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Gas exchange and chlorophyll fluorescence of pea (*Pisum sativum* L.) plants in response to ambient ozone at a rural site in Egypt

I.M. Ismail [a], J.M. Basahi [a], I.A. Hassan [a,b,*]

[a] *Air Pollution Laboratory (APL), Centre of Excellence in Environmental Studies (CEES), King Abdulaziz University, P. O. Box 80216, Jeddah 21589, Saudi Arabia*
[b] *Department of Botany, Faculty of Science, Alexandria University, 21526 El Shatby, Alexandria, Egypt*

HIGHLIGHTS

- Ozone (O_3) concentrations recorded were within the ranges of phytotoxicity.
- O_3 has a clear influence on the physiological parameters.
- O_3 decreased Photosynthetic rates, chlorophyll fluorescence parameters.
- The variability in stomatal response may be due to inherent differences.

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ABSTRACT

Egyptian pea cultivars (*Pisum sativum* L. cultivars Little Marvel, Perfection and Victory) grown in open-top chambers were exposed to either charcoal-filtered (FA) or non-filtered air (NF) for five consecutive years (2009–2013) at a rural site in northern Egypt. Net photosynthetic rates (P_N), stomatal conductance (g_s), intercellular CO_2 (C_i) and chlorophyll fluorescence were measured.

Ozone (O_3) was found to be the most prevalent pollutant common at the rural site and is suspected to be involved in the alteration of the physiological parameters measured in the present investigation.

P_N of different cultivars were found to respond similarly; decreases of 23, 29 and 39% were observed in the cultivars Perfection, Little Marvel and Victory, respectively (averaged over the five years) due to ambient O_3. The maximum impairment in P_N was recorded in the cultivar Victory (46%) in 2013 when the highest O_3 levels were recorded (90 nL L^{-1}). The average stomatal conductance decreased by 20 and 18% in the cultivars Little Marvel and Perfection, respectively, while the average stomatal conductance increased on average by 27% in the cultivar Victory. A significant correlation was found between P_N and C_i, indicating the importance of non-stomatal limitations of photosynthesis, especially in the cultivar Victory. The P_N vs. Ci curves were fitted to a non-rectangular hyperbolic model.

The actual quantum yield (Φ_{PSII}) and photochemical quenching coefficient (qP) were significantly decreased in the leaves of plants exposed to NF air. Non-photochemical quenching (NPQ) was increased in all cultivars.

Exposure to NF air caused reductions in chlorophyll (Chl *a*) of 19, 16 and 30% in the Little Marvel, Perfection and Victory cultivars, respectively.

1. Introduction

Current interest in investigating the impact of air pollutants on agricultural crops is now centered on the long-term low-level effects of the main phytotoxic gases on crop production (Weigel et al., 1987; De Temmerman et al., 1992; Schenone et al., 1992, 1994; Fiscus et al., 2005; Hassan, 2006, 2010). Chronic exposure to air pollutants can cause yield losses (Heagle et al., 1979; Pande and Mansfield, 1985; Weigel et al., 1987; Schenone and Lorenzini, 1992; Ali, 1993; Hassan et al., 1995, 1999; Dizengremel et al., 2008, 2009, 2012). Apart from crop yield losses, changes in plant development and reduced net growth can occur (Gould and Mansfield, 1988; Hassan, 2004; Hatata et al., 2013) as well as changes in crop quality (Peleijel et al., 1989; Fuhrer et al., 1990; Vandermerien et al., 1992; Hassan, 1998a), and plant physiology (Fuhrer et al., 1988; Hassan et al., 1994, 1999; El-Khatib, 2003; Hassan, 2004, 2006; Taie et al., 2013).

Ozone is the main phytotoxic air pollutant in the Mediterranean area (Lorenzini et al., 1994; Hassan, 1999; Hassan et al., 1994, 1995, 2013; Velissariou et al., 1996; Gimeno et al., 1999; Calatayud et al., 2002, 2004, 2011; Pellegrini et al., 2011).

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* Corresponding author at: Air Pollution Laboratory (APL), Centre of Excellence in Environmental Studies (CEES), King Abdulaziz University, P.O. Box 80216, Jeddah 21589, Saudi Arabia.
E-mail addresses: ihassan_eg@yahoo.com, iagadallah@kau.edu.sa (I.A. Hassan).

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Important physiological processes such as photosynthesis and stomatal conductance are known to be affected by air pollution (Hassan et al., 1994, 1999; Zhang et al., 2001; El-Khatib, 2003; Hassan, 1998 a, b, 2004; Thwe et al., 2014).

A considerable volume of published data exists that is useful for evaluating the response of agricultural crops to ambient air pollution in the USA and Northern Europe (e.g. NACLAN and EOTC). However, the data for the Middle East, and especially for Egypt are still extremely scarce, in spite of high population densities and rates of pollution emission, and the climatic conditions which favor photochemical air pollution (WHO/UNEP, 1992; El-Khatib, 2003; Hassan, 1999). To fill the above gap in knowledge, a specific research project was developed as a collaboration between Alexandria University and University of London to examine the effects of ambient air pollution effects on agricultural crops in rural (the main agricultural area in Egypt) and urban areas in northern Egypt.

Pea plants are sensitive to air pollutants. Hassan (2008) investigated the impact of O_3 on the growth and yield of three pea cultivars (*Pisum sativum* L. cvs. Little Marvel, Perfection and Victory) grown in ambient air, in northern Egypt. All cultivars showed significant yield reductions and severe foliar injury symptoms; these symptoms are characteristic of O_3 injury when compared with cultivars grown in filtered air. Moreover, the cultivar Victory showed the highest yield loss compared to the other two cultivars (cv. Little Marvel and cv. Perfection). Therefore it was concluded that Victory plants are more sensitive to air pollution (mainly O_3) than the other two cultivars (Hassan, 2008). The physiological characteristics that are responsible for these relative sensitivities have been elusive, but stomatal conductance (g_s) has been suggested to play a major role, as those species with higher g_s are likely to be less tolerant to pollutants (Pande and Mansfield, 1985). Similarly, plants in which stimulation of stomatal opening occurs in response to pollutant(s) are likely to be more sensitive to pollutants (Hassan et al., 1994). Previous work (Hassan et al.1995; Hassan, 1998a, 1999, 2006, 2010) showed that O_3 was the main air pollutant responsible for yield losses.

Ozone can cause damage to thylakoids leading to ion leakage and consequent change in Chl *a* fluorescence (Reichwnauer et al., 1998; Guidi et al., 1999a,b; Plazek et al., 2000). Chl *a* fluorescence which is a good indicator of photoinhibition of PSII (Thwe et al., 2014), shows changes in photosynthetic performance and allows localization of the primary sites of limitations. Therefore, Chl *a* fluorescence has been widely used for the assessment of environmental stresses on photosynthesis (Hassan, 2004; Pellegrini et al., 2011).

The present work was carried out to verify and characterize the effects of ambient air pollution on the photosynthetic processes of pea leaves through an analysis of Chl *a* fluorescence and gas exchange parameters. To our knowledge, this study is one of the very few investigations conducted in the Middle East attempting to clarify the potential mechanisms of reduced photosynthetic rate.

2. Materials and methods

2.1. Experimental facilities

The experimental site was located at Abbis village (a "rural" site) 35 km to the south of Alexandria in the Nile Delta, which is the main agricultural area of the country (30° 0′N and 31° 19′E). The experimental area was approximately 2 km from the nearest traffic road, and was surrounded by *Eucalyptus*, *Casuarina* and *Salix* trees (for full description see Hassan et al., 1995).

The soil texture at the experimental location was loamy clay with a pH 7.7 (Hassan, 1998 a).

The experiment was carried out in six portable open-top chambers (OTCs) constructed according to the design of Treshow and Stewart (1973). Briefly, chambers were constructed that could be placed over the plots in the field. An iron framework $2 \times 3\ m^2$ in size with a lid on the top was covered by a transparent, low-density polyethylene. Air was distributed into the chambers by means of two perforated semicircular annuli attached to the wall of the chamber at heights of 0.50 and 1.00 m above ground level. Ventilation was continuous at a rate of 1800 mh^{-1} (2.5 air changes a minute). The fans were switched on and operated for 8 h day^{-1} (09:00–17:00 h Egyptian local time) and switched off in the evening to allow for dew formation.

Six semi-open-top chambers were used in this experiment in a split plot design: three chambers received charcoal-filtered air (FA) and the other chambers received non-filtered (NF) air and an ambient air (AA) to determine responses to the chamber environment. Air filtration experiments with OTCs were carried out in between 2009 and 2013 (09:00–17:00 h Egyptian local time).

The AA plot was $3 \times 2\ m^2$ in size with four rows. The between-row distance was 0.50 m.

Air temperature, soil temperature, relative humidity and photosynthetic photon flux density (PPFD) were monitored regularly inside and outside the chambers. The microclimatic conditions were comparable over the five successive years (2009–2013) and there were no significant differences between chamber climate and open-field conditions.

2.2. Plant culture

Prior to sowing, dried cow manure was added to the soil. Seeds of three cultivars pea plants (*Pisum sativum* L. cultivars Little Marvel, Perfection and Victory) were hand sown directly into the soil, the sowing and harvest dates are shown in Table 1. There were six rows in each chamber, the between-row distance was 30 cm, and each cultivar was planted in two rows in each OTC. Two weeks after sowing, when the first true leaf expanded, the seedlings were thinned to one per lot. There were 20 plants per row, thus there were 40 plant/cultivar/chamber. The plants were irrigated manually with tap water once a week for the whole years.

No pesticides or fertilizers were applied.

2.3. Air pollution measurements

The concentrations of gaseous air pollutants (O_3, SO_2 and NO_x) were measured by AQM60 over the five successive years (2009–2013). The AQM60 contains a multichannel solenoid valve sampler which was used to sample different air pollutants sequentially from the top of the plant canopy.

This instrument contains an internal zero air scrubber and it is therefore, capable of periodically carrying out automatic zero and span calibrations and making its own zero and span self-adjustments to predetermined readings.

2.4. Physiological measurements

2.4.1. Net photosynthetic rate (P_N) and total CO_2 stomatal conductance (g_s)

Net photosynthetic rate (P_N) and total CO_2 stomatal conductance (g_s) were measured using the youngest fully expanded leaf of the main stem. Gas exchange measurements were carried out using the same leaf. Measurements were performed eighteen times at 5 days intervals to cover all growth stages using a *LI-6400* portable infrared gas analyzer (IRGA; *LI-COR*, Lincoln, USA) between 10:00 and 15:00 h (Egyptian Local time). All measurments were performed at the PPFD of 1200–1400 $\mu mol\ m^{-2}\ s^{-1}$ in air with an RH of 60–65% and a CO_2 concentration of approximately 345 mol mol^{-1}. Five plants were selected from each OTC, thus fifteen measurements were performed per treatment. The IRGA consists of a leaf cuvette, air supply unit (ASU) and data processor and logger. Air emerging from the cuvette and a sample of air emerging from the ASU are passed to the IRGA. The difference in CO_2 concentration between these two air streams is measured by the IRGA and the product of this and the flow/unit leaf area provide the assimilation rate (P_N) and g_s.

Table 1
Timetable of sowing and harvest dates.

Year	2009	2010	2011	2012	2013
Sowing date	5 Nov. 2009	5 Nov. 2010	29 Oct. 2011	25 Oct. 2012	15 Nov. 2013
Harvest date	5 Feb. 2010	3 Feb. 2011	25 Jan. 2012	3 Feb. 2013	10 Feb. 2014
Duration (days)	92	91	89	95	88

2.4.2. Chlorophyll (Chl) fluorescence

Chlorophyll (Chl) fluorescence was measured on the same leaf at the same time as P_N and g_s, using a portable Photosynthetic Efficiency Analyser PEA (Hansantech Instrumental, Hardwick, Norfolk, UK). The leaves were dark adapted for 30 min in leaf-clips before the measurements were performed. The ground (F_0), maximum (F_m), and variable (F_v) fluorescence were measured and used for calculations of the variable to maximum fluorescence ratio (F_v/F_m). The optimal quantum efficiency of PSII photochemistry (F_v/F_m) was calculated when the leaf was completely dark-adapted. Photochemical (Φ_{PSII} and ${}_qp$) and non-photochemical (NPQ) Chl quenching measurements were calculated when the leaf was completely adapted to light. (Hassan, 2004; Thwe et al., 2014).

2.4.3. Chlorophyll

Chlorophyll was extracted in cold acetone from all leaves on the main stems of nine plants per treatment were used (i.e. three chambers). The chlorophyll concentration was determined according to Khan and Khan (1994). Extraction was carried out in a dark room.

2.5. Statistical analysis

Gas exchange data were log-transformed prior to analysis to ensure that they were normally distributed. Then, these data were subjected to two-way ANOVA with blocking, with years as the blocks. The relationships among P_N, g_s and C_i were analyzed using the correlation coefficient test, while linear regression analysis was used to examine the relationship between P_N and g_s (using the STATGRAF statistical package). PPFD was used as a covariate in the ANOVA of gas exchange measurements. The least significant differences (LSDs) between the means were estimated at the 95% confidence level. Unless indicated otherwise, significant differences among the different treatments were determined at $P < 0.05$.

3. Results

3.1. Microclimate and air quality

There were slight differences (insignificant, $P > 0.05$) between AA plots and chambers in terms of microclimatic conditions and pollutant concentrations.

The light intensity inside chambers was reduced by 2%, on average due to the shadowing by the frames and the plastic sheets, while the mean air temperature and relative humidity were 1.6 °C and 4% higher than those of the outside chambers, (Table 2). Moreover, precipitation was slightly lower (ca 5%) inside the OTCs compared to the AA plots (Table 2).

The mean concentrations of major air pollutants (O_3, SO_2 and NO_x) throughout the years are shown in Table 3.

Air quality at the experimental site was characterized by very low concentrations of SO_2 and NO_x, where the mean 6-h concentrations of these gases throughout the years were 10.3 and 8.7 nL L^{-1}, respectively (Table 3). The mean 8-hour concentrations of O_3 were 70, 72, 80, 81 and 88 nL L^{-1} for the years 2009, 2010, 2011, 2012 and 2013, respectively (Table 3). The AOT40 were 22.816, 22.816, 26.344, 31.168 and 35.200 ppm · h throughout the five years, respectively. This increase in O_3 concentrations coincided with a slight increase in the seasonal mean temperature.

Table 4 shows the monthly mean and peak concentration of O_3 in the OTCs and AA plots. There were no significant differences in O_3 concentrations between AA plots and chambers ventilated with NF air (2–3% lower in NF compared to AA). The maximum concentration was recorded in Dec. 2013 (92 nL L^{-1}), while the lowest average concentration was recorded in Jan. 2009 (68 nL L^{-1}).

3.2. Physiological response

Fig. 1 shows the estimated values of P_N and g_s of the three pea cultivars. P_N of plants exposed to NF air was reduced by 23, 29 and 39% in the cultivars Perfection, Little Marvel and Victory, respectively, compared to the P_N of plants grown in FA (averaged between the five years) (Fig. 1A). In contrast, the effect of air filtration on g_s was very different in the three cultivars. The value of g_s decreased by 20 and 18% in the cultivars Little Marvel and Perfection, respectively, while the value of g_s increased by an average of 27% in the cultivar Victory (Fig. 1B).

The relationships between P_N and C_i for FA and NF plants are presented in Fig. 2. There was a strong significant negative correlation ($r = -0.317$) between P_N and C_i for NF plants of cultivars Little Marvel and Perfection, while a positive correlation ($r = 0.206$) was found in cultivar Victory (Fig. 2). Moreover, The P_N vs. Ci curve was fitted to a non-rectangular hyperbolic model, which indicates the importance of non-stomatal limitations of photosynthesis, especially in the cultivar Victory. Moreover, there was a significant correlation ($P < 0.01$) between g_s and C_i in the cultivars Little Marvel and Perfection ($r = 0.57$, 0.351, respectively), but not in the cultivar Victory ($r = 0.015$) (Fig. 2).

The regression of P_N against g_s was significant ($P < 0.01$) for the cultivars Little Marvel and Perfection grown in FA air ($R^2 = 0.406$ and 0.305) and NF air ($R^2 = 0.391$ and 0.213), respectively (Fig. 3). Moreover, the regression for the cultivar Victory grown in FA was

Table 2
Meteorological parameters recorded at the experimental location during the years.

Parameter	Air temperature (°C)		Soil temperature (°C)		Relative humidity (%)		PPFD (μmol m^{-2} s^{-1})		Precipitation (mm year^{-1})	
Year	Outside	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside	Inside
2009	21.3	22.5	21.0	22.5	60	62	1250	1230	143	141
2010	21.7	22.9	21.0	22.0	63	63	1310	1300	140	135
2011	22.0	24.0	21.0	22.0	62	65	1290	1260	133	130
2012	22.5	24.6	21.0	21.5	63	65	1325	1300	137	132
2013	25.0	25.1	21.7	22.4	60	65	1320	1300	157	149

Table 3
Mean concentrations of major pollutants during the five growing seasons at a rural site in northern Egypt in the open-field plot (AA), in the charcoal-filtered (FA) and non-filtered (NF) OTCs.

Pollutant					
Year		O_3 (8 h day^{-1}) ($nL\ L^{-1}$)	AOT4 (ppm·h)	SO_2 (6 h day^{-1}) ($nL\ L^{-1}$)	NO_x (6 h day^{-1}) ($nL\ L^{-1}$)
	AA	70	22.816	11	9
2009	NF	71	22.906	12	8
	FA	11	–	3	3
2010	AA	73	22.816	10	10
	NF	71	22.896	11	9
	FA	12	–	4	3
2011	AA	76	26.344	11	8
	NF	77	26.435	9	7
	FA	9	–	3	2
2012	AA	82	31.168	12	8
	NF	81	31.257	10	9
	FA	11	–	3	2
2013	AA	88	35.200	8	6
	NF	90	35.292	8	5
	FA	11	–	3	2

significant ($R^2 = 0.416$ at $P < 0.01$), but not for the plants grown in NF air ($R^2 = 0.0902$, $P > 0.05$) (Fig. 3).

Fig. 4 shows the annual trends in gas exchange parameters of all cultivars. Clearly, ambient O_3 caused reductions in P_N of all cultivars and the greatest effect was observed on the P_N of cv. Victory in 2013, when the highest O_3 levels were recorded.

Similarly, g_s showed a similar trend in cvs Little marvel and Perfection, while cv. Victory showed the opposite response; exposure to ambient air caused an increase in g_s (Fig. 4).

The variable to maximal fluorescence ratio (F_v/F_m), actual quantum yield (Φ_{PSII}), and photochemical quenching coefficient (qP) at steady-state kinetics decreased significantly by 10, 29 and 14%, respectively in the leaves of Little Marvel plants exposed to NF air. Moreover, these parameters decreased similarly in the other two cultivars, with cv. Victory, showing the maximum reductions in these parameters (29, 33 and 20%, respectively). Non-photochemical quenching (NPQ) was increased significantly by 60, 90 and 110% in the cultivars Marvel, Little Perfection and Victory, respectively (Table 5).

Chl *a* content decreased following exposure to non-filtered air by 16, 19 and 30% in cultivars Perfection, Little Marvel and Victory, respectively, but Chl *b* showed no significant response to air filtration ($P > 0.005$) (Table 6).

There was no significant difference ($P > 0.05$) in response to chlorophyll between plants exposed to ambient air (AA) and those exposed to non-filtered air (NF).

Table 4
Summary of ozone concentrations ($nL\ L^{-1}$) in OTCs during fumigation.

Year		Nov		Dec		Jan		Feb	
		Mean	Max	Mean	Max	Mean	Max	Mean	Max
2009	AA	73	77	70	75	68	70	69	75
	NF	72	77	70	76	67	69	70	74
	FA	11	13	12	13	11	12	13	14
2010	AA	73	76	75	77	72	75	74	80
	NF	72	74	74	77	74	76	73	79
	FA	11	13	12	13	13	15	13	16
2011	AA	75	79	75	76	74	77	79	81
	NF	74	74	74	77	73	76	74	79
	FA	10	11	10	12	8	11	8	13
2012	AA	80	85	83	87	77	80	80	85
	NF	77	80	77	81	74	78	73	78
	FA	11	14	10	12	10	13	9	13
2013	AA	87	90	89	92	81	88	80	85
	NF	85	88	84	87	79	82	77	80
	FA	10	11	11	14	12	15	9	14

4. Discussion

Open-top chambers (OTCs) have been used most extensively with herbaceous crops to assess impact of air pollution on plant performance (Kohut et al., 1977; Heagle et al., 1979; Roberts et al., 1983; Weigel et al., 1987, Vandermann et al., 1992; Maggs et al., 1996; Hassan et al., 1994, 1999; Wahid et al., 2001).

In the present study, which lasted for five consecutive years, only insignificant differences between environmental conditions in ambient air (AA) plots and OTCs were found. The most important effect of the chambers, although insignificant, was a reduction in light intensity and a small increase in temperature. These effects were of the same order of magnitude as those found by other researchers (Olszyk et al., 1980; Roberts et al., 1983; Weigel et al., 1987: Maggs et al., 1996).

In the present study, the concentrations of O_3 recorded, at the rural site, and the cumulative doses were within the ranges of phytotoxicity. In contrast, the concentrations of SO_2 and NO_x were so low, that we can certainly exclude any effect of these pollutants on the observed effect. This is in agreement with earlier results of Hassan et al. (1995) in Egypt and Schenone et al. (1994) in Italy.

Because no distinct trends in the response could be detected during a single day of measurement, all of the physiological measurements performed in the time period from 10:00 to 15:00 h were combined to provide a daily mean rate for each treatment. The lack of a daily trend in the observed response of pea plants to ambient air pollution suggests that the physiological effects were the results of medium and long-term modifications (chronic effects), which is in agreement with the results of Schenone et al. (1994) who studied *Phaseolus vulgaris*, and Zhang et al. (2001), who studied Swiss native plant species.

Most previous studies used growth, yield and visible injury symptoms as markers of the sensitivity of plants to O_3 toxicity (Fuhrer and Booker, 2003; Chappelka et al., 2003; Hassan, 2008). However, using physiological and biochemical responses to O_3 as biomarkers is helpful for detecting O_3 toxicity at early stages (Feng et al., 2007; Pellegrini et al., 2011; Thwe et al., 2014). An understanding of these physiological responses enhances our knowledge of the mechanisms involved in the plant response to O_3 (Baczek-Kwinta, 2002; Power and Ashmore, 2002; El Khatib, 2003; Nali et al., 2005, 2006; Pellegrini et al., 2011).

The net photosynthesis (P_N) of NF plants was negatively affected, and this result is in agreement with the general view reported in the literature (Darrall, 1989; Wolfenden and Mansfield, 1991; Hassan et al., 1994; Schenone et al., 1994; Zhang et al., 2001). Moreover, the results of the present investigation indicated that the percentage decrease was higher in cultivar Victory than in the other two cultivars (Little Marvel and Perfection). The decrease in P_N was recorded before the appearance of visible O_3 injury (chlorotic and necrotic spots) on the leaves of NF and AA plants. By that time most leaves of the FA plants were still green. Similar effects were recorded previously in other studies on plants exposed to air pollutants in OTCs (Sanders et al., 1992; Schenone et al., 1994; Zhang et al., 2001). The maximum impairment of P_N occurred in 2013 when the recorded ozone levels were high. Moreover, the maximum impairment occurred during pod filling (55 days after exposure) and this could have influenced the yield, which was significantly lower for NF plants of the three cultivars (Hassan, 2008).

Photosynthesis is a good indicator of environmental stress. Most studies have demonstrated stomatal closure under stress, with a consequent decrease in P_N (Calatayud et al., 2004). However, environmental stress may have direct effects on chloroplast that cause a decrease in P_N, thus non-stomatal factors have roles in restricting P_N (Zheng et al., 2000; Feng et al., 2007). Therefore, P_N restriction could occur due to stomatal and/or non-stomatal factors (Shangguan et al., 1999). The closure of stomata results in a shortage of CO_2 (Boyer 1976). Non-stomatal factors include the followings: (1) an increase in diffusive resistance to CO_2 in the mesophyll; (2) a reduction in PS II; and (3) a decrease in chlorophyll content and inhibition of electron transport (Mackerness

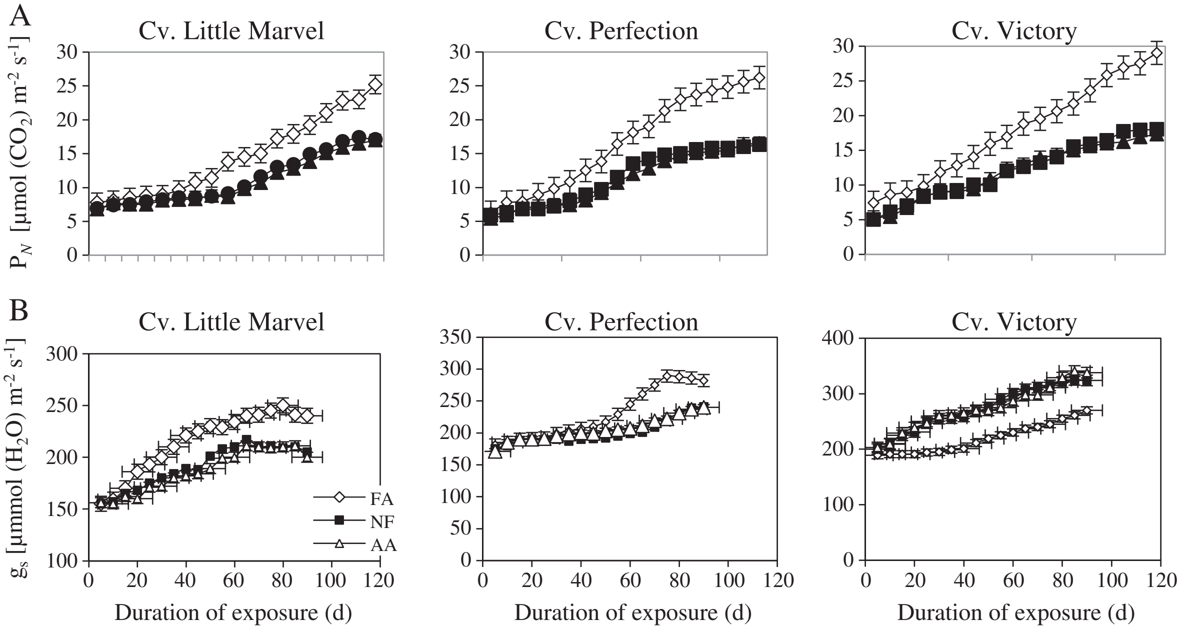

Fig. 1. Net photosynthetic rates (P_N) [µmol (CO$_2$) m^{-2} s^{-1}] (A), and stomatal conductance (g_s) [mmol (H$_2$O) m^{-2} s^{-1}] (B) of field-grown pea plants. The data shown in the figures are the means of five years. FA = filtered air. NF = non-filtered air, and AA = ambient air. (n = 15 ± 1 SE of the means).

2000; Hassan, 2006). If stomatal factors are the main factors, then P_N and g_s decrease owing to the decline in Ci. Otherwise, the decrease in P_N is dominated by non-stomatal factors.

The significant correlation between P_N and g_s indicated that stomatal closure could be the cause of the apparent reduction of P_N in the cultivars Little Marvel and Perfection. However, this was not the case in cv. Victory, where there was a negative correlation ($r = -1.45$, $P < 0.01$) between g_s and C_i (Fig. 2), suggesting that the reduction of P_N was not caused primarily by the lower g_s but rather by non-stomatal factors (Feng et al., 2007).

The regression of P_N versus C_i was analyzed to determine whether air pollutant(s) (mainly O_3 in the present study) had any effect on the amount of active ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco) or on the rate of ribulose 1,5 bisphosphate (RuBp) regeneration (Guidi et al., 2001). The results obtained in the leaves of cultivars Little Marvel and Perfection exposed to long-term non-filtered air seem to support the view that the decrease in quantum yield of electron transport may be the mechanism underlying the down regulation of photosynthetic electron transport. Thus, the production of ATP and NADPH would be in equilibrium with the decreased demand in the Calvin cycle, especially the reduction was related to a strong stomatal closure in these cultivars or to an altered mesophyll activity ascribed to the limitations of the dark reactions of the photosynthetic process. Amthor (1988) and Badiani et al. (1993) reported some biochemical modifications in NF plants such as a decrease in total ascorbic acid, an increase in total glutathione content and an increase in catalase activity.

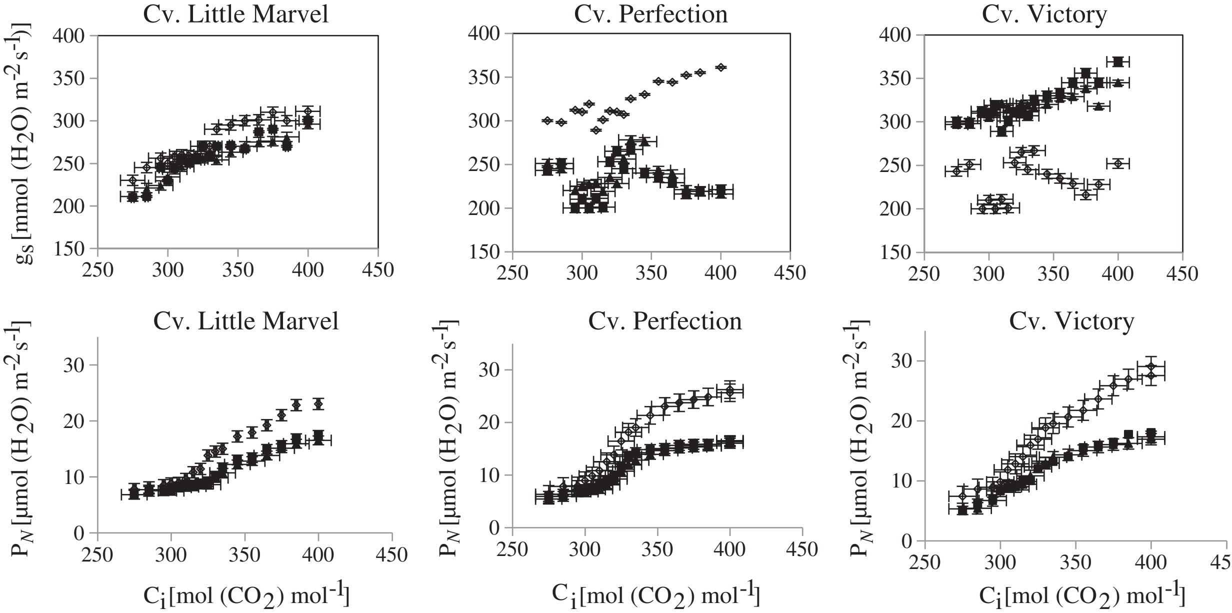

Fig. 2. Relationship between stomatal conductance (gs), photosynthetic activity (PN) and sub-stomatal CO2 concentration (Ci) [mol (CO2) mol^{-1}] in the three cultivars of pea (◊ = FA; ■ = NFA; ▲ = AA). (Abbreviations are the same as Fig. 1).

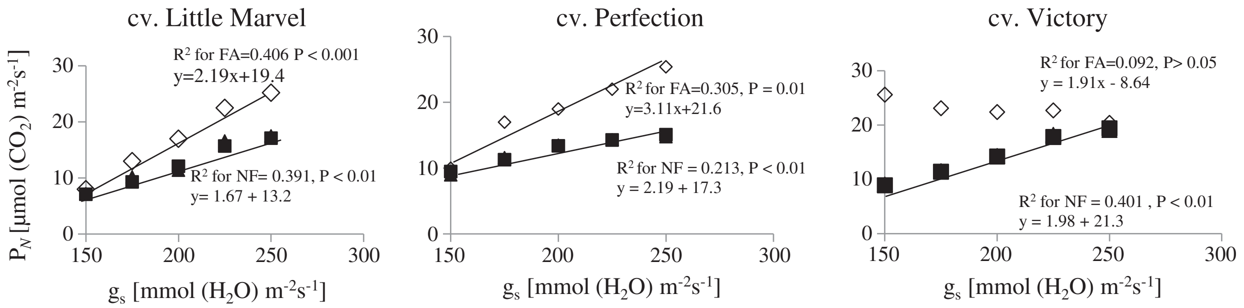

Fig. 3. Relationship between net photosynthetic rates (*P*N) and stomatal conductance (g_s) in pea leaves exposed to FA and NFA. (Abbreviations are the same as Fig. 2).

These changes suggest the occurrence of an oxidative stress, which causes an increased energy demand to support repair and maintenance processes, and which ultimately reduces net photosynthesis. Our results concerning the increased importance of the non-stomatal limitation of photosynthesis in pea plants exposed to air pollution are in agreement with similar observations in beans (Schenone et al., 1994) and in spring wheat (*Triticum aestivum* L.) (Lehnherr et al., 1988).

The variable to maximum fluorescence (F_v/F_m) ratio is a good indicator of the efficiency of the primary photochemistry of PSII and serves as a noninvasive indicator of the status of photosynthetic reaction centers (Plazek et al., 2000; Thwe et al., 2014). In contrast to the results of Farage et al. (1991) and Plazek et al. (2000), who found a reduction in P_N but no response in the photochemical efficiency of PSII (F_v/F_m), in wheat and barley, respectively, we found reductions in both P_N and the ratio of F_v/F_m in plants grown in NF or AA. These reductions indicated that ambient ozone inhibits the ability of crops to utilize photon energy and consequently changes the entire photosynthetic processes. These suggestions are supported by the alterations in other parameters such as Φ_{PSII}, and qP (Guidi et al., 1999; Calatayud et al., 2006; Thwe et al., 2014). However, plants exposed to NF had a higher NPQ,

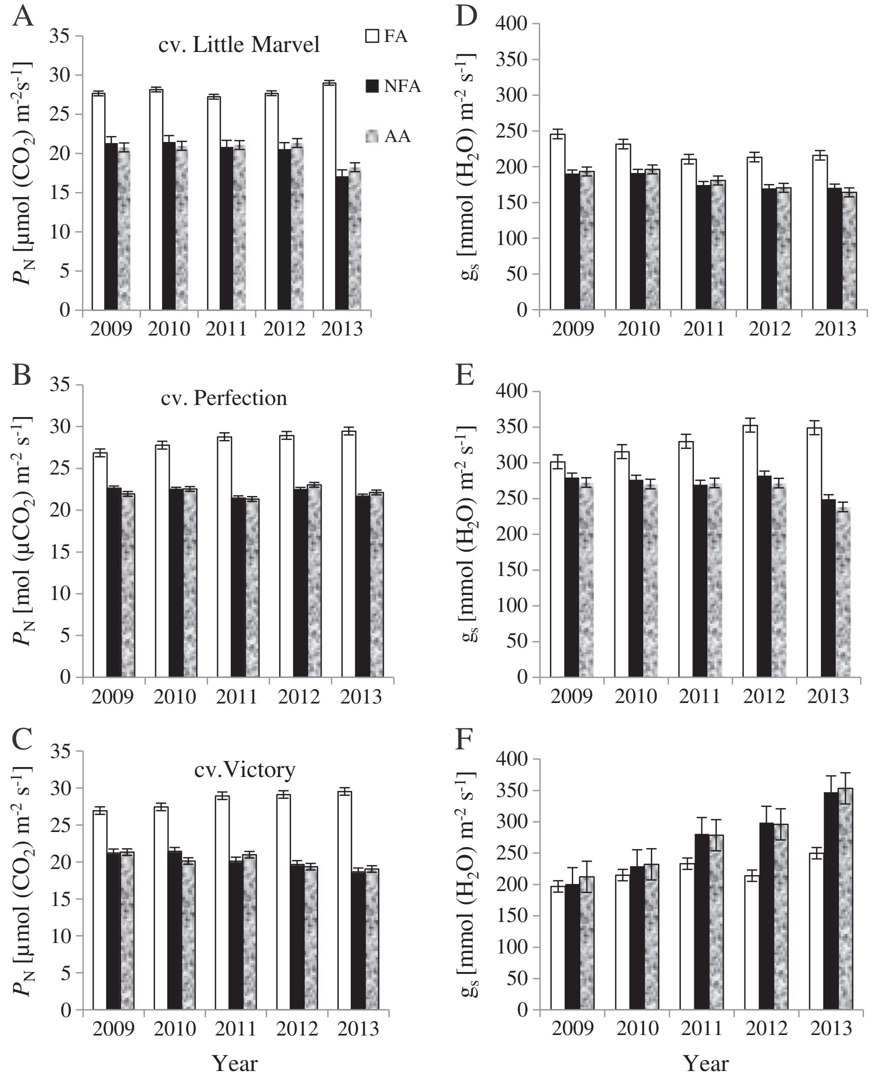

Fig. 4. Annual means of P_N [µmol (CO_2) m^{-2} s^{-1}] (A–C) and g_s [mmol (H_2O) m^{-2} s^{-1}] (D–F) of different pea cultivars of Pea, ($n = 15 \pm 1$ SE of the means).

Table 5

Chlorophyll a fluorescence parameters in the leaves of the three pea cultivars exposed to ambient ozone. Abbreviations: Fv/Fm, variable to maximal fluorescence ratio; Φ_{PSII} actual quantum yield of PSII; qP, photochemical quenching coefficient; and NPQ, non-photochemical quenching. The data represented are the means of five years ± SD. FA = filtered air. NF = non-filtered air. Means not followed by the same letter are significantly different from each other at $P < 0.05$ ($n = 10 \pm SD$).

Parameter	Treatment								
	Cv. Little Marvel			Cv. Perfection			Cv. Victory		
	FA	NF	AA	FA	NF	AA	FA	NF	AA
Fv/Fm	$0.82^{b} \pm 0.07$	$0.74^{a} \pm 0.05$	$0.75^{a} \pm 0.05$	$0.76^{b} \pm 0.06$	$0.65^{a} \pm 0.06$	$0.66^{a} \pm 0.07$	$0.82^{b} \pm 0.09$	$0.58^{a} \pm 0.06$	$0.59^{a} \pm 0.06$
Φ_{PSII}	$0.61^{b} \pm 0.06$	$0.43^{a} \pm 0.04$	$0.42^{a} \pm 0.04$	$0.59^{b} \pm 0.06$	$0.45^{a} \pm 0.05$	$0.46^{a} \pm 0.06$	$0.61^{b} \pm 0.07$	$0.41^{a} \pm 0.04$	$0.43^{a} \pm 0.04$
qP	$0.91^{b} \pm 0.05$	$0.78^{a} \pm 0.06$	$0.79^{a} \pm 0.08$	$0.88^{b} \pm 0.07$	$0.77^{a} \pm 0.08$	$0.75^{a} \pm 0.07$	$0.89^{b} \pm 0.08$	$0.71^{a} \pm 0.06$	$0.72^{a} \pm 0.07$
NPQ	$0.51^{a} \pm 0.03$	$0.97^{b} \pm 0.11$	$1.01^{b} \pm 0.09$	$0.63^{a} \pm 0.07$	$1.01^{b} \pm 0.09$	$0.99^{b} \pm 0.09$	$0.56^{a} \pm 0.07$	$1.13^{b} \pm 0.09$	$1.04^{b} \pm 0.08$

Table 6

Changes in chlorophyll (Chl *a*, *b*, total chl and Chl *a*/*b*) content [g kg^{-1} FW] in response to different treatments ($n = 5 \pm SD$). (Abbreviations are the same as those in Table 5).

Parameter	Treatment								
	Cv. Little Marvel			Cv. Perfection			Cv. Victory		
	FA	NF	AA	FA	NF	AA	FA	NF	AA
Chl *a*	$7.80^{b} \pm 1.1$	$6.30^{a} \pm 0.80$	$6.45^{a} \pm 1.11$	$6.20^{b} \pm 0.90$	$5.20^{a} \pm 1.05$	4.93 ± 0.87	$7.70^{b} \pm 0.80$	$5.41^{a} \pm 0.90$	$5.63^{a} \pm 1.02$
Chl *b*	5.70 ± 0.90	5.41 ± 0.60	5.21 ± 1.03	5.80 ± 0.80	5.60 ± 0.90	5.27 ± 0.76	$5.80^{b} \pm 1.10$	$4.10^{a} \pm 0.40$	$4.43^{a} \pm 0.91$
Tot. Chl	$13.50^{b} \pm 2.90$	$9.71^{a} \pm 1.90$	$11.66^{b} \pm 2.30$	12.0 ± 2.10	10.80 ± 2.10	10.20 ± 1.90	$13.50^{b} \pm 2.10$	$9.51^{a} \pm 1.60$	$10.06^{a} \pm 1.70$
Chl *a*/*b*	1.35 ± 0.20	1.16 ± 0.80	1.24 ± 0.09	1.11 ± 0.35	0.93 ± 0.22	0.93 ± 0.23	$1.33^{a} \pm 0.30$	$1.32^{a} \pm 0.30$	1.27 ± 0.50

which reveals lower photochemical quenching, indicating greater photoinactivation of PSII in plants grown under NF conditions (Park et al., 1995; Calatayud and Barreno, 2001; Guidi et al., 2001; Calatayud et al., 2004; Pellegrini et al., 2011). The higher value of NPQ indicates the ability to mitigate the negative effects of ambient O_3 at the chloroplast level, as these organelles have the ability to dissipate the excess excitation energy (Pellegrini et al., 2011). This finding was confirmed by the reductions in F_v/F_m ratios found in these plants.

Although, the three cultivars were exposed simultaneously to non-filtered air, their stomata responded differently to O_3, because exposure to NF air increased g_s in cv. Victory, but decreased it in the other two cultivars. This study is one of very few to demonstrate an opposite response of g_s to O_3 in closely related plants exposed simultaneously to non-filtered air under the same experimental conditions.

Reports in the literature on stomatal response to O_3 describe both increases and decreases in conductance (Darrall, 1989). It is not clear from these diverse reports whether these reported variations in stomatal responses to O_3 are related to the level and duration of exposure or to the climatic conditions during the experiment. In contrast, the present study provides clear evidence of a difference in stomatal response among cultivars. In the present experiment, the plants were exposed to relatively high levels of O_3 since emergence. Plant development and morphogenesis may therefore have been influenced, and the greater stomatal conductance observed in cv. Victory grown in NF air could be related to increased stomatal opening rather than increased stomatal number per leaf area, especially because the number of stomata on the three cultivars was very similar (data no shown). However, Schenone et al. (1994) related the increased in g_s to stomatal number rather than stomatal opening in bean plants. Moreover, stomatal opening could be the result of damage to epidermal cells. Importantly, visible injury symptoms (in the form of chlorotic and necrotic stipples) were more prominent in this cultivar. Hassan et al. (1994) attributed the increase in g_s in radish plants exposed to 50 nL L^{-1} O_3 to rupture of the epidermal cells adjacent to stomata, thus offering less mechanical resistance, exerting no back pressure on the guard cells, and allowing the stomata to open more widely. Recently, Paoletti and Grulke (2010) reported that a delay in stomatal responses (i.e. 'sluggish' responses) was due to O_3 exposure and they postulated that stomatal sluggishness is a cause of increased O_3-sensitivity in snap beans. This could also be the case in our study, however, further investigation is warranted.

In summary, photosynthetic CO_2 fixation in the chloroplast was inhibited by O_3 influx into the leaves. This inhibition induced an increase in C_i and stomatal closure (especially in the cultivars Little Marvel and Perfection), resulting in the reduction of g_s. The limitation of photosynthesis appears to be correlated with a reduction of the efficiency in the energy conversion of PSII. However, this was not the case in cultivar Victory, where increased g_s and can be considered as detrimental as it would increase air pollutant (O_3) flux and accelerate transpiration water loss. This may explain the greater sensitivity of the plants and indicates the importance of non-stomatal limitations of photosynthesis. In contrast, the decreased g_s in cvs Little Marvel and Perfection would reduce O_3 influx and consequently increase resistance to O_3-induced damage. Thus, the variability in the response of g_s to O_3 reported in the literature is not entirely due to differences in experimental conditions or methodology, but may be due to inherent differences between species.

The present study showed that ambient air pollution has a clear influence on the physiology of pea plants. Moreover, it is argued that the major air contaminant involved in causing the observed physiological effects was O_3, the presence of which at phytotoxic concentrations in the Mediterranean area has important impacts on vegetation.

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