. Fig. 2b shows the correlation between A_{max} and rubisco content of the pine needles ($r = 0.44$, $p < 0.05$). A positive relationship existed between the chlorophyll content of current-year pine needles and rubisco content ($r = 0.65$, $p < 0.01$; Fig 2c). Even though Na and Ca content of dried pine needles were not statistically different between PAHs and control treatments, the K content of seedlings in the PHE treatment was statistically different from that of the other treatments and the

control. In addition, Mg contents of seedlings in the FLU treatment showed significant differences when compared with that of the control and other PAHs treatments.

4. Discussion

Previous eco-physiological studies on Japanese red pine describe the actions of SO_2, NO_2, O_3 and hydroxyl (OH) radicals (Chiwa et al., 2005; Izuta et al., 2001; Kobayashi et al., 2002; Kohno, 2001; Kume et al., 2001; Naemura et al., 2000; Nakaji and Izuta, 2001). Reduction in A_{max} and g_s of Japanese red pine needles by the two PAHs in the current study are similar to results obtained by Kobayashi et al. (2002) and Yoon et al. (2006), who fumigated with OH radical-generating solutions (photo-Fenton reagent) and mist onto Japanese red pine needles and Japanese apricot (*Prunus mume)* leaves, respectively. Nakatani (2004) showed that negative effects of OH radical-generating solutions on Japanese red pine needles were diminished by adding mannitol to the solutions. In a different study conducted using O_3, it was found that the net photosynthesis rate of *P. densiflora* was reduced by O_3 (Nakaji and Izuta, 2001). There is therefore a similarity in results obtained by fumigation with the OH radical, O_3 and PAHs. This suggests that like the OH radical and O_3, which were shown to damage or inhibit the photosynthetic process (Farage et al., 1991; Nakatani, 2004), PAHs, may affect the pine seedlings in a similar way. The restriction of any step within the photosynthetic apparatus will decrease the ability of the photosynthetic membrane to utilize the light energy that it intercepts. This increases the potential for formation of reactive oxygen species within the chloroplast, causing photoinhibition and photooxidation. In addition, the decreased effect of g_s in this present study might also cause the decreased A_{max} values. Stomatal resistance, like resistance of boundary layers, is directly linked to the diffusion of the pollutant through the stomata. This stomatal limitation was absent in plants fumigated with O_3 (Farage et al., 1991). Our results on C_i mimic the earlier work carried out on *P. densiflora* using OH radical-generating solutions (Kobayashi et al., 2002; Kume et al., 2001). C_i represents a balance of CO_2 influx through the needles via the stomata aperture (g_s). In *P. mume,* the effect of OH radical-generating mists on Ci was masked as in *P. densiflora* (Yoon et al., 2006). The PAHs fumigated onto the pine seedlings took approximately three months to produce any significant effect on the photosynthetic rate and the stomatal conductance, however the previous research quoted above indicated significant results much earlier. The slow process of the rate-limiting step for the needle contamination process by PAH (Wenzel et al., 1997) may explain this disparity. The F_o value, which corresponds to the state when all reaction centers of PSII are open, showed a slight increase in seedlings treated with PAHs. This result contradicts that obtained by Kobayashi et al., (2002) where different sources of OH radicals were used as fumigants on pine seedlings. Their results suggested that the light harvesting and utilization system was not involved directly in the variation in A_{max}. In the present study, the negative effect of PAHs treatment on the F_o value of pine seedlings could be connected to the destruction of molecules of photosynthetic pigments. The effects of FLU in thylakoid membrane might have caused reversible inactivation of PSII which resulted in F_o increase (Huang et al., 1997; Mallakin et al., 2002). Our result for FLU-treated pine

seedlings agree with previous works using lichens *Lasallia pustulata* and *Umbilicaria hirsuta* by Kummerova et al. (2006a), Pea *Pisum sativum* by Kummerova et al. (2006b), Duckweed *Lemna gibba* L G-3 by Mallakin et al. (2002) and Canola *Brassica napus* by Huang et al. (1996) . These researchers recorded an increase in F_o values as well as decreased F_v/F_m values with exposure time. Even though the results were obtained from root exposure experiments in most cases, they are only slightly different from those reported from Kume et al. (2001) and Yoon et al. (2006) who carried out foliar-exposure experiments. The observed symptoms in our experiment may be due to the OH radicals formed during the transformation of the PAHs. Our result on decreased chlorophyll content in pine needles fumigated with FLU is consistent with that reported by Kummerova et al. (2006b) on their experiments using pea plants. A similar decrease in chlorophyll content was reported in a different work (Sakugawa and Cape, 2007) using N (III) in the form of HONO gas fumigated onto Scot pine (*Pinus sylvestris*). In the latter, the OH radical was generated in four-year-old Scot pine. The reactive oxygen species generated by the photolysis of HONO gas caused the observed negative effects. Likewise, in the present study, OH radical production may be the cause of the negative effects of these PAHs on *P. densiflora* needles. Total rubisco protein content of current-year pine needles showed a positive correlation with both A_{max} and total chlorophyll content of the pine needles (Table 2). The overall effects of reduction in chlorophyll content must have affected the rubisco content. It is also probable that PAHs have significant reduction effects on the synthesis of enzymes in the C_3 pathway, including rubisco, in fumigated plants. They may also cause a decrease of the rubisco level that existed in pine seedlings at the start of fumigation. OH radical production within the chloroplast may damage rubisco, thereby increasing photo-oxidative damage leading to widespread changes within the chloroplast. With lowered levels of rubisco, utilization of photochemically produced energy might have been limited or other components of the photosynthetic apparatus made unstable. This is a possible cause of the major reduction in the photosynthetic rate of the current pine needles in FLU and PHE-treated plants after 105 days of fumigation. Previous works by Nakatani et al. (2004) showed that the area-based Mg content of pine needles has a direct influence on their net photosynthetic capacity. The reduction was due to limitation or suppression of the nutrient absorption from roots, thereby also causing a reduction in the root biomass (Nakatani et al., 2007). Mg content of FLU treated pine seedlings in this study was highly decreased by FLU fumigation. We have not considered root biomass; however, the visible foliar damage observed in this study is a strong indication of foliar disorder (Plate 1). These symptoms are similar but less severe compared with the acute foliar SO_2 injury symptoms to white pine (*Pinus strobus*) and Scot pine (*P. sylvestris*) described by Legge et al. (1998). Conifers are considered to be less susceptible to foliar injury than broad-leaved species (Percy, 1991). The symptoms illustrated in Plate 1 clearly show that needles of the pine seedlings exposed to FLU and PHE have a high degree of chlorosis and reddish-brown necrosis compared with the control treatment. These supplement the evidence of injury infliction on the Japanese red pine needles. A possible mechanism for the observed PAHs effect in our work is comparable with an earlier description by Mallakin et al. (2002). Some PAHs being good photosensitizers (Greenberg et al., 1993), their mechanism of toxic action might have started with inhibition of photosystem I (PSI) or

the cytochrome-b6/f complex, followed by photo-oxidative damage to photosystem II (PSII). For example, Kummerova et al. (2006b) reported that FLU had negative effect on the activity of the water oxidizing complex (Oxygen evolving center, OEC). It was assumed that FLU could injure the primary electron donor Tyr Z. Probably, FLU might have intercepted electrons from the bond ferrodoxin acceptors and NADP and reduce O_2 to O_2^- . The super oxide ion in reacting with H_2O caused the formation of OH radical (Miller and Olejnik, 2001). The highly reactive O_2^- and OH radicals attack unsaturated membrane fatty acids, rapidly opening up and disintegrating the cell membranes and tissues. Babbs et al. (1989) observed a similar process with paraquat (N,N'-Dimethyl-4,4'-bipyridinium dichloride), a 'viologen'. This compound is one of the most widely used herbicides in the world. It is fast acting, non-selective, and kills green plant tissue on contact (Tomlin, 1994). From experiments where exogenous scavengers of the hydroxyl radical (OH), superoxide radical ($O_2^{\cdot -}$) and singlet oxygen ($^1O_2$) were applied to leaf discs, it appears that O_2^- and OH radicals are the main O_2 species which contribute to chlorophyll destruction by paraquat (Babbs et al., 1989; Cakmak and Marschner, 1992).

5. Conclusion

The symptoms of ecological and physiological properties found in the current-year needles of pine seedlings fumigated with FLU and PHE in our work show similar trends when compared with past studies involving OH radical and OH radical-generating mists or solutions. The extent of impacts of the two PAHs varied slightly. The difference in the observed negative effects of FLU and PHE may be attributed to their different lipophilicity (in terms of log K_{ow}) and availability. These two factors control the bioavailability of the PAHs in inner needle compartments and influence the uptake efficiency of PAHs into plants (Wenzel et al., 1997). Our work strongly suggests that the OH radical production is an important process involved in as a mechanism by which PAHs impact negative effects on pine trees and consequently on vegetation. Mannitol has been shown to mitigate the negative effects of the PAHs on pine trees. More so, FLU and PHE may be categorized as inducers of plant stress. Most of the eco-physiological symptoms displayed by plants fumigated with these PAHs (particularly FLU) were similar to those that would be obtained using a broad-spectrum herbicide. Further works to investigate the eco-toxicological impact of these groups of compounds on the biosphere should be encouraged.

References

ASTDR, 1995. Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAHs**)** Agency for Toxic Substances and Disease Registry, Division of Toxicology and Environmental Medicine. http://www.atsdr.cdc.gov/toxprofiles/tp69.html (updated February 27, 2007)

Awata, H., Bates, S., Knaub, D., Popelka R., 1998. Polynuclear Aromatic Hydrocarbons: Properties and Environmental Fate; Environmental Engineering Chemistry II: Environmental Organic chemistry. http://www.ces.clemson.edu/ees/lee/pahs.htm.

Babbs, C.F., Pham, J.A., Coolbaugh, R.C., 1989. Lethal hydroxyl radical production paraquat-treated plants. Plant Physiology 90, 1267–1270.

Cakmak, I., Marschner, H., 1992. Magnesium deficiency enhances resistance to paraquat toxicity in beans leaves. Plant, Cell & Environment 15, 955-960.

Chiwa, M., Matsuda, T., Nakatani, N., Sueki, Y., Kobayashi, T., Sakugawa, H., 2005. Physiological effects of hydroxyl radical (OH) generating solution as simulated dew on the needle surfaces of Japanese Red Pine (*Pinus densiflora* Sieb. et Zucc.). Phyton (Austria) 45, 237–244.

Dmuchowski, W., Bytnerowicz, A., 1995. Monitoring environmental pollution in Poland by chemical analysis of Scots pine *(Pinus Sylvestris* L.) needles. Environmental Pollution 87, 87–104.

Edwards, N.T., 1983. Polycyclic aromatic hydrocarbons (PAHs) in the terrestrial environment-a review. Journal of Environmental Quality 12, 427–441.

Evans, J.R., 1998. Photosynthesis and Nitrogen relationships in leaves of C3 plants. Oecologia 78, 9-19.

Farage, P.K., Long, S.P., Lechner, E.G., Baker, N.R., 1991. The sequence of change within the photosynthetic apparatus of wheat following short time exposure to ozone. Plant Physiology, 95, 529–535.

Gill, P.K., Sharma, A.D., Singh, P., Bhullar, S.S. 2002. Bulgarian Journal of Plant Physiology 28, 12-25

Greenberg, B.M., Huang, X.D., Dixon, D.G., Ren, L., McConkey, B.J., Duxbury, C.L., 1993. Quantitative structure activity relationships for the photoinduced toxicity of polycyclic aromatic hydrocarbons to plants-A preliminary model in: Gorsuch, J.W., Dwyer, F.J.,

Ingesoll, C.G., La Point, T.W. (Eds.). Environmental Toxicology and Risk Assessment, Vol. 2, American Society for Testing and Materials, Philadelphia, pp. 369–378

Harvey, R.G., 1997. Polycyclic Aromatic Hydrocarbons. Wiley-VCH, New York.

Huang, X.D., Zeiler, L.F., Dixon, D.G., Greenberg, B.M., 1996. Photoinduced toxicity of PAHs to the foliar regions of *Brassica napus* (Canola) and *Cucumbis sativus* (Cucumber) in simulated solar radiation. Ecotoxicology and Environmental Safety 35, 191–197.

Huang, X.D., McConkey, B.J., Babu, T.S., Greenberg, B.M., 1997. Mechanisms of photoinduced toxicity of photomodified anthracene to plants: Inhibition of photosynthesis in the aquatic higher plant *Lemna gibba* (Duckweed). Environmental Toxicology and Chemistry 16, 1707-1715.

Izuta, T., Matsumura, H., Kohno, Y., Shimizu, H., 2001. Experimental studies on the effects of ozone on forest tree species. Journal of Japan Society for Atmospheric Environment 36, 60–77.

Jensen, S., Eriksson, G., Kylin, H., Strachan, W.M.J, 1992. Atmospheric pollution by persistent organic compounds: Monitoring with pine needles. Chemosphere 24**,** 229–245.

Keymeulen, R., Görgényi, M., Héberger, K., Priksane, A., Langenhove, H.V., 2001. Benzene, toluene, ethyl benzene and xylenes in ambient air and *Pinus sylvestris* L needles: A comparative study between Belgium, Hungary and Latvia. Atmospheric Environment 35**,** 6327–6335.

Kobayashi, T., Nakatani, N., Suzuki, M., Miyake, T., Kim, D.H., Hirakawa, T., Kume, A., Nakane, K., Sakugawa, H., 2001. Diurnal patterns of needle gas exchange and chlorophyll fluorescence in Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) seedlings. Journal of the Japanese Society of Revegetation Technology 26, 343–348.

Kobayashi, T., Nakatani, N., Hirakawa, T., Suzuki, M., Miyake, T., Chiwa, M., Yuhara, T., Hashimoto, N., Inoue, K., Yamamura, K., Agus, N., Sinogaya, J.R., Nakane, K., Kume, A., Arakaki, T., Sakugawa, H., 2002. Variation in CO_2 assimilation rate induced by simulated dew waters with different sources of hydroxyl radical (OH) on the needle surfaces of Japanese red pine (*Pinus densiflora* Sieb. et Zucc.). Environmental Pollution 118, 383–391.

Kohno, Y., 2001. A review on effects of acidic deposition on trees by long-term exposure experiments. Journal of Japan Society for Atmospheric Environment 36, 47–59.

Kuhn, A., Ballach, H.J., Wittig, R., 2004. Studies in the biodegradation of 5 PAHs (phenanthrene, pyrene, fluoranthene, chrysene, and benzo (a) pyrene) in the presence of rooted poplar cuttings. Environmental Science Pollution Research International 11, 22–32.

Kume, A., Arakaki, T., Tsuboi, N., Suzuki, M., Kuramoto, D., Nakane, K., Sakugawa, H., 2001. Harmful effects of radicals generated in polluted dew on the needles of Japanese Red Pine (*Pinus densiflora*). New Phytologist 152, 53–58.

Kummerova, M., Kmentova, E., 2004. Photoinduced toxicity of fluoranthene on germination and early development of plant seedling. Chemosphere 56, 387-393.

Kummerová, M., Barták, M., Dubová, J., Tříska, J., Zubrová, E., Zezulka, S., 2006a. Inhibitory effect of fluoranthene on photosynthetic processes in lichens detected by chlorophyll fluorescence. Ecotoxicology 15, 121–131.

Kummerová, M., Krulová, J., Zezulka, S., Tříska, J., 2006b. Evaluation of fluoranthene phytotoxicity in pea plants by Hill reaction and chlorophyll fluorescence. Chemosphere 65, 489–496.

Kylin, H., Sjödin, A., 2003. Accumulation of airborne hexachlorocyclohexanes and DDT in pine needles. Environmental Science and Technology 37, 2350–2355.

Legge, A.H., Jäger, H.J., Krupa, S.V., 1998. Sulfur dioxide, in Flagler, R.B. (Ed.). Recognition of Air Pollution Injury to Vegetation: A Pictorial Atlas, 2nd Edition Air and Waste Management Association, Pittsburgh, PA, pp. 3-1–3-42.

Lin, C.C., Kao, C.H., 2002. Osmotic stress-induced changes in cell wall peroxidase activity and hydrogen peroxide level in roots of rice seedlings. Plant Growth Regulation 37, 177-184.

Makino, A., Mae, T., Ohira., K., 1986. Colorimetric measurements of protein stained with coomassie brilliant blue R on sodium dodecyl sulfate-polyacrylamide gel electrophoresis by eluting with formamide. Agricultural and Biological Chemistry 50, 1911–1912.

Mallakin, A., Babu, T.S., Dixon, D.G., Greenberg, B.M., 2002. Sites toxicity of specific photooxidation products of anthracene to higher plants: Inhibition of photosynthetic activity and electron transport in *Lemna gibba* L. G-3 (Duckweed). Environmental Toxicology 17, 462-471.

Matzner, E., 1984. Annual rates of deposition of polycyclic aromatic hydrocarbon in different forest ecosystems. Water, Air, and Soil Pollution 21, 425-434.

Miller, J.S., Olejnik, D., 2001. Photolysis of polycyclic aromatic hydrocarbon in water. Water Research 35, 233–243.

Naemura, A., Chiwa, M., Takeda, K., Nakane, K., Sakugawa, H., 2000. Acid deposition on pine needles in Mt. Gokurakuji, Hiroshima Prefecture, Japan. Japanese Journal of Biometeorology 37, 15–20.

Nakaji, T., Izuta, T., 2001. Effects of ozone and/or excess soil Nitrogen on growth, needle gas exchange rates and rubisco contents of *Pinus densiflora* seedlings. Water, Air and Soil Pollution 130, 971–976.

Nakatani, N., 2004. Study of hydroxyl radical photo-formation in environmental aqueous phase and its effects on plant. Dr. thesis, Graduate School of Biosphere Science at Hiroshima University, Higashi-Hiroshima, Japan. P. 140.

Nakatani, N., Kume, A., Kobayashi, T., Hirakawa, T., Sakugawa, H., 2004. Needle morphology related to chemical contents in the needles of Japanese Fir (*Abies Firma*) trees subjected to acidic deposition at Mt. Oyama, Eastern Japan. Water, Air and Soil Pollution 152, 97–110.

Nakatani, N., Akane, S., Chiwa, M., Kobayashi, T., Sakugawa, H., 2007. Roles of hydroxyl radical generating/scavenging mechanisms in pseudo polluted dew in reducing the foliar CO_2 assimilation rate and biomass production of Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) seedlings. Environmental and Experimental Botany 60, 159–169.

Porra, R.J., Thompson, W.A., Kriedemann, P.E., 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: Verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta 975, 384–394.

Percy, K., 1991. Effects of acid rain on forest vegetation: morphology and non-mensurational growth effects. In: *Effects of Acid Rain on Forest Resources*. Proceedings of a conference held in Ste. Foy. Quebec, Forestry Canada, Ottawa, 97–110.

Roháček, K., Barták, M., 1999. Technique of the modulated chlorophyll fluorescence: Basic concepts, useful parameters, and some applications. Photosynthetica 37, 339–363..

Safe, S., Brown K.W., Donnelly, K.C., Anderson C.S., Markiewicz, K.V., McLachlan, M.S., Reischl, A., Hutzinger, O., 1992. Polychlorinated dibenzo-*p*-dioxins and dibenzofurans associated with wood-preserving chemical sites: Biomonitoring with pine needles. Environmental Science and Technology 26, 394–396.

Sakugawa, H., Cape J.N., 2007. Harmful effects of atmospheric nitrous acid on the physiological status of Scot pine trees. Environmental Pollution 147, 532–534.

Samsoe-Peterson, L., Larsen, E.H., Larsen, P.B, Bruun, P., 2002. Uptake of trace elements and PAHs by fruit and vegetables from contaminated soils. Environmental Science and Technology 36, 3057–3063.

Tomlin, C., 1994. The Pesticide Manual, incorporating The Agrochemicals Handbook, A world compendium. British Crop Protection Council and the Royal Society of Chemistry, Farnham UK, 10th ed.1341p.

Tremolada, P., Burnett, V., Calamari, D., Jones K.C., 1996. Spatial distribution of PAHs in the UK atmosphere using pine needles Environmental Science and Technology 30, 3570–3577.

Upham, B.L., Jahnke, L.S., 1986. Photooxidative reactions in chloroplast thylakoids. Evidence for a Fenton-type reaction by superoxide or ascorbate. Photosynthesis research 8, 235-247.

Vaughan, B.E., 1984. State of research: Environmental pathways and food chain transfer. Environmental Health Perspectives 54, 353–371.

Wang, D., Chen, J., Xu, Z., Qiao, X., 1 Huang, L., 2005. Disappearance of polycyclic aromatic hydrocarbons sorbed on surfaces of pine (*Pinus thunbergii*) needles under irradiation of sunlight: Volatilization and photolysis. Atmospheric Environment 39, 4583–4591.

Wenzel, K.D., Weißflog, L., Paladini, E., Gantuz, M., Guerreiro, P., Puliafito, C., Schüürmann, G., 1997. Immision patterns of airborne pollutants in Argentina and Germany II. Biomonitoring of organochlorine compounds and polycyclic aromatics. Chemosphere 34, 2505–2518.

Wild, S.R., Jones K.C., 1991. Studies on the polynuclear aromatic hydrocarbon content of carrots (*Daucus carota*). Chemosphere 23, 243–251.

Wild, S.R, Jones K.C., 1992. Polynuclear aromatic hydrocarbon uptake by carrots grown in sludge-amended soil. Journal of Environmental Quality 21**,** 217–225.

Wild, E., Dent, J., Barber J.L., Thomas, G.O, Jones K.C., 2004. A novel analytical approach for visualizing and tracking organic chemicals in plants. Environmental Science and Technology 38, 4195-4199.

Wild, E., Dent, J., Thomas, G.O, Jones, K.C., 2005. Real-time visualization and quantification of PAH photodegradation on and within plant leaves. Environmental Science and Technology 39, 268-273.

Wild, E., Dent, J., Thomas, G.O, Jones, K.C., 2006. Visualizing the air-to-leaf transfer and within-leaf movement and distribution of phenanthrene: Further studies utilizing two-photon excitation icroscopy. Environmental Science and Technology 40, 907-916.

Yoon, J., Abe-Suzuki, M., Eko, P., Tamai, H., Hanamitsu, S., Nakane, K., 2006. Negative effects of hydroxyl radical-generating mists (simulated dew water) on the photosynthesis and growth of Japanese apricot seedlings (*Prunus mume*). Ecological Research 21, 117–125.

Table 1. Physiological parameters of current-year pine needles in November after 105th day fumigation.

Parameter /Treatment*	**MANN** (CONTROL)	**FLU**	**FLU+MANN**	**PHE**	**PHE+MANN**
A_{max} (µmol CO_2 $m^{-2}s^{-1}$)	10 ± 1^{a}	5 ± 1^{b}	9 ± 1^{ab}	7 ± 2^{ab}	11 ± 2^{a}
g_s (mmol H_2O $m^{-2}s^{-2}$)	0.19 ± 0.03^{a}	0.12 ± 0.02^{b}	0.16 ± 0.03^{ab}	0.15 ± 0.03^{ab}	0.20 ± 0.02^{a}
Fo	212 ± 7^{a}	247 ± 4^{b}	238 ± 7^{ab}	220 ± 7^{ab}	224 ± 5^{ab}
F_v/F_m	0.85 ± 0.01^{a}	0.82 ± 0.01^{a}	0.84 ± 0.01^{a}	0.80 ± 0.01^{a}	0.84 ± 0.01^{a}
Chl a (µmol m^{-2})	237 ± 16^{ab}	99 ± 15^{c}	277 ± 16^{a}	146 ± 21^{bc}	214 ± 46^{abc}
Chl b (µmolm^{-2})	62 ± 4^{ab}	26 ± 4^{c}	71 ± 3^{a}	37 ± 4^{bc}	56 ± 2^{ab}
Chl $_{(a+b)}$ (µmol m^{-2})	299 ± 20^{ab}	126 ± 19^{c}	348 ± 19^{a}	183 ± 25^{bc}	270 ± 57^{abc}
Chl a/Chl b	3.9 ± 0.2^{a}	3.8 ± 0.1^{a}	3.9 ± 0.1^{a}	3.9 ± 0.2^{a}	3.8 ± 0.1^{a}
Rubisco (g m^{-2})	0.7 ± 0.2^{a}	0.1 ± 0.0^{b}	0.3 ± 0.1^{ab}	0.2 ± 0.1^{ab}	0.4 ± 0.1^{ab}
Na (g m^{-2})	0.041 ± 0.001^{a}	0.008 ± 0.001^{a}	ND	0.015 ± 0.001^{a}	ND
K (g m^{-2})	2.4 ± 0.9^{a}	1.3 ± 0.3^{ab}	1.3 ± 0.4^{ab}	1.1 ± 0.3^{b}	1.2 ± 0.3^{ab}
Ca (g m^{-2})	1.0 ± 0.4^{a}	0.5 ± 0.1^{a}	0.9 ± 0.1^{a}	0.7 ± 0.2^{a}	1.3 ± 0.5^{a}
Mg (g m^{-2})	0.3 ± 0.1^{a}	0.2 ± 0.0^{b}	0.3 ± 0.0^{ab}	0.2 ± 0.1^{ab}	0.3 ± 0.1^{ab}

ND: Not detected or below detection limit.

Each value in the table represents a mean of determinations from five pine seedlings ± standard errors (S.E.).

Identical superscript letters indicate the same homogenous groups; different letters indicate significant difference at $p < 0.05$ (Fisher's least significant difference 'LSD').

Table 2. Correlation coefficients (*r*) among the eco-physiological traits of Japanese red pine needles after 105th day fumigation (*n* = 25).

	A_{max}	g_s	F_o	F_v/F_m	Chl. $_{(a+b)}$	Rubisco	Mg
A_{max}	-						
g_s	0.803**	-					
F_o	-0.404*	-0.221	-				
F_v/F_m	0.606**	0.374	-0.478**	-			
Chl $_{(a+b)}$	0.763**	0.562**	-0.312	0.578**	-		
Rubisco	0.443*	0.297	-0.129	0.313	0.652**	-	
Mg	0.421*	0.242	-0.289	0.296	0.526**	0.551**	-

** Significant at 0.01 level, * Significant at 0.05 level.

Figure Captions

Figure 1. Net photosynthetic rate at near-saturating irradiance (A_{max}), needle stomatal conductance (g_s), initial fluorescence values (F_o), photochemical efficiency of PSII in the dark (F_v/F_m) of pine current year needles of Japanese red pine (*P. densiflora*) after 90th day fumigation. Data are means of determinations from five pine seedlings, error bars are ± standard error (S.E.). Identical superscript letters indicate the same homogenous groups; different letters indicate significant difference between values at $p < 0.05$.

Figure 2. (a) Relationship between photosynthesis rate (A_{max}) and rubisco content (Rubisco) (b) relationship between photosynthesis rate and chlorophyll a and b content (Chl $_{(a+b)}$) (c) relationship between chlorophylls a and b content (Chl $_{(a+b)}$) and rubisco content (Rubisco) of current-year needles of Japanese red pine (*P. densiflora*) after 105th day fumigation (November). Data are means of determinations from five pine seedlings; error bars are ± S.E. Pearson's correlation coefficient (r, $p < 0.05, 0.01$). ● is MANN; ◊ is FLU+MANN; □ is FLU;

X is PHE+MANN and Δ is PHE.

Plate caption

Plate 1. Photos of pine seedlings before and after fumigation treatments. (a) Pine seedling before fumigation. (b, c and d) Pine seedlings after three months fumigations with FLU, control and PHE respectively.

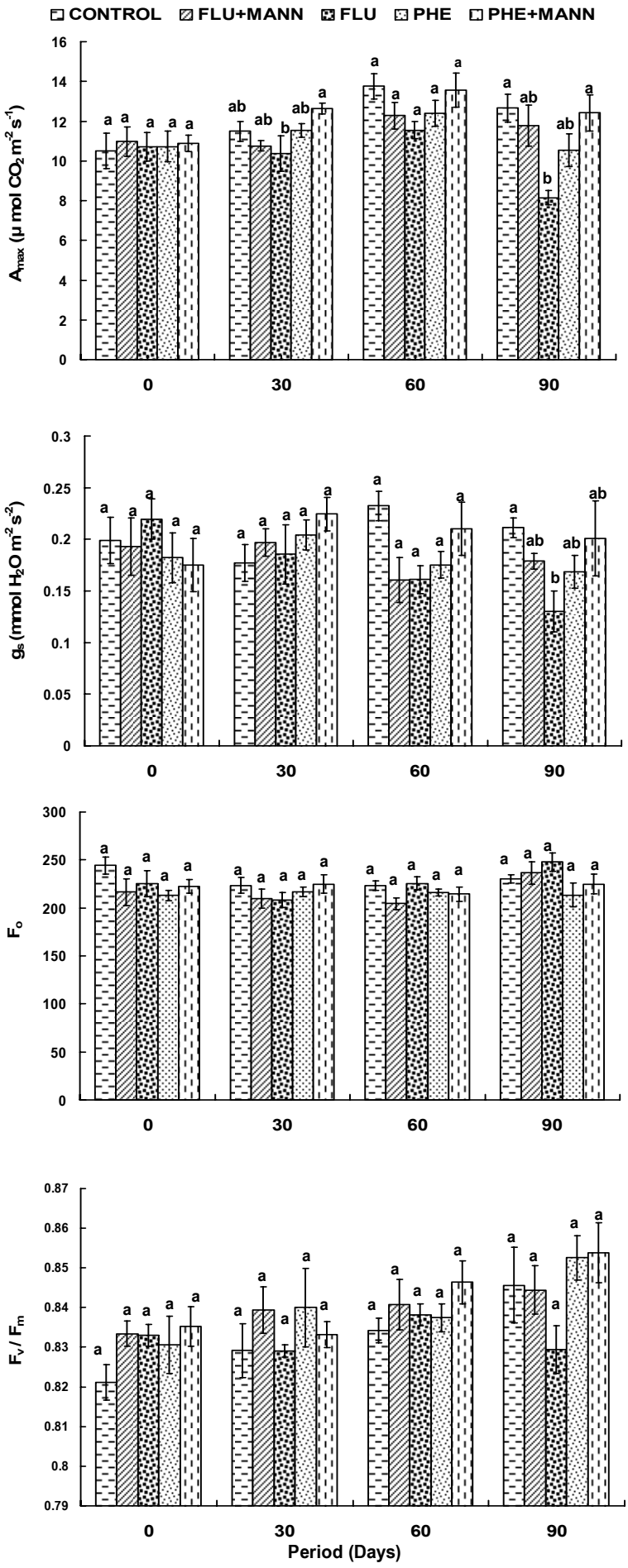
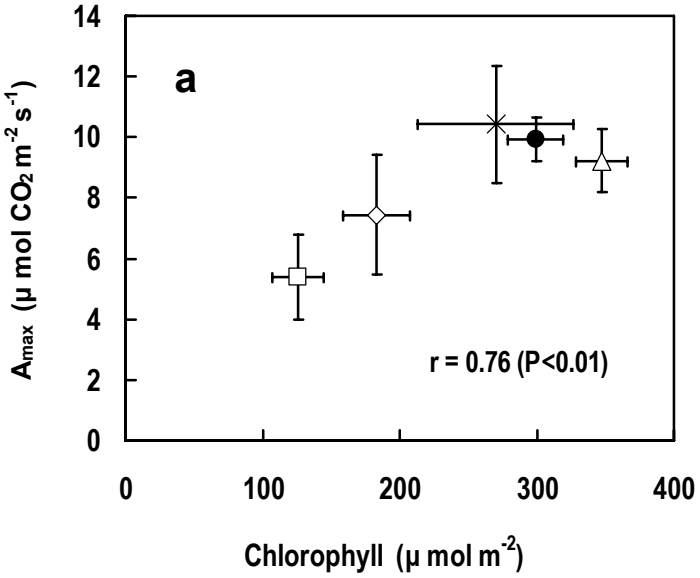
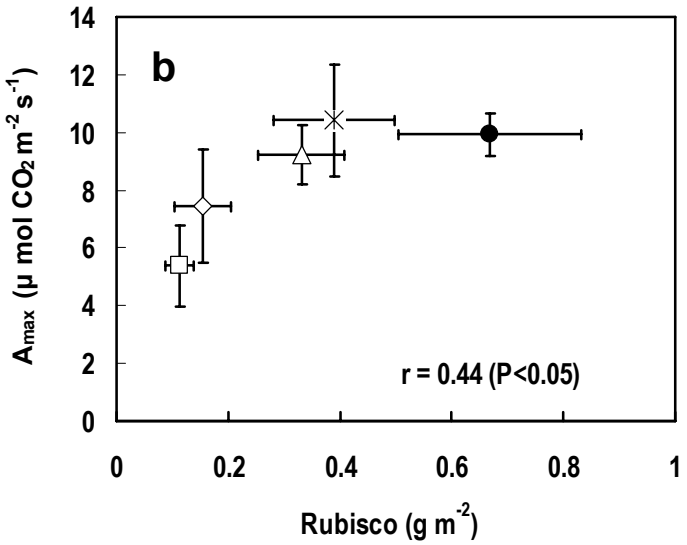

CONTROL
FLU+MANN
FLU
PHE
PHE+MANN
A_{max} (μ mol CO_2 m^{-2} s^{-1})
g_s (mmol H_2O m^{-2} s^{-2})
F_o
F_v / F_m
0
30
60
90
Period (Days)

Figure 1.


a
A_{max} (μ mol CO_2 m^{-2} s^{-1})
r = 0.76 (P<0.01)
Chlorophyll (μ mol m^{-2})


b
A_{max} (μ mol CO_2 m^{-2} s^{-1})
r = 0.44 (P<0.05)
Rubisco (g m^{-2})

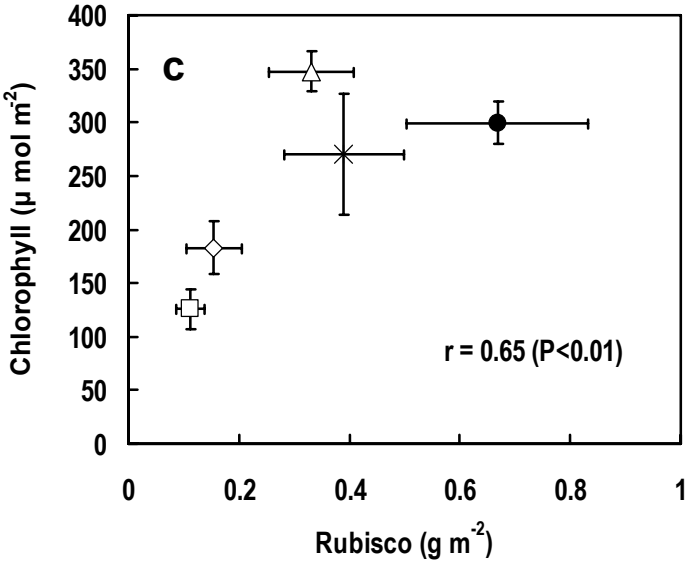

c
Chlorophyll (μ mol m^{-2})
r = 0.65 (P<0.01)
Rubisco (g m^{-2})

Figure 2

(a) **(b)** **(c)** **(d)**

Plate 1.