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Effects of ambient O_3 on wheat during reproductive development: Gas exchange, photosynthetic pigments, chlorophyll fluorescence, and carbohydrates

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Abstract

The current concentrations of O_3 have been shown to cause significant negative effects on crop yield. The present levels of ozone may not induce visible symptoms in most of plants, but can result in substantial losses in reproductive output. This paper considers the impact of ambient O_3 on gas exchange, photosynthetic pigments, chlorophyll (Chl) fluorescence and carbohydrate levels in the flag leaf of wheat plants during various stages of reproductive development using open-top chambers. Mean O_3 concentration was 45.7 ppb during wheat growth and 50.2 ppb after flag leaf development. Reproductive stage showed higher exceedence of O_3 above 40 ppb compared to the vegetative stage. Diurnal variations in net photosynthetic rate (P_N) and stomatal conductance (g_s), intercellular CO_2 concentration (C_i), F_v/F_m ratio, photosynthetic pigments, soluble sugars, and starch were measured at 10, 30, and 50 days after flag leaf expansion (DAFE). The results showed reductions in P_N, g_s, F_v/F_m ratio, photosynthetic pigments and starch, and increases in C_i, F_0, and soluble sugars in nonfiltered chambers (NFCs) compared to filtered chambers (FCs). Maximum changes in measured parameters were observed at 50 DAFE (*i.e.* grain filling and setting phase). Diurnal variation in P_N showed double peaked curve in both FCs and NFCs, but delayed peak and early depression in NFCs. Stomatal conductance was significantly lower in NFCs. The study suggests that higher prevalence of ambient O_3 during reproductive development led to significant alteration in physiological vitality of wheat having potential negative influence on yield.

Additional key words: ambient ozone; carbohydrates; chlorophyll fluorescence kinetics; photosynthesis; stomatal conductance; wheat.

Introduction

Tropospheric O_3 has been identified as a phytotoxic component of photochemical smog during investigations of leaf injury to grape vine (*Vitis vinifera* L.) in Southern California many years ago (Richards *et al*. 1958). O_3 have been rising at an annual rate of 0.5–2% (Vingarzan 2004). Rapidly growing economies of east, southeast and south Asia have experienced continued deterioration of air quality due to increased emissions of nitrogen oxides and hydrocarbons, which are linked with elevated surface O_3 levels. The atmospheric life time of tropospheric O_3 is long enough (1–2 weeks in summer to 1–2 months in winter) to be transported from polluted to other rural areas (Li *et al.* 2002). Numerous meta analytical studies on responses of wheat have reported deleterious effect of O_3 on premature leaf senescence, decrease in leaf interception, Chl content and photosynthesis, reductions in assimilate availability and alterations in assimilate partitioning (Biswas *et al.* 2008, Feng *et al.* 2008, 2009). The detrimental effects of O_3 are shown to depend on the genetic make up, developmental phase of the plants, O_3 doses and climate (Heath 1994).

The adverse effects of O_3 are initially perceived to be mediated indirectly through injury to the vegetative organs and consequent changes in assimilate production

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Abbreviations: C_i – intercellular CO_2 concentration; Chl – chlorophyll; F_m – maximal fluorescence of dark-adapted state; F_v – variable fluorescence; F_0 – initial fluorescence of dark-adapted state; FCs – filtered chambers; g_s – stomatal conductance; NFCs – nonfiltered chambers; OPs – open plots; OTCs – open-top chambers, P_N – net photosynthetic rate; PAR –photosynthetically active radiation; PSII – photosystem II.
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and distribution. Lee *et al.* (1988) suggested that the sensitivity of seed crops to O_3 was greatest during the period between flowering and seed maturity. Plant responses to O_3 are complex and specific and vary between different genera, species and cultivars (Keutgen and Lenz 2001). It has been recognized that peak O_3 concentrations are an important factor for examining plant injury (Heck *et al.* 1998, Sarkar and Agrawal 2010). After entering the stomata, O_3 reacts with the liquid components of the apoplast to create reactive oxygen species (ROS) (Kangasjarvi *et al.* 2005) that may oxidize the cell walls to initiate cascade reactions which lead to cell death. In the chloroplast these could directly or indirectly impair the light- and dark reactions of photosynthesis (Fiscus *et al.* 2005). The O_3 or ROS may alter the properties of thylakoids, thereby affecting the Chl *a* fluorescence leading to an over reduction of photosystem reaction centres (Biswas *et al*. 2008, Singh *et al.* 2009, Feng *et al.* 2011). Measurement of Chl *a* fluorescence provides information on light reaction photosynthesis and serves as a noninvasive indicator of the status of photosynthetic centres in the chloroplasts of green plants.

The central biochemical processes controlling photosynthesis are the maximum carboxylation efficiency (which suggests activity of Rubisco) and the maximum rate of RuBP regeneration. Physiological studies indicate that O_3 damages the photosynthetic machinery leading to reduction in carboxylation efficiency (Fiscus *et al.* 2005, Feng *et al.* 2011), and degradation of large and small subunits of Rubisco (Sarkar and Agrawal 2010). Clark *et al.* (2000) indicated that photosynthetic capacity is an ideal physiological activity to monitor the health and vitality of the plants. Therefore, the gas-exchange measurements provide direct correlation with the plant growth response under O_3 stress. O_3 has a typical diurnal profile with peak concentration during the afternoon. Hence, diurnal changes in O_3 may modify the response process of the plants at ambient O_3. The diurnal trend in gas exchange is often recognized as one of the best indications in reflecting the ability of plants to maintain their photosynthetic apparatus response to environmental stress (Geiger and Servaites 1994).

The grain dry matter of wheat originates mainly from (*1*) reserve photosynthates in the sheath and stem fixed prior to anthesis, and (*2*) current photosynthates after anthesis. Therefore, any negative impact on photosynthetic rate after anthesis usually affects grain yield. There are few studies that have explored impact of ambient O_3 on diurnal response of P_N and impact of perturbations in P_N rate at the reproductive development on contents of starch and soluble sugars of flag leaves during the reproductive development of wheat. The present study was conducted in open-top chambers under ambient O_3 concentrations on wheat (*Triticum aestivum* L. cv. M 533) during reproductive development with the objectives: (*1*) to assess the diurnal gas exchange responses, (*2*) to quantify the variations in photosynthetic pigments and chlorophyll fluorescence kinetics, and (*3*) to establish the relationship between changes in photosynthetic rate and soluble sugar and starch contents in flag leaves during reproductive development.

Materials and methods

Study area: The field experiments were conducted between December and March at a rural site in an agricultural area situated 20 km south of Varanasi city, in India (82°03'E longitude and 76.1 m a. s. l.). During the growth period of wheat, mean monthly maximum temperature ranged between 22.1–32.7°C and mean monthly minimum temperature varied from 18–28°C. Total rainfall 115.6 mm and sunshine hours varied from 6.2–8.4 h. Maximum relative humidity ranged between 72.7–86.7% and variations in minimum relative humidity was 33–55.7%.

Six open-top chambers (OTCs) were established at the experimental site and details of OTCs design described in Rai *et al.* (2007). There were three treatments *i.e.*: OTCs ventilated with ambient nonfiltered air (NFCs), OTCs ventilated with activated charcoal filters (FCs) and open plots (OPs) without chambers, respectively. Open plots (OPs) were kept for studying the chamber effects on plants. The treatments were distributed in a completely randomized design with three replicate of each treatment (n = 3). Microclimatic measurements were taken within and outside the chambers. The temperature and relative humidity respectively were 0.1 to 0.2 °C and 2 to 3% more in the chambers than the open plots. The light intensity in the chambers was 95% of the ambient level in the open plots. However, microclimatic measurements between FCs and NFCs were similar.

Plant material: Wheat (*Triticum aestivum* L.) cultivar M 533 chosen for the experiment is a highly recommended and widely grown variety for northeastern plain zone of India. It is a modern variety released in 2001 having UNNATH (306/HUW 81/K8027) parentage and a tall variety highly resistant against rusts and with a life cycle of 135 days.

Seeds of wheat were manually sown in OTCs and OPs prepared after using recommended agronomic practices including fertilizer doses given as urea (120 kg ha^{-1}), superphosphate (60 kg ha^{-1}) and muriate of potash (40 kg ha^{-1}). Half dose of N and full doses of P and K were given as basal dressing and another half dose of N was given as a top dressing after 40 days of germination. Plants were thinned to 1 plant every 15 cm after one week of germination. There were 30 plants in each OTC and

open plot. Manual weeding was performed three times over the life period of the plant. Uniform moisture status was maintained in all treatments.

Monitoring of O_3: Air samples were drawn through polytetrafluoroethylene tubes (0.25 cm in diameter) at canopy height from different chambers and open plots between 07:00 and 19.00 h throughout the growth of the plants. O_3 concentration was monitored using a UV absorption photometric ozone analyzer (*Model 400 A, API, Inc*. USA), which recorded the concentration every 5 min. The calibration of the instrument was performed frequently by a known concentration of O_3 generated through ozonator (*Standard Appliances, Model SA- 112-LP- 230C*, Varanasi, India).

Exposure index for ozone, *i.e*. AOT 40 (accumulated ozone over a concentration threshold of 40 ppb) was calculated by using the following formula (Mills *et al.* 2007):

$$AOT\ 40 = \sum^{n}[C_{O_3} - 40]_i \text{ for } C_{O_3} \geq 40 \text{ ppb},$$

[AOT 40 units: ppm h]

where C_{O_3} is the hourly O_3 concentration in parts per billion (ppb), i is the index, *n* is the number of hours with $C_{O_3} > 40$ ppb over the 3-month growing period that has been set as the evaluation period for respective crops.

Gas-exchange measurements: P_N, g_s, and C_i were measured using portable photosynthetic system (*Model LI-6200, LI-COR*, Lincoln, Nebraska, USA). Portable photosynthetic system (*LI-6200*) consists of three major components: a leaf chamber (measures air temperature, leaf temperature and relative humidity), infrared gas analyzer (*LI-6250*) (which measures CO_2 concentration) and flow valve to maintain a steady humidity in the chamber during measurement. Portable photosynthetic system monitors the rate at which the CO_2 concentration in the air changes for a short interval. The net amount of leaf area enclosed in leaf chamber, the volume of the enclosure, temperature and pressure. Measurements were recorded between 08:00 and 16:00 h on the flag leaves which were fully expanded and oriented to normal irradiation during cloud-free days. The system was calibrated using a known CO_2 source of 509 ppm concentration. During measurements of photosynthesis, the average diurnal variation in the photosynthetically active radiation (PAR) ranged between 400–1,100 µmol m^{-2} s^{-1}, mean temperature varied from 22.1–32.7°C, relative humidity ranged between 52–72% and CO_2 concentration was 383–385 ppm from 08:00 to 16:00 h (Fig. 1).

Three replicate measurements were conducted on three plants of each chamber and open plot after 10, 30, and 50 days of full expansion of flag leaves (DAFE). The days of sampling after germination and flag leaf expansion and their corresponding Zadoks scale (Zadoks *et al*. 1974) denoting the wheat growth stages are given in Table 1.

Chl fluorescence kinetics: Chl fluorescence was determined between 09:00 and 11:00 h using a portable plant efficiency analyzer (*Model, MK29414, Hansatech Instrument Ltd*., UK) on the same flag leaves, where P_N measurements were taken. Leaf clips for dark adaptation were placed on the adaxial side of the leaves 30 min before measurement and then exposed to red light of 650 nm through LED at excitation irradiance of 3,000 µmol m^{-2} s^{-1}. Minimum fluorescence (F_0) and maximum fluorescence (F_m) were measured from which variable fluorescence (F_v) and ratio of variable and maximal fluorescence (F_v/F_m) were calculated.

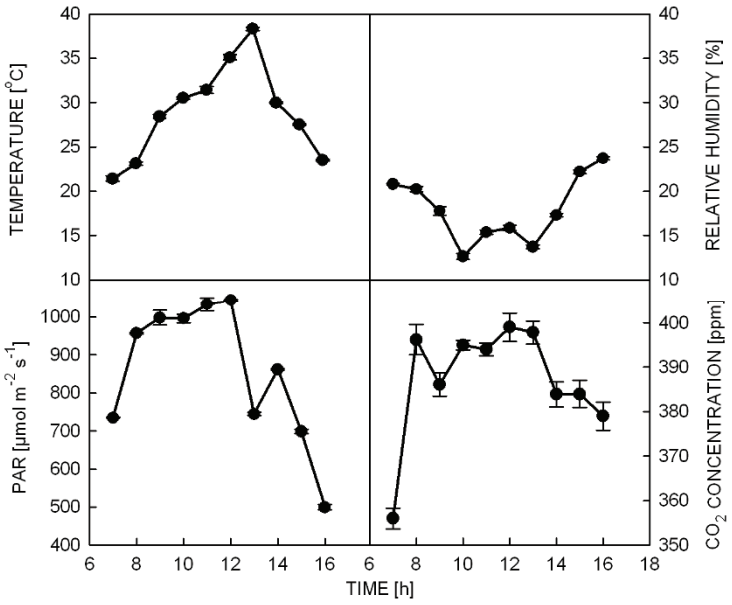

Fig. 1. Average diurnal variation in photosynthetically active radiation (PAR), relative humidity, CO_2 concentration, temperature during gas-exchange measurements in the leaf chamber of the photosynthetic system.

Table 1. Details of sampling schedule of wheat plants. DAFE – days after flag leaf expansion; DAG – days after germination.

Sampling schedule	Plant age [DAG]	Zadoks growth stage
Sowing		
Germination		
Flag leaf appearance	50	Z-37
Flag leaf fully expanded	60	
10 DAFE	70	Z-47
20 DAFE	80	Z-67
30 DAFE	90	Z-73

Photosynthetic pigments and carbohydrates: After photosynthetic rate is calculated using rate of change, taking the measurements of gas exchange and Chl fluorescence kinetics on the respective days, flag leaves from different chambers and open plots were cut for the estimation of photosynthetic pigments and carbohydrates. Photosynthetic pigments were extracted from leaf samples in 10 ml of 80% acetone. After centrifugation, the optical densities of the supernatant were measured at 480, 510, 645, and 663 nm wavelengths and the amounts of Chl *a*, *b* and carotenoids were calculated using the formulae of Machlachlan and Zalik (1963) and Duxbery and Yentsch (1956), respectively.

For extracting sugars and starch, 50 mg of the dry and powdered leaf sample was boiled with 5 ml of 80% ethanol (v/v) and then centrifuged (8,944 × *g*). The pellets were successively washed with 80% ethanol for 4 times and centrifuged after each washing. Finally the pellets were washed with distilled water and centrifuged again. The supernatant thus collected after each washing was used for estimating soluble sugars and pellets for extracting starch. Total soluble sugar was estimated following phenol/H_2SO_4 colorimetric assay (Dubois *et al.* 1956). For estimation of starch, pellets were washed twice with 52% perchloric acid (v/v) and centrifuged successively (McCready *et al.* 1950). The supernatant was made up to 50 ml with distilled water and a 1 ml aliquot was taken to determine the starch content following Dubois *et al.* (1956).

Statistical analyses: Data of photosynthetic pigments, P_N, g_s, Chl fluorescence kinetics and soluble sugar and starch contents were subjected to two-way analysis of variance (*ANOVA*) to examine the individual and combined effects of leaf age, treatment, and their interactions. The correlation coefficients and regression equations were also calculated between P_N, Chl content, parameters of Chl fluorescence kinetics and g_s at 50 DAFE. *Duncan*'s multiple range tests were performed as post hoc on parameters subjected to various *ANOVA* tests. All the statistical tests were performed using *SPSS* software (*SPSS Inc., version 16.0*, Chicago, USA).

Results

Monitoring of O_3: In the present study, the mean of the daily 12 h (M 12) O_3 was 45.1 ppb during entire study period (Fig. 2) and 50.2 ppb after flag – during entire study period and after flag leaf emergence were 560 h in FCs and 6.27 ppm h in NFCs during the growth period of wheat (Table 1). AOT 40 value recorded after flag and 329 h, respectively (Fig. 3). Accumulated O_3 exposure over a threshold of 40 ppb (AOT 40) was 0 ppm h

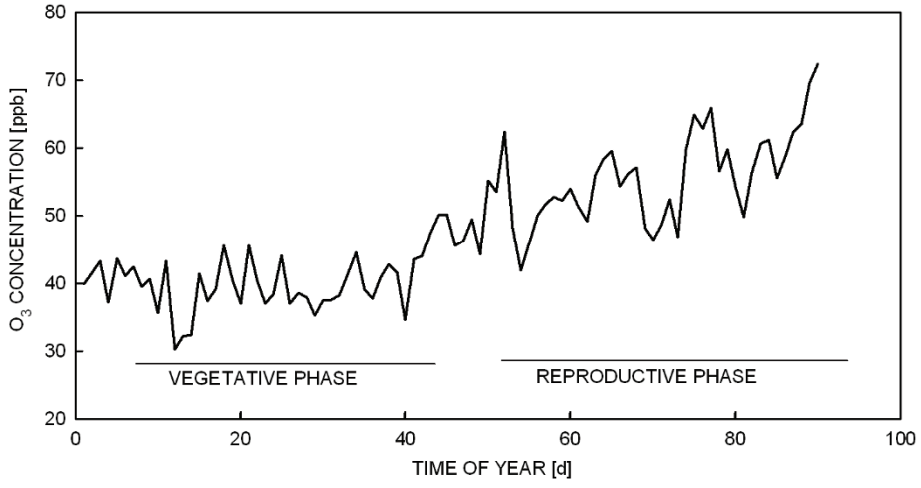

Fig. 2. Mean 12-h ozone concentration during the growth period of wheat.

Table 2. O_3-exposure indices and exceedance hours above 40 ppb in nonfiltered chambers during the whole fumigation (125 days) and after flag leaf development (85 days).

	Whole fumigation	Flag leaves development
M 12 (12 h) [ppb]	45.1	50.2
AOT 40 [ppm h]	6.27	5.31
Exceedance hours above 40 ppb [h]	560	329

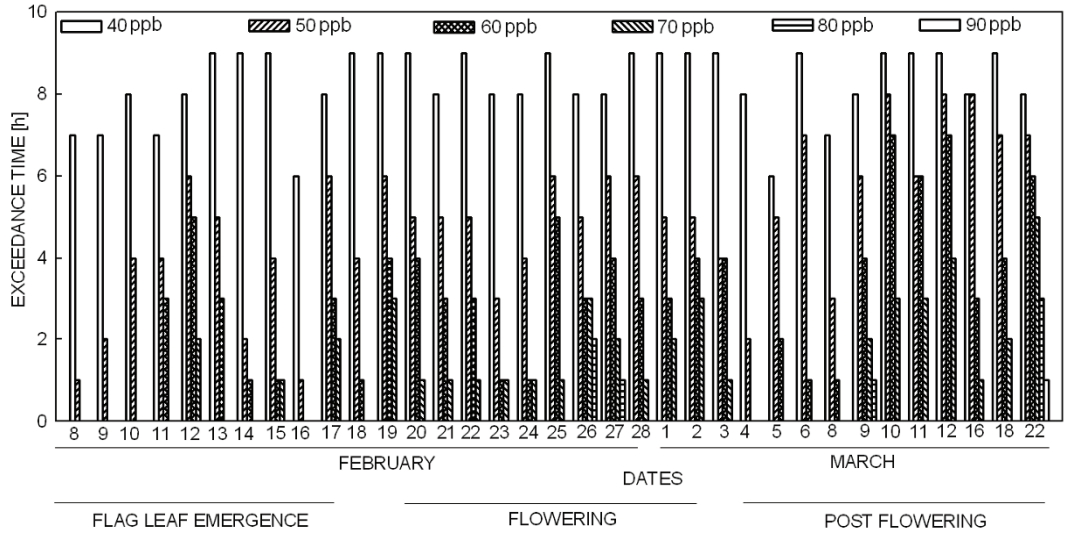

Fig. 3. Number of hours ozone concentration exceeding 40, 50, 60, 70, 80, and 90 ppb during reproductive development.

leaves emergence was 5.31 ppm h. In filtered chambers (FCs), air filtration with activated charcoal filters resulted in reduction of O_3 concentrations by 88% compared to nonfiltered chambers (NFCs) and open plots (OPs). At the experimental site, SO_2 (7.4–13.9 ppb) and NO_2 (11.2–28.9 ppb) were comparatively low and within the permissible limits.

Gas-exchange measurements: In general, P_N was higher in plants of FCs compared to NFCs and OPs (Table 3). The diurnal variation of P_N displayed a double-peaked curve in both FCs and NFCs (Fig. 4). Mean P_N reduced by 25.7, 28.6, and 32.3 at 10, 30, and 50 DAFE, respectively in NFCs compared to FCs (Table 3). The maximum rate of P_N was observed in the morning at 09:00 h in FCs, whereas a late peak at 10:00 h was recorded for P_N in NFCs during all the observations (Fig. 4). Variations in P_N between NFCs and OPs were not significant.

g_s showed lower values in plants of NFCs compared to FCs at all measurements (Fig. 5). The midday depression in g_s was observed in the afternoon in FCs, however, no specific trend of depression in g_s of plants was observed in NFCs. C_i was minimum at 09.00 h in FCs and 10.00 h in NFCs, however, at the time of depression in g_s, higher internal CO_2 concentration was recorded. Results of two-way *ANOVA* showed that P_N and g_s varied significantly due to leaf age and treatment (Table 4).

Chl fluorescence kinetics: Results of Chl fluorescence kinetics showed significant increase in F_0 in plants grown in NFCs compared to FCs (Fig. 6). Lower values of F_m and F_v were observed in NFCs compared to FCs. Reductions recorded in F_v/F_m ratio were 5, 8, and 14%, respectively at 10, 30, and 50 DAFE. Variations in F_0, F_m, F_v, and F_v/F_m were significant due to individual factors of leaf age, treatment, and their interactions (Table 4).

Photosynthetic pigments and carbohydrates: Photosynthetic pigments decreased significantly in NFCs compared to FCs (Table 3). Total Chl, Chl *a*, Chl *b*, and carotenoid contents decreased maximally at 50 DAFE (37, 34, 36, and 13.3%) followed by 30 DAFE (30, 26.2, 29, and 12%) and minimally at 10 DAFE (23.8, 17.1, 22.6, and 11.3%), respectively. Chl *a* reduced more than Chl *b* at all stages of observations. Results of two-way *ANOVA* showed that contents of Chl *a*, *b*, and total varied significantly due to leaf age, treatment, and their interactions (Table 3). Carotenoid content also varied significantly due to both the individual factors. Chl *a*/Chl *b* ratio was lower in NFCs compared to FCs, however these reductions were not significant. Soluble sugars increased significantly in leaves of wheat at 30 and 50 DAFE, however, starch content decreased at all observations (Table 3). A maximum increase in soluble sugar and decrease in starch was recorded at 50 DAFE. Variations in soluble sugars and starch were significant due to leaf age, treatment and their interactions (Table 4).

Table 3. Variations in net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), photosynthetic pigments, total soluble sugars and starch contents in wheat grown in filtered chambers (FCs), nonfiltered chambers (NFCs), and open plots (OPs) at 10, 30, and 50 days after flag leaf expansion (DAFE). Values are mean ± 1 SE ($n = 9$). *Different letters* within a group of column indicate significant differences among treatments differences at $p<0.05$ according to *Duncan*'s test. DM – dry mass; Chl – chlorophyll.

Parameters	10 DAFE			30 DAFE			50 DAFE		
	FCs	NFCs	OPs	FCs	NFCs	OPs	FCs	NFCs	OPs
P_N [µmol(CO_2) m^{-2} s^{-1}]	12.5 ± 1.58[a]	9.80 ± 0.52[b]	9.00 ± 0.51[b]	12.22 ± 1.19[a]	8.72 ± 0.75[b]	8.61 ± 0.71[b]	15.76 ± 1.61[a]	9.97 ± 0.80[b]	9.59 ± 0.67[b]
g_s [mol m^{-2} s^{-1}]	0.89 ± 0.15[a]	0.52 ± 0.03[b]	0.50 ± 0.04[b]	0.73 ± 0.37[a]	0.56 ± 0.05[b]	0.54 ± 0.06[b]	0.76 ± 0.14[a]	0.55 ± 0.06[b]	0.54 ± 0.07[b]
C_i [µmol mol^{-1}]	359.1 ± 5.39[a]	417 ± 4.9[b]	414 ± 2.66[b]	429.1 ± 2.47[a]	543.8 ± 4.06[b]	537.8 ± 4.5[b]	475.9 ± 2.3[a]	641.6 ± 3.55[b]	538.5 ± 4.14[b]
Chl *a* [mg g^{-1}(DM)]	1.89 ± 0.01[a]	1.44 ± 0.001[b]	1.72 ± 0.005[b]	2.48 ± 0.08[a]	1.74 ± 0.03[b]	1.73 ± 0.03[b]	2.49 ± 0.07[a]	1.58 ± 0.03[b]	1.56 ± 0.03[b]
Chl *b* [mg g^{-1}(DM).]	0.41 ± 0.05[a]	0.34 ± 0.01[a]	0.23 ± 0.02[a]	1.03 ± 0.03[a]	0.76 ± 0.06[b]	0.70 ± 0.05[b]	0.68 ± 0.07[a]	0.45 ± 0.05[b]	0.42 ± 0.02[b]
Total Chl [mg g^{-1}(DM).]	2.2 ± 0.06[a]	1.78 ± 0.03[b]	1.74 ± 0.02[b]	3.51 ± 0.05[a]	2.49 ± 0.07[b]	2.43 ± 0.05[b]	3.18 ± 0.12[a]	2.04 ± 0.02[b]	1.98 ± 0.05[b]
Carotenoids [mg g^{-1}(DM)	0.62 ± 0.02[a]	0.55 ± 0.01[b]	0.54 ± 0.006[b]	0.83 ± 0.03[a]	0.73 ± 0.03[b]	0.69 ± 0.02[b]	0.83 ± 0.05[a]	0.72 ± 0.006[b]	0.70 ± 0.03[b]
Chl *a*/Chl *b*	4.73 ± 0.36[a]	4.21 ± 0.11[a]	4.52 ± 0.26[a]	2.42 ± 0.14[a]	2.33 ± 0.19[a]	2.48 ± 0.17[a]	3.74 ± 0.39[a]	3.45 ± 0.16[a]	3.70 ± 0.11[a]
Soluble sugars [mg g^{-1}(DM)]	205.6 ± 3.1[b]	211.3 ± 3.4[a]	217.2 ± 2.3[a]	230.4 ± 4.9[b]	257.6 ± 2.9[a]	251.5 ± 2.9[a]	183.2 ± 0.5[b]	212.7 ± 3.3[a]	219 ± 2.35[a]
Starch [mg g^{-1}(DM)]	168.5 ± 2.2[a]	153.4 ± 3.0[b]	151.6 ± 1.6[b]	188.3 ± 2.4[a]	148.6 ± 2.9[b]	142.2 ± 1.9[b]	139.2 ± 2.2[a]	107.7 ± 3.0[b]	106.4 ± 2.8[b]

Discussion

In the present study, ambient concentration of O_3 was found to modify the diurnal pattern of gas exchange and Chl fluorescence kinetics during different stages of reproductive development. P_N displayed a double-peaked diurnal curve, with delayed peak and early depression in NFCs compared to FCs. In the present study, highest O_3 concentrations were recorded between 11:00 and 14:00 h, which coincided with depressions in g_s and P_N. It appears that diurnal cycles of P_N and g_s decouple with the diurnal profile of O_3. Carbon dioxide and O_3 uptake is coupled together with g_s, however, partial stomatal closure might be interpreted as avoidance or O_3-induced injury. Heath (1994) proposed that with increase in O_3 partial pressure in the substomatal cavity, the permeability of the guard cells is affected leading to a loss of osmotically active materials. As a consequence, internal water potential rises leading to withdrawal of water from the guard cells in favour of the subsidiary cells, causing closure of stomata (Heath 1994). Reduced uptake of CO_2 due to reduction in g_s has reduced P_N under ambient O_3 concentration in NFCs/OPs compared to FCs.

In the present study, P_N is positively correlated with g_s (R^2 = 0.64; p<0.01). But a significant negative correlation between P_N and C_i (R^2 = 0.77; p<0.001) suggests that the reduction in P_N was not merely due to lower g_s, but may be due to nonstomatal factors such as activities of photosystem II (PSII), inhibition of electron transport and decrease in Chl content. If stomatal factors alone had played a major significant role then reduction in P_N and g_s should have accompanied the decline in C_i (Feng *et al.* 2007). In the present study, C_i increased on stomatal closure, indicating reduced mesophyllic activity. The increased C_i suggests a more direct damage to photosynthetic mechanism. A positive correlation was observed between P_N and F_v/F_m ratio (R^2 = 0.85, p<0.001) suggesting that decrease in photosynthetic rate may also be attributed to inhibition of light reactions. In the present study, significant reductions in the quantum efficiency of open PSII centres represented by F_v/F_m ratio were recorded at all the observations, with maximum decrease at 50 DAFE. Reduction of F_v/F_m ratio indicates an alteration of PSII photochemistry (Calatayud and Barreno

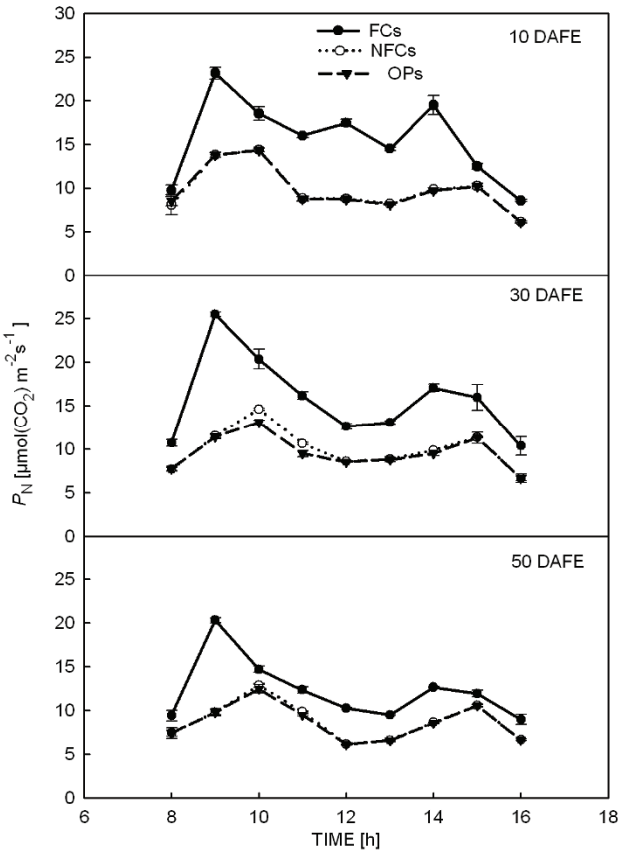

Fig. 4. Diurnal variations in net photosynthetic rate (P_N) at 10, 30, and 50 days after flag leaf expansion (DAFE) of wheat grown in filtered chambers (FCs), nonfiltered chambers (NFCs), and open plots (OPs). Values are mean ± 1 SE (n = 9).

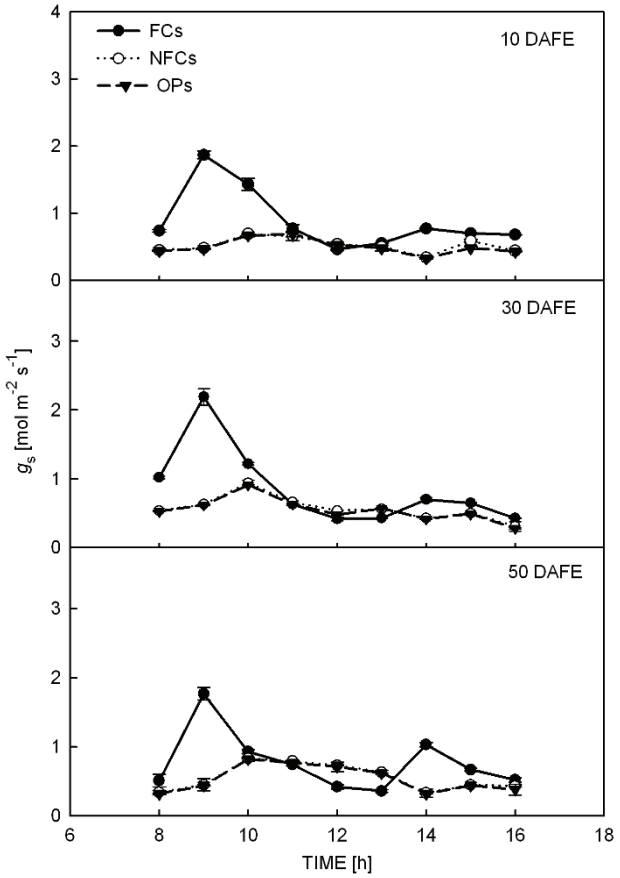

Fig. 5. Diurnal variations in stomatal conductance (g_s) at 10, 30, and 50 days after flag leaf expansion (DAFE) of wheat grown in filtered chambers (FCs), nonfiltered chambers (NFCs), and open plots (OPs). Values are mean ± 1 SE (n = 9).

Table 4. Results of two-way *ANOVA* test showing *F* values and level of significance for different measured characteristics of wheat grown in filtered chambers (FCs), nonfiltered chambers (NFCs), and open plots (OPs). P_N – net photosynthetic rate; g_s – stomatal conductance; Chl – chlorophyll. Level of significance: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS – nonsignificant.

Parameters	Leaf age	Treatment	Leaf age × treatment
P_N	3.1*	22.7***	0.5^{NS}
g_s	0.7^{NS}	6.9**	0.4^{NS}
F_0	258.7***	90.1***	3.8*
F_m	717.3***	395.9***	58.3***
F_v	314.5***	545.6***	62.2***
F_v/F_m	7.1**	307.1***	23.6***
Chl *a*	79***	307.4***	11.3***
Chl *b*	99.4***	25.7***	10.3***
Total Chl	161.1***	237.8***	10.3***
Chl *a*/Chl *b*	158.5***	0.67^{NS}	0.3^{NS}
Carotenoids	418.5***	0.83^{NS}	0.3^{NS}
Starch	270.1***	148.9***	10.1***
Total soluble sugars	177.8***	43.3***	4.6**

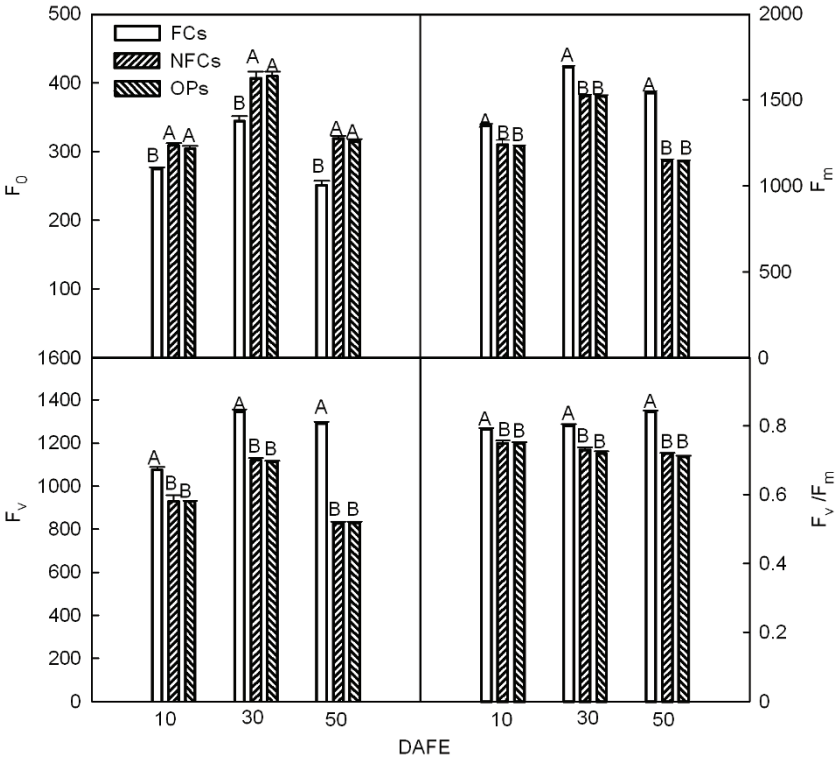

Fig. 6. Variations in chlorophyll (Chl) fluorescence kinetics in wheat grown in filtered chambers (FCs), nonfiltered chambers (NFCs), and open plots (OPs) at 10, 30, and 50 days after flag leaf expansion (DAFE) between 9:00–11:00 h. Values are mean ± 1 SE (n = 9). *Bars* showing *different letters* within a group of columns indicate significant differences among treatments at $p<0.05$ according to *Duncan*'s test.

2001). Reduction in P_N rate may influence the rate of NADPH and ATP utilization, hence altering the PSII efficiency (Guidi *et al.* 2001). Decline in F_v/F_m ratio was due to increase in F_0 and a parallel decrease in F_m, suggesting impaired PSII activity. A negative correlation between F_v/F_m ratio and F_0 (R^2 = 0.98, $p<0.001$) and a positive correlation between F_v/F_m and F_m (R^2 = 0.92, $p<0.001$) were recorded during the present study, suggesting modification at the antenna pigment level at the active centres of PSII, hence leading to a decrease in photochemical capacity (Guidi and Degl' Innocenti 2008).

The reduction in F_m in NFCs/OPs may be due to *n* decline in the ability to reduce the primary acceptor Q_A (Reiling and Davison 1994). Reductions recorded in variable fluorescence (F_v) are more strongly correlated with lowering of F_m during the present study, suggesting impairment of an electron transport, which involves a recombination reaction P680(+) and reduced phaeophytin Phaeo(-) within PSII or directly affecting a PSII antenna system (Ishii *et al.* 2004). At the stage of anthesis, Meyer *et al.* (1997) found reductions of 2 to 3% in F_v/F_m at 65 ppb and 5% at 110 ppb O_3 for 12 h after 14 days of exposure of flag leaves of wheat grown in closed chambers. In the present study, higher reductions

in F_v/F_m ratio were recorded at low mean ambient O_3 levels (45.3 ppb) compared to Meyer *et al*. (1997). Significant variations in F_0, F_m, F_v, and F_v/F_m due to leaf age × treatment interaction suggest differential response of light reaction with age to the ambient O_3.

Results of other nonstomatal factor, photosynthetic pigments showed that their destruction increased in NFCs at ambient O_3 exposure with maximum decrease at 50 DAFE. Total Chl content was positively and significantly correlated with photosynthetic rate ($R^2 = 0.96$, $p<0.001$). A positive correlation between F_v/F_m and total Chl ($R^2 = 0.87$, $p<0.001$) content signifies O_3-induced damage to the membrane bound organelles, chloroplasts, thus negatively affecting PSII (Kollner and Krause 2000). Accelerated Chl destruction is reported due to induced metabolic changes within the plant cells caused by the oxidative force of O_3.

In the present study Chl *a* reduced more than Chl *b*. Saitanis *et al.* (2001) reported greater reduction in Chl *a* than Chl *b* of tobacco plants exposed to O_3. Greater sensitivity of Chl *a* to O_3 implies a lower capacity for light harvesting (Leitao *et al.* 2007). Significant interaction between treatment and leaf age for reduction in Chl content may be caused by O_3-induced accelerated senescence with increasing age. Reduction in repair capacity of older leaves leading to chronic injury in wheat plants exposed to O_3 during anthesis was reported (Pleijel *et al.* 1995). Similar result was reported by Feng *et al*. (2011) after exposure to 27% higher ambient O_3 (52.1 ppb for 7 h) after flag leaf development of wheat cultivars Yanmai 16 (Y 16) and Yangfumai 2 (Y 2). In a study with 20 cultivars, Biswas *et al.* (2008) found 13% mean reductions in total chlorophyll content at 82 ppb O_3 for 7 h day^{-1} over 21 days in OTCs, however, in the present study 37% decrease in total Chl was observed at lower ambient O_3 concentration for longer duration (45.4 ppb throughout the whole fumigation). Carotenoids are vital photoprotective agents, which prevent photo-oxidative Chl destruction and act as light-harvesting complex (Lichtenthaler 1987). Carotenoid content decreased due to oxidative destruction under O_3 stress, leading to a decreased capacity to protect photosystem against photooxidation. Hence, the loss of Chl and carotenoids can produce a decrease in the light absorbing capacity to develop thermal dissipation energy under O_3-fumigation conditions (Calatayud and Barreno 2004).

Flag leaf acts as an active assimilate source during the anthesis stage of the plant. Flag and penultimate leaf sheath and peduncle photosynthesis provides assimilates for the grain, but flag leaf blade and spikes are the most important contributors to grain filling. In the present study, starch content decreased in NFCs compared to FCs, with maximum reduction at 50 DAFE. Decrease in starch may be associated with higher reductions in total Chl along with the lowest rate of photosynthesis at 50 DAFE. High levels of sugars were observed in the present study under ambient O_3. Meyer *et al.* (1997) found an increase in carbohydrate concentrations of flag leaves fumigated with O_3 at anthesis due to interference in transport processes (sugar transfer to conducting elements and/or phloem loading) caused by membrane damage of mesophyll or companion cells.

Conclusions: The ambient O_3 levels significantly reduced the physiological vitality of wheat plants in terms of loss of photosynthetic pigments, reductions in PSII activity and P_N, with maximum reductions at 50 DAFE. The results of the present study showed the highest ambient O_3 concentrations coincided with grain setting and filling stages of wheat plants. Depression in P_N was not completely resulted due to lowering of g_s, but also due to impaired PSII activity and reductions in total Chl content under ambient O_3 concentration compared to filtered chambers. The present study further suggested that ambient O_3 not only reduced the photosynthetic carbon assimilation and stomatal conductance. Higher reductions of P_N rate during late stage of reproductive development strongly influenced the newly produced assimilates required for grain filling. The changes occurring in flag leaf physiology and metabolism under ambient O_3 concentrations suggest significant negative repercussions for crop yield.

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