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Effects of Lead Stress on Germination, Seedlings Physiological and Biochemical Characteristics of *Withania somnifera*

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Abstract: Lead is a toxic heavy metal found in the environment and affects all organisms. The aim of this study was to investigate the responses of *Withania somnifera* to lead (Pb) stress. Increasing lead concentration caused a decrease in germination percentage and germination index significantly 20 and 40 mM Pb $(NO_3)_2$ resulted in a huge reduction in germination percentage (21 and 9%, respectively). The mean germination time was only affected under 20 mM Pb $(NO_3)_2$. lead significantly reduced roots and shoots length compared to the control, the inhibition effect of lead was increased with the increasing concentration. Seedlings grown under high levels of lead (40 mM) showed the shortest root and shoot lengths (0.7 and 1.0 cm, respectively). An increase in proline content and lipid peroxidation was observed in *W. somnifera* seedlings under Pb treatment. lead concentration in plant tissues increased with the increase in lead concentration in the growing medium. In contrast to Pb concentrations in plant tissues, P and K concentrations decreased by all lead dozes.

Key words: *Withania somnifera*, germination, lead, proline, lipid peroxidation, inhibition

INTRODUCTION

Withania somnifera which is known as Ashwagandha (Datta *et al*., 2010) is a short shrub (35-75 cm) with acentral stem which branches extend radially in a star form. Flowers are very small and green in color. On the other hand their fruits are orange to red (Umadevi *et al*., 2012). *Withania* has been used as medicinal plant worldwide for over 3,000 years (Mirjalili *et al*., 2009). More than 22 species of *Withania* are known and widely distributed in the dry arias of tropical and subtropical regions including North Africa and the Mediterranean Region (Warrier and Nambiar, 1993). High levels of flavonoids and alkaloids were observed in *Withania* (Praveen *et al*., 2015).

Soil contamination with heavy metals becomes a widespread problem that affects the environment worldwide. Heavy metals occur naturally in soil as a result of human activity such as traffic, paint and many other non specific civilian sources (Tyler *et al*., 1989; Alloway, 2013). Heavy metals are major enviromantal pollutants due to their ecological, evolutionary, nutritional and enviromantal consequences (Benavides *et al*., 2005). Heavy metals are toxic to almost all living organisms. They are non-biodegradable elements and can stay in nature for very long period of time. Heavy metals affect human and plant metabolic activities. They have the ability to inhibit photosynthesis, disrupt Xylem tissues, reduce growth rate and induce plant chlorosis and many other symptoms. Heavy metals include Pb, Cd, Cu, Zn, Mn, Ni, As.

One of the most important heavy metals is lead it is considerd to be very toxic element to living organisims. Lead is a non-essential elemante that is negatively affects plant growth and developmant it is broadly used in pigments industry, batteries and as to stabilize plastic (Di Toppi and Gabbrielli, 1999). Lead is a major soil pollutant, its very toxic and water solubile (Pinto *et al*., 2004; Van Assche and Clijsters, 1990). It has been shown that lead hamper the uptake, movement and metabolism of many elements (including Ca, Fe, Mg, P and K) (Das *et al*., 1997; Alcantara *et al*., 1994).

The sensitivity of plants to heavy metals such as lead depends on interrelated pathways of biochemical, physiological and molecular mechanisms (Verkleij and Schat, 1990). Lead toxicity symptoms including stunting and chlorosis (Haghiri, 1974). It has been shown that several cytogenetic consequences of lead toxicity were observed such as chromosomal aberration, formation of intramitochondrial granules filled with cadmium, mitochondrial degeneration and inhibition of cell proliferation (Silverberg, 1976; Rosas *et al*., 1984). Furthermore, lead toxicity affects permeability of plasma membrane and resulted in reduction of water content

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(Fodor *et al*., 1995). Found that lead stress affected membranes functions by inducing lipid peroxidation. Also, it has been shown that alteration in chloroplast metabolism was observed after Pb stress as a result of chlorophyll biosynthesis inhibition and reduction in enzymes involved in CO_2 fixation activity (De Filippis and Ziegler, 1993).

Many studies found Pb tolerant plant species including S. *cathayana* and L. *plyneura* that are able to accumulate high levels of lead in certain organs such as their roots (Sharma and Dubey, 2005). Plants use different mechanisms for lead tolerance including developing physical barriers that limits the uptake of Pb, detoxification and excretion of absorbed lead and using antioxidants defense mechanisms (Pourrut *et al*., 2011). The aim of this study was to examine the effect of lead stress on seed germination, seedlings growth and biochemical properties of *Withania somnifera*.

MATERIALS AND METHODS

Plant material and pb treatment: At the beginning, seeds were washed with distilled water then surface sterilized using 0.05% $HgCl_2$ for 5 min followed by 1 min washing with 70% ethyl alcohol. At the end seeds were washed with distilled water for 3 times, 1 min each time.

Different concentrations of Pb $(NO_3)_2$ (0, 5, 10, 20 and 40 mM) were prepared in distilled water; Distilled water was used as control. Seeds were germinated in Petri dishes (90 mm diameter) containing double layer of filter paper soaked with 7 mL treatment solution (either distilled water or various levels of Pb $(NO_3)_2$ Petridishes were incubated in the growth chamber at 25±1 C and under 8/16 h (dark/light) photoperiod using white fluorescent tubes. Germination percentage was recorded after 5 days. Seeds showing radical emergence was considered as germinated seed. Mean Germination Time (MGT) was calculated according to the following equation:

$$MGT = \Sigma f \cdot x/\Sigma f$$

where, f = Seeds germinated on day x. Germination Index (GI) was calculated according to the following equation:

$$GI = (10\times n1)+(9\times n2)+, \cdots, +(1\times n10)$$

where, n1, n2, . . . , n10 = No. of germinated seeds on the first, second and subsequent days until the 10th day; 10, 9, . . . and 1 are weights given to the number of germinated seeds on the first, second and subsequent day, respectively. Seedlings root and shoot length, number of roots, fresh weight were also measured.

Proline content determination: Proline content in *W. somnifera* shoots was measured according to Bates *et al*. (1973) protocol with few modifications. A mixture containing proline, ninhydrin acid and glacial acetic acid (1:1:1) was heated to 95°C for 45 min. Then, the reaction was stopped in an iced bath. After that the chromophore layer was removed by using toluene (3 mL). Finally, the absorbance was recorded at 520 nm using spectrophotometer.

Lipid peroxidation assay: Lipid peroxidation was determined using TBARS method according to (Heath and Packer, 1968). One gram of fresh *W. somnifera* shoots was homogenized in 50 mL of 0.1% trichloroacetic acid then centrifuged at 12000 rpm for 20 min. Then, 3 mL 0.5% thiobarbituric acid in 20% TCA was added to 1.5 mL supernatant and mixed by pippiting. The mixture was heated to 97°C for 20 min using water bath and then the reaction was stopped using ice bath. The mixture was centrifuged at 12,000 rpm for 5 min then the absorbance was measured at 532 nm. Finally, TBARS content was calculated using extinction coefficient of 155 mM^{-1} cm^{-1}.

Elements analysis: Seedlings were washed with 2.5 mM citrate solution for 3 times, 15 min each time at 4°C to remove the surface attached lead then seedlings were moved to the oven (70°C) for 72 h for complete dryness. Then, 10 mL concentrated HNO_3 was added to dry samples for digestion in heat blocks at 100°C for 30 min. Then, the sample volumes were completed to 20 mL using double distilled water. Finally, total Pb concentration was measured using atomic absorption spectrometer. Total phosphorus in dried plant samples was determined using ascorbic acid molybdate blue method (John, 1970) and for potassium content by flame photometer method.

Statistical analysis: Data are presented as means±SD. Five replications were used in this study and the whole experiment was repeated 2 times. Statistical analysis of this study was done using one-way Analysis of Variance (ANOVA) using SPSS 16.0 Software. Comparisons of means were performed using Fisher's LSD test at $p = 0.05$.

RESULTS AND DISCUSSION

Effect of Pb on seed germination: *Withania somnifera* seeds were treated with different levels of Pb (5, 10, 20 and 40 mM). The highest germination percentage was observed under control conditions (94%). Seeds treated with 5 mM showed similar germination as control (91%). Treating seeds with 10 mM significantly resulted in lower

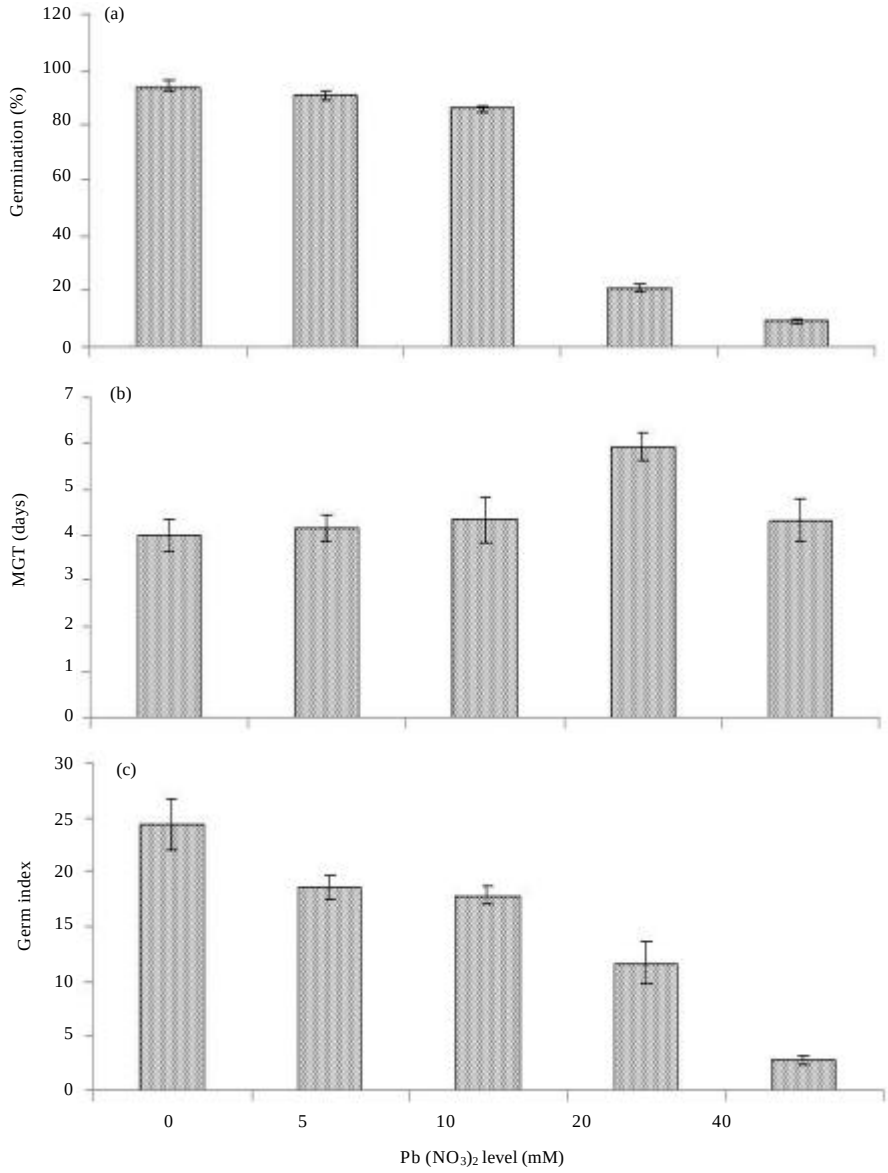

Fig. 1: Effects of different Pb $(NO_3)_2$ levels on germination %; a) Mean germination time; b) Germination index and c) The errors bars represent standard deviation values (n = 5)

germination (86%) than control. Higher levels of Pb (20 and 40 mM) resulted in huge reduction in germination percentage (21 and 9%, respectively) (Fig. 1a).

For mean germination time, no significant differences were observed between 5, 10 mM and control. Under 20 mM Pb $(NO_3)_2$ higher mean germination time was observed. Finally, seeds treated with 40 mM Pb $(NO_3)_2$ showed similar mean germination time to those grown under control conditions (Fig. 1b).

The effect of different Pb levels on germination index is presented in Fig. 1c. A significant difference between Pb levels on germination index was observed. Seeds treated with distilesd water (control) showed the highest germination index was found the control treatment. Seeds treated with intermediate levels of lead showed lower germination index than control seeds. Increasing lead concentration up to 40 mM significantly reduced germination index.

Effect of Pb on growth parameters: After 10 days of growth under different levels of Pb (0, 5, 10, 20 and 40 mM) results showed that Pb exhibited significant effect on shoot and root length. For root length, lead treated plants showed gradual decrease with the increasing Pb

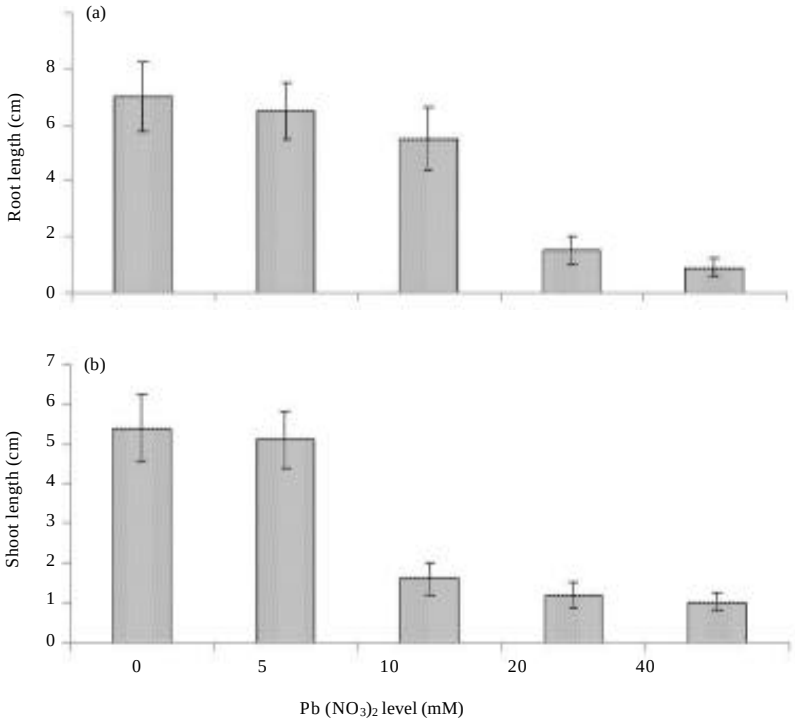

Fig. 2: Effects of different Pb $(NO_3)_2$ levels on root length (cm); a) Shoot length (cm) and b) The errors bars represent standard deviation values (n = 5)

level compared to control plants. Plants grown under control conditions showed the longest roots 7 cm. In contrast, plants grown under high level of Pb (40 mM) showed the shortest root length 0.7 cm (Fig. 2a).

Similar effect of Pb on shoot length was observed. High levels of Pb (10, 20 and 40 mM) significantly decrease shoot length compared to control plants. Shoot length of plants grown under 40 mM Pb showed more than 80% reduction compared to control. On the other hand, plants grown under low Pb levels (5 mM) showed similar shoot length as control (Fig. 2b).

Effect of Pb on biochemical content: A significant effect of Pb $(NO_3)_2$ on *Withania somnifera* proline content was observed. Plants grown under control conditions showed the lowest proline content (24 μg/mL). Plants grown under 5 and 10 mM Pb $(NO_3)_2$ showed significantly higher levels of proline (104 and 107 μg/mL, respectively) than control plants. Furthermore, growing *W. somnifera* plants under 40 mM Pb $(NO_3)_2$ significantly increased proline content and resulted in the highest level (135 μg/mL) compared to other treatments (Fig. 3a).

Lipid peroxidation of *Withania somnifera* plants under Pb $(NO_3)_2$ was also measured. Results showed that three different responses were observed; Plants grown under control conditions or low concentrations of Pb $(NO_3)_2$ showed the minimal LPO levels whereas plants grown under moderate levels of Pb $(NO_3)_2$ showed LPO values higher than the first group (around 15 μmol g FW^{-1}). Finally, plants grown under high levels (40 mM) of Pb $(NO_3)_2$ showed the highest amount of lipid peroxidations (24 μmol g FW^{-1}) (Fig. 3b).

Effect of Pb on Pb, P and K content: Growing *Withania somnifera* plants under Pb $(NO_3)_2$ treatment significantly increased Pb concentrations in plant tissues. A gradual increase in Pb concentration was observed in *Withania somnifera* tissues. Plants grown under 40 mM Pb $(NO_3)_2$ showed the highest Pb concentration in dry tissues (30.6 $mg.kg^{-1}$ DM) (Fig. 4a). K and P contents of *Withania somnifera* shoots grown under different levels of Pb $(NO_3)_2$ are shown in Fig. 4b and C. In general with the increase in Pb $(NO_3)_2$ concentration, K and P contents decreased. The highest K content was observed in plants grown under 5 mM Pb $(NO_3)_2$ but that was statistically similar to control plants. In contrast, the lowest concentration was observed under 40 mM Pb $(NO_3)_2$. For P content, lead significantly decrease phosphorus levels in *Withania somnifera* shoots. A sharp decrease in P concentration was observed when plants were grown under 5 mM Pb $(NO_3)_2$. The <0.5 mg/g P content was observed under 40 mM Pb $(NO_3)_2$.

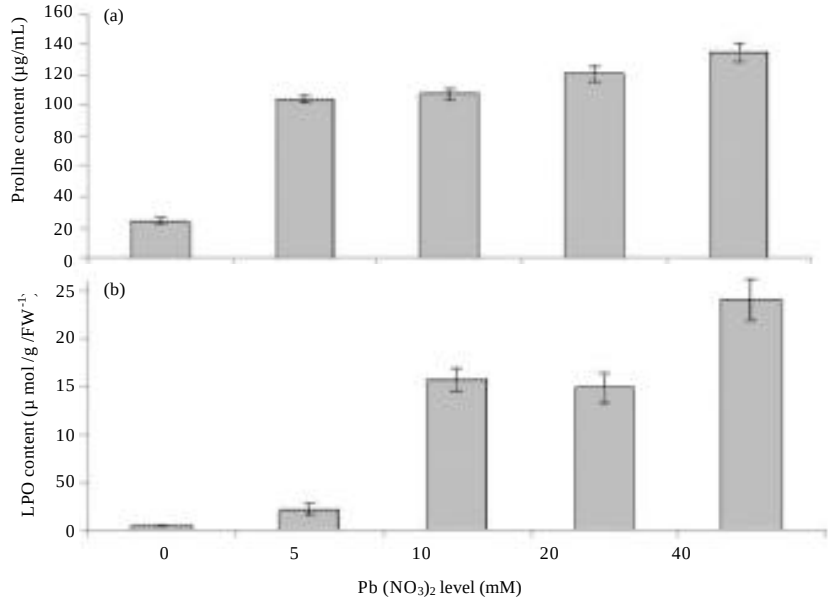

Fig. 3: Effects of different Pb $(NO_3)_2$ levels on Proline content; a) LPO and b) The errors bars represent standard deviation values (n = 5)

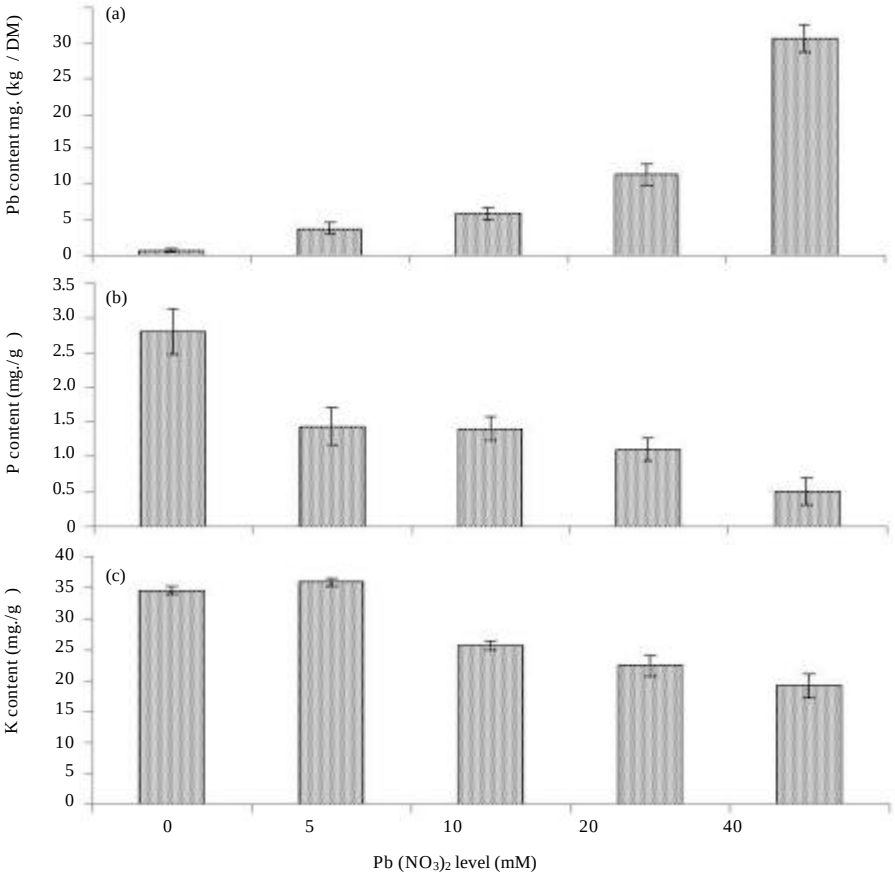

Fig. 4: Effects of different Pb $(NO_3)_2$ levels on Pb; a) P; b) K and c) Content and the errors bars represent standard deviation values (n = 5)

Results of this study showed that germination percentage and germination decreased significantly with the increase of lead compared to control treatment. These observations were in accordance with the findings of (Shafiq *et al*., 2008; Azmat *et al*., 2006) for different plant species. Ilic *et al*. (2015) studied the effect of Pb on different plant species; They found that high levels of Pb totally inhibited lettuce seeds germination, reduced germination percentage of carrots for 26% and for radish for 7.3% relative to control treatment. It has been shown that heavy metals reduced water uptake and movement to plant cells and this ultimately could reduce or delay the germination process (Poschenrieder *et al*., 1989). Vijayaragavan *et al*. (2011) found that high levels of heavy metals in *Vigna unguiculata* seeds germination medium decreased the availability of water to the embryo. Sharifah and Hishashi (1992) showed that the reduction in germination percentage of plants grown under heavy metals stress is a result of direct effect of these metals on amylase activity.

W. somnifera root and shoot length has been adversely affected after Pb treatment compared to control Fig. 2. Heavy metals could reduce rate of cell division, cellular turgor pressure, photosynthesis and respiration efficiency and all of these could affect plants growth and development (Kupper *et al*., 1996). Further more, different morphological and structural responses were also observed in plants grown in the presence of heavy metals including retardation in root elongation, damage of the root tip, reduction of number of roots, reduction in cell elongation rate (Marcano *et al*., 2002). In a pot experiment, *Brassica perkenensis* was treated with different levels of Pb, results showed a decrease in root and shoot lengths with the increase in Pb concentration in the growing treatment. Plants grown under high levels of Pb showed less than 12% root growth compared to plants grown under control conditions (Xiong, 1998). Akinci *et al*. (2010) studied the effect of Pb stress on tomato seedlings, they found that fresh and dry weights of roots and shoots were significantly reduced by increasing Pb levels. Verma and Dubey (2003) found that *Oryza sativa* L. shoots and roots lengths and weights decreased with the increase in Pb levels compared to control seedlings. Root growth of *Lens culinaris* was reduced after treatment with lead compared to control group, furthermore, the increase Pb concentrations reduced cell division rate and resulted in different mitotic alterations including chromosomes lagging and chromosome bridges formations (Kiran and Sahin, 2005).

Proline is considered as multifunctional amino acid it is important in signaling pathways as a starter of the waves during the signaling route. Proline acts as protein stabilizer and metal chelator to enhance tolerance of metals toxicity in plants (Sharma and Dubey, 2005). Here, an increase in proline levels was observed in *W. somnifera* plants grown under Pb stress. It has been shown that proline reduced proteins denaturation and membrane consistency it stabilize enzymes and reducing the harmful effect of ROS. Proline plays a crucial role in enhancing plant stress tolerance including heavy metals toxicities. Theriappan *et al*. (2011) studied the effect of different concentrations of heavy metals on Cauliflower seedlings, they found that proline accumulation rate in seedlings treated with heavy metals is 2 times higher than seedlings grown under control conditions.

Lipid peroxidation is considered as a biochemical indicator for the production of free radical inside the cells. The increase in lipid peroxidation is an indicator of cellular oxidative stress resulted from the unbalanced equilibrium between the amount of ROS produced and scavenged due to various defense mechanisms under stress conditions (Lima *et al*., 2006). Our results showed that lipid peroxidation rate was increase in *Withania* seedlings after treatment with lead (10-40 mM). Our results are in agreement with (Malecka *et al*., 2001) who found that pea seedlings treated with Pb stress showed high level of oxidative stress. Similarly in another study, the effect of Pb $(NO_3)_2$ on rice (*Oryza sativa* L.) seedlings was studied, they found that rice seedlings treated with Pb showed higher levels of lipid peroxidtion in addition to, increase in ascorbate peroxidise, glutathione reductase and superoxide dismutase enzymes activities compared to seedlings grown under control conditions (Verma and Dubey, 2003).

Results of this study showed that Pb concentrations in *W. somnifera* increased significantly with the increase in Pb concentration in the germination medium reaching 30.6 mg/kg^{-1} dry matter. In agreement with our results (Zhou *et al*., 2018) studied the effects of different levels of Pb ion content of privet plants they found that Pb concentrations were increased after treatment compared to control plants. Plants takes Pb from the soil solution using their roots. In a study aimed to investigate the effect of lead on cytoplasmic streaming of *Allium cepa* L. they found that most (p>97%) of Pb was accumulated as an insoluble form inside the roots epidermal cells (Wierzbick *et al*., 2007). It has been shown that there are differences in Pb accumulation rates between plant species. Mesmar and Jabor (1991) compared accumulation rates of Pb between T. *aestivum* and *Lens culinaris* seedlings they found that T. *aestivum* accumulate higher levels of Pb than L. *culinaris* they explained that due to the presence of nitrogen fixiation nodules on L. *culinaris* roots which would limit Pb absorbance by lentil plants. Also, results of the present study found that P and K contents in *Withania* seedlings were significantly lower

than control plants. Similarly, wheat (*Triticum aestivum*) and spinach (*Spinacia oleracea*) seedlings grown under high levels of Pb $(NO_3)_2$ showed low P content compared to control (Lamhamdi *et al*., 2013). P and K content in eggplant seedlings (*Solarium melongena*) were also, reduced due to lead stress (Yilmaz *et al*., 2009). It has been shown preveiously that Pb physically inhibits and some times totally prevents the tranfer of many elements from soil to the root system (Godbold and Kettner, 1991). Lamhamdi *et al*. (2013) found that Pb toxicity symptoms including reduction in growth rate could be a result of macroelements (especially K, P, Ca and Mg) deficiency which is a result inhibition of these elements uptake under Pb stress.

CONCLUSION

Results of this study showed that Pb stress reduced germination parameters, shoot and root elongation and increased lipid peroxidation and proline content. Higher levels of Pb accumulation were observed in *Withania* plants grown under Pb stress. In addition, Pb stress limited the uptake of P and K. Further studies should be conducted to clearly understand the mechanisms of interaction between these parameters.

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