DOCTORAL THESIS

Combined effects of hydrogen peroxide and ozone on growth and physiological functions of soybean plants

ダイズの生長および生理に対する過酸化水素とオゾンの複合影響

2013.9

Symbiotic Science of Environment and Natural Resources

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Abstract

Crop response to future air pollution stress is important information for crop production in the future. Ozone (O_3) and peroxides are recognized as significantly damaging air pollutants to plants. Presently information on ambient peroxides both hydrogen peroxide (H_2O_2) and organic peroxide concentrations is limited not only in Thailand and South East Asia but also in Japan. The objectives of the present study were to accumulate data of H_2O_2 concentration in Tokyo and Thailand for understanding annual and seasonal characteristics of ambient H_2O_2 in Japan and Thailand, and to investigate the effect of single O_3 and combined O_3 and peroxides on physiological responses and growth of two Japanese and two Thai soybean cultivars.

Hydrogen peroxide concentrations were measured in Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan (lat. 35.7 ^{o}N long. 139.5 ^{o}E), Kasesart University, Kamphaeng Saen, Nakhon Pathom (lat. 14.0 ^{o}N long. 100.0 ^{o}E) and Mae-On, Chiang Mai (lat. 18.8 ^{o}N long. 99.2 ^{o}E), Thailand in 2009, 2010 and 2011. During the measurement periods, the monthly average H_2O_2 concentration in Tokyo ranged from lower than detection limited to 2.2 ppbv, and showed lower values in May, June and November 2010 and become zero in January to May 2011. In Chiang Mai, the monthly average H_2O_2 concentration was slightly higher than that of Tokyo, ranging from 1.2 to 3.1 ppbv. The monthly average H_2O_2 concentrations in Nakhon Pathom were three to four

times higher than those of Tokyo and Chiang Mai in the same period except March to July 2011. The diurnal characteristics of H_2O_2 concentrations from October to December 2009 at Tokyo and Chiang Mai were similar and high around noon and low in the morning and late afternoon. Nevertheless, the pattern of diurnal characteristics in 2010 at Chiang Mai was high in the morning and gradually decreased in the afternoon and it was different from Tokyo. In the case of Nakhon Pathom, the variation was high each time. Thus, the diurnal characteristics varied from place to place and year to year. A positive correlation between atmospheric H_2O_2 and O_3, and air temperature in Tokyo was found. The monthly average levels of H_2O_2 concentration in Nakhon Pathom measurement site ranged from 2 to 10 ppbv in 2010 and 2011, and these concentrations reach the levels that are harmful to soybean plants when combined with O_3 of 50 ppbv or higher.

The study of effects of peroxides and O_3 on physiological responses and growth of four soybean cultivars was conducted at Tokyo University of Agriculture and Technology, Fuchu, Tokyo. Two Japanese soybean cultivars, Tachinagaha (TC), and Chamame (CM) and two Thai soybeans, A75 and Sorjor 5 (SJ5) were selected as plant materials. Four treatment plots were set up; those are a control plot (C plot: free of O_3 and peroxides), O_3 50 ppbv (O plot), O_3 50 ppbv and peroxides 2-3 ppbv (OP1 plot) and O_3 50 ppbv and peroxides 4-5 ppbv (OP2 plot). We found that combined O_3 and 4-5 ppbv of peroxides (OP2 plot) caused severer damage than the OP1 plot and single O_3 (O plot) to leaf injury, chlorophyll content and photosynthetic rate, and reduced total dry weight and pod dry weight. The net photosynthetic rate (A) in OP plots was reduced by both low stomatal conductance (gs) and high CO_2 concentration in intercellular space (Ci). While A in O plot was reduced by the low gs only. In combined O_3 and peroxides exposure, SJ5 was the most sensitive cultivar in leaf injury, photosynthetic rate, biomass and pod dry weight,

while CM showed less sensitivity for photosynthetic rate and pod dry weight. In addition, the sensitive soybean cultivar (SJ5) showed the highest gs in C, O, and OP1 plots while the tolerant cultivar (CM) show the lowest gs in O, and OP1 plots on 10 days after exposure.

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CHAPTER 1

General introduction

1.1 Ozone (O_3) and peroxides

1.1.1 Ozone

1.1.1.1 Ozone formation

Tropospheric ozone (O_3) is a secondary air pollutant and has been recognized as one of the most damaging air pollutants in recent years (Tompson, 1992). The photochemical formation of O_3 in the troposphere proceeds through the oxidation of nitric oxide (NO) to nitrogen dioxide (NO_2) by organic (RO_2) or hydro-peroxy (HO_2) radicals. The photolysis of NO_2 yields nitric oxide (NO) and a ground-state oxygen atom, $O(^3P)$, which then reacts with molecular oxygen to form O_3. Free radicals oxidizing NO to NO_2 are formed during the oxidation of VOCs (EPA 2006). Carbon monoxide (CO) is also important for O_3 formation in background areas. The formation of O_3 is complex and depends on many factors consisting of (1) sunlight intensity and spectral distribution; (2) concentrations of precursors and the rate of chemical reaction in atmosphere; (3) atmospheric mixing and (4) processing on cloud and aerosol particles. These processes also lead to the formation of other photochemical products such as peroxyacetyl nitrate (PAN), HNO_3, H_2SO_4, peroxides and other compounds (EPA, 2013). The NOx, CO, and VOC precursors of ozone have major anthropogenic sources from fuel combustion as well as natural sources including lightning, wildfires, and plants. The residence time of O_3 is about 100 days in the atmosphere and it is known as a moderately long lived species (Hobbs, 2000). Ozone and its precursors can be also transported over thousands of kilometers. Then, O_3 can be persistent and accumulated in some areas until impacting on plants and human health. Ozone has been attaining high concentrations during the past 60 years, especially in

summer, due to near exponential increases in precursor emissions (Horowitz, 2006; Lamarque *et al*., 2005).

1.1.1.2 Ozone concentrations

In the last century, the concentrations of O_3 increased up to 80 ppbv in China (Wang *et al*., 2006) and have been increasing in many places in the world (Voulgarakis *et al.*, 2011). Ozone concentrations over North America were 50-70 ppbv (Thompson *et al*., 2007; Zhang *et al*., 2010), and frequently observed above 80 ppbv in plumes from intercontinental pollution, fires, and stratospheric intrusion*s (*Liang *et al*., 2007; Oltmans *et al*., 2010). Environmental Protection Agency (EPA), U.S.A, (2013) reported that the O_3 concentrations in California in 2010 were exceeding 80 to 100 ppbv, however trend of O_3 concentration was gradually decreasing from 1990. In Europe, European Environment Agency (EEA) (2012) reported that the daily maximum of 8-hours running mean values of O_3 exceeded 70 ppbv on more than 25 days per year at a large number of stations across Europe in 2010. The highest concentrations were found in the Mediterranean countries. At 66% of stations, a slight decreasing trend of less than 1 ppbv per year was apparent, while 28% of the stations showed an increasing trend from 2001 to 2010 (EEA, 2012). Presently, East Asia has the fastest growing ozone precursor emission. Zhang *et al.* (2009) found that the increasing trend during 2001-2006 of NOx and VOC in China were 55% and 29%, respectively. The increase in NOx and VOC in China not only raises O_3 concentrations in China but also leads to an increase in O_3 over Japan through a long-distance transport of air pollution (Yoshitomi *et al*., 2011). The air quality standard for O_3 in Japan is 0.06 ppmv per hour while that of Thailand is 0.10 ppmv per hour (ADORC, 2006). In remote islands in west Japan such as Oki-Island and Tsusima Island, O_3 concentrations in the

regionally polluted continental outflow are 41-46 ppbv in winter and 54-61 ppbv in spring (Pochanart *et al*., 2002). Kohno *et al.* (2007) and Takeda and Aihara (2007) found that relatively high concentrations of O_3 above 100 ppbv have been frequently observed from spring to autumn in several mountainous areas of Japan. Sato (2011) reported that the annual trend of O_3 concentration in Japan and Thailand from 2001 to 2010 has been increasing (Fig 1.1).

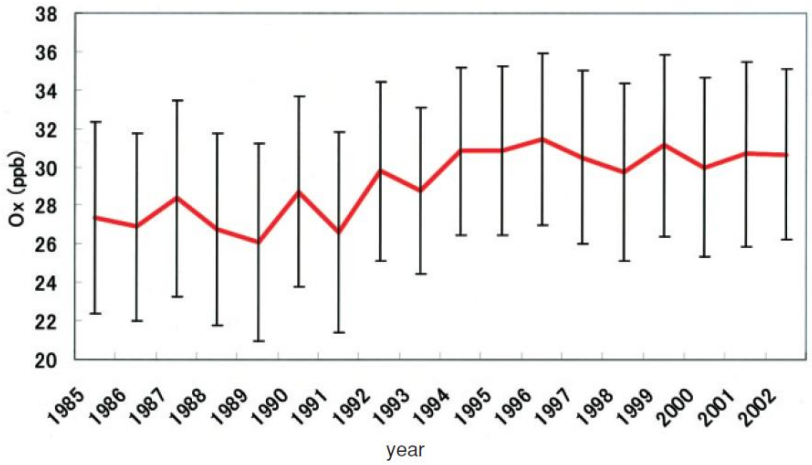


Fig. 1.1. Yearly average daytime concentration of O_3 in Japan (326 continuous ambient air pollution monitoring stations nationwide) Vertical bars show standard deviation (Based on Ohara and Sakata 2003).

1.1.1.3 Effects of O_3 on plants so far

Tropospheric O_3 is considered an important phytotoxic air pollutant that can reduce growth and productivity of many crops and natural vegetation (Akhtar et al. 2010; Cho *et al*., 2011; Yamaguchi *et al*., 2011). Since O_3 formation in the air is accelerated in higher air temperature condition, it tends to reach a level which causes enormous decrease in growth and yield under global warming condition (Booker *et al*., 2009). Crop losses by O_3 damage in 2000 have been estimated as 1.8 to 3.9 billion US dollars in the United States

and 3.0 to 5.5 billion US dollars in China (Van Dingenen *et al.*, 2009). In Europe, various plant species have shown the symptom of O_3 damage and the occurrence of O_3 induced injury to crops and forest species in Italy and other Mediterranean areas had been reported (Bossotti and Ferritte, 1998; Inclan *et al*., 1999). Bhatid *et al*. (2012) demonstrated that O_3 concentration in Asia caused high yield loss on several kinds of economic crops such as wheat, rice, soybean, barley and tomato. Takagi and Ohara (2003) reported that O_3 caused economic loss for rice in Kanto areas, Japan about 14 billion yen a year and seriously affected on many kinds of plants particularly vegetables. In addition, the decrease of the dry weight of Japanese cedar in the northern Kanto area was found. Yamaguchi *et al*. (2011) reviewed that the current levels of O_3 in Japan is high enough to adversely affect the growth of Japanese forest tree species with relatively high O_3 sensitivity such as Japanese beech. However, Takami *et al*. (2003) reported that severe forest decline was seen at Oku-Nikko Mountains in the central Japan where O_3 concentration was not so high while the peroxides concentration in the area was high. Then, the severe plant damage in the areas cannot be fully explained by the effects of O_3 alone.

Ozone is highly reactive and it binds to plasma membranes and alters metabolism (Taiz and Zeiger, 2010). As a result, chloroplast thylakoid membranes are injured, ribulose-1,5-bisphosphate carboxylase/ oxygenase (Rubisco) is degraded and stomatal aperture is poorly regulated (Fiscus *et al.*, 2005; Lu *et al*., 2009). Long-term exposure to ozone also can lead reduction of growth and yield. Ozone generally enters the leaf mainly through the stomata in case of the higher plants (Taiz and Zeiger, 2010). It can react with organic molecules in the apoplast and produce reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and singlet oxygen ($^1O_2$) (Mudd, 1996). The secondary oxidants react preferentially with protein components

of the cell membrane (Mudd, 1996). The ROS can also damage other cellular components and initiate signaling cascades that alter gene expression, activate antioxidant defenses, induce stress ethylene and cause cell death (Baier *et al*., 2005; Cho *et al*., 2011; Pasqualini *et al*., 2003; Rao and Davis, 2001). These reactions are prevented to some extent by the presence of radical scavengers, such as ascorbic acid and polyamines, and antioxidant enzymes (Srivastava, 1999). Other defense responses involving jasmonic acid for example limit lesion development in some plant species (Kangasjarvi *et al*., 2005). There is also a genetic component to plant responses to O_3 that can differentially transduce and amplify O_3 effects (Kangasjarvi *et al*., 2005). As a result, photosynthesis, growth and yield are inhibited (Cho *et al*., 2011; Fiscus *et al*., 2005).

Plant responses to O_3 have been studied on several parameters. The important parameters consist of visible leaf injury, photosynthetic capacity, biomass and yield.

1.1.1.3.1 Visible leaf injury

Foliar injury symptom is one of the parameters used worldwide for studying the impact of O_3 on plants (Booker *et al*., 2009). Several factors should be considered when attempting to determine if O_3 is causing the observed injury. The symptom of foliar injury is variously dependent on plant species and cultivars. Moreover, different types of symptoms can occur on the same plant and sometimes even on the same leaf. In soybean, acute O_3 induced visible foliar injury can result in a variety of symptoms. The symptoms is usually seen as pigmented stippling or necrotic flecking, and often preceded by water-soaked areas (Krupa *et al*., 1998). Occasionally, O_3 exposure may cause extensive bronzing, bleaching or bifacial necrosis of the leaf. Chronic response may be seen as leaf yellowing and may result in premature senescence (Krupa *et al*. 1998).

1.1.1.3.2 Photosynthetic capacity

Photosynthesis has been the mostly studied aspect of plant responses to O_3. Generally, O_3 exposure results in decreased photosynthetic rate (Morgan *et al*., 2003; Watanabe *et al*., 2010; Yamaguchi *et al*., 2011). In many case loss of photosynthetic capacity was shown to be due primarily to reduction of carboxylation efficiency which was directly related to loss of Rubisco activity (Ficus *et al*., 2005; Inada *et al*., 2008). Another possible point of attack by O_3 that cannot be ignored is guard cells (Hoshika *et al*. 2012). However, some scientists indicated that the reduction of stomatal conductance (gs) by O_3 was not a direct cause of reduced assimilation (Ficus *et al*., 1997; Long and Naidu, 2002).

1.1.1.3.3 Biomass and yield

Biomass is the total quantity or weight of organisms in a given area or volume which corresponds to net photosynthetic rate of a whole plant [Equations (1)]. Many scientists found that both acute, short-term exposure to O_3 at concentration >120 ppbv and chronic, long-term exposure (throughout growth) at elevated O_3 concentration can lead the reduction of plant production and biomass (Morgan *et al*., 2003; Ficus *et al*., 2005; Betzelberger *et al*., 2010; Yamaguchi *et al*., 2011).

$$\text{Dry weight} = \text{Photosynthesis-Respiration-Loss} \qquad (1)$$

1.1.2 Peroxides

A peroxide is a compound containing an oxygen–oxygen single bond or the peroxide anion ($[O{-}O]^{2-}$). Peroxide compounds can be roughly classified into organic and inorganic. Atmospheric peroxides consist of both inorganic and organic peroxides including;

Hydrogen peroxide (H_2O_2)	inorganic peroxide
MHP (methyl hydroperoxide; CH_3OOH)	organic peroxides
EHP (ethyl hydroperoxide; CH_3CH_2OOH)	organic peroxides
HMHP (hydroxymethyl hydroperoxide; $HOCH_2OOH$)	organic peroxides
PAA (peroxyacetic acid; $CH_3C(O)OOH$)	organic peroxides

Hydrogen peroxide and the organic peroxides play important roles in the chemistry of the troposphere and serve as an important link between gas phase radicals and aqueous phase chemistry in the atmosphere. Gas-phase H_2O_2 is one of the most important precursors of hydroxyl radicals in the atmosphere and plays an important role as a radical reservoir and sink and a major oxidant of sulfur dioxide (SO_2) to sulfuric acid (H_2SO_4) and sulfate aerosol (SO_4^{2-}) (Calvert *et al*., 1985). The behaviors of peroxides in the atmosphere are shown in Fig. 1.1 and Fig 1.2. Hewitt and Kok (1991) demonstrated that "the organic peroxides were also observed in the aerosol phase but at insignificant concentrations. A range of organic peroxides were found in rain water and constituted 5-20% of the total peroxide content".

1.1.2.1 Formation of peroxides

In unpolluted troposphere, H_2O_2 is generated by photochemical reactions and mainly produced by the disproportionation reaction of two HO_2 radicals [Equations (2)].

$$HO_2 + HO_2 \longrightarrow H_2O_2 + O_2 \qquad (2)$$

Methyl hydroperoxide is formed by the reaction of methylperoxy radical and HO_2 radical [Equations (3)].

$$CH_3O_2 + HO_2 \longrightarrow CH_3OOH + O_2 \qquad (3)$$

Another organic peroxide, hydroxymethyl hydroperoxide is known to be formed by the addition of H_2O_2 to aldehydes under acidic conditions [Equations (4)].

$$HCHO + H_2O_2 \longrightarrow HOCH_2OOH \qquad (4)$$

It has also been found as a product of the photooxidation of formaldehyde and is also formed by the reaction of alkenes, including some biogenic alkenes, with ozone.

Several sources for HO_2 radical in the gas phase have been identified as follows.

$$O_3 + h\nu\ (\leq 320\ nm) \longrightarrow O(^1D) + O_2$$

$$O(^1D) + H_2O \longrightarrow 2OH$$

$$CO + HO \longrightarrow H + CO_2$$

$$O_2 + H \xrightarrow{M} HO_2$$

$$HCHO + h\nu \longrightarrow H + HCO$$

$$H + O_2 \longrightarrow HO_2$$

$$HCO + O_2 \longrightarrow HO_2 + CO$$

$$HCHO + HO \longrightarrow HCO + H_2O$$

In the nighttime, NOx leads to the formation of NO_3 which can react to produce HO_2 as follow;

$$NO_3 + HCHO \longrightarrow HNO_3 + HCO$$

$$HCO + O_2 \longrightarrow HO_2 + CO$$

$$R\text{-}CH_3 + NO_3 \longrightarrow R\text{-}CH_2 + HNO_3$$

$$R\text{-}CH_2 + O_2 \xrightarrow{M} R\text{-}CH_2O_2$$

$$R\text{-}CH_2O_2 + NO \longrightarrow R\text{-}CH_2O + NO_2$$

$$R\text{-}CH_2O + O_2 \longrightarrow R\text{-}CHO + HO_2$$

Besides, the thermal decomposition of peroxyacetyl nitrate (PAN) releases peroxyacetyl radical and yields HO_2 and HCHO.

$$CH_3C{=}OO_2NO_2 \longrightarrow CH_3C{=}OO_2 + NO_2$$

$$CH_3C{=}OO_2{+}NO \longrightarrow CH_3C{=}OO + NO_2$$

$$CH_3C{=}OO \longrightarrow CH_3 + CO_2$$

$$CH_3 + O_2 \xrightarrow{M} CH_3O_2$$

$$CH_3O_2 + NO \longrightarrow CH_3O + NO_2$$

$$CH_3O + O_2 \longrightarrow HCHO + HO_2$$

Moreover, the temperature-dependent decomposition of peroxynitric acid HO_2NO_2 will yield HO_2. The photo-oxidation of isoprene also yields HO_2 (Gu *et al.*, 1985) which is a main contributor for the enhancement of H_2O_2 levels in forest areas (Gaffney *et al.*, 1987). Atkinson and Lloyd (1984) suggested that the production of HO and HO_2 radical occur via alkene oxidation initiated by O_3.

Therefore, the production of gas-phase H_2O_2 is dependent on the ambient concentrations of hydrocarbons, aldehydes, CO, NO, NO_2 and O_3, and light intensity (Longan *et al.*, 1981; Kleinman, 1986). The most sensitive parameter of H_2O_2 production is the rate of the reaction of HO with NO_2 which removes both HO and nitrogen from the photochemical system (Stockwell, 1986). The second most important pathway of H_2O_2 production is HO_2 produced from HCHO photolysis as well as the rate constant for the reaction of HO_2 with NO because the HO_2 self-reaction to form H_2O_2 competes with HO_2-NO reaction. Moller (2009) summarized the atmospheric H_2O_2 sources by the following processes:

a) Direct emission through combustion (e.g. biomass burning)
b) Photochemical initiated formation via the HO_2 radical as a product of photolysis of O_3 and aldehyde
c) Thermal direct formation in gas phase via ozonolysis of alkene

d) Decay of O_3 in (alkaline) aqueous solution

e) Photosensitized formation (‘photocatalysis’) via electron transfer onto O_2 in the aqueous phase

Principally the peroxides always coexist with ozone (O_3) in the air by the reaction of O_3 and volatile organic compounds (VOCs). Presently O_3 is gradually increasing in several regions around the world and industry emits much VOCs. Therefore, the concentrations of atmospheric peroxides can be expected to increase in the future.

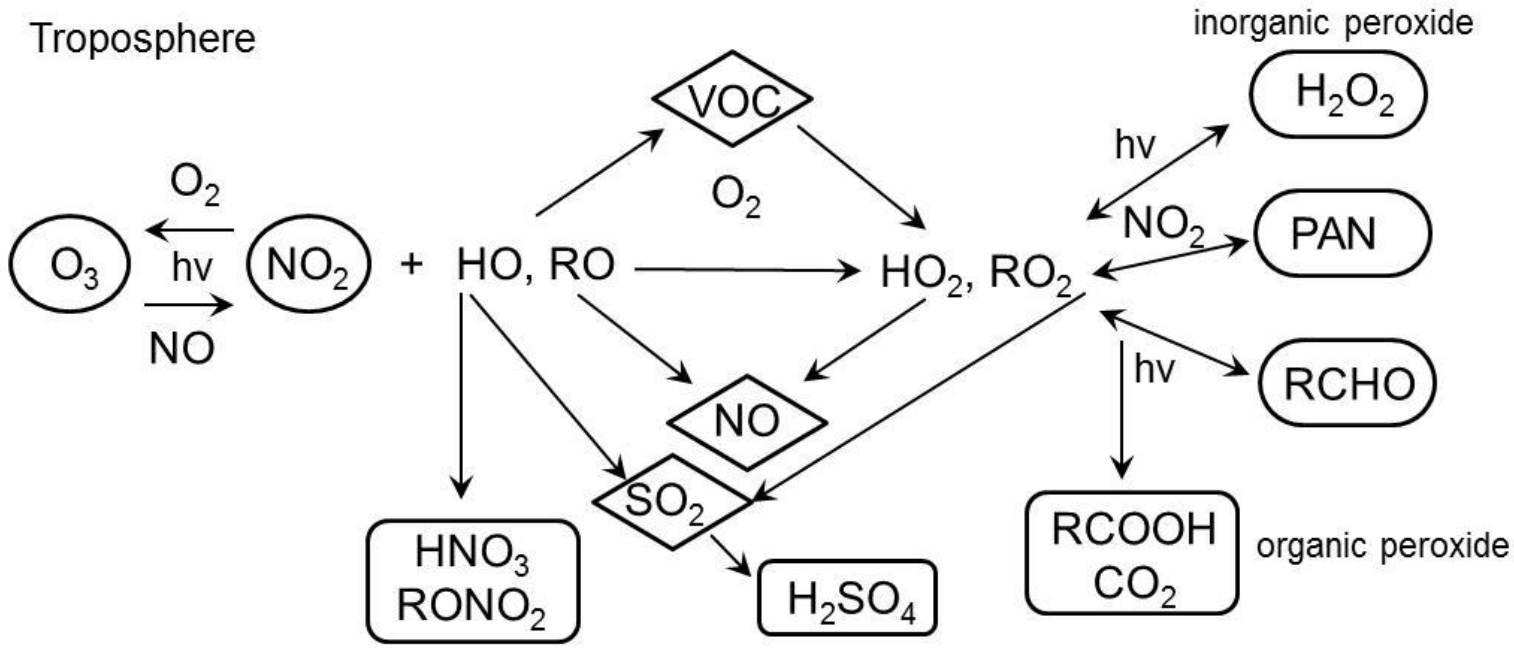


Fig. 1.2. Schematic representation of gas-phase chemistry resulting in the generation of ozone and peroxides in troposphere. Primary pollutants emitted from anthropogenic sources, are shown in diamond-shaped boxes; secondary pollutants, formed as a result of atmospheric reactions, are shown in circular boxes (from Karnosky *et al*. 2003).

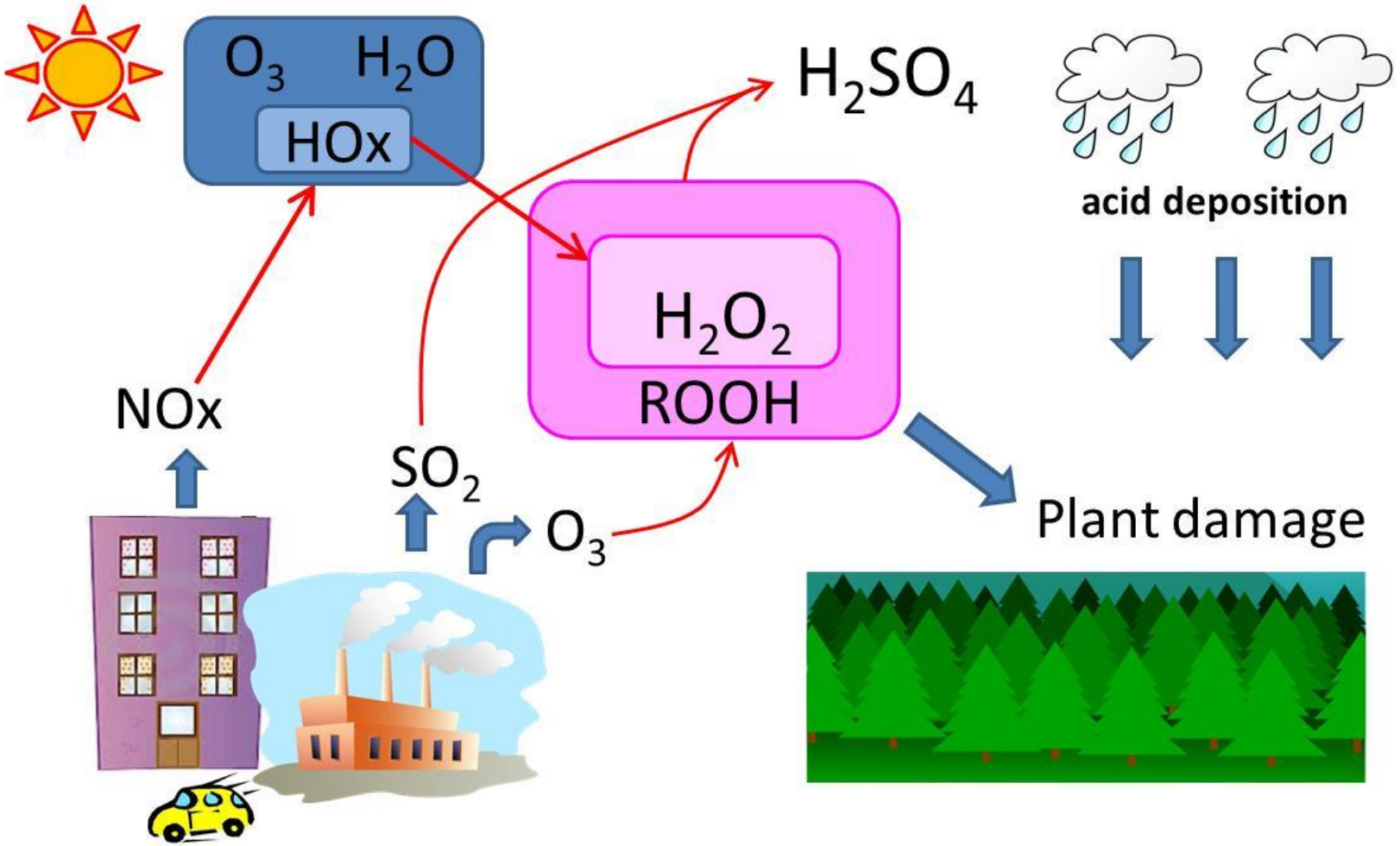


Fig.1.3. The behaviors of peroxides in the atmosphere (modified from Chen, 2006)

1.1.2.2 Peroxide concentrations

The atmospheric H_2O_2 was studied in the 20th century (Gunz and Hoffmann, 1990,). The H_2O_2 concentrations are in the level of several ppbv and dependent on several environmental factors such as air temperature, humidity, solar radiation, seasons and areas (Gunz and Hoffmann, 1990; Takami *et al*., 2003; Chen *et.al.*, 2008). In recent years, field measurements of H_2O_2 were carried out in several areas in the US, Europe and Japan. Daum (1989) reported that the H_2O_2 concentration at Columbus, Ohio, USA ranged from <0.2 to 8 ppbv which was under strong daily variation. Das and Husian, (1999) found that the H_2O_2 concentration in Whiteface Mountain lodge, New York, USA ranged from 0.1 to 4.6 ppbv, while Hasson and Paulson (2003) revealed that the concentration of H_2O_2 in Los Anglelis, USA, was from 0.5 to 3.5 ppbv. In Brazil, Jacob *et al*. (1988) reported that H_2O_2 concentration ranged from 0.2 to 3.9 ppbv. In Europe, the H_2O_2 and MHP in the costal environment of Ireland ranged from 0.01 to 7.10 and 0.01 to 0.30 ppbv, respectively (Morgan and Jackson, 2002). Moller (2009) found that H_2O_2 concentration in summer at Hohenpeissenberg, Germany showed a trend about +0.02 ppbv yr^{-1}. In Japan, Watanabe *et al*. (1995), Yamada *et al*. (2002) and Takami *et al*. (2003) reported that the concentration of H_2O_2 in the daytime is usually higher than that at night and ranged from 0.1 to 4.5 ppbv. However, the information on atmospheric H_2O_2 concentration is very limited not only in Thailand and South East Asia but also in Japan.

1.1.2.3 Effects of peroxides on plants so far

Peroxides are strong oxidizing agents and are much more water-soluble than O_3 (the Henry's Law constants for H_2O_2, MHP, and O_3 are 1 x 10^5, 2.8 x 10^2, and 1 x 10^2, respectively; (Zhou and Lee, 1992; O'Sullivan *et al*., 1996). Then, H_2O_2 and MHP have a

greater potential to damage plant tissues. Generally, gases enter the leaf through stomata and react with other molecules in intercellular space or cell membrane (Taiz and Zeiger, 2010). Hewitt *et al*. (1990) found the peroxides in the leaves of isoprene-emitting plant after exposure to O_3 but are not found in control plants grown in clean air. Then, they suggested that the reaction of ozone with biogenic alkenes to produce toxic peroxides could be one of the mechanisms by which damage to plants occurs. And this model may explain in part the die-back of tree species producing reactive alkenes, such as the red spruce, which emits isoprene and monoterpenes. Masuch *et al*. (1986) reported that wet deposition of H_2O_2 in acidic cloud or fog water damaged the leaf cell structure, decreased resistance to drought stress and decreased the ability to transport assimilation of young spruce and beech. In contrary, dry deposition of H_2O_2 alone did not result in significant uptake into the foliage and damage on the coniferous species (Claiborn and Aneja, 1993; Claiborn *et al.,* 1993). However, they suggested that H_2O_2 is possible to reach the stomata and even in the inner tissues of broadleaf plants which do not have sunken stomata. Terry *et.al*., (1995) suggested that peroxides damage macromolecules such as nucleic acids, proteins and lipid, and may highly impact to plant growth by affecting source and sink strength, reducing photosynthetic capacity, inhibiting transportation and declining yield accumulation.

1.1.3 Combined effect of O_3 and peroxides on plants

Until now there are few reports and little information about the study of combined effects of O_3 and peroxides on plants. Chen *et al*. (2005) reported that combined O_3 and peroxides can cause higher visible leaf injury on several kinds of plants such as Japanese radish and Chinese cabbage than single O_3. Chen *et al.* (2010) demonstrated that only 2-3

ppbv gas-phase peroxides combined with 50 ppbv O_3 can cause severer damage to Japanese radish than 100 ppbv O_3. Na-ngern *et al*. (2011) found that combined presence of peroxides and O_3 in Japan can cause higher reduction of net photosynthetic rate and leaf injury on rice. Chutteng *et al*. (2012) also reported that combined O_3 and peroxides caused severer leaf injury, decrease in chlorophyll content and growth rate on beech seedling than single O_3 and these damages increased year by year. In addition, the increases in H_2O_2 and organic peroxides were reported to be associated with forest decline in some places in Japan (Hatakeyama *et al*., 1993; Takami *et al*., 2003; Aoki *et al*., 2012). Presently, there is no information about the combined effect of peroxides and O_3 on soybean plant which is important economic crop in the world.

1.2 Soybean

Soybeans *(Glycine max)* are one of the most valuable crops in the world not only as an oil seed crop and feed for livestock and aquaculture, but also as a good source of protein for the human diet and as a biofuel feedstock. Soybeans were planted on 75 million acres (30.3 million hectares) in 2011, producing 3.06 billion bushels (83.18 million metric tons) of soybeans (Soy Stats, 2012). The average price paid to farmers was 11.70 US dollars per bushel (430 US dollars per metric ton) (Soy Stats, 2012). The total 2011 crop value exceeded 35.7 billion US dollars. In 2011, soybeans represented 56 percent of world oilseed production. The world's five largest producers in 2011 were United States of America, Brazil, Argentina, China and India (Soy Stats, 2012). Soybean is one of the major crops in Japan, too. In 2011 land allotted for soybean farming was down by 1% to 55,442 hectares and production also fell by the same percentage to reach 220,000 tons

(MAFF, 2012). As for Thailand, soybeans were planted on 48,220 hectares and producing 83,222 tons (OAE, 2013).

Soybean is known to be sensitive to O_3 (Heagle *et al*., 1998; Morgan *et al*., 2003). Several scientists reported that an above-ground biomass of soybean was reduced by 40% when grown at 60 ppbv O_3 (Morgan *et al*., 2003). Ashmore (2002) supported that an increase in 7 h mean daytime O_3 concentration from 30 to 60 ppbv was estimated to reduce soybean yields by 16%. Additionally, Wahid *et al*. (2001) found that yield loss of soybean due to surface O_3 in Pakistan was about 60%. Betzelberger *et al*. (2010) demonstrated that many physiological and biochemical parameters correlated with soybean yield and there were significant intraspecific variability in soybean yield response to elevated O_3. The sensitivities of soybean to O_3 varies among genotypes (Burkey and Carter, 2009;Akhtar *et al*., 2010; Betzelberger *et al*., 2010).

1.3 Objectives of this study

The increasing of O_3 and sources of peroxides in several places around the world especially in Asia may cause the plant damages in some areas. Thailand is agricultural areas and high emission of air pollutants due to the increasing industrial activity, populations, transportations and biomass burning. The information of atmospheric peroxides concentration is necessary to investigate and accumulate particularly long-term measurement to know the concentration and its impacts in Thailand. Generally the major component of peroxides in the air is H_2O_2. Therefore, the first objective of this study is to accumulate data of H_2O_2 concentration for long periods in Tokyo and Thailand to understand the annual and seasonal characteristic of ambient H_2O_2 in Japan and Thailand. It is well known that soybean is an important crop not only in Japan and Thailand but also

all around the world and sensitive to O_3. Chen *et al*. (2010); Na-ngern *et al*. (2011); Chutteang *et al*. (2012) found that combined O_3 and peroxides cause severer damage on several kinds of plants than single O_3. Importantly, there is no information about the effects of combined O_3 and peroxides on soybean plants and less report about the physiological responses of Japanese and Thai soybean cultivars to single and combined O_3 and peroxides which are necessary to investigate for future crop productivity assessment since atmospheric peroxides concentration would increase in the future. Thus, the second objective of this study is to investigate the effects of single O_3 and combined O_3 and peroxides on leaf injury and physiological responses of Japanese and Thai soybean cultivars to understand the impact of combined effect of O_3 and peroxides in soybean plant.

CHAPTER 2

Atmospheric H_2O_2 concentrations in Japan and Thailand

2.1 Background

Peroxides including H_2O_2 always coexist with ozone (O_3) in the air because they are produced by the photochemical reactions to form O_3 from volatile organic compounds (VOCs) and NOx. The H_2O_2 concentrations are in the level of several ppbv (Gunz and Hoffmann, 1990; Takami *et al.*, 2003; Chen *et.al.*, 2008). At present, O_3 is gradually increasing in several regions around the world and industry emits much VOCs. Therefore, the concentrations of atmospheric peroxides can be expected to increase in the future.

From the agricultural perspective, peroxides are very harmful to plants especially when combined with O_3 (Chen *et.al*., 2005; Chen *et.al*., 2010). Chen *et al.* (2010) demonstrated that only 2-3 ppbv peroxides combined with 50 ppbv O_3 can cause severer damage to Japanese radish than 100 ppbv O_3. Moreover increases in H_2O_2 were reported to be associated with forest decline (Hatakeyama *et al*., 1993; Takami *et al*., 2003). Therefore, the knowledge about the concentration of H_2O_2 in the field is very important for agriculture and terrestrial ecosystems.

In recent years, short period field measurements of hydrogen peroxide (H_2O_2) have been carried out in several areas in the US, Europe and Japan (Watanabe *et al*., 1995; Jackson and Hewitt, 1996; Das and Husain, 1999; Yamada *et al*., 2002; Takami *et al*., 2003). In Japan, Watanabe *et al*. (1995), Yamada *et al*. (2002) and Takami *et al*. (2003) reported that the concentration of H_2O_2 in the daytime is usually higher than that at night and ranged from 0.1 ppb to 4.5 ppbv. The ambient H_2O_2 concentration depends on several environmental factors such as air temperature, humidity, solar radiation, seasons and areas (Gunz and Hoffmann, 1990). However, the information on atmospheric H_2O_2 concentration is very limited in South East Asia, and in Japan.

2.2 Objectives

To accumulate data of H_2O_2 concentration Japan and Thailand to understand the annual and seasonal characteristic of ambient H_2O_2 in Thailand and Japan

2.3 Materials and Methods

2.3.1 Study site description

H_2O_2 concentrations were measured at three measurement sites (Fig. 2.3.1), that is, 1) urban area, Tokyo site, Japan, 2) mixed area of residential and crop production areas, Nakhon Pathom site, Thailand and 3) rural valley area, Chiang Mai site, Thailand.

2.3.1.1 Urban area, Tokyo site, Japan

The first location is in Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan (lat. 35.7 ^{o}N long. 139.5 ^{o}E). This measurement site, Tokyo site, is in a city located 20 km west of central Tokyo and 28 km from Tokyo Bay (Fig.2.3.2 and Fig.2.3.3). The measurement tower was located in the farm of Tokyo University of Agriculture and Technology.

2.3.1.2 Mixed area of residential and crop production areas, Nakhon Pathom site, Thailand

The second location is in Kasesart University Kamphaeng Saen Campus, Kamphaeng Saen, Nakhon Pathom (lat. 14.0 ^{o}N long. 100.0 ^{o}E). It is located 80 km from Bangkok and 30 km from down town Nakhon Pathom Province, and is surrounded by industrial areas of Ayutthaya with a distance of 70 km and Saraburi with a distance of 120 km (see Fig. 2.3.4). This measurement site, Nakhon Pathom site, is in mixed areas of

residential and crop production areas in the central part of Thailand (Fig. 2.3.5). The pollutants emitted by the industrial areas of Ayutthaya and Saraburi may affect the measurement site in the dry season from November to January due to the steady prevailing wind from north east. The industrial areas of Rachaburi locates about 45 km south west of the measurement site, and the emitted pollutants may affect the measurement site by the steady prevailing wind of Monsoon in rainy season from the south west in May to October. Within the period of March-April, the prevailing wind direction is south. In this period no big industrial area affects Nakhon Pathom site.

2.3.1.3 Rural valley area, Chiang Mai site, Thailand

The third location is in the northern part of Thailand in Mae On, Chiang Mai, Thailand (lat. 18.8 ^{o}N long. 99.2 ^{o}E). This site, Chiang Mai site, is in a rural valley area and surrounded by mountains. The measurement site is located 45 km east of central Chiang Mai (Fig. 2.3.6 and Fig. 2.3.7).

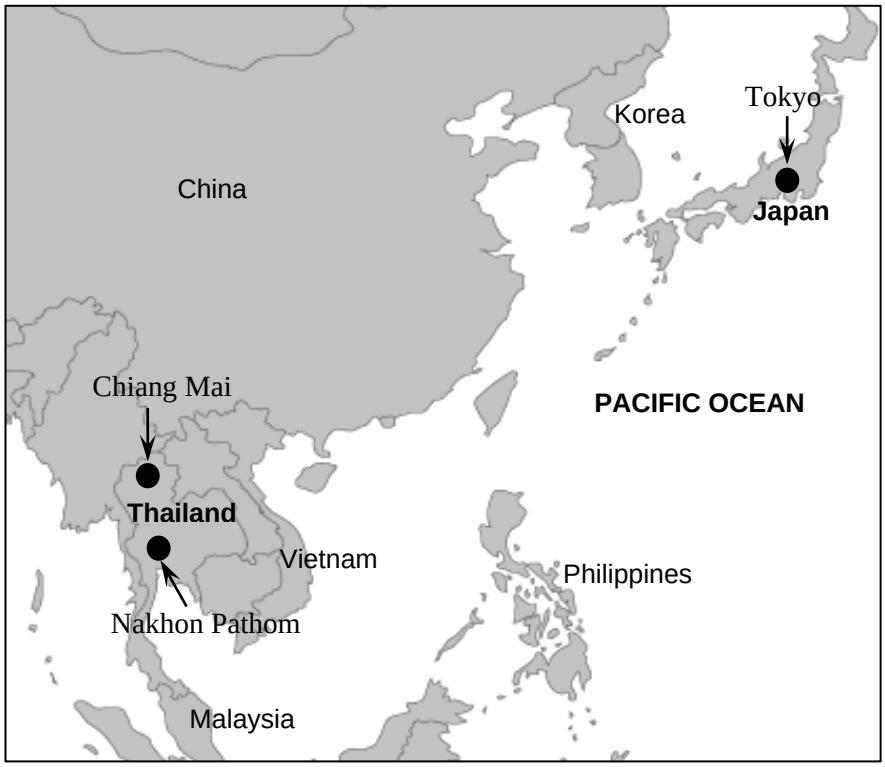


Fig. 2.3.1. Locations of three measurement sites in Tokyo, Nakhon Pathom and Chiang Mai

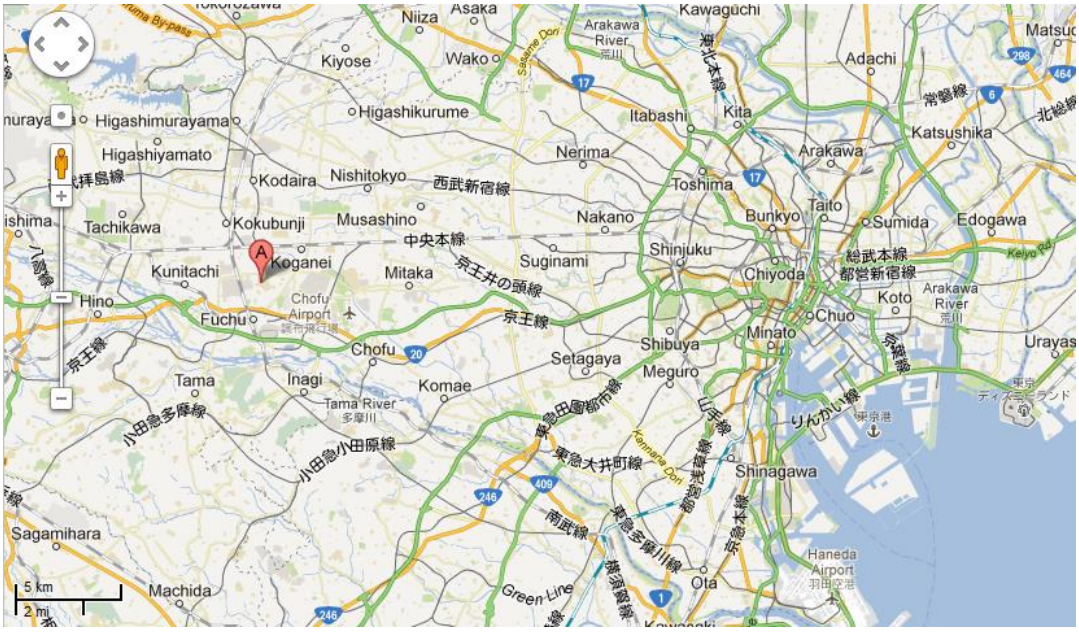


Fig. 2.3.2. Location of Tokyo measurement site

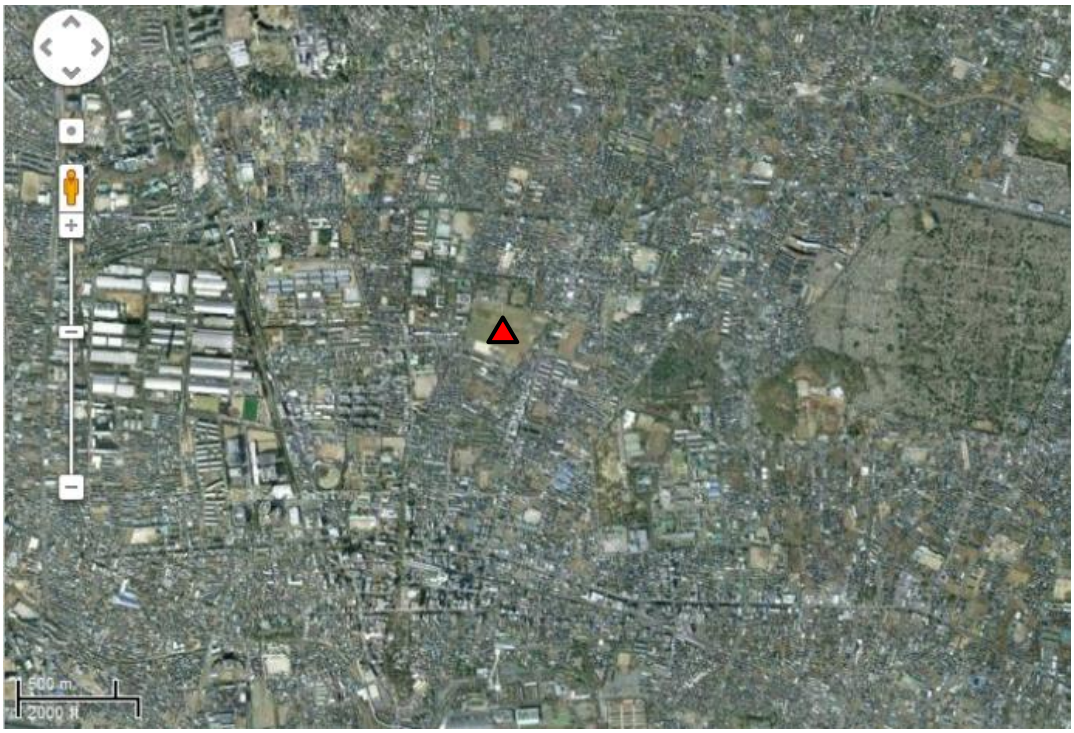


Fig. 2.3.3. Top view of Tokyo measurement site located in urban area in Japan

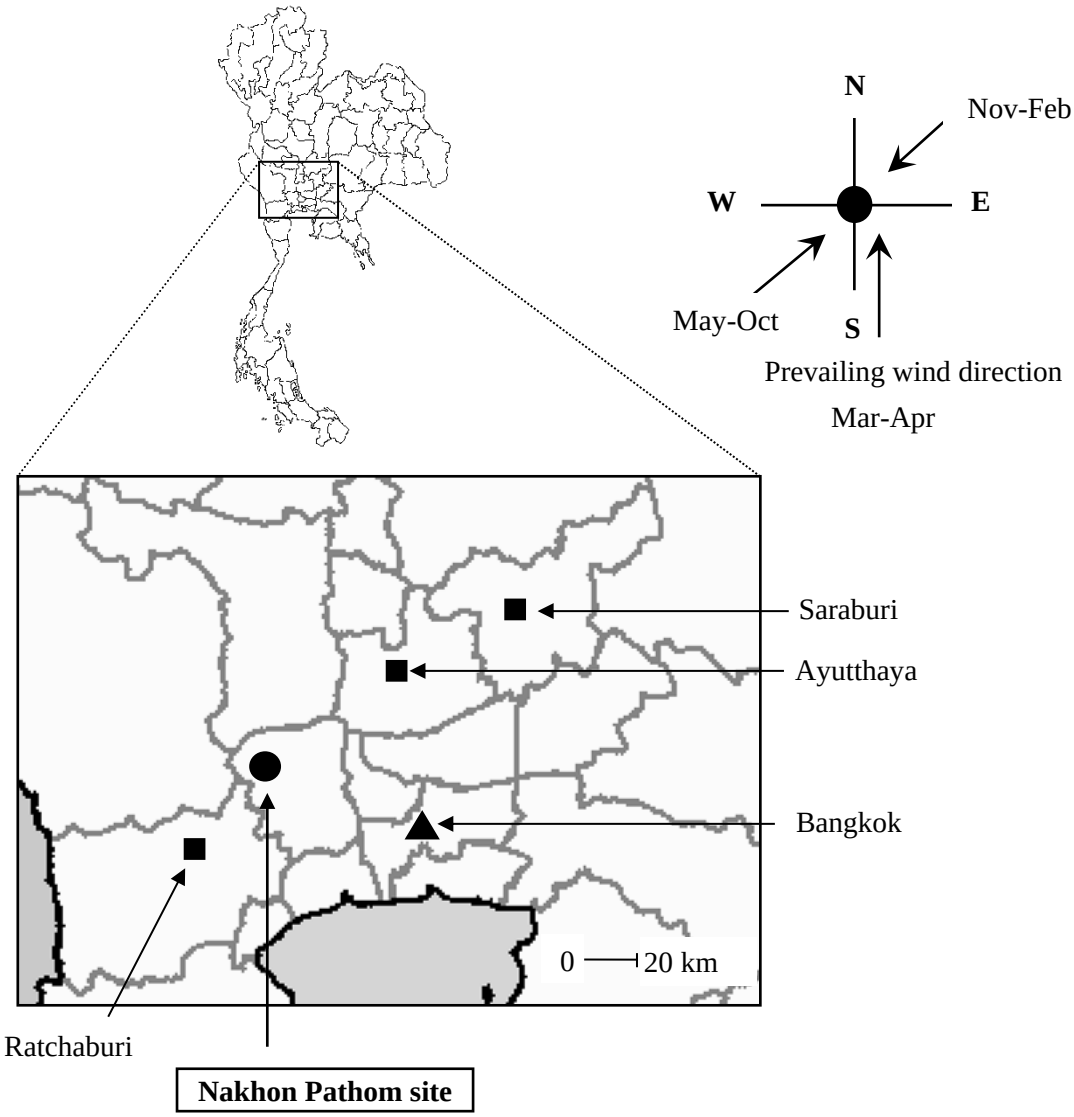


Fig. 2.3.4. Location of Nakhon Pathom measurement site and surrounding big cities and industrial areas

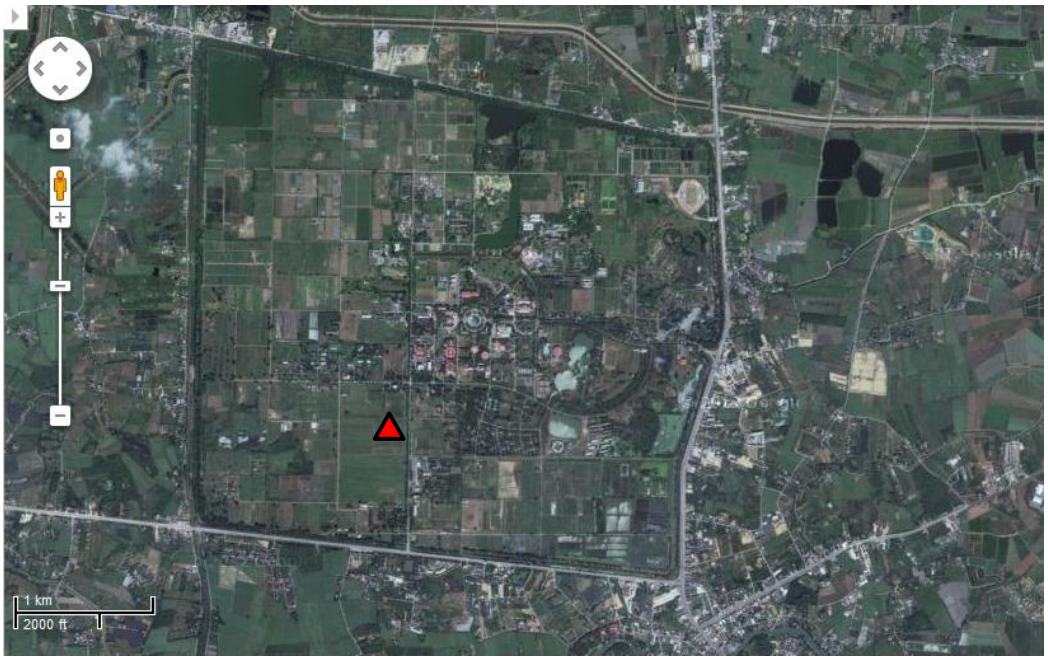


Fig. 2.3.5. Mix area of residential and agricultural areas at Nakhon Pathom measurement site

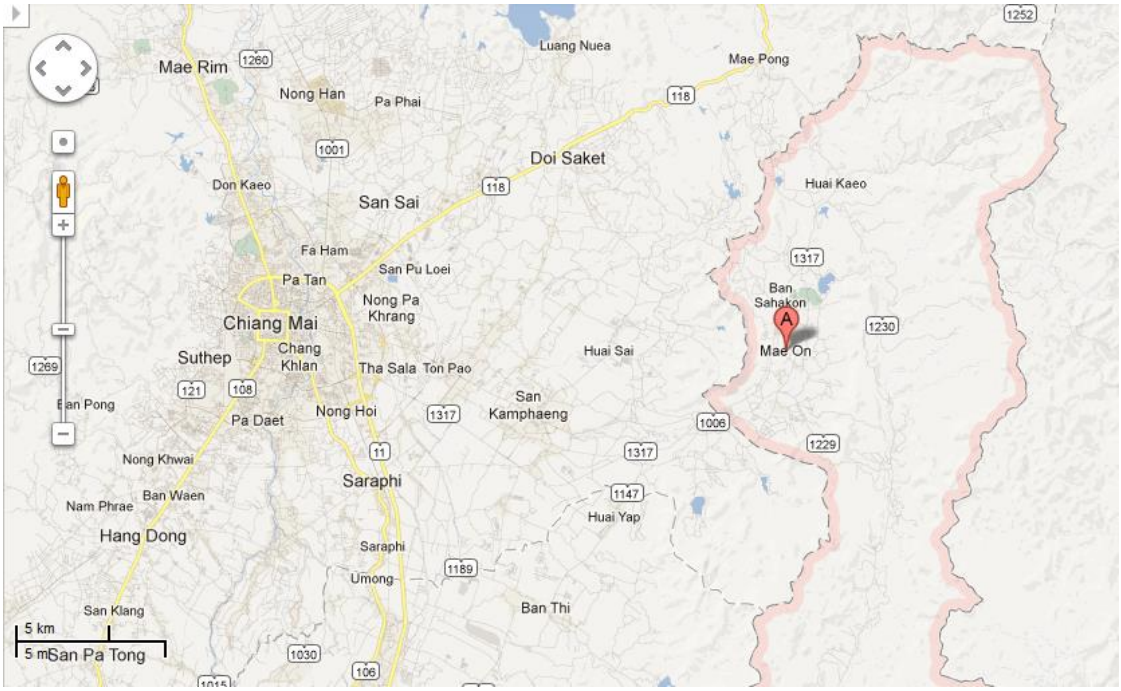


Fig. 2.3.6. Location of Chiang Mai measurement site 45 km from the Chiang Mai city

Fig. 2.3.7. Rural valley area at Chiang Mai site surrounding mountainous areas

2.3.2 Measurement of gaseous H_2O_2

Gaseous H_2O_2 in the air was sampled for 30-60 min with Pyrex glass mist chamber (Cofer *et al.*, 1985) filled with about 5 ml distilled water (Fig. 2.3.11) The mist chamber was placed at 2 m above ground (Fig.2.3.8, Fig.2.3.9, and Fig. 2.3.10). In order to prevent photolysis of peroxides during sampling, the mist chamber was shielded with aluminum foil. The Teflon filter with pore size of 1 μm was mounted at the sample air inlet of the mist chamber to eliminate the dust. Samples were collected four days a month and three to five times a day in the morning, afternoon and evening from 2009 to 2011. Samples were analyzed by portable equipment of RQflex 10 reflectometer (MERCK KGaA, Dannstadl, Germany) see Fig. 2.3.12 which measures the difference in intensity of emitted and reflected light and allows a quantitative determination of the concentration of H_2O_2. This equipment is reliable to measure H_2O_2 concentration as shown in Fig. 2.3.14. which is calibrated against HPLC system (JASCO, LCSS-905, Tokyo, Japan) equipped with a fluorescence detector (Fig. 2.3.13) by using standard H_2O_2 solution. An ODS-2 column (GL, Science; 5 μm, 4.6×250 mm2, Tokyo, Japan) was employed to separate H_2O_2 and organic peroxides. A polymer column and polymer tubing were used to eliminate decomposition of hydroperoxides on metal surfaces, and the column was cooled at 4°C to minimize decomposition of peroxides within the column. H_2O_2 was detected by fluorescence from the dimer of *p*-hydroxyphenylacetic acid formed by the post-column reaction of *p*-hydroxyphenylacetic acid and peroxides in the presence of horseradish peroxidase. The H_2O_2 value is the mean of three time analyses by RQflex 10. Other pollutant gases and meteorological factors at Tokyo site were provided by a monitoring site in Fuchu which is one of the sites of Atmospheric Environmental Regional

Observation System (AEROS) of Japan. The Fuchu AEROS site is at 2-24 Miyanishi, Fuchu, Tokyo, Japan and located about 2 km south west of Tokyo site.

Fig.2.3.8. Measurement tower in Tokyo measurement site

Fig.2.3.9. Measurement tower in Nakhon Pathom measurement site

Fig.2.3.10. Measurement tower in Chiang Mai measurement site

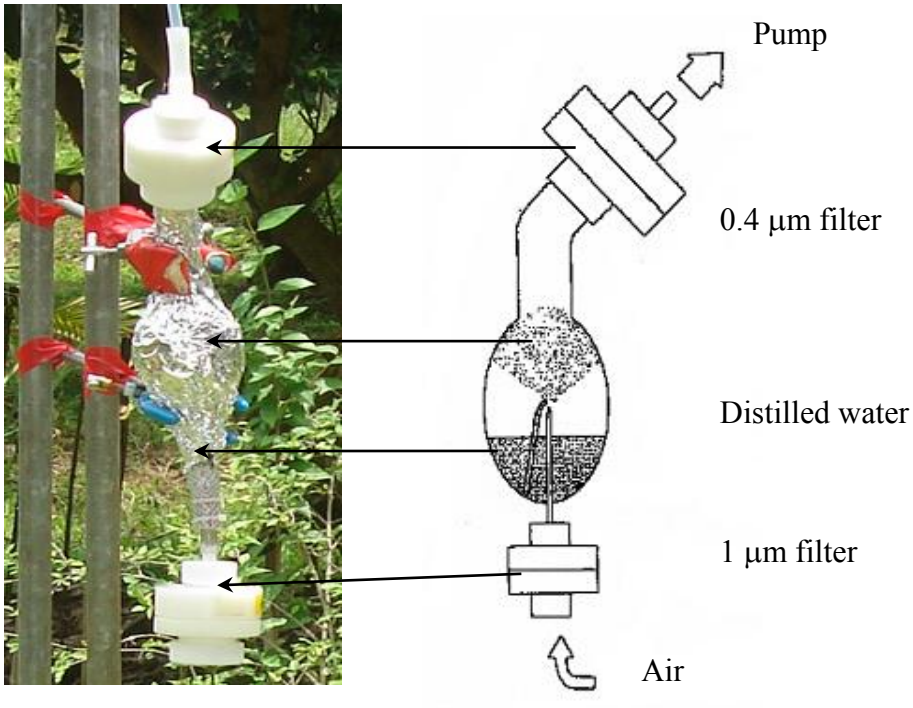
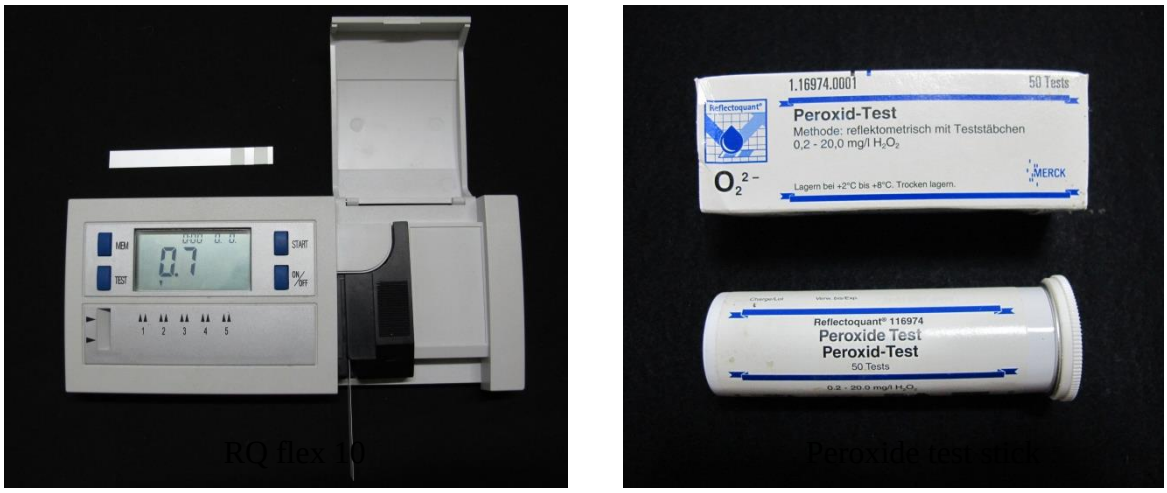


Fig. 2.3.11. Mist chamber for collecting peroxides in water



RQ flex 10 Peroxide test stick

Fig. 2.3.12. RQ flex 10 system for peroxides measurement

Fig. 2.3.13. HPLC system for peroxides measurement

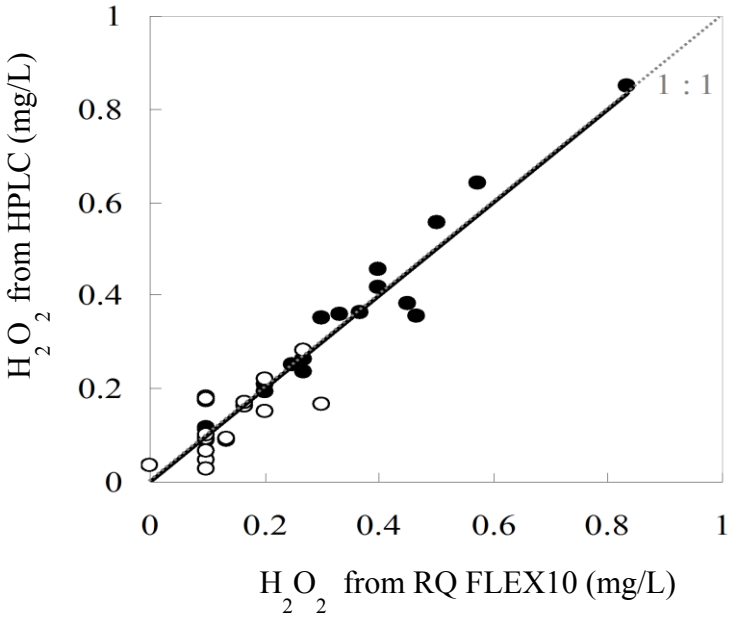


Fig. 2.3.14. Calibration of H_2O_2 concentration from RQ FLEX 10 versus HPLC system (JASCO, LCSS-905); Different symbols refer to different measurement series.

2.4 Results

2.4.1 Monthly H_2O_2 concentrations in Tokyo, Chiang Mai and Nakhon Pathom

During the measurement period, the monthly average H_2O_2 concentration in Tokyo site ranged from 0.0 to 2.2 ppbv (Fig. 2.4.1), showed lower concentrations in May, June and November 2010 (0.6, 0.6, and 0.0), and become zero in January, February and April 2011. The range of minimum and maximum air temperatures in May-June 2010 were 7-28 °C, -3 - 14 °C in November 2010-February 2011 and 8-28 °C in April 2011.

The monthly average H_2O_2 concentration in Chiang Mai, was from 1.2 to 3.1 ppbv which is slightly higher than that of Tokyo (Fig. 2.4.1). However the levels in March and April 2011 were high. The air temperature during measurement time in November 2010 to January 2011 ranged from 12.5-31 °C while that of March-July 2011 ranged from 18-36 °C. The total rainfall in 2010 and 2011 was 1,440 and 1,706 mm respectively.

In case of Nakhon Pathom, the monthly average H_2O_2 concentrations from August 2010 to February 2011 were about three to four times higher than those of Chiang Mai in the same period except from March to July 2011 (Fig. 2.4.1). The daytime minimum and maximum air temperature in August and October 2010 were 26-35°C while those in November 2010 to February 2011 were 19.5-34°C. The air temperature in March to July 2011 ranged from 28 to 39°C. The total rainfall in 2011 was about 1,296 mm which was 5% higher than that in 2010. In 2011, the rain began to fall in March earlier than normal year.

The gas-phase H_2O_2 concentration measured in 2009, 2010 and 2011 are summarized in Table 2.4.1. The H_2O_2 concentration ranged from below the detection limit of 0.1 to 5.1 ppbv in Tokyo. In Chiang Mai and Nakhon Pathom, the ranges of H_2O_2

concentration were from below the detection limit of 0.1 to 6.2 ppbv and 1.4 to 15 ppbv, respectively.

The monthly mean H_2O_2 concentration in Tokyo was high in early summer and low in winter and early spring. The monthly mean values of H_2O_2 concentration in Chiang Mai were high in July to November 2010 and March to July 2011 whereas the monthly mean concentration in Nakhon Pathom was low in March and July 2011 and high in August 2010 to January 2011.

2.4.2 Diurnal characteristics of H_2O_2 concentration in 2009 and 2010

The diurnal variations of H_2O_2 concentrations from October to December 2009 at Tokyo and Chiang Mai sites showed similar patterns, which were high around noon and low in the morning and in the evening (Fig. 2.4.2a). However, the pattern of diurnal characteristics of Chiang Mai in 2010 was high in the morning and gradually decreased in the afternoon (Fig. 2.4.2b), and the daily pattern was different from that of Tokyo. In case of Nakhon Pathom, the monthly variation in concentration was high. Thus, the diurnal characteristics were different from place to place and year to year.

2.4.3 Correlation between H_2O_2 and air pollutants and meteorological data

Due to the limitation of air pollutants and meteorological database in measurement sites in Thailand, correlation between H_2O_2 and air pollutants and meteorological data was analyzed only in Tokyo. The correlation of H_2O_2 with other pollutant gases and meteorological factors in Tokyo measurement site are shown in Fig. 2.4.3. Among them, O_3 and air temperature showed a positive correlation (Fig. 2.4.3d and Fig. 2.4.3f). However, the slopes of air temperature and H_2O_2 were different for July and October, 2010

(Fig. 2.4.3f). These trends were similar to the correlation between O_3 and air temperature (Fig. 2.4.4). Relative humidity showed weak negative correlation with H_2O_2 in July and October 2010 (Fig. 2.4.3g). As for NO, a negative correlation was observed (Fig. 2.4.3a). NO_2 and NO_x showed similar patterns which were positive correlation in July and negative correlation in October 2010 (Fig. 2.4.3b and Fig. 2.4.3c). Whereas, no clear correlation was found between H_2O_2 and SPM, Rs and wind speed (Fig. 2.4.3e, Fig. 2.4.3h and Fig. 2.4.3i).

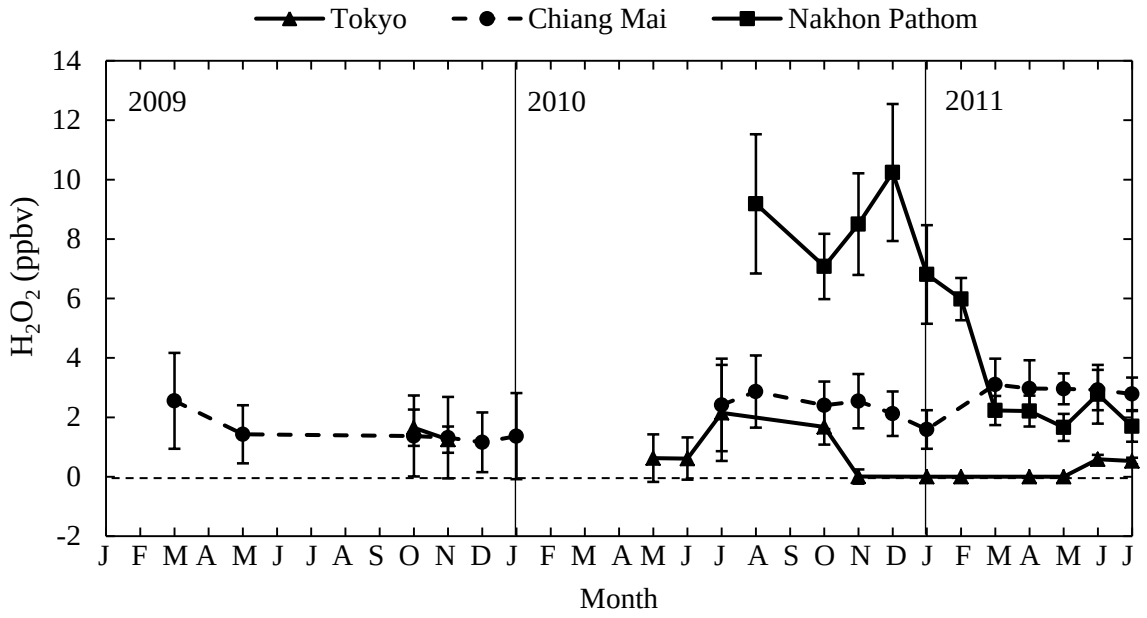


Fig. 2.4.1. Monthly average H_2O_2 concentration in three measurement sites. The error bars show the standard deviation.

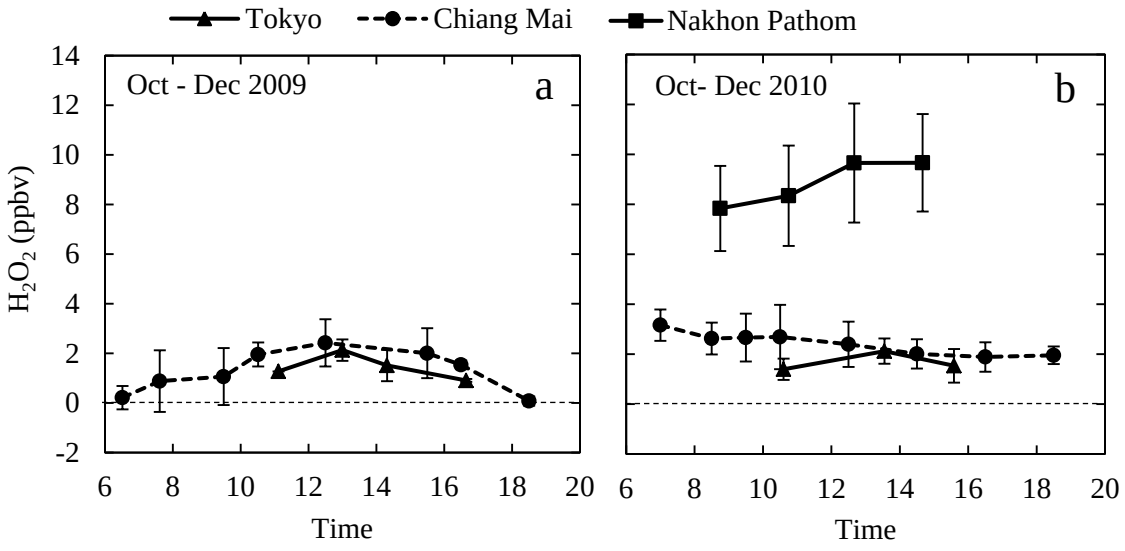


Fig. 2.4.2. Diurnal variation of H_2O_2 from October to December in 2009 (a) and 2010 (b). The error bars show the standard deviation.

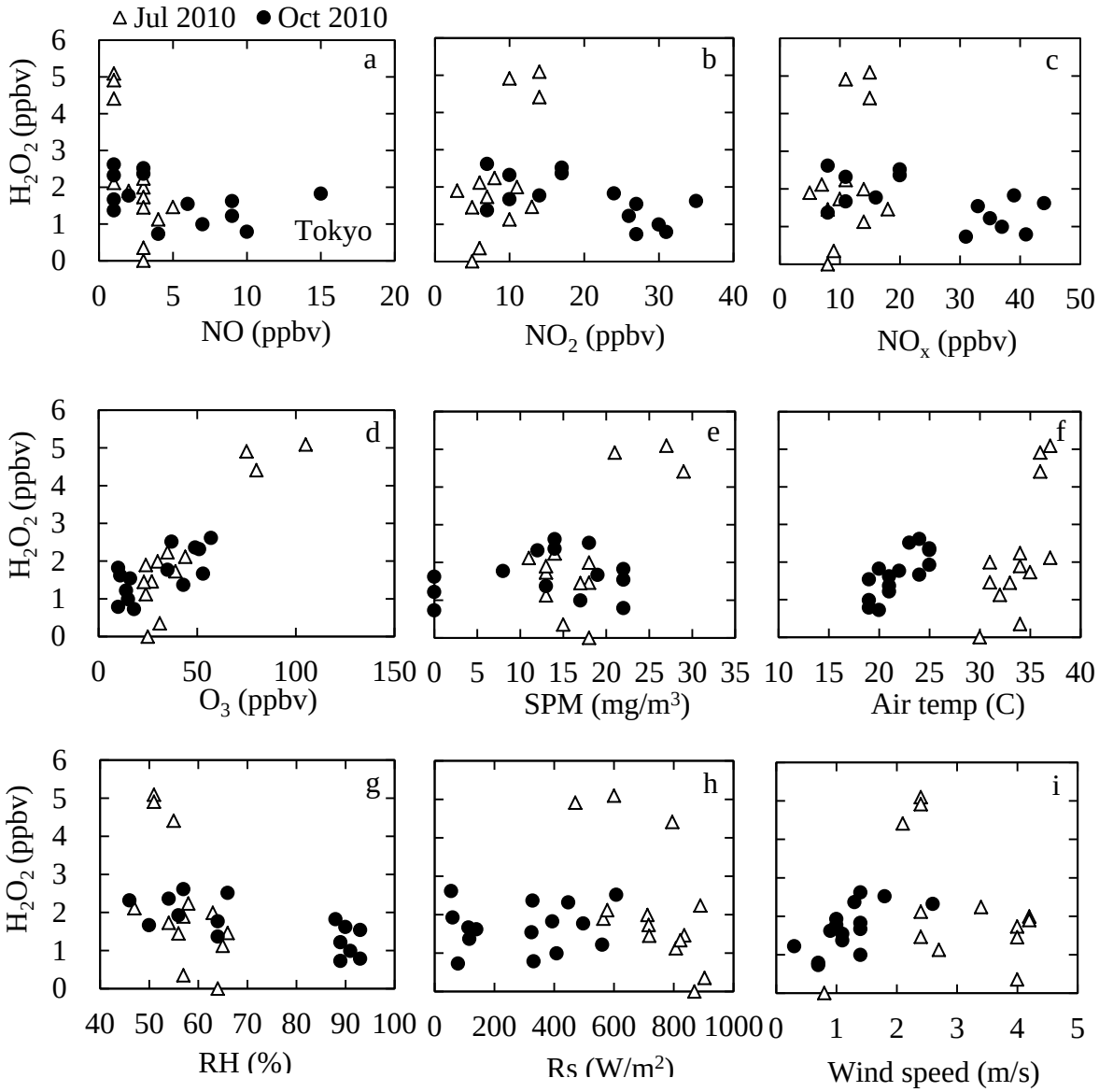


Fig. 2.4.3. Correlation of peroxides with NO (a), NO_2 (b), NO_x (c), O_3 (d), SPM (e), air temperature (f), relative humidity (g), solar radiation (h) and wind speed (i) in July (△) and October (●) 2010. All plots are based on each measured data in Tokyo site and Fuchu AEROS site.

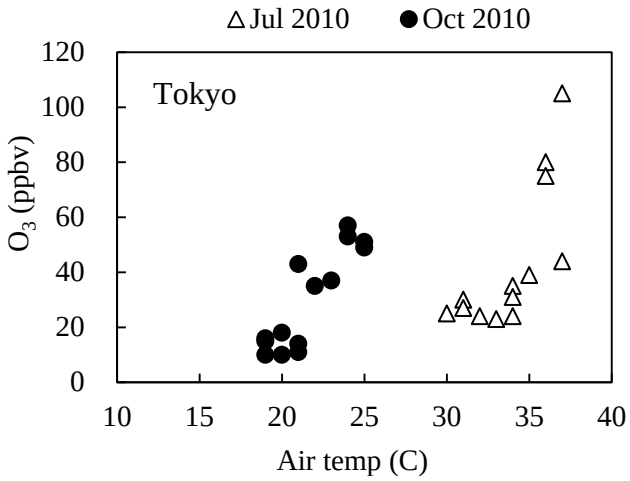


Fig. 2.4.4. Correlation of ozone with air temperature in Tokyo in July (△) and October (●) 2010. All plots are based on each measured data.

Table 2.4.1. Observed atmospheric H_2O_2 concentrations in 2009, 2010 and 2011 in Tokyo, Chiang Mai and Nakhon Pathom based on each measured data

* average of all measurement data

Site	Measurement periods	Number of measurements	Min (ppbv)	Max (ppbv)	Mean (ppbv)	SD (ppbv)
Tokyo	Oct. 29-30, 2009	5	1.0	2.4	1.7	0.6
	Nov. 1-4, 2009	4	0.9	1.8	1.3	0.4
	AVG 2009*	**9**	**-**	**-**	**1.5**	**0.5**
	Apr. 30-May 3, 2010	12	<0.1	2.4	0.6	0.8
	Jun. 5-7, 2010	9	<0.1	1.4	0.6	0.7
	Jul. 15-20, 2010	14	<0.1	5.1	2.2	1.6
	Sep. 30-Oct. 4, 2010	15	0.7	2.6	1.7	0.6
	Nov. 18-21, 2010	8	<0.1	0.1	<0.1	0.0
	AVG 2010*	**58**	**-**	**-**	**1.2**	**1.2**
	Jan. 19-22, 2011	12	<0.1	<0.1	<0.1	0.0
	Feb. 22-25, 2011	12	<0.1	<0.1	<0.1	0.0
	Apr. 14-17, 2011	12	<0.1	<0.1	<0.1	0.0
	May 18-21, 2011	16	<0.1	<0.1	<0.1	0.0
	Jun. 21-24, 2011	16	0.5	0.8	0.6	0.2
	Jul. 23-26, 2011	19	0.5	0.6	0.5	0.1
	AVG 2011*	**87**	**-**	**-**	**0.2**	**0.0**
Chiang Mai	Mar. 15-17, 2009	29	<0.1	5.4	2.6	1.6
	May 2-5, 2009	16	0.2	4.0	1.4	1.0
	Oct. 12-14, 2009	15	<0.1	4.0	1.4	1.4
	Nov. 20-22, 2009	10	<0.1	3.6	1.3	1.4
	Dec. 23-26, 2009	21	<0.1	2.7	1.2	1.0
	AVG 2009*	**91**	**-**	**-**	**1.7**	**1.4**
	Jan. 23-26, 2010	8	<0.1	4.7	1.4	1.5
	Jul. 8-12, 2010	15	0.3	6.2	2.4	1.6
	Aug. 12-16, 2010	25	1.0	5.7	2.9	1.2
	Sep. 30- Oct. 4, 2010	23	1.1	4.1	2.4	0.8
	Nov. 2-6, 2010	25	1.3	4.8	2.6	0.9
	Dec. 25-29, 2010	25	0.5	3.6	2.1	0.8
	AVG 2010*	**121**	**-**	**-**	**2.4**	**1.1**
	Jan. 25-29, 2011	25	0.6	3.5	1.6	0.7
	Feb. 27-Mar. 3, 2011	25	1.1	4.4	3.1	0.9
	Apr. 4-8, 2011	25	0.8	5.4	3.0	1.0
	May 2-6, 2011	25	2.0	3.9	3.0	0.5
	Jun. 1-5, 2011	25	1.8	4.5	2.9	0.7
	Jul. 12-16, 2011	25	1.8	4.0	2.8	0.6
	AVG 2011*	**150**	**-**	**-**	**2.7**	**0.9**

Table 2.4.1. Observed atmospheric H_2O_2 concentrations in 2009, 2010 and 2011 in Tokyo, Chiang Mai and Nakhon Pathom based on each measured data

* average of all measurement data

Site	Measurement periods	Number of measurements	Min (ppbv)	Max (ppbv)	Mean (ppbv)	SD (ppbv)
Nakhon Pathom	Aug. 24-27, 2010	15	6.0	14.7	9.2	2.3
	Oct. 30, 2010	4	6.0	8.1	7.1	1.1
	Nov. 12-15, 2010	16	5.8	12.1	8.5	1.7
	Dec. 18-20, 2010	12	6.1	13.3	10.2	2.3
	AVG 2010*	**47**	**-**	**-**	**9.0**	**2.2**
	Jan. 14-17, 2011	16	4.1	9.5	6.8	1.7
	Feb. 11-14, 2011	20	4.8	7.1	6.0	0.7
	Mar. 12-15, 2011	20	1.6	3.2	2.2	0.5
	Apr. 21-24, 2011	16	1.4	3.1	2.2	0.5
	May 16-19, 2011	19	1.0	2.4	1.7	0.5
	Jun. 15-18, 2011	18	1.5	4.9	2.8	1.0
	Jul. 28-31, 2011	19	1.0	3.2	1.7	0.5
	AVG 2011*	**128**	**-**	**-**	**3.3**	**2.1**

2.5 Discussion

2. 5.1 Monthly H_2O_2 concentrations in Tokyo, Chiang Mai and Nakhon Pathom

Gnauk *et al*. (1997) and Hua *et al*. (2008) reported that atmospheric H_2O_2 concentration in urban areas in Germany and China ranged from 0 to 5.3 ppbv and mean value of 2.1 to 2.4 ppbv. These levels are similar to those of Tokyo site (monthly mean ranged from <0.1 to 2.2 ppbv). The H_2O_2 concentrations in Tokyo were high in July and October 2010 and autumn, and lower than detection limit of RQ-flex10 from November 2010 until April 2011. The lower H_2O_2 may be primarily due to lower O_3 concentration and effect of low temperature. Sakugawa *et. al*. (2005) reported that the H_2O_2 concentration in the mountainous forest area, Mt. Oyama, Kanagawa, ranged from 0.8 to 4.0 ppbv. These values are similar to the concentration of Chiang Mai measurement site located in natural areas and surrounded by mixed-deciduous forest where monthly average level ranged from 1.2 to 3.1 ppbv. Moreover, Watanabe *et al*. (1995) demonstrated that natural olefins such as isoprene and monoterpenes are emitted from the forest into the atmosphere, and produce H_2O_2 and organic peroxides such as MHP in mountainous area in Mt. Norikura, Japan. Nakhon Pathom measurement site locates in mixed areas of crop production and residential areas and close to big metropolitan city of Bangkok. The site is surrounded by industrial areas such as Ayutthya, Saraburi and Ratchaburi (Fig. 2.3.5). Monthly average levels from August to February were three to four times higher than those of Chiang Mai. There is a possibility that transport of air masses from heavily polluted areas affected Nakhon Pathom site by steady north east and south west prevailing winds. Monthly average level in October 2010 was slightly low because of the high amount of rainfall in this period which can wash out hydrogen peroxides. In March and April 2011, the site showed a low H_2O_2 concentration. This may be attributed to wind

direction change to south in March and April (Fig. 2.3.5). Gunz and Hoffmann (1990) showed that the highest H_2O_2 concentration was found in heavily polluted areas such as Los Angeles. According to the air pollutant data of Pollution Control Department, Ministry of Natural Resources and Environment of Thailand, the values for O_3 concentration in Bangkok and Chiang Mai were similar to Tokyo and ranged from 0-125 ppbv in 2010. But in Bangkok, annual average level of some VOCs such as benzene was about 6.2 $\mu g\ m^{-3}$ which is about 10 times higher than that of Tokyo (annual average level in 2008 around 0.5 $\mu g\ m^{-3}$) (Table 2.5.1.). In addition, the biomass burning from agricultural activities such as pre-harvesting of sugarcane and post-harvesting of rice (one of the sources of VOCs and other air pollutants) was incident around Naknon Pathom measurement site during September to December 2010. This may cause high precursors of H_2O_2 in these areas. Very high concentration of H_2O_2 in Nakhon Pathom is probably due to high concentration of VOCs and also to high air temperature. Thai Meteorological Department, Ministry of Information and Communication Technology of Thailand reported that monthly average air temperature in 2010 was higher than that of the past especially in lowland areas. However, monthly average air temperature was low in January and February 2011, resulting in the lower monthly H_2O_2 concentrations. We also found that several plants in Nakhon Pathom measurement site had leaf injury symptoms (Fig. 2.5.1). The low H_2O_2 concentration from May to July 2011 in Nakhon Pathom measurement site may be caused by the early and high amount of rainfall in this period and no biomass burning from agricultural activities.

Fig. 2.5.1. Visible leaf injury symptom caused by peroxides on rice (a) alligator weed (b) and broad leaf plant (c) was found at Nakhon Pathom measurement site taken photo on September 2010.

Table 2.5.1. Yearly range of O_3 and annual average of volatile organic compounds (VOC) concentration in Tokyo and Thailand in 2010 provided by AEROS of Japan and Pollution Control Department, Ministry of Natural Resources and Environment of Thailand

Site	O_3 (ppbv) range	Benzene $\mu g\ m^{-3}$	Chloroform $\mu g\ m^{-3}$	1,3 Butadiene $\mu g\ m^{-3}$
Tokyo	0-115	0.5*	-	0.0*
Chiang Mai	0-125	4.6	0.2	0.4
Bangkok	0-100	6.2	0.9	0.6

*Annual average in 2008 reported by Tajima *et al*. (2010) in Hachioji city, Tokyo, which is located 12 km south west of Tokyo site.

2.5.2 Diurnal characteristics of H_2O_2 concentrations in 2009 and 2010

Several observations of H_2O_2 concentrations showed that the concentration of H_2O_2 at ground level was higher in the day time and lower at night due to photochemical process (Gunz and Hoffmann, 1990). Chen *et al*. (2008) suggested that the photochemical reactions are accelerated by the increase in solar radiation, temperature, and O_3 concentration that begin in the morning and the large amount of HO_2 is generated along with OH. Then H_2O_2 is mainly produced by means of photochemical reactions during the mid-day period. The concentration of H_2O_2 in our observation in Tokyo and Chiang Mai was low in the morning, gradually increased around noon time and became low in the evening in 2009. The diurnal patterns of H_2O_2 concentration in Tokyo and Nakhon Pathom in 2010 was similar to those of 2009. And these patterns are similar to those of several H_2O_2 observations by Gnauk *et al*. (1997) and Hua *et al*. (2008). While Chiang Mai measurement site in 2010 showed high values in the early morning and gradually decreased through the daytime. This pattern is similar to reported H_2O_2 observations in mountain areas which was high at night and low in the daytime by Watanabe *et al*. (1995) and Das and Husain (1999). The high concentration of H_2O_2 at night is possibly due to the transport of higher H_2O_2 air mass from the high altitude atmospheric layer where the H_2O_2 concentration is higher than at ground level. It is not clear why the diurnal pattern from October to December 2009 was not the same as October to December 2010 (see Fig.2.4.2). The air temperature in Chiang Mai measurement site was not different from year to year, then, it may be caused by the differences in the concentrations of precursors of H_2O_2 and other meteorological conditions.

2.5.3 Correlation between H_2O_2 and air pollutants and meteorological data

Strong positive correlations with H_2O_2 were observed for ozone (Fig. 2.4.3.d) and air temperature (Fig. 2.4.3.f) and a weak negative correlation was observed between H_2O_2 and NO (Fig. 2.4.3.a). Correlations of H_2O_2 with other pollutants or meteorological parameters such as NO_2 (Fig. 2.4.3.b), NOx (Fig. 2.4.3.c), SPM (Fig. 2.4.3.e), relative humidity (Fig. 2.4.3.g), solar radiation (Fig. 2.4.3.h) and wind speed (Fig. 2.4.3.i) were not clear.

The correlation between O_3 and H_2O_2 seems to be the strongest among the factors we examined. Several reports showed the same positive correlation (Jackson and Hewitt, 1996; Takami *et al*., 2003; Chen *et al*., 2008). The reasons for this are twofold. One is that H_2O_2 is mainly formed in the atmospheric photochemical reactions to form hydroperoxyl (HO_2) radicals. Self-reactions of HO_2 radicals produce H_2O_2. As discussed in Chapter I, HO_2 is the main precursor of H_2O_2 and also it plays an important role in the formation of photochemical ozone. In addition O_3 produces H_2O_2 by reacting with VOC (Gunz and Hoffmann, 1990; Hatakeyama *et al*., 1993, 2001). Thus, correlation between H_2O_2 and O_3 is very straightforward.

A negative correlation is observed for nitric oxide (NO). Hydrogen peroxide is a product of the self-reactions of HO_2 radicals. Nitric oxide reacts with HO_2 radicals to form NO_2. So, high concentration of NO brings about the reduction of HO_2 radicals (Gunz and Hoffmann, 1990; Aneja and Das, 1994). Thus, it is reasonable that there is a negative correlation between H_2O_2 and NO.

The strong correlation between H_2O_2 and air temperature can be explained as follows. Chemical reactions are generally accelerated under high temperature. Therefore, high temperature can cause high formation of HO_2 radicals and O_3 which are the key components for H_2O_2 production (Gunz and Hoffmann, 1990). Similar results are reported

by Takami *et al*. (2003) and Chen *et al*. (2008). However, relations between H_2O_2 and air temperature were significantly different in July and October (Fig. 2.4.4). A steep slope of H_2O_2 against air temperature was found in July when the range of air temperature was 30 - 38 °C. On the contrary, a gentle slope was observed in October when the range of air temperature was only 18 - 26 °C. This suggests that the range of air temperature may be important for accelerating formation and removal process of H_2O_2 in the atmosphere.

2.5.4 Trends of atmospheric H_2O_2 concentrations

According to previous studies in Table 2.5.2., the data showed that the H_2O_2 concentrations in 2009 and 2010 were higher than that in 2004. Takami *et al*. (2003) reported that the H_2O_2 concentrations in Okku-Nikko area from 1998 to 2000, located at about 130 km north of Tokyo measurement site, ranged from 0.2 to 1.6 ppbv. Chen *et al*. (2008) reported that the H_2O_2 concentrations in Japanese red pine forest in Nagano Prefecture, located at about 160 km west of Tokyo measurement site raged from below detection limit 0.01 to 1.64 ppbv. It implies that the H_2O_2 concentrations in Tokyo have been increasing, recently. Moreover, in Tanzawa Mountains ridge, where serious decline of beech forest occurs, H_2O_2 concentrations are higher than in Tokyo (Table 2.5.2). The increasing of H_2O_2 in Tokyo may be mainly caused by the increasing of O_3 concentration, the precursor of H_2O_2. The Ministry of the Environment, Japan reported that the O_3 concentrations in Kanto areas have been gradually increasing from 1980.

In addition the H_2O_2 concentrations were measured by the author in North Carolina State University, NC, USA from September to December 2011 by the same method (unpublished results). The data showed that atmospheric H_2O_2 concentration ranged from

less than detection limit to 6.4 ppbv. High H_2O_2 concentrations were found while O_3 concentration was higher than 50 ppbv on sunny day and air temperature was higher than 28 ^oC. These H_2O_2 levels were three times as high as that measured in 1991 ranged from less than 0.5 to 2.0 ppbv (Aneja and Das, 1994). As a whole, although the number of cases examined is not so large but we can consider that the atmospheric peroxide concentration is increasing in the world and such atmospheric peroxides will highly impact on crops and natural resources in the future. The impact of atmospheric peroxides will be discussed in later chapters.

Table 2.5.2. Peroxides concentrations in central Japan (Aoki *et al*., 2012)

Measurement sites	times	H_2O_2 (ppbv)		
		Max	Min	AVG
Tanzawa Mountains (Ridge) (29 Sep to 1 Oct 2009)	13	2.8	0.5	1.7
Tanzawa Mountain (Middle slope) (28 to 30 Jul, 29 Sep to 1 Oct 2009)	23	2.9	nd	1.2
TUAT (Sep to Nov 2004)	189	1.8	nd	0.3
TUAT (Sep to Nov 2009)	15	2.4	0.9	1.2
TUAT (Sep to Dec 2010)	54	2.5	nd	1.3

nd = non detectable

2.6 Summary

The monthly average level of H_2O_2 in Chiang Mai ranged from 1.2 to 3.1 ppbv which was slightly higher than those in Tokyo. In Nakhon Pathom it was about three to four times higher than that of Chiang Mai and Tokyo. The diurnal characteristics in H_2O_2 concentration were found to be dependent on place, year and season. H_2O_2 concentration showed strong correlation with O_3 concentration. Air temperature also showed positive correlation with H_2O_2 concentration. More extensive and systematic measurement data of H_2O_2 should be obtained to determine how the concentration will increase in the future and how it affects agricultural and natural plants.

CHAPTER 3

Effects of ozone and peroxides on leaf injury, physiological responses, and growth of two Japanese and two Thai soybean cultivars

3.1 Background

Ozone has been noticed as one of the most damaging air pollutants (Tompson, 1992) in recent years. In the last century, the concentrations of O_3 increased up to 80 ppbv (Wang *et al*., 2006) and have been increasing in many places in the world (Voulgarakis *et al.*, 2011). Since O_3 formation in the air is accelerated in higher air temperature condition, it tends to reach a level which causes enormous decrease in growth and yield under global warming condition. The damages of plant caused by high O_3 concentration have been reported since 1961. Van Dingenen *et al.* (2009) reported that economic losses by the O_3 damage in four economic crops including wheat, rice, maize, and soybean of the world in 2000 have been estimated as 26 billion US dollars which were India 23%, China 21%, North United State of America 19% and Europe 4%. Yamaguchi *et al*. (2011) revealed that several kinds of forest species were damaged by high concentrations of O_3. However, Takami *et al*. (2003) reported that severe forest decline was seen at Oku-Nikko Mountains in Japan where O_3 concentration was not so high. They also reported that peroxides concentration in the area was high. Then, the severe plant damage in the areas cannot be fully explained by the effects of O_3 alone.

It is well known that peroxides always coexist with O_3. Atmospheric peroxides generally comprise H_2O_2 and organic peroxides such as MHP and HMHP (Hatakeyama *et al.*, 1993; Takami *et al.*, 2003). In fact, combined O_3 and peroxides are much harmful to some vegetables and other plants than single O_3 (Chen *et al*., 2005; Chen *et al*., 2010). Recent investigations showed that peroxides concentrations have been increasing (Chen *et al*., 2008; Chutteang *et al.*, 2012b). Terry *et al.* (1995) suggested that peroxides can damage cell membrane which may impact on biomass and crop production. Chen *et al*. (2010) reported that exposure to O_3 of 50 ppbv in combination with peroxides of 2-3 ppbv

caused much severer decrease in leaf photosynthetic rate and much higher foliar injury of radish than the exposure to 100 ppbv O_3 alone.

Soybean (*Glycine max* (L.) Merr) is an important crop species which is sensitive to O_3 (Morgan *et al.* 2003). Heagle *et al.* (1998) reported that moderately high level of O_3 (60-100 ppbv) in mid-Atlantic states of USA may cause as much as 40% loss of soybean yield. However, the sensitivity of plants to O_3 and air pollutants varies among genotypes (Akhtar *et al.*, 2010; Betzelberger *et al.*, 2010). Genetic variation in O_3 sensitivity was commonly observed in several plant species, for example, snap bean (Reitnart and Eason, 2000), tomato (Temple, 1990), potato (Heagle *et al.*, 2003), and soybean (Burkey and Carter, 2009). However, there is no information about cultivar sensitivity of soybeans under combined exposure of O_3 and peroxides. Moreover, the physiological responses to combined O_3 and peroxides are necessary to investigate for future crop productivity assessment since atmospheric peroxides concentration would increase in the future.

3.2 Objectives

The objectives of the present research are to investigate the single and combined effects of O_3 and peroxides on leaf injury and physiological responses of soybean, and to compare the cultivar sensitivities.

3.3 Materials and Methods

These experiments were conducted at Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan using four phytotrons as shown in Fig. 3.3.1.

3.3.1. Plant materials

Two Japanese soybeans, Tachinagaha (TC) and Chamame (CM), and two Thai soybean cultivars, A75 and Sorjor 5 (SJ5) were selected and used as plant materials. CM and A75 are relatively early maturity cultivars, while TC and SJ5 are relatively middle maturity cultivar in Japanese and Thai cultivars. The experiments were conducted two times from December 2010 to March 2011 and December 2011 to March 2012. In these two experiments, methods and procedures were the same except fumigation period which will be described in 3.3.2. Soybean seeds were seeded in plastic pots of 0.8L on 16 December 2010 and 22 December 2011 filled with Cumulic Andosols (Melanudand) soil. Fertilizer (N-P-K=14-14-14) was applied 5 g per pot before seeding, and pest control was applied. The plants were grown under charcoal–filtered air in the glass house for 20 days before the exposure experiments, and the exposure was begun at the third trifoliate leaf stage. Ten plants in ten pots were randomly chosen for each treatment. The potted plants were watered twice a day before and after exposure every day.

3.3.2. Exposure treatments

Four outdoor growth chambers (Koito Ltd., Yokohama, Japan), in which the air temperature (day: 06.00 to 18.00 25 ^{o}C; night: 18.01 to 05.59 18 ^{o}C) and the relative humidity (70 %) were controlled, were used. The air in the chambers was homogenized by fans. Four experimental plots were control plot which is O_3 and peroxides free (C plot), 50 ppbv O_3 plot (O plot), 50 ppbv and 2-3 ppbv peroxides (+α-pinene) plot (OP1 plot), and 50 ppbv O_3 and 4-5 ppbv peroxides (+α-pinene) plot (OP2 plot). C plot was controlled with charcoal-filtered air. Ozone was generated from air by a silent electrical discharge O_3 generator (MO-5A, Nihon Ozone Ltd., Kagoshima, Japan). The oxides of nitrogen were

completely removed by water filter. The concentration of O_3 was regulated by the automatic controlling system (HMC-2000/S; Koito Ltd., Yokohama, Japan) and monitored every 90 seconds. This measurement was done automatically and continuously. Peroxides, which are the mixture of H_2O_2 and organic peroxides, were produced by similar formation way to that taking place in the real atmosphere; namely by the reaction of O_3 with vaporized α-pinene, which is the same way as Chen *et al.* (2010). The system of peroxide synthesis was shown in Fig. 3.3.2. Loreto *et al*. (1998) suggested that monoterpene such as α-pinene can protect plant from high temperature by stabilizing the cell membrane and protecting photosynthesis system. In addition Chen *et al*. (2005) and the results of our previous (unpublished) study revealed that α-pinene caused no visible foliar injury on several kinds of plants. Therefore, α-pinene is not harmful to plants and can be used in these experiments. The gaseous peroxides in each chamber was sampled with a Pyrex glass mist chamber (Cofer *et al*., 1985) three times a day in the morning, early afternoon, and late afternoon. The collection of gaseous-peroxides was shown in Fig. 3.3.3. The concentration of peroxides was measured by a portable RQflex 10 reflectometer (MERCK KGaA, Darmstadt, Germany). Details of the above procedures are explained in Chapter 2, Chen *et al*. (2005, 2010) and Chutteang *et al*. (2012b). The exposure was carried out from 9:00 to 16:00 every day.

3.3.3. Evaluation of visible leaf injury

Visible foliar injury was evaluated on first trifoliate leaves every two to three days after exposure. Three lateral leaves at the same position for each plant from four plants per plot were evaluated. Visible injury was expressed by its intensity of 0, 1, 2, 3 and 4, which represent no injury, chlorosis < 50% of individual leaf area, chlorosis > 50% of individual

leaf area, necrosis < 50% of individual leaf area, and necrosis > 50% of individual leaf area, respectively.

3.3.4. Measurements of SPAD index and chlorophyll content

SPAD index was measured by chlorophyll meter (SPAD502, Minolta Camera Co., Ltd., Japan) every 2-3 days. Chlorophyll content was evaluated by the equation from the correlation of SPAD index and chlorophyll a, b and total chlorophyll content for each cultivar (Fig.3.4.1). The chlorophyll a, b and total chlorophyll content were measured from leaf disc extract with N-N Dimethyl formamine (DMF) by Moran (1982) method.

3.3.5. Measurements of leaf gas exchange

Leaf gas exchange was measured on first trifoliate leaf by a photosynthesis measurement system (LI6400-40 by LI-COR, Nebraska, U.S.A.) on 10 (1^{st} measurement), 20 (2^{nd} measurement), and 30 (3^{rd} measurement) days after exposure in the second experiment from four plants in each plot and cultivar. The leaf chamber environment conditions were set to optimal conditions for stomatal opening and light saturation condition which are under PAR 1500 μmol PPFD m^{-2} s^{-1}, CO_2 400 μmolCO_2 mol^{-1}, temperature 25 ^{o}C, and RH 65-70 %. The light-saturated photosynthetic rate (A), transpiration rate (E), stomatal conductance (gs), and intercellular CO_2 concentration (Ci) were evaluated. The 1^{st} and 3^{rd} measurements were carried out under sunny condition, but the 2^{nd} measurement was under cloudy condition, then in the present research the results of 1^{st} and 3^{rd} measurement were used in the case of correlations between A and gs and Ci.

3.3.6 Measurement of biomass and plant growth

The biomasses of root, leaf, stem, and pod were measured on 50 days (CM) and on 55 days (A75, TC and SJ5) when the growth stage was R6 and pod containing green seed in the pod cavities (Fer and Caviness, 1977). Four plants in each plot and cultivars were randomly chosen. We could not continue the experiment further because some plants started to die due to severe damage. Each organ was weighed after oven drying at 80 ^{o}C for 3 days.

Plant height was measured in the 3rd experiment every two weeks after exposure from eight plants per plot per cultivar. Specific leaf weight (SLW) defined as the leaf dry weight per unit leaf area also was evaluated on the 3rd experiment on 50 days after exposure.

3.3.7 Data analysis

Statistical data analysis was performed with R 2.13.1 Software. Analysis of variance (ANOVA) was used to test the effects of experimental plots and cultivars. Tukey's HSD test was performed to identify significant differences in a cultivar.

Fig. 3.3.1. Phytotrons and control room were used for exposure of plants

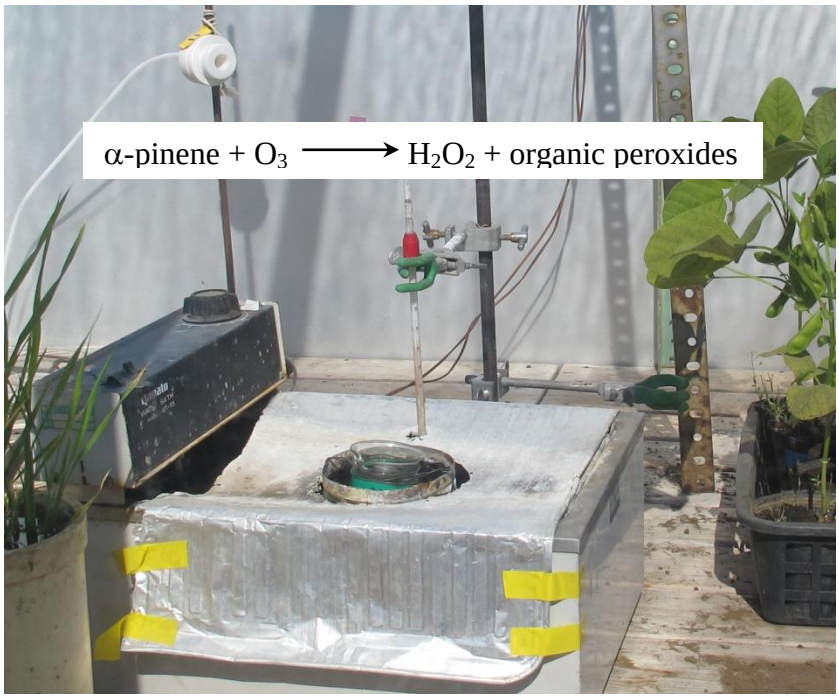


Fig 3.3.2. The generated peroxides system in OP plot;

α- pinene was vaporized by maintaining the water temperature

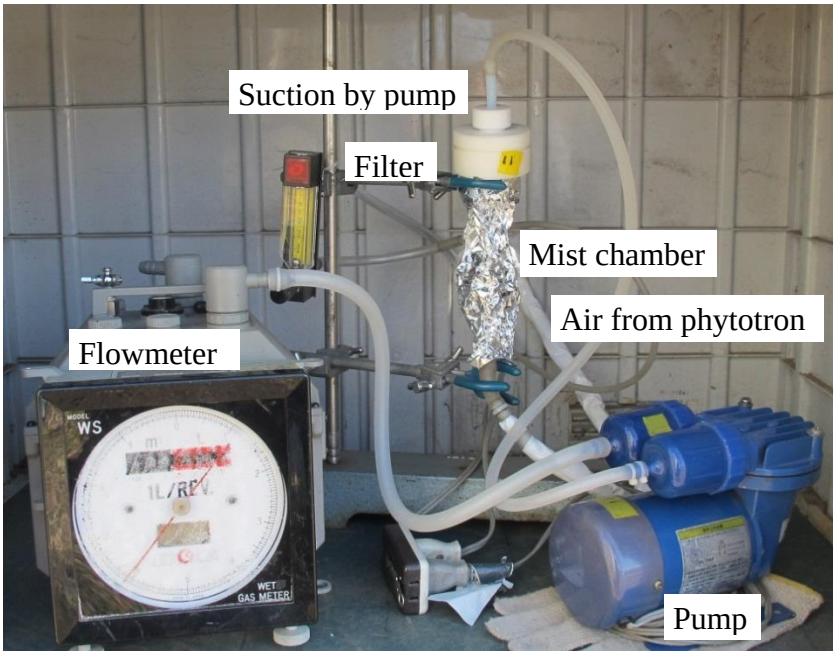


Fig.3.3.3. Sampling system: gaseous peroxides collected by mist chamber; air from phytotron was sucked by pump and the collected the gaseous peroxide in mist chamber water

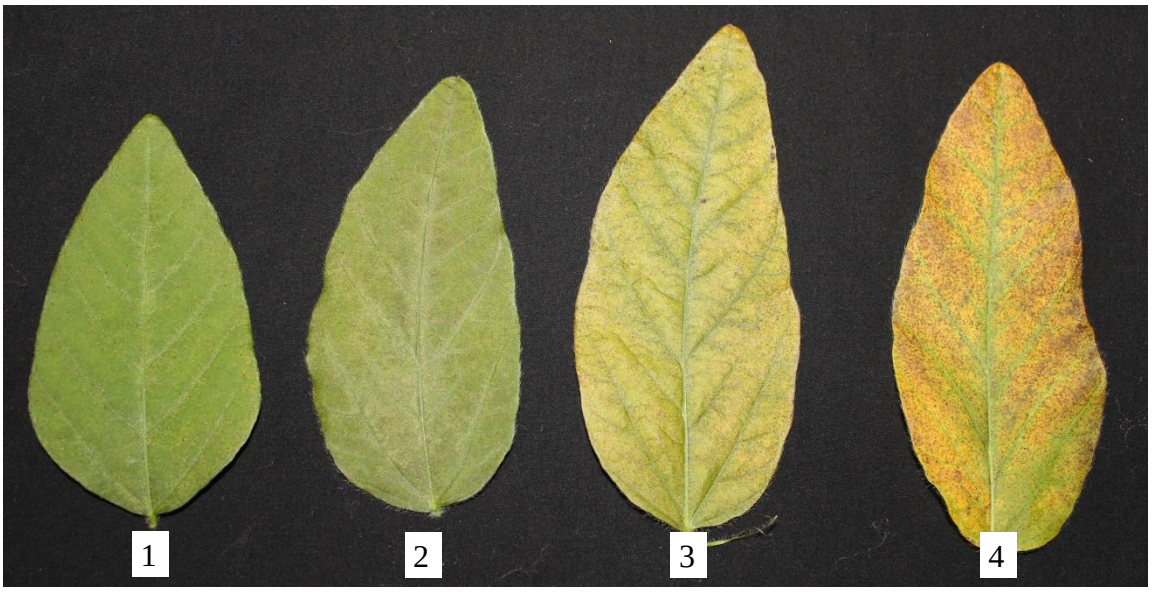


Fig.3.3.4. Visible foliar injury score of soybean. The intensity was valuated as 0, 1, 2, 3, and 4. Zero was no injury, 1 was chlorosis less than 50%, 2 was chlorosis more than 50%, 3 was necrosis less than 50%, and 4 was necrosis more than 50% as show on this picture.

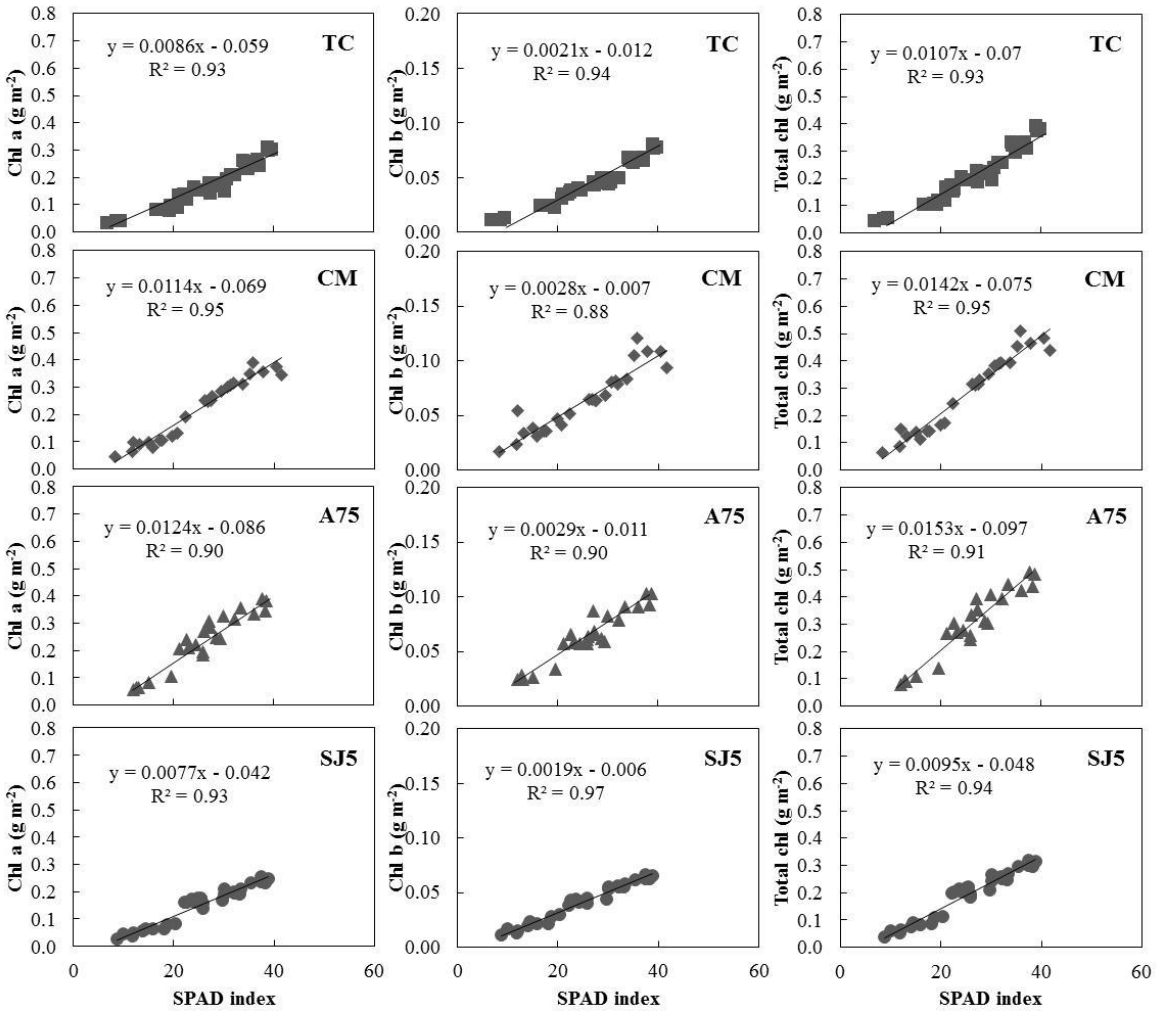


Fig. 3.3.5. Correlation between SPAD index and chlorophyll a, b and total chlorophyll content of four soybean cultivars

3.4 Results

3.4.1 Visible foliar injury

In O plot, the silver stipples were found in the early stage after fumigation and they turned to brown spot, and necrosis was gradually appeared. However, brown spot and necrosis were appeared much earlier in OP1 and OP2 plots than O plot. Some typical symptoms of visible foliar injury in combined O_3 and peroxides (OP2) are shown in Fig. 3.4.1. Although the intensity of visible foliar injury of four soybean cultivars seemed similar, OP2 plot caused severer damage than those of OP1 plot and O plot (Fig. 3.4.2). The visible leaf injury of all four soybean cultivars in OP2 plot showed early appearance on lower O_3 dose (less than 5000 ppbv.hr) than those of OP1 (about 5000-7500 ppbv.hr) and O plots (more than 9000 ppbv.hr) (Fig. 3.4.2). Comparing the visible leaf injury among four soybean cultivars, SJ5 showed the earliest visible leaf injury appearance starting at 200 ppbv.hr peroxide dose and it rapidly increased, while the visible injury in TC started at 300 ppbv.hr peroxide dose and gradually increased in both OP1 and OP2 plots. Therefore, SJ5 was concluded more sensitive than CM, A75 and TC in OP1 and OP2 plots. In O plot, the visible leaf injury of CM occurred early at around 9000 ppbv.hr O_3 dose, while A75 showed the latest appearance at about 15000 ppbv.hr. O_3 dose. Therefore, A75 cultivar was less sensitive than TC, SJ5 and CM (Fig. 3.4.3) to O_3.

3.4.2 Chlorophyll content

Total chlorophyll content of OP1 and OP2 plots showed different response with that of O plot (Fig. 3.4.4). OP1 and OP2 plots showed linear response to peroxide dose while O plot showed two step responses to O_3 dose as shown in Fig. 3.4.4. At first, total chlorophyll content of O plot was gradually decreased from 5000 to 13000 ppbv.hr O_3

dose and then rapidly decreased when the O_3 dose was higher than 13000 ppbv.hr. Total chlorophyll content of OP1 and OP2 plots rapidly decreased from just after exposure (Fig. 3.4.4). In case of O plot, CM showed the most sensitive cultivar that the total chlorophyll content was decrease more than 80% on 20000 ppbv.hr O_3 dose, while A75 seem to be less sensitive which was shown only 20% decrease in total chlorophyll content (Fig.3.4.5). In OP1 and OP2 plots, SJ5 showed high sensitivity which the steepest slope was shown in both OP1 and OP2 plot. TC showed less sensitivity which was less decrease in total chlorophyll content about 70% (Fig. 3.4.5). The order of cultivar sensitivity in total chlorophyll content in O, OP1 and OP2 plots was similar to those of visible leaf injury.

3.4.3 Leaf gas exchange

OP2 plot showed highly reduced net photosynthetic rate than OP1 and O plots on 10 and 20 days after exposure (Fig. 3.4.6). SJ5 was the most sensitive in O, OP1 and OP2 plots except on 30 days. The percentage of net photosynthetic rate (A) of O plot to C plot of SJ5 was 35% while those of OP1 and OP2 plots were 20% and 24%, respectively, on 20 days after exposure. TC showed less sensitive in O plot; 73% of A in O plot compared to C plot. CM showed less sensitive in both OP1 and OP2 plots which was 51% and 40% of A of OP1 and OP2 plots (Fig. 3.4.6).

Correlations between A and gs and Ci were shown in Fig. 3.4.7. A versus gs relationship in C plot which was shown by dot line (·····) circle in Fig.3.4.7a did not change the position in the figure with Fig. 3.4.7c. It means that A versus gs relationship in C plot was almost the same during 20 days from 10 days and 30 days after exposure. On the contrary in OP1 and OP2 plots, shown respectively by two-dot chain line (–··–) circle and solid line (——) circle, both of A and gs values of all cultivars decreased during 20

days. It means that A values of all cultivars decreased followed by the decrease in gs when the exposure continued more than 20 days. In this case the decreases in A and gs values in OP2 plot were more than OP1 plot.

As for the position of A versus gs relationship in O plot shown by dashed line (- - - -) circle, A and gs values of some cultivars changed much during 20 days, and then A versus gs relationship became positive correlation on 30 days after exposure as shown in Fig. 3.4.7c.

In case of C plot, A versus Ci relationship did not change during 20 days as shown in Fig. 3.4.7b and Fig. 3.4.7d, while in OP1 and OP2 plots, A of all cultivars decreased but Ci increased during 20 days. Especially this tendency of OP2 plot was much more than OP1 plot. In O plot such tendency was much smaller than OP1 plot (See Fig. 3.4.7b and Fig. 3.4.7d). As a whole, in O plot, A was reduced by gs, but not by Ci. In OP plots, A was reduced by both low gs and high Ci.

It was also found that the sensitive cultivar, SJ5, showed the highest gs value in C, O and OP1 plots on 10 days after exposure and became lowest on 30 days after exposure. Tolerant cultivars, TC and CM, gs showed lower values in each plot except OP2 plot on 10 days after exposure as shown in Fig.3.4.7a. As for A versus Ci, SJ5 showed the highest Ci in each C, O and OP1 plot groups on 10 days after exposure (see Fig. 3.4.7b). The present research results showed that sensitive cultivar (SJ5) highly reacted with single O_3 and combined O_3 and peroxides much more than tolerant cultivars (TC and CM).

3.4.4 Biomass and plant growth

The biomasses of four soybean cultivars after 50 days (CM cultivar) and 55 days (TC, A75 and SJ5 cultivars) exposure are shown in Table 3.4.1. In TC cultivar, the total

dry weights of C and O plots were not significantly different, and OP1 plot was not clearly different from O plot, whereas 44% decrease in total dry weight and 91% decrease in pod dry weight compared to C plot were found in OP2 plot. Moreover, root dry weight significantly reduced in OP2 plot. The results of CM cultivar showed that dry weight of all organs was significantly different among four treatments. The total dry weight was not statistically different between C and O plots**,** while both of OP1 and OP2 plots caused 29% and 55% reduction in total dry weight compared to C plot. Both OP1 and OP2 plots caused 47% and 49% reduction in total dry weight of A75 and 45% and 59% in that of SJ5, respectively. As for total dry weight in O plot, TC showed less reduction, and thus, it was the most tolerant cultivar. Other three cultivars showed no significant difference. In OP1 and OP2 plots, TC was less sensitive while SJ5 was the most sensitive cultivar. Considering pod dry weight, we found that SJ5 was the most decreasing cultivar in O (30%), OP1 (61%), and OP2 (96%), while CM was the least decreasing cultivar in O (14%), OP1 (45%) and OP2 (52%). Then, it may imply that SJ5 was the most sensitive cultivar on total dry weight and pod dry weight, while CM was the less sensitive cultivar on pod dry weight (Table 3.4.1).

Comparison of specific leaf weight (SLW) in C plot among four soybean cultivars showed that TC had the highest SLW followed by A75, CM, and SJ5. The SLW of TC and CM slightly decreased, while that of A75 and SJ5 significantly decreased after exposure in O, OP1, and OP2 plot (Fig. 3.4.8). In terms of plant height, OP2 caused high reduction of plant height than OP1, and O plot. SJ5 was the most sensitive cultivar while A75 showed less sensitivity (Fig.3.4.9).

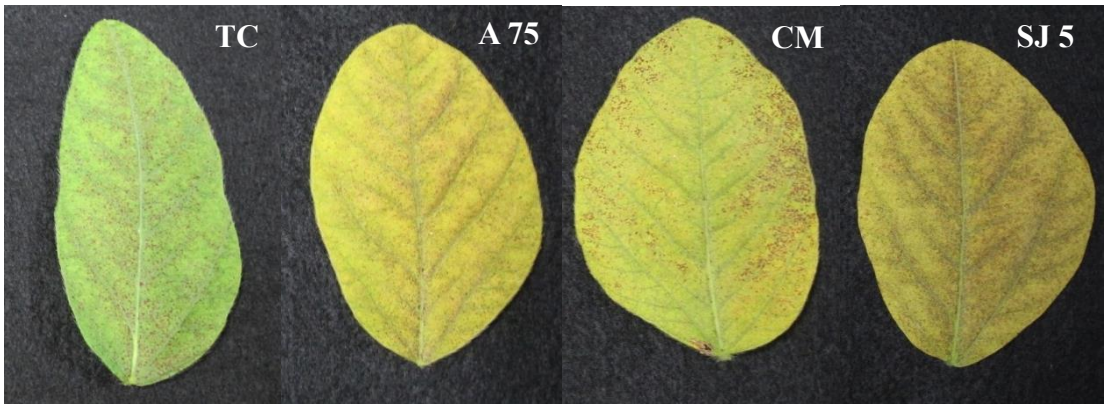


Fig. 3.4.1. A typical symptom of visible leaf injury under combined O_3 and peroxides after 30 days of exposure in OP2 plot

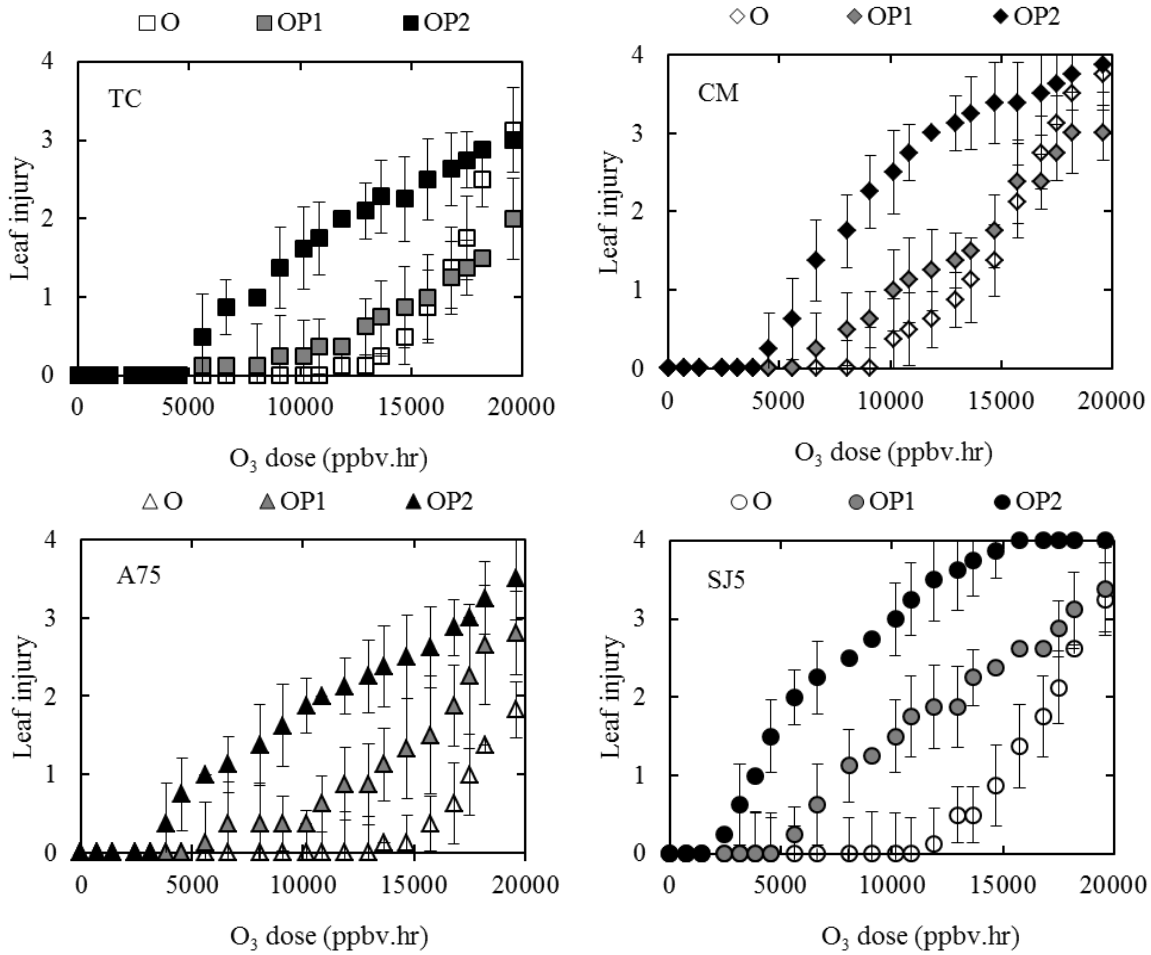


Fig. 3.4.2. O_3 dose responses of visible leaf injury in four soybean; the vertical bars of symbols are standard deviations (average from 8 leaves).

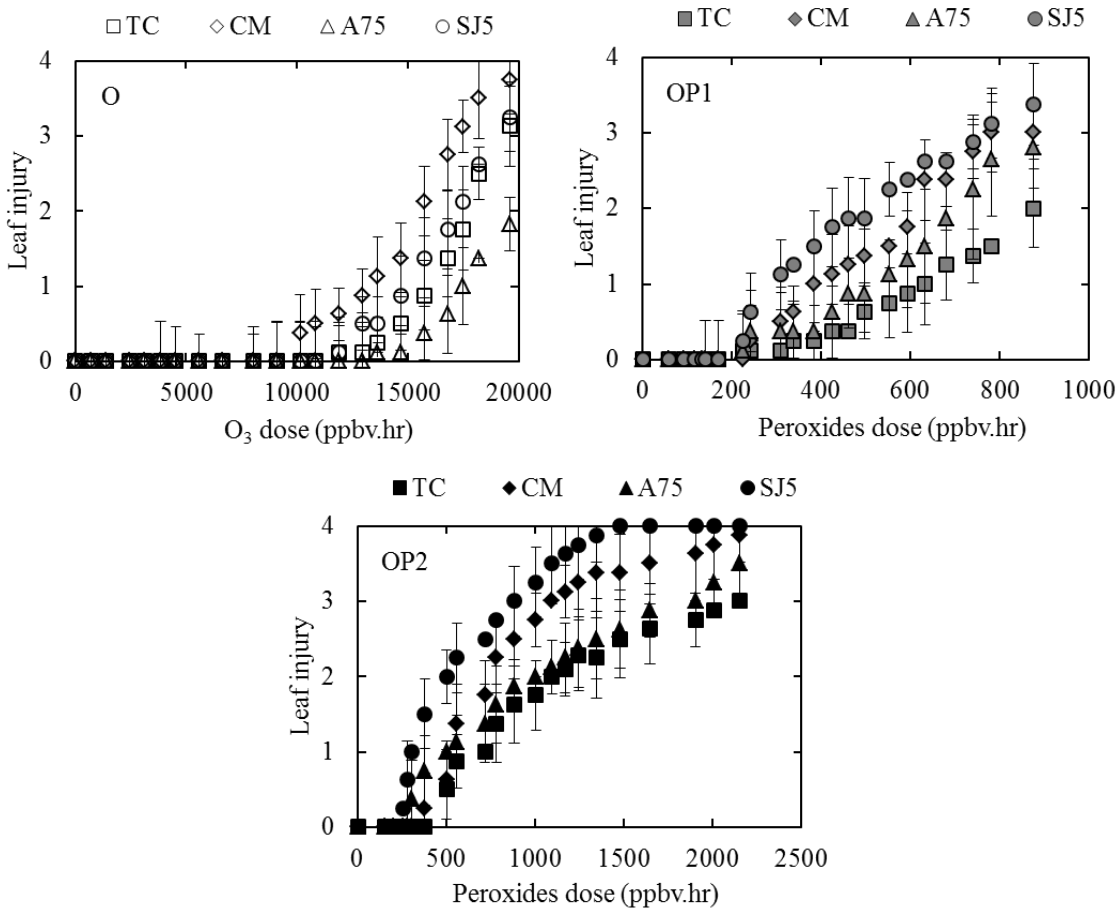


Fig. 3.4.3. Comparison of visible leaf injury sensitivity; the vertical bars of symbols are standard deviations.

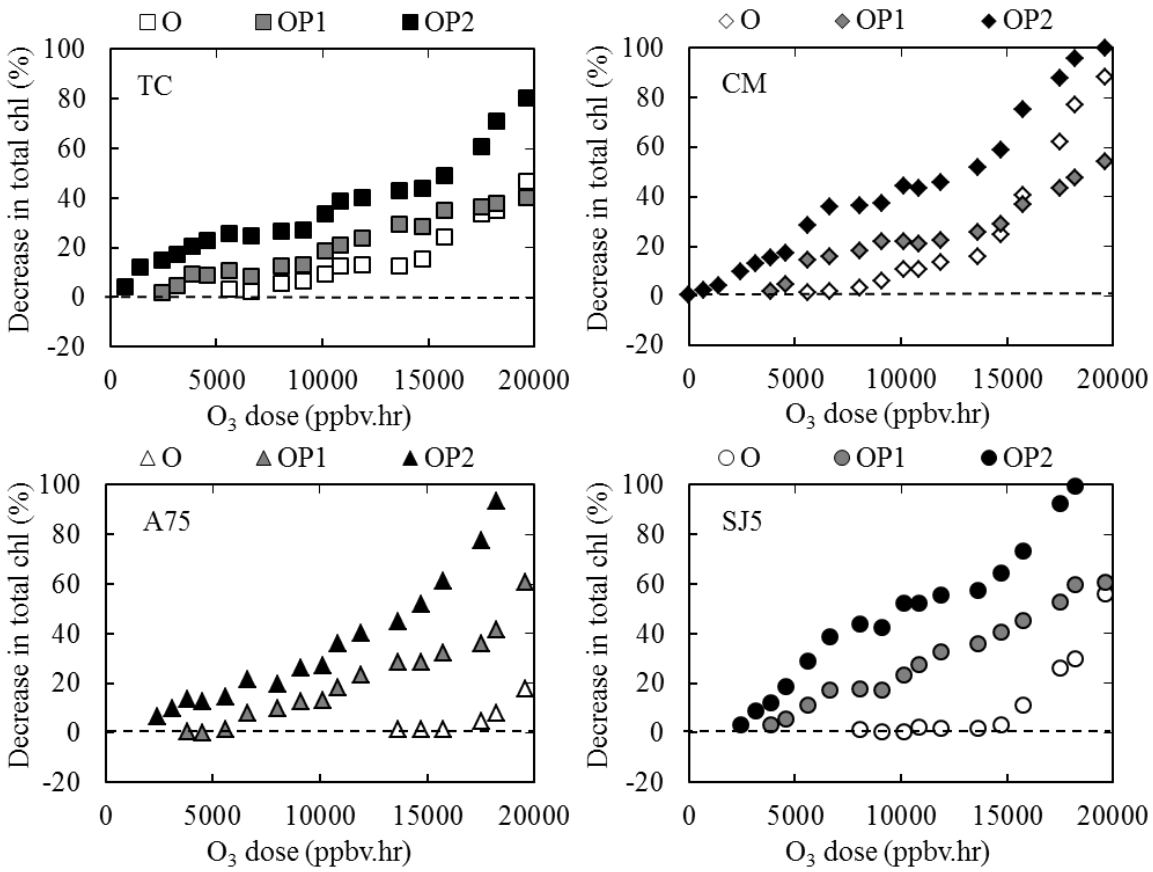


Fig. 3.4.4. O_3 dose responses of total chlorophyll content in four soybean cultivars; the vertical bars of symbols are standard deviations (average from 8 leaves).

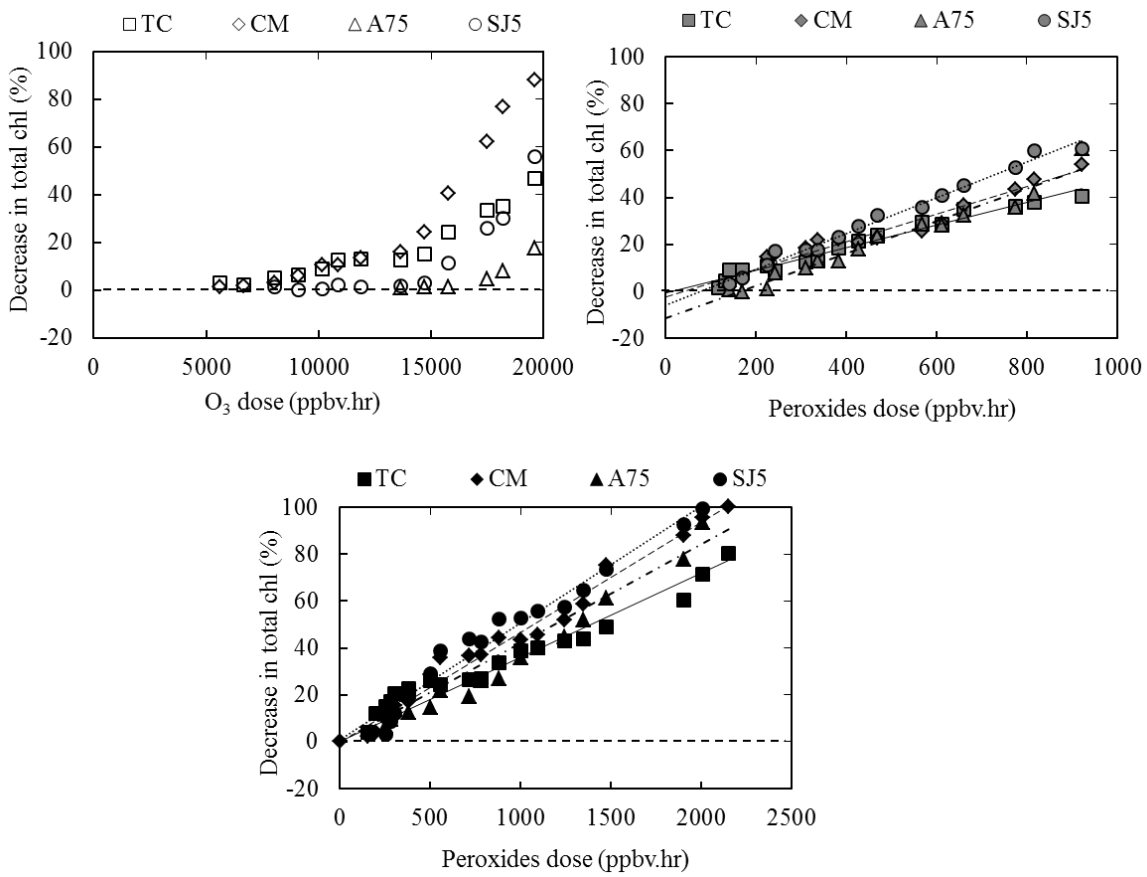


Fig. 3.4.5. Comparison of decrease in total chlorophyll content (Chl)

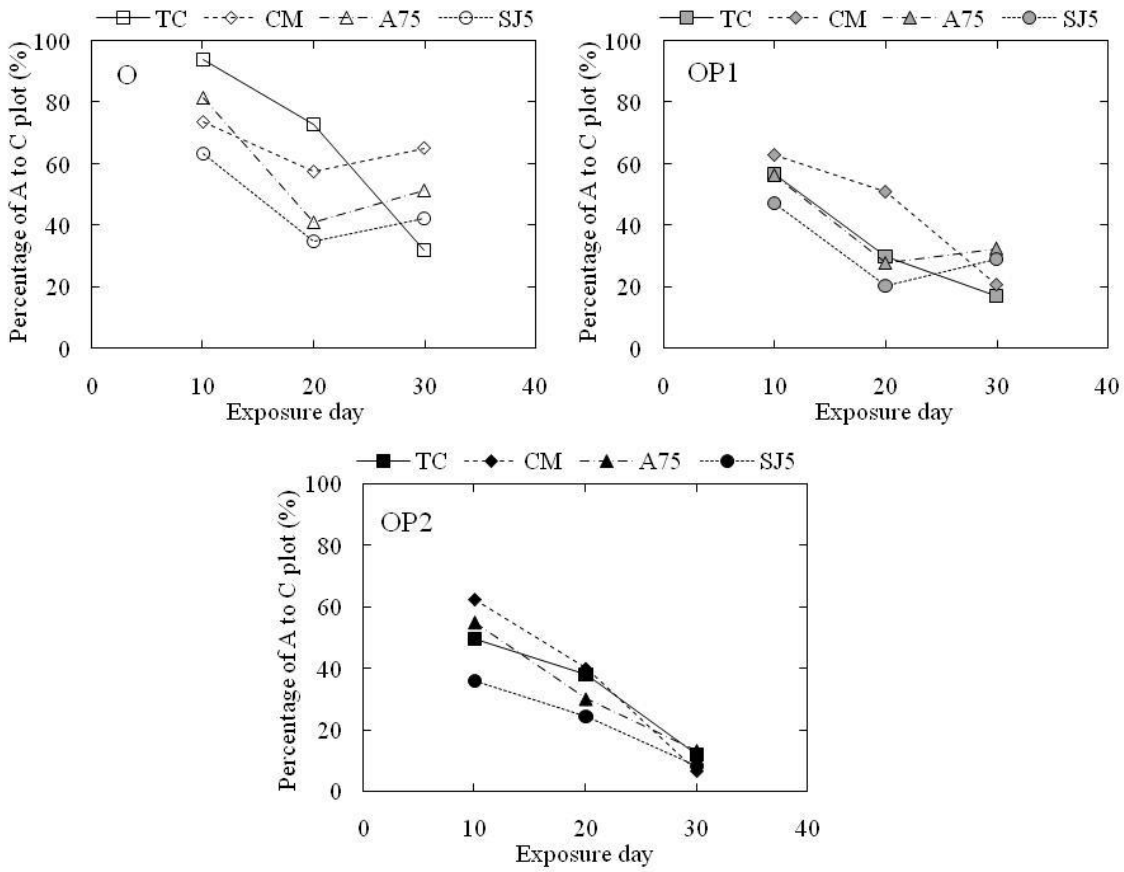


Fig. 3.4.6. Percentage of net photosynthetic rate (A) of each treatment to C plot of four soybean cultivars; The data were measured only in the second year experiment.

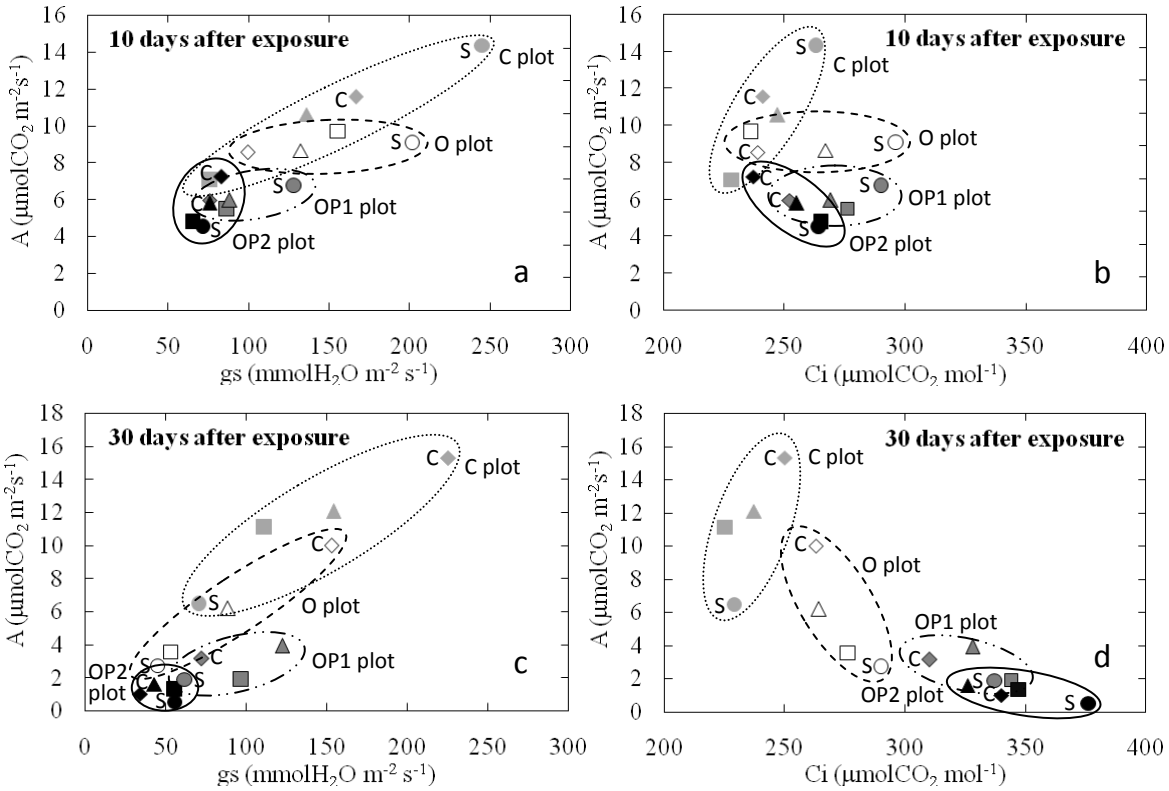


Fig. 3.4.7. Correlations between net photosynthetic rate (A) and stomatal conductance (gs) (Fig. 6a and Fig. 6c) and CO_2 concentration in intercellular space (Ci) (Fig. 6b and Fig. 6d) of four soybean cultivars

C and S show CM and SJ5 cultivars, respectively. The data were measured only in the second year experiment.

C plot : TC (■) CM (◆) A75 (▲) SJ5 (●)

O plot : TC (□) CM (◇) A75 (△) SJ5 (○)

OP1 plot : TC (■) CM (◆) A75 (▲) SJ5 (●)

OP2 plot : TC (■) CM (◆) A75 (▲) SJ5 (●)

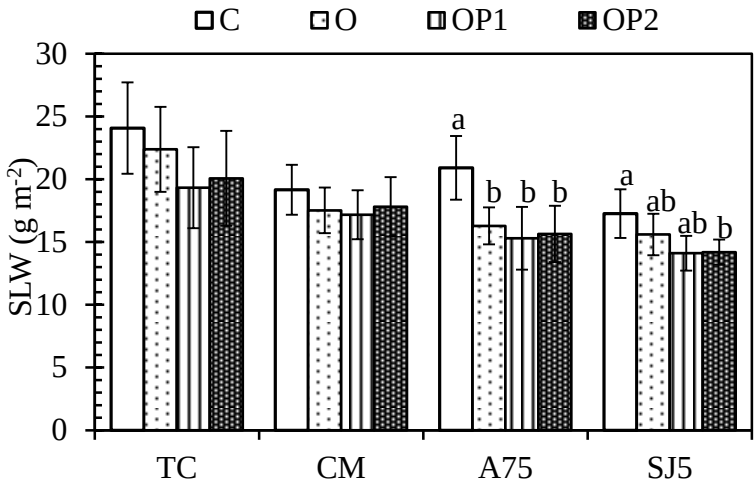


Fig. 3.4.8 Specific leaf weight (SLW) of four soybean cultivars (average from ten leaves)

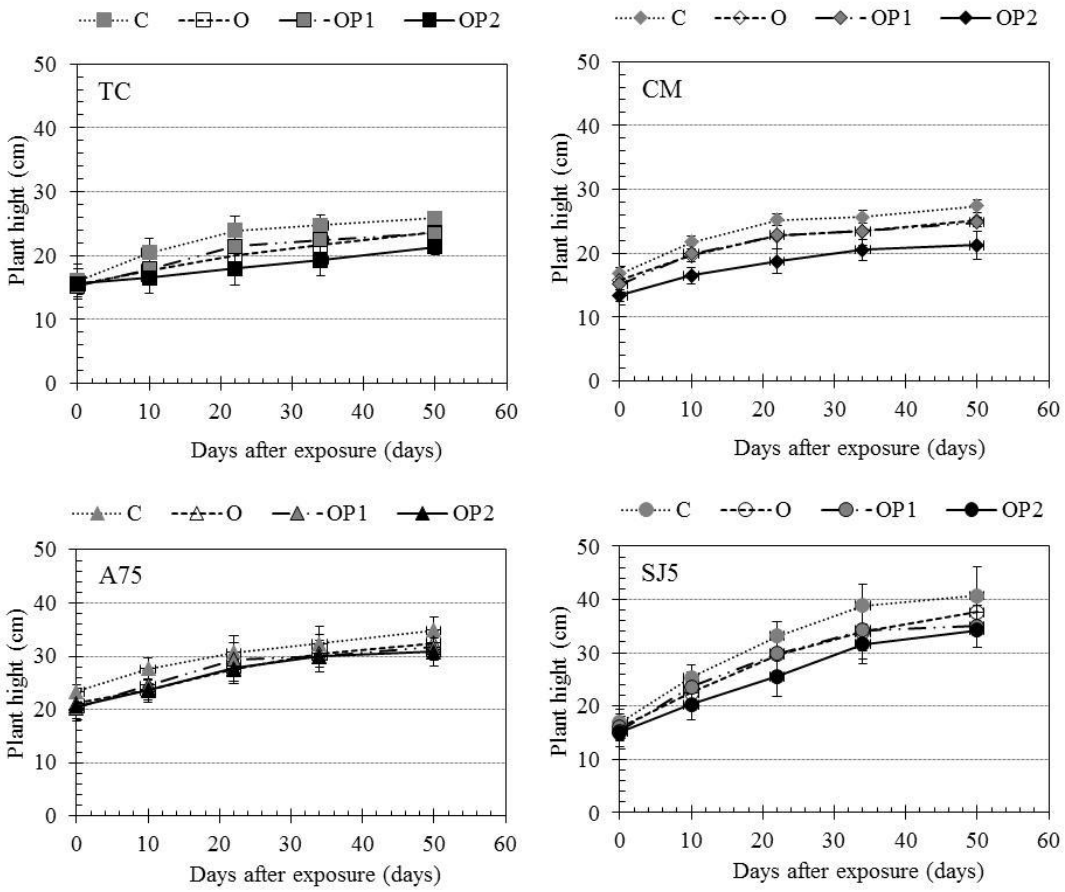


Fig. 3.4.9 Plant height of four soybean cultivars (average from eight plants in the 2nd experiment)

Table 3.4.1. Effect of O_3 and peroxides on dry weight of four soybean cultivars in winter 2010 and 2011 (Mean of eight replicates ±SD)

Cultivars	Plots	Dry weight (g plant^{-1})							Root/Shoot ratio	Reproductive/ Vegetative ratio
		Root	Petiole	Stem	Leaf	Flower	Pod	Total		
TC	C	1.88±0.59^{a}	0.34±0.19	1.15±0.42	1.48±0.78	0.12±0.14	2.80±0.81^{a}	7.77±1.29^{a}	0.31±0.08^{b}	0.74±0.41^{a}
	O	1.81±0.67^{a}	0.31±0.15	1.02±0.42	1.41±0.67	0.10±0.14	2.35±0.95^{ab}	6.99±1.17^{ab}	0.36±0.15^{ab}	0.70±0.56^{a}
	OP1	1.51±0.27^{ab}	0.29±0.06	1.09±0.32	1.14±0.27	0.17±0.13	1.45±0.85^{b}	5.65±1.65^{bc}	0.41±0.16^{a}	0.40±0.31^{b}
	OP2	1.11±0.40^{b}	0.29±0.09	1.04±0.16	1.49±0.29	0.16±0.06	0.25±0.17^{c}	4.33±0.80^{c}	0.34±0.10^{ab}	0.11±0.07^{b}
	ANOVA	**	ns	ns	ns	ns	**	**	*	**
CM	C	1.18±0.19^{ab}	0.35±0.17^{a}	1.01±0.22^{a}	1.31±0.67^{a}	-	3.73±0.66^{a}	7.58±1.82^{a}	0.19±0.04	1.00±0.13^{a}
	O	1.24±0.20^{a}	0.23±0.05^{ab}	0.90±0.13^{a}	0.87±0.27^{ab}	-	3.22±0.24^{a}	6.45±0.49^{ab}	0.24±0.03	1.02±0.20^{a}
	OP1	0.91±0.18^{b}	0.29±0.08^{a}	0.86±0.17^{a}	1.25±0.17^{a}	-	2.07±0.62^{b}	5.37±1.18^{b}	0.21±0.02	1.09±0.22^{a}
	OP2	0.58±0.16^{c}	0.12±0.04^{b}	0.47±0.10^{b}	0.46±0.20^{c}	-	1.79±0.44^{b}	3.42±0.86^{c}	0.20±0.03	0.62±0.10^{b}
	ANOVA	**	**	**	**		**	**	ns	**
A 75	C	1.57±0.16^{a}	0.34±0.17^{a}	1.19±0.47^{a}	1.78±0.91^{a}	-	4.27±0.39^{a}	9.15±1.95^{a}	0.21±0.04	0.98±0.38^{a}
	O	1.34±0.21^{a}	0.30±0.12^{ab}	1.13±0.19^{a}	1.45±0.60^{ab}	-	3.49±0.55^{a}	7.70±0.73^{a}	0.21±0.04	0.87±0.26^{ab}
	OP1	0.74±0.24^{b}	0.20±0.06^{b}	0.72±0.19^{b}	0.86±0.33^{b}	-	2.26±0.47^{b}	4.78±1.06^{b}	0.18±0.06	0.91±0.10^{a}
	OP2	0.81±0.14^{b}	0.25±0.07^{ab}	0.90±0.23^{ab}	1.13±0.36^{ab}	-	1.55±0.48^{b}	4.63±0.59^{b}	0.21±0.03	0.55±0.28^{b}
	ANOVA	**	*	*	**		**	**	ns	**
SJ 5	C	1.59±0.61^{a}	0.50±0.35	1.12±0.52^{a}	1.71±0.88	0.00±0.01	3.01±0.12^{a}	7.92±1.97^{a}	0.25±0.02	0.73±0.29^{a}
	O	1.42±0.38^{a}	0.44±0.25	1.10±0.46^{ab}	1.64±0.78	0.01±0.02	2.11±0.90^{b}	6.72±1.11^{a}	0.27±0.05	0.60±0.43^{ab}
	OP1	0.87±0.19^{b}	0.34±0.15	0.77±0.18^{b}	1.21±0.37	0.01±0.03	1.17±0.35^{c}	4.37±1.11^{b}	0.26±0.07	0.37±0.07^{b}
	OP2	0.68±0.22^{b}	0.34±0.09	0.79±0.15^{ab}	1.27±0.22	0.02±0.03	0.11±0.06^{d}	3.25±0.57^{b}	0.27±0.06	0.05±0.05^{c}
	ANOVA	**	ns	**	ns	ns	**	**	ns	**

CM was harvested on 50 days after exposure, while other cultivars were harvested on 55 days after exposure. Two-way ANOVA: **P<0.01, * P<0.05, NS=not significant. Values with different letters are significantly different at P<0.05.

3.5 Discussion

3.5.1 Visible foliar injury

Several scientists found that soybean had the genetic variation in O_3 responses (Foy *et al.*, 1995; Robinson and Britz, 2000; Morgan *et al.*, 2003). Burkey and Carter (2009) reported that ancestral lines of soybean in USA and Canada showed wide ranges of visible leaf injury to high O_3. The symptom of visible leaf injury in O, OP1 and OP2 plots was similar to that in Burkey and Carter (2009). The intensity of visible leaf injury showed severe damage in combined O_3 and peroxides more than single O_3. Similar results were found by Chen *et al*. (2010), Chutteang *et al.* (2011) and Na-ngern *et al.* (2011). Moreover the sensitivity of visible leaf injury was different among four soybean cultivars and orders of cultivar sensitivity were different between single O_3 and combined O_3 and peroxides. The cultivar sensitivity was identified as three groups consisting of tolerant, moderately tolerant, and sensitive in both single O_3 and combined O_3 and peroxides. It implies that some injury mechanism may be different between single O_3 and combined O_3 and peroxides.

3.5.2 Chlorophyll content

Chen *et al.* (2010) reported that the total chlorophyll content of Japanese radish was reduced by the fumigation of combined 50 ppbv O_3 and sub-ppbv peroxides as well as by high concentration level of O_3. Similar results were found in the present research that much severer reduction was observed in OP1 and OP2 plots than O plot (Fig. 3.4.4). It implies that low concentrations of O_3 causes less reduction in chlorophyll content but sub-ppbv peroxides concurrent with 50 ppbv O_3 caused severe damage in photosynthetic

pigment. Similar results were found by Inada *et al.* (2008) that low O_3 condition did not reduce chlorophyll content in two Japanese rice cultivars.

3.5.3 Leaf gas exchange

As for soybean, Robinson and Britz (2000) revealed that short term exposure to moderate level of ozone did not significantly affect CO_2 assimilation and stomatal conductance per unit leaf area. Fiscus *et al.* (2005) reported that reduction in CO_2 fixation under long period O_3 exposure is mainly restricted by Rubisco activity. The present result showed similar result in TC of which cultivar was less sensitive. Net photosynthetic rate slightly decreased in 10 days after single O_3 exposure and was highly reduced in 30 days after exposure. On the other hand, the relative net photosynthetic rate in C plot of sensitive cultivar (SJ5) was about 40% on 10 days after exposure by single O_3. OP2 plot showed much severe decrease in net photosynthetic rate. Similar result was found in Japanese radish by Chen *et al.* (2010). Although we cannot fully explain the mechanisms for the severe reduction of photosynthetic rate occurred in OP plots than O plot at present, the report by Reid *et al*. (1998) that chronic O_3 condition of 80 ppbv O_3 affected carbon fixation by decreasing the activity and content of Rubisco in soybean is very suggestive. It implies that 50 ppbv O_3 may not cause severe decrease in carbon fixation, but combined peroxides and O_3 is very harmful and causes the reduction of carbon fixation significantly.

It was found that the sensitive cultivar had larger gs value than tolerant cultivar in each experimental plot. But these tendencies were not shown on 30 days after exposure as shown in Fig. 3.4.7a and Fig. 3.4.7c. This may be because the leaves were getting senescence on 30 days after exposure. Flux of O_3 and other gases into the leaf is controlled by stomatal conductance (Fiscus *et al.*, 1997). As for the results mentioned above that the

sensitive cultivar had larger gs value than tolerant cultivar, O_3 and peroxides have higher possibility to enter into the leaf in sensitive plants than tolerant cultivar and cause severer damage. Fiscus *et al.* (2005) suggested that tolerant plants will close the stomata to protect the absorption of O_3 while sensitive plants cannot regulate the stomata. Our results (Fig. 3.4.7a) support the suggestion of Fiscus *et al.* (2005).

The present research results also suggest as shown in Fig. 3.4.7 that the net photosynthetic rate of soybean leaf under 50 ppbv O_3 alone was reduced by the reduction of gs or low stomata aperture, but those of combined 50 ppbv O_3 and peroxides reduced by both low gs and high Ci which relates to low carbon fixation. Farquhar and Sharkey (1982) proposed that whether the stomatal or non-stomatal factors are the main causes of the reduction in net photosynthetic rate can be judged by the change pattern of gs and Ci. If gs decreases and Ci increases simultaneously, the decrease of net photosynthetic rate can be described by non-stomatal factors. As a whole, combined O_3 and peroxides caused great reduction in net photosynthetic rate by decreasing stomata aperture and carboxylation efficiency while the net photosynthetic rate in chronic O_3 50 ppbv was mainly limited by the stomatal factor.

3.5.4 Biomass and plant growth

O_3 exposure results in decreased photosynthetic carbon assimilation (Morgan *et al*. 2003; Betzelberger *et al*. 2010). The present research results showed that total dry weight was not significantly decreased under 50 ppbv O_3 which is an average O_3 concentration in Japan. Obviously, combined O_3 and high peroxides condition caused decreases in total dry weight of more than 40% and pod dry weight of more than 50% in four soybean cultivars (Table 3.4.1). Similar research result was reported by Chen *et al.* (2010) that the combined

O_3 and 2-3 ppbv peroxides reduced the biomass of Japanese radish by about 30%. Generally biomass is primarily regulated by source and sink strength. Then, high reduction in biomass would be caused by severer damage on visible leaf injury and higher decrease in photosynthetic rate by combined O_3 and peroxides than by single O_3. Peroxides and O_3 can oxidize some protein and enzyme involved in photosynthesis (Chen *et al.*, 2005; Lu *et al.*, 2009). Then, the structure and function of photosynthetic organ were destroyed after the exposure of the soybean plant to combined O_3 and peroxides. The chlorophyll content was also degraded and stomatal aperture could not be regulated. These caused a significant decline in the photosynthetic rate and synthesis of dry matter, resulting in a considerable decrease in biomass. TC cultivar showed less reduction in total dry weight but much decrease in pod dry weight while CM showed more reduction in total dry weight than TC but could maintain pod productivity (Table 3.4.1). Similar results were found by Betzelberger *et al*. (2010) who suggested that there were various mechanisms of tolerance to high O_3 concentration within soybean germplasm such as the decrease in photosynthetic rate, chlorophyll content, and leaf area index was shown in tolerant cultivar during seed filling while the significant decreases in photosynthetic carbon gain was shown in sensitive soybean cultivars during the seed filling simultaneously with accelerating senescence.

For possible future high O_3 and peroxides condition, SJ5 may not be a suitable cultivar since reduction in crop yield is high, but CM may have less damage on production and would be suitable cultivar (Table 3.4.1). Our results showed that CM could maintain biomass and pod yield, although it induced severer damage on leaf injury and more decrease in total chlorophyll content than those of other cultivars.

In terms of dry matter distribution, TC, A75 and SJ5 showed that dry matter of pod was decreased, while fraction of dry matter of leaf, petiole and stem dry mass was

increased in OP plots. Marcelis (1996) demonstrated that the dry matter partitioning among the sinks of a plant is primarily regulated by the sink organs and depends on plant genetic and environment. The TC, A75 and SJ5 cultivars could not produce pods and seeds, but could produce leaf and stem biomass.

Specific leaf weight (SLW) is known as the thickness of leaf. The sensitive cultivar SJ5 had the lowest SLW among four soybean cultivars or the leaf of SJ5 was thinner than other cultivars. The O_3 and peroxides may have high possibility to get through the leaf and spread out in the plant cell of SJ5 more than other cultivars which causes much severer damage on visible injury, degradation on photosynthetic apparatus, and decrease in biomass and pod yield. Paakkonen *et al*. (1997) reported that high O3 tolerance of birch is related to thicker leaves as compared to sensitive species. Tuomainen *et al.* (1996) indicated that in thick leaves, the high reactivity of ozone and detoxification mechanisms are likely to suppress the harmful effects of ozone before reaching the palisade tissue where the main photosynthesis occurs. Leaf anatomy characteristics are important for future research.

The decrease in plant height in O and OP1 plot was similar and less than OP2 plot. It implies that the combined O_3 and high concentration of peroxides cause higher reduction in soybean growth than single O_3.

3.6 Summary

The results of the present study showed that single O_3 caused leaf injury, chlorophyll degradation, low stomatal conductance, and low photosynthetic rate, but single O_3 did not cause more total biomass reduction than the C plot. Several ppbv peroxides coexisting with 50 ppb O_3 induced a significant increase in leaf injury, high intercellular CO_2

concentration, and also a significant reduction in net photosynthetic rates, plant height and biomass than single 50 ppbv O_3. It was found that SJ5 is the most sensitive cultivar while CM was the least sensitive in both single O_3 and combined O_3 and peroxides from the perspective of pod yield. The sensitive cultivar showed higher gs and higher Ci than tolerant cultivars in each experimental plot on A versus gs and A versus Ci relationships after 10 days of exposure.

C plot **O plot** **OP1 plot** **OP2 plot**

Fig. 3.4.10. Visible injury of Tachinagaha (TC) after 55 days of exposure

C plot = Control plot (free O_3 and peroxides),

O plot = 50 ppbv of O_3,

OP1 plot = 50 ppbv of O_3 and 2-3 ppbv of peroxides, and

OP2 plot = 50 ppbv of O_3 and 4-5 ppbv of peroxides

C plot **O plot** **OP1 plot** **OP2 plot**

Fig. 3.4.11. Visible injury of Chamame (CM) after 50 days of exposure

C plot = Control plot (free O_3 and peroxides),

O plot = 50 ppbv of O_3,

OP1 plot = 50 ppbv of O_3 and 2-3 ppbv of peroxides, and

OP2 plot = 50 ppbv of O_3 and 4-5 ppbv of peroxides

C plot **O plot** **OP1 plot** **OP2 plot**

Fig. 3.4.12. Visible injury of A75 after 55 days of exposure

C plot = Control plot (free O_3 and peroxides),

O plot = 50 ppbv of O_3,

OP1 plot = 50 ppbv of O_3 and 2-3 ppbv of peroxides, and

OP2 plot = 50 ppbv of O_3 and 4-5 ppbv of peroxides

C plot **O plot** **OP1 plot** **OP2 plot**

Fig. 3.4.13. Visible injury of Sorjo5 (SJ5) after 55 days of exposure

C plot = Control plot (free O_3 and peroxides),

O plot = 50 ppbv of O_3,

OP1 plot = 50 ppbv of O_3 and 2-3 ppbv of peroxides, and

OP2 plot = 50 ppbv of O_3 and 4-5 ppbv of peroxides

CHAPTER 4

General discussion

4.1 Variations of H_2O_2 concentrations in Japan and Thailand

In the present study, the H_2O_2 concentrations and the diurnal characteristics in H_2O_2 concentration were found to be dependent on place, year and season (Fig. 2.4.2). High H_2O_2 concentrations were found in the polluted areas during high temperature and dry air condition. Gunz and Hoffman (1990) described that the high H_2O_2 concentration was found in heavily polluted areas such as Los Angeles and New York, and in strong diurnal and seasonal variation areas such as California. In present study, the H_2O_2 concentration was dependent on O_3 directly or, in other words, if O_3 concentration is high the H_2O_2 concentration will be high. Air temperature indirectly affected H_2O_2 concentrations by controlling the O_3 formation reaction. Several reports showed the similar results that O_3 was positively correlated with H_2O_2 concentrations (Jackson and Hewitt, 1996; Takami *et al*., 2003; Chen *et al*., 2008). This should be because O_3 is the key compound produced in the photochemical reactions to produce OH and HO_2 radicals involving NOx and VOC (Gunz and Hoffman, 1990) as precursors. Higher temperature brings about acceleration of photochemical reaction and O_3 formation; therefore, it affects H_2O_2 concentrations. Chen *et al*. (2008) revealed that H_2O_2 increased with increasing O_3 and air temperature. In tropical developing countries such as Thailand, the emission of air pollutants such as NOx and VOC increases because of increasing of industrial areas, populations, transportations, and biomass burning, which results in the increase of atmospheric peroxide concentrations in polluted areas. The H_2O_2 concentrations in rural areas at Chiang Mai measurement site in Thailand were not so high, but these concentrations may impact on crops and forest decline in the future when combined with O_3 in the air as shown in the chapter 3. In addition the high H_2O_2 concentrations in Nakhon Pathom measurement site which is the mixed areas between crop production areas and residential areas, and surrounded by

industrial areas can cause the leaf injury on several kinds of plants as shown in the chapter 2. As a whole these H_2O_2 concentration may highly relate with yield loss of crops and forest decline in Thailand. Thus, the atmospheric H_2O_2 concentration is also one of the important air pollutants data that should be considered. Aoki *et al*. (2012) and Takami *et al*. (2003) reported that high H_2O_2 concentration was found in the area with severe forest decline in Japan. Therefore, a systematic measurement data of H_2O_2 and organic peroxides are strongly required to realize the concentration in the future and to evaluate the impact of peroxides to agriculture and forest.

4.2 Effects of single O_3 and combined O_3 and peroxides on physiological responses in tolerance and sensitive soybean genotype

In present study, it was clarified that the single 50 ppbv O_3 caused leaf injury, chlorophyll degradation, low stomatal conductance (gs), and low net photosynthetic rate (Fig. 3.4.2, Fig. 3.4.4, Fig. 3.4.6, Fig 3.4.7), and single O_3 for 50-55 days fumigation caused about 10%-15% reduction of total biomass comparing to C plot and it was not large total biomass reduction (Table 3.4.1). Morgan *et al*. (2003) found similar results that the O_3 concentration about 40-60 ppbv caused the reduction in seed yield, shoot and root dry weight of soybean about 10%, and the decrease in net photosynthetic rate and stomatal conductance. Whereas the combined 4-5 ppbv peroxides in the present study with 50 ppbv O_3 exposure induced a significant increase in leaf injury, high intercellular CO_2 concentration (Ci), and also a significant reduction in net photosynthetic rates and biomass compared to single 50 ppbv O_3 exposure. The reduction of total dry weight was about 40%-55% of C plot. Similar results were found by Chen *et al*. (2010) that the exposure of

combined 50 pbbv O_3 and 2-3 ppbv peroxides caused severer damage such as visible injury, reduction of photosynthetic rate, and decrease of dry mass compared to exposure of single 100 ppbv O_3 in Japanese radish. Na-ngern *et al*. (2011) also found that 50 ppbv O_3 coexisted with 4-5 ppbv peroxides caused high damage of visible injury and reduction of photosynthetic capacity in Thai and Japanese rice cultivars. Elstner (1987) reported that the wax of the bean epidermis leaf was degraded by the H_2O_2 produced by the reaction between ethylene and O_3. Masuch and Kettrup (1986) indicated that the mesophyll cell both palisade and spongy cells of beech collapsed after exposure to H_2O_2-containing acidic-fog. The results of this research suggested that atmospheric peroxides can be the main contributor to cause the severe plant damage when combined with present level of O_3.

In addition, the present study showed that the net photosynthetic rate of soybean leaf under 50 ppbv O_3 was mainly limited by the gs, but that under combined 50 ppbv O_3 and peroxides was limited by both low gs and high Ci. Similar results were found by Belzelberger *et al*. (2012) that the low O_3 dose of an accumulated exposure over a threshold of O_3 40 ppbv (AOT40) caused slight reduction in carboxylation efficiency in soybean plant. He *et al*. (2007) also reported that the decline of photosynthesis in *Gingo biloba* leaf in the beginning of O_3 exposure was especially caused by the stomatal factors. However, long term exposure of AOT40, chronic moderate O_3 conditions, and acute O_3 conditions caused the significant decrease in carbon fixation (Belzelberger *et al*. 2012, Ficus *et al*., 2005, He *et al*., 2007, Reid *et al*. 1998,). Singh *et al*. (2009) reported that the long period of elevated O_3, 70 and 100 ppbv, caused the increase in Ci, which leaded the large reduction in photosynthesis on soybean plants and suggested that the carbon fixation is more severely affected in O_3-stressed plants. Therefore, these research results suggested

that the short term exposure of chronic 50 ppbv O_3 may not cause severe decrease in carbon fixation, but combined O_3 and peroxides is harmful to both the carboxylation mechanism and stomata aperture.

Differential physiological responses of soybean genotype under single O_3 and combined O_3 and peroxides were found. The sensitive cultivar showed higher gs and higher Ci than tolerant cultivars in both single O_3 and combined O_3 and peroxides exposure for a short term (Fig. 3.4.7a, Fig. 3.4.7b). The high gs in sensitive cultivar showed that O_3 and peroxides had higher possibility to enter the leaf of sensitive cultivar plants than that of tolerant cultivar plants. Then, it caused severer damage on visible appearance, photosynthetic capacity, and production in sensitive cultivar than in tolerant cultivar. Ficus *et al*. (2005) described that tolerant soybean plants will close the stomata aperture under elevated O_3 to protect the absorption of O_3 while sensitive plant cannot regulate the stomata. These results suggested that the tolerant cultivar of soybean can regulate the stomata aperture during the early fumigation of both single O_3 and combined O_3 and peroxides. In addition, the other characteristics such as less reduction in net photosynthetic rate and crop yield are shown in the tolerant cultivar.

4.3 Crop improvement for possible future high O_3 and peroxides conditions

The variation of peroxide concentrations based on place, time, and environmental conditions was found in the present research. Concentrations of ozone and peroxides can increase in developing countries such as Thailand in the future. High H_2O_2 concentration (monthly average level raged from 2-10 ppbv) and visible leaf injury probably caused by combined O_3 and peroxides on several kinds of plants were found in Nakhon Pathom

measurement site, Thailand. In addition, the O_3 concentration strongly related with H_2O_2 in positive way. If H_2O_2 is high as OP2 plot, the O_3 in reality is much higher, and, then, the injury of plant can be much more serious. These can be one of the reasons for the loss in crop yield and forest decline in some places especially in highly polluted areas and with high O_3 concentrations. Then, the information of atmospheric concentrations of peroxides and O_3 is necessary for evaluating the loss in crop yield and planning a suitable time for planting by considering the concentration and accumulation of those air pollutants during growth season. The plant damage and the loss in crop yield are dependent on the concentrations of both O_3 and peroxides, and meteorological or weather conditions. The tropical area which is generally warm or even hot and humid year-round may have higher H_2O_2 concentration and, thus, greater damage on crop and forests can be anticipated especially in industrial areas with busy transportation.

The results of present study also showed that the damage and the loss in crop yield of soybean under combined O_3 and peroxides were different with that under single O_3 exposure, and that they were dependent on cultivar. Then, the information about the cultivar sensitivity should be studied in more detail and more research works on many plant species and soybean cultivars are required to improve crop production in the future. Moreover, plant breeding program for finding tolerant crop cultivars is important for crop production for future conditions under increasing peroxides and O_3 concentrations under global warming. The well regulated stomatal aperture in the early exposure to O_3 and peroxides was shown in the tolerant cultivars. This may be one of the characteristics for a desired cultivar. The early maturity of soybean plant is also important characteristic to avoid the severe plant damage on high O_3 and peroxides dose. Basically the high

production is very important for the crop improvement, and, thus, the less reduction in crop production against the exposure to O_3 and peroxides is required for a desired cultivar.

Acknowledgements

I would like to extend my special appreciation to Prof. Dr. Masatoshi Aoki and Prof. Dr. Shiro Hatakeyama, my research supervisors, for their excellent continuous guidance, valuable time and support, and constant encouragement given in regard to my thesis and study life. I am grateful for their teaching and advice. I could not have achieved this far and this thesis would not have been completed without all the supports that have always received from them.

I am thankful to my advisory committee, Prof. Dr. Bellingrath-Kimura Sonoko Dorothea, Prof. Dr. Takeshi Izuta, Prof. Dr. Hitoshi Honjo (Utsunomiya University), Prof. Dr. Toshihiko Narusawa (Ibaraki University) for their valuable suggestion during my Ph.D tenure.

I would like to express my sincere thanks to Dr. Fitzgerald L. Booker and Dr. Kent O. Berkey, United States Department of Agriculture-Agriculture Research Services (USDA-ARS), Plant Science Research Unit, and Department of Crop Science, North Carolina State University (NCSU), Raleigh, North Carolina, the internship host, for invaluable advice in both the research methodologies and meaning of life.

I would like to express my deep gratitude to Prof. Dr. Poonpipope Kasemsap and Dr. Tiwa Pakokthom, Kasetsart University, Thailand for their contribution of time and resources.

I am thankful to Prof. Dr. Onwana-Agyeman Siaw for providing English correct. I am thankful to Prof. Dr. Isao Ogiwara of Horticulture laboratory for technical and equipment support. I would also like to thank Prof. Dr. Ruangchai Juwattanasamran, Maejo University, Thailand for supporting the soybean seeds in this research.

I would like to express my appreciation to all the past and present members in Aoki's laboratory (Atmospheric Environmental Laboratory) and Hatakeyama's laboratory for good advice and collaboration. Thanks to Ms. Nongpat Chaichana for her kind support to measure and collect the data in Thailand and other aspects on this research. I am especially grateful for the immense pleasure that we had during our research hour and my study life from great friends like Mr. Pathomrak na-ngern, Mr. Fumiaki Takemasa, Mr. Ryota Marushima, Mr. Ge Zhenrui, Mr. Tuvshinbayar, Mr. Hakushi Masuzawa and other lab-mates. Thanks all of Thai, Japanese and international friends for their friendship and encouragements for all the time in Japan.

I am sincerely grateful to all teachers in my life especially Prof. Dr. Suntaree Yingjajaval for the things they have taught and guided me. And a special thanks to my boss and our staffs in Kasetsart University, Thailand for giving me a chance to carry out this Ph.D.

I would like to extend my appreciation to the Ministry of Education, Culture, Sports, Science and Technology (MEXT) through the Education Program for Field-Oriented Leaders in Environmental Sectors in Asia and Africa (FOLENS program) in Tokyo University of Agriculture and Technology for the financial support and providing other invaluable experiences during my Ph.D. program for three years.

Last but not least, I would like to express my eternal appreciation towards my father, mother and sisters for all unconditional support and patience. Thank you for their love, understanding and encouragement, and for being a constant source of my inspiration throughout my study. It is a pleasure for me to thank all of my friends in Thailand, Japan and so on for their support throughout the period of my study life and have been inspirational to me.

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