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LEAD TOLERANCE IN DATE PALM (*PHOENIX DACTYLIFERA* L.) PLANTLETS DERIVED FROM TISSUE CULTURE, ANATOMICAL STUDY

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Abstract

Soil pollution by heavy metal including lead (Pb) is a widespread problem; and the toxicity of lead as a major heavy metals pollutant to plants has gained a considerable attention around the world. Our study revealed the negative impacts of Pb at various concentrations (2.5, 5 and 10 mg.L^{-1}) on the in vitro growth of date palm plantlets; and mostly on the basis of anatomical analysis. Results showed that the Pb treatment significantly reduced the height and fresh weight of date palm plantlets compared to untreated ones; fresh weight and height of plantlets were 252.33 mg and 4.46 cm in control plantlets reduced to 147.33 mg and 3.50 cm when plantlets grown in medium contain 2.5 mg.L^{-1}. Interestingly, both fresh weight and height were increased to 272.33 mg and 4.03 when plantlets passed from 2.5 to 10 mg.L^{-1} Pb. Results of anatomical analysis showed no significant difference among all treatments on abaxial and adaxial epidermis, while the upper and lower cuticle layer increased when plantlets were exposed to Pb, these traits decreased when plantlets passed from low to high concentration of Pb. Mesophyll thickness was decreased from 218.90 µm in plantlets grown in medium without Pb to 162 µm in plantlets grown in medium supplemented with Pb at 2.5 mg.L^{-1}, the mesophyll thickness increased in the plantlets passed from 2.5 to 5 mg.L^{-1} and reached to 465.16 µm. The same pattern of results was observed in the blade thickness; as well as; the number of vascular bundles and the width and height of vascular bundles. Our results demonstrate the usefulness of in vitro technique in testing and generating a tolerant clone of date palm to lead stress.

Key words : Anatomical, date palm, heavy metals, mesophyll, tolerance, vascular bundles.

Introduction

All the dynamic development of anthropogenic activities during the 20th century resulted in high level of the environment pollution (Gavrilescu *et al*., 2015), the heavy metals among dangerous pollutants are considered hazardous chemicals because of their high toxicity which resulted from their bioaccumulation and non-biodegradable characteristics (Verma and Dwivedi, 2013). Lead (Pb) is one of the most toxic metals that pollute the environment and threating living organism's health (Taghizadeh *et al*., 2015). Plant biotechnology has been used to improve plant genotypes resistance to several stresses such as heavy metals (Kaeppler *et al*., 2000), in this context, tissue culture technique rise as auseful procedure in screeningof metal tolerance in plant (Toan, 2004; Gatti, 2008). This technique has been used to screen tolerant genotypes of Pb for numerous plants for example, *Ailanthus altissima* (Gatti, 2008), *Cynodon dactylon* L. (Taghizadeh *et al*., 2015) and *Daphne* species (Wiszniewska *et al*., 2015). Date palm *Phoenix dactylifera* L. belonging *Arecaceae* family, propagating by three main methods, sexually through seeds, vegetatively by offshoots and the third method is tissue culture multiplication (Abass, 2013). Tissue culture technique is effective method to date palm propagation, and it is a rapid system for large number production of genetically uniform plantlets (Aslam and Khan, 2009). Thus, this technique can be used in the production of plants which have resistant characteristics to biotic and abiotic stresses (Abass, 2016). Al-Mansoori *et al*. (2007) and Aldhebiani *et al*. (2018),applied this technique to screen *P. dactylifera* L. genotypes for salt tolerance. To the best of our knowledge thereis no previous reports onscreening Pb-tolerance in *P. dactylifera* L. genotypes by using tissue culture

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technology. Due to the increase level of Pb in the agricultural soils of Basrah province (southern of Iraq), which exceeded the permissible limits of EU standards up to 100 mg/kg of soil (2006) and the Pb level was found to be three folds more than the permissible limits(277 mg/kg of soil) (Abass *et al*., 2015; Al-Jabary *et al*., 2016), also *P. dactylifera* L. is a widespread tree in this city, and considered an important source of income and nutrition to the population, therefore, there is an urgent need to produce plants that can tolerate the high levels of pollution of heavy metals, especially lead. The present work is aimed at screeningand generating Pb- tolerant date palm clones by using tissue culture technology.

Materials and Methods

Plant materials

Plant materials used in our experiments were shoots of *Phoenix dactylifera* L. derived by tissue culture provided from tissue culture laboratory in date palm research Centre- Basrah university. Cultures were established using 1 cm shoot long; culture medium consisted MS (Murashigi and Skoog, 1962). Cultures were maintained in a growth chamber at 24^{o}C, under 16 h photoperiod.

Experimental design

Two different experiments have been designed to screen and generate date palm Pb- tolerance clones as below:

First experiment : Exposing date palm issue cultured shoots to several Pb concentrations (2.5, 5 and 10 mg.L^{-1}), Pb was added to MS medium as lead acetate prior autoclaving, and pH adjusted to 5.8. Plants materials have been incubated for four weeks.

Second experiment : Similar medium (MS)was used in the second experiment, briefly, after 4 weeks of incubation in the first experiment, the incubated shootstransferred from low to high concentration of Pb; more details, the incubated plantlets in medium supplemented with Pb at 2.5 mg.L^{-1}in 1st experiment transferred into a culture medium contain 10 mg.L^{-1} Pb, and the plantlets passed from medium contain 2.5 to 10 mg.L^{-1}Pb. This experiment alsoterminated after four weeks.

At the end of each experiment, plantlets were weighted and their height was measured to evaluate their responses to Pb stress. Additionally, anatomical sectioning was done by preparing permanent cross-section of date palm leaves, the samples of leaf sections were cut from the middle and fixed in Formlin-Glacial acetic acid- ethyl alcohol (FAA) and preserved in 70% alcohol. Willey (1971) method was followed to prepare anatomical sections, and fast green dye was used in tissues staining, the sections were examined with Olympus light microscope and photographed with a digital camera.

Statistical analysis

Randomized design was used in this experiment, each treatment with triplicates, all obtained data subjected to one way ANOVA analysis and the least significant difference (LSD) was used to test significant difference among the treatments mean at 0.05 significant level ($P \leq$ 0.05), analyzed statistical was done by using SPSS-22 statistical software (SPSS In., Chicago, IL., USA).

Results

Growth parameters

At the end of incubation period of date palm tissue cultured shoots in MS medium supplemented with Pb-acetate either in 1st or 2^{end} experiment; fresh weight and plantlets height were analyzed as shown in figs. 1 and 2. The fresh weight of plantlets grown on MS medium without Pb (as control treatment) was 252.66 mg, and the supplying of MS medium with Pb led to decrease the FW to 147.33, 180.66 and 122.33 mg at 2.5, 5 and 10 mg.L^{-1} Pb treatments; respectively. Results depicted in fig. 1 revealed that the fresh weight of plantlets passing from 2.5 to 10 was less than the fresh weight of control plants, interestingly, when plantlets passed from 2.5 to 10 mg.L^{-1}the fresh weight increased to 273.33 mg and this value significantlyhigher than other treatments with an exception was observed at control treatment. Regarding plantlets height the results depicted in fig. 2 showed that, the plantlets grown on medium without Pb recorded the highest average which was 4.46 cm and this treatment significantlyhigher than other treatments except plantlets passing from medium supplemented with Pb at 2.5 to 10 mg.L^{-1} of Pb.

Plantlets responses to Pb treatments

Leaf anatomy characteristics

Epidermis and cuticle thickness

Results obtained from cross section of *P. dactylifera* L. plantlets leavesgrown in medium supplemented with Pb at different concentrations and those werepassed from low to high concentration described in the table 1. Results showed there was no significant effect of Pb treatment on the thickness of both abaxial and adaxial epidermis, however, the upper and the lower cuticle thickness were significantly increased when medium supplemented with Pb at 5 mg.L^{-1}, which the upper and lower cuticle thickness was recorded the average of 3.90 and 2.60 µm at this treatment, respectively.

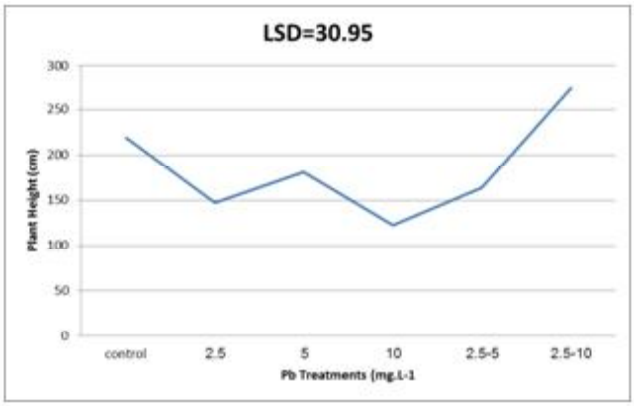

Fig. 1 : The effect of different concentrationsof Pb(mg.L^{-1}) and passing treatments from low to high concentration onthe fresh weight of date palm plantlets.

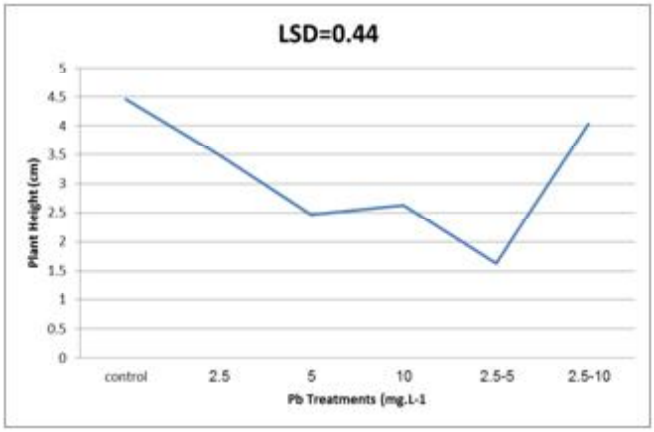

Fig. 2 : The effect of different concentrations of Pb (mg.L^{-1}) and passing treatments from low to high concentration on the height of date palm plantlets.

to other medium containing higher concentration of Pb, especially when plantlets passed from 2.5 to 5 mg.L^{-1} Pb concentration which was 465.16 µm, compared with 218.90 µm in control plantlets and 162.50 µm in plantlets exposed to Pb at 2.5 mg.L^{-1}.

Number of mesophyll row reduced significantly when Pb supplemented to medium at all examined concentrations, the number of row was 12 in control plantlets, and reduced to 9 when plantlets exposed to Pb at 2.5 mg.L^{-1}, but it reached 12.33 rows when plantlets passed from medium contain Pb at 2.5 to 5 mg.L^{-1} and 8 when plantlets passed from 2.5 to 10 mg.L^{-1} Pb. The highest average of mesophyll cell diameter was 36.30 µm which observed in the plantlets transferred from medium contain 2.5 to another contain 5 mg.L^{-1} of Pb. Additionally, this treatment led to increase the number of mesophyll cell in square millimeter from 144.00 cells in plantlets exposed to Pb at 2.5 mg.L^{-1} to 200.00 cells, while it was 160.00 cells in plantlets grown in medium without Pb. The results showed that, the increase of blade thickness was consistent with the increase in the thickness of mesophyll tissue, the plantlets that were transferred from medium with 2.5 mg.L^{-1} Pb to medium with 5 mg.L^{-1} Pb showed the highest average of blade thickness and reached to 478.20 µm, while it was 260.66 µm in control plantlets and 213.00 µm in 2.5 mg.L^{-1} treatment.

Vascular bundles (VB)

The effect of Pb treatments on the large vascular bundles of *P. dactylifera* L. presented in table 3. No significant effect observed when the medium supplemented with Pb at (2.5, 5 and 10) mg.L^{-1} also when plantlets passes from 2.5 to 5 mg.L^{-1}Pb, but a significant increase was observed when plantlet passing from 2.5 to 10 mg.L^{-1}Pb, the number of VB was 20in this treatment. Results describing the width and height of VB revealed that, the plantlets passing from medium supplemented with Pb at 2.5 to 10 mg.L^{-1}, recorded the highest values of width and height of VB with significant differences

Table 1 : Effect of Pb treatments on leaf epidermal and cuticle anatomical characteristics.

Treatments	Abaxial epidermis thickness	Adaxial epidermis thickness	Upper cuticle thickness	Lower cuticle thickness
Control	13.40± 5.62*	7.80± 1.27	2.56± 0.41	1.30± 0.17
2.5	7.80± 2.26	6.50± 0.72	2.56± 0.50	1.10± 0.26
5	12.30± 0.52	10.20± 1.06	3.90± 0.43	2.60± 0.26
10	10.40± 1.11	8.40± 1.24	2.60± 0.26	1.30± 0.26
2.5-5	11.47± 0.90	9.75± 0.37	1.93± 0.07	1.56± 0.21
2.5-10	13.26± 0.39	12.50± 1.05	2.60± 0.10	1.30± 0.10
LSD	6.00	6.05	0.55	0.35

* Values represent the mean of triplicate per treatment ±SD

Mesophyll and blade thickness

The results presented in table 2 described the mesophyll and blade thickness and some mesophyll cells traits. The results showed clearly that, the thickness of mesophyll decreased significantly in the plantlets leaves grown in MS medium contained Pb at 2.5, 5 and 10 mg.L^{-1}, but the mesophyll thickness increased significantly up to 2 folds when these plantlets transferred after a month

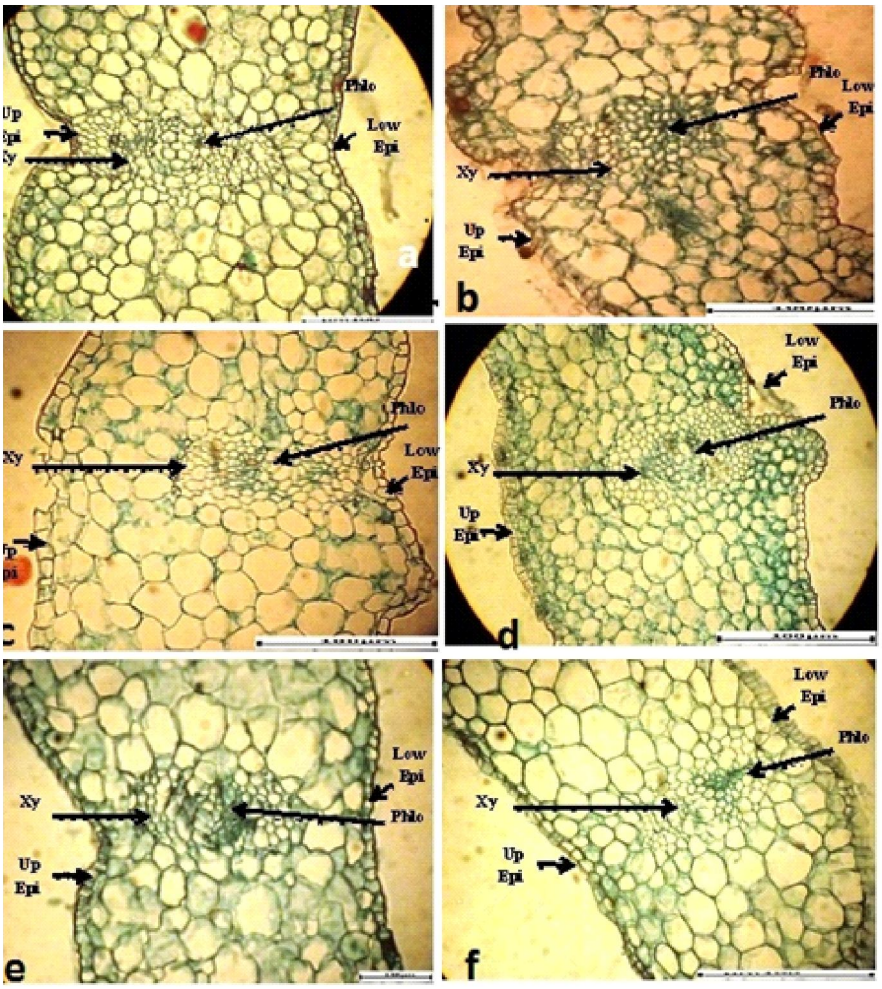

Plate 1 : Effect of Pb treatments on anatomical characteristic of date palm leaves (X 40).
a: control; b: 2.5 mg.L^{-1} Pb; c:5 mg.L^{-1} Pb; d: 10 mg.L^{-1} Pb; e: 2.5 to 5 mg.L^{-1} Pb; f: 2.5 to 10 mg.L^{-1} Pb
Xy: Xylem; Phlo: Phloem; Up Epi: Upper Epidermis; Low Epi: Lower Epidermis

Table 2 : Effect of Pb treatments on Mesophyll and blade anatomical characteristics.

Treatment	Mesophyll thickness	Number of mesophyll cell row	Diameter of mesophyll cell	Number of mesophyll cell in mm^2	Blade thickness
Control	218.90 ± 5.50*	12.00 ± 0.62	18.20 ± 1.08	160.00 ± 3.60	260.66 ± 35.85
2.5	162.50 ± 3.96	9.00 ± 1.80	17.80 ± 1.32	144.00 ± 10.81	213.00 ± 7.21
5	187.00 ± 12.12	8.00 ± 1.00	23.00 ± 2.64	140.00 ± 2.64	236.00 ± 10.14
10	178.00 ± 5.29	12.00 ± 1.00	22.36 ± 1.05	151.09 ± 3.18	226.00 ± 9.16
2.5-5	465.16 ± 19.55	12.33 ± 1.52	36.30 ± 1.30	200.00 ± 2.64	478.20 ± 22.58
2.5-10	117.00 ± 28.00	8.00 ± 2.64	14.70 ± 2.19	108.00 ± 6.02	163.60 ± 12.57
LSD	26.66	1.00	2.80	8.03	24.66

* Values represent the mean of triplicate per treatment ±SD

compared to other treatments, which was 130.00 and 281.26 µm, respectively.

The results of distance between adjacentVB showed a different pattern, the highest distance between VB recorded in the plantlets cultured in medium supplemented with Pb at 5 mg.L^{-1}, which was 1731.33 µm with significant difference to other treatments, while the lowest values appeared in Pb treatment at 2.5 mg.L^{-1}. Pb treatment at 10 mg.L^{-1} significantly ($P \leq 0.05$) led to increase the xylem thickness from 52.00 in control plantlets to 67.60 µm; it is noteworthy, that the supplementing of Pb to medium at 2.5 and 5 mg.L^{-1} led to reduce the xylem

Table 3 : Effect of Pb treatments on Vascular bundles anatomical characteristics.

Treatment	Number of VB	Width of VB	Height of VB	Distance between adjacent VB	Xylem thickness	Phloem thickness	Upper cap thickness	Lower cap thickness
Control	14±2.64*	78.00±3.60	169.93±8.01	1080.00±30.26	52.00±4.58	26.00±4.58	54.93±6.73	39.00±6.08
2.5	13±2.64	80.13±3.55	147.40±4.81	939.33±105.10	44.20±4.08	21.90±1.90	46.80±4.01	33.53±2.43
5	11±1.00	81.80±1.24	144.80±9.03	1731.33±39.80	42.64±2.16	30.93±1.98	41.60±1.90	28.51±2.27
10	14±1.00	83.60±2.25	165.60±6.50	1512.00±40.63	67.60±2.33	28.60±2.40	36.40±2.23	31.20±1.87
2.5-5	11±1.00	91.00±2.64	201.40±3.55	1080.00±26.45	59.80±2.25	33.80±1.57	67.60±2.43	43.30±0.81
2.5-10	20±2.64	130.00±2.64	281.26±4.47	1328.00±34.82	57.20±1.70	31.20±2.72	96.20±2.36	65.00±2.64
LSD	3.00	4.80	10.49	86.79	5.18	4.10	6.01	5.18

* Values represent the mean of triplicate per treatment ±SD.

thickness, while when the plantlets passed from 2.5 to 5 and 10 mg.L^{-1} Pb treatments, xylem thickness was increased.

Phloem thickness in plantlets leaves that grown in medium without Pb was 26.00 µm, this thickness reduced when Pb supplemented to medium at 2.5 mg.L^{-1} to 21.90 µm, per contra, the phloem thickness increased when Pb added at 5 and 10 mg.L^{-1} compared to control plantlets, the Pb-tolerance plantlets showed the highest averages of phloem thickness, which were 33.80 and 31.20 µm to plantlets passing from 2.5 to 5 and 10 mg.L^{-1}, respectively. The results describe the upper and lower VB cap thickness showed that, the plantlets passing from medium contain Pb at 2.5 to 10 mg.L^{-1}showed the highest values, which the upper cap thickness was 67.60 and the lower cap thickness was 43.30 µm under this treatment.

Discussion

The current study was designed on the basis of exposing date palm plantlets to Pb at different concentrations and then transferring stressed plants from the lowest to the highest concentrations of Pb and evaluatingtheir adaptation to the stress and the possibility of generatingPb tolerance clones. The results revealed that, the growth parameters of *P. dactylifera* L. plantlets exposed to various concentrations of Pb were affected significantly; the fresh weight of plantlets was reduced to 41.68, 28.49 and 51.58% at 2.5, 5 and 10 mg.L^{-1} Pb respectively, compared to control plantlets. Interestingly, the plantlets passed from 2.5 to 10 mg.L^{-1} medium contain Pb recorded an increase up to 8.18% compared to control treatment, while the plantlets passed from medium at 2.5 to 5 mg.L^{-1} Pb showed a reduction up to 35.35 and 40.37% for plantlets passed from 5 to 10 mg.L^{-1}.

Shoot height was reduced about 21.52, 44.84 and 41.03% when medium supplemented with Pb at 2.5,5 and 10 mg.L^{-1}, respectively, the plantlets passed from 2.5 to 5 mg.L^{-1} Pb showed a reduction in the shoot height to 63.45%, but when passed to 10 mg.L^{-1} the reduction percentage was 9.64%. Plant tolerances to heavy metals are usually estimated on the inhibition growth responses of their root and shoot by metal present in a nutrient solution (Parlak, 2016). The presence of Pb, even at small concentrations in culture media can induce a number of toxicity symptoms including the non-specific symptoms such as stunted growth (Sharma and Dubey, 2005).

The reduction ofplant growth is a common responsein plant exposed to Pb stress (Wiszniewska *et al*., 2015), the reduction in growth parameters may be attributed to the presence of Pb inhibition of enzymatic activities, water imbalance, altered hormonal status and membrane permeability (Wang *et al*., 2014; Muszynska *et al*., 2018). Plantlets height reduced when medium supplied with Pb at all examined concentrations; this is due to accumulationof Pb in the cell wall components such as pectic substances and hemicellulose (Tomar *et al*., 2000). The ability of Pb in retarding cell division was reported, as well as, the differentiation and that led to reduce elongation (Kastori *et al*., 1993).

Under tissue culture conditions, plant growth reduction was reported previously when culture media supplemented with Pb (Babu

et al., 2014; Muszynska *et al*., 2018). Our findings indicate that, the growth parameters were improved when plantlets passed from medium supplied with low to high concentration of Pb (as tolerant lines) compared to those treated with Pb in first experiment.This could be due to the adaptation of plants to the presence of Pb and this was on anatomical basis, our results confirmed that as seen in tables 2 and 3, the plantlets in tolerance lines showed alteration in the mesophyll thickness, number of mesophyll cell row, diameter of mesophyll cell, number of mesophyll cell in mm^2, blade thickness, vascular bundles and conductive elements characteristics. The noted results revealed, mesophyll thickness was increased up to 5.25 folds, number of mesophyll cell row to 3.11 fold, blade thickness to 4.34 fold, number of VB to 1.53 fold, VB width1.62 fold, VB height to 1.41 fold, upper VB cap thickness to 2.05 fold and lower VB cap thickness to 1.93fold in plantlets passed from medium contain 2.5 $mg.L^{-1}$ Pb to one contain 10 $mg.L^{-1}$ Pb compared to their growth in medium contain 2.5 $mg.L^{-1}$ Pb.

The reduction of mesophyll thickness cell size, number and size of vascular bundles, conducting elements and collapse of parenchyma cells are pronounced indicators of stressed plants (Sandalio *et al*., 2001; Sridhar *et al*., 2005; Gomez *et al*., 2011; Al-Saadi *et al*., 2013). These anatomical changes could be considered as a adaptation mechanisms of plant to heavy metals toxicity (Singh *et al*., 2015), such adaptation mechanisms helped plantlets to recover their health; or could be a consequence of genome template instability which reulsted from heavy metals damage into plant DNA as shown in Abass *et al*. (2018). The increased in mesophyll size could be explained by the increase of the vacuoles' size, as well as an increase in the size and diameter of the mesophyll cells. Ma *et al*. (2005) suggest that, sequestration of heavy metals in vacuoles and cuticle layers is important mechanisms to detoxification of heavy metals, which our results in tables 1 and 3 proved that.

Ciamporova (2002) reported an increase in intercellular spaces and/or in the number of parenchyma cells and that led to increase the leaf blade thickness. Vascular bundles structure may be altered when plant exposed to toxic metals (Mukhtar *et al*., 2013) and this changes ions and water status (De Jesus *et al*., 2016). The increase of mesophyll size could be explained by the increase in the distance between adjacent vascular bundles. Based on alteration in anatomical structure in plantlets examined as Pb-tolerance lines we assuming that this mechanism is related to the acquisition of tolerance by date palm cells exposed to Pb.

Conclusion

Here, we confirmed the negative effect of Pb at examined concentrations (2.5, 5, 10 $mg.L^{-1}$ Pb) on the growth parameters of *P. dactylifera* L., plantlets height and fresh weight; as well as; most analyzed anatomical characteristics were significantly reduced compared with untreated plantlets. Interestingly, theses parameters improved when plantlets passed from low to high concentration especially from 2.5 to 10 $mg.L^{-1}$.

Our results revealed that the Pb treatment led to an alteration of anatomical characteristics in stressed plantlets line, the reduction of both upper and lower cuticle layer, mesophyll features, blade thickness and vascular bundles were noted, only abaxial and adaxial epidermis did not affected. These results have changed to be close or superior to the control treatment when plantlets tested as Pb-tolerance clone by passing from low to high Pb concentrations, the best results were appeared in plantlets passed from culture media contain Pb at 2.5 $mg.L^{-1}$ to another one contain 10 $mg.L^{-1}$, these plantlets showed anatomical alteration as defense mechanism to tolerance Pb stress. This study confirmed the suitability of *in vitro* selection for generating of Pb tolerant plantlets.

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