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Nitric Oxide improves plant tolerance under zinc treatment in *Lepidium sativum* L.

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1 ABSTRACT

Nitric oxide (NO) is dynamic molecule implicated in diverse biological functions demonstrating its protective effect against damages induced by abiotic stresses. The present study investigated that exogenous NO (100 and 300 mM sodium nitroprusside) prevented the injurious effect of Zn- metallic stress (300 and 750 µM of ZnSO4) on plant growth. *Lepidium sativum* exposed to different Zn doses (300 and 750 µM) reduced plant growth, decreased chlorophyll content and reduced the nitrate reductase activity (NR) in leaves. Exogenous NO alleviated Zn toxicity in *Lepidium sativum* L., especially under 750 µM -Zn dose combined to 300 mM of NO donor. The applications of NO also improved the nitrogen assimilation especially in plants treated with 300 mM of NO donor. These results indicate that NO treatment mitigated Zn toxicity trough with proline and sugar content reduction.

2 INTRODUCTION

Heavy metal stress has become a major concern in various terrestrial ecosystems worldwide. The extensive industrialization imparts detrimental effects on soil as well as on crop productivity by accumulating heavy metals (Shahid *et al,* 2015). Damage to soil texture, pH of soil, presence of different elements, and accumulation of heavy metals cause a reduction of plant growth by adversely affecting various physiological and molecular activities of plants (Panuccio *et al.* 2009; Hassan *et al,* 2017). Heavy metals such as Zn, Cu, Mo, Mn, Co, and Ni are essential for crucial biological processes and developmental pathways (Salla *et al,* 2011; Shahid *et al.* 2015). Metals are among the oldest known toxic substances; this is related to their ancient use. An important toxicological characteristic of metals is that they have the ability to react in biological processes after the loss of electron(s). They also have the particularity of being low-dose for many of them essential in metabolism, but they can become toxic at higher doses. For example, zinc (Zn), at millimolar concentration, is a trace element that is involved in many enzymatic reactions (dehydrogenases, proteinases, peptidases) and plays an important role in the metabolism of proteins, carbohydrates and lipids (Kabata-Pendias, 2004). Plants are, throughout their lives, in constant interaction with their environment. They are confronted with different types of stress affecting their physiology. Over the course of evolution, all these constraints have led to the implementation of effective defence responses allowing plants to better adapt to their environment. Indeed, plants have a range of defences, from morphological barriers to the implementation of specific cellular mechanisms. *Lepidium sativum L.* has

been considered as important medicinal plant since Vedic era. In numerous countries, seedlings of *L. sativum* L. are utilized in salads because of their pungent taste. In addition, the seeds are utilized as a seasoning with a spicy flavour. Boiled seeds are used in drinks by Arabs, moreover milled in honey or as an infusion in heated milk. The seed can be used for soap preparing (Wadhwa *et al,* 2012). *Lepidium sativum L.* is a rapid developing plant that is characterize by little nutrition necessity. As showed by OECD, this plant exposed to metals during germination, under standardized conditions, is an appropriate model of environmental stress. The main objectives of the current investigation were to describe the response of Garden cress (*L. sativum* L.) in presence of four metals at different levels of toxicity, and to investigate hormesis effect and metal stress in *L. sativum* L. seeds germination and seedlings grown. Nowadays, the application of sodium nitroprusside (SNP), a precursor to the formation of nitrogen monoxide (NO), is a recent biological approach to improving plant tolerance (Seabra and Oliveira, 2016; Nabi *et al.,* 2019; Garcia-Marti *et al,* 2019). Under normal temperature and pressure conditions, nitrogen monoxide (NO) is a colour less gas in its pure state. NO is a radical consisting of a nitrogen atom and an oxygen atom linked by a double bond (Parankeson *et al,* 2017; Ahmed *et al,* 2019). Its solubility in water is comparable to the solubility of carbon monoxide (CO) and molecular oxygen (O2). The zero charge of NO makes it soluble in non-polar solvents, which facilitates its diffusion through cell membranes. NO chemistry is characterized by its radical nature (NO●), NO has an unpaired electron on its orbital and its half- lifetime is in the order of a few seconds. The loss of this electron causes the formation of the nitrosonium cation (NO^+) while the gain of an electron will form the nitroxyl radical ($R2N\text{-}O^*$), each of these compounds having its own properties and reactivity (Vanin, 2020). Biochemical, molecular and genetic studies indicate that NO is responsible for multiple cellular responses both rapidly (cyclic GMP production, mobilization of the second Ca^{2+} messenger, kinase activation) and late (defence gene induction, hypersensitive reaction and systemic resistance (Wendehenne *et al,* 2004; Backer *et al,* 2018). Understanding the molecular mechanisms by which NO contributes to plant resistance opens new perspectives on strategies to stimulate natural plant defence reactions, strategies currently used by firms in the plant protection industry where sodium nitroprusside (SNP) is used as a precursor to nitrogen monoxide (NO) formation. It is in this context that this work is being carried out; it aims to study the target role of nitrogen monoxide (NO) in the tolerance of *Lepidium sativum* under metallic stress conditions by zinc.

3 MATERIAL AND METHODS

3.1 Plant material and growth conditions: The plant material concerned by this study is the garden cress (*Lepidium Sativum* L.). The seeds are disinfected by washing with 10% bleach for 15 minutes, and then rinsed thoroughly with distilled water; as a result, they are germinated in petri dishes on filter paper soaked in water at room temperature and in the dark. Five days after germination, seedlings are transplanted at a rate of 12 per bucket of 1.5 litres of nutrient solution containing: KNO3 3 mM, Ca (NO3)2 1 mM, KH2PO4 2 mM, MgSO4 0.5mM, Fe-Ethylene diamine tetra acetic acid (EDTA) 32.9 µM, and micronutrients: H3BO4 30 µM, MnSO4 5 µM, CuSO4 1 µM, ZnSO4 1 µM, and (NH4)6Mo7O 1µM. The solutions are constantly aerated and renewed regularly every 5 days in order to avoid PH changes (5 to 6) and variations of ionic concentrations. The cultures are conducted in air-conditioned room; the photoperiod is 8 hours of light / 16 hours of darkness. After 20 days of culture on basic nutrient medium, garden cress seedlings are placed in buckets containing nutritive solutions of different composition, depending on the dose of the metal and the dose of SNP. Harvests are made after 5 days of treatment. The plants are subdivided into leaves

and shoots. These are rinsed with distilled water in order to eliminate the superficial mineral elements and then quickly wiped with filter paper and then measure the length of their aerial part and those of the root part. Part of the samples are used for the determination of their fresh material (FM) and placed in the oven at 60°C for 3 days and reweighed to determine the mass of dry matter (DM) and another part is kept at - 80°C for enzymatic assays or certain metabolites.

3.2 Determination of soluble sugar: The method used for the determination of soluble sugars is that described by Dubois and al (1956). The extraction is carried out starting from 25 mg of dry matter in the presence of 5 ml of 80% ethanol. The samples are placed in a water bath at 70°C for 30 minutes. After cooling, the samples are centrifuged at 6000 g for 15 minutes. Subsequently, 25 µl of supernatant is removed and added to 5 ml of anthrone solution in test tubes under a fume hood.

Then these tubes are put in a water bath at 100°C for 10 minutes. The tubes are directly placed in the ice, and finally the reading is made at 640 nm.

3.3 Determination of proline: An amount of 25 mg of dry matter (DM) (leaves and shoots) already ground with the mortar is added to 1 ml of 3% sulfosalicylic acid. The extracts are then centrifuged at 12000 g for 20 minutes at 4°C. The assay is carried out by mixing 500 µl of the extract, 500 µl of sulfosalicylic acid, 1 ml of concentrated acetic acid and 1 ml of ninhydrin.

The tubes are incubated in a water bath at 100°C for 1 hour, cooled to 4°C, and then the contents are mixed with 2 ml of toluene. The tubes are allowed to stand before taking the upper phase (containing proline) for a spectrophotometer reading at 520 nm.

3.4 Extraction of proteins: Protein extraction 100mg of fresh plant material already preserved in liquid nitrogen is mixed with 700 µl of extraction buffer containing a phosphate buffer (50 mM, pH = 7) EDTA-Na2 (1 mM), MgCl2 (20 mM), KCl (50 mM)and PMSF (0.5 mM). Centrifugation is performed at 14000g for 30 minutes at 4°C. The obtained supernatant contains the soluble proteins and will be used for the determination of proteins and for enzymatic activity tests.

3.5 Determination of soluble proteins: Soluble protein assay is performed according to the method of Bradford (1976) which is based on the attachment of Coomassie blue to proteins. For 25 µl of plant material extract, 975 µl of Bradford 5X diluted reagent are added. After developing the reaction, the optical density of each sample is determined at 595 nm, which is proportional to the amount of protein. Protein concentration is determined by a standard curve established from standard serum albumin (BSA) concentrations. The results are expressed in mg. g^{-1}FW.

3.6 Determination of ammonium $NH4^+$: Ammonium was extracted from plant material at 4 °C with 0.3 mM H2SO4 and 0.5% (w/v) polyclar AT. Ammonium content was quantified according to the reaction of Berthelot modified by Weatherburn1976.

3.7 Determination of nitrate NO_3^-: The same extract used for the determination of the mineral elements was used for the determination of the nitrate. Incubation for 2 h at 60°C. of 1 ml of mineral extract and the mixed solution which is composed of (8.3 ml HCl, 192 ml H2O, 0.5 g Vanadium III chloride, 0.2 g Sulphanilamide and 0.01 g N-naphthylethylenediamine (NNED). The readings are performed at a wavelength of 540 nm and the results are expressed in µmol $NO3^-$. g^{-1}FW.

3.8 Measurement of nitrate reductase (NR) activity: The nitrate reductase activity is measured by Robin's method 1979. This method consists in extracting the enzyme from the plant tissue by grinding in an extraction buffer (8 ml per gram of fresh material) in the presence of 1mM EDTA, 7.5mM cysteine and 2.5% w / v casein. The grinding is carried out at 4°C. In a mortar in the presence of an extraction medium of pH 7.4 composed of 100 mM potassium phosphate, 1N KOH, casein and EDTA. The ground material, filtered on a Bultex cloth, is centrifuged for 15 minutes at 30000 g. The supernatant constituting the enzymatic extract is stored in ice until measurement. The NADH-

NR activity is measured by determination of the nitrite formed in the reaction medium. The diazotization of the nitrites formed is carried out by the addition of sulphanilamide and N-naphthyl- ethylene diamine dichloride. The optical density of the supernatant is read at 540 nm after 20 min of colour development.

3.9 Statistical Analysis: Each value is the mean of three or five independent measurements. The values of the analysed parameters were expressed as the mean ± standard deviation (SD). The two-way analysis of variance (ANOVA) and Bonferroni post-test between groups were performed at a p value of <0.05 to evaluate the significance of differences between values. Statistical analyses were performed using XLSTAT (2014).

4 RESULTS AND DISCUSSION

4.1 Combined effects of zinc and NO on growth: Morphological aspects: The basic nutrient medium, containing all minerals necessary for plant development, was used as a control for zinc and sodium nitroprusside treatments. Morphological observation of the plants shows toxicity due to Zn exposure, which manifests itself in a decrease in stem length and distance between nodes, as well as a reduction in the length of the main root. Other studies showed that in high concentrations, Zn induced chlorosis and necrosis, and reduced the shoot biomass in *Brassica rapa* and Populus species (Blasco *et al,* 2015; Todeschini *et al,* 2011). Under simultaneous treatment of Zn- NO (SNP: NO donor), growth is improved in the shoot and root. Nitric oxide (NO) is a simple molecule that acts as a signalling molecule used in many biological processes in plants. The use of NO donors or inhibitors indicates that NO serves as a signal molecule in the induction of metal stress resistance (Kopyra and Gwozdz, 2003; Nabi *et al.*2019).Plants co-treated by Zn-NO develop better than those treated only with Zn^{2+} (Fig. 1).

Figure 1: Morphology of *Lepidium sativum* plants treated with 0, 300 and 750 µM ZnSO4 in the presence of SNP as NO donor (300mM).
A: control; B: 300µMZnSO4;C: 300µM ZnSO4 + 300mM NO D: 750µMZnSO4
E: 750µM ZnSO4 + 300mM NO

The NO addition improved photosynthesis and growth in several plant species such as Indian Mustard, also its presence has a significant strengthening of the anti-oxidant defence system and decreased Cu-caused oxidative stress parameters (Rather B *et al.,*2020). Effect on shoot and root length: After 5 days of 300 µM-Zn exposure, shoot and root length decreased by about 21% and 26% respectively referring to control. Exogenous application of sodium nitroprusside (NO donor) induced a partial improvement in seedling length, especially 300mM-SNP addition (Fig. 2). The 300 mM SNP dose gave an optimal growth under Zn exposure. These results are in agreement with several studies which showed an improve effect of NO on seed germination and growth parameters (Beligni and Lamattin+a, 2000) (Kopyra and Gwozdz, 2003). Effect on fresh weight (FW) production: Results showed that leaf and root FW production is affected under different doses of Zn. Indeed, we noticed that leaf FW production is reduced progressively by 42% and 54% in plants treated respectively with 300 and 750 µM Zn. The root FW decrease was also about 40% and 55%, respectively under 300 and 750 µM Zn exposure. Results showed too, that ZnSO4 application caused visual symptoms of toxicity, which are leaf chlorosis and plant decrease. Thus, the **shoot and** root length were reduced under Zn stress, mainly under 750µM Zn treatment. In fact, the root growth inhibition could be explained by zinc interference with hydrocarbon metabolism, which prevented root supply with assimilates. Besides visual symptoms, FW production showed growth inhibition in Zn-treated seedlings. These results are consistent with those found in *Lablab purpureus* (Myrene *et al,* 2012) and *Brassica juncea* (Prasad *et al.* 1999). Exogenous application of SNP induced a significant increase in leaf and root FW production referring to plants treated only with zinc (Fig.2).

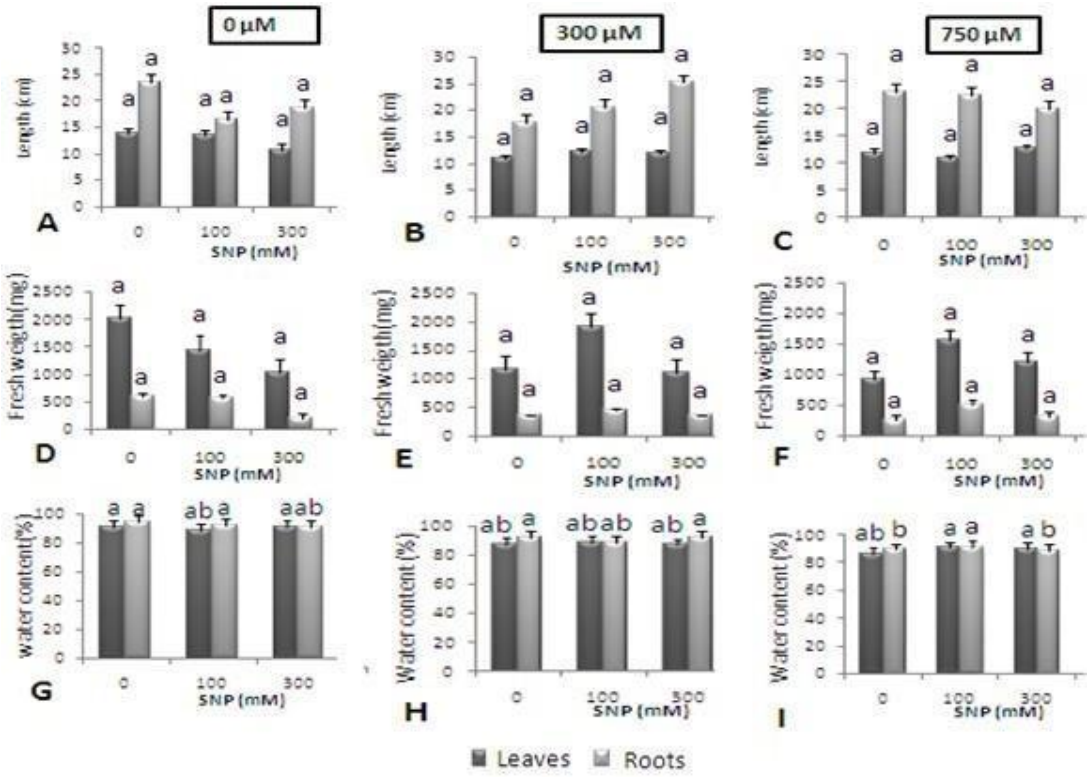

Figure 2: Variation in the length of aerial part and roots under 0,300 and 750µM ZnSO4 stress (A, B and C), fresh weight under 0, 300 and 750 µM ZnSO4 (D, E and F) and water content under 0, 300 and 750 µM (G, H and I) in the presence of different doses of NO donor (0, 100 and 300).

The growth enhancing- effect of NO was reported too in *Trifolium repens* L.under Cd stress. In Cd treated plants, NO elevated the activity of the PMH^+ ATPase in both shoots and roots, enhanced activity of V-H^+ ATPase in roots and enhanced the uptake of minerals (shoots: Mg and Cu; roots: Ca, Mg and Fe) (Liu SL *et al.*,2015).

4.2 Effect on water content: Results showed that water content was not affected by different treatments neither in shoot nor in roots. A slight decrease water content was reported in leaves treated by 750 µM ZnSO4 (Fig. 2). Generally, heavy metal induced a decrease in plant water content, such as Cd and Zn. Raklami *et al.* 2021 reported a water content decrease in *Medicago Sativa* under Cd and Zn stress. Effect on Chlorophyll content: We noticed that chlorophyll a and chlorophyll b in primary leaves were reduced respectively by 25% and 41% in plants treated with 300 µM ZnSO4. In 750µM, Zn- treated plants, the content of chlorophyll a and b decreased respectively by 54% and 28% referring to control. Zn treatment decreased chlorophyll contents and affect biosynthesis by inhibition of CO2 fixation and electron transfer in the photosynthetic chain (Upadhyay and Panda 2010). The Zn- electron transfer inhibition resulting in overproduction of reactive oxygen species (Upadhyay and Panda2010). Exogenous application of SNP induced a significant increase in the chlorophyll a and b contents, especially in plants co-treated by 750µM and 300mM of SNP (Fig. 3).

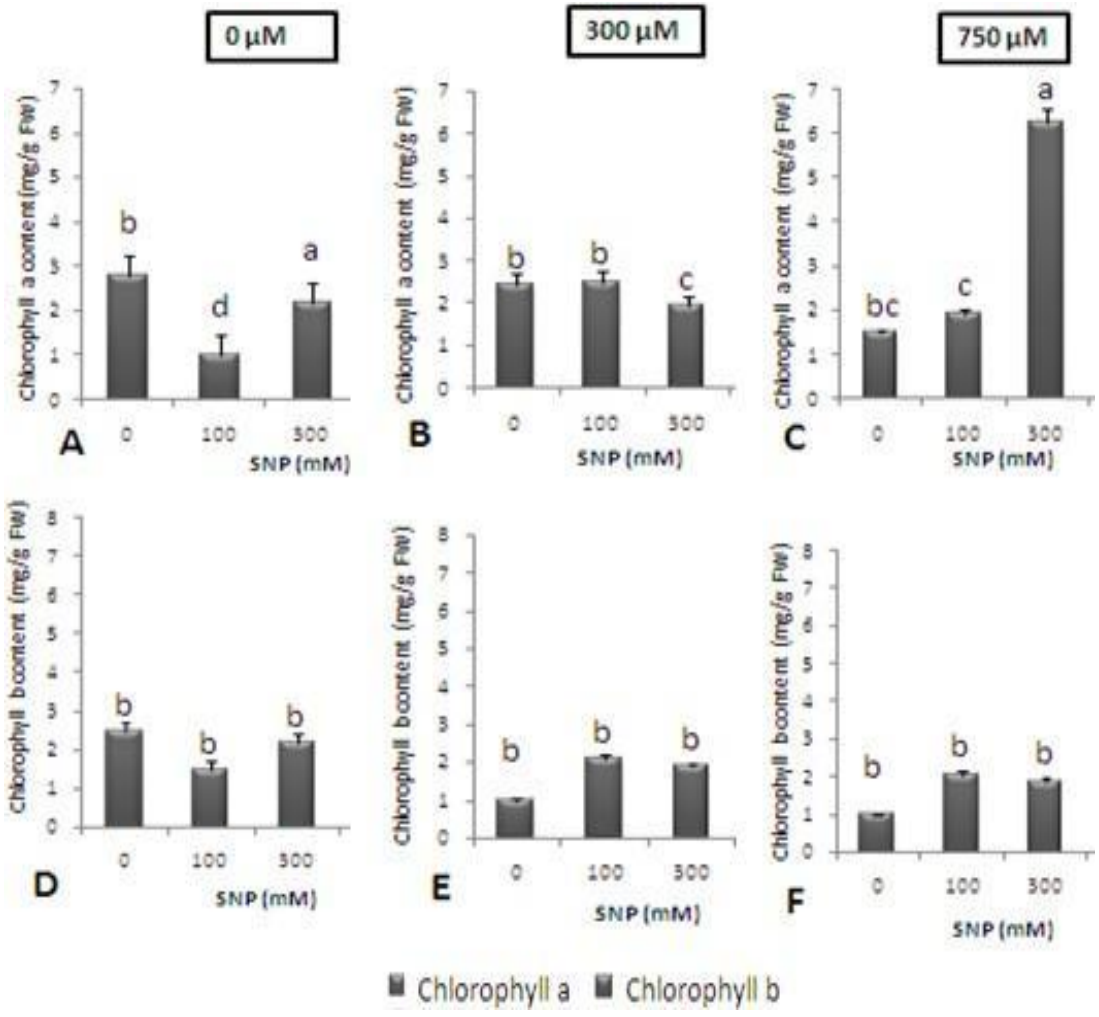

Figure 3: Variation in the chlorophyll (A, B and C) and B content (D, E and F) in Lepidium sativum plants under stress of 0, 300 and 500 µM ZnSO4 treated with 0, 100 and 300 mM of NO

donor.

These data are consistent with those found in other plants, such as lettuce, red cabbage and Arabidopsis (Beligni and Lamattina, 2000; Mannai *et al,* 2014).

4.3 Zinc accumulation: The Leaf and root Zn accumulation in *Lepidium sativum* plants exposed to different Zn treatments is illustrated in figures 6. Results showed a progressive increase of leaf and root Zn contents. Indeed, leaf Zn content increased significantly and steadily. Root Zn content followed the same pattern as in leaves but at much lower levels (Fig.4).

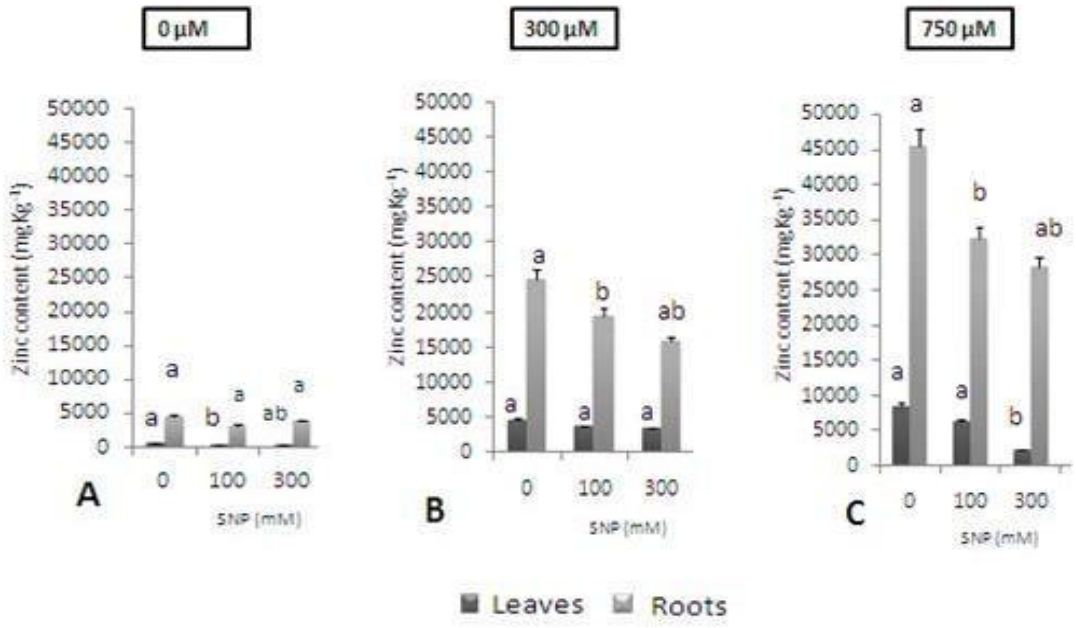

Figure 4: Variation Zinc content (A, B and C) in leaves and roots of *Lepidium sativum* plants under stress of 0, 300 and 750µM ZnSO4 treated with 0, 100, 300 and 500mM of NO donor.

The results showed that the plant accumulated a large amount of Zinc. The Zn accumulation was proportional with Zn concentration in culture medium. Zinc accumulation is mainly root-based, which explain the growth reduction more pronounced in roots than in leaves. Roots would therefore be a Zn storing organ. However, there is too a translocation to leaves. Therefore, *Lepidium sativum* could be an interesting plant for Zn phyto-extraction from contaminated soils. The SNP addition in the presence of ZnSO4 decreased Zn^{2+} content in both leaves and roots. In roots, the 300mM-SNP co-addition decreased Zn content by 17% and 40% respectively under 300 and 750 µM ZnSO4 treatments referring to Zn-treated plants. The application of SNP modified the Zn profile accumulation and induced a decrease in the Zn content in leaves and roots. This decrease was more significant in leaves under 300 mM SNP and 750 µM ZnSO4 co- treatment. This result was reported in *Plantago major L.* (Nasiri-savadkokhi *et al.* 2017).

4.4 Combined effect of zinc and NO on C-N status

4.4.1 Total soluble sugar content: According to figure 5, zinc treatment for 5 days induced a significant accumulation of sugars in *Lepidium sativum* plants. Thus, in300 µM Zn-treated plants, soluble sugar content increased by 64% in roots. While in leaves, soluble sugar content variation remained not significant referring to control.

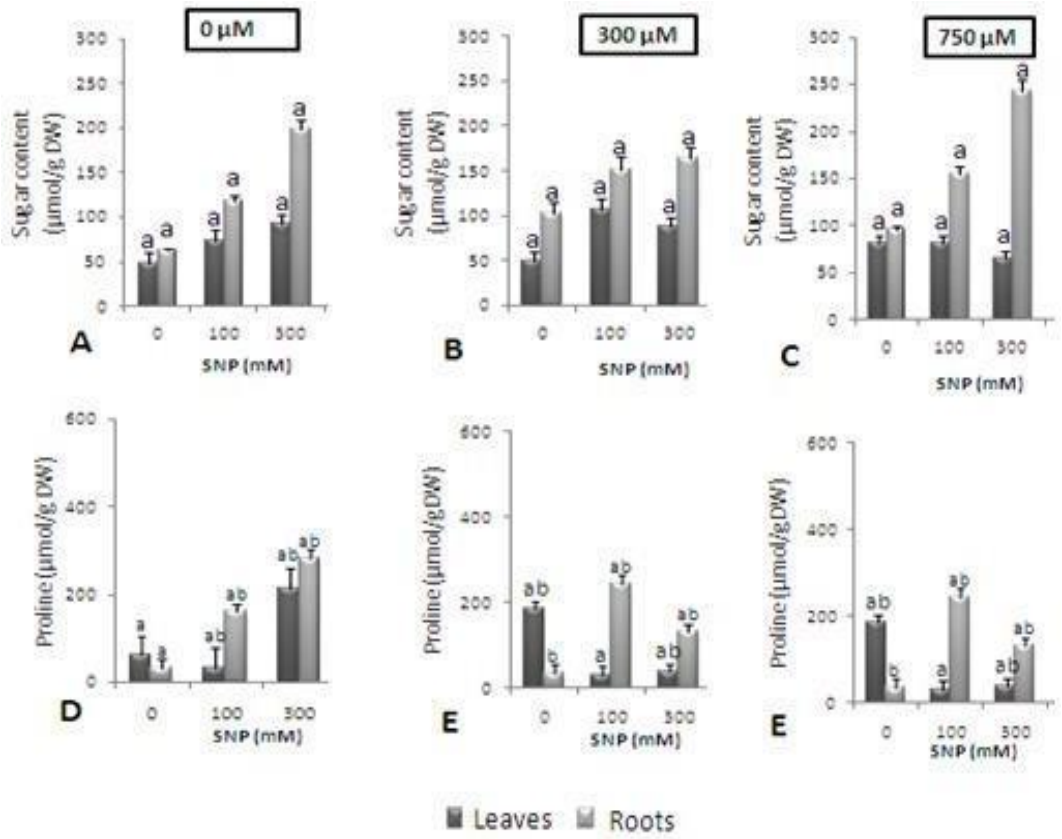

Figure 5: Variation sugar (A, B and C) and proline (D, E and F) content in leaves and roots of *Lepidium sativum* under stress of 0, 300 and 750 ZnSO4 treated with 0,100 and 300 mM of NO donor.

In 750 µM Zn- treated plants, the soluble sugar content was 1.6 times higher in leaves and roots than in control. The application of300 mM SNP to Zn-treated plants, the soluble sugar content increased significantly in leaves (about 2 times compared to control) and in roots (about 4 times compared to control) mainly under 750 µM zinc treatment. Previous studies showed that low level of SNP application significantly enhanced soluble sugar content in Lettuce leaves. Studies showed too, that SNP induced the accumulation of glucose, fructose and sucrose under UV-B stress in Lettuce leaves (Esringu A, 2015). It is well known that soluble sugars had an essential role in plant metabolism. They act as typical osmoprotectants and stabilize cellular membranes, maintain turgor. They are also signal molecules in sugar sensing and signalling system. It has been also reported that there is a relation between sugar accumulation and ROS balance (Couee *et al.,* 2006). Proline content: In *Lepidium sativum* plants, results showed a significant proline accumulation in both leaves and roots in Zn-treated plants (figure 5). Thus, there is a progressive proline accumulation within Zn dose increase in culture medium. In 750 µM Zn-treated plants, leaf and root Proline content showed an increase respectively by 63% and 51% referring to control. The application of 300 mM SNP increased significantly the proline content in zinc- treated plants, mainly with the 750-µM Zn dose. Indeed, the co-treatment 300 mM-SNP and 750 µM-Zn increased leaf and root proline content by respectively 78% and 120% compared to zinc-treated plants. In SNP-treated plants, proline content and sugar content increased considerably. These results were shown in *Perennial ryegrass* under cadmium stress (Wang *et al.* 2016). Heavy metal exposure often induced various metabolites synthesis such as

proline and carbohydrates. Our results showed a significant increase in soluble sugar content in leaves and roots of *Lepidium sativum*. In addition, results showed too, a significant accumulation of proline. In addition to its recognized role as an osmoticum, proline could eliminate or reduce the production of toxic oxygen species. Thus, plants treated with SNP accumulated more proline, which explain growth parameter improving of nitric oxide. Similar results were observed in *Triticum aestivum* treated with aluminium (Zhang *et al* 2008; Balafrej *et al.* 2020). Soluble protein content: The variation in soluble protein content in leaves and roots of *Lepidium sativum* treated with increasing doses of Zn and/or SNP for 5 days was illustrated in figure6.

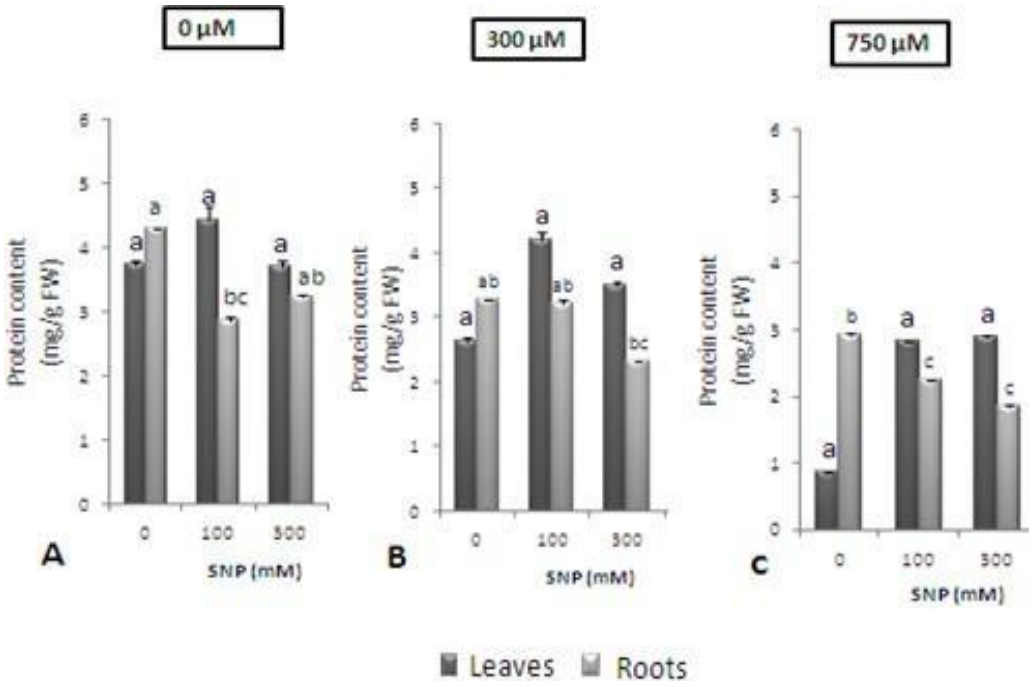

Figure 6: Variation in protein content (A, B and C) in leaves and roots of *Lepidium sativum* plants under stress of 0, 300 and 750 µM ZnSO4 treated with 0,100 and 300 mM of NO donor.

Results showed that ZnSO4 addition in culture medium induced protein content decrease in the leaves and roots. The protein content decrease was more important in leaves. Leaf protein content decreased by about 50 % under 300 µM Zn treatment and more than 65 % under 750 µM Zn treatment. The root protein content decrease was less significant. Indeed, root protein content decreased by 23% under 300 µM Zn exposure and 38% under 750 µM Zn exposure. The nitric oxide addition alleviated negative effect of zinc treatment on soluble protein content. The SNP addition increased leaf protein content especially under 300 µM and 750 µM Zn treatment, compared to Zn-treated plants. Previous studies showed that NO alleviated the soluble protein content decrease induced by Zn treatment (Nasiri-Savadkoohi *et al.,*2017). Nitrate content: Results illustrated by figure 7 showed that leaf and root $NO3^-$ contents were strongly affected by zinc exposure. After 5 days of treatment, leaf and root $NO3^-$ ion levels were reduced respectively by 46% and 50% in300 µM Zn-treated plants compared to control. In 750 µM Zn-treated plants, the nitrate content reduction was about 95% and 65% respectively in leaves and roots compared to control. The Zn treatment as several heavy metals could affect nitrate absorption by roots (Maaroufi *et al* 2019). Recent study showed that Zn treatment triggered NRT1.1-mediated nitrate uptake (Pan *et al.* 2020). Indeed, NRT1.1-regulated Zn accumulation in stressed-plants (Pan *et al.,* 2020).

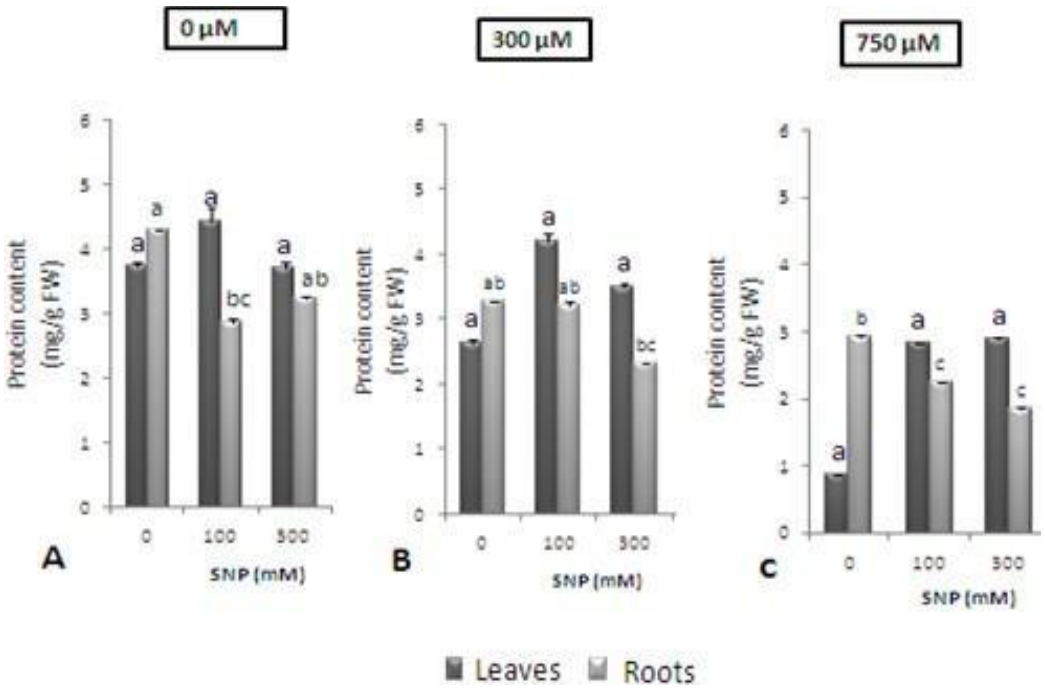

Figure 7: Variation in nitrate (A, B and C) and ammonium (D, E and F) content in leaves and roots of *Lepidium sativum* plants under stress of 0,300 and 750 µM ZnSO4 treated with 0, 100 and 300 mM of NO donor.

The 300 mM-SNP application induced an increase in nitrate content in control plants. Under 300 µM Zn exposure, The SNP addition enhanced the Zn-induced inhibitory effect on leaf nitrate content. Under 750 µM Zn exposure, 300 mM of SNP improved nitrate content compared to Zn-treated plants. On the other hand, in roots, the presence of SNP induced an improvement in nitrate levels compared to plants treated with Zn only. This improvement was less marked than that of the leaves. The SNP addition to medium improved nitrate absorption. In addition, oxidation of NO from SNP, through an enzymatic or non-enzymatic mechanism, could lead to an increase in $NO3^-$ content at the cytosol level (Wilson *et al.,* 2008). Ammonium content: The variations in ammonium ($NH4^+$) content in leaves and roots of *Lepidium sativum* following the different treatments were shown in figure 7. In Zn-treated plants, leaf and root ammonium content decreased mainly under 750 µM Zn exposure. This decrease was more significant in leaves than roots. The ammonium content decreased despite of protease activity enhancement and protein degradation detected under Zn stress. Ammonium derived from the primary nitrate reduction as well as other metabolic pathways such as protein catabolism, is converted first to glutamine-by-glutamine synthetase (GS) then to glutamate-by-glutamate synthase (GOGAT). Ammonium can be directly incorporated into glutamate by the aminating reaction of glutamate dehydrogenase (GDH). Several studies showed that abiotic stress decreased glutamine synthetase activity while GDH activity was improved such as under Cd stress (Maaroufi *et al.,* 2019) and salinity (Maaroufi *et al.,* 2011). Therefore, ammonium could be assimilated by GS/GOGAT pathway or by GDH activity often enhanced by abiotic stress. The co-treatment 300mM SNP-750 M Zn induced ammonium accumulation mainly in roots. The co-treatment SNP- Zn enhanced ammonium accumulation, especially under 750 µM Zn and 300mM SNP co-exposure. When SNP is introduced into culture medium, the $NH4^+$ content is lower than that induced by only Zn. This behaviour is

explained by the decrease in protease activity in plants that have undergone combined treatment. These results are proven in *O. sativa* treated with cadmium (Yi and Ching, 2004; Terron-Camero *et al.* 2019). Effect on NR activity: The results showed that in control plants, nitric oxide reduced nitrate reductase (NR) activity, mainly in leaves (Fig. 8).

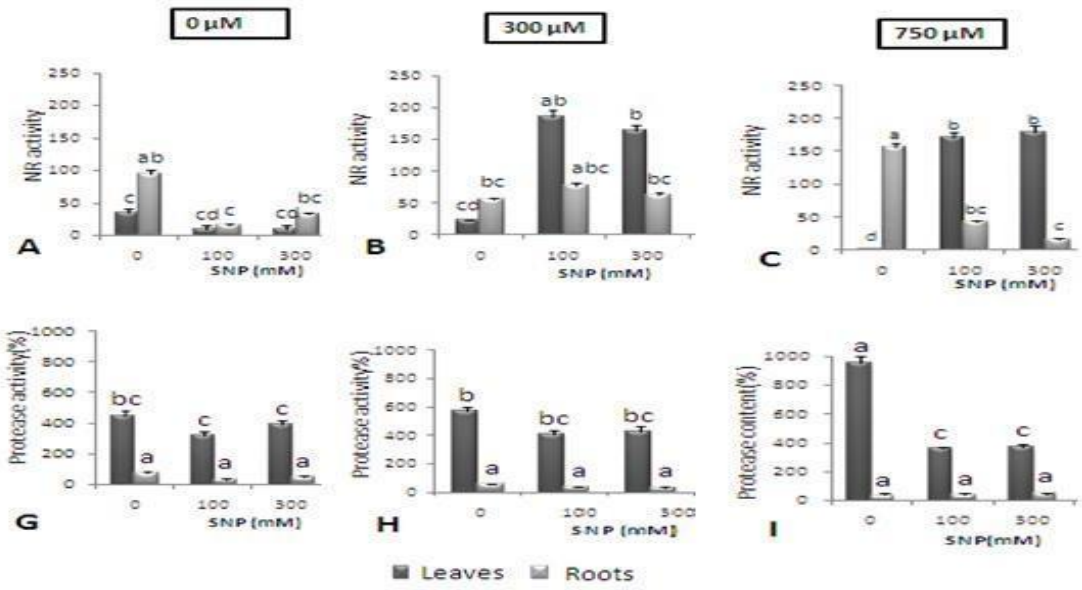

Figure 8: Variation in NR (reduced µmol $NO2^{-}$ g^{-1} FW. h^{-1}) (A, B, C, D, E and F) and protease activity (G, H and I) in leaves and roots of *Lepidium sativum* plants under the stress of 0,300 and 750 µM ZnSO4 treated with 0, 100 and 300 mM of NO donor.

The Zn exposure caused a decrease in nitrate reductase activity in both leaves and roots. This NR activity decreased mainly in leaves under high dose of ZnSO4 (750 µM). Following the introduction of NPS into the culture medium of stressed plants, a significant increase in NR activity in both leaves and roots was recorded. The 100mM SNP treatment induced a significant leaf NR activity increase in 300 µM Zn-treated plants. Under 750 µM Zn- 300mM SNP co-treatment, leaf NR activity increased to exceed control values (Fig. 8). In roots, NR activity remained affected by Zn exposure despite NSPco-treatment. Several studies suggest that nitrate reductase (NR) plays an important role in NO production in plants (Yamasaki and Cohen, 2006). Under our experimental conditions, exogenous 100 mM-SNP application had no stimulating effect on NR activity neither in leaves nor in roots of control plants. Under 300mM-SNP exposure, NR activity increased significantly in leaves. In Zn-treated plant, nitric oxide (SNP) induced leaf NR activity enhancement mainly with the high Zn-dose (750 µM). Similar behaviour has been reported in other species (Rockel *et al,* 2002). This result suggested that NR activity enhancement allowed a counteract of Zn- toxic effects. These data suggested a relationship between NO, nitrate uptake and NR activity enhancement under Zn treatment.

4.4.2 Effect on protease activity: After 5 days of 300 µM of ZnSO4 treatment, we observed an increase in leaf protease activity. However, in roots, Zn treatments had no significant effect on protease activity, referring to control. This result agrees with those presented by Pena *et al.* 2006 in which, he showed that several metals increased protease activity (Cu, Pb, Al, Ni, Cd, Hg, Co, Cr) except Zn. In Zn-treated plants, SNP application decreased leaf protease activity mainly under 300mM NSP treatment (Fig. 8). Several studies signalled the NO- modulation effect under

abiotic stress (Bilibana MP 2010; Mannai *et al.* 2014).

5 CONCLUSION

In last decades, soil-heavy metals pollution has become a major concern for which finding a solution is becoming necessity to conserve soil for future generations. Several studies tried many techniques to alleviate heavy metal stress in many plant species. Recently researchers tried the compost and arbuscular mycorrhizal fungus as a solution to reduce harmful effects of Cd and Zn in *Medicago sativa*. This study focusses to NO use to alleviate Zn toxicity on plant growth. Presented results showed that NO treatment significantly improved growth and could reduce Zn-toxic effect in *Lepidium sativum*. NO treatment improve growth, chlorophyll content, proline and sugar contents, nitrate content and NR activity in Zn-treated seedlings. Results are encouraging and promising for further research into NO use to alleviate heavy metal phytotoxicity. Results need more research to understand NO mechanisms in mitigating zinc's effect on growth plants.

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