

2 **Instrumental Approach to the Determination of Ozone**
3 **Phytotoxicity**

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7 **Abstract**

8 Ozone (O₃) phytotoxicity is a serious concern all over the globe. O₃ can cause both biochemical and
9 physiological changes to plants that could lead to reduction in growth and yield. Its effect on
10 agricultural crops is usually detected as foliar injury. Although foliar injury has frequently served as
11 physiological basis for monitoring performance of agricultural crops and assessing air quality, its
12 visibility depends on period of exposure, concentration of ozone, and environmental conditions. The
13 present study focused on the determination of biochemical indicators of ozone phytotoxicity including
14 Rubisco (biochemical indicator for carbon assimilation), photo-pigments [carotenoids and chlorophyll
15 (a & b)], and chlorophyll photochemical efficiency on a judiciously selected plant-*Nicotiana tabacum*
16 (Tobacco), variety: Little Havanna. An enclosed computer controlled growth chamber was used for
17 cultivation of the experimental plant groups (ozone exposed and control). Spectrophotometric
18 measurements of the biochemical indicators were carried out to evaluate the exposure-response
19 behaviour of the plant. Our results showed a significant (P< 0.05) decrease of the biochemical
20 indicators that is reproducible even when foliar injury is not detected. Besides, experiments also
21 showed that chlorophyll a is more sensitive to ozone than chlorophyll b. The instrumental readings
22 were supported by a reduction in total organic nitrogen and starch content and ultimate decrease in
23 growth rate and root: shoot ratio. While significant variations in the intensities of foliar injury can be
24 detected among plants within a group, a reproducible exposure-response data were obtained from
25 instrumental measurements. Such measurements can also serve as basis for the development of
26 implantable sensors for in-situ monitoring of ozone pollution in sensitive crops.

27
28 **Key words:** *Nicotiana tabacum*, ozone phytotoxicity, biochemical indicators, growth chambers

Introduction

Tropospheric ozone, a photo-oxidant produced from nitrogen oxides and volatile organic compounds in the presence of sunlight, is toxic to sensitive plant species at relatively low concentrations. It is one of the most phytotoxic air pollutants that cause considerable damage to vegetation throughout the world. The effects of O₃ on plants are numerous and vary with the intensity and duration of exposure, concentration, and presence of compounding stresses, such as drought, competition, nutrient deficiency, and pathogens. O₃ enters plants through stomatal pores and reacts with plant cells, generates other reactive oxygen species and causes oxidative stress [1]. It causes biochemical and physiological changes which lead to decrease in photosynthetic rate and crop yield [1-5]. There can be visible foliage symptoms associated with ozone injury, including chlorotic mottle, tip or margin burn, stipple, and necrosis [6-7]. Such symptoms have served as a basis for monitoring performance of agricultural crops and assessing air quality. However, foliar ozone injury symptoms are only an indication of previous exposure to elevated ozone concentrations for a certain time period, and when environmental conditions were conducive to ozone uptake and cellular injury. There is, therefore, continuing interest to establish more reliable methods for monitoring performance of agricultural crops and assessing air quality related to O₃. Studies have shown that exposure to ozone often leads to reduction in growth and yield of a plant even if signs of any visual injury are not observed [8]. Both growth and yield results from optimum photosynthetic rate and chlorophyll photochemical efficiency. Photosynthetic rate is linked to carbon dioxide assimilation. Exposure to O₃ causes reduction in assimilation and increased respiratory loss of carbon dioxide. The diminished assimilation of carbon dioxide is induced by a decline in Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and causes impairment of the processes of photosynthesis and alter allocation of carbon to different organs and leads to altered growth responses of these organs [9-11]. In agricultural crops, this results in reduced grain or seed yield, and restricts the plant's ability to tolerate additional stresses, such as stress due to drought or low temperatures. Measurements of CO₂ gas exchange, carboxylation rate of ribulose bisphosphate by Rubisco and chlorophyll photochemical efficiency could thus serve as a basis to monitor performance of agricultural crops.

The objective of this work was to determine the impact of ozone exposures on selected plant cultivars through measurement of CO₂ gas exchange, Rubisco, chlorophyll photochemical efficiency and related parameters such as biomass accumulation, stomatal conductance, carotenoids, total organic nitrogen and starch contents. Tobacco variety, Little Havanna, SR1 (*Nicotiana tabacum*) was selected because of its sensitivity to ozone. Experiments were carried out in enclosed computer controlled growth chambers. Such chambers provide climatic conditions that are similar to those outside and provide the

68 best available source of data for plant responses to O₃ exposure owing to rapid air exchange. In addition
69 to a reliable exposure-response data, instrumental measurements reported herein can also serve as a
70 basis for the development of implantable sensors for in-situ monitoring of ozone pollution in sensitive
71 crops.

72 2. Materials and methods

73 2.1 Plant material and environmental conditions

74 *Nicotiana tabacum* plants (Tobacco), variety Little Havanna, SR1 were subjected to O₃ exposure.
75 Tobacco plants grown from seed were germinated in the research garden. Eight tobacco plants (four
76 plants for each group) were later transplanted into pots in standard soil medium (peat: soil: sand =
77 2:1:1). Plants were watered daily with tap water to provide adequate soil moisture and fertilization was
78 done three times before the plants were transferred to the chamber. The required amount of nutrient per
79 sq. ft was calculated by dividing the recommended amount of the nutrient (lbs per sq. ft) from a prior
80 soil test by the percentage of the nutrient in the fertilizer.

81 There was no requirement for minimum plant size, although all plants were required to have healthy
82 appearance and at least five leaves in terms of leaf coloration. The eight tobacco plants were divided
83 into two groups (control and exposed to ozone) and from each group, two plants were used for
84 photosynthesis and biomass determination and other two plants were analysed for biochemical analysis.
85 Plants for each treatment were divided into two replicate chambers so that each chamber contained one
86 'biochemistry plant' and one 'photosynthesis and biomass' plant (i.e. two plants per group). O₃
87 exposures were conducted in enclosed computer controlled growth chambers for 9 days and 8 hours per
88 day under the following environmental conditions: 22⁰ C / 18⁰ C (day/ night), 18 h: 6 h (light: dark, 6
89 am to 12 midnight) photoperiod, light intensity about 250 μmol photons/m²/sec. O₃ concentration was 0
90 ppb in control chamber and 120 ppb in ozone chamber.

91 2.2 Qualitative (Visible) observations

92 O₃ exposed tobacco plants exhibited foliar injury during the 8 days exposure. Specific characteristics
93 recorded for leaves included abnormal coloration (especially chlorosis), mottling, necrosis, pigmented
94 stipple, curling, and senescence. The appearance of visible injury was evaluated at the end of ozone
95 exposure from the onset of lesion development and served as basis for subsequent experiments.

96 **2.3. Instrumental Measurements**

97 **2.3.1 Gas exchange measurement**

98 Net photosynthetic rate (P_n) and stomatal conductance (g_s) of ozone exposed and of control tobacco
99 plants were assessed using an open gas exchange system (LICOR -6400). Three fully developed leaves
100 from each tobacco plant were selected and measured under saturating irradiance. Photosynthetic photon
101 flux density (PPFD) was fixed at 1200 $\mu\text{mol photons/m}^2/\text{sec}$, using a red-blue LED light source built
102 into the leaf cuvette, though other environmental factors, such as air humidity was not controlled, i.e.
103 natural variation was permitted to replicate the conditions that occur in the real environment.

104 **2.3.2 Chlorophyll fluorescence measurement**

105 The chlorophyll fluorometer, a multi-purpose tool which provides dark adaptation for the sample
106 (required for the measurement of maximum photochemical efficiency) was used to measure the
107 maximal apparent efficiency of PSII i.e. $F_v/F_m = (F_m - F_0) / F_m$, where F_0 is minimal fluorescence yield
108 and F_m is maximal fluorescence yield of dark adapted leaves. Four fully expanded leaves were selected
109 from each plant (four plants, two from each treatment) and a dark leaf clip was placed on the leaf for 20
110 minutes prior to the measurements. The instrument utilized a time resolved method to determine F_0
111 with light-emitting diodes that also delivered a saturating pulse of 6000 $\mu\text{mol photons/m}^2/\text{sec}$ to obtain
112 F_m . It is vitally important that the illumination should not vary in intensity during measurement
113 otherwise the F_0 and peak fluorescence will have been determined using different light levels and
114 therefore cannot be compared. By measuring the yield of chlorophyll fluorescence, information about
115 changes in the efficiency of photochemistry and heat dissipation can be gained. Dark-adapted values of
116 F_0 , F_m and F_v/F_m were obtained prior to the first light period.

117 **2.3.3 Determination of chlorophyll concentration, total carotenoids and Rubisco**

118 Chlorophyll extraction and total carotenoids were determined in 80% acetone from three leaf discs of 2
119 cm^2 diameter (Ca. 70 mg altogether) according to the method described by [12], with modifications by
120 [13]. Leaf discs were carefully removed from non-necrotic areas of leaves, weighed and immediately
121 frozen in liquid N_2 and then stored at -80°C . The leaf discs were ground and the carotenoids and
122 chlorophyll pigments were extracted by immersion of the crude extract in 80% acetone (1600 μl of 100
123 % acetone and 400 μl crude extract makes 80 % acetone). Absorbance of extracts was read by
124 spectrophotometer (Shimadzu UV-2401PC, UV-VIS recording spectrophotometer) at 646 nm, 663nm

and 470nm. Chlorophyll a (mg/cm^2), chlorophyll b (mg/cm^2) and total chlorophyll content (mg/cm^2) were calculated according to Lichtenthaler, and Welburn equations [13].

The concentration of Rubisco was determined by native polyacrylamide gel electrophoresis using Thermo EC120 Mini Vertical Gel System [14], using purified spinach Rubisco with a known concentration as a standard. With this procedure, the concentrations of leaf Rubisco can be determined from a fresh leaf sample in a single crude extract. Fresh tobacco leaf samples were used, with three leaf discs of 2 cm^2 (ca. 70 mg altogether) per 2 ml of extraction buffer.

2.3.4 Determination of starch

Determination of starch concentration is based on enzyme reactions in which starch is first broken down to glucose, glucose then converted to gluconate-6-phosphate, and on light absorption of NADPH (at 340 nm) formed in the reactions. Boehringer Mannheim Kit describes how to analyse starch (common polysaccharides in plants) concentration with enzymatic methods from freeze-dried plant material. The leaf discs were ground in liquid nitrogen with a mortar and pestle. Ca. 300 mg powder, 20 ml DMSO and 5 ml 8 M HCl were taken respectively to a 100 ml Erlenmeyer flask with wide opening. Flasks were capped with a piece of Para film and, placed into a water bath (60°C) for 30 min. The flasks were allowed to cool down to room temperature and ca. 50ml ultra purified water was added. pH was adjusted to 4-5, and the sample was moved to 100 ml volumetric flask. Water was added to the volume mark and well mixed.

The sample was filtered with suction bottle and Büchner funnel. Sample solution and the solutions from the enzyme kit (bottles 1, 2 and 3) were pipetted to the cuvettes as follows, 0.10 ml of sample solution into sample and sample blank and 0.2ml of solution 1 into sample. The cuvettes were capped with Para film, well mixed and kept in the water bath at 60°C for 15 min. 1.00 ml and 1.20 ml of water was added to sample and sample blank respectively. Then 1.00 ml of solution 2 was added to both sample and sample blank. Absorbance was measured with the Shimadzu UV-2401PC, UV-VIS recording spectrophotometer.

2.3.5 Determination of total organic nitrogen

Total organic nitrogen is often analysed from leaf samples to assess the general nitrogen (and nutrient) status of the plants. Total organic nitrogen was determined by the Kjeldahl method followed by digestion, steam distillation and titration.

3. Results and discussion

3.1. Qualitative Results

A preliminary examination of ozone effect on the *Nicotiana tabacum* (Tobacco), variety Little Havana, plant was investigated through qualitative experiments involving observation of foliar symptoms of ozone exposure vis-à-vis control. Our results showed significant physiological changes including necrotic and chlorotic spots, and acceleration of leaf senescence with 85 % of the ozone fumigated plant groups and the intensities of such effects varied among plants within the group. However, the intensity of the observed symptoms depended on the period of exposure. No visible deleterious effects of ozone were observed with the remaining 15 % of the plants. The plants remained in similar vigor and growth rate as those in the control group.

3.2. Quantitative (Instrumental) results

3.2.1. Photosynthetic rate, stomatal conductance, and chlorophyll photochemical efficiency

Quantitative determination of the biochemical indicators were carried with both groups (ozone fumigated and control) as well as those with which physiological changes were not observed after ozone exposure. Photosynthetic rate, stomatal conductance, and chlorophyll photochemical efficiency are interdependent and are determined by health state of the plant. In this study, the above parameters were measured to determine the phytotoxic effect of ozone and results are summarized in Table 1.

Table 1. Mean values with SD for net photosynthesis, stomatal conductance, and chlorophyll photochemical efficiency (F_v/F_m) of control and ozone fumigated groups of *Nicotiana tabacum* plants.

Experiment	Net Photosynthesis ($\mu\text{mol}/\text{m}^2/\text{sec}$)		Stomatal Conductance $\text{mol H}_2\text{O}/\text{m}^2/\text{sec}$		F_v/F_m	
Type	control	ozone	Control	Ozone	control	ozone
Instrumental	12.08 ±	10.15 ±	0.175	± 0.125	± 0.85 ± 0.01	0.65 ± 0.01
readings (mean, sd)	2.95	1.50	0.025	0.011		

As indicated in the Table above, reduction in all of the three parameters was recorded with ozone treated plant groups as compared to that of control group. The results can be explained based on interaction of ozone with constitutive plant cells and consequent effect on biochemical processes. Photosynthetic rate is usually measured by CO_2 gas exchange rate [15]. In this study, a decrease in gas exchange was observed (Table 1) and suggested a decrease in photosynthetic rate. The other secondary

response to ozone is a reduction in stomatal conductance, as the stomata close in response to increased internal CO₂ that occurs because of the reduced photosynthetic activity caused by the ozone [16-18]. It has been suggested that the decrease in stomatal conductance caused by O₃ is similar in magnitude to the 10 % decrease caused by CO₂ increases since preindustrial conditions [19]. A reduction of net photosynthesis and a reduction in stomatal conductance observed in this study, agrees with the results of other researchers, who also found reduced photosynthesis and reduced stomatal conductance in O₃ exposed plants [20]. Chlorophyll photochemical efficiency is measured by chlorophyll fluorescence. Chlorophyll fluorescence is a red and far-red light emitted by photosynthetic tissue when it is excited by a light. O₃-induced reduction in photosynthetic rate can thus indicate reduction in chlorophyll activity and/or quantity. A decrease in chlorophyll fluorescence in turn indicates decreased concentration of chlorophyll (Beer's law). Therefore photosynthetic rate and chlorophyll fluorescence are interrelated [21, 22] and their measurement can serve as a basis to monitoring O₃ pollution. Interestingly, all the O₃ treated plants including those which **did not** show foliar injury responded to the exposure.

3.2.2. Rubisco, Chl a, Chl b, and **carotenoid concentrations**

Results from instrumental measurements of the carbon assimilation indicator (Rubisco), and photosynthetic pigments (chl a, chl b, and carotenoids) for O₃ pollution are summarized in Table 2. A general decrease in concentration of Rubisco and the pigments can be observed with all of the plants previously exposed to ozone as compared to that of the control. While the decrease in Rubisco concentration in O₃ polluted plants could be due to O₃-induced oxidation which enhances its susceptibility to proteolytic degradation [23] that of chlorophyll and carotenoid is explained by their redox reaction with ozone. Reductions in stomatal conductance (g_s), net photosynthetic CO₂ assimilation, and carboxylation efficiency have all been associated with O₃ exposure [24-27]. These reductions reveal a loss of ribulose-1, 5-bisphosphate carboxylase/ oxygenase (Rubisco), and decreased Rubisco activity [28, 29]. **C**onditions where additional synthesis should be minimal showed a substantial decline in Rubisco content suggesting that the primary cause of the decline in Rubisco due to O₃ exposure was enhanced degradation rather than reduced production. Rubisco activity and/or quantity is thus a good bioindicator for oxidative stresses in plants. Accelerated loss of Rubisco in mature leaves reflect O₃-stimulated proteolytic degradation.

Chlorophyll a (Chl a) is universally found in all photosynthesizing cells and plays **an** active role in photosynthesis functioning as **a photoenzyme that converts and stores** incoming excitation energy in the form of an oxido-reduction potential. Chl b is the second common but not universal kind of

chlorophyll, in addition to chl a, and represents a locally oxidized derivative of chl a. The observed decrease in chl a concentrations is explained by the oxidative damage to the chlorophyll by reaction with O₃. Experiments also showed that chlorophyll a is more sensitive to ozone than chlorophyll b (Table 2).

Carotenoids [carotenes (non-oxygenated) and carotenoids referred to as xanthophylls (oxygenated)], on the other hand, are long chain conjugated olefinic molecules synthesized by a wide variety of photosynthetic (plants, algae) and non-photosynthetic (some fungi and bacteria) organisms. Carotenoids are involved in several aspects of photosynthesis, notably light absorption, energy transfer to photosynthesis system, and protection of the photosynthetic apparatus from damage by strong illumination. Previous studies have shown that carotenoids are also susceptible to oxidative damage by O₃ [30]. Thus the observed decrease in the pigments in the ozone treated plants as compared to the control is consistent with previous reports.

Table 2. Mean values with SD for Rubisco concentration (µg/mg f.wt), concentrations of chlorophyll a, b and carotenoids (µg/mg f.wt) of control and ozone fumigated groups of *Nicotiana tabacum* plants

Experiment	Rubisco, µg/mg f.wt		Chl a, µg/mg f.wt		Chl b, µg/mg f.wt		Carotenoids	
Type	control	ozone	control	Ozone	control	ozone	control	ozone
Instrumental readings (mean, sd)	8.85± 2.05	6.25 ± 2.01	1.75 ± 0.25	1.35 ± 0.05	0.42± 0.025	0.40± 0.01	0.43 ± 0.05	0.35 ± .01

3.2.3. Total nitrogen and starch concentrations

Generally, reduction in photosynthesis would lead to a reduction in starch and total organic nitrogen production. This is verified in our experiment and result is indicated in Figure 1.

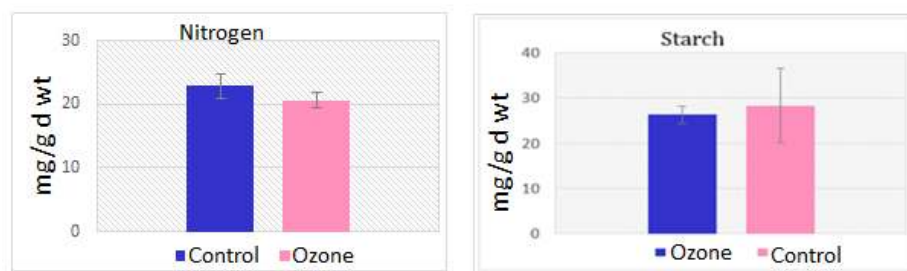


Figure 1. Total organic nitrogen and starch content in milligram per gram of dry weight (mg/g d wt) of ozone fumigated vs control of *Nicotiana tabacum* plants.

As can be seen in the Figure above, a decrease in total nitrogen and starch content was observed with O_3 fumigated plant groups. The decrease in starch content expected from a decrease in photosynthetic rate due to O_3 . The degree of O_3 induced accelerated senescence could be responsible for decline in N availability. Previous studies [31, 32] suggested that when available organic N in the plant is declines, the intensity of O_3 induced accelerated senescence may be increased and vice versa. Data reported herein support these observations.

3.2.4. Biomass accumulation

Allocation of carbon to different organs may be altered under conditions of reduced assimilate supply through photosynthesis leading to altered growth responses of these organs. This results in reduced root:shoot weight ratios or in a reduction of the ratio between seed yield and total biomass production. In order to verify this experimentally, measurements were conducted and results are summarized in Figure 2.

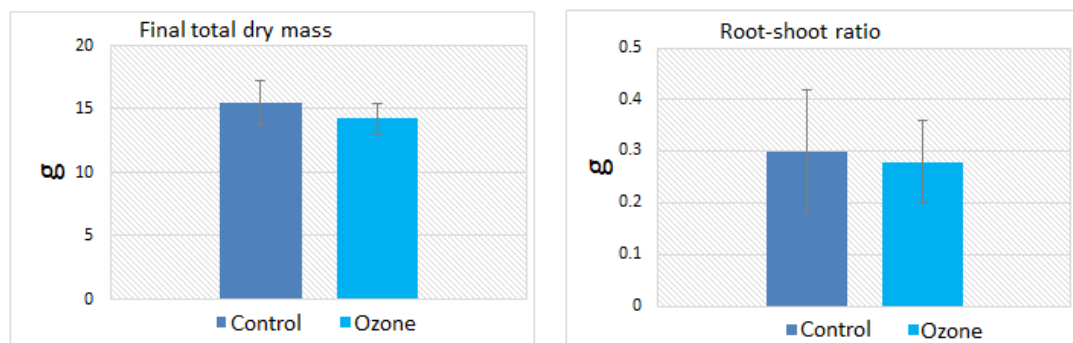


Figure 2. Total dry mass and root:shoot ratio of ozone fumigated vs control groups of the *Nicotiana tabacum* plants.

As can be seen from the Figure a decrease in both total dry mass and root:shoot ratio was recorded. The observed reduction in the dry weight was probably the consequence of reduced P_n . The greater reduction of P_n in the lower leaves is much more important for plant growth than an equal percentage reduction in the upper leaves, since, due to their greater size, their contribution to plant assimilation is greater.

The reduction of the root to shoot ratio suggests that, although the root was not directly exposed to ozone, it was the most severely affected part of the exposed plant. This observation is in agreement with those of [33] who found that ‘the respiratory activity of roots began to decrease well before visible injury appeared on the leaves, and the percent of reduction of respiration was much greater than the percent of leaf injury. The number of healthy looking leaves per plant was significantly decreased in

ozone treated plants. In contrast, old, yellow leaves (%), leaves with brown, grey, yellow and white stippling (%) and leaves with necrosis (%) were increased significantly in O₃ exposed plants.

4. Conclusions

In conclusion, a reliable exposure-response data were obtained by instrumental measurement of biochemical indicators [Rubisco, carotenoids and chlorophyll (a & b), and chlorophyll photochemical efficiency] of ozone pollution on a judiciously selected plant-*Nicotiana tabacum* (Tobacco), variety: Little Havanna. While significant variations in the intensities of foliar injury can be observed among plants within a group, a reproducible exposure-response data were obtained from instrumental measurements of the biochemical indicators. Exposure to ozone caused a decrease in photosynthetic rate and concentrations of biochemical indicators (Rubisco, Chl a, Chl b, and carotenoid). The observed decrease in bioaccumulation, root: shoot ratio, total nitrogen, and starch were also consistent with the above findings. Experiments also showed that chlorophyll a is more sensitive to ozone than chlorophyll b. Further research at the biochemical level using sensor technologies is needed to decipher the mechanism and improve sensitivities of the measurement for field applications and in situ monitoring.

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Author Contributions

We, the two authors, of this paper have made equal contribution to the work. We have read and approved the revised manuscript submitted.

Conflict of Interest

We declare that we don't have any competitive conflict of interest with anybody.

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