Effect of Ozone on Nitrogen Fixation, Nitrogenase Activity and Rhizobium of Cowpea (*Vigna unguiculata* (L.) Walp)

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Abstract

The research aimed to investigate the effects of ozone on nitrogen fixation in cowpea (*Vigna unguiculata* (L.) Walp) following the growing period e.g. seedling, vegetative (V3), flowering (R1) and harvesting (R5). Plant samples were grown in the fumigating chambers of which the temperature, light, and ozone concentration were controlled. The treatment groups were given two levels of ozone at 40 and 70 ppb for 8 hours per day. The control group, ozone in the ambient air was charcoal filtered less than 10 ppb before entering to the chambers. The results showed the effects of ozone on significant reduction in the total biomass particularly root dry weight, the number of nodule, distribution of nodule size over 2 mm and the nodule dry weight. Moreover, the total nitrogen in plant tissues and the nitrogenase activity were significantly decreased when plant samples were in the stages of vegetative, flowering and harvesting due to ozone exposure. Slow growing rhizobium well established in the rhizophere of the control group rather than ozone fumigated plant. Indeed, continuing ozone fumigating concentration both levels at 40 and 70 ppb decreased the total soil nitrogen 3.42-3.91% as compared with the control group. Finally, the reduction in the total nitrogen in plant tissue and soil were due to decreasing the number of nitrogen fixation bacteria hosted nodule at the rhizosphere and deficiency of nitrogenase activity.

Keywords: Ozone; Rhizobium; Nitrogenase acivity; Cowpea (*Vigna unguiculata* (L.) Walp)

INTRODUCTION

The global climate change was considered as the threat to human civilization. The ambient air quality was continuing deteriorated by increasing of emission gases from human activities such as traffic and industry. One of the important polluted gases was ozone. Tropospheric ozone had been continuingly reported significant increasing in the industrial area (Ezzel, 2002). Recently, the air quality monitoring in Thailand during January-March 2008 was deteriorated when compared with the data during October-December 2007. The areas where under being monitored and recorded exceeded the standard of the Department of Pollution Control were Bangkok, Rachaburi, Rayong and Chiangmai (Thai Environmental Engineering Magazine, 2008). Ozone is one of the polluted gases which had been monitored and predicted increasing in those areas. Basically, ozone was a toxic gas which harmed to human and vegetation. Plants commonly uptake ozone through the stomata during photosynthesis and respiration. Consequently, ozone caused decreasing of stomata aperture and reducing carbon dioxide exchange which affected to photosynthesis rate. The certain concentration of ozone would directly inhibit plant growth and production (Calatayud & Barreno, 2004; Heck & Miller, 1994; Sharma & Davis, 1997) including re-duce nitrogen accumulation in plant and soil (Agrawal & Agrawal, 1990). Indeed, root development and water uptake were found affected by ozone (Grantz, 2003). Poor root development might retard rhizobium inoculation to root system reducing nitrogen fixation (Farhharn et al., 1985). According to the reduction in agricultural production, farmers had to increase fertilizer for retaining their crop production. Therefore, symbiotic nitrogen fixation in legume was important to maintain global food production (Bordeleau & Prévost, 1994; Herridge & Rose, 1999). The legume was mostly help to contribute nitrogen in soil and reduce using chemical fertilizer. Moreover, nodules where hosted rhizobium bacteria which fix nitrogen from the air reduce amount of green house gases and carbon dioxide along with the benefit to crop fields (Carr et al., 1995; Jensen, 1996). However, legume were tremendous affected by ozone which caused reduction in growth, shoot and root dry weight, amount and weight of nodule, rhizopheric nitrogen, rhizobium in root nodule and activities of nitrogenase (Agrawal & Agrawal, 1990; Blum & David, 1977; Manning, 2003; Ezzel, 2002). Cowpea (*Vigna unguiculata* (L.) Walp) was recently reported by Umponstira et al. (2006) sensitive to ozone and suitable for this experiment. Furthermore, cowpea was widely introduced as soil improver plant which recommended by the department of land development due to having a short life cycle crop and growing capability in several soil types (Kowasurat, n.d.).

improver plant which recommended by the department of land development due to having a short life cycle crop and growing capability in several soil types (Kowasurat, n.d.). This research will intend to investigate the effects of ozone on nitrogen fixation mechanism in legume particularly in the rhizosphere environment.

MATERIAL AND METHODS

Experiment design

The experimental design was conducted in complete block design which treatments were allocated as control, ozone concentration at 40 and 70 ppb group respectively. Plants were exposure to ozone through at the growing period which was in 4 stages; seedling (7 days), vegetative (V3; 17 days), reproductive (R1; 45 days) and harvesting (R5; 81 days). The total ozone exposure period was 74 days.

Dry weight

Dry weight was determined at every stage of the growing period. Root and shoot of plant samples were washed and placed in the hot air oven for 48 hours at 70 °C before weighing.

Nitrogenase activity and Nitrogen in plant tissues and soil

Root of plant samples was washed and nodules were collected by separated in two sizes, smaller and bigger than 2 mm. The number of both sizes was counted and dried in a hot air oven at 70 °C for 48 hours (Wongnai, 1998).

Total nitrogen in plant tissues and soil was determined by Micro KeJeldahl method (Black, 1965). Soil samples from the pot were placed in the room with well ventilation for 2 days before analysis. Notably, nitrogen in soil was measurement both before and after conducting the experiment.

Nitrogenease activity was determined by Acetylene Reduction Activity method (Hardy et al., 1973). Fresh shoot and washed root were separately placed in plastic airlock bags with gas-proof serum stopper filled by a small piece of soak filter paper. Air inside the bag was pumped out 10 % of the total volume. Added acetylene 10% was filled back to the bag. The samples were incubated for 1 hour. After that 3 air samples of 1 mm were collected by syringe and analyses by gas chromatography. Ethylene purified standard gas (99.5%; Supleco) was injected to gas chromatography for creating a standard peak.

Rhizobium bacteria analyses

Isolates of rhizobium were isolated from root of cowpea from both ozone and the control groups. Regarding the bacterium physiology standard identification e.g. colony, size, colour, mucus and gram stain, only 6 isolates were considered differences which were coded as CF1, CF5, OZ 40/2, OZ 70/3, OZ 70/4, OZ 70/5. Furthermore, all isolates were identified into two main groups as fast-growing nodule bacteria and slow-growing nodule bacteria according to Bergey's manual of Systematic bacteriology Vol.1 by culturing in the yeast extract mannitol agar (YMA) added congo red and capability of acidity and alkalinity produce in YMA added with bromthymol blue. The further test was carried out by sugar consumption capability as a carbon source to produce acidity from glucose, lactose, sucrose and maltose including growth in NaCl 1-5% in YMA.

Statistic analysis

One-way ANOVA with Duncan's Multiple Range Test (DMRT) was analysis of treatment differences between control and ozone treatment groups. Lease significant difference (LSD) was tested during the growing period of different treatments.

RESULTS

Dry weight

Fumigation cowpea plants with ozone had effects on root, shoot and the total dry weight. Shoot dry weigh was significantly decreased after exposure to ozone concentration at 40 and 70 ppb when compared with the control group during growing at V3 (0.97, 0.72, 0.35 g) and R5 (16.70, 12.84, 10.66 g; CF, O_3 40 and 70 ppb) respectively (Figure 1a). While at R1 ozone concentration at 70 ppb caused significant decreasing of shoot dry weight (6.22 g) when compared with both the control group and ozone 40 concentration (11.24 and 10.32 g). Like shoot dry weight during V3, R1 and R5 ozone concentration at 70 ppb caused a significant effect to root dry weight when compared to the control and mind ozone concentration at 40 ppb (Figure 1b). Both ozone concentration at 40 and 70 ppb significantly affected to the total weight of plant at V3 (1.20, 0.90, 0.45 g) and R5 (18.70, 14.38, 1.18 g; CF, O_3 40 ppb, O_3 70 ppb) respectively. However, only R1 total dry weight of plant was significantly decreased by ozone concentration at 70 ppb and no effect of ozone at 40 ppb when compared with the control group 13.03, 11.72 and 7.37 g; CF, O_3 40 ppb, O_3 70 ppb (Figure 1c).

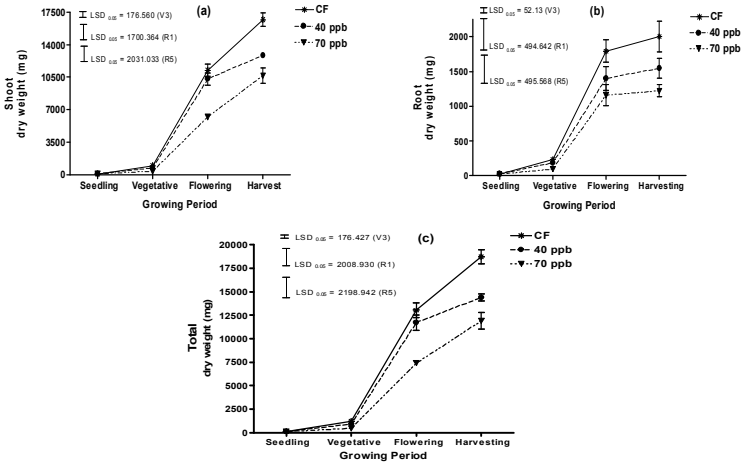

Figure 1. Shoot (a), Root (b) and Total dry weight (c) (mg) of cowpea when exposure to ozone at 40 and 70 ppb and the control group (CF) with ozone concentration ≤10 ppb. The growing stages were seedlings, vegetative (V3), flowering (R1) and harvesting (R5). Data represent the mean ±SE (n=5). Lease significant difference at p≤0.05.

Nodule dry weight and the number of nodule

The nodule dry weight was significantly affected by both ozone concentration at 40 and 70 ppb when plants were at V3 and R5 stages as 33.42, 13.00, 3.10 mg and 531.76, 306.80, 236.24 mg (CF, O_3 40 and 70 ppb) respectively. However, during R1 only ozone concentration at 70 ppb showed the effect on nodule dry weight as 180.88, 392.80, 314.28 mg (CF, O_3 40 and 70 ppb) respectively (Table 1). Nodules size over 2 mm were observed after V3 (Figure 2). The number of nodules size over 2 mm found significantly decreased after exposure to ozone concentration at 40 and 70 ppb during V3, R1 and R5 stage as 14.80, 5.40, 3.20 nodules, 114.80, 63.20, 28.80 nodules and 72.60, 35.80, 35 nodules (CF, O_3 40 and 70 ppb) respectively (Table 2). There were no effects of ozone on node size smaller than 2 mm.

Table 1 Nodule dry weight of cowpea (mg) when exposure to ozone at 40 and 70 ppb and the control group (CF) with ozone concentration ≤10 ppb. The growing stages were seedlings, vegetative 3 (V3), flowering (R1) and harvesting (R5). Data represent the mean ±SE (n=5). The different letter (a-b) indicated significant difference at p ≤0.05

Growing stage	Nodule dry weight (mg)		
	CF	40 ppb	70 ppb
Seedlings	NN	NN	NN
Vegetative (V3)	33.42±9.42[a]	13.00±1.45[b]	3.10±1.35[b]
Flowering (R1)	480.88±47.58[a]	392.08±34.03[ab]	314.28±42.09[b]
Harvesting (R5)	531.76±78.48[a]	306.80±47.96[b]	236.24±46.53[b]

Remark: NN = No Nodule observed

(a) (b) (c)

Figure 2. The nodulation of cowpea; control group (CF) (a), ozone 40ppb (b) and ozone 70 ppb (c) when exposed to ozone at 40 and 70 ppb and with ozone ≤10 ppb. Photos were taken during flowering (R1) stage.

Table 2 The number of nodule of cowpea when exposure to ozone at 40 and 70 ppb and the control group (CF) with ozone concentration ≤10 ppb. The growing stages were seedlings, vegetative 3 (V3), flowering (R1) and harvesting (R5). Data represent the mean±SE (n=5). The different letter (a-b) indicated significant difference at $p \leq 0.05$

Growing period	The number of nodule			
	Size	CF	40 ppb	70 ppb
Seedlings	>2 mm	NN	NN	NN
	<2 mm	NN	NN	NN
Vegetative (V3)	>2 mm	14.80±1.39[a]	5.40±2.40[b]	3.20±1.46[b]
	<2 mm	NN	6.60±0. 67[a]	7.60±1.54[a]
Flowering (R1)	>2 mm	114.80±22.60[a]	63.20±6.56[b]	28.80±3.55[b]
	<2 mm	57.80±35.22[ns]	30.20±7.65[ns]	36.60±10.94[ns]
Harvesting (R5)	>2 mm	72.60±8.91[a]	35.80±5.54[b]	35.00±1.92[b]
	<2 mm	15.40±9.05[ns]	15.00±5.98[ns]	5.80±2.65[ns]

Remark NN = No Nodule observed

[ns] = No significant difference

Rhizobium isolation

The results showed that the fast growing nodule bacteria were isolated from the ozone additional treatment groups from isolates OZ 70/3, OZ 70/4, OZ 70/5. These isolates were well grown in YMA incubated at 30°C in 3 days. The colony size was 2-5 mm with round, curve and transparency with mucus production. Moreover, they had a capability of acidity production when cultured in YMA added with bromthymol blue. All isolates were identified to be genus *Rhizobium* sp. (Jordan, 1938). According to biochemistry determination the isolation was confirmed as *R. meliloti* (OZ 70/3, OZ 70/4 and OZ 70/5) and *R. leguminosarum* (OZ 70/4 and OZ 70/5 (Krieg & Holt, 1984). The slow growing nodule bacteria isolate CF1, OZ 40/2 and CF5 grew in YMA with bromthymol blue at 30°C within 5-7 days. The colony size was 1 mm, pale white, round curve and mucus production. Isolations were identified to be genus *Bradyrhizobium* sp. (Jordan, 1938) or alkali-producing bradyrhizobium.

Nitrogen in plant tissues and soil

The nitrogen in plant shoot was significantly affected by ozone concentration at 40 and 70 ppb at V3 (64.34, 62.24, 55.02 milligram Nitrogen/gram dry weight (mg N/g dry weight)) and R1 (46.16, 45.52, 43.15 mg N/g dry weight (CF, O_3 and 70 ppb)) respectively when compared with the control group. However, there was no significant difference observed at R5 (Table 3). The nitrogen of plant root was also significantly decreased by ozone concentration at 40 and 70 ppb at V3 (57.94, 50.83, 40.86 mg N/g dry weight (CF, O_3 40 ppb, O_3 70 ppb) and R5 (43.95, 41.92, 40.08 mg N/g dry weight). However, during R1 only ozone concentration at 70 ppb had significant reduction of nitrogen in root as 50.98, 50.16, 38.06 mg N/g dry weight (CF, O_3 40 ppb, O_3 70). The total nitrogen in plant tissues during V3 as 122.28, 113.07, 95.89 mg N/g dry weight (CF, O_3 40 ppb, O_3 70 ppb) and R5 80.38, 75.01, 73.77 mg N/g dry weight was significantly affected by ozone when compared with the control group. Furthermore, during R1 ozone concentration at 70 ppb caused significant difference of nitrogen as 97.14, 95.68, 81.21 mg N/g dry weight (Table 3). However, during the R5 stage the nitrogen of all treatments was decreased.

Ozone concentration had a significant effect on the total soil nitrogen when compared with the control group. During V3 and R1 the soil nitrogen was 2.69, 2.41, 2.05 mg N/g dry weight, 2.83, 2.59, 2.49 mg N/g dry weight (CF, O3 40 and 70 ppb) respectively (Figure 3). The total reduction of soil nitrogen was calculated as 3.42-3.91%. There was no significant difference of the soil nitrogen during the R5 stage.

Nitrogenase activity

Additional ozone concentration at 40 and 70 ppb showed significant differences of nitrogenease activity when compared with the control group R1 as 5.13, 3.01, 4.29 micro ethylene/gram dry weight/hour (μC2H4 /g dry weight /h) and R5 as 1.27, 0.88, 0.84 μC2H4 /g dry weight/h (CF, O_3 40 ppb, O_3 70 ppb). However, during V3 ozone concentration 70 ppb caused significant difference when compared with the control group. There was no effect of ozone concentration at 40 ppb (Figure 4).

Table 3 The total of nitrogen of cowpea (mg N/g dry weight) when exposure to ozone at 40 and 70 ppb and the control group (CF) with ozone concentration ≤ 10 ppb. The growing stages were seedlings, vegetative (V3), flowering (R1) and harvesting (R5). Data represent the mean ± SE (n=5). The different letter (a-c) indicated significant difference at $p \leq 0.05$

Growing period	The total nitrogen in plant tissue (mg N/g dry weight)			
	Plant tissue	CF	40 ppb	70 ppb
Seedlings	Shoot	27.88±0.06^{ns}	28.06±0.42^{ns}	27.78±0.76^{ns}
	Root	16.40±0.11^{ns}	16.56±0.22^{ns}	16.25±0.08^{ns}
	Total	44.29±0.11^{ns}	44.56±0.45^{ns}	44.03±0.70^{ns}
Vegetative (V3)	Shoot	64.34±0.15^{a}	62.24±0.55^{b}	55.02±0.67^{c}
	Root	57.94±1.41^{a}	50.83±1.50^{b}	40.86±0.62^{c}
	Total	122.28±1.40^{a}	113.07±1.88^{b}	95.89±0.09^{c}
Flowering (R1)	Shoot	46.16±0.68^{a}	45.52±0.59^{ab}	43.15±0.95^{b}
	Root	50.98±0.56^{a}	50.16±1.57^{a}	38.06±0.73^{b}
	Total	97.14±1.02^{a}	95.68±1.95^{a}	81.21±1.32^{b}
Harvesting (R5)	Shoot	36.53±1.60^{ns}	33.09±0.80^{ns}	33.68±0.65^{ns}
	Root	43.95±0.37^{a}	41.92±0.25^{b}	40.08±0.22^{c}
	Total	80.38±0.19^{a}	75.01±0.09^{b}	73.77±0.52^{b}

Remark: ^{ns} = No significant difference

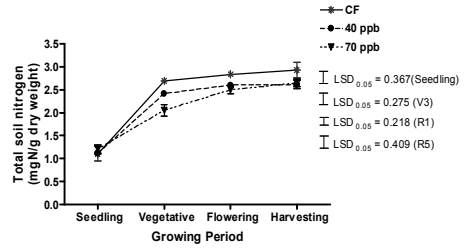

Figure 3. Total soil nitrogen (mg N/g dry weight) of cowpea when exposed to ozone at 40 and 70 ppb and control group (CF) with ozone ≤ 10 ppb. The growing stages were seedlings and vegetative (V3), flowering (R1) and harvesting (R5). Data represent the mean ±SE (n=5). Lease significant difference at $p \leq 0.05$.

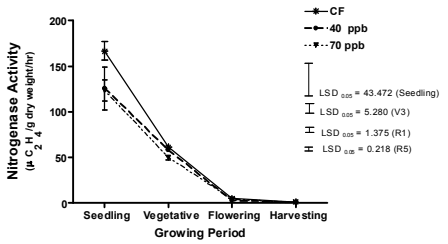

Figure 4. Nitrogenase activity (μC2H4/g dry weight/h) of cowpea when exposed to ozone at 40 and 70 ppb and control group (CF) with ozone≤ 10 ppb. The growing stages were seedlings and vegetative 3 (V3), flowering (R1) and harvesting (R5). Data represent the mean ± SE (n=3). Lease significant difference at $p \leq 0.05$.

DISCUSSION

Dry weight

Ozone fumigated plants had effects on root, shoot and the total dry weight. The physiological responses of plants was naturally reacted when ozone enter through the stomata. Excessive ozone would decrease stomatal conductance causing less gas exchange lead to affect to photosynthesis rate. Generally, ozone caused the deficiency of carboxylation during photosynthesis which played an important role during photosynthesis which affected to plant growth (Calatayud et al., 2003). Indeed, most of the ozone affected plant show retarding of the root development rather than stem (Didier & Sirkku, 2002).

Nodule dry weight and the number of nodule

The nodule dry weight was significantly affected plants were at V3 and R5 stages. Like the report from Nasser (2002) found decreasing the weight of root nodule of Faba Bean (*Vicia fabia*) after expose to ozone 100 ppb for 5 h day^{-1} for two week. The number of nodules size over 2 mm found significantly decreased after exposure to ozone concentration at 40 and 70 ppb during V3, R1 and R5 stage. There were no effects of ozone on nodule size smaller than 2 mm. Several researchers also found the same effect of ozone on decreasing of the number of nodule. For instance, Agrawal & Agrawal (1990) found reduction of nodulation of *Vicia faba* and *Cicer arietinum* after exposure to ozone 196±5.0 μgm^{-3} for 2 h daily for 30 days. Similarly, the research on Pinto bean (Manning et al., 1971). Rhizobium nodules were found on the root of plants grown in charcoal-filtered air, but were not found on the root of plants grown in the ozone chamber.

Rhizobium isolation

Isolates were identified into two main groups as Fast-growing nodule bacteria and Slow-growing nodule bacteria. The Fast-growing nodule bacteria I solates were identified in Genus *Rhizobium* sp. (Jordan, 1938). According to biochemistry deter mination the isolation was confirmed as *R. meliloti* and *R. leguminosarum*. The slow growing nodule bacteria isolations were identified in genus *Bradyrhizobium* sp. (Jordan, 1938) or alkali-producing bradyrhizobium bradyrhizobium. *Bradyrhizobium* was commonly found attaching to plant root in tropic and sub-tropical zone. Particularly, cowpea (*Vigna unguiculata* (L.) Walp) showed strong relation hosted with *B. elkanii* and *B. japonicum* (Jerri et al., 2004; Laity et al., 2003; Zhang et al., 2007; Zhang et al., 2008). Moreover, the fast growing group, *R. meliloti* and *R. leguminosarum*, were naturally found in cowpea and siratro. However, they might be able to establish nodule in plant root but was considered as an ineffective rhizobium due to low capability of nitrogen fixation. In contrast *Bradyrhizobium* which found in cowpea also can grow in the other legumes such as lima bean, peanut and siratro with low nitrogen fixation efficiency (Zhang et al., 2007; Dokora & Vincent, 1984). Also during the seedling stage cowpea could fix more nitrogen than the other stage due to the cooperation of indigenous natural bacteria living in plant tissue, root cell membrane and soil from Endophyte and Diazotrophs. Those bacteria could fix N_2 from ambient air to NH_3 with electron reduction and protonation associated by nitrogenase enzyme complex inside nitrifying bacteria (Theaumrung, n.d.)

Nitrogen in plant tissues and soil

The nitrogen in plant shoot, root and total nitrogen was also significantly decreased by ozone concentration at 40 and 70 ppb. This can be explained by the normal physiological change of cowpea during the growing period which nodules at the core root were decreased causing reduction of nitrogen fixation capability (Boonkeod et al., 1977). Similarly, Marschner (1986) also found the nitrogen fixation rate of cowpea was sharply decreased after flowering stage. Ozone concentration had a significant effect to the total soil nitrogen when compared with the control group. The total reduction of soil nitrogen was calculated as 3.42-3.91%. Obviously, there were significant decreasing of the number of root nodes and the quality of node which was directly affected to nitrogen in plant tissues and soil. Indeed, ozone also showed the significant impact to the efficiency of nitrogenase activity during growing period. This caused the reduction of rhizobium hosted by root plant which directly affected to nitrogen fixation in plant tissues and soil (Farhharn et al., 1985). Also Mulchi et al. (1992) found leaf N concentration of soybean was significantly reduced by the NF+O_3 treatment. Indeed, Pausch et al. (1996) used ^{13}C and ^{15}N to investigate ozone effects on C and N metabolism in soybeans, the results showed ozone exposure plants reduced the amount of N derived from N-fixation, but did not significantly affect total N or % N for organ and whole plants.

Nitrogenase activity

Additional ozone concentration at 40 and 70 ppb showed significant differences of nitrogenease activity when compared with the control group. Indeed, the ozone up taken through stomata affected root and nodules development (Blum & David, 1977) which caused decreasing the number of node and root nitrogenase activity and total nitrogen in plant tissues (Wathanaluck, 2008; Agrawal & Agrawal, 1990; Mulchi et al., 1992). This could explain by exposure of foliage to air pollution causing the effect to the nodule activities. Similarly, Jones et al. (1985) found the nodule activity of soybean reduced by 16.4 and 27.4% from the greenhouse and field house due to plant exposure to the air pollution. Also high concentration of ozone could suppress the nitrogenase activity which was similarly found in Faba Bean (Nasser, 2002).

CONCLUSIONS

Ozone had direct effects on plant physiology which was determined by decreasing of plant dry weight particularly root dry weight. Decreasing the nodule size over 2 mm in ozone treated plants at rhizosphere could reduce the capability of nitrogen fixation of legume. Consequently, reduction in total nitrogen in plant tissues and soil were found. The concentration of additional ozone at 70 ppb level was severely affected to the development of rhizosphere which reduced the number of nodule size over 2 mm rather than 40 ppb. The slow growing rhizobium group well established in the root system of the control group plant but not found in ozone fumigated plants.

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