EDTA AMPLIFIES OXIDATIVE STRESS INDUCED BY LEAD AND NICKEL IN *Pisum sativum* L.

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Abstract. Soil contamination by heavy metals is considered as one of the major environmental issues in recent years as it poses a threat to agricultural production due to their adverse effects on plant growth, food quality and environmental health. Pollution by heavy metals involves metal ions which are not always essential to plants as they affect growth and development. The application of a chelating agent, EDTA, increases the absorption of heavy metals by increasing their phytoavailability. Our results showed that lead and nickel have a negative impact on peas (*Pisum sativum*) as they affect their germination rate, plant growth and protein content. EDTA itself also causes the same negative effects as heavy metals since it represents a toxic xenobiotic substance. The combination of this chelator with Ni or Pb amplified the action of these heavy metals on the various parameters studied. The Oxidative stress is manifested through the accumulation of reactive oxygen species and MDA which is an indication of cellular membranes alteration. The enzymatic activities of catalase, ascorbate peroxidase and superoxide dismutase were stimulated by EDTA, heavy metals or a combination of the two. In addition, the activity of the glutathione S-transferase was greatly increased in response to EDTA and both heavy metals.

Key words: EDTA; lead; nickel; oxidative stress; *Pisum sativum*; pollution.

INTRODUCTION

Over the last few decades, human activities have induced a colossal environmental pollution which has become a major threat to human health and ecosystems. Among the most widespread pollutants, heavy metals are the most dangerous. According to Baize (1997) [6], the contamination of an environment by heavy metals means an increase in the total content of these elements in the environment, following significant anthropogenic inputs. Heavy metals, known as metallic trace elements (MTE), are serious environmental pollutants, especially in areas with high anthropogenic pressure. Their presence in the atmosphere, soil and water, even in the form of traces, can cause serious problems for all organisms [1]. Heavy metals are one of the most severe types of pollutants and are difficult to remove, and even small amounts can pose a great threat to the eco-environment and human health [18]. Accumulation of heavy metals in soils poses a threat to agricultural production because of their negative effects on plant growth, food quality and environmental health [17].

Pollution by heavy metals concerns metallic ions which are not always essential for plants. Thus, metals such as cadmium, nickel, lead or mercury play no role in plant growth and development. They are said non-essential and are toxic even in trace amounts [56]. These heavy metals cannot be biodegraded and therefore persist in the environment for long periods of time. Furthermore they are continuously added to the soil by various activities: in agriculture by the application of pesticides or in the metallurgical industry or by natural means by runoff. Their concentrations continue to increase which prompted the WHO (World Health Organization) to sound the alarm about release of such pollutants in industrial effluents.

At the plant level, heavy metals affect growth and development of plants by disrupting the photosynthetic and respiratory chains [21]; inhibiting some enzymes by interfering with the metals involved in the functioning of these enzymes [78]; creating a nutritional deficiency by antagonism with mineral elements essential for the plant [22] and generating harmful oxidative stress for cellular macromolecules [55]. In addition to the concentration, the degree of toxicity towards plants depends on the availability and mobility of heavy metals. The application of chelating agents can increase the absorption of heavy metals by increasing their phytoavailability. For this purpose, EDTA is very often used as a suitable chelator for the induction of absorption and heavy metal translocation, which largely depends on plants and heavy metals studied [44].

The aim of our work is to examine the effects of nickel (Ni) and lead (Pb) without or with supplementation of EDTA on germination rate, plant growth, some aspects of oxidative stress and glutathione S-transferase (GST) activity of *Pisum sativum*, a species widely used for food and for physiological and molecular studies [5, 67].

MATERIALS AND METHODS

Plant material

Pisum sativum L. was the species of choice because it had been proven to accumulate significant amount of heavy metals in the shoots, hence it is considered as hyper-accumulator species particul arly for lead and zinc [80]. It had also been selected for its rapid rate of growth and its substantial biomass.

Experimental protocol

To identify the level of toxicity of lead (Pb) and nickel (Ni), preliminary experiments were performed. The toxicity threshold was 1 mM for both metals. EDTA concentration was also taken at 1 mM. In order to conduct the experiments, five stock solutions containing the targeted heavy metals and EDTA were prepared: $NiCl_2$, $Pb(C_2H_3O_2)_2$, EDTA, $NiCl_2$+EDTA, $Pb(C_2H_3O_2)_2$+EDTA in which the concentrations of Pb, Ni and EDTA were 1 mM.

Pea seeds (*Pisum sativum*) were surface sterilized using sodium hypochlorite then soaked in distilled water for 3 hours. After that, the seeds were transferred into germinating trays (20 x 20 cm). Hundred mL of each of the solutions of heavy metals (Pb, Ni) or EDTA were applied. A control sample received only 100 mL of distilled water. The trays were put to germination at 23 °C for 48 hours. After germination, seedlings were transferred into pots, filled with a soil mixture (1/3 clay, 1/3 sand and 1/3 potting soil). Six sample patches were prepared. Each sample was watered with 400 mL, every two days for 45 days, with one of the prepared solutions. The sixth sample patch was watered with H_2O and considered as a control sample.

Measurement of germination and growth

Germination rate was studied by measuring the capacity of germination which represents the maximum germination percentage of seeds [51]. According to Kofler (1980) [39], the process of plant growth is a quantitatively measurable phenomenon in which, the augmentation of weight and dimension is irreversible. In order to follow the linear growth in aerial parts, measurements of size were taken at the 4-leaf stage, in cm, using millimetre paper. Each value represents the average of ten measures.

Biochemical analyses

Lipid Peroxidation: Measurement of Malondialdehyde Content (MDA)

Lipid peroxidation is measured by quantification of malondialdehyde (MDA), a breakdown product of lipid peroxidation reactions formed during attack of polyunsaturated lipids by reactive oxygen species. A molecule of MDA in acid medium and hot is condensed with two molecules of thiobarbituric acid (TBA) to form a pink-colored complex susceptible to spectrophotometric assay at a wavelength λ = 532 nm. This technique has excellent qualities in terms of sensitivity, except that it lacks specificity because of interference of molecules not relevant to oxidative stress but reacting with TBA. However, it remains the simplest test and the most classic which allows the evaluation of lipid peroxidation. Besides, some authors do not speak of MDA, but of substances reacting with TBA (TBARS) [8].

Determination of total water-soluble proteins content

The total water-soluble proteins content was determined according to the Bradford method [9]. This method is based on the colorimetric assay due to the change in the colour of the blue of Coomassie after its reaction with aromatic amino acids (tryptophan, tyrosine and phenylalanine) and the hydrophobic residues of amino acids present in proteins [79].

Antioxidant Enzymatic Activities Measurement

Catalase (CAT)

According to Dorey et al. (1998) [20], the measurement of the catalase activity is related to the degradation of H_2O_2 (ε=36 $M^{-1}\cdot cm^{-1}$) at 240 nm. Hydrogen peroxide (H_2O_2) has an absorption peak in the UV (between 230 and 250 nm). This absorption is easily measured. The decomposition of H_2O_2 results by a decrease in optical density and the enzymatic activity can thus be measured.

Superoxide Dismutase Activity (SOD)

The superoxide dismutase activity was measured according to the method of Marklund and Marklund (1974) [47] slightly modified by Boucelha et al. (2019) [8]. The auto-oxidation of pyrogallol, in the presence of EDTA, is inhibited by SOD from pH 7.9 to 9.1. The reaction is inhibited up to 90% by SOD, which is an indicator of a total dependence of the anion superoxide (O_2^-) in this autooxidation. The % of inhibition was calculated according to the following equation:

% of inhibition = (ΔDO control-ΔDO assay) * 100/ ΔDO control

Ascorbate Peroxidase Activity (APX)

The ascorbate peroxidase activity (APX) was measured according to the method of Nakano and Asada (1981) [58], following the oxidation of ascorbate by hydrogen peroxide, at the wavelength of 290 nm (ε =2.8 $mM^{-1}\cdot cm^{-1}$). The enzymatic activity was expressed in mmol of oxidized ascorbate $min^{-1}\cdot mg^{-1}$ protein. This activity was calculated using the extinction coefficient of ascorbate which is 2.8 $mM^{-1}\cdot cm^{-1}$.

Glutathione S-Transférase (GST)

Glutathione transferases (GSTs) constitute a ubiquitous superfamily of enzymes multifunctional involved in cellular detoxification processes by metabolizing exogenous substrates called xenobiotics and in secondary metabolism. For this, these enzymes can catalyze the conjugation of a molecule of glutathione (GSH) on the targeted compounds or simply bind them through a ligandin function. The measure of the activity of this enzyme is carried out by the method of Habig et al. (1974) [32] which consists in making react GST activity on a mixture of 1-chloro-2,4-dinitro benzene (CDNB) and glutathione reduced (GSH). The variation of the optical density is measured every 30 seconds for 4 min at 340 nm. GST activity is expressed as $\mu moles\cdot min^{-1}\cdot mg^{-1}$ protein.

Hydrogen Peroxide H_2O_2

Hydrogen peroxide was measured according to the method of Alexieva et al. (2001) [2], using photospectrometry. After a reaction with potassium iodide (KI), a yellow coloration was obtained. A calibration curve was realised using increasing concentrations of H_2O_2, prepared from a stock solution of 100 µmoles.

Variation percentage

The percentage variation was calculated for each parameter studied according to the following relation: Variation percentage (Assay- Control) * 100 / Control

Percentage points are the numerical difference between two percentages.

Statistical Test

The bars represent the standard errors of five repetitions. The statistical Student test was carried out using the Excel 2007 software.

RESULTS

The germination rate of *Pisum sativum* seeds after the application of EDTA decreased significantly (12%), compared to the germination rate of the control samples. Results have also proven that lead caused a significant decrease (17%) in the germination rate of the seeds, in comparison with the germination rate of the control. The association Pb+EDTA provoked 22% diminution in the germination rate with a cumulative effect of 5 percentage points compared to the lead alone. It was also shown that the germination rate decreased significantly (31%) in the seeds treated with nickel, compared to the germination rate of the control samples. The EDTA associated with nickel decreased the germination (36%) with an additive effect of 5 percentage points compared to the nickel alone (Fig. 1).

Plant Growth

The results have shown a significant decrease, of 12%, in the growth of the plants treated with EDTA compared to the growth of the control samples. The lead provoked a significant decrease, of 24%, in the plants growth compared to control samples. The Pb+EDTA caused a diminution, of 5.5%, in the plants growth compared to the ones treated with lead. It was also observed that nickel caused a significant diminution, of 30%, in the plants growth compared to the growth of the control samples. The Ni+EDTA association had no effect on the plants growth compared to the control samples (Fig. 2).

Lipid Peroxidation: Measurement of Malon-dialdehyde Content (MDA)

Results have shown an increase (83%) in MDA content in plants treated with EDTA compared to the control samples. The lead provoked an increase, of 47%, in the MDA content while, the nickel caused an increase of 200% compared to the control samples. It was also observed that MDA content increased by 47%, for the plants treated with Pb+EDTA compared to the plants treated with only Pb. The association Ni+EDTA showed an increase (7%) in the MDA content compared to the control samples (Fig. 3).

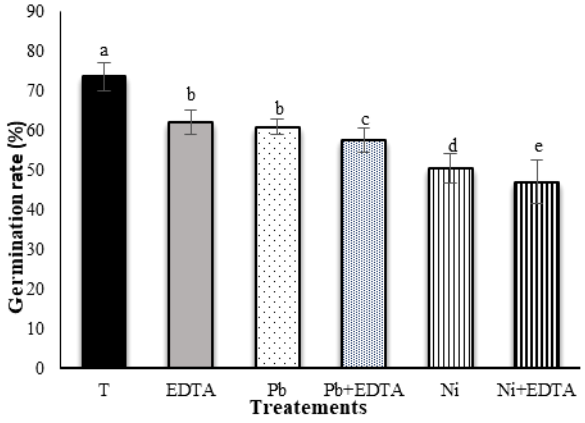

Figure 1. Effect of EDTA, Pb and Ni on germination rate of *Pisum sativum*

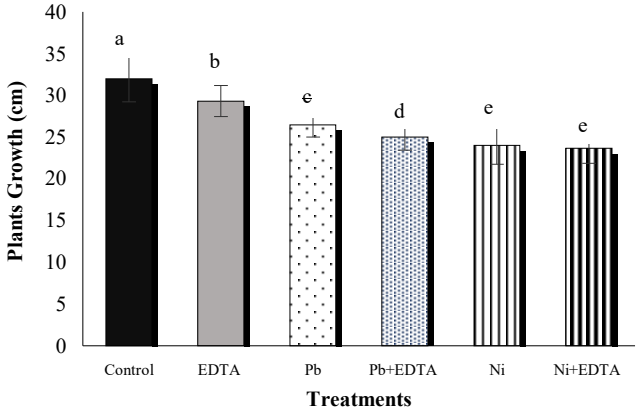

Figure 2. Effect of EDTA, Pb and Ni on plant growth of *Pisum sativum*

Total Water-Soluble Proteins

The results have shown a significant decrease, of 17%, in the proteins content of the plants treated with EDTA compared with the control samples. The lead provoked a significant decrease, of 14%, in the proteins content compared to the control samples. The Pb+EDTA association caused a diminution, of 26%, in the proteins content compared with the plants treated with lead only. It was also observed that the plants treated with nickel underwent a significant decrease, of 22%, in the proteins content compared with the control samples. The EDTA associated with nickel provoked a diminution, of 23%, in the proteins content, compared with plants treated with only nickel (Fig. 4).

Catalase Activity (CAT)

Plants treated with EDTA presented a significant CAT activity increase, of 21.5%, compared to the control samples. The lead also caused a significant enzyme activity augmentation of 20% while the nickel provoked an increase of 32% of enzyme activity compared to the control samples. However, when comparing the CAT activity between plants treated with EDTA and plants treated with Pb, it was observed that the CAT activity is slightly higher in the case of EDTA by 1.5%. The cumulative effect was observed in the plants treated with heavy metals after adding the EDTA. In fact, the EDTA associated with lead caused a very significant increase of +109% in the CAT activity compared to the plants treated with lead only. While Ni+EDTA association provoked an increase of 63.5% in this enzyme activity in comparison with the plants treated with nickel only (Fig. 5).

Ascorbate Peroxidase Activity (APX)

The EDTA provoked a significant increase, of 83%, in the APX activity compared to the control samples. It was observed that the plants treated with lead display a strong APX activity (150%) compared to the control samples. Plants treated with nickel increase of (180%) compared to the control samples. It was also observed that the association Pb+EDTA provoked an augmentation of 127% in the APX activity compared to the plants treated with only lead. The EDTA associated with nickel caused an increase, of 114%, in the enzyme activity in comparison with the plants treated with only nickel (Fig. 6)

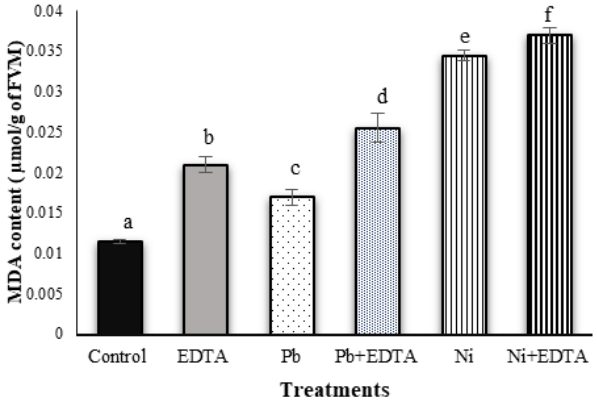

Figure 3. Effect of EDTA, Pb and Ni on MDA content in *Pisum sativum* (FVM is fresh vegetable mass)

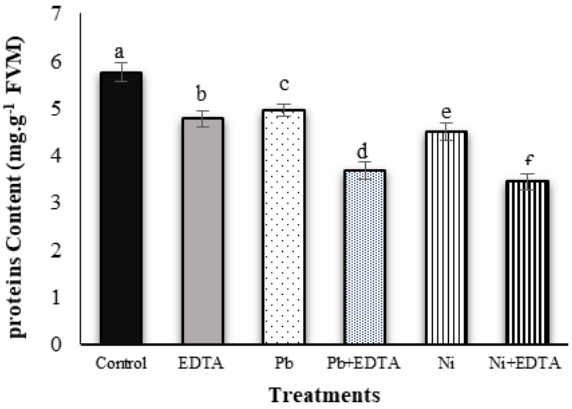

Figure 4. Effect of EDTA, Pb and Ni on Proteins content in *Pisum sativum* (FVM is fresh vegetable mass)

Glutathione S-Transférase (GST)

Results have shown a significant increase, of 130%, in the GST activity in the plants treated with EDTA compared to the control samples. The lead provoked a slight augmentation, of 7%, in the GST activity, while the nickel caused a very significant increase, of +108%, compared to the control samples. The results also shown that the addition of EDTA caused an augmentation, of 22%, in the GST activity compared to the plants treated with lead only. While Ni+EDTA association provoked 19% augmentation in this enzyme activity in comparison with the plants treated with nickel only (Fig. 8).

Hydrogen Peroxide (H_2O_2)

Results have shown different contents of H_2O_2 in the plants treated with Pb, Ni, EDTA plants and the control samples. In Plants treated with EDTA, the H_2O_2 content doubled compared to the control samples. However, plants treated with Pb showed a significant increase of 140% compared to the control samples. Plants treated with Ni also showed an increase (140%) compared to the control samples. Similarly, it was observed that the association Pb+EDTA produced 20% more hydrogen peroxide compared to the plants treated with only Pb. The Association Ni+EDTA also caused an increase (22%) in the hydrogen peroxide content compared to the plants treated with only Ni (Fig. 9).

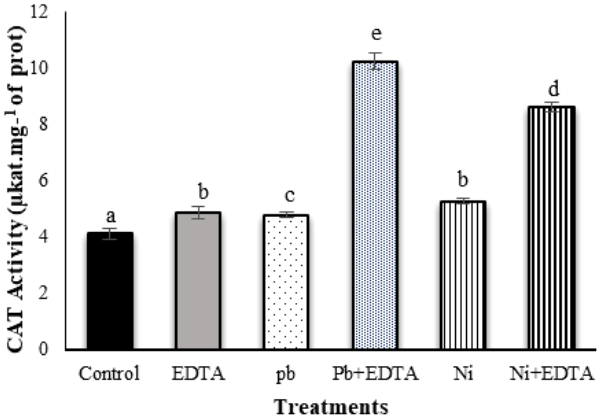

Figure 5. Effect of EDTA, Pb and Ni on catalase activity in *Pisum sativum* (µkat=µmoles H_2O_2 consumed per mn)

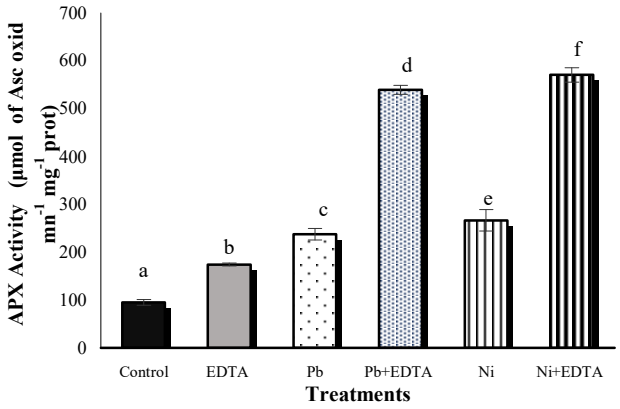

Figure 6. Effect of EDTA, Pb and Ni on APX activity in *Pisum sativum*

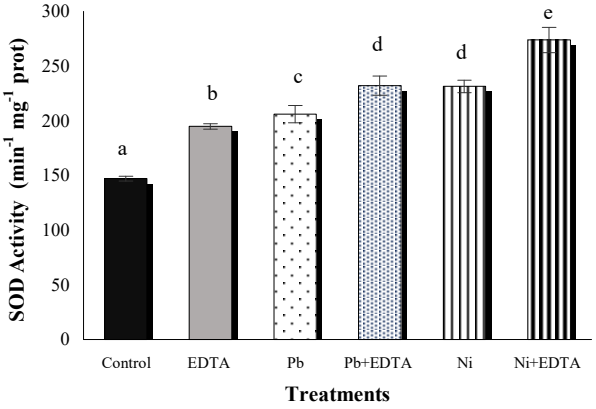

Figure 7. Effect of EDTA, Pb and Ni on SOD activity in *Pisum sativum*

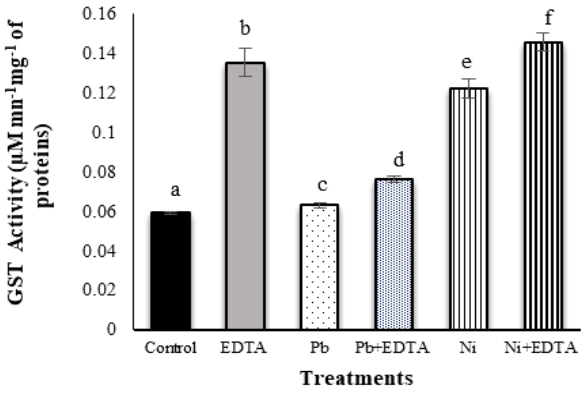

Figure 8. Effect of EDTA, Pb and Ni on GST activity in *Pisum sativum*

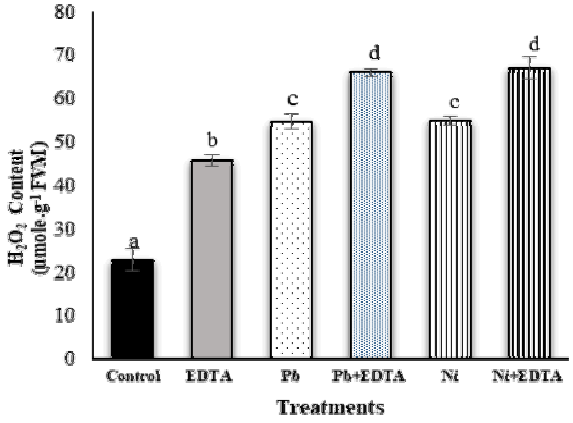

Figure 9. Effect of EDTA, Pb and Ni on H_2O_2 content in *Pisum sativum* (FVM is fresh vegetable mass)

DISCUSSION

Effects of lead and nickel

Heavy metals, in general, and lead as well as nickel, in particular, affect the plants in many different ways. These two metals can cause a nutrition deficiency due to the competition between these elements and the mineral elements needed for the plant's growth and development [22]. They have also, as a consequence, the inhibition of metal-dependant enzymes following the antagonism between the heavy metals and the enzymes implicated in the metabolism [78]. Furthermore, heavy metals interfere in the respiratory and photosynthetic chains [21] and induce an oxidative stress provoked by the accumulation of reactive oxygen species.

Based on our results, the partial inhibition of germination rate was due to the high level of toxicity of lead and nickel. Similarly, Shalini (2003) [71] revealed that the decrease in the percentage and the mean germination time is due to the high level of toxicity of lead and nickel. This decrease of germination rate is attributed to the fact that toxic concentrations of heavy metals alter the structure of membranes of root cells and reduce water absorbent surfaces [35]. Lead presents affinity for cellular proteins notably the enzymes which control the germination process. These results are confirmed by many researchers [26, 68, 81]. For the nickel, our results show similar effects as those of lead. According to Cheng (2003) [12], nickel affects the activity of many hydrolases (amylases, ribonucleases and proteases), which delay the germination.

For the plant growth, results indicated that lead and nickel caused a significant decrease in the plant growth. Other studies on corn show that edaphic pollution with lead and nickel, affect the plant growth [40, 57, 65]. According to Zhang et al. (2020) [81], both Pb and Ni inhibit stem growth. Concerning the effect of lead, many studies have explained the role of this metal in the decrease of seedlings' growth, by inhibiting the enzymes and the overall metabolism of the plant [14, 59, 73, 76].

Beside the effects cited above, heavy metals have an effect on the proteins content. The decrease in the proteins content can be explained by the alteration of the photosynthesis which is the energetic source for the protein biosynthesis. Many studies have shown that the pollution by heavy metals cause a diminution in the chlorophyll pigments [24, 25, 27, 30]. This diminution is probably due to the inhibition of a key enzyme in chlorophyll biosynthesis (aminolevulinic acid dehydratase) [64]. On the other hand, the low proteins content found in the plants treated with nickel and lead can be related to the oxidative stress which is

responsible for the oxidation and the degradation of proteins [75].

It is well known that lead and nickel induced pollution provoke an oxidative stress resulted of ROS [55, 69, 72]. According to Dias et al. (2019) [19], lead concentration induces higher oxidative damages. For this purpose, our results confirmed the installation of this secondary stress following contamination by these two metals. The accumulation of reactive oxygen species destroy membranes and macromolecules and also affect the cellular metabolism [59, 73, 76]. This can cause plants to show symptoms of toxicity or death, and eventually losing their tolerance and resistance to pollutants [41].

These damages, caused by ROS, were observed in our study through the formation of MDA which is the final product of lipid peroxidation and is used to evaluate cell membrane damage [81]. Our study showed that MDA content was higher under Ni stress which is an indication of higher plant membrane cell damage. This also indicated that *Pisum sativum* tolerance to Ni toxicity is not as strong as that of Pb toxicity. Other plants, such as *Myriophyllum aquaticum* and *Egeria densa*, show similar results [11].

In order to diminish these deleterious consequences, enzymatic antioxidant systems are activated. This will help to maintain homeostasis of the redox state [16, 48, 63]. Thus, our study showed the activation of three antioxidant enzymes, namely catalase, ascrobate peroxidase (APX) and superoxide dismutase (SOD). Our study showed that the catalase activity slighlty increased in the plants treated with lead. The same results are found by Malecka et al. (2012) [45] in *Pisum sativum*. However, Dias et al. (2019) [19], in *Pisum sativum*, and Zhang et al. (2019) [82], in *Hydrilla verticillata*, showed that an increase in lead concentration induce a decrease in catalase activity. This might explain the slight difference in the catalase activity between the plants treated with lead and the control plants. As for the plants treated with lead, our results showed a slight increase in the catalase activity in plants treated with nickel. Zhang et al., (2019) [82] observed in *Hydrilla verticillata* an increase in the catalase activity and then a decrease but after 4 days of the exposure to nickel.

APX oxidizes ascorbate (reductant) in the presence of H_2O_2 to give monohydroascorbate and water. This enzyme is present in all cellular compartments, in the cytosol and in the apoplast, in soluble form or bound to membranes (Asada, 1999) [4]. Thus the strong APX activities can be linked to the high H_2O_2 contents recorded during our study. These same results were observed by Pourrut (2008) [66] who noted a strong activation of APX following treatment with heavy metals.

Similarly, superoxide dismutase (SOD) is the third enzyme involved in the detoxification and elimination of ROS. It allows the dismutation of superoxide into O_2 and H_2O_2. It is activated following the accumulation of superoxide (O_2^-) [10]. Our results are in line with those obtained by Metwally et al. (2003), Milone et al. (2003), Hsu and Kao, (2004), Cho and Seo (2005), Singh et al. (2010) and Martinez et al. (2010) [13, 34, 50, 52 ,54, 74].

Glutathione S-transferase, a key enzyme in detoxification, is strongly activated by nickel but weakly by lead, probably because much of the latter is accumulated at root level. It is known that this enzyme is strongly activated during chemical pollution [36]. Glutathione S-transferases (GSTs) are a superfamily of enzymes that catalyze the conjugation between reduced glutathione and electrophilic pollutants [49]. It has been shown that this enzyme is activated following cadmium pollution in an aquatic plant *Elodea canadensis* [83]. A study on *Allium cepa*, also showed that heavy metals (Cd, Cr, Cu, Hg, Pb and Zn) activate GST [23].

Effects of EDTA

EDTA has a partially inhibiting effect on the germination of *Pisum sativum* seeds. In general, this inhibition could be explained by the toxicity of EDTA as a xenobiotic chemical [28]. This effect has already been observed in *Oryza sativa* L. by Nazmul et al. (2021) [60] as well as Grčman et al. (2001) [28] who mentioned the toxic effect of this chelator on the species *Trifolium pratense*. Singh et al. (2021) [74] showed a decrease in germination correlated with EDTA concentration and they explain this by the cytotoxic effect of EDTA on cell division. Researchers have found that concentrations above 25 µM are harmful to *Oryza sativa* L. plants [60]. These observations are consistent with the results of the growth measurements in the batches treated with EDTA since we observed a decrease of 12% compared to the control plants. The same researchers found a strong accumulation of carotenoids for high concentrations of EDTA; which means the installation of oxidative stress knowing that these molecules are antioxidants [77]. This toxicity effect is also found at the protein level, the content of which decreases following treatment with EDTA. This has already been observed in rice [60]. This inhibitory effect on protein synthesis can be explained by the installation of an oxidative stress which has resulted in an accumulation of H_2O_2 and the superoxide anion (O_2^-) and an activation of three antioxidant enzymes (CAT, APX and SOD). This improvement of antioxidant enzymes activities by EDTA have already been reported by Mahmud et al. (2019) [44]. The activation of these enzymes makes it possible to attenuate the consequences of oxidative stress which resulted, among other things, in an accumulation of MDA, an indicator of the membrane alteration. This accumulation of MDA as well as that of H_2O_2 were also observed in maize and bread wheat [61]. However, Arabaci and Usluoglu (2013) [3] showed that, in presence of 3 mM EDTA, 55% activity of catalase, an essential antioxidant enzyme, was inhibited in *Malva sylvestris*. EDTA inhibited the enzyme activity because it was known as a metal chelator that could

make complex with iron in the active site of catalase [29]. This difference in the results can be explained by the plant species itself and its stage of development, the experimental protocol, etc. Our results showed also a highly significant increase in GST activity. It is known that this enzyme is involved in the detoxification of organisms contaminated by xenobiotics. Indeed, GST participates in the conjugation of a molecule of glutathione (GSH) on the targeted compounds or simply bind them through a ligandin function [33].

Combined effects of EDTA and Heavy Metals

For all the parameters studied, the addition of EDTA amplifies the action of lead and nickel on pea plants. Similarly to our results, Kanwal et al. (2014) [37] showed that the addition of EDTA increased MDA content and antioxidant enzymes activity (CAT, SOD and APX) in *Brassica napus*. EDTA is considered an efficient antioxidant and it can improve the oxidative stability, and some studies indicate that EDTA has the ability to protect against oxidation [43]. In addition to its toxicity, as has been shown by this study, it is known that EDTA promotes the bioavailability and translocation of heavy metals [7]. EDTA also plays a role in the sequestration of heavy metals, and particularly lead, in the vacuole [15, 70]. Ultimately, EDTA causes heavy metals accumulation in the plant [38]

The objectives set for this work were to study the effects of two heavy metals, nickel and lead as well as the addition of a chelator, EDTA, on the morpho-physiological and biochemical characters as well as parameters related to oxidative stress. The results obtained showed that lead and nickel have a negative impact on germination, growth and protein content of plants. This is due to the disruption of photosynthetic and respiratory chains and to their interference with the ions involved in enzymatic activity and nutritional deficiency, which is the consequence of the antagonism between metals and essential elements. In addition to these effects, heavy metals induced also oxidative stress. EDTA itself also caused the same negative effects and be considered as toxic xenobiotic substance. The combination of this chelator with the Ni or Pb amplified the action of these heavy metals.

The antioxidant activity of at least three enzymes (CAT, APX and SOD) was stimulated by EDTA, heavy metals or a combination of both. GST is a key enzyme in plant detoxification and, indeed, its activity was greatly increased in respnse to EDTA and both heavy metals.

The lack of measurements of nickel and lead levels in *Pisum sativum* plants did not allowed us to know if these heavy metals accumulated or not and if the EDTA had a role in that. This is the first perspective that looms for the continuation of this work. The second perspective is to measure the activities of other antioxidant enzymes (guaiacol peroxidase, glutathione reductase) and non-enzymatic systems (ascorbate and glutathione). Genotoxicity is another way to assess the toxicity of heavy metals. Its study will enrich our knowledge of the effects of these pollutants on the genetic equipment.

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Conflict of Interests. There is no actual or potential conflict of interest in relation to this article.

REFERENCES

[1] Akujobi, C.O., Odu, N.N., Okorondu, S.I., (2012) : Bioaccumulation of lead by *Bacillus* species isolated from pig waste. Journal of Research in Biology, 2: 83-89.

[2] Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E., (2001): The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ, 24: 1337-1344.

[3] Arabaci, G., Usluoglu, A., (2013): Catalytic properties and immobilization studies of Catalase from *Malva sylvestris* L. Journal of Chemistry, Article ID 686185, 6 pages.

[4] Asada, K., (1999): The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. Annual Review of Plant Physiology and Plant Molecular Biology, 50(1): 601-639.

[5] Atta, S., Maltese, S., Marget, P., Cousin, R., (2004): (NO_3)-N-15 assimilation by the field pea *Pisum sativum* L. Agronomie, 24: 85-92.

[6] Baize, D., (1997): Teneurs totales en éléments traces métalliques dans les sols (France). Références et stratégies d'interprétation , Paris: INRA éditions, 410 p.

[7] Bareen, F.E., Tahira, S.A., (2010): Efficiency of seven different cultivated plant species for phytoextraction of toxic metals from tannery effluent contaminated soil using EDTA. Soil and Sediment Contamination, 19: 160-173.

[8] Boucelha, L., Djebbar, R., Abrous-Belbachir, O., (2019): *Vigna unguiculata* seed priming is related to redox status of plumule, radicle and cotyledons. Functional Plant Biology, 46(6): 584-594.

[9] Bradford, M.M., (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. Analytical Biochemistry, 72: 248-254.

[10] Cadenas, E., Davies, K.J., (2000): Mitochondrial free radical generation, oxidative stress, and aging. Free Radical Biology & Medicine, 29(3–4): 222–230.

[11] Carlos, A., Harguinteguy, M., Pignata, L., Fernández-Cirelli, A., (2015): Nickel, lead and zinc accumulation and performance in relation to their use in phytoremediation of macrophytes *Myriophyllum aquaticum* and *Egeria densa.* Ecological Engineering, 82: 512-516.

[12] Cheng, S., (2003): Effect of heavy metals on plants and resistance mechanisms. Environmental Science and Pollution Research, 10(4): 256-264.

[13] Cho, U.H., Seo, N.H., (2005): Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. Plant Science, 168: 113-120.

[14] Clijsters, H., Cuypers, A., Vangronsveld, J., (1999): Physiological responses to heavy metals in higher plants: defense against oxidative stress. Zeitschrift fur Naturforschung, 54: 730-734.

[15] Colzi, I., Arnetoli, M., Gallo, A., Doumett, S., Del Bubba, M., Pignattelli, S., Gonnelli, C., (2012): Copper tolerance strategies involving the root cell wall pectins in *Silene paradoxa* L. Environmental and Experimental Botany, 78: 91-98.

[16] Corpas, F.J., Gupta, D.K., Palma, J.M., (2015): Production sites of reactive oxygen species (ROS) in organelles from plant cells. pp. 1–22. In Gupta, D.K, Palma, J.M., Corpas,

F.J., (Eds.): Reactive Oxygen Species and Oxidative Damage in Plants under Stress. Springer.
[17] Costa, A.C.A., Duta, F.P., (2001): Bioaccumulation of copper, zinc, cadmium and lead by *Bacillus sp*., *Bacillus cereus*, *Bacillus spheaerecus* and *Bacillus subtillus*. Brazilian Journal of Microbiology, 32: 32-50.
[18] Demarco, C.F., Afonso, T.F., Pieniz, S., Quadro, M.S., Camargo, F.A.O, Andreazza, R., (2018): *In situ* phytoremediation characterization of heavy metals promoted by *Hydrocotyle ranunculoides* at Santa Bárbara stream, an anthropogenic polluted site in southern of Brazil. Environmental Science and Pollution Research International, 25: 28312–28321.
[19] Dias, M.C., Mariz-Ponte, N., Santos, C., (2019): Lead induces oxidative stress in *Pisum sativum* plants and changes the levels of phytohormones with antioxidant role. Plant Physiology and Biochemstry, 137: 121-129.
[20] Dorey, S., Baillieul, F., Saindrenan, P., Fritig, B., Kauffmann, S., (1998): Tobacco class I and II catalases are differentially expressed during elicitor-induced hypersensitive cell death and localized acquired resistance. Molecular Plant-Microbe Interactions, 11(11): 1102-1109.
[21] Ebrahim Zadeh, H., (1994): Plant physiology (topic of nutrition and absorption). Teheran University Press, 689 p.
[22] Evlard, A., Campanella, B., (2013): Impact des éléments-traces métalliques sur les plantes et les techniques de phytoremédiation. pp. 59-75. In Druart, P., Husson C., Paul, R., (Eds.): Renaturation des berges de cours d'eau et phytoremédiation. Gembloux, Belgium: Presses agronomiques de Gembloux.
[23] Fatima, R.A., Ahmad, M., (2006): *Allium cepa* derived EROD as a potential biomarker for the presence of certain pesticides in water. Chemosphere, 62(4): 527-537.
[24] Gajewska, E., Sktodowska, M., (2007): Relations between tocopherol, chlorophyll and lipid peroxides content in shoots of Ni-treated wheat. Journal of Plant Physiology, 164: 364-366.
[25] Ghani, A., Shah, A.U., Akhtar, U., (2010): Effect of lead toxicity on growth, chlorophyll and lead (Pb^{2+}) contents of two varieties of maize (*Zea mays* L.). Pakistan Journal of Nutrition, 9: 887-891.
[26] Gholinejad, B., Khashij, S., Ghorbani, F., Isa Bandak, I., Farajollahi, A., (2020): Effects of lead ions on germination, initial growth, and physiological characteristics of *Lolium perenne* L. species and its bioaccumulation potential. Environmental Science and Pollution Research volume, 27: 11155-11163.
[27] Gopal, R., Rizvi, A.H., (2008): Excess lead alters growth, metabolism and translocation of certain nutrients in radish. Chemosphere, 70: 1539-1544.
[28] Grčman, H., Velikonja-Bolta, Š., Vodnik, D., Kos, B., Leštan, D., (2001): EDTA enhanced heavy metal phytoextraction: metal accumulation, leaching and toxicity. Plant and Soil, 235: 105-114.
[29] Guarrera, P.M., (2005): Traditional phytotherapy in Central Italy (Marche, Abruzzo, and Latium). Fitoterapia, 76(1): 1-25.
[30] Gupta, D.K., Sandalio, L.M., (2011): Metal toxicity in plants: perception, signaling and remediation. Springer Science & Business Media, 274 p.
[31] Habig, W.H., Pabst, M.J., Jakoby, W.B., (1974): Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry, 249(22): 7130-7139.
[32] Habig, W.H., Pabst, M.J., Jakoby, W.B., (1974): Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry, 249(22): 7130-7139.
[33] Hayes, J.D., Flanagan, J.U., (2005): Glutathione transferases. Annual Review of Pharmacology and Toxicology, 45: 51-88.
[34] Hsu, Y.T., Kao, C.H., (2004): Cadmium toxicity is reduced by nitric oxide in rice leaves. Plant Growth Regulation, 42(3): 227-238.
[35] Kabir, M., Iqbal, M.Z., Shafiq, M., (2009): Effects of lead on seedling growth of *Thespesia populnea* L. Advances in Environmental Biology, 3: 184-190.
[36] Kammerscheit, X., Chauvat, F., Cassier-Chauvat, C., (2019): First *in vivo* evidence that glutathione S-transferase operates in photo-oxidative stress in *Cyanobacteria.* Frontiers in Microbiology, 10: 1899.
[37] Kanwal, U., Ali, S., Shakoor, M.B., Farid, M., Hussain, S., Yasmeen, T., Abbas, F., (2014): EDTA ameliorates phytoextraction of lead and plant growth by reducing morphological and biochemical injuries in *Brassica napus* L. under lead stress. Environmental Science and Pollution Research, 21(16): 9899-9910.
[38] Khan, A.H.A., Butt, T.A., Mirza, C.R., Yousaf, S., Nawaz, I., Iqbal, M., (2019): Combined application of selected heavy metals and EDTA reduced the growth of *Petunia hybrida* L. Scientific Reports, 9: 4138.
[39] Kofler, L., (1963): Croissance et développement des plantes. Gauthier-Villars, Paris, 234 p.
[40] Krupa, Z., Siedlecka, A., Maksymiec, W., Baszynski, T., (1993): *In vivo* response of photosynthetic apparatus of *Phaseolus vulgaris* L. to nickel toxicity. Journal of Plant Physiology, 142(6): 664-668.
[41] Ladislav, T., Lgor, M., Veronika, Z., (2017): Heavy metal-induced reactive oxygen species and cell death in barley root tip. Environmental and Experimental Botany, 140: 34-40.
[42] Lebourg, A., Sterckeman, T., (1996): Intérêt de différents réactifs d'extraction chimique pour l'évaluation de la biodisponibilité des métaux en traces du sol. Agronomie, 16: 201-215.
[43] Let, M.B., Jacobsen, C., Meyer, A.S., (2007): Ascorbyl palmitate, γ-tocopherol, and EDTA affect lipid oxidation in fish oil enriched salad dressing differently. Journal of agricultural and food chemistry, 55(6): 2369-2375.
[44] Mahmud, J., Hasanuzzaman, M., Nahar, K., Rahman, A., Fujita, M., (2019): EDTA reduces cadmium toxicity in mustard (Brassica juncea L.) by enhancing metal chelation, antioxidant defense and glyoxalase systems. Acta Agrobot., 72: 17-72.
[45] Malecka, A., Piechalak, A., Mensinger, A., Hanć, A., Baralkiewicz, D., Tomaszewska, B., (2012): Antioxidative defense system in *Pisum sativum* roots exposed to heavy metals (Pb, Cu, Cd, Zn). Polish Journal of Environmental Studies, 21(6): 1721-1730.
[46] Marek, B., (1985): Influence of lead on the chlorophyll content and on initial steps of its synthesis in greening cucumber seedling. Acta Societatis Botanicorum Poloniae, 54(1): 95-105.
[47] Marklund, S., Marklund, G., (1974): Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European Journal of Biochemistry, 47: 469-474.
[48] Marques, D.N., Carvalho, M.E.A., Piotto, F.A., Batagin-Piotto, K.D., Nogueira, M., Gaziola, S.A., Azevedo, R.A., (2019): Antioxidant defense response in plants to cadmium stress, pp. 423-461. In Hasanuzzaman, M., Prasad, M.N.V., Nahar, K., (eds): Cadmium tolerance in plants: agronomic, molecular, signaling, and omic approaches. Academic Press, New York.
[49] Marrs, K.A., (1996): The function and regulation of glutathione S-transferases in plants. Annual Review of Plant Physiology and Plant Molecular Biology, 47: 127-158.
[50] Martínez Domínguez, D., Córdoba García, F., Canalejo Raya, A., Torronteras Santiago, R., (2010): Cadmium-induced oxidative stress and the response of the antioxidative defense system in *Spartina densiflora*. Physiologia Plantarum, 139(3): 289-302.

[51] Mazliak, P., (1982): Physiologie végétale. II, Croissance et développement. Collection Méthodes Herman, Paris, 243 p.
[52] Metwally, A., Finkemeier, I., Georgi, M., Dietz, K.J., (2003): Salicylic acid alleviates the cadmium toxicity in barley seedlings. Plant Physiology, 132: 272-281.
[53] Michalak, A., (2006): Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Polish Journal of Environmental Studies, 15: 523-530.
[54] Milone, M.T., Sgherri, C., Clijsters, H., Navari-Izzo, F., (2003): Antioxidative responses of wheat treated with realistic concentration of cadmium. Environmental and Experimental Botany, 50(3): 265-276.
[55] Mourato, M.P., Moreira, I.N., Leitão, I., Pinto, F.R., Sales, J.R., Martins, L.L., (2015): Effect of heavy metals in plants of the *Genus Brassica*. International Journal of Molecular Sciences, 16(8): 17975-17998.
[56] Moussavou Moudouma, C.F., (2010): Etude des mécanismes d'accumulation du cadmium chez *Arabidopsis thaliana* (écotype Wassilewskija) et chez un mélèze hybride (*Larix xeurolepis*) par des approaches moléculaire et développementale. PhD Thesis. Université de Limoges, France.
[57] Moya, J.L., Ros, R., Picazo, I., (1993): Influence of cadmium and nickel on growth, net photosynthesis and carbohydrate distribution in rice plants. Photosynthesis Research, 36: 75-80.
[58] Nakano, Y., Asada, K., (1981): Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant and Cell Physiology, 22: 867-880.
[59] Navari-Lazzo, F., Quartacci, M.F., (2001): Phytoremediation of metals tolerance mechanisms against oxidative stress. Biotechnology, 13: 73-85.
[60] Nazmul Huda, K.M., Hossain., M., Mukta., R.H., Khatun, M.R., Md., Anwarul Haque, M.A., (2021): EDTA-enhanced Cr detoxification and its potential toxicity in rice (*Oryza sativa* L.). Plant Stress, 2: 100014.
[61] Neugschtner, R.W., Tlustos, P., Komarek, M., Szakova, J., Jakoubkova, L., (2012): Chemically enhanced phytoextraction of risk elements from a contaminated agricultural soil using *Zea mays* and *Triticum aestivum*: performance and metal mobilization over a three year period. International Journal of Phytoremediation, 14: 754-771.
[62] Nowack, B., Kari, F.G., Krüger, H.G., (2006): Critical assessment of chelatant-enhanced metal phytoextraction. Environmental Science & Technology, 40: 5225-5232.
[63] Palma, J.M., Gupta, D.K., Corpas, F.J., (2019): Hydrogen peroxideandnitric oxide generation in plant cells: Overview and queries. pp. 1-16. In Gupta, D.K., Palma, J.M., Corpas, F.J., (eds.): Nitric Oxide and Hydrogen Peroxide Signaling in Higher Plants. Springer: Chemistry, Swizerland.
[64] Pereira, W.E., Siqueira, D.L., Martînez, C.A., Puiatti, M., (2000): Gas exchange and chlorophyll fluorescence in four citrus root stocks under aluminum stress. Journal of Plant Physiology, 157: 513-520.
[65] Piccini, D.F., Malavolta, E., (2008): Effect of nickel on two common bean cultivars. Journal of Plant Nutrition, 15: 2343-2350.
[66] Pourrut, B., (2008): Implication du stress oxydatif dans la toxicité du plomb sur une plante modèle *Vicia faba*. PhD Thesis, Université de Toulouse, France.
[67] Repetto, O., Bestel-Corre, G., Dumas-Gaudot, E., Berta, G., Gianinazzi-Pearson, V., Gianinazzi, S., (2003): Targeted proteomics to identify cadmium-induced proteins modifications in *Glomus mosseae* - inoculated pea roots. New Phytologist, 157: 555-567.
[68] Rouibi, A., (1992): Etude des effets du nitrate de plomb sur la germination et la productivité primaire nette chez *Phaseolus vulgaris*. Thèse de Magister, Université d'Annaba, Algérie.
[69] Seregin, I.V., Ivanov, V.B., (2001): Physiological aspects of cadmium and lead toxic effects on higher plants. Russian Journal of Plant Physiology, 48: 523-544.
[70] Shahid, M., Pinelli, E., Pourrut, B., Silvestre, J., Dumat, C., (2011): Lead-induced genotoxicity to *Vicia faba* L. roots in relation with metal cell uptake and initial speciation. Ecotoxicology and Environmental Safety, 74: 78-84 .
[71] Shalini, R.S., (2003): Lead toxicity induces lipid peroxidation and the activities of antioxidant enzymes in growing rice plants. Plant Science, 164(4): 645-655.
[72] Sharma, P., Dubey, R.S., (2005): Lead toxicity in plants. Brazilian Journal of Plant Physiology, 17: 35-52.
[73] Shaw, K., Kumar, R.G., Verma, S., Dubey, R.S., (2001): Effect of Cadmium and activities of antioxydant enzymes in growing rice seedling. Plant Science, 161: 1135-1144.
[74] Singh, S.P., Ma, L.Q., Harris, W.G., (2001): Heavy metal interactions with clay: Sorption and desorption behavior. Journal of Environmental Quality, 30(6): 1961-1968.
[75] Stadtman, E.R., (2004): Role of oxidant species in aging. Current Medicinal Chemistry, 11: 1105-1112.
[76] Stohs, S.J., Bagchi, D., (1995): Oxidative mechanism in the toxicity of metal ions. Free Radical Biology and Medicine, 18(2): 321-336.
[77] Strzałka, K., Kostecka-Gugała, A., Latowski, D., (2003): Carotenoids and environmental stress in plants: significance of carotenoid-mediated modulation of membrane physical properties. Russian Journal of Plant Physiology, 50: 168-173.
[78] Tyler, L.D., McBride, M.B., (1982): Mobility and extractability of cadmium, copper, nickel and zinc in organic and mineral soil columns. Soil Science, 134: 198-205.
[79] Weckbecker, G., Cory, J.G., (1988): Ribonucleotide reductase activity and growth of glutathione depleted mouse *leukemia* L 1210 cells *in vitro*. Cancer Letters, 40(3): 257-264.
[80] Wysokinski, A., Kuziemska, B., Lozak, I., (2023): Heavy metal allocation to pea plant organs (Pisum sativum L.) from soil during different development stages and years. Agronomy, 13(3): 673.
[81] Xiong, Z.T., (1998): Lead uptake and effects on seed germination and plant growth in a Pb hyperaccumulator *Brassica pekinensis* Rupr. Bulletin of Environmental Contamination and Toxicology, 60: 285-291.
[82] Zhang, H., Zhang, L.L., Li, J., Chen, M., An, R.D., (2020): Comparative study on the bioaccumulation of lead, cadmium and nickel and their toxic effects on the growth and enzyme defence strategies of a heavy metal accumulator, *Hydrilla verticillata* (L.f.) Royle. Environmental Science and Pollution Research, 27: 9853-9865.
[83] Zouainia, S., (2017): Evaluation de la toxicité de deux métaux lourds (Cadmium et Zinc) sur le métabolisme énergétique (Respiratoire et Photosynthétique) d'une plante aquatique *Elodea canadensis*. PhD Thesis, Université Badji Mokhtar, Annaba, Algeria.

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