PHYSIOLOGICAL, BIOCHEMICAL AND MICROMORPHOLOGICAL CHANGES IN EPICUTICULAR WAXIN LEAVES OF CITRUS LEAVES (*Citrus aurantium* L.) INDUCED BY OZONE IN EGYPT

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Abstract

Macro- and micromorphological changes in epicuticular wax, the peroxides activity, chlorophyll contents, photosynthetic rates (A), stomatal conductance (gs) and transpirational rates were investigated in the leaves of citrus plants (*Citrus aurantium* L.) grown in rural area un northern Egypt characterised by high levels of ambient ozone (O_3).

Exposure to ambient O_3 caused an increased rates of weathering of epicuticular wax, peroxidase activity, stomatal conductance and transpirational rate by43, 40, 37 and 39%, respectively, while photosynthetic rates and chlorophyll contents were decreased by 35 and 26%, respectively.

Keywords: ambient ozone (O_3), Egypt, citrus leaves, epicuticular wax, photosynthetic rates, peroxidase activity.

INTRODUCTION

Decline of trees observed in Europe, the USA and other industrialised parts of the world has led to speculation on possible effect of atmospheric pollution [4].

Citrus tress (*Citrus aurantium L.*.) are distributed in the Delta, Northern Egypt, the main agricultural land. However, a significant decline (defoliation and/or dieback) has been observed in recent years (Awad, person. Communication). Although several factors have been suggested to explain this phenomenon, the direct effect of oxidants was initially suspected [21], since the area of decline coincides well with the distribution of such air pollutants [20,22]. Soil acidification is also a possible factor, especially near tree bases. In addition, water deficiency may be a significant factor due to urbanisation [12, 20,42]

Nashinoto *et al* [29] reported a decline in cedar trees (*Cryptomeria japonica* D Don) in Japan due to oxidant pollutants, soil acidification and water stress [27 & 33]

Structural degradation of epicuticular wax has been attributed to the influence of O_3 [17, 35, 36 & 37], which may imply, that this degradation is coupled with a reduction of wax.

Increased leaching of ions has been reported to be another effect of O_3, which would lead to higher conductivity of leaves [17]

To the present knowledge, however, there has been little information about the physiological and functional changes in citrus trees in area of decline.

With regard to physiological changes, it has been suggested that oxidant air pollutants could affect the cuticle layer, especially the epicuticular wax, directly or indirectly [2, 3, 26, 35, 36 & 39]. In their studies Mengel *et al*.[28]; Barnes & Brown [2,3] ; Cape [9], reported that in *Picea abies* (L.) Karst, acid deposition and oxidant air pollutant accelerates melting and/or erosion of epicuticular

wax and change its chemical composition, thus increasing surface wettability [2, 7]. Since the cuticular layer plays an important role against water loss and pathogen infection, these changes may have serious effects on physiological functions of plants such as increase the cuticular transpiration rate [28, 35 & 36]

Plants tend to produce a large quantity of epicuticular wax under drought conditions, probably to compensate the lost water. Bondada *et al.* [6] reported that cotton plants (*Gossypium hirsutum* L.) grown under drought conditions showed increased amounts of epicuticular wax. These results were supported by results of Saneoka & Ogata [34], who reported similar results with forage crops.

Therefore, degradation of wax may case serious water stress and sometimes lethal damage to plants [35, 36]

Amount of epicuticular wax may be affected by leaf age [8, 9 & 31] and by its morphology and chemical composition [16]. Nevertheless, structural degradation of epicuticular wax has been attributed to the influence of O_3 [37], which might imply that this degradation is coupled with a reduction of wax [2, 3 & 17]. However, several studies indicated that the properties of epicuticular wax may be affected not only by oxidant air pollution but also by natural environmental factors such as leaf age, morphology and composition of wax [2, 3, 8, 9, 10, 17, 31 & 36].

There is a lake of experimental evidence concerning the effects of O_3 on growth, physiology, biochemistry, and the micrmorphology of epicuticular waxes of Egyptian trees.

The present study was undertaken to fill the above mentioned gap of knowledge and to clarify the alteration in epicuticular wax, photosynthetic rates, stomatal conductance and peroxidase levels (as biochemical marker in response to O_3 stress) in leaves of citrus (*Citrus aurantium* L.).

MATERIALS AND METHODS

Sampling of citrus leaves under Egyptian filed conditions

Leaves of *Citrus spp*. were collected from a rural site in northern Egypt (Abbis village). The description of the site and topography are discussed elsewhere [22]. Sampling of leaves was carried out from 5 years old trees and was carried out from October to April for three consecutive years (2001 – 2003). Controlled samples were collected in parallel with samples from trees grown in unpolluted glasshouse.

The leaves were collected in icebox and transferred to the laboratory and kept at 5 ^{0}C overnight until analysis in the following day.

There was no any microbial infection or morphological changes in the wax under these conditions [32, 35 &36]. Moreover, under these conditions, leaves remained fresh without any water loss.

During transportation and keeping of leaves, care was taken not to abrade the wax.

Photosynthetic rates (A) and stomatal conductance (gs) measurements

A and gs were measured once a week, regularly on the same leaf throughout the entire course of the experiment over the three successive years using LICOR-1600 (Licor, Nicolin, USA)

Extraction of Epicuticular wax

Epicuticular wax was extracted according to [8] and the modified method [35 & 36] Forty grams of fumigated and non-fumigated leaves was taken from different branches on the trees. Leaves were washed with deionised water for about 5 min (the washed leaves showed no loss in the wax due to washing). The washed leaves were dried in an oven at 39 ^{o}C for 45 min and allowed to dry on a laboratory bench at room temperature (25 – 30 ^{o}C). The weight of prepared leaves was regarded as fresh mass, since the water content of leaves remains constant throughout the whole process, including storage and washing [35 & 36]. Three grams of prepared leaves was shaken for 25 sec with 30 ml chloroform to extract epicuticular wax. The wax extracts were then filtered through quartz wool and a filter paper (Watmann No. 3) to remove aerosols adsorbed on the leaf surface. Six batches were prepared initially for each set of fumigated and non-fumigated leaves, and two of them were combined to make triplicate composite extract. The chloroform was then evaporated and

the residual wax was determined gravimetrically (Barnes, J.D., Pers. Communication). The amount of wax was expressed on a fresh mass (mg/g fresh leaf weight).
The wax samples were refrigerated using a filter Cartridge (Millipore, pore size 0.5 μm) and stored in liquid nitrogen (- 80 ^{o}C) for analysis of chemical composition.

Measurement of cuticular transpiration

Twenty leaves rinsed with distilled water and dried gently against a paper towel at a room temperature. The cut part of leaves was covered with grease to avoid excessive water loss.
Leaves were weighed and then stored in a desiccator at (25 – 30 ^{o}C) I the laboratory. Leaves were weighed daily at 12:00 h until the weight was constant (after 7 days) which indicates cuticular transpiration [28].

Determination of elemental composition of epicuticular wax

The C, N and O contents were determined according to Sase *et al*. (1998) with an elemental analyser (Fisons, EA 1110). Sulfanilamide ($C_6H_8O_2N_2S$, Fisons) was used as a standard sample for N, 1-Docosanol ($C_{22}H_{46}O$, Fluka Chemie) was used for O and benzoic acid ($C_6 H_5$ COOH, Fisons) for N.

The analyses were repeated twice for each sample.

Statistical analysis

One-way ANOVA was applied to log-transformed data. There was no covariate used except for photosynthetic rates and stomatal conductance, where photosynthetic flux density (PPFD) was used as a covariate. Significant difference between means tested by LSD (STTATGRAPHICS Statistical Package).

RESULTS

The mean concentrations of major air pollutants (O_3, SO_2 and NO_x) through the growing seasons are shown in table 1.

		Pollutant		
Year	**Experiment**	O_3 (8h d^{-1})	SO_2 (6h d^{-1})	NO_x (6h d^{-1})
2001	NF	71	10	7
	FA	11	3	3
2002	NF	79	8.7	6
	FA	10	3	2
2003	NF	91	7	5
	FA	11	3	2

Table 1. Mean concentrations (nl l^{-1}) of major pollutants during the growing seasons at Abbis village in the charcoal-filtered (FA) and non-filtered (NF) OTCs.

Air quality at the experimental site was characterised by very low concentrations of SO_2 and NO_x, where the mean 6-h concentrations of these gases through the four growing seasons were 8.5 and 6.4 nl l^{-1}, respectively table 1). The mean 8-hour concentrations of O_3 through the growing seasons (2001 –2003) averaged 80 nl l^{-1} (table 1).

Table 2 shows the amount of wax collected from leaves of citrus plants grown in filtered and non-filtered air. The amount of epicuticular wax (mg/g dry leaves) was found to decrease year by year

due to exposure to unfiltered air: it was 35, 42 and 51% reduction in 2001, 2002 and 2003, respectively. Moreover, there was a strong positive correlation between the loss of wax and the ambient O_3 levels in the study area ($R^2 = 1.10$, data not shown).

Experiment			
tment	)/ 2000 – 4/2001)	0/2001 – 4/2002)	10/2002 – 4/2003)
	19.3	20.1	19.9
	12.6 (-35%)	11.7 (-42%)	9.8 (-51%)

Table 2. Amount of wax (mg/g fresh leaf); CF = Charcoal-filtered air, NF = non-filtered air; Figures between parthenses are percentage difference from the control.

The chlorophyll content (mg/g) of leaves was shown in table 3. It was indicated that chlorophyll contents of plants grown in non-filtered air was significantly lower than that collected from plants grown in filtered air (averaged 35% for the three seasons). Moreover, the highest percentage reduction in chlorophyll was recorded to be –42% in 2003 and this was also significantly correlated to O_3 levels recorded in the region ($R^2 = 0.97$).

Experiment			
tment	)/ 2000 – 4/2001)	0/2001 – 4/2002)	10/2002 – 4/2003)
	8.2	7.4	7.3
	5.8 (-29%)	5.1 (-31%)	4.4 (-42%)

Table 3. Chlorophyll content (mg/g fresh leaf); Legends as table 1.

Table 4. shows that C,N and O contents of epicuticular wax. Parallel with the decreased in chlorophyll contents of leaves and the loss of wax, the concentrations of C,N and O contents were significantly lowered in non-filtered plants.. The lowest percentage reduction was in C content (ca. 20%), while that of N and O contents was 56% each (averaged between the three experimental seasons).

Experiment									
	)/ 2000 – 4/2001)			0/2001 – 4/2002)			10/2002 – 4/2003)		
tment									
	4						3		
	5			8			7		

Table 4. Elemental composition of epicuticular wax; Legends as table 1.

To evaluate the effect of wax reduction on plant physiology, cuticular transpiration rate was examined (Table 5). Exposure to non-filtered air caused an increase in cuticular transpiration, and the highest percentage increase (ca.64%) was found in 2003 and this was correlated to the increase in O_3 levels ($R^2 = 0.85$) and the loss in wax ($R^2 = 0.98$).

Experiment			
tment	)/ 2000 – 4/2001)	0/2001 – 4/2002)	10/2002 – 4/2003)
	0.45	0.53	0.61
	0.61	0.82	1.36

Table 5. Cuticular transpiration rate (%h); Legends as table 1.

Table 6. Shows the there was an increase in peroxidase activity in leaves exposed to non-filtered air (averaged ca. 40%) compared to that extracted from plants grown in filtered air.

Experiment			
tment	)/ 2000 – 4/2001)	0/2001 – 4/2002)	10/2002 – 4/2003)
	5.0	8.4	4.5
	5.6	8.9	9.4

Table 6. Peroxidase activity (μmol g^{-1} fresh weight) in leaves of *Citrus aurantium* L.; Legends as table 1.

Figure 1 a. Shows that photosynthetic rate (A) of plants grown in non-filtered air was decreased by about 26%. On the other hand, stomatal conductance (gs) was found to be increased by about 37% (Fig 1b).

DISCUSSION

The concentrations of O_3 recorded in the present study further a support for previous results [22 & 42] that suggested that it is unlikely to have SO_2 and NO_x in a rural site in Egypt, which has been supported recently [20]. Moreover, the results of the present investigation are in agreement with the results of Anjea *et al*. [1] who reported relatively high levels of O_3 in a rural site in the USA and Schenone & Lorenzini [38] who had similar results in Italy.

It seems very reasonable to conclude that ambient O_3 played a major role in causing the observed alterations in morpho-functional parameters examined here (epicuticular wax and peroxidase) in plants exposed to non-filtered air.

The observed changes in epicuticular wax structure are in agreement with the effects of pollutants until now described for coniferous species [25, 35, 36 & 37], as there are only very few studies investigating the effects of air pollutants on degradation of waxes on broad-leaved plants [6 & 26]. Manes et al [26] found a serious alteration of the epicuticular waxes in *Nicoaiana tabacum* leaves collected from urban areas, where there was a greater accumulation of particulate matter and epicuticular waxes formed an amorphous fused layer.

In general, waxes decrease with age [7, 8, 31, 35 & 36]. This reduction occurs and is sometimes accelerated by factors such as wind [18, 19 & 41] as well as by air pollution [2, 23, 24 & 40].

It was suggested that air pollution contribute to the erosion of wax. Therefore, the relatively low amount of epicuticular wax in leaves collected from non-filtered plants. Moreover, the amount of epicuticular waxes collected in 2003 was lower than that collected in the other two seasons, this may have resulted from aging of leaves, affected mainly by higher O_3 levels recorded in 2003.

The increased stomatal conductance (reduced electrical conductivity) was suggested to be resulted from impeded uptake or plant-internal distribution of the ions, especially there was loss in elemental composition of leaves, especially in N content. However, the decreased contents of measured elements cannot alone cause the total reduction. It is also possible that fewer organic substances had leached into the diffusate especially there was degradation in epicuticular wax [26].

The negative correlation (R^2 = -0.98) between cuticular transpiration and the amount of wax, could provide a strong evidence for the potential stress due to O_3 in the region, which may have altered the characteristics of epicuticular wax in citrus tress grown in non-filtered conditions and this is in agreement with the results of O'Toole et al. [30]; Bengston et al. [5] and Jordon et al. [24] on rice, oat and sorghum, respectively. These authors reported that cuticular transpiration rates increased after removal of wax. It is suggested that the amount of wax plays a key role in regulating the cuticular transpiration rate in citrus plants. Moreover, the increased stomatal conductance (g_s) may cause an increase in cuticular transpiration especially there was stomatal malfunction observed (data not shown), this is in agreement with the results that reported an increase in g_s of radish leaves after exposure to 89 nl l^{-1} [20] and they attributed this increase in g_s to destruction of epidermal cells and stomatal malfunction. This phenomenon

has been suggested to cause incomplete stomatal closure of stomata, resulting in water loss [11 , 13, 14 & 35].

A rise in peroxidase activity due to ozone stress, as occurs in leaf senescence [5, 10 & 26), would represent an induced protective reaction. This enzymatic system plays an important role in the maintenance of an adequate redox potential in leaf cells and may protect the cell membrane from active oxidant arising from exogenous sources [15]. Although peroxidase test has proved to be a valid marker of acid stress, more work is needed to evaluate this test as marker of a stress attributable to atmospheric pollution.

In Egypt, the temperature is increasing, while the precipitation has decrease year after year and thus the atmosphere has shifted towards dry conditions. Therefore, it is likely that citrus trees suffer significant water stress, not only because of enhanced transpiration through the combined effect of degraded wax layer and partial malfunctioning of stomata, but also because the recent dry conditions. This stress may prove lethal at times, especially there are some unexplained symptoms appeared on trees in the field such as dieoff and/or defoliation of treetop, even though the lower part of trees may be apparently healthy.

Nevertheless, the parameters examined in he present study showed that the plants respond to O_3 with a pattern similar to that described for senescence. Therefore, the action stress may be interpreted as an accelerative process of aging.

In general, the results of the present investigation are important not only as basic information on the physiological properties of Citrus plants, but also for future evaluation of the effect of anthropogenic environmental factors on plants.

In conclusion, O_3 accelerate the natural aging of wax and increased frequency of stomata occluded by an amorphous plug, which would be expected to have far reaching physiological consequences in vivo, such as increased number of diseased and dying trees in Egypt.

More work, however, is urgently needed to identify the effects of season and the relationships between the impact of O_3 on tree species growing in natural sites, which differ in exposure and average concentrations of major gaseous pollutants.

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