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Effects of ozone on cell organelles of alfalfa (*Medicago sativa* L.) seedlingsMohamed El-Zaidy^{a,*}, Abdulrahman Almusalim^a, Abdulaziz Alsahli^a, Abdullah Doaigey^a, Sobhy M. Yakout^b, Ibrahim A. Arif^c, Ibrahim A.S. Saleh^c, Omar Shair^a^a Department of Botany and Microbiology, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia^b Department of Biochemistry, College of Science, King Saud University, Riyadh 11451, Saudi Arabia^c Prince Sultan Research Chair for Environment and Wildlife, Department of Botany and Microbiology, College of Sciences, King Saud University (KSU), Riyadh, Saudi Arabia

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ABSTRACT

Seedlings of alfalfa (*Medicago sativa* L.) were exposed to different concentrations of atmospheric ozone (20–30 (control), 40–60, 65–80, and 85–120 ppb) in four distinct areas in the Riyadh region, so as to decide how ozone affected some of the seedling cellular organelles. Results acquired utilizing transmission electron microscopy demonstrated certifiable impacts to exist on the cell organelles in the tissues of both the leaf mesophyll and stem cortex; contrasted with control plants, the chloroplasts seemed enlarged, irregular, different sizes, decomposed, and possibly dissolved, while the plastoglobules seemed deformed, more widely spaced, and enlarged, also the vacuoles contained no clear non-living components. Moreover, some parts of the cytoplasmic membranes were ruptured, with only a few vesicles created at all concentrations, particularly in plants exposed to concentrations of 65–80 and 85–120 ppb, while no effects were noted in these organelles in control plants or plants exposed to 40–60 ppb. High concentrations (85–120 ppb) led to enlarged, irregularly shaped nuclei and chromatin intensification; however, no clear effects of ozone were noted on the shapes of chloroplast starch grains or the mitochondria in leaf mesophyll and cortex cells in the stem. The high ozone concentrations can cause negative effects on the growth of alfalfa seedlings, leading to imbalances in their vital functions and acceleration of aging, thus potentially decreasing the total plant yield. The discoveries hence propose that alfalfa plants should not be planted near polluted areas, and that they can be utilized as bioindicators of air pollution by ozone.

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1. Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important fodder crops because of its excellent fodder value (Radovic et al., 2009). Ozone gas is an air pollutant that has significant adverse effects on vegetation (Bytnerowicz et al., 2007). Several studies have been led on the effect of ozone on various plants. Plants exposed to high concentrations of ozone gas have been shown to accumulate oxidizing substances, leading to the appearance of bulges on the cell walls and protoplast separation, a lack of intensification of chromatin material in nuclei, and an increase in vacuole size that accel-

erates cell aging and leads to chloroplast damage in mesophyll layer cells (Vollenweider et al., 2003). A study of the effect of different ozone gas concentrations on *Betula pendula* has revealed that high ozone concentrations cause a decrease in the size of starch grains and an increase in the size of plastoglobules and chloroplasts, as well as thylakoid deformation, tannin accumulation in vacuoles, and increased cellular wall thickness (Repo et al., 2004). Plants exposed to high ozone concentrations show clear effects on the internal structure of their leaves, including: an apparent thickening of the outer walls of epidermal cells; enlarged, irregular and inflated chloroplasts within cells; impurities and accumulation of tannins in the vacuoles; cell walls showing abnormalities such as bulging; and an increase in intercellular spaces. These factors all result in an acceleration of cell aging, and consequent damage to the plant (Reig-Armiñana et al., 2004; Zahalan et al., 2009). In other studies of the effect of ozone on different crop types, a reduction in chloroplast size, thylakoid swelling, and an increase in the size of the spherical globules has been observed (Amparo et al., 2010; Bréhélin and Kessler, 2008; Gravano et al., 2003; Miyake

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et al., 1989; Oksanen et al., 2004; Pääkkönen, 1995; Violini et al., 1992). In similar studies, exposing the plant to ozone gas leads to thylakoid separation and a decrease in the number and size of starch grains in chloroplasts in the mesophyll of new leaves (Pechak et al., 1986). It also leads to the accumulation of starch grains and vacuoles around the nuclei, as well as a modification of the shape and number of chloroplasts (Kivimaenpaa, 2003). Ying-ying et al. (2008) have found that exposing wheat to high ozone levels resulted in anatomical effects, such as the rupture and separation of cell walls from cytoplasm, and irregular grana in leaf chloroplasts. Giacomo et al. (2010) studied the anatomical characters of leaf palisade mesophyll tissue cells in two different plants in order to determine their tolerance and resistance to ozone gas, and found that ozone caused apoptosis in sensitive plant leaves; the authors conclude that nucleic shrinkage, chromatin intensification, and cell wall shrinkage are indicators of apoptosis.

Because of its worldwide financial significance, and the rareness of studies exploring the effects of ozone gas on the anatomical characteristics of the cell organelles of alfalfa, we conceived the idea of this present research. Our work was aim to study the effect of ozone on some cell organelles of alfalfa seedlings.

2. Materials and methods

Measurements of ozone gas concentrations using air pollution measurement system (Aeroqual Series 200 Monitor Multi sensors), that were carried out using part per billion (ppb) units daily beginning from germination to harvest the crop. Average readings were calculated monthly during the experiment period to confirm ozone concentrations prior to determining the places of study, planting dates and growth periods of crop. Four sites were identified based on their ozone concentrations (Table 1). The pots were filled with soil at the same time and at one level, to prevent water from pooling and to ensure an even water distribution. The soil was sterilized with fungicides (dose of 1.2 g L⁻¹ water). The alfalfa seeds were washed with distilled water and then planted in the autumn of 2017. The experiment was distributed in 10 replicates of 52 cm pots per site; 10 to 15 seeds were placed in each pot. The pots were irrigated daily with 200 ml water in winter. After the seedlings had grown and reached a height of 2–3 cm, five plants were retained in each pot, and the fertigation program was set at 4 g per liter of water every 15 days, using Norus (20:20:20 + TE) fertilizer for all stages of plant growth. The experimental plants were treated with malathion insecticide once every 10 days at a dose of 1 cm³ pesticide per liter of water. At three months after planting (i.e., once the plants had reached maturity and were just flowering), the plants were harvested and samples prepared for anatomical study by Transmission Electron Microscopy (TEM) in several steps according to the method of Guzmán et al. (2014), including killing and fixation, washing with buffer solution, dehydration, clearing, infiltration, embedding, trimming, sectioning, loading on to copper nets,

and staining. The internal structures of leaves and stem cell organelles, such as the chloroplasts, vacuoles, nuclei, and mitochondria, were investigated with digital photography using a TEM (JEOL JEM1011) equipment connected to a computer, in the Central Laboratory, Faculty of Science, King Saud University.

3. Results and discussion

The results of this study into the effects of different ozone concentrations on the components of living cells (microstructures) indicated that some parts of the palisade tissue cell walls in the leaves ruptured at ozone concentrations of 85–120 ppb (Table 2; Fig. 12) when compared with the control plants (Table 2; Fig. 9). This finding is consistent with the results of Reig-Armiñana et al. (2004), but not with those of Repo et al. (2004), where the authors determined that high ozone concentrations led to increased thickness of cellular walls. Ruptures occurred in some sections of the cytoplasmic membranes of the leaf palisade tissue cells, and a number of bulges were noted in some plant cells when exposed to ozone concentrations of 65–80 and 85–120 ppb (Table 2; Fig. 11,12). Ruptures and bulges were noted in the cytoplasmic membranes of collenchyma tissue cells in plant stems under all ozone concentrations, when compared with the control plants (Table 2; Fig. 17–20). This finding agreed with results obtained in other studies (Evans and Ting, 1974; Faoro and Iriti, 2005). In comparison with the control treatment, the palisade tissue cell chloroplasts in the leaves and collenchyma tissue cells in the cortex were affected by all concentrations of atmospheric ozone gas, appearing irregular in shape (often round to sometimes oval), and having different sizes and being often overgrown; these effects all increased with increasing ozone concentrations (Table 2; Fig. 1–4, and Fig. 13–16, respectively). This finding is consistent with previously reported findings (Madkour and Laurence, 2002; Miyake et al., 1989; Pääkkönen, 1995; Reig-Armiñana et al., 2004). In comparison with the control treatment, the effect on the degradation of granum groups in the chloroplasts was evident at ozone concentrations of 65–80 and 85–120 ppb (Fig. 3, 4); this is consistent with results noted in other studies (Gravano et al., 2003; Oksanen et al., 2004; Rinnan, 2004).

There was no clear effect observed on starch granules in the chloroplasts in any of the ozone concentrations. There was, however, a demonstrable effect on the plastoglobules, which increased in size with increasing ozone concentration levels, compared with the control plants (Table 2; Fig. 13–16); these results were consistent with that of the other studies (Miyake et al., 1989; Repo et al., 2004; Violini et al., 1992), in which the authors observed that chloroplast plastoglobules increased in size when the plants were exposed to high concentrations of ozone. No significant effect was observed on the mitochondria, which were observed to be of different sizes, and often round to oval in shape (Table 2). However, there was an obvious effect on the nucleus, which appeared enlarged and irregular in shape, with dense chromatin, whereas in the control plants it appeared almost oval (Table 2; Fig. 5–8); this is supported by the findings of Vollenweider et al. (2003), who suggest that cell exposure to ozone gas affects the nuclei and intensifies chromatin. Additionally, the vacuoles containing non-living components were shown to be large in palisade tissue cells exposed to ozone concentrations of 65–80 and 85–120 ppb, when compared with those of the control plants (Table 2; Fig. 5,7,8). The results of our study are consistent with the findings of Reig-Armiñana et al. (2004), which suggest that exposure to ozone gas leads to the degradation of some components of the cell and the vacuoles becoming filled with obvious impurities. Inter-cellular spaces in the spongy tissue were also more extensive under exposure to all concentrations of ozone compared with that in

Table 1
Ozone concentrations (ppb) at four sites.

O ₃ concentration at sites (ppb)	Distance from the control site (km)	Coordinates
30–20 (control)	—	11,52,24,45 Lat. 42,07,46,21 Lon.
40–60	15.15	41,57,24,44 Lat. 38,24,46,30 Lon.
65–80	33.95	34,76,24,40 Lat. 14,91,46,41 Lon.
85–120	59.66	21,21,24,32 Lat. 11,29,46,54 Lon.

Table 2
The effect of ozone concentration (ppb) on cell microcomponents in alfalfa leaves and stems.

Cell organelles		Ozone concentration (ppb)			
		Control (20–30)	(60–40)	(80–65)	(120–85)
Cell wall		Clear and obvious in leaf and stem	As control	As control	Ruptured in some parts
Cytoplasmic membrane		Appear particularly in leaf and stem	As control in leaf, but in stem some bulges appear in the cytoplasm	Ruptured in some parts and some bulges formed inside the cytoplasm of leaf and stem	Ruptured in some parts and some bulges formed inside the cytoplasm of some leaf and stem cells
Cytoplasm	Chloroplasts	Shape and size	Oval shape, different sizes in leaf and stem	Irregular to often round and sometimes oval; different sizes and often overgrown in leaf and stem	Irregular to often round and sometimes oval; different sizes and overgrown in leaf and stem
	Granum and thylakoid	Chloroplast contains some grana (containing thylakoid) in leaf and stem	As control in leaf and stem	Contains few grana (contains thylakoid) in leaf and stem, but sometimes not clear in leaf	Contains few grana (contains thylakoid) in leaf and stem, but sometimes not clear in either leaf or stem
	Starch grains	Few, different sizes in leaf and stem	As control in leaf and stem	As control in leaf and stem	As control in leaf and stem
	Plastoglobules	Small size in leaf and stem	Large in size compared with control leaf and stem	Large in size compared with control leaf and stem	Large in size compared with control leaf and stem
Mitochondria		Shape and size	Round to often oval shape; different sizes in leaf and stem	As in control leaf and stem	As in control leaf and stem
Nuclei		Shape and size	Oval shape in leaf only	Overgrown, irregular shape, chromatin was dense in leaf only	Overgrown, irregular shape, chromatin was dense in leaf only
Vacuoles		Shape and size	Large, do not contain clear non-living components clearly, in leaf only	As control, in leaf only	Large, clearly contain non-living components, in leaf only

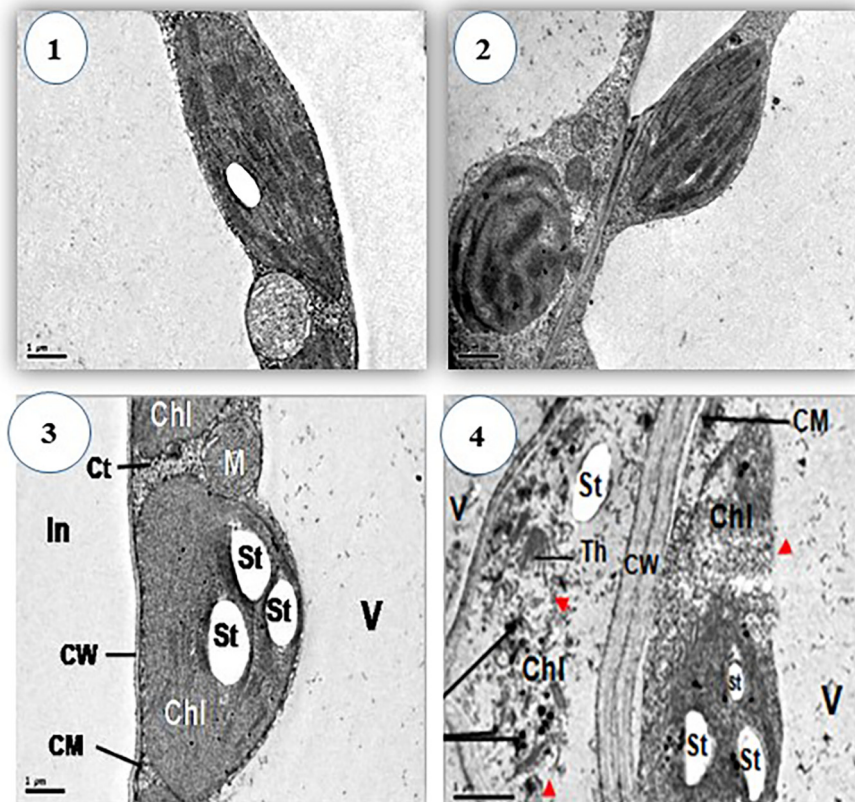


Fig. 1–4. Chloroplast structure of leaf blade mesophyll. 1; Control treatment (Ozone gas, 20–30 ppb). 2; (Ozone gas, 40–60 ppb). 3; (Ozone gas, 65–80 ppb). 4; (Ozone gas, 85–120 ppb).

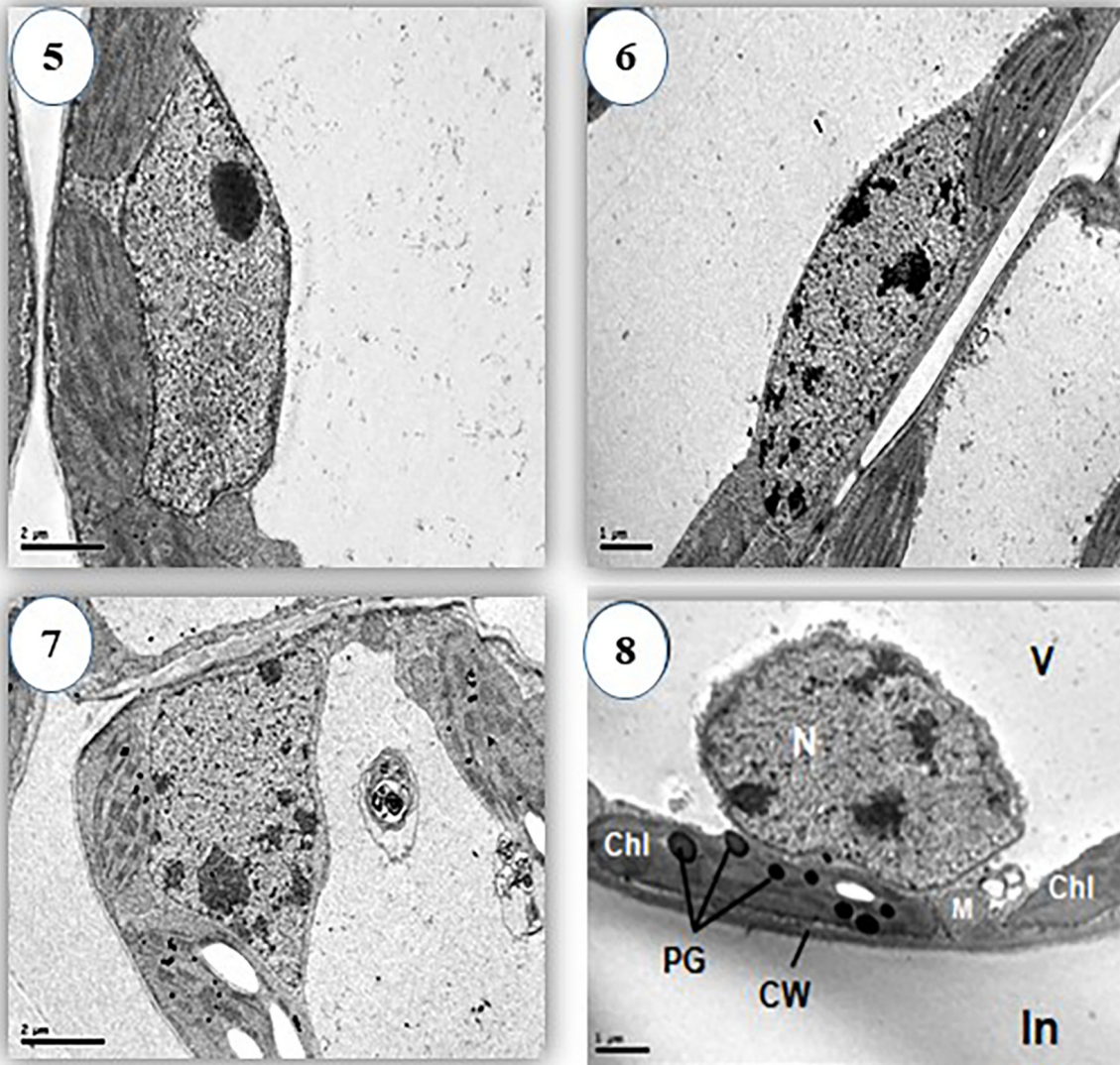


Fig. 5–8. Nucleus structure of leaf blade mesophyll. 5; Control treatment (Ozone gas, 20–30 ppb). 6; (Ozone gas, 40–60 ppb). 7; (Ozone gas, 65–80 ppb). 8; (Ozone gas, 85–120 ppb).

the control plants (Table 2). This finding was in agreement with Al-Muwayhi et al. (2014), who have determined that exposing eggplant (*Solanum melongena*) to high concentrations of ozone affects the internal structure of the leaf cells (such as by an increase of the intercellular spaces in the spongy tissue).

Overall, the results of our study determined that high ozone concentrations have a negative effect on alfalfa plant growth. This is consistent with several reports indicating that ozone exposure leads to: accelerated plant aging (Booker et al., 2009); imbalances in vital cell functions resulting from different anatomical effects, such as cell wall fragmentation (Ying-ying et al., 2008); and nucleus enlargement, irregularity, and chromatin intensification, which is an indicator of apoptosis, as illustrated by visual damage marks (Giacomo et al., 2010). High concentrations of ozone gas also affect chloroplasts in the plant cells, changing their micro composition, thereby damaging their total chlorophyll content; this leads to a decline in the rate of photosynthesis (Zouzoulas et al., 2009), which in turn decreases the yield (Amparo et al., 2010).

4. Conclusions

As indicated by the results of our study, ozone exposure causes obvious effects on cell organelles in the tissue cells of both the leaf mesophyll and stem cortex; chloroplasts appeared enlarged and irregular, were of different sizes, decomposed, and possibly dissolved. The plastoglobules seemed deformed, spaced, and enlarged. The vacuoles contained unclear non-living components, some parts of the cytoplasmic membranes were ruptured, and only a few vesicles were created at all ozone concentrations, particularly in plants exposed to concentrations of 65–80 and 85–120 ppb. High ozone concentrations led to enlarged, irregularly shaped nuclei and to chromatin intensification; however, no clear effects of ozone were noted on starch grain shapes in chloroplasts or the shapes of the mitochondria in the leaf mesophyll and stem cortex cells. Finally, a high ozone concentration was observed negatively influence the growth of alfalfa seedlings, which creates imbalance in the vital functions of the plants and accelerated aging, and thus decreases

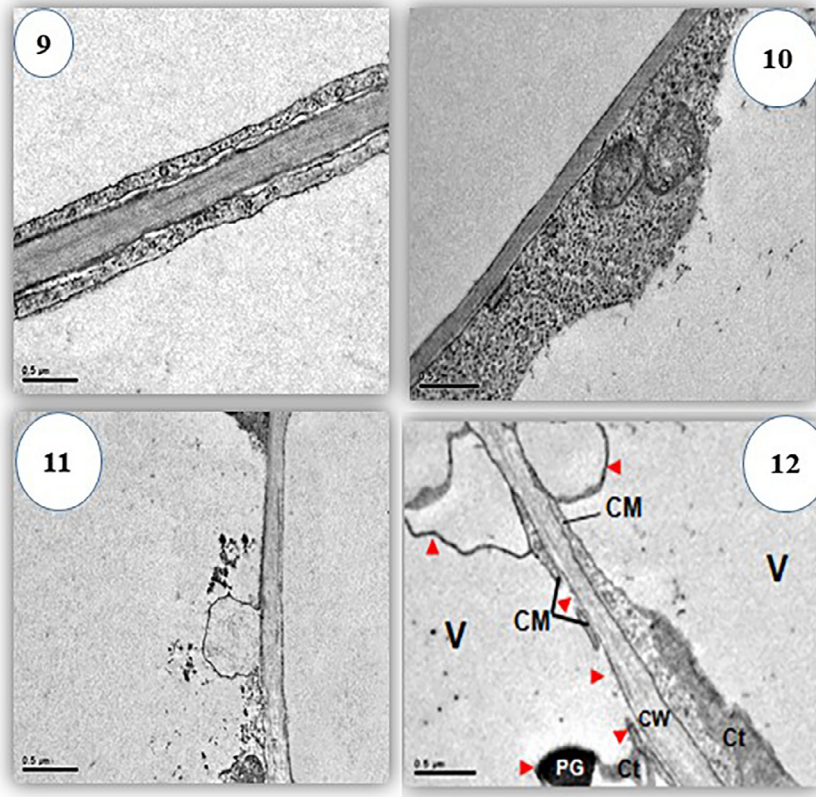


Fig. 9–12. Cell wall structure of leaf blade mesophyll. 9; Control treatment (Ozone gas, 20–30 ppb). 10; (Ozone gas, 40–60 ppb). 11; (Ozone gas, 65–80 ppb). 12; (Ozone gas, 85–120 ppb).

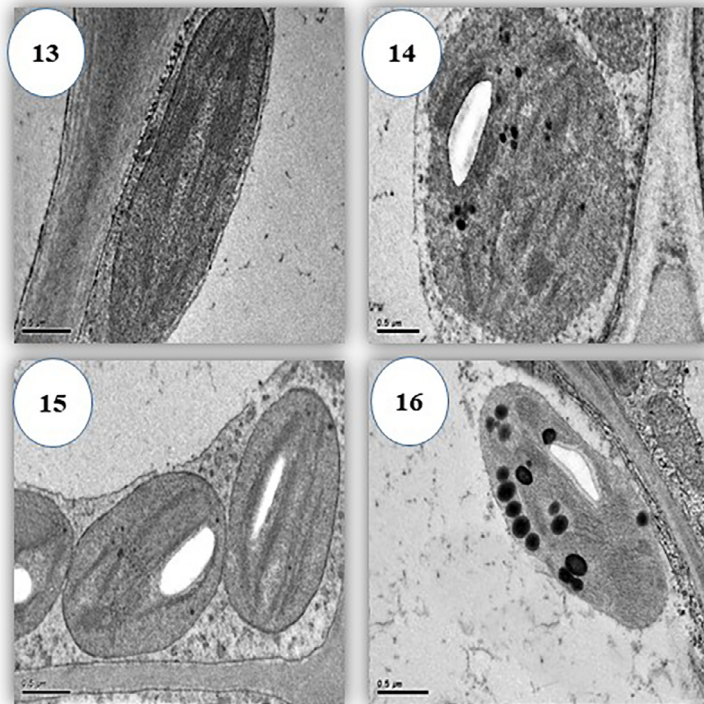


Fig. 13–16. Chloroplast structure of stem cortex. 13; Control treatment (Ozone gas, 20–30 ppb). 14; (Ozone gas, 40–60 ppb). 15; (Ozone gas, 65–80 ppb). 16; (Ozone gas, 85–120 ppb).

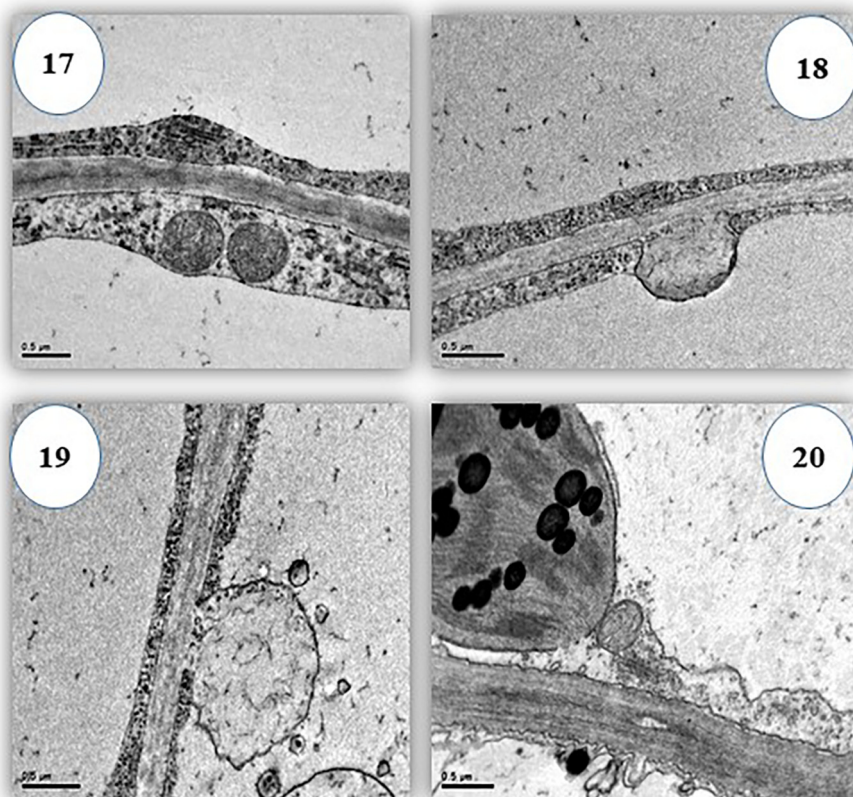


Fig. 17–20. Cell wall structure of stem cortex. 17; Control treatment (Ozone gas, 20–30 ppb). 18; (Ozone gas, 40–60 ppb). 19; (Ozone gas, 65–80 ppb). 20; (Ozone gas, 85–120 ppb).

the total plant yield. Therefore, the results of our study suggest that alfalfa should not be planted in close proximity to polluted areas, and when planted, can be used as a bioindicators of ozone-induced air pollution.

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