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Ultrastructural Responses of Faba Bean (*Vicia faba* L.) Plants Irrigated by Treated and Untreated Sewage Water

Suzan A. Abdallah[1] • Hassan M. Emara[1*] • Awatif I. El-Nahas[2]

[1] Department of Botany, Faculty of Science, Benha University, Benha, Egypt
[2] Department of Biological Sciences and Geology, Faculty of Education, Ain Shams University, Cairo, Egypt
Corresponding author: * **hassanmemara@yahoo.com**

ABSTRACT

Faba bean (*Vicia faba* L. cv. 'Giza 2') seeds were cultivated in untreated (effluent) and treated (influent) sewage water, while control seeds were cultivated in tap water. The ultrastructure of 9-week old leaves and roots was investigated. The effects of biotic agents (fungal endophyte and/or a fungal pathogen(s)) and heavy metals were also demonstrated. Chloroplasts from plants growing in effluent water exhibited ill-defined thylakoids in a very dense stroma whereas those from plants growing in influent sewage water displayed a normal ultrastructure compared to control stroma. Moreover, transmission electron micrographs showed other effects such as advanced vacuolation, dilated rough-endoplasmic-reticulum cisternae and separation of the plasma membrane from the cell wall. Dense cytoplasm rich in ribosomes and lipids, small vacuoles with electron-dense granules was also observed. The common host reactions to the invading fungus were also investigated. Intracellular penetration of the epidermal, cortical, and mesophyll cells by fungal hyphae was also observed. In addition, the host cell wall had a loosely organized fibrillar appearance at the contact sites with the fungal hyphae.

Keywords: faba bean, light microscope, sewage water, transmission electron microscope

INTRODUCTION

Rainfall is scarce in Egypt; even the small amount which normally occurs over the Delta comes during winter when crop demands are low. Consequently, agriculture almost entirely depends on irrigation from the Nile River besides the ground water. These farmlands are so-called "old lands". Some additional land (semi-arid areas) has been and is being reclaimed to satisfy the increase in population. Therefore, it is imperative to find an alternative source for irrigation such as sewage water or water recycling after treatment.

Using treated sewage water is economically important especially in arid and semi-arid lands (El-Ameen *et al*. 2005). The basic advantages of using treated sewage water are preventing the pollution of growing plants. Hence, eliminating the hazardous effect on the surrounding community may sustain the biological diversity in and between a population of plants. Little work has been done on the safety of using untreated and treated sewage water with respect to the context of human, feed and plant health (Rank and Nielsen 1998; El-Nahas 1999). This is due to the growing utilization of wastewater sludge as a fertilizer in agriculture; however, genotoxic chemicals such as heavy metals, which can harm organisms in an ecosystem as well as humans during accumulation of the food chain, should be considered and evaluated. Moreover, the heavy metal content in plants can also be affected by other factors such as the application of fertilisers, sewage sludge or irrigation with wastewater (Devkota and Schmidt 2000; Frost and Ketchum 2000; Muchuweti *et al*. 2006).

It is known that certain heavy metals cause DNA damage and their carcinogenic effects in animals have been assumed to be related to their mutagenic activities (IARC 1976). This DNA damage was extended to include humans and plants. Knasmüller *et al*. (1998) tested the genotoxic effects of heavy metals and their salt-contaminated soils in meristematic root tip cells of faba beans. Zhang and Xiao (1998) reported that Cd^{2+} toxicity resulted in a decrease in the mitotic index of root cells and production of chlorophylic mutations.

Herbaceous leguminous plants can be used as pioneer species, which solve the problem of nitrogen deficiency in soils, improve soil quality, and reduce weed occurrence (Yang *et al*. 2003). Some herbaceous legumes (e.g. soybean, Adzuki bean, mung bean and peanut) could successfully grow on contaminated soils (Liu *et al*. 2005).

High concentrations of metals can cause severe damage to plants, hence inhibiting their growth and reproduction. The leguminous *Vicia faba* L. might be of interest to grow on metal-contaminated sites with deficient nitrogen and/or irrigated by sewage water. But since it is considered as sensitive to metal uptake (AFNOR 2004; White and Claxton 2004; Probst *et al*. 2009), it is thus of interest to check if it develops any special mechanisms to grow on sewage water and to inhibit toxic metal uptake and storage.

For this purpose, a pot experiment on soil was carried out to: (i) identify metal translocation from the substrate to plant tissues of *V. faba* L.; (ii) determine the ultrastructural cell changes under extreme experimental conditions; (iii) discuss toxic metal uptake and storage in the root and leaf cells, and (iv) assess the presence of endophytic fungi and/or plant fungal pathogen(s) and their effect on morphological responses of plants as a survival strategy.

MATERIALS AND METHODS

Water sources

Sewage water (treated/influent and untreated/effluent) was obtained from the El-Berkah plant, El-Salam City, Cairo, Egypt. Tap water was used for watering control plants. For measuring heavy metals in irrigation water, water samples were filtered via 0.45 µm Millipore filter paper, dried, acid-digested, and measured by an atomic absorption spectrophotometer (Z-6100, Hitachi, Tokyo, Japan).

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Original Research Paper

Plant materials and the treatments involved

Seeds of faba bean (*Vicia faba* L. cv. 'Giza 2') were obtained from the Agricultural Research Center, Legume Research Department, Giza, Egypt. Homogeneous seeds (similar size) were soaked for 24 hours in control, untreated (effluent) and treated (influent) sewage water, separately then cultivated in 15-cm diameter pots containing a mixture of clay and sandy soil (2: 1) irrigated regularly once a week until the maturation stage (9 weeks), under greenhouse conditions at 22-25°C. The third leaf from the apical tips was collected and the sections were done near the midrib. Root samples were collected 3 cm before the tips of the main tape root. Leaves and roots samples were subjected to histological and ultrastructural studies using a light microscope (LM) and transmission electron microscope (TEM).

Metal analysis in leaves and roots of faba bean plants

For measuring heavy metals in leaves and roots of faba bean plants in different treatments, leaf and root samples were oven dried at 85°C until constant weight and 1.0 g dry weight was ashed in a muffle furnace at 550°C for 6 hrs. Ash was acid digested with nitric acid and diluted with 2 N HCl to a constant volume. Heavy metals were determined using an atomic absorption spectrophotometer (Z-6100, Hitachi, Tokyo, Japan) according to Hernandez *et al.* (2003).

Tissue processing for TEM

Both leaves and roots of different treatments were cut into 3 mm pieces and processed as described by El-Nahas (1999). Samples were excised into a fixative of 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) at room temperature (18-20°C) and post fixed overnight in 2% osmium tetroxide (Sigma-Aldrich, St. Louis, MI, USA) in 0.1 M phosphate buffer for 30 min at 4°C. After rinsing in the buffer, tissues were dehydrated in an ethanol series and placed into two changes of propylene oxide (Sigma-Aldrich) for 30 min each. The tissues were infiltrated overnight in a 50: 50 mixture of propylene oxide: araldite on an agar specimen rotator, rotating at 3 revs/min and then transferred to meat araldite (25: 75 mixture of propylene oxide: araldite), which was polymerized at 60°C overnight (Luft 1961). Ultrathin sections were cut on a Reichert Jung ultramicrotome with glass knives (Leica UK, Milton Keynes, UK). The sections were collected on 100 mesh copper grids and stained with Reynolds lead citrate for 20 min (Reynold 1963). The sections were examined in a JEM Jeol, 1200 transmission electron microscope (Jeol UK Ltd, Welwyn Garden City, UK).

Light microscopy

Semi-thin sections (1.0-1.5 μm) of resin-embedded material prepared as for TEM were stained for 2 min with 1% toluidine blue in 1% borax at 60°C and examined with a Zeiss ultraphotomicroscope (Carl-Zeiss photomicroscope III, Germany).

RESULTS

Metal concentrations in treated and untreated sewage water

Metal concentrations in control, T1, and T2 water are listed in **Table 1**. T1 was characterized by high concentrations of Al, Cd, Ni, and Zn as compared with control. In contrast, the concentrations of Cr, Co, Cu, Fe, and Pb were low; so, the effects of these metals were not supposed to significantly affect plant growth. The concentrations of these metals were reduced in the treated sewage water.

Metal concentrations in tissues of faba bean

The most accumulated metals in the leaves and roots tissues of faba bean plants were Fe, Cu, Zn, and Al (**Table 2**). Among the four elements, Fe concentration in all faba bean tissues was the highest, followed by Cu in all treatments. Zn and Al concentrations were quite low. The results showed that faba bean roots contained higher Fe, Cu, and Al more than leaves, ranked as Fe > Cu > Al. Zn concentration was higher in leaves than roots in all treatments.

Table 1 The concentrations of heavy metals in dechlorinated tap water (control), treated sewage water (T1), and untreated sewage water (T2) at the beginning of the experiment.

Heavy metals (ppm)	Control	T1	T2
Al	0.0	0.01	0.20
Cd	0.03	0.10	0.30
Cr	0.00	0.02	0.03
Co	0.00	0.00	0.02
Cu	0.00	0.02	0.04
Fe	0.00	0.01	0.02
Pb	0.00	0.02	0.05
Ni	0.00	0.1	0.20
Zn	0.00	0.00	0.1

Table 2 The concentrations of accumulated heavy metals (mg/g dry weight) in leaves and roots of faba bean plants germinated and irrigated with tap water (control), treated sewage water (T1), and untreated sewage water (T2).

Heavy metals	Control		T1		T2	
(ppm)	Leaves	Roots	Leaves	Roots	Leaves	Roots
Al	0.09	0.19	0.03	0.37	0.11	0.36
Cd	0.03	0.00	0.01	0.02	0.01	0.03
Cr	0.01	0.06	0.01	0.08	0.02	0.12
Co	0.01	0.03	0.00	0.02	0.01	0.02
Cu	0.25	0.35	0.60	0.60	0.30	1.00
Fe	0.21	0.91	3.48	10.26	6.93	9.62
Pb	0.01	0.09	0.09	0.06	0.05	0.28
Ni	0.01	0.07	0.05	0.11	0.03	0.13
Zn	0.12	0.43	0.62	0.58	0.61	0.55

Changes in the tissues of faba bean plant grown on treated and untreated sewage water

The ultrastructure of leaves derived from T1 and T2 showed marked differences compared to that irrigated by control water (**Fig. 1A-C**). The nucleus is larger in cells of T2 plants than of other samples. The chromatin material in the nucleus of leaves cells of T2 plants was more electron dense than those of control and T1 plants. In addition, the nucleus in T1 and T2 plant leaves was irregular in shape (**Fig. 1B**).

The ultrastructure of chloroplasts from T2 plants leaves displays abnormalities where its stroma lamella was compacted and not well defined as compared to control and T1 plants leaves (**Fig. 2A**). The grana stacks were compacted and had high electron dense particles. Numerous plastoglobules (lipid droplets) were seen in the chloroplasts of T1 plants as compared to T2 (**Fig. 2A**, **2B**). The chloroplasts from the control samples were oval and round-shaped, characterized by distinct starch grains, which were more abundant as compared to T1 and T2 samples. These ultrastructure characteristic features of the chloroplasts indicate well-developed grana stacks and also there were numerous, small, highly contrasted plastoglobules present in the stroma. An increased number of chloroplasts was also observed in T2 samples and they were small and elongated with poorly defined stroma (**Fig. 1A**). In T1 plants, the chloroplasts had deformed shape; they were curved shape and swollen with few starch grains (**Fig. 1B**). The thylakoid membranes can hardly be distinguished as compared to those of control and T1 plants, but in T1 plants they were arranged irregularly in the stroma and few observed starch grains present in the deformed chloroplasts associated with small and fine electron dense particles (**Fig. 2B**).

When root cells were examined by electron microscopy, mitochondria revealed no differences among the tested plants (**Fig. 3A-C**). The proplastids in all samples were different with respect to: 1) the electron density of their mem-

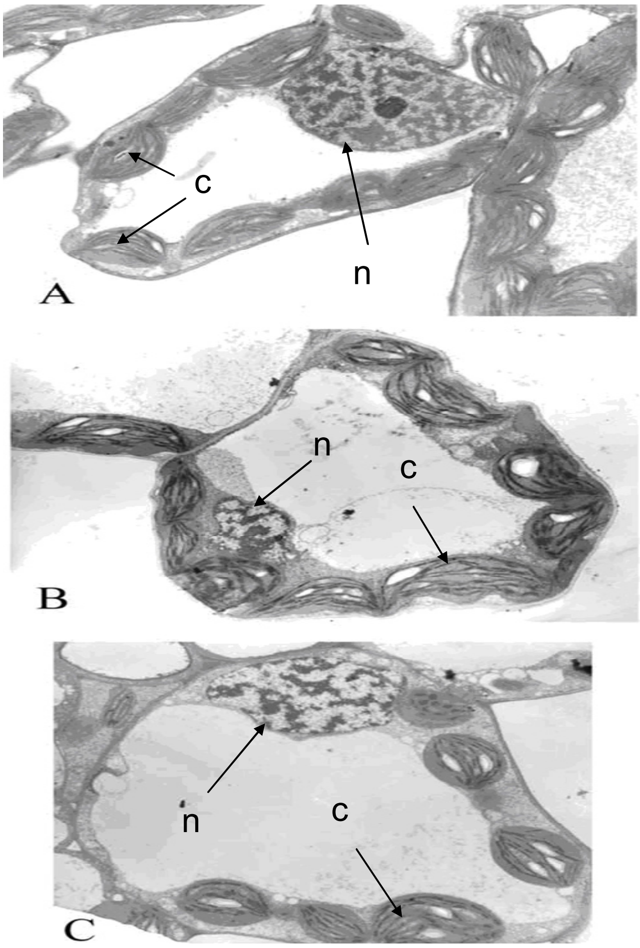

Fig. 1 Ultrastructure of epidermal cells of mature leaves of plants irrigated by untreated sewage (**A**), treated sewage water (**B**), and control water (**C**) under greenhouse conditions. The size of nucleus (n) in **A** is larger than those in **B**, and **C**. In **A** and **B**, the nuclei have irregular shape, while in **C** the nucleus is normal. There are large number of chloroplasts (c) in **A** more than **B** and **C**.

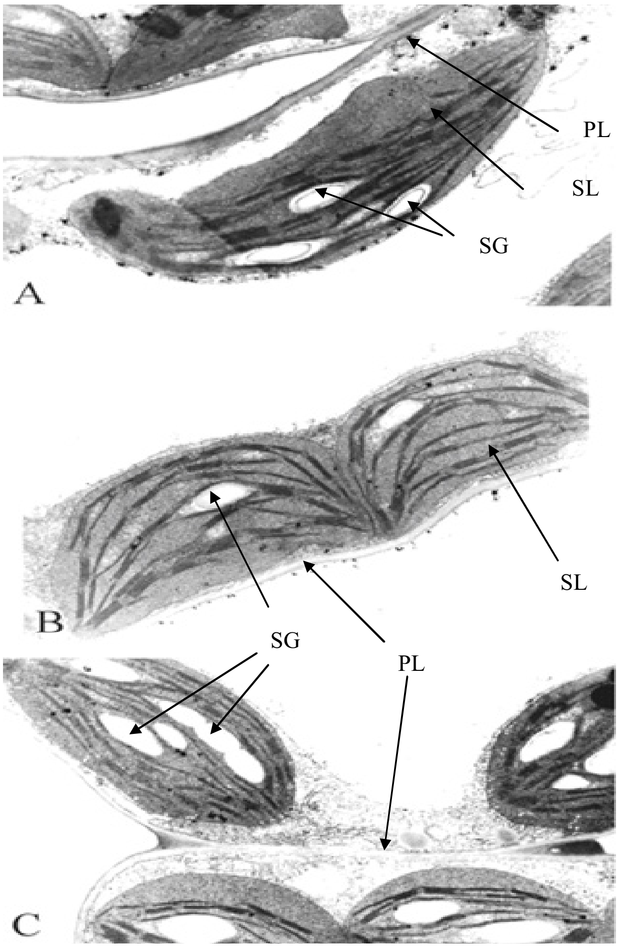

Fig. 2 The changes in the chloroplasts in leaves of plants irrigated by untreated sewage (**A**), treated sewage water (**B**), and control water (**C**) under green house conditions. Few phospholipids droplets are scattered in **A** and **B** as compared to **C**. The chloroplast was oval and round in **C**, while it was not close to the normal case in **A** and **B**. In **A**, the stroma lamellae (SL) were arranged irregularly in stroma and had more electron dense particles. The starch granules (SG) in **A** and **B** were compact and have more electron dense particles as compared to **C**. The plasto-globules (PL) in the chloroplasts are numerous, small, and highly contrasted in stroma in **C**; these characters were reduced in **A** and **B** and deformed.

brane looked more electron dense in the control as compared to untreated and treated samples; 2) the number of lipid droplets were rarely seen in the untreated and treated ones (**Fig. 3D, 3E**). A difference in the proliferation of the proplastids was also observed. Ribosomes, endoplasmic reticulum, and Golgi apparatus were also seen very distinct in the plants irrigated by treated sewage water as compared to the untreated and control ones. On the other hand, various vesicles were only abundant in the untreated ones and invagination of the plasmalemma (**Fig. 3D**).

Light microscopy observations showed that leaves epidermal cells of control plant were regular, intact, and bulliform. The penetration and colonization by fungal hyphae in the epidermal cells of the leaves of T2 only were evident. However, epidermal, bulliform, mesophyll, and colorless cells lost their turgidity, chloroplasts were not visible, and the plasma membrane of the cell was invaginated. Some chloroplasts of mesophyll cells adjacent to cells contained fungal hyphae were destroyed, few vascular complexes were seen away from the penetration sites, and they were normal and intact. Adjacent cells which did not invade or affect by hyphae or its product (s), seemed to be intact and normal (**Fig. 4B**).

Further hyphal growth seen in the mesophyll and the penetrated cells were disorganized. Lignified cells seen as they retained the toluidine blue stain in their walls (**Fig. 4A**). Most fungal infections occurred on the abaxial surface. But penetration and colonization of abaxial and adaxial surface were similar. The fungus grew in mesophyll and parenchyma cells. Hyphae were also near the midrib of the leaves (**Fig. 4A**, **4C**).

Hypertrophy and/or hyperplasia of leaves host cells were observed in T1 and T2 plants (**Fig. 4A**, **4C**), but were more abundant and distinct in T1 samples (**Fig. 4C**). In T1 leaves, the xylem and phloem were not invaded. The bundle sheath seems to act as barrier to the growth of the penetrating hyphae (as they were restricted in lateral growth). The diseased or stressed areas are seen in **Fig. 4C**. Some of the spongy cells were degenerated and contained vesicular structures associated with fine cytoplasmic granules (**Fig. 4D**).

In root tissues of T2 plants, the parenchyma cells surrounding the vascular bundle were penetrated by fungal hyphae (**Fig. 5A**, **5B**). Furthermore, remnance of fungal hyphae were also evident as seen in hyperplasia of root tissue.

DISCUSSION

The response of faba bean to metal contamination

The chemical and biological characterization of sewage water can help in providing an adequate assessment of their genotoxicity and potential hazard. Faba bean plants in the present study have been shown to accumulate several heavy metals that cause genotoxic effects on the roots and

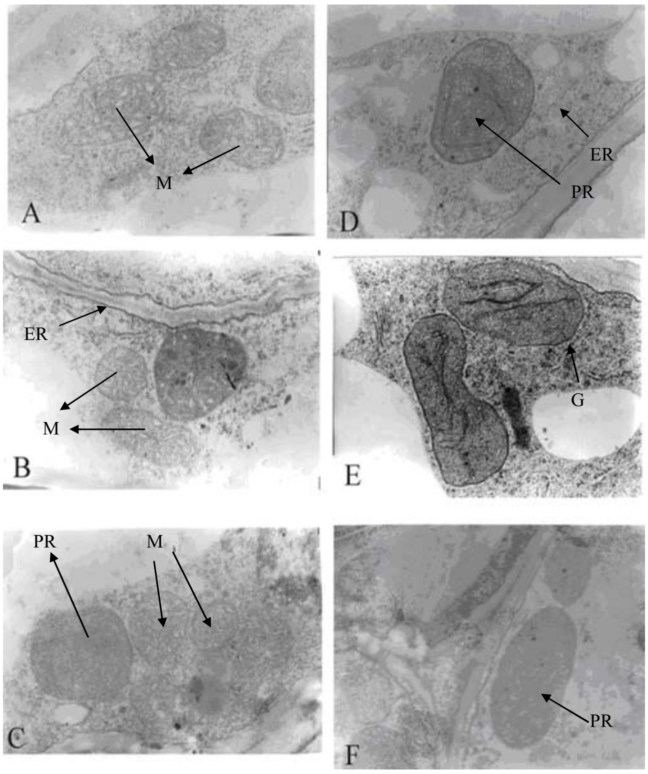

Fig. 3 Ultrastructure of epidermal cells of root tissues of plant irrigated by untreated sewage (**A**, **D**), treated sewage water (**B**, **E**), and control water (**C**, **F**) under green house condition. There were no changes in the mitochondria (M) among **A**, **B**, and **C**. Note the different proliferation of the proplastids (PR) present in **D**, **E**, and **F**. In **D** and **E**, the proplastids (PR) were not fully developed. Note also the electron dense membrane of the proplastids (PR) in **E** and it is in less density in **D** as compared to **F**. Electron-dense particles associated with ribosomes in **E** in addition to distinct Golgi apparatus (G) and abundant endoplasmic reticulum (ER) with electron-dense ribosomes.

leaves of the tested plants, as well as by other researchers (IARC 1976; Snow 1992; Rossman 1995). Moreover, the obtained data herein clearly showed both chemical and morphological plant responses depending on the kind of metal and water conditions. In this experiment, faba bean accumulated the metal inside its leaves and roots (**Table 2**). However, this response was not linear and depended on metal, water characteristics, and plant tissue. Similar results were obtained by Probst *et al.* (2009) who studied the response of *Vicia faba* L. to metal toxicity on mine tailing substrate and investigated the metals in the substrates and their translocation in root, stem and leaf tissues. They reported that metal concentration, and generally bioaccumulation, was in the order: roots > leaves > stems, except Pb and Cd. However, metal concentration in root and leaf was not proportional to that in the substrates.

Up to a certain critical limit of metal concentration, some mechanisms of plant uptake regulation take place regarding metal contamination. This was attested by the metals concentrations, which generally decreased from roots to leaves, and with increasing level of water contamination. Nevertheless, metal concentration in plant depends not only on water metal concentration level but also on metal type and on the water characteristics. Fe and Cu, and Al were significantly stored in plant roots more than leaves, whereas Zn was particularly concentrated in leaves more than roots. Metal accumulation is in the order of root > leaf, except for Zn (leaf > root); this indicated the plant ability to regulate metal transfer. In this regard, Marcato-Romain *et al.* (2009) found genotoxic effects of Cu and Zn on faba bean and phytotoxic symptoms appeared at higher concentrations of both metals. Probst *et al.* (2009) reported that Pb, Cd, Cu, and Zn were the most severe contaminants, which affected the ultrastructure of faba bean tissues in leaves, roots, and stem.

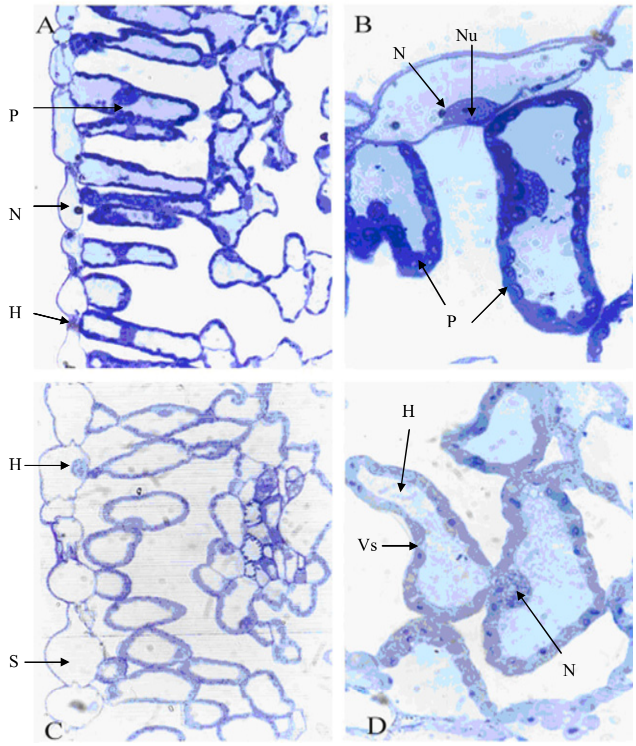

Fig. 4 Resin-embedded transverse sections of leaves tissues of faba bean plant irrigated by untreated sewage water showing the observed stained tissues with toluidine blue. (**A**) Leaf of plants irrigated by untreated sewage water showing, destruction of mesophyll cells; note leaf vein region (LV), damage disorganized palaside cells (P); note the presence of fungal hyphae (H) in spongy cells of the mesophyll tissue (X250). (**B**) A higher magnification of A showing disrupted-epidermal cells and palaside cells; note, the plasmolosis of the epidermal cells and the difference in staining intensity and the nucleus shape (X320). (**C**) Leaf tissues of plants irrigated by treated sewage water; note breaking and swelling of the epidermal cells (S), fungal colonization showing hyphae filling the epidermal cells (H). The differences in the shape and size of the epidermal cells and the palaside and spongy cells are clearly seen (X250). (**D**) A higher magnification of C showing large nucleus (N) with nuclei (Nu) in disorganize cell and small vesicles (Vs) at the site close to penetrating hyphae (H) (X320).

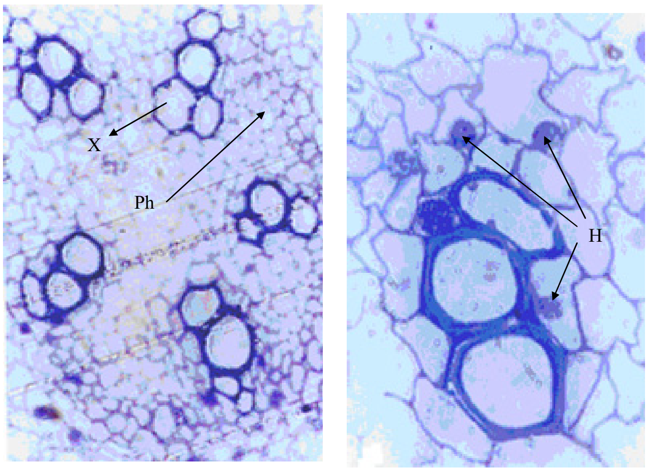

Fig. 5 Transverse sections in roots tissues of faba bean plant irrigated by untreated sewage water. Note the phloem (Ph), xylem (X), and the surrounding parenchyma cells (in **A** and **B**) showing the penetrating fungal hyphae (H). Magnification was X320 in **A** and X440 in **B**.

Damage in the leaves of faba bean grown in treated and untreated sewage water

The irrigation by sewage water could result in severe damage to the crops and severe economic losses. The present study shows that, based on light and electron microscopy, the existence of fugal hyphae in both leaf and root tissues may represent plant fungal pathogen(s) derived from the untreated sewage water and transmitted to faba bean plants. In this context, the existence of fungal hyphae in the examined organs (**Figs. 4**, **5**) indicates that faba bean could be naturally infected as a result of using the untreated sewage water.

Since the disorders in plasma membrane could be a central event in allowing the access to nutrients from the host. The damage of plasma and chloroplasts membranes observed in T2 plants could possibly be attributed to the production of a toxin by certain pathogen(s). These findings were commonly reported with some pathogens such as *Botrytis fabae* and *Alternaria alternata* (Nishimura *et al.* 1974) that produced toxins result in damage of the plasma membrane and chlorosis. Several researchers found similar observations involving the determination of the onset changes occurring during the pathogenesis of microbial invasion to the plants (Zimmer 1970; Heath 1972; Abdallah 1981, 1989).

Of the main findings of the current study are the heavy metals which should be taken to account for the genotoxicity observed in the examined tissues. In this regard, El-Nahas (1999) and Rank and Nielson (1998) demonstrated that faba bean and *Allium cepa* accumulated several heavy metals when water sludge was used for irrigation. The toxicity of the metals accumulated was manifested as chromosome aberrations in both the faba bean and *Allium cepa* root tissues. Moreover, Williams *et al.* (1990) found that the presence of heavy metals affected the genotoxicity of the roots and leaves of faba bean seedlings watered by the sewage water at molecular level using RAPD-PCR.

The detection of fungal hyphae in the parenchyma cells in the mesophyll tissue of the leaves (**Fig. 4**) suggests that the fungal hyphae release protein-bound iron. This was strongly evident from the detection of electron dense particles deposited along the cell wall of the leaves tissues from T2 plants. El-Nahas (1999) has found that the accumulation of few electron-dense particles was detected in T1 samples as compared to T2 ones. She also found other ultrastructural changes at the cell wall of faba bean leaves in T1 samples beside a reduction of the thickening of the cell walls. In T2 samples, the plasma membranes were seen disrupted and electron dense particles were accumulated near the plasma membranes. But the cell walls of T1 samples possess smaller numbers of those globules; these globules have been implicated in the micronutrient uptake and utilization by plants. Moreover, the increased number of electron dense particles in T1 plants (**Fig. 2A**) is similar to previous findings by El-Nahas (1999).

Damage in the roots of faba bean grown on treated and untreated sewage water

Excessive damage of root tissues was manifested in T2 plants (**Figs. 3D, 3E**, **5**). This damage may be attributed to the existence of fungal hyphae in the root tissues and may represent a fungal pathogen(s), causing this damage to the root tissues. However, this study does not exclude the possibility of the presence of a fungal endophyte in the treated and untreated sewage water. Likewise, the damage of root tissues observed in T1 and T2 plants could possibly be attributed to the production of a toxin by certain pathogen(s), as stated before.

In addition, this damage may be attributed to the heavy metals found in untreated sewage water (**Table 1**). These observations suggest that heavy metals entered within the root cells. Similar Pb deposits have also been shown along plasma membranes of *Sesbania* root cells by Sahi and Sharma (2005).

In the roots tissues, one of the ultrastructural modifications induced by high-metal content in untreated sewage water concerns the increase of cell wall thickness. This is one of the mechanisms that plant could develop to limit metal absorption in roots. In this regard, Probst *et al.* (2009) studied the morphological changes in faba bean seeds grown in a pot experiment on soil, mine tailings, and a mixture of both to mimic field situations in cultivated contaminated areas near mining sites. They found an increase in root cell wall thickening and suggested that the decrease uptake of toxic metals, a possible control of metal transport from roots to leaves by synthesizing phytochelators–toxic metal complexes. Moreover, thickening of cell wall was observed in shoots of faba bean exposed to Cd or Cu (Liu *et al.* 2004) and in marine macroalgus exposed to Cu (Andrade *et al.* 2004). This phenomenon seems to be associated with an increased activity of peroxidase (Liu *et al.* 2004). This enzyme is able to catalyze lignin synthesis (Arduini *et al.* 1995) and is induced in higher plants exposed to toxic metals (Prasad 1996) as well as with biotic stress (Reddy *et al.* 2007; Cernadas *et al.* 2008).

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