Physiological and biochemical responses of elevated ozone on *Pterocarpus indicus* under well-watered and drought conditions

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**ABSTRACT**

Seedlings of *Pterocarpus indicus* were grown in both well-watered and drought stress conditions in phytotron. Seedlings grown under well-watered and drought stress conditions were exposed to either combined or without ozone of 200 ppb for one month. First, the physiological responses to elevated ozone levels indicated a decreased biomass. The seedlings grown in arid soil and exposed to ozone showed less biomass than those grown in arid soil but not exposed to ozone. Moreover, all the seedlings except the well-watered and unexposed ones showed a significantly lower photosynthetic rate \((\text{PN})\) over time. However, with the accumulation of ozone injuries, the antioxidant enzyme activities increased overall. In the study results, when exposed to ozone, the well-watered seedlings exhibited more antioxidative enzyme activity than did the seedlings grown in arid soil. Generally, *P. indicus* in arid soil suffered less damage from elevated ozone than did the well-watered plants.

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Antioxidant enzyme; biomass; ozone; photosynthetic rate; *Pterocarpus indicus*

**Introduction**

Air pollution in Philippine cities has become a serious problem during the last few decades. In particular, gas emissions from the increasing number of automobiles in the cities have contributed to increased SO\(_2\) and ozone levels. Emissions have not been successfully restricted: the air-quality standards for SO\(_2\) and O\(_3\) in Philippine cities rose by 4–5% by the end of 2004 (Woo et al. 2007). Matyssek and Innes (1999) reported that air pollution has been caused by industrialization and fossil fuel consumption and ozone is of greatest concern among the various types of air pollutants which is currently affecting urban areas and forests in the world.

Environmental problems arise from both primary pollutants and photochemically generated secondary pollutants. Ozone is produced by photochemical reactions with the NO\(_x\) and SO\(_2\) pollutants formed by automobile emissions, and can harm both plants and animals (Lee et al. 2005).

Ozone causes differences in leaf injury, growth, and yield, reduces carbon assimilation because of stomatal change, and decreases stomata conductance by changing the rates of photosynthesis and internal CO\(_2\) concentration (Andersen 2003). Moreover, plant injuries may induce many antioxidant mechanisms to eliminate the reactive oxygen species (ROS). Plant resistance to ozone may be measured in terms of the changes in the antioxidant enzymes and concurrently the extent of physiological effects on plants exposed to increasing levels of ozone (Moraes et al. 2006).

When drought-stressed trees are exposed to high levels of ozone, they reduce the uptake of ozone by means of stomatal resistance. Therefore, water stress may protect plants from ozone injury (Pearson and Mansfield 1994; Karlsson et al. 1997). Many studies have found a much smaller negative effect of ozone on tree biomass in trees in arid soil than in well-watered trees (Karlsson et al. 1995). Others have found that both the stomatal conductance and the photosynthetic rate \((\text{PS})\) were decreased during drought stress (Wallin and Skärby 1992). In general, water-stressed plants tolerated ozone exposure better, because stomatal closure reduced the effective dose reaching the mesophyll rather than because of a change in the sensitivity of these tissues (Darrall 1989).

Among the tree species grown in the Philippines, *P. indicus* is one of the best known, because it is the national tree and a roadside tree species in Metro Manila. *P. indicus* is dominated by subtropical and tropical regions where shows 22–32 Celsius degree of annual temperature with low humidity and it is well adapted to the harsh environmental conditions such as barren soils and strong winds. The direct effect of ozone on stomata can be explained by either the stimulation of stomatal closing or the inhibition of stomatal opening (Lee and Kim 1997). Karlsson et al. (1997) reported that drought stress can protect trees...
against ozone-induced growth reductions. Therefore, the interactive effects of O₃ and water stress on P. indicus may have ecological implications.

The objectives of this study were (1) to examine the extent to which P. indicus leaves, exposed to elevated ozone, show an antioxidative defense metabolism and differences in physiological response, and (2) to investigate how P. indicus responds to different environmental conditions, i.e., well-watered and soil drought treatment, when it is exposed to elevated ozone.

Materials and methods

Experimental design

One-year-old seedlings of P. indicus, acquired as seeds from research areas in the Philippines, were grown in phytotron. They were pre-adapted to natural sunlight a week before ozone exposure. The ozone fumigation experiment was carried out for one month at a concentration level of 200 ppb. The exposure regimen was a square wave of 200 ppb ozone for 8 hours from 8:00 to 16:00. Seedlings were grown under two water regime treatments of well-watered (28/26 °C day/night, 80% RH, natural sunlight) and drought stress conditions (28/26 °C day/night, 10 ± 5% RH, natural sunlight). Next, combined treatments of ozone and drought were as follows: (1) SW (seedlings grown in well-watered soil), (2) SWO (SW seedlings that were exposed to a high concentration of ozone), (3) SD (seedlings grown in drought stressed soil), and (4) SDO (SD seedlings that were exposed to a high concentration of ozone). During the treatment period, the drought-stressed seedlings were irrigated only when the soil moisture was in the range of 3–7%, while the well-watered treatment was sufficiently irrigated every day. Analyses were carried out after every week of ozone exposure.

Growth and biomass

At the end of the experiment, the seedlings were harvested. The entire plant was separated into root, stem, and branch by removing the leaves, and was then dried for 72 h at 70 °C. The shoot height, basal diameter, total biomass, root, shoot, and root/shoot ratio (R/S) were measured (Woo 1997).

Photosynthesis rate

Net photosynthesis was measured with a Li-6400 photosynthesis system (Li-Cor, Inc. USA). The gas exchange of fully expanded leaves from the fourth to sixth branch from the top of each seedling was measured using an infrared gas analyzer (Li-6400, Li-COR, USA). Light-response curves were measured at 25 °C and 400 μmol mol⁻¹ CO₂. Leaves were acclimated for 2 min prior to measurement at the following photon flux densities (PPFD): 0, 30, 50, 100, 300, 500, 800, 1000, 1500, and 2000 μmol m⁻² s⁻¹.

The cumulative ozone uptake

The cumulative ozone uptake rate was calculated using

\[ Q = Z_a \times \frac{g_{sw}}{1.68}, \]

where \( Q \) is the ozone inhalation, \( Z_a \) the exposed ozone level, \( g_{sw} \) the stomatal conductance, and 1.68 the ratio of diffusion coefficient on vapor and ozone under one atmosphere (Laisk et al. 1989). It was calculated weekly as the ozone exposed time multiplied by the calculated uptake rate.

Antioxidant enzyme activities

SOD, POD, and CAT

Preparation of enzyme extracts for SOD, POD and CAT activities was obtained according to previously described methods (Zhang and Kirkham, 1994).

The SOD activity was evaluated Giannopolitis and Ries (1977). Activities of POD and CAT were determined according to the method described by Chance and Maehly (1995).

APX, GR, DHAR, and MDHAR

Preparation of enzyme extracts for APX, GR, DHAR and MDHAR activities was obtained according to previously described methods (Cakmak et al. 1993).

The APX and DHAR activity were taken using the method of Nakano and Asada (1981), the GR activity was evaluated taken Cakmak et al. (1993). The MDHAR activity was obtained by method of Hossain et al. (1984).

Statistical analyses

Data were submitted to compare the effect of the ozone and drought treatment using two-factor analysis of variance (ANOVA) using SAS statistical software package (Systat 9.2, Systat Software Inc., Richmond, USA). Variance was related to the main treatments (ozone and drought stress) and to the interaction between them.

Results and discussion

Physiological parameters

Ozone stress generally affected the growth and biomass of plants (Woo 1997). Many other studies have reported that ozone can affect plant growth and physiological function (Miller et al. 1994; Landolt et al. 2000). Biomass was measured at the end of the experiment. The effects of ozone on the biomass and R/S of P. indicus seedlings grown under different environmental conditions (well-watered and arid soil) were observed. In the SWO plants, elevated ozone stress significantly decreased the shoot height, root collar diameter, total biomass, shoot, and root, but not R/S (Table 1), perhaps because of reduced nutrient uptake (Oksanen and Saleem 1999). The shoot height, total biomass, shoot, and R/S significantly decreased more for the SDO plants than for the SD plants. Although there were no large differences between SDO and SD
Table 1. Shoot height, root collar diameter, total biomass, shoot, root and R/S of Pterocarpus indicus in well-watered (combined or without ozone of 200 ppb) and drought stress conditions (combined or without ozone of 200 ppb).

| Treatment         | Shoot height (cm) | Root collar diameter (mm) | Total biomass (g dry wt.) | Shoot (g dry wt.) | Root (g dry wt.) | R/S |
|-------------------|-------------------|---------------------------|---------------------------|-----------------|-----------------|-----|
| Well-watered      |                   |                           |                           |                 |                 |     |
| SW1               | 146.6 ± 6.88bc    | 13.9 ± 1.69a             | 107.6 ± 1.7a              | 80.9 ± 2.7b     | 27.0 ± 2.4a     | 3.0 ± 0.4a     |
| SWO               | 116.3 ± 6.33c     | 9.8 ± 0.73b              | 39.7 ± 14.0c             | 29.8 ± 10.8b    | 9.9 ± 3.8b      | 3.2 ± 0.2b     |
| Soil drought      |                   |                           |                           |                 |                 |     |
| SD1               | 216.6 ± 0.52a     | 15.0 ± 0.52a             | 70.8 ± 5.6a              | 5.9 ± 3.7a      | 19.3 ± 3.6a     | 2.4 ± 0.3a     |
| SDO               | 168.6 ± 10.6b     | 13.9 ± 0.50a             | 74.1 ± 6.4b              | 42.9 ± 3.4b     | 30.9 ± 3.4a     | 1.3 ± 0.3a     |
| Ozone (O)         | *                 | *                         | ***                      | ***             | *               | ns  |
| Drought (D)       | **                | ns                        | ns                       | *               | ns              | ns  |
| Interaction (O x D) | ns               | ns                        | ns                       | ns              | *               | ns  |

1SW, seedlings grown in well-watered soil.
2SWO, SW seedlings that were exposed to a high concentration of ozone.
3SD, seedlings grown in drought stressed soil.
4SDO, SD seedlings that were exposed to a high concentration of ozone. Data were analyzed using multifactor two-way ANOVA interactions between ozone and drought. Values are means ± SD for 3 samples.

\( p < 0.05; \quad ** p < 0.01; \quad *** p < 0.001; \quad ns, not significant \)

The effects of ozone on plant response include an actual inhibition of carbon fixation and a closure of the stomata (Heath 1994). Numerous experiments have demonstrated that ozone can cause significant reductions in \( P_N \) (Heath 1994; Miller et al. 1994; Bortier et al. 2000; Woo et al. 2004, 2007; Lee et al. 2005). The \( P_N \) of the SWO plants was slightly increased initially, and then was significantly decreased over time, exhibiting the lowest \( P_N \) at the end of the experiment (Figure 1). \( P_N \) was decreased by almost 45% at the end of the experiment over that before ozone exposure. During ozone exposure, the seedlings showed slightly increased \( P_N \), which was attributed to an improved photosynthesis in undamaged leaves or leaf parts for a whole-plant response that made up for small losses of photosynthetic leaf tissue (Heath 1994; Nali et al. 2004). This ozone-induced decrease in photosynthetic leaf tissue can be explained by the impact on carbon-dioxide fixation and the loss in Rubisco activity (Pell et al. 1994). SD and SDO seedlings had exposed to a pre-adaptative period of water shortage for a week, after that they exposed to 200 ppb ozone. The \( P_N \) of the SDO seedlings slightly decreased over time. The \( P_N \) for the SD seedlings increased about 5% at the end of the period, but for the SDO plants, the \( P_N \) decreased about 55% at the end of the period from what it had been before ozone exposure. However, there were no significant differences between SD and SDO plants. The SD seedlings showed more of a decrease in \( P_N \) than the SW seedlings did. In addition, the SDO seedlings showed a slightly greater reduction in \( P_N \) than did the SWO (Figure 1). The principal mechanism by which plants control their water loss is by regulating the stomata apertures, and several investigators have found that regulation of stomatal movement can be affected by ozone (Karlsson et al. 1997; Beyers et al. 1992). This can be explained by stomatal regulation. Karlsson et al. (1997) found that a high ozone treatment might reduce stomatal closure during drought, which explains why the stomatal conductance decreased more in the SDO seedlings than in the SWO seedlings (Pearson and Mansfield 1993, 1994). Andersen (2003) reviewed the effects of ozone using the flow of carbon from the atmosphere through the plant to the soil. Moreover, drought protects trees from the negative effects of ozone (Karlsson et al. 1995). Experimentally induced drought stress reduced the impact of high ozone, but also reduced growth and nutrient use efficiency. Trees growing in strong drought conditions may be protected to some degree from ozone damage, but their growth will be limited by stomatal closure (Beyers et al. 1992).

The ozone uptake rate in this study was calculated by using the standard of stomatal conductance used by Laisk et al. (1989) and Lee et al. (2002). Figure 2 shows the cumulative ozone uptake during ozone exposure over time. For the SWO seedlings, the uptake gradually increased over time to a maximum at the end of the treatment period, and the SDO plants showed the highest level in the second week. The value in SWO was larger than that in SDO. The uptake was approximately 90% lower in the SDO seedlings than in the SWO seedlings, because of stomatal narrowing (Kronfusz et al. 1998). The stomatal conductance indicated that the ozone uptake rate and \( P_N \) were still significantly coupled in \( P. indicus \) seedlings. Tissue responses to ozone damage include protective and repair processes, as well as changes in carbon allocation. There may also be an interaction between carbon and ozone uptake. Accordingly, an increased uptake of
carbon may benefit trees that have been damaged by increased ozone uptake (Panek and Goldstein 2001).

**Detoxification metabolism**

Under environmental stress, plants produce protective cellular components and, together with the enzymatic detoxification cycle, ascorbate, glutathion, POD, CAT, and superoxide are essential compounds of the antioxidative defense (Herbinger et al. 2002) that render it flexible and dynamic (Noctor and Foyer 1998). In plant cells, ascorbate POD (APX) uses two molecules of ascorbate to change H$_2$O$_2$ to water. MDHA can also be reduced directly to ascorbate via catalysis by monodehydroascorbate reductase (MDHAR) (Noctor and Foyer 1998). APX and MDHAR levels were significantly elevated following ozone exposure over time, but then were partially decreased in the SWO and SDO plants. However, there were no significant differences between the SWO and SDO plants (Figures 3 and 4). Some dehydroascorbate (DHA), which was produced from oxidized ascorbate, is reduced to ascorbate by the action of DHA reductase (DHAR), using GSH as the reducing substrate. This reaction induces glutathione disulphide (GSSG), which is in turn reduced to GSH by NADPH, a reaction catalyzed by glutathione reductase (GR) (Noctor and Foyer 1998). DHAR activity decreased in the SWO plants over time, but partially increased after three weeks; the pattern was similar for the SD and SDO plants. In addition, GR was significantly decreased by ozone over time in the SWO plants, but increased after two weeks in the SWO plants. DHAR and GR activities tended to be higher in the SWO plants than in the SDO plants (Figure 3 and 4). SOD activity started to increase initially. In the SWO plants, it showed increased activity after 2 weeks of ozone exposure. In the arid-soil treatment, SOD activity was similar to that of SD and SDO plants over time, but was higher in SDO plants than in SD plants. Also, SD seedlings had lower SOD activity than SW seedlings, as also observed by Kronfusz et al.
POD and CAT independently eliminate H₂O₂. The activity of the POD enzyme in SWO and SDO plants was significantly increased by the ozone over time, and was higher in SWO plants than in the SDO plants. In addition, CAT activity in SWO plants was increased, but subsequently decreased. After two weeks, a remarkable increase was shown. The CAT activity in the SDO plants did not change over time (Figures 3 and 4).

In this study, most of the antioxidative enzymes were increased by ozone stress over time, and the antioxidative enzyme activities were more suppressed in the SDO seedlings (Figures 3 and 4). Similarly, Alonso et al. (2001) also found that the combined effects of ozone and drought stress did not increase the activation of the antioxidative enzymes. Generally, ozone induced significant increases in the activity of antioxidative enzymes (Wu and Tiedemann 2002). Antioxidant changes are induced to take part in cellular repairs, which reduce the initial oxidative damage caused by ozone, especially on plasma membrane structure and function (Nali et al. 2004). Two studies have examined antioxidant metabolites and enzymes (Herbinger et al. 2002; Nali et al. 2004). Being able to increase antioxidant levels in response to ozone might have contributed to protecting the photosynthetic machinery and membrane function from oxidative stress. Indeed, acclimation to drought, requiring an adequate level of antioxidants, may have protected plant cells from the oxidative damage caused by ozone (Nali et al. 2004). Moreover, ascorbate is the major water-soluble antioxidant (Foyer et al. 1994) that protects plants against ROS and oxidative damage, and a drought-induced
decrease in carbon fixation (through stomatal closure) may limit ascorbate synthesis, which is directly connected to carbon metabolism. Glutathione plays a major role in the regeneration of ascorbate in the ascorbate-glutathione cycle (Smirnoff 1996; Herbinger et al. 2002).

Conclusions
This study showed that elevated ozone exposure caused the decrease of biomass and photosynthesis capacity. The photosynthesis capacity of P. indicus seedling was considerably decreased in SDO plants caused by stomata closure in order to minimize the evaporation as compared to SWO plants. However, biomass showed decreasing tendency in SWO plants rather than SDO plants. The ozone induced these morphological changes, so that less severe cytological injuries were observed in SDO plants than in SWO plants (unpublished data). Moreover, the less severe cytological injuries of SDO seedlings were related to the detoxification metabolism. To remove the oxidative substances, P. indicus exposed to elevated ozone had higher antioxidative enzyme activities. However, the SDO plants decreased antioxidative enzyme activities slightly more than SWO plants did. Accordingly, P. indicus, which is well adapted to dry environments, suffered less damage from ozone when it was grown in arid soil than when it was grown in well-watered soil.

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Disclosure statement

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References

Andersen CP. 2003. Source-sink balance and carbon allocation below ground in plants exposed to ozone. New Phytol. 157(2):213–228.

Alonso R, Elvira S, Castillo FJ, Gimeno BS. 2001. Interactive effects of ozone and drought stress on pigments and activities of antioxidative enzymes in Pinus halepensis. Plant Cell Environ. 24(9):905–916.

Beyers JL, Riechers GH, Temple PJ. 1992. Effects of long-term ozone exposure and drought on the photosynthetic capacity of ponderosa pine (Pinus ponderosa Laws.). New Phytol. 122(1):81–90.

Bortier K, Ceulemans R, Temmerman LE. 2000. Effects of ozone and drought stress on pigments and activities of antioxidative enzymes in Pinus halepensis. New Phytol. 122(1):81–90.

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