Spanish Journal of Agricultural Research
14(4), e0807, 13 pages (2016)
eISSN: 2171-9292
http://dx.doi.org/10.5424/sjar/2016144-9210
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Effects of sewage sludge on bio-accumulation of heavy metals in tomato seedlings

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Abstract

The proposal to use sewage sludge (SS) on agricultural fields as a sustainable way to dispose of the waste is based on its high organic and nutrients content. However, the presence of heavy metals (HMs) in sludge can contaminate crops and accumulate in the food chain. The aim of this study was to assess changes in soil fertility, biochemical responses of tomato (*Solanum lycopersicum* L. cv. Rio Grande) seedlings and the availability of HMs with increased rate application of SS (0, 2.5, 5 and 7.5%). Leaf chlorophyll content, nutritional status, proline, membrane peroxidation, stomatal conductance and HM accumulation were investigated. Results showed that the soil pH decreased, whereas soil salinity, organic carbon, total N, available P and exchangeable Na, Ca, K and HM content increased significantly with increasing application rates of SS. Among the three HMs (Zn, Cu and Cr), Zn had the highest capacity for transferring from soil into plants. Low metal translocation was observed from roots to leaves. The 7.5% SS dose decreased biomass production and caused a decline in chlorophyll content and stomatal conductance. However, lipid peroxidation and proline contents increased. Therefore, the use of 2.5 and 5% doses of sewage sludge in agriculture would be an efficient and cost-effective method to restore the fertility of soil and an environment-friendly solution for disposal problems.

Additional key words: sewage sludge amendment; soil fertility; biochemical responses; *Solanum lycopersicum*.

Abbreviations used: BCF (biological concentration factor); DW (dry weight); EC (electrical conductivity); FW (fresh weight); HM (heavy metal); MDA (malondialdehyde); OM (organic matter); ROS (reactive oxygen species); SS (sewage sludge); SSA (sewage sludge amendment); TBA (thiobarbituric acid); TCA (trichloroacetic acid); TF (translocation factor).

Authors' contributions: Conceived and designed the experiments: NE, MZ and MK. Performed the experiments: NE and DB. Analyzed the data: NE and MZ. Contributed reagents/materials/analysis tools: NE and BJ. Wrote the paper: NE and MK.

Citation: Elloumi, N.; Belhaj, D.; Jerbi, B.; Zouari, M.; Kallel, M. (2016). Effects of sewage sludge on bio-accumulation of heavy metals in tomato seedlings. Spanish Journal of Agricultural Research, Volume 14, Issue 4, e0807. http://dx.doi.org/10.5424/sjar/2016144-9210.

Received: 29 Dec 2015. **Accepted:** 27 Oct 2016.

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Increasing soils pollution caused by heavy metals (HMs), due to agricultural and industrial activities, is becoming a serious environmental problem to the present world (Li *et al.,* 2013; Imran *et al.,* 2015). The direct and indirect discharges of industrial and urban wastes have resulted in the chemical contamination of the soil by organic pollutants and HMs (Sun *et al.,* 2012; Elloumi *et al.,* 2015). The presence of HMs in sewage sludge (SS), used as agricultural fertilizer is a major problem for soil and crop qualities. The SS contains some nutrients and organic matter (OM), and it may be used to replace commercial fertilizers for plant. In many regions in the world, particularly in arid and semi-arid regions, agricultural soils are poor in OM. Therefore, the use of SS as a fertilizer can be considered a sustainable way for the management of these wastes. The use of SS as a fertilizer show increases in plants productivity, which may be equal and in some cases, higher than the chemical fertilizer. Restrictions placed on the agricultural use of SS arise due to its HMs content. HMs characterization of SS is an important requirement prior to its application to soil because there is a risk of accumulation and transfer to plants and to groundwater. Consequently, plants are directly affected by HMs accumulation in roots and their translocation to the upper parts (Daud *et al.,* 2015). HMs such as Cr, Pb and Ni have no known biological function and are extremely toxic. The phytotoxic effects of HMs can probably be a consequence of their interference with a number of metabolic processes (Lin *et al.,* 2007; Daud *et al.,* 2015). Growth reduction as a result of changes in biochemical and physiological processes in plants growing on HMs polluted soils has been recorded (Lux *et al.,* 2011; Luković *et al.,* 2012). This may be due to a reduction of cell water content (Daud *et al.,* 2015) and mal functioning of plasma membrane (Romero-Puertas, 2002). HMs toxicity causes the generation of reactive oxygen species (ROS) including superoxide, hydroxyl radicals, hydrogen peroxide and singlet oxygen and associates changes in antioxidative enzyme activities (Gill *et al.,* 2012). The excessive ROS reacts with lipids, pigments and proteins, resulting in membrane damage, inhibition of photosynthesis and enzyme inactivation (Scandalios, 2005; Gill &Tuteja, 2010).

The availability and uptake of HMs in plants is affected by a variety of factors such as pH, redox potential, solubility, contents of OM, soil mineralogy, texture and chemical speciation of the metal (Čásová *et al.,* 2009; Nayak *et al.,* 2015). Some researchers have showed that OM contributes to the reduction of metal availability by decreasing the labile metal in soil (Gao *et al.,* 2003).

Several studies showed different plants responses to HMs accumulation in soil (Santos *et al.,* 2011; Gonçalves *et al.,* 2014). It has been shown that crop production is favored by the land application of SS (Antolín *et al.,* 2005; Singh & Agrawal, 2008). Morera *et al.* (2002) reported that sludge amendment at the rate of 80, 160 and 320 t/ha DW in soil increased the average dry weight of sunflower plantlets (*Helianthus annuus* L.). Sludge amendment (SSA) (30, 45, 60, 90 and 120 t/ha) enhanced stomatal conductance and photosynthetic rate of rice (*Oryza sativa* L.) (Singh & Agrawal, 2010). This can be attributed to the increase in plant photosynthesis and correlated to increases in the total chlorophyll content of plants grown under various SSA rates. However, the increase in stomatal conductance may be due to a high nutrient availability through SSA which reduces HM toxicity (Singh & Agrawal, 2010). Mata-Gonzalez *et al.* (2002) indicated that the increase in SS rates (0, 7, 18, 34 and 90 t/ha DW) on growing tobosa grass (*Hilaria mutica* L.) and blue grama (*Bouteloua gracilis* L.) produced a significant increase in leaf area. This evolution did not always correspond to an increase in photosynthesis rate. Experiments carried out by Chandra *et al.* (2008) on soil amended with SS (10, 20, 40, 60, 80 and 100%) on seed germination and growth parameters of *Phaseolus mungo* L. showed that soil amended with 10% SS is favorable to growth, however >10% was inhibitory for plant growth. Rrong *et al.* (2015) showed that under different application levels of SS (2, 4, 6, 8, 10, 12, 14, and 16%) the dry weights were all higher than that in the control and reached the maximal levels when treated with the amount of SS at 4% and 10%. Therefore, we have developed our study with 2.5, 5 and 7.5% of SS application to determine the beneficial effect on growing plant and to identify the eventual toxicities of HMs and salinity. In this way changes of the soil properties and the biochemical responses of tomato seedling were examined. Heavy metals uptake and transfer from soil within plant tissues were also investigated.

Material and methods

Physico-chemical characterization of materials

SS was supplied from a municipal waste water treatment plant of Sfax (Tunisia) which processes domestic and industrial wastewater amounting to 48,000 m^3 per day. The SS treatment was done by aerobically digested stabilization. Uncontaminated garden soil was collected and served as control. The particle size grading of the soil samples was determined by gravimetry through 2 mm to 63 µm meshed sieves.

The sludge was mixed with the soil at 2.5%, 5% and 7.5% (DW) proportions and laid into 3-L pots. Control pots (0%) were also prepared as well as pots containing only control soil.

Control soil, SS and the soils treated with SS were dried, ground, passed through a 2 mm sieve and processed for chemical analysis. The soil pH at different treatments was determined in the suspension of 1:5 (w/v) using a pH meter (Model EA940, Orion, USA) and conductivity was measured by a conductivity meter (Model WTW LF 90). Organic carbon was determined according to the method of Kalra & Maynard (1991). Total nitrogen was determined by Kjeldahl's procedure. The total concentrations of HMs were measured using an Atomic Absorption Spectrometer (Thermo Scientific EC 3200), after the digestion of the samples with HNO_3-HCl (McGrath & Cunliffe, 1985).

The physico-chemical characteristics of the control soil are shown in Table 1. The soil is a sandy soil with a neutral pH, and has a low OM and low N content. The HM contents in the soil did not exceed the limit values for metals concentrations in the soil set by the European Union (EC, 1986). Selected physico-chem-

Table 1. Physicochemical properties of control soil and sewage sludge used in the experiments.

Properties	Control soil	Sewage sludge	Limit values		
			[a]	[b]	[c]
Sand (%)	90	–			
Silt (%)	10				
Texture	Sandy				
pH (1:5)	7.06	7.16	–	5.5-8.5	6-7
EC (dS/m)	0.40	4.2			
Organic matter (%)	0.67	43.4			
Organic C (%)	0.39	34.1			
Total N (%)	0.04	2.8			
Available P (mg/kg)	0.5	404			
Ca (g/kg)	0.45	102			
Mg (g/kg)	0.84	18			
K (g/kg)	5.81	4.38			
Na (g/kg)	0.09	7.97			
Fe (mg/kg)	54	2160			
Mn (mg/kg)	22.5	415			
Zn (mg/kg)	3.5	1825	2000	2500-4000	150-300
Cu (mg/kg)	ND	550	1000	1000-1750	50-140
Pb (mg/kg)	ND	23	800	750-1200	50-300
Cr (mg/kg)	ND	665	500	500	–

[a] Limit values for heavy metals in sewage sludge (NT, 2002). [b] Limit values for heavy metals in sewage sludge (EC, 1986). [c] Limit values for heavy metals in soil (EC, 1986).

ical characteristics of the SS applied at this study are given in Table 1. The value of EC (4.24 dS/m) was higher in sludge. The physicochemical characterization of SS showed high OM content (43.4%), total N (2.8%), available P (404 mg/kg), Ca (102g/kg) and total Fe (2160 mg/kg) contents. However, SS contains not only beneficial elements for plant growth but also HMs. Zn (1825 mg/kg) and Cr (665 mg/kg) were the most common HMs in SS.

Phytotoxicity test

Tomato (*Solanum lycopersicum* L.) was selected as recommended by many previous methods as well as due to its importance as a food crop. Prior to the germination test, tomato cv. Rio Grande seeds were surface-sterilized in H_2O_2 (3%) and then rinsed with distilled water. Soil (10 g) was extracted with 100 mL of deionized water, stirred for 2 h and then centrifuged at 9000 rpm. Filter paper was placed on a Petri dish and moistened with 10 mL of the soil sample extracts. Ten seeds of each treatment were then placed on a dish, which was covered by a lid and incubated in the dark at 25 °C for 7 days. The number of germinated seeds was then counted and the length of the roots was measured (Hoekstra *et al.,* 2002). When the root extended more than 2 cm from the stem-root junction, germination was confirmed (Al Harbi *et al.,* 2008). Triplicate sets were performed for each treatment. The germination percentage and root length were estimated using the following equations:

$$\text{Germination (\%)} = $$
$$= (\text{seeds germinated/total seeds}) \times 100$$

$$\text{Root length} =$$
$$= \text{mean length of root/number of germinated seeds}$$

Experimental setup

Tomato seeds were germinated on wet filter paper in darkness at 25 ±2 °C for 7 days. Following germination, seedlings of approximately equal size were transferred into 3-L pots containing soil – SS mixtures.

Pots were maintained in a greenhouse with the temperatures controlled at 28/18 °C ± 3 °C day/night, relative humidity 60-70%. A total of 40 pots were randomly divided into four groups (control and three treatments). The plants were irrigated, in accordance with their water demand, with distilled water during the growing period, the irrigation scheduling, and water quantity being equal for all treatments. The water level was made up as and when required. A total of five pots were used for analyses of plant growth parameters and the others pots were used for the others parameters. Each parameter was analysed in triplicate.

The plants were harvested after 30 days after sowing. All the plants were free from any disease in the whole duration of the experiment.

Stomatal conductance measurement

Stomatal conductance was determined at the end of the experiment on expanded leaves from the median part of the shoots from 10:00 to12:00 a.m. using a portable porometer (Steady State Porometer model MK III, Delta-TDevices). The measurements were done on a sunny day with 890 µmol/m^2·s photosynthetic active radiations, 25 °C air temperature and at 70% relative air humidity.

Measurement of plant biomass

At the end of the experiment and after the plant harvest, measurement of plant biomass was determined. Plants dry weights were recorded after drying the samples in a hot air oven at 60 °C until a constant weight. The plant tissues were weighed using an electric weighing balance.

Estimations of heavy metal concentrations in plants

After 30 days of growth, plants were divided into shoots and roots, washed extensively in distilled water to remove the mechanically adhering impurities, dried on filter paper and either immediately used for analyses.

The latter were oven-dried at 60 °C until reaching a constant weight. Heavy metal concentrations were determined by atomic absorption spectrophotometer (Thermo Scientific EC 3200) after digestion with a mixture of acids (HNO_3:HCl/ 2:1).

Biological concentration factor (BCF) was calculated as metal concentration ratio of soil to plant roots (Yoon *et al.,* 2006). Translocation factor (TF) was described as the ratio of metal concentration in plant shoots to roots (Cui *et al.,* 2007; Li *et al.,* 2007).

Estimations of physiological and biochemical parameters

Chlorophylls were extracted in 80% acetone and estimated according to the method of Arnon (1949).

Leaf samples used for proline content determination were immediately frozen in liquid nitrogen. Proline content was determined according to the method of Bates et al. (1973). A total of 0.5 g of frozen powder was mixed with a 5 mL aliquot of 3% (w/v) sulfosalicylic acid in covered glass tubes and boiled in a water bath at 100 °C. The mixture was centrifuged at 2000×g for 5 min at 25 °C. A 200 µL of the extract was mixed with 400µL distilled water and 20 mL of the reagent mixture (30 mL glacial acetic acid, 20 mL distilled water and 0.5 g ninhydrin) and boiled at 100 °C for 1 h. After cooling the mixture, we added 6.0 mL of toluene. The chromophore-containing toluene was separated and absorption at 520 nm was read, using toluene as a blank. Proline concentration was calculated using L-proline for the standard curve (0-50 mg/mL).

The level of lipid peroxidation in the leaf tissues was measured in terms of malondialdehyde content (MDA, a product of lipid peroxidation) determined by the thiobarbituric acid (TBA) reaction using the method of Heath & Packer (1968), with minor modifications as described by Zhang & Kirham (1994). A 0.25 g leaf sample was homogenized in 5 mL 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000×g for 5 min. Then 4 mL of 20% TCA containing 0.5% TBA was added to 1 mL aliquot of the supernatant. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000×g for 10 min, the absorbance of the supernatant was read at 532 nm and the value of the nonspecific absorption at 600 nm was subtracted. The MDA content was calculated by using an extinction coefficient of 155 mM/cm.

Statistical analysis

All statistical analyses were performed using analysis with SPSS version 17 software. Tukey's multiple range test was performed to test the significance of difference between the treatments.

Results

Characteristics of the growing media

Changes in pH and EC, and other physicochemical properties of SS-treated soils, are summarized in Table 2. Addition of SS led to immediate reductions in soil pH proportional to the SS concentrations added. A significant pH decrease was noted following addition of 7.5% SS. The highest pH value was found for control soils (7.08) and the lowest for the soils treated with 7.5% SS (6.66). Also, EC was affected by SS treatments, showing significant increases in comparison with control soil.

Table 2. Physicochemical properties of soil following sewage sludge (SS) supply at 2.5, 5and 7.5% at 0 d after sowing of tomato seedlings. Data are the means of three replicates. Means with different letters indicate a significant difference at $p \leq 0.05$ using Tukey multiple range test.

Variables	2.5% SS	5% SS	7.5% SS
pH (1:5)	6.78±0.05[a]	6.74±0.03[a]	6.66±0.06[a]
EC (dS/m)	0.58±0.07[c]	0.69±0.05[b]	0.80±0.08[a]
Organic matter (%)	1.74±0.04[c]	2.81±0.08[b]	3.88±0.08[a]
Total N (%)	0.11±0.02[b]	0.2±0.01[a]	0.26±0.05[a]
Available P (mg/kg)	10.58±0.08[c]	20.67±0.06[b]	30.76±0.05[a]
Ca (g/kg)	3.05±0.07[c]	5.4±0.06[b]	8.2±0.03[a]
Mg (g/kg)	0.88±0.06[a]	0.93±0.05[a]	0.98±0.03[a]
K (g/kg)	5.77±0.2[a]	5.73±0.11[a]	5.70±0.3[a]
Na (g/kg)	0.287±0.02[c]	0.484±0.02[b]	0.681±0.02[a]
Fe (mg/kg)	75.5±5[b]	97.13±2[a]	109.09±9[a]
Mn (mg/kg)	32.31±2[c]	42.12±2[b]	51.93±3[a]
Zn (mg/kg)	49.03±0.49[c]	94.57±1.03[b]	140.11±2.86[a]
Cu (mg/kg)	13.75±0.5[c]	27.5±0.8[b]	41.25±1.75[a]
Pb (mg/kg)	0.575±0.105[c]	1.15±0.1[b]	1.725±0.175[a]
Cr (mg/kg)	16.62±0.63[c]	33.25±1.75[b]	49.87±1.13[a]

Table 3. Effect of different sewage sludge (SS) concentrations on seed germination and root elongation. Data are the means of three replicates.

Substrate	Germination (%)	Root growth (mm)
0% SS	73.3 [b]	25±4.5 [c]
2.5% SS	76.6 [b]	42±3.5 [b]
5% SS	76.6 [b]	58.4±2.51 [a]
7.5% SS	90 [a]	56.8±3.6 [a]

Means with different letters indicate a significant difference at $p \leq 0.05$ using Tukey multiple range test.

Organic matter, total N, available P, Na, K, Ca, and Mg contents increased in soil amended with SS due to higher levels of these nutrients in SS (Table 2). SS treatments in soil led to higher concentrations of HMs as compared to unamended soil. Zn, Cr and Cu concentrations in soil were highest at 7.5% SS (Table 2).

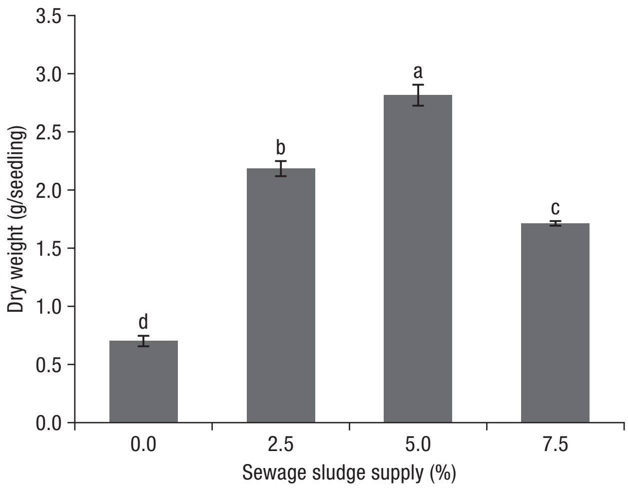

Figure 1. Effect of sewage sludge supply on dry weight of tomato seedlings. Means with different letters indicate a significant difference at $p \leq 0.05$ using Tukey multiple range test.

Effects of SS addition to soil on seed germination and plant growth

Additions of SS to soil adversely affected the seed germination and root elongation tests of tomato seedlings (Table 3). Treatment of soil with 2.5% and 5% SS had no significant effect on seed germination of tomato seedlings. In contrast, addition of 7.5% SS leads to a significant increase in seed germination of ~ 17%. Results of root elongation tests revealed an increase in values for treatments with SS compared to the control soil. In comparison with the control, treatment with 2.5% SS increased the root growth by about 40%. Seed root length for 5% and 7.5% SS was significantly greater than that for 2.5% SS treatment ($p<0.05$).

Present data showed also the beneficial effects of SS addition to soil on plant growth, in that a significant increase in the biomass production of tomato cultivated in the presence of SS was observed compared with control plants (Fig. 1). The maximum increase in the biomass production was 280% at 5% SS treatment, compared to control. However, at 7.5% SS the increase in the biomass production was only 140%.

Heavy metals accumulation in tomato seedlings

Metal accumulation in tomato seedlings grown at different sludge concentrations of amended soil showed different magnitude and relative distribution (Table 4).

Table 4. Trace element concentrations (mg/kg DW) in leaf and root of tomato.

	Zn	Cu	Cr
Leaf			
Control	22.19[c]	5.86[c]	ND[c]
2.5% SS	33.67[b]	7.15[b]	ND [c]
5% SS	40.54[a]	10.86[a]	0.12 [b]
7.5% SS	45.71[a]	12.57[a]	0.81[a]
Root			
Control	13.63[c]	6.82[c]	ND [c]
2.5% SS	22.09[b]	19.65[b]	5.07[b]
5% SS	24.31[a]	32.34[a]	6[b]
7.5% SS	25.54[a]	31.15[a]	25[a]
Normal range[1]	15-150	3-20	0.02-14

[1] Normal ranges in plants (Ostos *et al*., 2008). For each organ and metal, means with different letters(a,b,c) indicate a significant difference between sewage sludge treatments at $p \leq 0.05$ using Tukey multiple range test. ND: non-detected.

Table 5. Mean values of translocation factor (TF) and biological concentration factor (BCF) for Zn, Cu and Cr in tomato seedlings grown in SS-treated soils.

Treatment	Factor	Heavy metals		
		Zn	Cu	Cr
0% SS	TF	1.62	0.85	–
	BCF	3.89	–	–
2.5% SS	TF	1.53	0.36	–
	BCF	0.27	0.49	0.15
5% SS	TF	1.66	0.33	0.02
	BCF	0.23	1.17	0.18
7.5% SS	TF	1.78	0.40	0.03
	BCF	0.18	0.75	0.5

Table 6. Content of chlorophyll a (Ca), chlorophyll b (Cb) and total chlorophyll (Ca+b) in different sewage sludge supply rates (mg/g FW). Data are the means of three replicates.

Treatments	Ca	Cb	Ca+b
Control	0.54 [a]	0.26 [a]	0.80 [a]
2.5% SS	0.64 [a]	0.30[a]	0.94 [a]
5% SS	0.32 [b]	0.17 [b]	0.48 [b]
7.5% SS	0.15 [c]	0.10 [b]	0.25 [c]

Values with different letters are significantly different at $p \leq 0.05$ using Tukey multiple range test.

The concentration of Zn was higher than that of Cu and Cr. The relative concentration of these HMs depended on their concentration in SS and the ability of plant to uptake HMs. In SS, Zn concentration was 2.7 times that of Cr and 3.3 times that of Cu (Table 1). According to these conditions, the Zn concentration in plant was higher than that of Cu and Cr. However, the plant concentration of Cr was lower than that of Cu despite the SS concentration of Cr was higher than Cu. On the other hand, the accumulation of metals in the roots and leaves varied from one metal to another. Therefore, the ability of plants to transfer metals from leaves to roots was determined by calculating the TF (Table 5). The TF for HMs from plant leaf to root was <1 for all HMs, except for Zn. The distribution of the metals within the leaves and roots was different: Zn was found in the leaves, while the greatest amount of Cu and Cr was observed in the roots. The BCF for HMs from soil to root was <1 for all HMs. Values were <1 for all HMs, which indicates that translocation was allowed from soil to plant roots.

Physiological and biochemical responses

Tomato seedlings treated with different amounts of SS amendments (2.5, 5 and 7.5%) showed variations in photosynthetic pigments production. For instance, plants treated with 2.5% of SS treatment exhibited a non-significant increase in the concentrations of Chl*a*, Chl*b* and total chlorophyll. However, a decline in Chl*a*, Chl*b* and total chlorophyll contents was noticed following 5 and 7.5% of SS treatments (Table 6).The decrease of total chlorophyll was higher at 7.5% (69%) than at 5% (40%) SS treatment. Decreases in chlorophyll concentration have been noted as indicators of leaf damage produced by HMs.

Changes in the content of lipid peroxides, expressed as MDA, an indicator of lipid peroxidation and oxidative damage to membrane, are shown in Fig. 2. Addition of 2.5, 5 and 7.5% SS to soils led to significant increase in the quantities of MDA in tomato plants, but without showing significant differences between 2.5 and 5% SS treatments. Proline also increased significantly in plants grown in the 7.5% SS-treated soil, although quantities in the 2.5 and 5% SS treatments were not significantly different from those in the control and 7.5% SS treated soil. The maximum leaf conductance was observed for control plants which decreased significantly under SS treatments, but without significant differences between the three treatments.

Discussion

The SS characteristics vary with wastewater composition as well as treatment processes. High quantities of OM (43.4%), total N (2.8%), available P (404 mg/kg), Ca (102g/kg) and total Fe (2160 mg/kg) in SS make this material an important soil amendment. The considerable amount of these elements highlights the benefits of using SS as an agricultural fertilizer. However, SS contains not only beneficial elements for plant

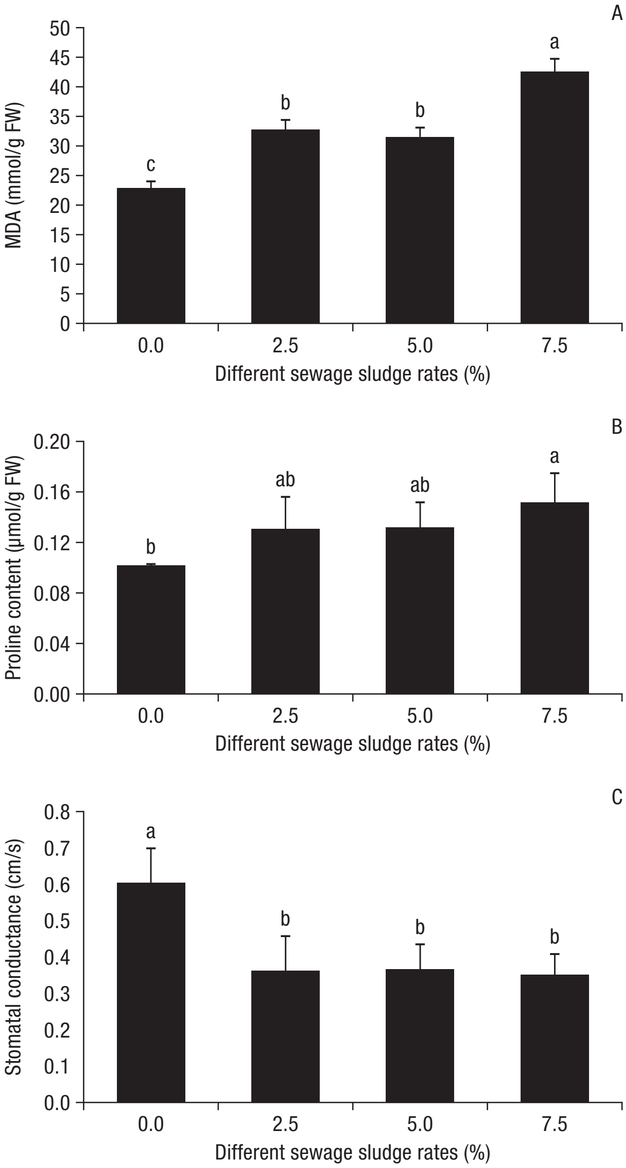

Figure 2. Lipid peroxidation (A) and proline (B) contents and stomatal conductance (C) of tomato seedlings grown at different sewage sludge treatment rates. Data are the means of three replicates. Means with different letters indicate a significant difference at $p\leq 0.05$ using Tukey multiple range test.

growth but also HMs. According to Tunisian standards for sewage sludge reuse (NT, 2002), the permissible levels for potential toxic elements such as Zn, Cu, Pb and Cr in sludge to be used in agricultural soils are 2000, 1000, 800 and 500 mg/kg, respectively. The sludge used for study contains 1825, 550, 23 and 665 mg/kg of Zn, Cu, Pb and Cr, respectively. Thus only Cr was above the permissible limit. Babel & Dacera (2005) and Gupta & Sinha (2007) found that due to its origin, *i.e.*, urban- and industrial-used water, the sludge could contain high concentrations of HMs.

The value of EC (4.24 dS/m) was higher in SS than in the control soil where the EC was 0.4 dS/m. High values of EC in the sludge may be due to the presence of high concentrations of soluble salts.

Addition of SS to soil at 2.5, 5 and 7.5% led to changes in pH, EC, and other physico-chemical properties. A significant pH decrease was noted following addition of 7.5% SS. This evolution of soil pH was related to the important increase on the soil OM and the biological activity. The decrease in pH was due to the degradation of OM, especially organic acids production. Soil pH in this study was in the range at which plants grow well. Most agricultural crops grow well in soil with a pH between 5 and 7.5 (Khoudi *et al.,* 2013). In the same case, EC was affected by SS treatment, showing significant increases (EC=0.8 dS/m with 7.5% SSA) in comparison with control soil (0.4 dS/m). However, EC values remained below the salinity threshold of 4 dS/m. Many authors (*e.g.* Taws, 2003; Gasco & Lobo, 2007) have reported that the soil salinity concentrations are classified as 'moderate' for 2 to 6 dS/m, 'high' for 6 to 15 dS/m and 'extreme' for over 15 dS/m. In our study, SS application caused slight changes in soil EC, apparently due to textural class of the soil (sandy texture), which could favor leaching (Gascó & Lobo, 2007; Angin *et al.,* 2012). Yadav *et al.* (2011) showed that the accumulation of salts in the root zone resulted in an EC <1.0 dS/m, which did not cause salt toxicity for plants, and thus did not influence plant growth. Yilmaz & Temizgül (2012) also found an increase in EC and a reduction in pH due to the SSA at different rates in the soil. Organic matter, total N, available P, Na, K, Ca, and Mg contents increased in soil treated with SS due to higher levels of these nutrients in SS. Organic matter plays a major role in maintaining soil quality and improves soil structure which can enhance infiltration rate and reduce soil erosion (Gao *et al.,* 2008). The SS treatment in soil led to higher concentrations of HMs as compared to control soil. Zn, Cr and Cu concentrations were higher at 7.5% SS treatment. Accumulation of HMs in agricultural soils has become an important problem due to food safety issues and potential health risks. Some vegetative responses such as seed germination test, elongation of root and seedling growth are commonly used to assess the overall toxicity of organic and inorganic compounds in different substrates (Di Salvatore *et al.,* 2008). Our results showed that the different SS levels increased both seed germination and seedling growth. The most impressive increase in root growth and seed germination was recorded in seeds incubated in 7.5% SS. The positive effect of SS was more pronounced with regard to the stimulation of root growth than the seed germination. Furthermore, the increases in percent germination rates and root growth may be as a result for increases in plant nutrients supplied by SS compared to control. This could indicate that most metals present in SS remained in chemical forms of low bioavailability in extract of SS treated soils. Morera *et al.* (2002) also reported a reduction in the HM toxicity due to adsorption of HMs by additional sources of OM and humic substances in sludge. Araùjo & Monteiro (2005) showed that seed coats constitute a barrier between the embryo and its immediate environment. According to these authors, the metals occurring in the substrate could be adsorbed by the seed coat, which would thus not affect the growth of the embryonic root. Li *et al.* (2005) support also the idea that tissues covering the embryo play a role in selective penetration of different HMs into seeds.

In the present study, we noted a significant increase in biomass production in soils treated with SS. The maximum increase in the biomass production was 280% at 5% SS treatment, compared to control. However, at 7.5% SS treatment the increase in the biomass production was only 140%. The treatment with 7.5% SS decreased growth seedling but remained higher than control. Li *et al.* (2005) showed that seeds still germinated in the presence of high concentrations of HMs, but the subsequent seedling growth (after the breakage of seed coat) was severely inhibited at much lower concentrations of HMs. Furthermore, the increases in seedling growth may be a result of increases in plant nutrients supplied by SS compared to control. Mishra & Behera (1991) reported that high sludge content was suppressive for plant growth hormone(s) (auxin and gibberellin) which are responsible for the growth and development of plants. Chandra *et al.* (2008) showed that the reduction in plant growth at high concentrations of sludge might be due to the entrance of the metal into the protoplasm resulting in the loss of intermediary metabolites which are essential for further development and growth of plants. Oleszczuk (2006) reported that contaminants present in SS, due to the mineralisation of OM, are subject to continuous processes of remobilization and repeated binding by newly formed organic structures, which affects their bioavailability and toxicity. The reason for this smaller increase in biomass production at higher concentrations of SS could therefore be attributed to bioavailability of contaminants due to the mineralisation of OM present in soil amended with SS. This degradation of OM increased with time.

Metal accumulation in tomato seedlings grown at different sludge concentrations of treated soil showed different magnitude and relative distribution. Cr, Zn and Cu concentrations in leaves and roots of plants grown in SS-treated soils were significantly higher as compared to those in control soil. In all treatments, Zn and Cu contents in plant tissues remained below the toxicity levels. Except for Cr, concentration in roots of tomato seedlings grown in 7.5% SS-treated soils were significantly higher as compared to normal ranges in plants (Ostos *et al.,* 2008).

The difference in seedlings biomass between the lowest and the highest SS treatment seems to be related to the presence of phytotoxic concentration of Cr in roots and increased concentrations of organic and inorganic compounds in the highest SS treatment. In this context, the mineralisation of organic compounds in the soil is more important with time and this degradation decreases the soil pH and consequently increases the bioavailability of HMs. Rowell *et al.* (2001) stated that OM introduced together with the sludge underwent mineralisation very quickly. As a result of that process, formerly unavailable pollutants related with OM undergo remobilization. An increase in the phytotoxicity with time was most probably related to the fact that the organic contaminants, initially adsorbed to the SS/soil mixture, were temporarily less available. As a result of OM mineralization, the strength of these bonds could weaken, and hence, there was an increase in the bioavailability of pollutants which had not been bioavailable earlier (Oleszczuk, 2006). Singh *et al.* (2011) reported that insoluble OM inhibits the uptake of metals, which are tightly bound to OM, thus reducing bioavailability. However, soluble OM increases bioavailability of HMs by forming soluble metal organic complexes (Singh *et al.,* 2011). Paschke *et al.* (2006) reported that evaluating metal phytotoxicity thresholds is difficult because of complex interactions between metal elements and other biogeochemical factors. Our results for seed germination and seedling growth show that the different SS levels support germination, however with 7.5% SS growth seedling decreased but remained higher than control. Our results support the idea of other authors that tissues covering the embryo play a role in selective penetration of different HMs into seeds. This was first suggested by the fact that seeds still germinated in the presence of high concentrations of HMs, but the subsequent seedling growth (after the breakage of seed coat) was severely inhibited at much lower concentrations of HMs (Li *et al.,* 2005). In accordance to our results Tauqeer *et al.* (2016) showed that plant growth characteristics and biomass gradually increased under lower metal stress (0.5 and 1.0 mM Cd or Pb) as compared to control while decreased under higher metal stress (2.0 mM).

In order to estimate the transfer of HMs from the sludge to the plants several parameters such as TF and BCF were used. In the present study, BCF values were mostly less than 1 except for Zn at control soil (0% SS). For all treatment with SS, values of BCF are <1 indicating a low translocation from soil treated with SS to plant roots. The bioavailability of metals in soil to plant is further influenced by soil properties such as pH, OM content, as well as sludge application rate (Hue & Ranjith, 1994). Parkpain *et al.* (2000) showed that immobilization of metal increased with time in soil subjected to heavy applications of SS; however, a small amount of bioavailable Cu, Zn and Mn were measured in soil solution after. Morera *et al.* (2002) also reported a reduction in the HM toxicity due to adsorption of HMs by additional sources of OM and humic substances in sludge.

The pH of soil is an important factor that affects the bioavailability to plants of HMs in contaminated soils (Kashem *et al.,* 2007). At pH>6.5, uptake of HMs from the soil into the plants is diminished. A TF>1 suggests that HMs are readily translocated from roots to leaves, whereas values <1 signify more accumulation of HMs in the roots than the leaves (Singh & Agrawal, 2007). The TF for HMs from plant leaf to root was < 1 for all HMs, except Zn. The distribution of the metals was different within the leaves and roots: Zn was found in the leaves, while the greatest amount of Cu and Cr was observed in the roots. Mobility of HMs in soil is very important for plant uptake. Most of HMs are immobile in soil and their high concentration was in roots rather than in shoot (Chen *et al.,* 2004). McGrath (1987) reported that Zn, Ni and Cd were the most bioavailable metals, whereas Pb and Cr were scarcely available, after estimating the metal uptake of plants grown on sludge-treated plots. Sinha *et al.* (2005) reported that most of the Cr in *Pistia stratiotes* was found in the roots, which is probably due to binding of metals to the ligands and thus reducing its mobility from roots to aerial parts. The same authors reported that this behaviour is a strategy of the plants to limit metal translocation to the aerial parts. Our results showed that the availability of metals in control soil and in treated soil is not similar. Logan *et al.* (1997) suggested that the chemistry of the sludge affect plant uptake. Probably metals added to soil in organic forms are not more available than native metals as reported by Leita *et al.* (1999). Richards *et al.* (2000) found that soil OM had a more pronounced effect on metal leaching than pH in controlling the leaching of metals from sludge. In addition, the comparison of HMs TF values of the different treatments showed that these values were similar. These results show that tomato seedlings are unable to actively avoid the transport of HMs from roots to leaves and this transport to aerial organs is independent on the amount of HMs.

Tomato seedlings treated with different SS levels exhibited some physiological and biochemical modifications. Photosynthesis system is sensitive to environmental stress. The chlorophyll content and stomatal conductance have proved to be key limiting factors on photosynthesis. A decrease in chlorophyll amount is a bioindicator of HMs phytotoxicity which induces an inhibition of metabolic enzymes in the chlorophyll biosynthesis pathways (Xu *et al.,* 2013). In the present study, chlorophyll concentration of tomato seedlings decreased significantly with an increase in HMs concentrations at SS treatments. The reduction in chlorophyll amount in the stressed leaves could be due to structural alterations in chloroplasts (Gratao *et al.,* 2009). Furthermore, referring to Sandalio *et al.* (2001) the decrease in Chl contents in stressed leaves was attributed to the inhibition of chlorophyll biosynthesis or increased chlorophyll degradation. According to Gomes *et al.* (2015) the decrease in chlorophyll content could also be related to the increase in ROS as they can induce PSII and chlorophyll alteration. The decrease in chlorophyll is one of the most commonly observed consequences of HMs stress and can partly explain the decrease of stomatal conductance. According to Gajewska *et al.* (2013), the decrease in stomatal conductance is a common response to HMs stress. This reduction under stress condition may be attributed to the reduced stomatal pore size that induces lower photosynthetic rate (Elloumi *et al.,* 2014; Zouari *et al.,* 2016). Sipos *et al.* (2013) showed that the lower Chl concentration may have resulted in lower photosynthetic performance that required a lower gas exchange rate leading to stomatal closure. Hence, strong decrease in leaf conductance of plants grown in SS-treated soil was detected. These results show that approximately the same reduction efficiency, on the average 50%, of stomatal conductance occurred in plants grown at 2.5, 5 and 7.5% SS treatments. The treatment of tomato seedlings with 7.5% SS can cause a negative effect on photosynthesis and biomass production and this effect becomes more clear at long term.

Damages on membranes are also an important manifestation of HMs stress. In our study, the loss in chlorophyll content could be due to the peroxidation of chloroplast membranes. A decreased rate of photosynthetic pigment accumulation in association with SS treatment may be the consequence of peroxidation of chloroplast membranes due to increased level of ROS generation. The increased MDA content in leaves with the different SS treatment rate constitutes an index of lipid peroxidation and, therefore, of oxidative stress. No significant difference in MDA levels was observed between 2.5 and 5% SS treatments. A high lipid peroxidation level was recorded at higher SS treatment rates. The peroxidation of cell membranes severely affects its integrity and can produce an irreversible damage to the cell function (Gunes *et al.,* 2007; Elloumi *et al.,* 2015). Like that of lipid peroxidation, proline level in tomato grown under different SS rates was relatively higher than controls. The maximum proline accumulation was recorded at 7.5% SS. Besides, high proline concentration measured in tomato seedlings treated with 7.5% SS could also contribute to a protective role as scavenger of ROS (Türkan & Demiral, 2009; Antolín *et al.,* 2010). Investigations carried out by Tripathi & Gaur (2004) showed that the protective action of proline was probably connected with an ability to detoxify ROS and to inhibit lipid peroxidation.

It may be concluded from the present study that the application of sewage sludge enhanced significantly the soil characteristics such as organic C, total N, available P and exchangeable nutrients. This effect was accompanied with increased HMs in the soil at different SS rates. Low metal translocation was observed from roots to leaves. The 7.5% SS dose decreased biomass production and caused a decline in chlorophyll content and stomatal conductance. However, lipid peroxidation and proline contents increased. Collectively, these results strongly support the hypothesis that HMs of soil amended with 7.5% SS are responsible for this toxicity in tomato seedlings.

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