

Instrumental Approach to the Determination of Ozone Phytotoxicity

Abstract

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

Key words: *Nicotianatabacum*, Ozone phytotoxicity, biochemical indicators, growth chambers

32 Introduction

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

60
 61 The objective of this work was to determine the impact of ozone exposures on selected plant cultivars
 62 through measurement of CO₂ gas exchange, Rubisco, chlorophyll photochemical efficiency and related
 63 parameters such as biomass accumulation, stomatal conductance, carotenoids, total organic nitrogen
 64 and starch contents. Tobacco variety, Little Havanna, SR1 (*Nicotiana tabacum*) was selected because of
 65 its sensitivity to ozone. Experiments were carried out in an enclosed computer controlled growth

66 chambers. Such chambers provide climatic conditions that are similar to those outside and provide the
67 best available source of data for plant responses to O₃ exposure owing to rapid air exchange. In addition
68 to a reliable exposure-response data, instrumental measurements reported herein can also serve as basis
69 for the development of implantable sensors for in-situ monitoring ozone pollution in sensitive crops.

70 **2. Materials and methods**

71 **2.1 Plant material and environmental conditions**

72 *Nicotianatabacum* (Tobacco), variety Little Havanna, SR1 were subjected to O₃ exposure. Tobacco
73 plants grown from seed were germinated in the research garden. Eight tobacco plants (four plants for
74 each group) were later transplanted into pots in standard soil medium (peat: soil: sand = 2:1:1). Plants
75 were watered daily with tap water to provide adequate soil moisture and fertilization was done three
76 times before the experiment started to add nutrients in the soil. There was no requirement for minimum
77 plant size, although all plants were required to have healthy appearance and at least five leaves in terms
78 of leaf coloration. Selected eight tobacco plants were divided into two groups (control and exposed to
79 ozone) and from each group, two plants were analysed for photosynthesis and biomass determination
80 and other two plants were analysed for biochemistry analysis. Plants for each treatment were divided
81 into two replicate chambers so that each chamber contained one 'biochemistry plant' and one
82 'photosynthesis and biomass' plant (i.e. two plants per group). O₃ exposures were conducted in
83 enclosed computer controlled growth chambers for 9 days and 8 hours per day under the following
84 environmental conditions: 22⁰ C / 18⁰ C (day/ night), 18 h: 6 h (light: dark, 6 am to 24 pm) photoperiod,
85 light intensity about 250 μmol photons/m²/sec. O₃ concentration was 0 ppb in control chamber and 120
86 ppb in ozone chamber.

87 **2.2 Qualitative (Visible) observations**

88 O₃exposed tobacco plants exhibited foliar injury during the 8 days exposures. Specific characteristics
89 recorded for leaves included abnormal coloration (especially chlorosis), mottling, necrosis, pigmented
90 stipple, curling, and senescence. The appearance of visible injury wasevaluated at the end of ozone
91 exposure from the onset of lesion development and served as basis for subsequent experiments.

92 **2.2. Instrumental Measurements**

93 **2.1 Gas exchange measurement**

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

100 **2.2 Chlorophyll fluorescence measurement**

101 The chlorophyll fluorometer, a multi-purpose tool which provides dark adaptation for the sample
 102 (required for the measurement of maximum photochemical efficiency) was used to measure the
 103 maximal apparent efficiency of PSII i.e. $F_v/F_m = (F_m - F_0) / F_m$, where F_0 is minimal fluorescence yield
 104 and F_m is maximal fluorescence yield of dark adapted leaves. Four fully expanded leaves were selected
 105 from each plant (four plants, two from each treatment) and a dark leaf clip was placed on the leaf for 20
 106 minutes prior to the measurements. The instrument utilized a time resolved method to determine F_0
 107 with light-emitting diodes that also delivered a saturating pulse of 6000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ to obtain
 108 F_m . It is vitally important that the illumination should not vary in intensity during measurement
 109 otherwise the F_0 and peak fluorescence will have been determined using different light levels and
 110 therefore cannot be compared. By measuring the yield of chlorophyll fluorescence, information about
 111 changes in the efficiency of photochemistry and heat dissipation can be gained. Dark-adapted values of
 112 F_0 , F_m and F_v/F_m were obtained prior to the first light period.

113 **2.5 Determination of chlorophyll concentration, total carotenoids and Rubisco**

114 Chlorophyll extraction and total carotenoids were determined in 80% acetone from three leaf discs of 2
 115 cm^2 diameter (Ca. 70 mg altogether) according to the method described by [12], with modifications by
 116 [13]. Leaf discs were carefully removed from non-necrotic areas of leaves, weighed and immediately
 117 frozen in liquid N_2 and then stored at -80°C . The leaf discs were ground and the carotenoids and
 118 chlorophyll pigments were extracted by immersion of crude extract in 80% acetone (1600 μl of 100 %
 119 acetone and 400 μl crude extract makes 80 % acetone). Absorbance of extracts was read by
 120 spectrophotometer (Shimadzu UV-2401PC, UV-VIS recording spectrophotometer) at 646 nm, 663 nm

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

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.

2.6 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. After capping flasks with piece of Para film, were placed into water bath (60°C) for 30 min. Then 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 then 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.

2.7 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 *Nicotianatabacum* (Tobacco), variety Little Havanna, 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 remaining 15 %. 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 determined by health state of the plant. In this study, the above parameters are 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 *Nicotianatabacum* plants.

Experiment	Net Photosynthesis		Stomatal Conductance		F_v/F_m	
	$(\mu\text{mol}/\text{m}^2/\text{sec})$		$\text{mol H}_2\text{O}/\text{m}^2/\text{sec}$			
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, 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 have 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 didn't 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], under conditions 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 play active role in photosynthesis functioning as photoenzyme-convert and store 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 represent a locally oxidized derivative of chl a. The observed decrease in chl a concentrations is explained by the oxidative damage to the chl by reaction with O₃. Besides, experiments also showed that chlorophyll a is more sensitive to ozone than chlorophyll b.

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 involve in several aspects of photosynthesis, notably light absorption, energy transfer to photosynthesis system, and protection of the photosynthetic apparatus from damage by strong illumination. Recent 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 *Nicotianatabacum* 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.

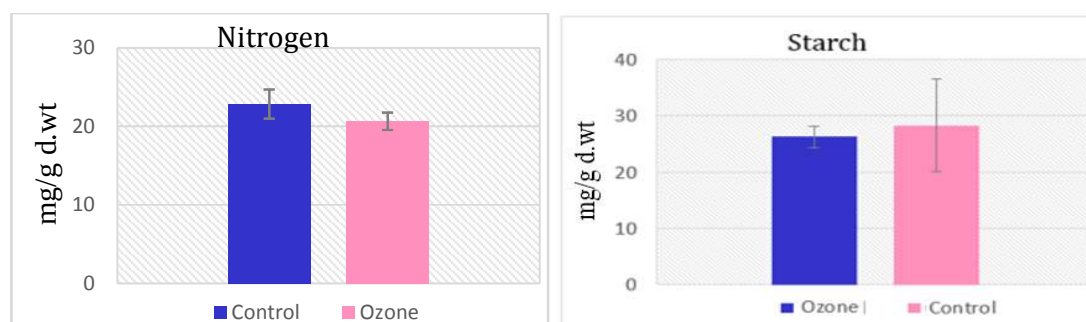


Figure 1. Total organic nitrogen and starch content of ozone fumigated vs control of *Nicotianatabacum* plants.

As can be seen in the **Figure** below, a strong decrease in total nitrogen and starch content was observed with O₃ fumigated plant groups. The degree of O₃ 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 O₃ 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.

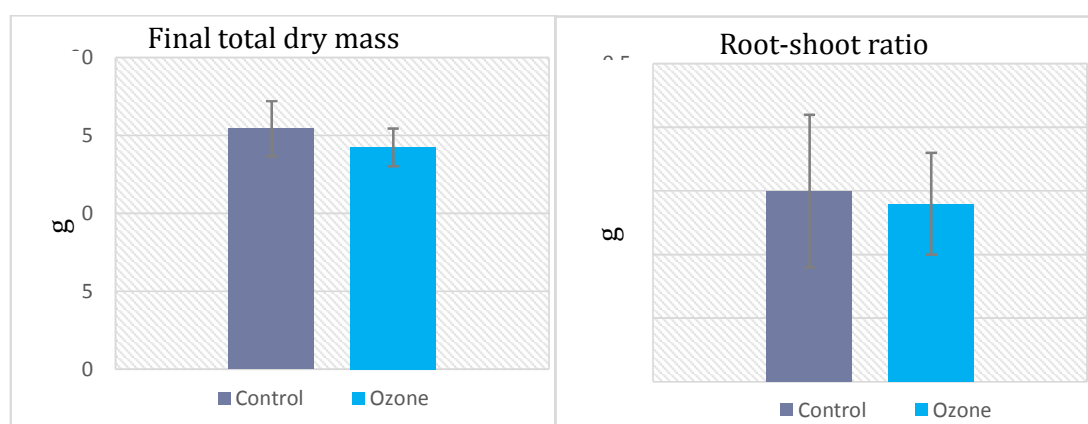


Figure 2. Total dry mass and root:shoot ratio of ozone fumigated vs control groups of the *Nicotianatabacum* plants.

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-*Nicotianatabacum* (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. Besides, 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.

5. References

1. Fiscus EL, Fitzgarrald LB, Burkey KO. Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning, *Plant Cell Environ.* 2005; 28: 997–1011.
2. Guderian R, Tingey DT, Rabe R. Effects of photochemical oxidants on plants. In: Guderian R, ed. *Air pollution by photochemical oxidants. Formation, transport, control and effects on plants.* Berlin: Springer-Verlag. 1985; 129–334.
3. Fumigalli I, Gimeno BS, Velissariou D, De Temmerman L, Mills G. Evidence of ozone-induced adverse effects on crops in the Mediterranean region. *Atmos Environ* 2001; 35:2583–2587.
4. Ashmore MR. Assessing the future global impacts of ozone on vegetation. *Plant Cell Environ.* 2005; 28:949–964.
5. Ainsworth EA, Yendrek CR, Sitch S, Collins WJ, Emberson LD. The effects of tropospheric ozone on net primary productivity and implications for climate change. *Annu Rev Plant Biol.* 2012; 63:637–661.
6. Temple PJ, Reichers GH, Miller PR. Foliar injury responses of ponderosa pine seedlings to ozone, wet and dry acidic deposition, and drought. *Environmental and Experimental Botany.* 1992; 32: 101-113.
7. Treshow M, Anderson FK. *Plant stress from air pollution.* New York: John Wiley and Sons. 1991.

- 283 8. Steinberger EH, and Naveh Z. Effects of recurring exposures to small ozone concentrations on
284 bel w3 tobacco plants. *Agriculture and environment*. 1982; 7: 255—263.
- 285 9. Doughty C, Asner G, Martin R. Predicting tropical plant physiology from leaf and canopy
286 spectroscopy. *Oecologia*. 2011; 165:289–299.
- 287 10. Serbin SP, Dillaway DN, Kruger EL, Townsend PA. Leaf optical properties reflect variation in
288 photosynthetic metabolism and its sensitivity to temperature. *J Exp Bot*. 2012; 63:489–502.
- 289 11. Mudd JB. USDA Forest Service Gen.Tech.Rep. PSW-GTR. 1998;166: 1998
- 290 12. Arnon DI. Copper enzymes in isolated chloroplasts: Polyphenol oxidase in *Beta vulgaris*. *Plant*
291 *Physiol*. 1949; 24: 1-15.
- 292 13. LichtenthalerHK, and Welburn AK. Determinations of total carotenoids and chlorophylls a and
293 b of leaf extracts in different solvents. *Photosynthesis Research*. 1983; 73: 149-156.
- 294 14. Rintamäki E, Keys AJ, Parry MAJ. Comparison of the specific activity of ribulose 1, 5-
295 bisphosphate carboxylase-oxygenase from some C3 and C4 plants. *PhysiologiaPlantarum*.
296 1988;74: 326-331.
- 297 15. Elizabeth AA, Shawn PS, Jeffrey AS, Philip AT. Using leaf optical properties to detect ozone
298 effects on foliar biochemistry *Photosynth Res*. 2014; 119:65–76.
- 299 16. Reich PB, Amundson RG. Ambient levels of ozone reduce net photosynthesis in tree and crop
300 species, *Science*. 1985; 230: 566–570.
- 301 17. Reich PB. Quantifying plant response to ozone: a unifying theory, *Tree Physiol*. 1987;3: 63–91.
- 302 18. Runeckles VC, Chevone BI. Tree responses to ozone, in: A. S. Lefohn (Ed.), *Surface level*
303 *ozone exposures and their effects on vegetation*, Lewis Publishers Inc, Chelsea, MI, USA, 1992;
304 pp. 189–270.
- 305 19. Taylor GE, Johnson DW, Andersen CP. Air pollution and forest ecosystems: a regional to
306 global perspective, *Ecol. Appl*. 1994; 4: 662–689.
- 307 20. Pasqualini S, Della TG, Ferranti F, Ederli L, Piccioni C, Reale L, Antonielli M. Salicylic acid
308 modulates ozone-induced hyper sensitive cell death in tobacco plants. *Physiol. Plant*. 2002; 115:
309 204–212.
- 310 21. Espinosa-Calderon A, Torres-Pacheco I, Padilla-Medina JA, Osornio-Rios RA, Romero-
311 Troncoso RJ, Villaseñor-Mora V, Rico-Garcia E, Guevara-Gonzalez RG. Description of
312 photosynthesis measurement methods in green plants involving optical techniques, advantages
313 and limitations. *Afr. J. Agric. Res*. 2011; 12: 2638–2647.
- 314 22. Fedack V, Kytaev O, Klochan P, Romanov V, Voytovych I. Portable Chronofluorometer for
315 Express-Diagnostics of Photosynthesis. In *Proceedings of Intelligent Data Acquisition and*

- 316 Advanced Computing Systems: Technology and Applications, Sofia, Bulgaria, 5–7 September
317 2005.
- 318 23. Laurie C, Landry' and Eva J. Pell. Modification of Rubisco and Altered Proteolytic Activity in
319 O₃-Stressed Hybrid Poplar (*Populusmaximowizii* x *trichocarpa* Plant Physiol. 1993; 101: 1355-
320 1362.
- 321 24. Pell EJ, Eckardt N, Enyedi AJ. Timing of ozone stress and resulting status of
322 ribulosebiphosphate carboxylase/ oxygenase and associated net photosynthesis. New Phytol.
323 1992; 120: 397–405.
- 324 25. Fiscus EL, Reid CD, Miller JE, Heagle AS. Elevated CO₂ reduced O₃ flux and O₃-induced yield
325 losses in soybeans: possible implications for elevated CO₂ studies. J. Exp. Bot. 1997; 48: 307–
326 313.
- 327 26. Guidi L, Degl'Innocenti E, Soldatini GF. Assimilation of CO₂, enzyme activation and
328 photosynthetic electron transport in bean leaves as affected by high light and ozone. New
329 Phytol. 2002; 156: 377–388.
- 330 27. Morgan PB, Ainsworth EA, Long SP. How does elevated ozone impact soybean? A meta-
331 analysis of photosynthesis, growth, and yield. Plant Cell Environ. 2003; 26: 1317–1328.
- 332 28. Long SP, and Naidu SL. Effects of oxidants at the biochemical, cell and physiological levels,
333 with particular reference to ozone. In Air Pollution and Plant Life (eds J. N. B. Bell & M.
334 Treshow). 2002; pp. 69–88.
- 335 29. EckardtNA, and Pell E. O₃-induced degradation of Rubisco protein and loss of Rubisco mRNA
336 in relation to leaf age in *Solanumtuberosum* L. New Phytologist. 1994; 127:741–748.
- 337 30. de Jesus Benevides CM, da Cunha Veloso MC, de Paula Pereira PA, de Andrade JB. A
338 chemical study of β -carotene oxidation by ozone in an organic model system and the
339 identification of the resulting products. Food Chemistry. 2011; 126: 927–934.
- 340 31. Pell EJ, Sinn JP, Vinten-Johansen C. Nitrogen supply as a limiting factor determining the
341 sensitivity of *Populustremuloides*Michx. to ozone stress. New Phytologist. 1995; 130: 437–446.
- 342 32. Brendley BW, Pell EJ. Ozone-induced changes in biosynthesis of Rubisco and associated
343 compensation to stress in foliage of hybrid poplar. Tree Physiology. 1998; 18: 81–90.
- 344 33. Hofstra G, Ali A, Wukasch RT, Fletcher RA. The rapid inhibition of root respiration after
345 exposure of bean (*Phaseolus vulgaris* L.) plants to ozone, Atmos. Pollut. 1981; 15: 483-487.