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Comparative Evaluation of Zinc and Lead and their Synergistic Effects on Growth and Some Physiological Responses of Hassawi Okra (*Hibiscus esculentus*) Seedlings

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ABSTRACT

Soil contamination with heavy metals has become a worldwide problem, leading to a reduction of plant growth and productivity. The objective of this study was carried out to compare the effect of different levels of zinc (Zn^{2+}) or lead (Pb^{2+})and their interactions on growth and physiological changes of Hassawi okra seedlings. These seedlings were grown in a soil, to which different levels (0, 5, 10, 20, 40, 80 mM) of Zn^{2+} or Pb^{2+} were added with water (control), singly or in combinations. The results revealed that, okra seedlings growth were reduced gradually with increasing Zn^{2+} or/and Pb^{2+}. These parameters were completely inhibited at 80 mM of Zn^{2+} or Pb^{2+}, when they added in combination. While, 5 mM Zn^{2+} had a favorable effect on these growth parameters. The contents of chl. a and chl. b were significantly reduced as a result of exposure to Zn^{2+} or/and Pb^{2+}. However, carotenoids content was increased at lower levels as compared with control. The increased of Zn^{2+} or/and Pb^{2+} levels inhibited sugars accumulation while the contents of protein, total free amino acids and proline were promoted. Pb^{2+} had more toxic effect than Zn^{2+}. The toxicity caused by each metal alone was lower than that caused when they were added in combinations. Our data suggested that Zn^{2+} and Pb^{2+} exhibited synergistic effects on the growth and physiological responses of Hassawi okra seedlings, leading to injurious effects followed by death of these seedlings at 80 mM. Combined exposure to Zn^{2+} and Pb^{2+} enhanced or inhibited some of the effects, that were induced when only one metal was applied to growth medium.

Key words: Free amino acids, membrane stability index, photosynthetic pigments, proline, proteins, sugars

INTRODUCTION

Heavy metal pollution in air, water and agricultural soils is one of major ecological concern due its impact on human health through the food chain and its high persistence in the environment (Valko *et al*., 2005; Jiang *et al*., 2010). Environmental pollution by toxic metals originate from many activities, largely industrial (Waisberg *et al*., 2003). Soil contamination with Heavy Metals (HM) is a worldwide problem, leading to agricultural losses and hazardous health effects as metals enter the food chain. HM can infect soils in town areas, between many of them, Zn^{2+} and Pb^{2+} deriving from galvanized metals, rubber production batteries, petrol, steel mill residues and paints (Garg and Aggarwal, 2011).

The phytotoxicity of the heavy metals due to industrial pollution has serious implications in soil degradation (Friedland, 1990). This may reduce both the quality and productivity of plants. Although, Zn is an essential element to plants, excess Zn inhibits plant growth and impairs some important cellular processes. Zinc concentrations in plant above than 300 mg kg^{-1} are considered toxic (Marschner, 1995). It is an essential trace element for the normal healthy growth and reproduction of some plants and present in enzymatic system. Genc *et al.* (2006) reported that zinc has vast members of functions in plant metabolism. Although, Zn^{2+} required in low concentrations in the plant organs (Parker *et al.*, 1992). It is highly toxic at elevated concentrations and can slow down plant growth and disrupt various essential physiological processes (Clemens, 2006). Zn^{2+} was decreased respiratory rate and increased of membrane damage in sunflower and safflower plants and affected the accumulation of other nutrients in different plants (Ismail and Azooz, 2005). Zn^{2+} at high levels leads to chlorosis in young leaves, inhibits photosynthesis at various steps and decreases the growth and metabolic activity in various plant species (Panda *et al.*, 2003; Vaillant *et al.*, 2005). Excess zinc in the soil can alter certain physiological equilibrium because the similarities in ion radius of bivalent cations and their local competition at various sites (Monnet *et al.*, 2001). It has been reported that phototoxic concentrations of Zn^{2+} induce lipid peroxidation, thereby enhancing antioxidative activity in plants (Weckx and Clijsters, 1997).

Lead (Pb^{2+}) is a heavy metal, with characteristic toxic action and main sources of environmental pollution. It inhibits some of metabolic activities in the plant such as the biosynthesis of nitrogenous compounds, carbohydrate metabolism and water absorption (Sharma and Dubey, 2005; John *et al.*, 2008; Hamid *et al.*, 2010). Plants exposed to Pb^{2+} showed a considerable decrease in their dry weight and a decline in the total chlorophyll and those photosynthetic efficiency (Kosobrukhov *et al.*, 2004; Kambhampati *et al.*, 2005). The plant processes are adversely affected by increasing Pb^{2+} levels in soils and even at very low concentration (Patra *et al.*, 2004).

Plants have developed defense strategies against heavy metals. They have developed two basic strategies to resist the toxicity of heavy metals: avoidance and accumulation (Tang *et al.*, 2009). Most heavy metal-tolerant species have the capability of preventing heavy metal accumulation in their shoots and therefore are called excluders while others can take up heavy metals, translocate them into the shoots and requisition them in non-metabolic-active tissues and organs in less harmful forms and these plants, called hyper-accumulators (Kupper *et al.*, 2007).

Okra Hassawi plant (*Hibiscus esculentus* L., member of Malvaceae) is cultivated widely in Al-Hassa region (eastern region) in Saudi Arabia. The plant organs, such as flowers and green tissues, may be consumed as foods while the seed oil may be used for industrial or food purposes (Andras *et al.*, 2005).

The knowledge about the toxic effects of Zn^{2+} and Pb^{2+} on the physiological processes in Hassawi okra is potentially useful to establish criteria for selection of plants to be used as bio-indicators. Therefore, the purpose of this investigation was to evaluate the influence of an additional supply of Zn^{2+} and Pb^{2+} (in nitrate form) on: (1) growth parameters and (2) some physiological activities in Hassawi okra seedlings. This may be allowed to find differential characteristics suitable to be used as bio-indicators of toxic levels of Zn^{2+} and Pb^{2+}. In addition, this study will provide better understanding to the tolerance mechanisms of Hassawi okra to the toxicity of Zn^{2+} and Pb^{2+}. The treatments were added to the soil in solution with water only (control), one metal and with a mixture of both. The mixture of metals was included in order to determine the occurrence of any antagonistic or synergistic interactions between Pb^{2+} and Zn^{2+}.

MATERIALS AND METHODS

Plant growth and treatments: The experiments were carried out within the period of September 2010-February 2011, in the Faculty of Science, King Faisal University. Homogenous Hassawi okra seeds were chosen and surface sterilized with 0.1% sodium hypochlorite for 5 min and washed three times with sterile water. Sterile seeds were germinated in dark on plastic trays covered with wet filter papers and were kept wet by spraying with sterile water. After complete germination, ten uniformly germinated seeds were sown in the prepared soil at a depth of 1 cm in each pot. Seedlings of okra (*Hibiscus esculentus* cv. Hassawi) were grown in plastic pots filled with soil composed of air dried clay and sand (1:1 by volume) in growth chamber under constant conditions. The Hassawi okra germinated seeds were divided into 4 groups. The first group was supplied with water (control). The second and the third groups were supplied with 5, 10, 20, 40 and 80 mM Zn $(NO_3)_2$ and Pb $(NO_3)_2$, respectively. The forth group was supplied with 5, 10, 20, 40 and 80 mM of both Zn $(NO_3)_2$ and Pb $(NO_3)_2$. The soil was irrigated with the applied concentrations of Zn^{2+} and Pb^{2+} or both. Three replicates for each treatment were prepared. The plants were watered with normal water to field capacity by weighing the pots every two days and left to grow until the end of experiment (30 Days after Sowing (DAS)).

Determination of growth parameters: At the end of experiment, Hassawi okra seedlings were harvested and washed with distilled water three times. Leaf area, root and shoot length of seedlings were determined. To determine fresh and dry weight of seedlings, the freshly seedlings are rinsed with deionized water and blotted on paper towels before being weighed (fresh weight). To determine the dry matter yields, the freshly tissues were dried in an aerated oven at 80°C to constant weight.

Photosynthetic pigments content (chl a and chl b and total carotenoids) were estimated in 80% acetone extracts using the spectrophotometric method according to Lichtenthaler and Wellburn (1983).

Membrane stability index (MSI): Leaf Membrane Stability Index (MSI) was determined according to the method of Premchandra *et al.* (1990) as modified by Sairam (1994).

Determination of some metabolic activities: Sugars were extracted from the plant tissues and quantified by the anthrone sulphuric acid method described by Fales (1951). The soluble and insoluble proteins were determined according to Bradford (1976). Free amino acids were extracted from plant tissues and determined according to the method of Lee and Takahashi (1966). Proline was determined according to Bates *et al.* (1973).

Statistical analysis: Statistical analysis of data was conducted using one-way Analysis of Variance (ANOVA) using SPSS 12.0 software. Values in the figures indicate the mean values±SD based on independent three determinations (n = 3). Least Significant Difference (LSD) test was used to assess the differences between control and different treatments; $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Growth parameters: The growth parameters were used as a useful bio-indicators of zinc or/and lead toxicity in okra seedlings. These parameters are expressed as leaf area, root and shoot length and fresh and dry weight (Fig. 1a-e). Increasing of Zn^{2+} or/and Pb^{2+} levels exhibited gradual reduction in these parameters. While, they were exhibited injurious effects followed by the death of the okra seedlings, when they added in combination at the highest (80 mM) level (So, data do not appear in the all figures at this level). It is worthy to note that, Zn^{2+} at low level (5 mM) promoted the growth parameters in comparing to their respective control. Inhibition of growth and reduction of biomass production are general responses of some higher plants to Zn^{2+} or/and Pb^{2+} toxicity and are often a reliable indication of plant's sensitivity to their stresses (Kosobrukhov *et al*., 2004; Vaillant *et al*., 2005; Sharma *et al*., 2009). The reduction in growth could be due to the suppression of the elongation growth of cells as reported by John *et al*. (2008). The inhibition of root growth could lead to the slowing down of the water absorption and mineral elements and consequently affects the growth of okra seedlings.

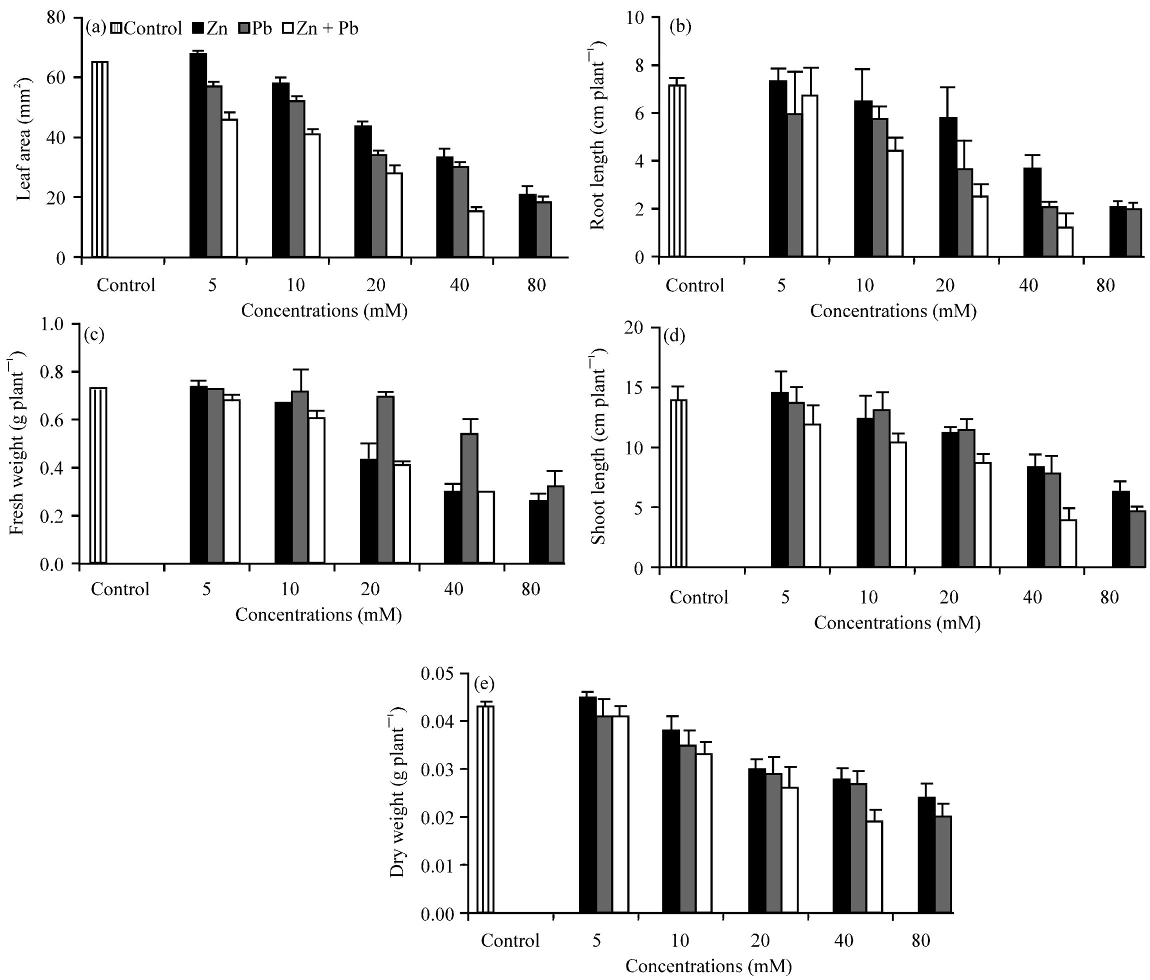

Fig. 1(a-e): Effect of Zn^{2+} or/and Pb^{2+} treatment individually or in combination on (a) leaf area (mm^2), length (cm $plant^{-1}$) of (b) root and (c) shoot and (d) fresh and (e) dry weight (g $plant^{-1}$) of Hassawi okra seedlings. Vertical bars represent ±SD

The inhibitory effects on growth parameters caused by Pb^{2+} was more phytotoxic than that of Zn^{2+}, suggesting that the okra seedlings were more sensitive to the toxicity of Pb^{2+} than Zn^{2+} metal. In addition, Zn^{2+} is an essential element for plants and may be absorbed and become involved in metabolic pathways more readily than Pb^{2+} (Hardiman *et al.*, 1984). The effects of Zn^{2+} and Pb^{2+} in combination were much severe than that of Zn^{2+} or Pb^{2+} alone, leading to the injurious effects followed by the death of the okra seedlings at the highest levels which may suggest a synergistic effect between Zn^{2+} and Pb^{2+} on okra seedlings growth. Similar inhibitory effects in co-presence of metals have also been observed in other plant species (Ruley *et al.*, 2006; January *et al.*, 2008; Israr *et al.*, 2011). Guo *et al.* (2007) showed that the synergistic effects of heavy metal burdens are significantly more toxic than the individual heavy metal exposure. The enhancement effect of Zn^{2+} at the lower concentration on growth parameters was recommended by Ismail and Azooz (2005) and Sharma *et al.* (2009). This may be attributed to the beneficial vital role of Zn^{2+} in plant growth and development. Wierzbicka and Obidziska (1998) reported that Zn^{2+} is a micronutrient and indispensable for plant growth. Bagci *et al.* (2007) suggested that adequate applied of Zn^{2+} improved the water use efficiency of wheat plant. However, Zeid (2001) concluded that, at critical level of Zn^{2+} could behave as toxic metal like other heavy metals. These reports are in a good agreement and supported our results in respect to the biphasic effects of Zn^{2+} on the growth parameters of Hassawi okra seedlings.

Photosynthetic pigments: The contents of photosynthetic pigments such as chl. a, chl. b, total chlorophyll and carotenoids of Hassawi okra leaves, grown under different levels of Zn^{2+} or/and Pb^{2+} (Fig. 2a-e) indicated that, there were no significant changes on the contents of chl. a, chl. b up to the level of 10 mM Zn^{2+} or Pb^{2+}, thereafter, a significant decrease were obtained. On the other side, the content of chl. a (at lower concentration of Zn) and carotenoids (at the most concentrations of Zn^{2+} or Pb^{2+}) was increased as compared with control. The reduction in the content of chl. b was higher (43.3 and 60.9%) than chl. a (34.5 and 50.6%) below the control, when Hassawi okra seedlings were exposed to the highest Zn^{2+} and Pb^{2+} levels respectively, resulting in a higher chl. a/b ratio than in control. The reductions in photosynthetic pigments were more obvious in case of Pb^{2+} than $Zn^{2+}$and in binary than in individually. Reduction in chlorophyll contents by excess Zn^{2+} and Pb^{2+} and increased of carotenoid content have been documented by other investigators (Ismail and Azooz, 2005; Sharma *et al.*, 2009; Hamid *et al.*, 2010). The biosynthesis of photosynthetic pigments is believed to be due to the adequate supply of metal ions. Thus, the excess supplies of Zn^{2+} and/or Pb^{2+} may be prevent the incorporation of Fe in phytoporphyrin molecule resulting in the reduction of chlorophyll pigments (Nyitrai *et al.*, 2002; Jaleel *et al.*, 2009). Sharma and Dubey (2005) reported that plants exposed to Pb^{2+} showed a decline in the photosynthetic rate as a result of restrained synthesis of chlorophyll. This may be as a result of increased in chlorophyllase activity (Drazkiewicz, 1994). Increasing of carotenoids content in the Hassawi okra seedlings at the most levels of Zn^{2+} or Pb^{2+} could be enhanced their capacity to minimize the toxic effect caused by these metals. Singh *et al.* (2006) reported that the enhancement in carotenoid content in heavy metal-treated plants, is probably a part of strategy adopted by the plant to alleviate the toxic effect of free radicals generated under heavy metal toxicity. Hu and Sparks (1991) reported that Zn^{2+} plays a key role in photosynthesis affecting chlorophyll synthesis. The observed increased in chlorophyll content at lower level of Zn^{2+} may be attributed to the better growth of okra seedlings. Sharma *et al.* (2009) suggested that increased of chlorophyll content at lower concentration of Zn^{2+} in *Cicer artietinun* might be due to eagerly gathering of Zn^{2+} in the leaf which significantly affects metabolic processes in the chloroplast.

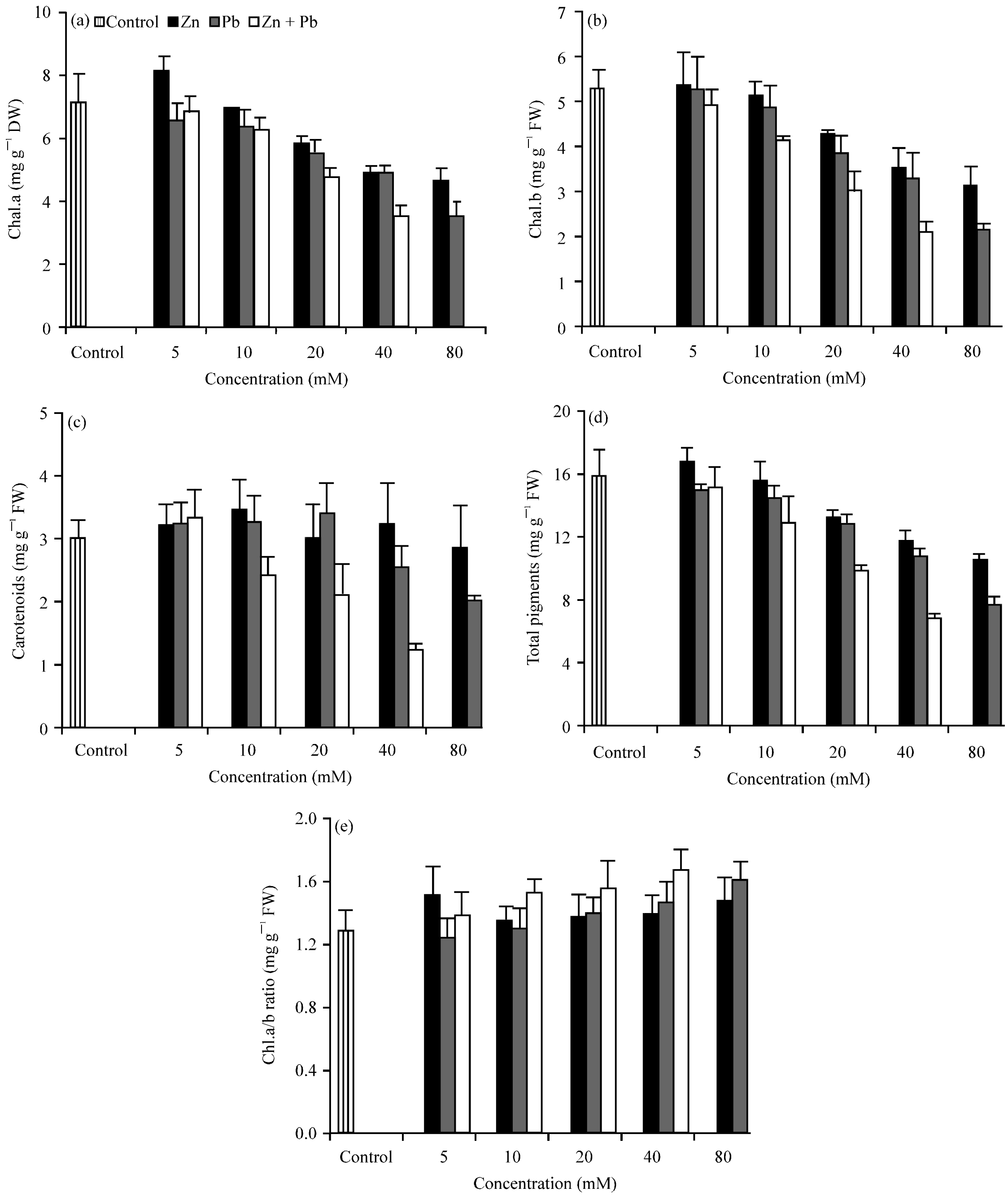

Fig. 2(a-e): Effect of Zn^{2+} or/and Pb^{2+} treatment individually or in combination on the leaf contents (mg g^{-1} fresh weight) of (a) chl. a (b) chl. b (c) carotenoids, (d) total chlorophyll and (e) chl. a/chl. b ratio in leaves of Hassawi okra seedlings. Vertical bars represent ±SD

Sugar contents: The effect of Zn^{2+} or Pb^{2+} individually or in combination in Hassawi okra seedlings on reducing, non-reducing and total sugars (Fig. 3a-c) showed that, Zn^{2+} exhibited positive effect on the reducing sugars at most levels while a decreasing trend in the content of other sugar fractions was observed as a result of increasing in Zn^{2+} or/and Pb^{2+} levels when compared

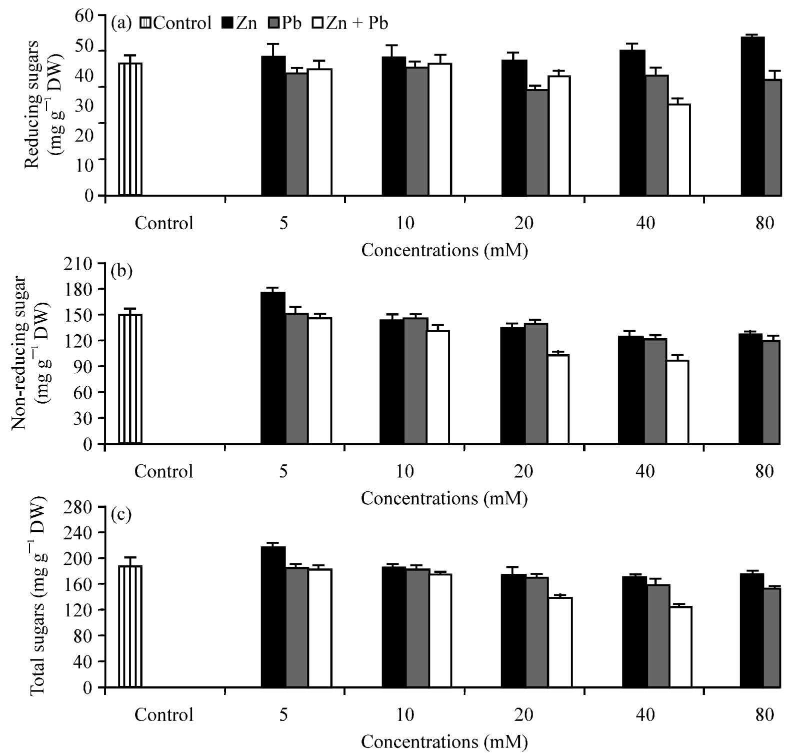

Fig. 3(a-c): Effect of Zn^{2+} or/and Pb^{2+} treatment individually or in combination on the contents (mg g^{-1} dry weight) of (a) soluble (b) insoluble and (c) total sugars of Hassawi okra seedlings. Vertical bars represent ±SD

with control. This decrease was more obvious when Zn^{2+} and Pb^{2+} were applied binary than individually; similarly with early other reports (Farshian *et al.*, 2007; Jaleel *et al.*, 2009; Hamid *et al.*, 2010). The increases of reducing sugar at most levels of Zn^{2+} could be improve the tolerance of Hassawi okra seedlings to zinc toxicity. Verma and Dubey (2001) suggested that higher concentration of soluble sugar could possibly provide an adaptive mechanism in maintaining favorable osmotic potential under toxicity of heavy metals. This was in a good coincidence with our results and explains the slighter toxicity caused by Zn^{2+} in comparing to Pb^{2+} on the growth parameters of Hassawi okra seedlings. The reduction in sugar fractions may be due to the decrease observed in chlorophyll content and the abnormal structure of chloroplast which led to decrease photosynthesis efficiency (Farshian *et al.*, 2007). A synergistic effect of both metals was detected in the seedlings exposed to the combination effect of both Zn^{2+} and Pb^{2+} leading to a higher decrease of sugar fractions as compared to the seedlings exposed to Zn^{2+} or Pb^{2+} alone.

Protein contents: Application of Zn^{2+} individually or in combination with Pb^{2+} increased protein content especially under the higher levels. While in most cases, excess supply of Pb^{2+} did not affect significantly insoluble and total protein while a decreased in soluble protein content was observed (Fig. 4a-c). The stimulation effect was more pronounced in seedlings exposed to Zn^{2+} alone than in combination with Pb^{2+}. Heavy metal stress has been shown to induce a variety of proteins resulting in an overall increase in protein content (Shah and Dubey, 1997). Our results coincide with the results obtained by John *et al.* (2009) in *Brassica juncea* L. and Hamid *et al.* (2010) in

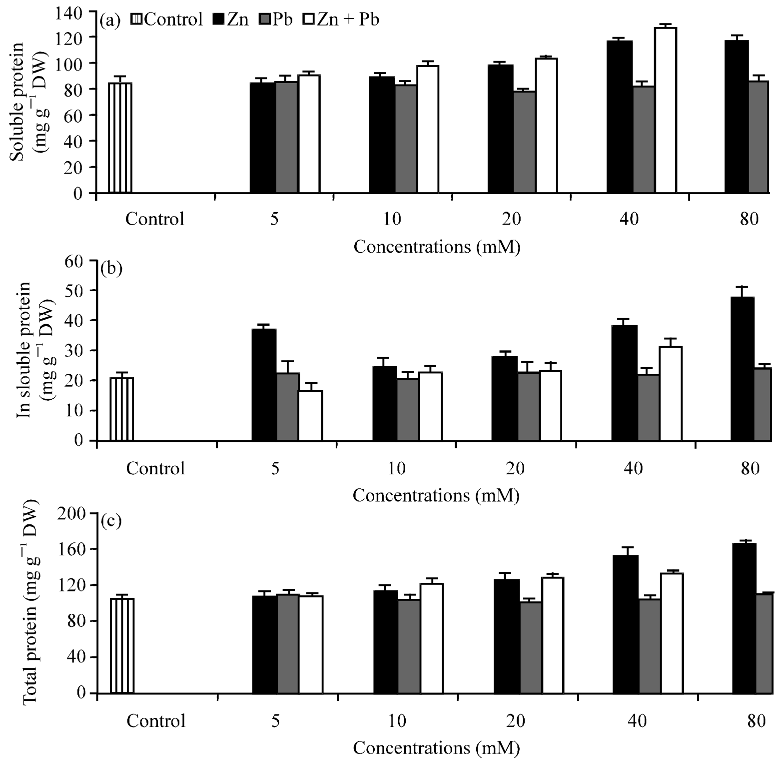

Fig. 4(a-c): Effect of Zn^{2+} or/and Pb^{2+} treatment individually or in combination on the contents (mg g $^{-1}$ dry weight) of (a) soluble (b) insoluble and (c) total proteins of Hassawi okra seedlings. Vertical bars represent ±SD

Phaseolus vulgari, who also reported a decrease in a soluble protein content in response to Pb^{2+} stress, suggesting that these heavy metals may have induced lipid peroxidation and fragmentation of proteins due to toxic effects of reactive oxygen species which led to reduced protein content. The decrease in protein content in Hassawi okra seedlings grown under Pb^{2+} may be due to the enhanced of protein degradation process as a result of increased protease activity (Palma *et al.*, 2002) which is found to increase under stress conditions. While, the increased in protein content in okra seedlings exposed to Zn^{2+} or Pb^{2+}alone and in combination may be due to the increase in nitrate resource in soil produced by these metals, because they were applied in nitrate form, leading to release of N in the soil and consequently increased the biosynthesis of protein, or may be due to *de novo* synthesis of stress proteins (Verma and Dubey, 2003; Mishra *et al.*, 2006).

Total free amino acids and proline: The contents of total free amino acids and proline (Fig. 5a, b) were significantly increased as Zn^{2+} or/and Pb^{2+} increased while proline content was reduced at the highest (80 mM) level of Zn^{2+} or Pb^{2+}. The stimulatory effect of Zn^{2+} individually or in combination with Pb^{2+} was more than Pb^{2+}. The considerable accumulation of proline and total free amino acids as a result of heavy metals stress has been reported by Dhir *et al.* (2004), John *et al.* (2008) and Pant *et al.* (2011). Total free amino acids are regarded to play a significant role in metal chelation, by which heavy metal detoxification and tolerance in plants take place (Hall, 2002). Accordingly, the increase of total free amino acids in our research may be a detoxification response of Hassawi okra seedlings to Zn or/and Pb treatments. Proline was found

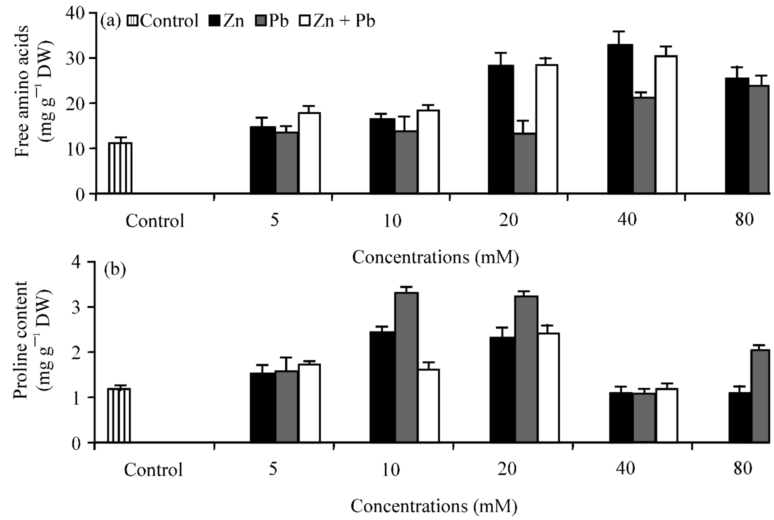

Fig. 5(a-b): Effect of Zn^{2+} or/and Pb^{2+} treatment individually or in combination on the contents ($mg\ g^{-1}$ dry weight) of (a) total free amino acids and (b) proline of Hassawi okra seedlings. Vertical bars represent ±SD

to accumulate in *Helianthus annuus* due to Pb^{2+} and Zn^{2+} (Kastori *et al*., 1992). It suggests that physiological dryness might be an initial consequence of Zn^{2+} or Pb^{2+} stress as a result of inhibition of root growth which leads to the slowing down of the water absorption. The reduction of proline at the highest level of Zn^{2+} or Pb^{2+} may be attributed to that, these levels may be lead to either stimulation of proline degradation or inhibited its synthesis (Qureshi *et al*., 2005). Interestingly, our results showed that increasing of Zn^{2+} or Pb^{2+} up to 20 mM, did not alter the chlorophyll and carotenoids content. Therefore, it could be concluded that the increase in proline content explains the stability of photosynthetic pigments in Hassawi okra leaves under these levels. This results are in agreement with Backor *et al*. (2004) who found that proline treatment increased the content of chlorophyll and carotenoids of *Trebouxia erici* exposed to heavy metal stress. Further, proline contributes to stabilizing sub-cellular structures, scavenging free radicals under stress conditions (Ashraf and Foolad, 2007). This is in accordance with the alleviating effect of proline under excess of heavy metals (Mehta and Gaur, 1999). Increasing of proline content may be either due to *de novo* synthesis or decreased degradation or both (Kasai *et al*., 1998). Proline accumulation may serve as a means of osmotic adjustment and storing carbon and nitrogen when stress leads to slower growth. Thus, it is worthy to note, that the pattern of changes of total sugars was opposite to that obtained for changes in total protein, free amino acids and proline. This gives a considerable reason to believe that, heavy metals tolerance seems to be linked with an equilibrium and intereconversion between total sugars and nitrogen metabolism in Hassawi okra seedlings exposed to Zn^{2+} or/and Pb^{2+} toxicity. So, it could be concluded that, proline increases the stress tolerance of plants through such mechanisms as osmoregulation, protection of enzymes against denaturation and stabilization of protein synthesis (Schat *et al*., 1997).

Membrane stability index (MSI): Membrane Stability Index (MSI) which was estimated as electrolyte leakage has been used as indicator to assess tolerance of various plant species exposed to stress Sudhakar *et al*. (2001). Linear decrease occurred in MSI (Fig. 6) with the increase of Zn^{2+}

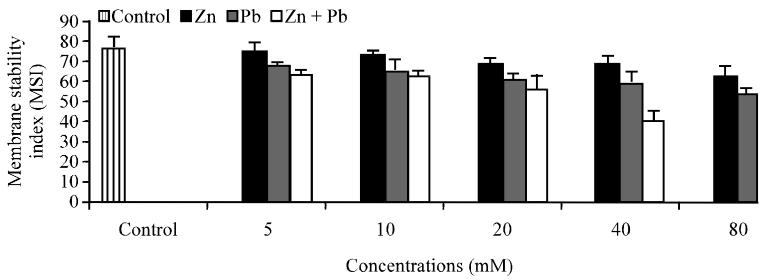

Fig. 6: Effect of Zn^{2+} or/and Pb^{2+} treatment individually or in combination on Membrane Stability Index (MSI) of Hassawi okra seedlings. Vertical bars represent ±SD

or/and Pb^{2+} in agreement with the result obtained by Zeng *et al.* (2011). This correlation was more notable in case of Pb^{2+} than Zn^{2+} and in binary than in individually. This is important sign of lower oxidative damage caused by ROS under Zn^{2+} toxicity leading to maintenance of the membrane integrity as well as reduction of membrane injury. The significant decrease in MSI at Pb^{2+} alone or in combination with Zn^{2+} may be due to plasmalemma injury caused by ROS. This supports our results which showed that, Hassawi okra seedlings exhibited higher Zn^{2+} tolerance than Pb^{2+}, resulting in slighter toxicity caused by Zn^{2+} on their growth parameters in compared with Pb^{2+} when added alone or in combinations.

CONCLUSION

The results in the present work suggest that, level of 5 mM Zn^{2+} only had a favorable effect on growth parameters and some related physiological activities of Hassawi okra (*Hibiscus esculentus* L.) seedlings. While these parameters were significantly decreased as exposure to Zn^{2+} or Pb^{2+} separately or in combination. These were associated with a decreased of total sugars and increased in most cases of soluble sugar and nitrogenous compounds such as protein, total free amino acids and proline. The results obtained indicated that, Zn^{2+} was less toxic than Pb^{2+}. The effect of toxicity caused by each metal alone was lower than that caused when they were used in combination. The combination effect of Zn^{2+} and Pb^{2+} produced an additive inhibitor effect on the growth parameters of Hassawi okra seedlings, suggesting synergistic or additive response between Zn^{2+} and Pb^{2+} leading to injurious effects followed by death of these seedlings, when they used in combination at 80 mM.

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