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Influence of nitrate fertilization on Cd uptake and oxidative stress parameters in alfalfa plants cultivated in presence of Cd

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Abstract

Plant nutrients might affect the activity and bioavailability of heavy metals in the soil –plant environment thus their accumulation in the plant. Little is known about the effect of nitrogen fertilization on cadmium (Cd) uptake by alfalfa "*Medicago sativa*". This work aims to characterize the oxidative status, the physiological stress parameters and the Cd uptake in alfalfa « *Medicago sativa* » exposed to Cd and supplied with nitrogen fertilizers. The experiment was carried out in a green house with alfalfa grown in Cd-polluted soil (3.6 ppm) and amended with two different fertilizers: without NO_3^- (PK) or with NO_3^- (NPK) in a sand:peat (v:v) mixture. The following parameters were monitored: Chlorophyll content, Photosynthesis rate, Catalase (CAT) activity, Thiobarbutiric Acid Reactive Species (TBARS) content and Cd bioaccumulation. It was found that NPK fertilization increased mean Cd uptake as well as plant biomass in Cd-exposed plants. Nitrogen supply was also effective in reduction Cd-induces phytotoxicity (Photosynthetic pigments and rate) and oxidative stress alterations. Our results suggest that nitrogen supply may improve the uptake rate of Cd by alfalfa and provide new insights on the importance of nitrogen fertilization towards future phytoremediation applications using alfalfa.

Keywords: Phytoremediation, alfalfa, cadmium, nitrogen fertilization

1. Introduction

Cadmium (Cd) is considered to be potent toxic heavy metals, causing adverse effects to humans and all other living organisms (Sarwar *et al.*, 2010). It enters the environment from natural and, essentially, anthropogenic sources (Schützendübel *et al*., 2002). Majority of the emissions to the environment are through industrial waste from processes such as mining, paint pigments, power stations, metalworking industries and waste incineration (Suzuki *et al*., 2001). Cadmium is also used in some agricultural treatments as fungicides and phosphate fertilizer industry. Tunisia plays an important role in the global phosphate market and Tunisian phosphate rocks contain high level of Cd (50.3 $mg.kg^{-1}$) (Da Silva *et al*., 2010) comparing to the permissible limit established by the Soil EC Directive (1.5 $mg.kg^{-1}$) and the Average World Phosphorite by Altschuler (1980) (18 $mg.kg^{-1}$).

When in the soil, Cd could be transferred to edible parts of the plants and reach up to 0.5 $mg.kg^{-1}$ DW in lettuce and spinach (Jackson and Alloway 1992). This may affect human health since Cd is classified as a Class 1 human carcinogen (Group B) by the International Agency for Research on Cancer and the World Health Organization (WHO, 1992).

Cadmium is a non-essential for plant metabolism and exhibit varied degrees of phytotoxicity. As a non-redox active metal, Cd can replace essential metals or cofactors at enzyme active site causing imbalance in cellular redox status. Plants exposed to Cd showed changes in photosynthetic rate due to distortion of chloroplast ultra-structure, impairments of chlorophyll synthesis and reduced minerals uptake (Hattab *et al*., 2009). Cd interacts with respiration and nitrogen metabolism and causes oxidative damage (Hattab *et al*., 2009). Indeed, Cd has been found to generate reactive oxygen species (ROS) like ($O_{.2}^{-}$, H_2O_2 et OH.) (Qian *et al*., 2005). Failure to control ROS accumulation leads to lipid peroxidation (TBARS), protein oxidation, DNA damage and even cell death (Fu and Huang 2001). However, plant cells are protected against ROS by a complex antioxidant system capable of scavenging ROS in a process known as redox homeostasis. The defense systems of plants to reactive oxygen species include enzymes like catalase (CAT), one of the first-induced enzymes responsible for the hydrogen peroxide (H_2O_2) quenching. Increasing of CAT activity and TBARS content can be considered as sensitive biomarkers for oxidative stress inducers such as Cd (Sun and Zhou 2008).

Phytoextraction is the use of plants to remove metals and other contaminants from soil, sediments and water (Salt *et al*., 1995). In most cases, heavy metal tolerance is achieved through sequestration in vacuoles by their complexation of cadmium with phytochelatins (Carrasco Gill *et al*., 2012). Plant nutrients play a very important role in developing plant tolerance to Cd toxicity and thus, high Cd accumulation in different plant parts (Sarwar *et al.*, 2010). Alfalfa is one of the plants that have the ability to accumulate heavy metals (Carrasco Gill *et al*., 2012). Alfalfa is known to be an important forage crop that has several advantages like high amount of proteins, fast growth rate, drought tolerance and adaptation to a wide range of climates. Alfalfa is also characterized by its well-developed root system and nodules rich in nitrogen-fixing bacteria i.e. (*Sinorhizobium meliloti*) that contributes to the improvement of fertilization. Several studies have shown that alfalfa can bind various heavy metal ions (Parsons *et al.*, 2005). Moreover, alfalfa has been shown to tolerate and take up heavy metals from soil (Carrasco-Gill *et al*., 2012). Thus, alfalfa is an ideal natural resource for the remediation of contaminated soils (Su *et al*., 2004). However, alfalfa plants used in the phytoremediation programs must be destroyed to avoid the entry of metals into the food chain through grazing livestock. Relatively few studies have investigated the effects of nitrogen fertilization on the uptake and distribution of metals in alfalfa (Carrasco-Gill *et al.*, 2012). Hence, optimizing fertilizer strategies could be crucial to enhance the heavy metal extraction capacity by this plant.

This study aimed to investigate the Cd-induced oxidative stress parameters in Medicago sativa grown in Cd-contaminated soils. To shed some light into how nitrogen fertilization can contribute to the improvement of alfalfa phytoextraction capacity as well as it's physiological response we considered soils amended with two different fertilizers: without NO_3^- (PK) or with NO_3^- (NPK). Effects on growth, biomass, chlorophyll concentration, rate of photosynthesis, catalase activity (CAT), TBARS content and Cd bioaccumulation were investigated.

2. Materials and Methods

2.1. Plant material and growth condition

Plants were grown in the greenhouse (mean T°: 20.5±1.5 ◦C, with 14 h light- period) in plastic pots (1 plant/pot) filled with 3kg dry mixture of peat, perlite

and quartz sand (3:3:3 w/w) for 4 weeks. Pots were watered with 0.5 L of water to initiate the drainage. A first batch of the plants (30 pots) were fertilized with a NPK mixture (30:110:100; adequate NO_3^-), where nitrogen was added as NH_4NO_3. The first batch was divided into two sub-batches where the first one (15 pots) was supplied with CdCl2 (3.6 ppm) and the second was maintained unsupplied. A second batch (30 pots) was only fertilized with a PK mixture (110:100, poor NO_3^-). Phosphate and potassium were also added as K_2HPO_4 and KH_2PO_4 forms. The second batch was also divided into 2 sub-batches where the first one (15 pots) were supplied with CdCl2 (3.6ppm) and the second was maintained unsupplied. CdCl2 was dissolved in distilled water (Stock solutions) and added as a single application to the pots at sowing. Following the agronomic practices in central eastern Tunisian area, plants were sown in February. Two weeks before sowing, the pots were ground fertilized with phosphate and potassium doses. Nitrogen was applied at the moment that alfalfa seedlings were sown to avoid excess drainage due to the high solubility of NH_4NO_3.

Alfalfa (*Medicago sativa* cv. Aragon) seeds were surface sterilized for 5 min in 5% (v/v) commercial bleach. After rinsing several times with distilled water, seeds were soaked overnight at 4 ºC. Ten homogeneous selected seedlings were transferred to each pot where they were germinated. Periodic irrigations with distilled water were performed according to the water requirements of the culture.

2.2. Sampling

Sampling was performed at seedling stage (4 weeks after sowing). Plants were collected in 5 pools (NPK, PK, NPK-Cd, PK-Cd and Cd). Each pool contains 15 pots. Plants were divided in shoot and root and placed into beakers and rinsed several times with 10 mM Na_2EDTA solution to remove superficial Cd. Then length and fresh weight of roots and shoots were measured and stored at -80 ºC until analysis.

2.3. Cadmium accumulation

The alfalfa tissues were carefully washed with deionized water and oven-dried at 105 °C for 60 min and 60 °C for 24 h, then ground into fine powder, and sieved through 1 mm nylon sieve. 0.5 gram samples were then digested by HNO_3:$HCIO_4$ (3:1 v/v) in the microwave system. The concentrations of Cd were determined by a graphite furnace atomic absorption spectrophotometer (Perkin-Elmer 3300, Perkin-Elmer, Wellesley, MA). Standard material consisting of known concentration of CdCl2 was included for assurance control. Means of Cd concentrations were calculated from three replicates. The limit of detection (LOD) of Cd was 0.05 $\mu g.g^{-1}$ dry weight.

2.4. Cadmium stress indexes

Chlorophyll content and rate of photosynthesis

Chlorophylls were extracted from 0.05 g of frozen leaves with 10 ml 80% (v/v) acetone using a mortar and pestle. Homogenates were filter and absorbance was measured in a UV-6705/ Vis. Spectrophotometer JENWAY at 645 and 663 nm. Total chlorophyll concentrations were calculated according to the formula described by Porra (2002). Total Chlorophyll ($mg.g^{-1}$ FW) = Chlorophyll a + Chlorophyll b, where:

$$\text{Chl}a\left(\frac{\text{mg}}{\text{gFW}}\right) = [(12.7\text{x}A_{663}) - (2.69\text{x}A_{645})]\frac{\mu\text{g}}{\text{ml}} \text{ x } \frac{10\text{ml x mg}}{0.05\text{g FW x } 10^3\mu\text{g}}$$

$$\text{Chl}b\left(\frac{\text{mg}}{\text{gFW}}\right) = [(22.9 \text{ x } A_{645}) - (4.68 \text{ x } A_{663})]\frac{\mu\text{g}}{\text{ml}} \text{ x } \frac{10\text{ml x mg}}{0.05\text{g FW x } 10^3\mu\text{g}}$$

Photosynthetic activity based on the rate of light-dependent oxygen evolution of excised leaves was determined at 30 °C following the procedure of Greef *et al*. (1971) using a Biological Oxygen Monitor (YSI 5300A, Yellow Springs Instruments, USA).

2.5. Lipid peroxydation

Lipid peroxidation was estimated by the formation of malondialdehyde, a by-product of lipid peroxidation that reacts with thiobarbituric acid (TBA) according to Ortega-Villasante *et al.* (2005). The resulting chromophore absorbs at 535 nm, Ground frozen tissue (0.1g) was transferred to a screw-capped 1.5 ml Eppendorf tube, and the concentration was calculated directly from the extinction coefficient of 1.563105 $M.cm^{-1}$ homogenized following addition of 1 ml of TCA–TBA–HCl reagent (15% (w/v) trichloroacetic acid (TCA), 0.37% (w/v) 2-TBA, 0.25 M HCl, and 0.01% butylated hydroxytoluene). After homogenization, samples were incubated at 90 ºC for 30 min in a hot block, then chilled in ice, and centrifuged at 12 000 g for 10 min. Absorbance was measured in a UV-6705/ Vis. Spectrophotometer JENWAY at 535 nm and 600 nm, the last one to correct the non-specific turbidity.

TBARS (nmol /g FW)=((DO535-DO600)*1.56.10-6)/106

2.6. Proteins extraction and Catalase activity

For the determination of catalase activity, proteins extraction were prepared by homogenizing 0.5 g of intact frozen samples in pre-chilled mortar and pestle under ice-cold condition in 1 ml of extraction buffer freshly prepared by mixing 10 mL extraction buffer – 30 mM MOPS at pH 7.5, 5mM Na2-EDTA, 10 mM DTT, 10 mM ascorbic acid, 0.6% PVP, 10 µL 100 mM PMSF and 1 mL protease inhibitors cocktail. After centrifugation (14,000g) for 15 min at 4 °C, the supernatant was stored as single use 200–300 µL aliquots at -80 °C. Protein concentration in the extracts was determined with the BioRad Protein reagent). CAT (EC 1.11.1.6) activity was determined by consumption of H_2O_2 in absorbance at 240 nm according to method of Aebi (1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 500 mM H_2O_2 and 50 µl of enzyme extract. Activity was calculated using the extinction coefficient (0.036 mM^{-1} cm^{-1}).

2.7. Statistics

All investigated parameters were expressed as mean values ± standard deviations. For statistical analysis, percentage data were subjected to arcsine root square transformation. The Statistica Software, version 6.0, computer software package (Statsoft. Inc. 2002) was used for statistical analysis. The normality of the distribution was tested using the Shapiro-Wilk test. To assess multiple comparisons, a parametric one-way analysis of variance (ANOVA) was performed on data, with a Tukey's test.

3. Results

3.1. Plant growth parameters and Cd accumulation in roots and shoots

Cd accumulation in the roots and shoots of plants exposed to Cd and supplemented or/not with fertilizers are reported in Table 1. Cd loads in roots were 12-15 times higher than in shoots. In plants exposed to Cd and supplemented with NPK, Cd loads in roots was up to 1.423±0.136 µg/g DW and 0.111±0.043 µg/g DW in shoots. However, in plants exposed to Cd but not supplemented with N (PKCd), the Cd loads were respectively respectively in shoots and roots 1.047 ± 0.055 and 0.68 ±0.007 (Table 1).

Table1. Cd uptake (µg/g DW) by alfalfa's roots and shoots after 30 d post-germination exposure to NPK (30.110.100), PK (110.100), NPK (30.110.100)-Cd 3.6 ppm, PK (110.100)-Cd 3.6 ppm. *p<0.05 significantly different, Tukey's test ANOVA Multiple Comparison test versus control group (NPK).

Treatment	Roots	Shoots
NPK	ND	ND
PK	ND	ND
Cd-NPK	1.432±0.136*	0.112±0.043*
Cd-PK	1.074±0.055*	0.068±0.007*

On the other hand, PK addition did not affect Cd uptake by alfalfa shoots and roots when compared to Cd-exposed plants.

Table 2 reports the roots and shoots length (cm) of plants from the different experimental conditions. Our data may indicate that the most pronounced effect was observed in plants exposed to Cd without any fertilizer supply, falling to 8.38±0.76 cm and 3.11±0.22 respectively for root and shoot lengths when compared to NPK group (11.79±1.17 cm and 3.94±0.21cm respectively for roots and shoots). Similar inhibitory trend was observed in Cd-PK treated plants.

Table 2. Effect of Cd on alfalfa's roots and shoots lengths (cm) after 30 d post-germination exposure to NPK (30.110.100), PK (110.100), NPK (30.110.100)-Cd 3.6 ppm, PK (110.100)-Cd 3.6 ppm. *p<0.05 significantly different, Tukey's test ANOVA Multiple Comparison test versus control group (NPK).

Treatment	Root length (cm)	Shoot length (cm)
NPK	11.79±1.17	3.94±0.21
PK	11.85±1.09	3.53±0.28
Cd-NPK	10.31±1.08	3.49±0.17*
Cd-PK	8.52±0.60*	3.16±0.21*

Data relative to shoots and roots fresh weight is illustrated in Table 3.

Table 3. Effects of Cd on alfalfa's roots and shoots weigh after 30 d post-germination exposure to NPK (30.110.100), PK (110.100), NPK (30.110.100)-Cd 3.6 ppm, PK (110.100)-Cd 3.6 ppm. *p<0.05 significantly different, Tukey's test ANOVA Multiple Comparison test versus control group

Treatment	Root weigh (g)	Shoot weigh (g)
NPK	0.277±0.014	0.295±0.029
PK	0.240±0.016	0.246±0.027
Cd-NPK	0.125±0.015*	0.186±0.014*
Cd-PK	0.133±0.021*	0.170±0.012*

The highest Fresh weight was observed in NPK-fertilized plants (0.277±0.014 0 g and 295±0.029 g respectively in roots and shoots) compared to the rest of the treatments. However, no significant difference was retained significantly different in NPK-Cd exposed plants and Cd-PK exposed plants.

3.2. Effects of cadmium on chlorophyll content and photosynthetic rate

The exposure of alfalfa to Cd resulted in a reduction of chlorophyll content in leaves (Figure. 1). However in plants exposed to Cd +NPK, Chlorophyll-a content is similar to that of control plants. On the other hand, the Chlorophyll-b content was altered in all the plants grown in presence of Cd comparing to controls.

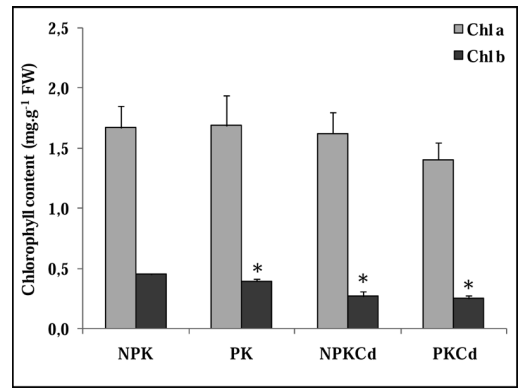

Figure 1. Photosynthetic pigments (Chlorophyll a and chlorophyll b in leaves of M. sativa exposed to NPK (30.110.100), PK (110.100), NPK (30.110.100)-Cd (3.6 ppm), PK (110.100)-Cd (3.6 ppm). Data are average of three 15 independent replicates (±SD), and different letters denote significant differences at p<0.05. *p<0.05 significantly different, Tukey's test ANOVA Multiple Comparison test versus control group (NPK).

The rate of photosynthesis as measured from the rate of light dependent CO_2 consumption per m^2 leaf area (Figure 2) was minimal in all alfalfa plants exposed to Cd/NPK and Cd/PK comparing to control (NPK) plants with respectively 19.29±1.33 µmol CO_2 consumed /

$cm^2.s^{-1}$ and 15.72±1.62 µmol CO_2 consumed / $cm^2.s^{-1}$ when compared to the control group (NPK) (23.78±3.75 µmol CO_2 consumed /$cm^2.s^{-1}$)

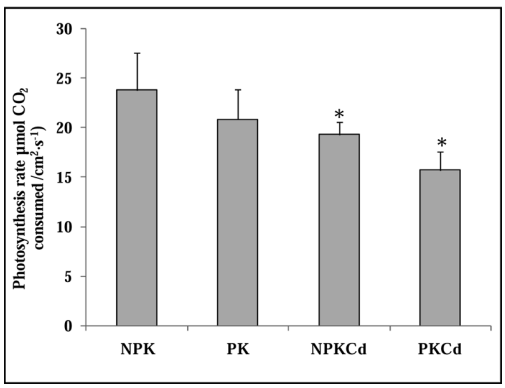

Figure 2. Effect of NPK (30.110.100), PK (110.100), NPK (30.110.100)-Cd (3.6 ppm), PK (110.100)-Cd (3.6 ppm) application on the rate of photosynthesis of treated alfalfa plants expressed as µmol CO_2 consumed/(cm2•s). Data are average of three 15 independent replicates (± SD), and different letters denote significant differences at $p<0.05$. $^a p<0.05$ significantly different, Tukey's test ANOVA Multiple Comparison test versus control group (NPK).

3.3. Lipid peroxydation

The level of lipid peroxidation was measured in terms of TBARS. As shown in Figure 3.A, in shoots lipid peroxidation's content increased significantly in PK, NPK-Cd and PK-Cd exposed plants. However, in roots TBARS accumulation remained almost unchanged compared to control (NPK). (Figure 3.B).

3.4. CAT activity

To better understand the plant stress response in Cd contaminated soil the antioxidant enzymatic defense system of *M. sativa* plants was evaluated by measuring the activity of CAT in the leaves and roots of plants.

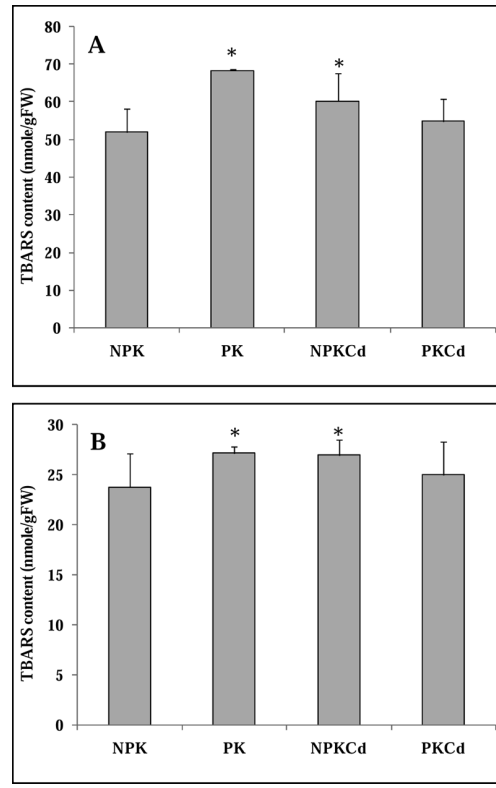

Figure 3. TBARS content (nmol.g^{-1} FW) in shoots (A) and roots (B) of Medicago sativa exposed to NPK (30.110.100), PK (110.100), NPK (30.110.100)-Cd (3.6 ppm), PK (110.100)-Cd (3.6 ppm). Data are average of three 15 independent replicates (± SD), and different letters denote significant differences at $p<0.05$. $^a p<0.05$ significantly different, Tukey's test ANOVA Multiple Comparison test versus control group (NPK).

The CAT activity in leaves (Figure 4.A) was enhanced at PK-Cd treated plants (106.86±16.73 µmole/min/mg of proteins) comparing to control (NPK). However in roots (Figure 4.B), the CAT activity was decreased in plants treated with Cd and associated with fertilizers (NPK-Cd and PK-Cd) showing respectively 44.85%, 59.53% and 42.31% inhibition in comparison with control plants (NPK).

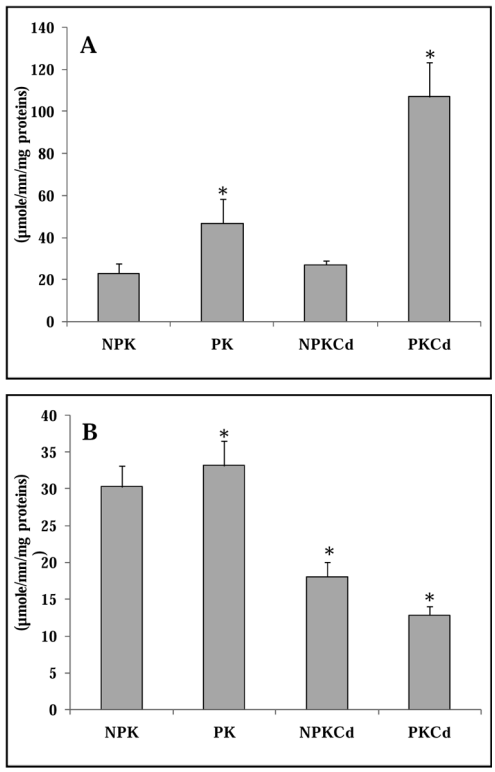

Figure 4. CAT activity (µmol/min/mg of proteins) in shoots (A) and roots (B) of Medicago sativa exposed to NPK (30.110.100), PK (110.100), NPK (30.110.100)-Cd (3.6 ppm), PK (110.100)-Cd (3.6 ppm). Data are average of three 15 independent replicates (± SD), and different letters denote significant differences at $p<0.05$. $^{a}p<0.05$ significantly different, Tukey's test ANOVA Multiple Comparison test versus control group (NPK).

4. Discussion

Cadmium accumulated to a higher extent in roots, as found in many wild and crop plants (Rellan-Alvarez *et al.*, 2006). In our case, the ratio of Cd accumulated in roots to shoots was around 14. This is in accordance with the results obtained in *Zea mais* where higher proportion of Cd was found in roots than in shoots and the ratio was 35 (Rellán-Álvarez *et al*., 2006). Similar results were observed by Suzuki *et al.* (2001), in Arabidopsis paniculata where The Cd concentrations in roots were 8 times higher than that in shoots.

The addition of nitrogen fertilizer augmented significantly the concentration of Cd by 37.5% and 25.27% respectively in alfalfa shoots and roots. This result was somehow surprising, as increased N supply would decrease heavy metal concentration due to a dilution effect (Landberg and Greger 2003). Other reports dealing with the effects of N nutritional status on plants grown in soils observed that fertilization with NO_3 had a positive effect in increasing the concentration of Cd in roots and plant aerial parts Carrasco Gill *et al*. (2012). Wangstrand *et al*. (2007) evaluated the effect of nitrogen fertilizers on Cd concentration in the grain of durum wheat, and found also that the supply of nitrogen fertilizer increased Cd accumulation in different parts of the plant. Similarly, Carrasco Gill *et al*. (2012) found that Alfalafa seedling grown in a metalliferous soil were capable of accumulating higher concentrations of Hg if supplied with NPK fertilization. Additionally, Barrutia *et al*. (2009) found that Rumex spp. accessions grown in a metalliferous soil were capable of accumulating higher concentrations of Zn and Cd when amended with NPK fertilization.

It is well known that the nitrogen fertilization plays a crucial role in plant growth and development. However, these treatments could affect rhizosphere composition, root growth that in turn modifies the availability, absorption and accumulation in different plant parts (Sarwar *et al*., 2010). To explain the increase in Cd concentration in plants fertilized with N, several hypotheses have been drawn. Metal concentrations in soils, soil pH, cation exchange capacity, organic matter content, types and varieties of plants, and plant age are some factors that could affect metal uptake by plants. It is generally accepted that the metal concentration in soil is the dominant factor but many studies confirm that the pH of the soil is usually the most important factor that controls uptake, with low pH favoring Cd accumulation, and that phosphate and zinc decrease Cd uptake (Kirkham, 2006).

Conversely, Zaccheo *et al*. (2006) suggested that the effect NH_4^+ from N fertilizers could acidify the soil pH by the extrusion of H^+ and increases metal solubility. Thus, application of some fertilizers, such as NH_4^+ fertilizers including urea, ammonium sulfate and mono ammonium phosphate (MAP), can enhance Cd availability by lowering pH (Zaccheo *et al.* 2006).

Growth of *Medicago sativa* plants was significantly affected by Cd exposure. The Cd inhibited alfalfa's biomass production and caused a decrease in the chlorophyll content as well as in the stomatal conductance and transpiration level (Table 2, Figure 2). These results are in agreement with those obtained by Hattab *et al*. (2009) in *Pisum sativum* L. where Cd induced a reduction in the biomass production and the chlorophyll content. Similarly, Carrasco-Gill *et al*. (2012) found that alfalfa growth was inhibited by addition of Hg and Cd, as reflected by the general trend of decreased plant size with increasing metal concentration and Chlorophyll content diminished in the treated plants. Cadmium-induced growth reduction might be explained on the basis of inhibition of carbon fixation due to a decrease in photosynthetic rate and chlorophyll content (Hattab *et al*., 2009). Cadmium in soils could induce water stress in plants by decreasing stomatal conductance and transpiration (Chen and Huerta 1997).

On the other hand, our results show that the nitrogen supply significantly improved alfalfa's growth in Cd treated plants. This may suggesting that N application would result in more biomass production by increasing the photosynthetic rate (Landberg and Greger 2003) It have been reported that heavy metal stress induced by Cd generate Reactive Oxygen Species (ROS) production in plants which lead to different damages. Lipid peroxydation is considered as the most damaging process known to occur due to ROS production (Qian *et al*., 2005). Our results showed that TBARS content is two times higher in shoots than in roots. Similar results were found in *Zea mays* (Rellan-Alvarez *et al*., 2006) and *Triticum aestivum* (Guo *et al*., 2004) exposed to Cd contamination.

In this study, TBARS content augmented in shoots of plants grown in presence of nitrogen in the Cd contaminated soil but decreased in those grown in contaminated but non fertilized soil. In agreement with this observation, N-supplied plants accumulate Cd higher than non N-supplied ones. Indeed, Nitrogen fertilization promote alfalfa's Cd uptake which in turn generate ROS responsible of the lipid peroxidation. It has been described that TBARS level was shown to increase under heavy metal stress such as Cd and Hg (Carrasco-Gill *et al*. (2012). However, in the present experiment, no correlation between the Cd uptake and the stress marker TBARS was observed. This is in agreement with the study of Lyubenova *et al*. (2009) where the nitrogen fertilizer didn't affect the lipid peroxidation level in *Nicotiana tabacum* under Cd stress.

Antioxidants play an important role in adaptation and ultimate survival of plants under heavy metal stress conditions (Qian *et al*., 2005). One of the protective mechanisms is the enzymatic antioxidant system, which involves catalase (CAT), an enzyme which catalyses hydrogen peroxide (H_2O_2) dismutation into oxygen(O_2) and water (H_2O) (Sun and Zhou 2008)

Our results indicate that CAT activity significantly increased in shoots of plants exposed to Cd combined with PK fertilization. This may suggest that application of PK fertilizers under Cd stress may promote CAT activity. This is in agreement with the results obtained by Wang *et al*. (2009), where CAT and POD activities increased in the *E. nuttallii* treated with Cd and P. The result showed that using P can protect plants from the toxicity and oxidative stress caused by Cd.

However, in roots CAT activity increased only in presence of Cd and N. This could be explained by the higher Cd level accumulated by these plants. In fact, Cd could stimulate the antioxidative defense as found by (Wang *et al*., 2009) where Vici faba grown in a hydroponic system shows an increase of CAT activity in roots exposed to Cd. Another explanation is that the nitrogen may alleviate Cd stress through

improving antioxidant enzymes activity as reported by Jalloh *et al*. (2009) where superoxyde dismutase (SOD) and peroxydases (POX) activities increased in rice (*Oryza sativa* L.) grown in a Cd polluted soil fertilized with nitrogen. Similarly, Lyubenova *et al*. (2009) found that CAT and SOD activities were significantly increased in *N. tabacum* grown in Cd-N treated soil.

Additionally, our results indicated that CAT activity is inhibited in shoots and roots of Cd treated alfalfa, when compared to plants grown in NPK-Cd treated soil. This confirms the toxic role of the Cd in the CAT inhibition and may shed some light into the possible implication of N amendment in reducing Cd-induced toxicity in plants, thus improving phytoremediation ability of some plants.

5. Conclusion

Phytoextraction tests on pot experiment showed the efficiency of common agronomic practices where fertilization with N augmented plant biomass and increased Cd uptake from the soil. A proper N-management should be considered in case plants being cropped in Cd-polluted soils, as the metal can accumulate in edible parts of the plants above safety consumption. Moreover, alteration of CAT's activity indicates that PK supply on Cd-polluted soil may increase the antioxidant enzymes activity in alfalfa plants. These results provide novel insights about the improvement of Cd tolerance and detoxification in plants supported with nitrogen fertilization.

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