Effect of Some Heavy Metals on Seed Germination of *Medicago arborea* L. (*Fabaceae*)

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Summary

The present work deals with the effect of three heavy metals (zinc, copper and lead) on germination of *Medicago arborea* seeds. Solutions of four concentrations (25, 50, 75 and 100 ppm) of each heavy metal were tested separately, and deionised water was used as a control treatment. The experiments were conducted during 14 days, under strictly controlled laboratory conditions according to a completely random design with three replicates of 20 seeds/Petri dish, for each treatment. The following germination indices: Final germination percentage (FGP), Mean daily germination (MDG), Mean germination time (MGT), Germination index (GI) and Germination value (GV) were estimated. The results showed that FGP, MDG, GI and GV were significantly affected by heavy metal stress. In contrast, the increase of applied heavy metal dose resulted in prolongation of MGT, and therefore, in significant increase of its value. It should be noted that *M. arborea* seeds were able to germinate even at 100 ppm, which is a concentration higher than critical limits for agricultural soils and irrigation water. This suggests that *M. arborea* could be considered as a moderately tolerant species, at least during the germination phase, to metal stress and as a candidate with acceptable potential for phytoremediation.

Key words

abiotic stress, heavy metal, germination, *Medicago arborea*, seeds

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Received: December 12, 2018 | Accepted: January 29, 2019

Introduction

Due to rapid industrialization, urbanization and intensive agriculture, increasing contamination of heavy metals in soil has become of great interest for research purpose (Samuilov et al., 2014). Heavy metal toxicity is one of the major abiotic stresses causing deleterious effects on living beings (human, animals and plants) (Maksymiec, 2007). For plants, an excessive level of heavy metals in soils can have a negative impact on the germination capacity of seeds, roots growth and dry biomass evolution (Pavel et al., 2013). Also, it may interfere with many physiological and biochemical processes such as cell membrane permeability, nitrogen assimilation, photosynthesis, enzymatic activities, growth regulation and cell reproduction (Tuna et al., 2002).

Seed is a stage in life cycle of plant that is well protected against various external stresses. However, soon after imbibition and during the remainder of the germination process, they become stress-sensitive in general (Li et al., 2005). Seed germination is a fundamental trait at both, individual and population levels (Al Khateeb et al., 2010). It is a critical phase which is considered as a determining factor in the success of crop production especially in arid and semi-arid regions. According to Swapna and Rama Gopal (2014), germination is an important criterion for the evaluation of germplasm tolerance to various stresses including heavy metals. Therefore, it is necessary to understand seed germination process under adverse environmental conditions (Hatamzadeh et al., 2012) as well as heavy metal stress.

Fodder shrubs have received considerable attention in recent years and they have been extensively used in dry regions of the Mediterranean basin due to their many advantages (high resistance to aridity, ability to produce biomass in extreme environmental conditions, control of soil erosion, maintenance of soil fertility, supply of useful products and ecological services: fuel wood, game, shading, etc.) (Mulas and Mulas, 2004).

Medicago is a genus of the legume family (*Fabaceae*) that includes agriculturally and economically important species (e.g. *M. sativa*) and model organisms for legume biology (*M. truncatula*) (Rosato et al., 2008). Among its 83 annual and perennial species, growing predominately around the Mediterranean basin (Bena et al., 2005), *Medicago arborea* L. (2n = 4x = 32) is phylogenetically the oldest species in this genus and the only characterized by woody growth habit which can adapt to extreme ecological conditions (Boughalleb et al., 2011). It is one of the most important native shrubs in arid and semiarid Mediterranean regions (Travlos, 2009) with a great phenotypic variability and dispersal area (González-Andrés et al., 1999). The main features of agronomic interest in *M. arborea* are drought resistance and the absence of summer and winter dormancy (Guerrouj et al., 2013), which gives it the capacity to guarantee an appreciable forage yield in winter and in summer when the perennial *Medicago* species, like *M. sativa*, are dormant and the annual ones have already completed their biological cycle (Nenz et al., 1996). This species has also been tested as a source of biologically fixed nitrogen in N-deficient ecosystems (González-Andrés et al., 1999) whose preferred symbiont is *Ensifer meliloti* (Guerrouj et al., 2013). In addition, it represents one of the most interesting plants cultivated to prevent soil erosion (Tava et al., 2005), or for revegetation purposes under semiarid conditions (Valdenegro et al., 2001).

Medicago arborea is one of the most promising forage shrubs for Algeria. It can be remarkably useful for several purposes (grazing, regeneration of degraded ecosystem, combating desertification, etc.). However, in order to fully exploit this potential species, it is absolutely necessary to understand its germination behaviour under various abiotic factors such as metallic stress.

To the best of our knowledge, the present study is the first report describing the germination behaviour of *M. arborea* seeds under heavy metal stress. It was carried out to evaluate the effect of increasing concentrations, ranging from 25 to 100 ppm, of zinc, copper and lead on *M. arborea* seed germination.

Material and Methods

Seed material preparation

Medicago arborea (*Fabaceae*) seeds were kindly provided by the state pastoral nursery of Moudjbara, 26 km East of Djelfa province, capital of the Algerian steppe (2°39'E longitude, 34°50' N latitude and 934 m elevation).

After manual decortication of their pods, seeds were surface sterilized 5 min in a 1% solution of hypochlorite sodium. Then, they were thoroughly washed several times with deionised water to remove all traces of disinfecting solution (Bajji et al., 2001). Only intact, fully developed seeds were selected for the germination experiment.

Preparation of heavy metal solutions

Four concentrations with an increment of 25 (25, 50, 75 and 100 ppm) of zinc, copper and lead were prepared with deionised water using zinc sulphate [$ZnSO_4.5H_2O$], copper (II) sulphate [$CuSO_4.5H_2O$] and lead (II) Nitrate [$Pb(NO_3)_2$], respectively.

Germination assay

The selected seeds were equispacially placed in 9 cm diameter sterile Petri dishes covered with filter paper previously imbibed with various concentrations of different heavy metal solutions or by deionised water (control treatment) (Panuccio et al., 2014). For each treatment, three replicates (Petri dishes) of 20 seeds were maintained. The experiment was carried out in an incubator (LMS Cooled Incubator) under 16h day/8h night cycle, at 25°C day and 15°C night temperature. Seeds were daily irrigated with 5 ml of the appropriate solution of heavy metal or with deionised water (control seeds) (Saedipour, 2010) and germination was recorded for a period of 14 days. Seeds were considered as germinated if a radicle of at least 2 mm was visible to the naked eye (Panuccio et al., 2014).

Studied parameters

Five germination indices were calculated to investigate the effect of heavy metal (Cu, Pb and Zn) stress on *M. arborea* seed germination. They were chosen based on their relevance and extensive use in similar research studies. The assessed parameters are: final germination percentage (FGP), mean daily germination (MDG), Mean germination time (MGT), germination index (GI) and germination value (GV).

Final germination percentage was calculated according to the following equation:

$$FGP = (n / N) \times 100$$

where 'n' is the number of germinated seeds and N is the complete number of tested seeds.

Mean daily germination (MDG) was estimated as described by Almaghrabi et al. (2014):

$$MDG = N / D$$

where N is total number of germinated seeds and D is total number of days.

Mean germination time (MGT) was obtained by the following expression (Akinci and Akinci, 2010):

$$MGT = \Sigma (n \times d) / \Sigma n$$

where 'n' is the number of seeds which were germinated on day 'd' after imbibing.

Germination index (GI) was determined by the formula given by Salehzade et al. (2009):

$$GI = \Sigma (n_i / D_i)$$

where 'n_i' is number of germinated seeds at day 'i' and D_i is day 'i'.

Germination value (GV) is a composite value which combines both germination speed and total germination. It was computed following the formula of Djavanshir and Pourbeik (1976):

$$GV = (\Sigma DGS / N) \times FGP \times 10$$

where DGS is daily germination speed, FGP is final germination percentage and N is the number of DGS that were calculated during the test.

Statistical analysis

All obtained data were subjected to statistical analysis (ANOVA, one-way analysis of variance) using STASTICA software (version 10, StatSoft, INC). Treatment means were discriminated using Least Significant Difference (LSD) test at a probability $p<0.05$.

Results

The obtained results showed that the application of heavy metal stress, using Zn, Cu and Pb elements, exerted a toxic effect on *M. arborea* seed germination. In fact, all concentrations (25 to 100 ppm), whatever the used metal, significantly reduced FGP, MDG, GI and GV and increased MGT.

Treatments with Zn, Cu and Pb caused a significant inhibition ($p< 0.05$) of the final germination percentage (Fig. 1) and the mean daily germination (Fig. 2) of *M. arborea* seeds. This inhibition was dependent on the used heavy metal and its dose in the germinative media. Control seeds exhibited the maximum FGP and MDG (i.e. 86.24 % and 5.75% day^{-1} respectively). Treatments with 50 ppm of zinc, 75 ppm of copper and 75 or 100 ppm of lead resulted in similar values of FGP and MDG (48.33% and 3.22% day^{-1} respectively), suggesting that *M. arborea* seeds were more sensitive to zinc than copper and lead.

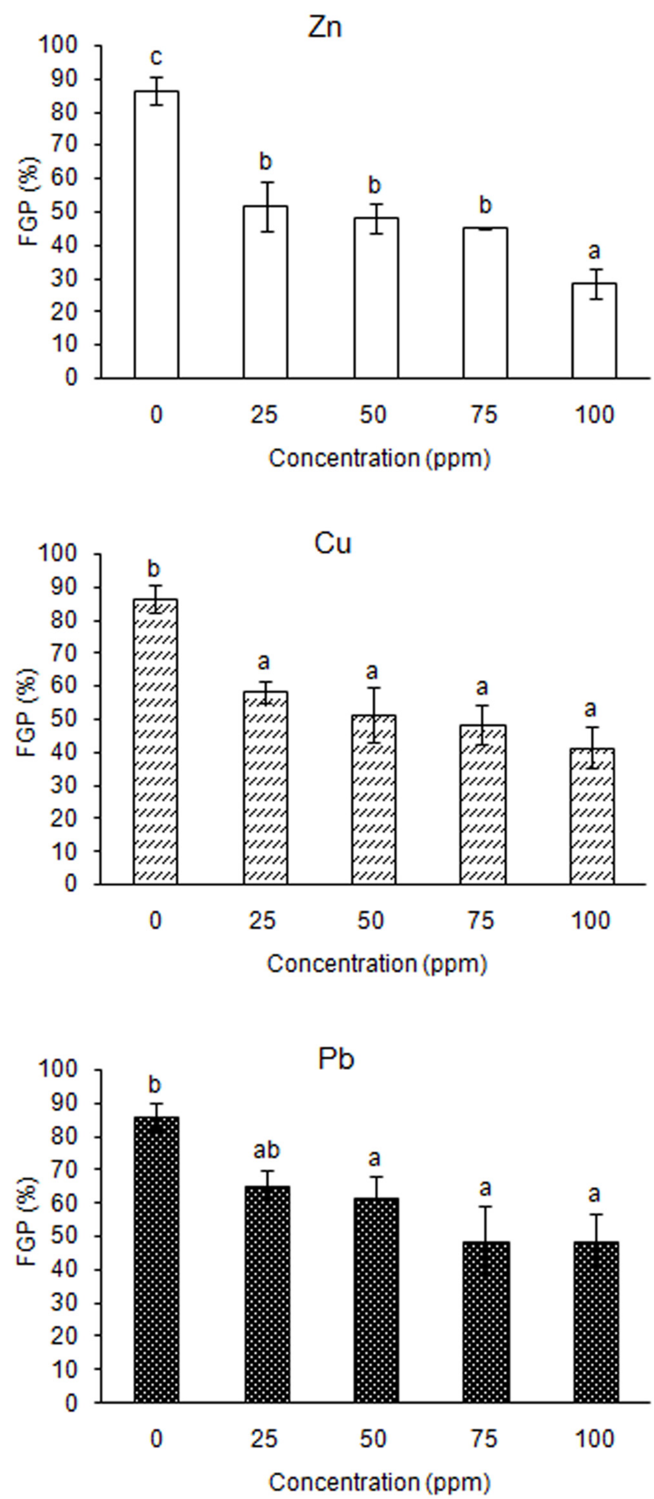

Figure 1. Effect of different concentrations of Zn, Cu and Pb on final germination percentage of *M. arborea* seeds. Results are means ± SE (n = 3). Different letters denote significant differences between treatments ($p < 0.05$)

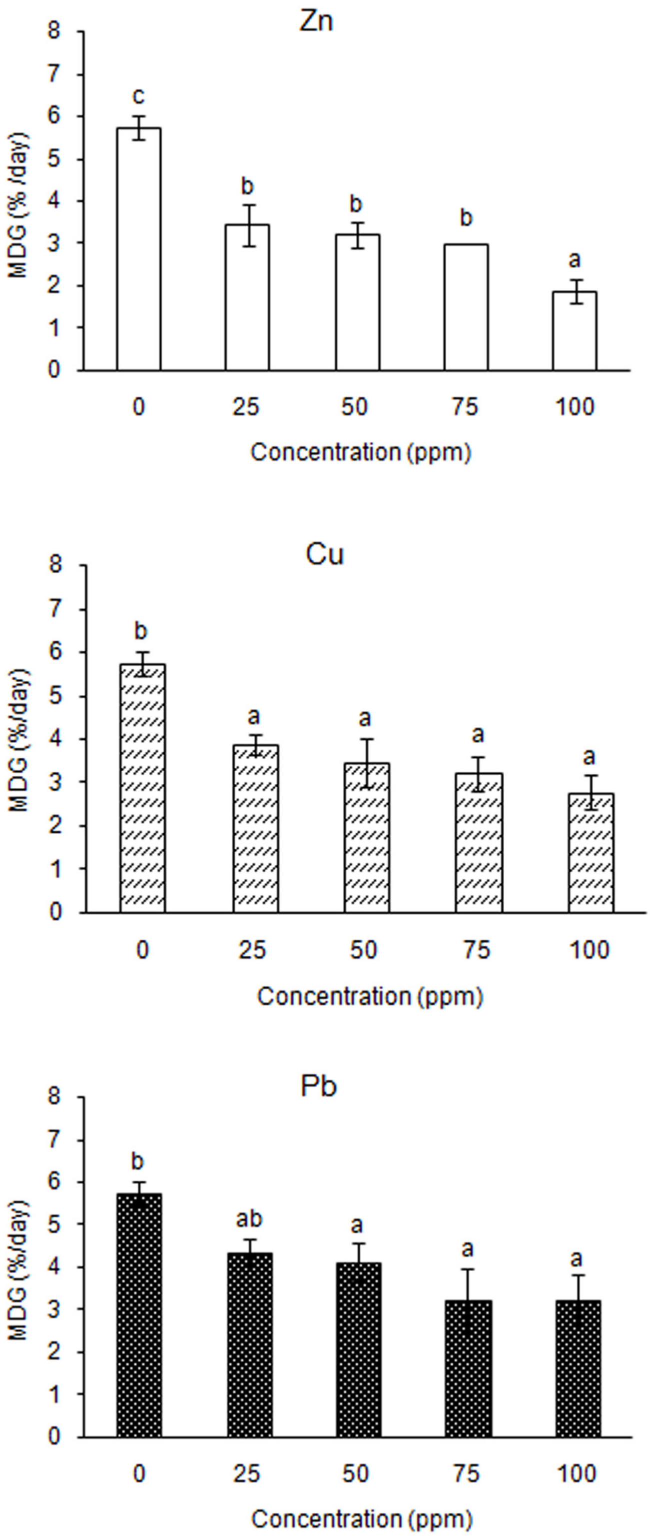

Figure 2. Effect of different concentrations of Zn, Cu and Pb on mean daily germination of *M. arborea* seeds. Results are means ± SE (n = 3). Different letters denote significant differences between treatments ($p < 0.05$).

Despite the negative effect of heavy metal stress, *M. arborea* seeds can, to a certain degree, germinate under the highest concentration of studied metals. Thus, FGP was respectively 28.33, 41.67 and 48.33% under 100 ppm of Zn, Cu and Pb. This concentration is more or less close to the maximum admissible limits for agricultural soils used in many countries. However, it is considerably higher than the permissible limits in irrigation water set by various standards.

Seeds irrigated with deionised water (control) had the lowest MGT value (3.70 days) than their homologues treated by various heavy metal solutions (Table 1). The MGT was 1.38 to 5.63 days longer in treated than in control seeds depending on applied treatment. The longest MGT (9.33 days) was recorded with 100 ppm of Zn, while the shortest (5.07 days) was observed at 50 ppm of Pb.

Table 1. Effect of different concentrations of Zn, Cu and Pb on mean germination time of *M. arborea* seeds. Results are means ± SE (n = 3). Different letters denote significant differences between treatments at 5 % (LSD, $p < 0.05$)

Treatment (ppm)	Zinc	Copper	Lead
0	3.70 ± 0.16 a	3.70 ± 0.16 a	3.70 ± 0.16 a
25	7.10 ± 0.23 b	6.29 ± 0.61 b	5.93 ± 0.69 bc
50	8.77 ± 1.07 bc	6.36 ± 0.33 b	5.08 ± 0.48 ab
75	7.33 ± 0.36 bc	7.49 ± 1.01 b	7.76 ± 0.45 d
100	9.33 ± 1.07 c	7.77 ± 1.32 b	7.27 ± 0.83 cd

GI and GV were significantly ($p<0.05$) decreased by all heavy metal treatments in comparison to unstressed seeds (control) which had a GI and GV of 4.78 seed day^{-1} and 97.36% respectively (Fig. 3; Fig. 4). Similarly to FGP and MDG, the negative effect of lead on GI (3.30 to 1.70 seed day^{-1}) and GV (42.68 to 21.98 %) was less pronounced than that of copper and zinc.

For Zn, similar GI (1.62 seed day^{-1}) was recorded under 50 and 75 ppm. However, GV was slightly higher (21.90%) at 75 ppm than 50 ppm (19.31%), due to an increase, under 75 ppm comparatively to 50 ppm treatment, of daily cumulative germination between the 5th and 12th day. Regarding Pb treatments, analogous GI or GV values have been recorded between 25 and 50 ppm (~ 3.1 seed day^{-1} and 42.6 % respectively) and between 75 and 100 ppm (~ 1.6 day^{-1} and 22% respectively).

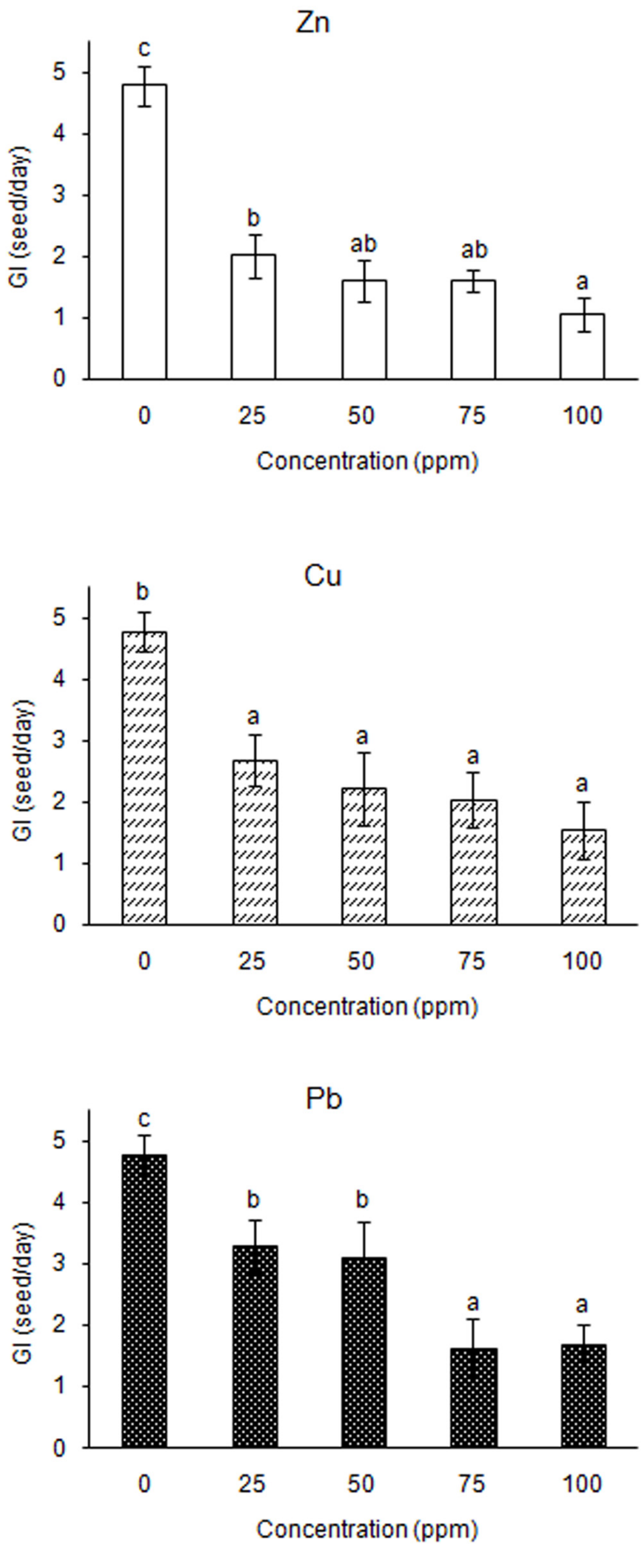

Figure 3. Effect of different concentrations of Zn, Cu and Pb on germination index of *M. arborea* seeds. Results are means ± SE (n = 3). Different letters denote significant differences between treatments ($p < 0.05$)

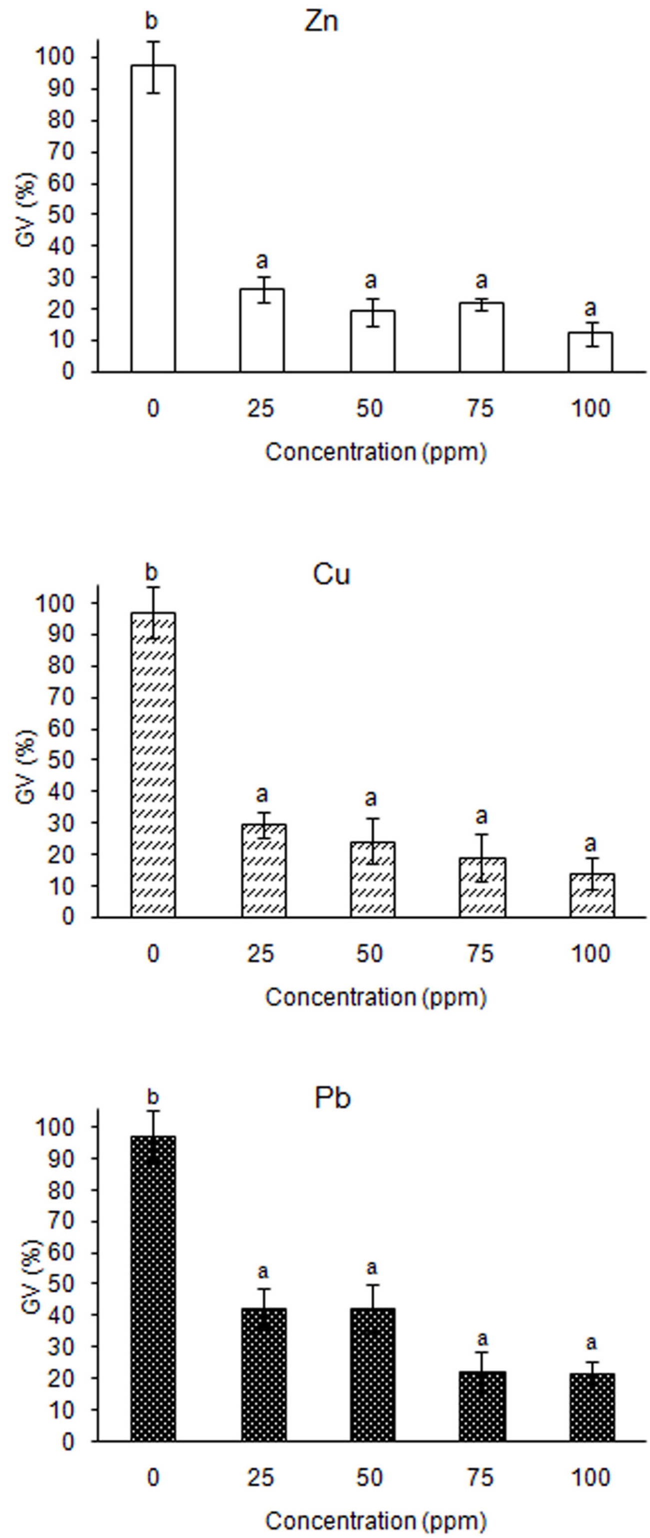

Figure 4. Effect of different concentrations of Zn, Cu and Pb on germination value of *M. arborea* seeds. Results are means ± SE (n = 3). Different letters denote significant differences between treatments ($p < 0.05$)

Discussion

Germination test is a basic procedure to assess heavy metals toxic effects on plants (Kaduková et al., 2015). Seed germination is the first exchange interface with the surrounding medium and has been considered as highly sensitive to environmental changes (Solanki and Dhankhar, 2011).

The results of the present study revealed that zinc, copper and lead adversely influenced the germination indices of *M. arborea* seeds. This has been reflected by a gradual and significant ($p<0.05$) decrease in FGP, GI, GV and MDG indices with concentration increase of selected heavy metals. In contrast, the MGT was 1.38 to 5.63 days longer in treated than in control seeds.

A similar effect using various heavy metals treatments has been noted for seed germination of several leguminous species such as *M. sativa* under Cd, Cu, Ni and Pb (Abusriwil et al., 2011), *A. tortilis*, *A. Raddiana* and *P. Juliflora* under Cu and Pb (Abbas et al., 2017), *A. auriculiformis* under Pb (Zerkout et al., 2018). Likewise, similar finding has been found for other species belonging to different plant families including, for example: *L. perenne* and *F. rubra* (*Poaceae*) under Cu, Zn and Co (Taghizadeh and Solgi, 2017), *A. halimus* (*Amaranthaceae*) under Cd, Zn and Pb (Fatarna et al., 2017), *R. sativus* and *B. oleracea* (*Brassicaceae*) under As (Dutta et al., 2014).

However, several studies have shown that some heavy metals can have a positive impact on germination. It has been reported that germination of *M. sativa* seeds is stimulated between 40 and 160 ppm of Zn (Abusriwil et al., 2011). Also, germination of *Ulmus pumila* has been enhanced by Cd and Pb treatment (Đukić et al., 2014).

Although the extent of adverse effect was greater as the applied concentration increased, the studied elements presented different degree of toxicity. In plant ecophysiology, the establishment of the toxicity ranking order of heavy metals is very complex and remarkable differences can be noticed depending on both: i) the metal (considered element, chemical characteristics and applied dose), ii) the studied plant (species, development stage,...) and iii) the experimental approach (duration, conditions, substratum,...).

In this work, the toxicity order of studied metals on FGP, GI, MDG and GV of *M. arborea* seeds was Zn > Cu > Pb. This result is more or less in line with previous researches. Abusriwil et al. (2011) reported an order of toxicity for metals on seed germination of *M. sativa* as Cd > Cu > Ni > Pb > Zn. Munzuroglu and Geckil (2002) ranked six heavy metals depending on increasing degree of germination inhibition as Hg > Cd > Cu > Pb > Co > Zn in *Triticum aestivum* grains and as Hg > Cu > Cd > Pb > Zn > Co in *Cucumis sativus* seeds.

The inhibitory effect of the studied heavy metals (Zn, Cu and Pb) on *M. arborea* seed germination could be the result of ionic toxicity on embryo viability and/or due to an osmotic effect of tested solutions which impairs water uptake by seeds, the most important factor for germination process. In our study, the proportion of each type of effect in the final inhibition percentage of germination could not be determined with certainty. These two types of effect (osmotic and ionic) have been widely debated not only under heavy metal stress (Ahsan et al., 2007) but also in response to various abiotic stresses such as salinity (Panuccio et al., 2014).

Many other possible causes of germination inhibition under heavy metal stress have been reported in previous studies: alteration of selection permeability properties of cell membrane, accelerated breakdown of stocked nutrients in the endosperm (Shafiq et al., 2008), decrease of enzymatic hydrolysis of starch, especially of α-amylase, which impairs the supply of sugar to developing embryo axes (Mittal et al., 2015), disturbance of cellular homeostasis causing oxidative stress including alterations in enzymes of the antioxidant defence system (Patel et al., 2013) and an up-regulation of some stress-related proteins (Ahsan et al., 2007).

Finally, the harmful effect of one or the other selected heavy metals (Zn, Cu or Pb) on *M. arborea* seed germination can be attributed to hormonal or nutritional imbalances. Heavy metals may interact with other toxic and essential macro and microelements which may cause the deficiency of other elements essential for germinating seeds, thereby resulting in poor germination (Fodor, 2002). In addition, the hormonal balance of seeds is frequently affected by stress factors including heavy metals, which results in more or less significant inhibition of germination. Earlier study revealed an increasing of abscisic acid and zeatin concentration and a decreasing of gibberellic acid and zeatin riboside in chickpea seeds germinating under lead and zinc stress (Atici et al., 2005).

Conclusion

The present research work was conducted to investigate whether or not the supply of aqueous concentrations (25 to 100 ppm) of zinc (Zn), copper (Cu) and lead (Pb) elements influence seed germination parameters of *M. arborea*. The obtained results showed that *M. arborea* seed germination was affected by heavy metal stress. However, despite this negative effect, *M. arborea* seeds were capable to germinate, at various degrees depending on the used heavy metal, even at 100 ppm. This concentration is greatly above the permissible limits especially for irrigation water. The obtained results suggest that *M. arborea* is a moderately tolerant species to heavy metal stress, which has, to some extent, a phytoremediation potential. Additional research using *Medicago arborea* is recommended in order to evaluate its phytoremediation potential in contaminated soils especially with Cu, Zn and Pb.

Acknowledgements

This study was supported by the Algerian Ministry of Higher Education and Scientific Research (CNEPRU Project, code: D04N01UN 170120150002). The authors appreciate Professor PhD Maria Magdalena ZAMFIRACHE (Department of Biology, Alexandru Ioan Cuza University, Romania) for her valuable comments on the manuscript.

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