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EFFECT OF NICKEL ON GERMINATION, SEEDLING GROWTH, CHLOLOROPHYLL CONTENT AND AMYLASE ACTIVITY OF *LENS CULINARIS* L.:AN APPLICATION OF APPROPRIATE TOOL FOR ANALYSIS OF GERMINATION DATA

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ABSTRACT

Life cycle stages of plants reflect the environment to which they are subjected to varied factors including abiotic and biotic. They are often exposed to various heavy metals throughout their life span. Certain metals support their growth (usually in small amounts) whereas sometime they suppress their growth pattern. Present study examines the effect of one of the heavy metals i.e. Nickel on seed germination and early seedling growth of *Lens culinaris* L.at different concentrations. In addition, amylase activity and chlorophyll content of *Lens culinaris* was investigated during germination and seedling development. Germination data at various time intervals was analysed using Repeated Measures Design instead of commonly but incorrectly used Analysis of Variance (ANOVA) as the former is the appropriate procedure when the successive observations are dependent rather being random and independent. The results reveal that different concentrations of Ni, particularly at higher concentrations (200 and 400ppm) effectively decreased the final germination percentage, speed of germination, photosynthetic pigments (in cotyledons and leaves) and amylase activity of germinating seeds, whereas it slightly increased the amylase activity at 50 and 100 ppm. These findings are discussed in the light of metabolism during germinating seeds and early seedling growth.

Keywords: Nickel, heavy metal, germination, photosynthetic pigments, chlorophylls, carotene, amylase activity.

INTRODUCTION

Micronutrients, in general, play an eminent role in the growth of plant but beyond optimum limits they becomes highly toxic (Hopkins and Huner, 2009; Shahzad *et al*., 2018; Taiz *et al.*, 2008). Heavy metals are omnipresent in the environment, including air, water and soil, due to unabated industrial, agricultural and municipal discharges (Adrino, 1986; Seregin and Kozhevnikova, 2006). Nickel is easily absorbed in ionic form from soil solution though not so easily when it occurs in chelated form (Aschmann and Zaroski, 1987) or in conjunction with organic pollutants in soil (Khan *et al*., 1991). Ni enters the plant both by passive diffusion and active transport (van Assche and Clisters,1990). Nickel (Ni) is included in the list of essential micronutrient for plants as it is the active constituent of the enzyme urease involved in the nitrogen metabolism of many plant species and also required for glyoxalase-I activity (Hopkins and Huner, 2009; Shahzad *et al*., 2018) though in higher concentration it is phytotoxic. Ni expresses its toxicity owing to its particular physical and chemical characteristics. Similar to the behavior of many other heavy metals Ni binds to various ligands, in particular, binding to SH-group modifies protein (enzyme) conformation resulting in loss (or significant decrease) of enzyme activity (Seregin and Kozhevnikova, 2006).Since argenine and urides are the principal reserves of N-source in the seeds, Ni levels absorbed by the seeds are of great significance for N-mobilization during germination and early seedling development. On the other hand, certain plants (e.g., *Alyssum inflatum*) are known to be hyper accumulators of Ni (Ghaderian *et al*., 2007; Ghasemi*et al*., 2009), accumulating more than 1000 μg Ni g^{-1} dry weight of leaves (Vander-Ent *et al*., 2013). Gardea-Torresdey *et al*. (2005) listed some plants as hyper accumulators of Ni and can be employed for phytoremediation.

Heavy metals are invariably present in the atmosphere, as a result of both natural and anthropogenic activities, particularly owing to plants and animals (Wilson and Pyatt, 2007). The mechanism of Ni toxicity comprises of disturbance of mineral nutrition, impaired hydrological regime, suppression of photosynthesis, reduction in chlorophyll biosynthesis, reduction in leaf surface area, cell responses that detoxify the metal ions (Seregin and Kozhevvnikeva, 2006; Srekanth *et al*., 2013).

Accumulation of high concentrations of heavy metal in plants leads to the generation of reactive oxygen species (ROS) resulting in oxidative stress that arises when the balance between ROS generation and removal is disturbed (Yan *et al*., 2008). Nickel is a unique and essential heavy metal which is required at low concentration for the

germination of seed and growth of plant (Shweti and Verma, 2018) but at higher levels causes varied toxic effects similar to those of other heavy metals (Mishra and Kar, 1978; Parlak, 2016).

As pointed out above Nickel acts as an essential element in the process of nitrogen metabolism, it also contributes to the growth and germination of plant. The mechanism of Ni toxicity primarily involves disruption of plant mineral nutrition, water balance, photosynthesis and morphogenesis; also disrupting the specific cell responses that detoxify the heavy metals entering the cells and tissues (Seregin and Kozhevnikova, 2006). Ni triggers chlorosis and necrosis of leaves and releases free radicals in the environment (Halliwelland Gutteridge, 1999).Ni is widely distributed and exists in different combinations and chemicals in various soil types. It is known to occur abundantly in serpentine rocks, but their existence might be spoiled due to dumping of various industrial waste and other activities such as industrial digging, purification of Nickel ores, burning of fossil fuels while the remaining agrochemicals usually become contaminated due to exposure to Nickel ores (Lyaka, 2011). It is pertinent to mention here that normal range of Ni in soil is between 02 to 750 ppm and any increase in or uncontrolled spread of Ni damages the fertility of soil leading to necrosis in plants, downsizing the leaves and entirely damages the structural growth of plant (Lyaka,2011).

An investigation conducted by Leon *et al.,* (2005) showed that Ni hasa tendency to accumulate in living system and also exhibits strong resistance against pathogens. This unique property might be commonly present in other metals that can be enhanced if these elements are limited to a small area of the outer surface of leaves and roots. Ni has been observed in the epidermal cells of leaves of a number of plant species as a red stained nickel-dimethyl glyoxime complex (Martens and Boyd, 2002).Numerous species of *Alyssum* are known as hyper accumulator of nickel. Often such group of plants are commonly used to eliminate Ni-contamination from the soils. Nickel (Ni) is an essential element for plants (in minute concentration) that may be toxic and even carcinogenic at high level in plants (Nriagu, 1988). Further, if consumed by humans, such huge concentration of Ni can cause permanent damage to human vital systems (Srivastava *et al.,* 2005). Madhavaa nd Sresty (2000) conducted a study which demonstrated that excessive concentration of Ni in soil surely restricts the seedling growth processes, germination and disturbs the growth of plant in general.

The objectives of this study were: 1) to examine the effect of Nickel on germination and early seedling growth of *Lens culinaris* L., 2) to investigate the effect of Nickel on photosynthetic pigments, and 3) to observe the effect of various nickel concentrations on amylase activity of germinating seeds.

MATERIALS AND METHODS

Effect of different concentrations of Nickel on germination and seedling growths of *Lens culinaris*

The seeds of *Lens culinaris* L. (var, NIAB Masoor) were obtained from Pakistan Agricultural Research council Office, Karachi. Solutions of Ni $(NO_3)_2$. $6H_2O$ were prepared in deionized distilled water. The different concentrations of $Ni(NO_3)_2$. $6H_2O$ used were :0 (control) , 50, 100, 200 and 400 ppm based solely on Ni. Seed germination was examined in nine cm diameter sterile glass Petri plates using Whatman filter paper No.1. Seeds of *Lens culinaris* were washed with 1% $NaOCl_2$ solution and then with sterile distilled water and 10 seeds were placed on Whatman No. 1 filter paper fitted inside the Petri plates which also contained 5 ml of Nickel aqueous solution of a concentration or sterile distilled water for controls. The concentrations of Ni were based on the fraction of heavy metals in Nickel nitrate salt. Treatments and controls were replicated thrice. The temperature was maintained at 28 ± 1^oC during daytime (14 h) and 24 ± 1^oC during night (10h) time. In order to ensure that Petri plates do not dry out during the experiment, small amounts of respective solutions or sterile distilled water (for controls) were added.

Germination percent was recorded daily and the experiment was considered complete after one week when there was no further chance of germination. Subsequently, the length of root and shoot were measured. Seedlings were weighed and then placed in the oven at $75^oC$for 24h and the dry weights were recorded. The speedof germination was calculated as suggested by Khandakar and Bradbeer (1983):

$$S = [N1/1 + N2/2 + N3/3 \ldots \ldots .Nn/n] \times 100 /1$$

Where N1, N2, N3 . . . Nn = proportion of seeds which germinated on day 1, 2,3 . . . n following the setup of the experiment. Program GERMVEL.FOR was developed to compute the speed of germination and is available on request.

Estimation of Chlorophylls

Chlorophylls were extracted from leaves of *Lens culinaris* and its determination was performed by the method of Arnon (1949). To start with this method,1 g of leaf tissue was macerated in 10 ml of 100% acetone using a pestle and a mortar and then the mixture centrifuged at a speed of 568g for 10 minutes. Later by using a spectrophotometer (UV-Vis 1240 Shimadzu, Japan), absorbance was read at 663 and 645 nm for chlorophylls a and b respectively, 470

nm for beta carotene against 100% acetone as blank. Chlorophylls and carotene were estimated using Arnon's equation to convert the absorbance values for the pigments to mg Chlorophyll g^{-1} leaf tissue:

Chl a (mg/g) = $[(12.7 \times A_{663}) - (2.64 \times A_{645})]$ x ml acetone / mg plant tissue

Chl b (mg/g) = $[(22.9 \times A_{645}) - (4.68 \times A_{663})]$ x ml acetone / mg plant tissue

Total Chl = Chl a + Chl b

C x = $1000A_{470} - 1.90$Chl a – 63.14Chl b / 214, (x = carotenes)

Treatments and controls were replicated thrice.

Measurement of Amylase activity

Aamylase activity in germinating seeds was determined by agar-gel diffusion method developed by Clum (1967) was selected with a slightly modified process. One g of soluble starch was used as substrate and added to 20 g of agar (Oxoid No 3, BDH England) and poured in one liter boiling distilled water. Subsequently, it was stirred for 5 min. and then poured in 9 cm diam. sterile Petri plates. Each plate received about 25 ml of the agar mixture. Thereafter, an amylase extract was obtained by crushing 10 seeds (from one Petri plate) in 25 ml of chilled 0.2 N acetate buffer (pH 5.3). This was later centrifuged at a speed of 568 g for 10 min. duration. The r supernatant was made up to 49 ml with the buffer and 1 ml chloramphenicol solution (500 ppm) was added. Chloramphenicol was used to control the growth of organisms without affecting changes in metabolism of the tissue extract (Sabota *et al*., 1968). Filter paper discs (each 6 mm in diameter) were evenly placed on agar-gel plates. Each disc received 10 µl of amylase extract. The plates were incubated at 21 $\pm 1^{o}$C. They were then developed by 5% I_2 KI Sol. Clear zone diameters were taken at 48 and 72h subsequent to imbibition. Treatments and controls were replicated three times each.

Statistical Methods

The means and standard errors of mean (SE) were computed for all treatments and controls of each variable examined. The germination data was subjected to Repeated Measures Design (Howell, 2002). This is the appropriate analytical method when successive observations are dependent (the usually employed analysis of variance ANOVA is invalid in this situation). MINITAB Ver. 18 was employed for Repeated Measures Design. The data on chlorophylls a and b and carotene as well as amylase activity were subjected to analysis of variance (ANOVA) (Zar, 2008). Post-hoc test was performed using Duncan's multiple range test. However, for amylase activity Fisher's least significant test (LSD at 0.05) was also performed to test the significance of the two factors: time and concentration of Ni, following Zar (2008). For ANOVA and its follow-up, computer programs in FORTRAN were developed by the senior author (S.S.S.).

RESULTS AND DISCUSSION

Germination

In general, the percentage germination of controls and the treatments increased during the observations at various days. Thus observations are dependent and the usual analysis of variance is invalid, hence the Repeated Measures Design that is appropriate for the dependent observations was employed. Different concentrations of Nickel used during the experiment showed that the seed germination percentages of *Lens culinaris* was slightly decreased at lower concentrations (50 and 100 ppm though significantly) while at 200 and 400ppm germination percentage was significantly reduced compared to controls (p at the most 0.05)(Fig.1).The result of Repeated Measures Design showed multivariate tests Pillai's trace, Wilk's lambda and Hotelling's trace to be significant ($p<0.001$) ; between treatment test was also significant ($F=808.2$, $p<0.001$). Germination percentage, in general, increased significantly over days as evidenced by multivariate tests ($p<0.001$) (Fig. 1). The speed of germination was also consistently suppressed by the increasing concentration of Ni (Table 1). Shweti *et al.,* (2018) conducted a series of experiments in which they found the rate (velocity) as well as germination percentage was gradually decreased with higher concentrations of nickel in soil. Khan and Khan (2010) found that Ni markedly reduced seed germination, plant growth and biomass production.

Root and shoot Length

The root length of *Lens culinaris* was significantly suppressed (p at the most 0.01) at all the concentrations of Nickel compared to controls (p at the most 0.05) (Table 1).Significant reduction occurred at 50 ppm and 100 ppm as compared to control ($p < 0.05$) while at200 and 400 ppm suppression of growth was much higher relative to controls ($p < 0.001$).

The shoot length slightly increased at two lower concentrations of Nickel (50 and 100 ppm) but it wasnon-significant compared to controls. However, at Nickel concentrations of 200 and 400 ppm there was significant reduction compared to control ($p < 0.01$) (Table 1). A study stated that the fertilizers that are formulated from manure slush, mining waste and other discarded sources directly increase the contamination of heavy metals including Nickel in the soil and the outcome of leakage on these contaminated sites in turn pollutes the underwater table (Gratao *et al*., 2005) increasing Ni level. This nickel is toxic to the growth of crops as well as natural vegetation. Khan and Khan (2010) obtained significant growth reduction of gram seedlings following Ni treatment. Likewise Selvaraj (2018) found marked growth reduction in *Phaseolus mungo* L. seedlings. The results of these studies corroborate the findings of the present paper.

Fresh and Dry weights

As shown in Table 1, fresh weight of *Lens culinaris* decreased slightly but not significantly at 50 ppm Nickel compared to control. Whereas significant reduction in fresh weight occurred at 200 ppm and 400 ppm relative to the controls ($p < 0.001$).

It was observed that in Nickel concentrations of 50 and 100ppm, dry weight of *Lens culinaris* was not significantly influenced. However, significant decline in the dry weights was observed at 200 and 400 ppm (p at the most 0.01) (Table 1). Jagetiya *et al*., (2013) found significant suppression in the growth of green gram following Ni treatment. Mishra and Kar (1978) in a review also reported substantial growth reduction by high concentrations of nickel.

Table 1.Effect of concentrations of Nickel on germination and seedling growth of *Lens culinaris.*

Treatment Ni concentrations ppm	Speed of Germination	Mean root length (cm)	Mean shoot length (cm)	Mean fresh weight (g)	Mean dry weight (g)
0 Control	81.26	3.8 ± 0.41a	3.2 ± 0.26a	0.23 ± 0.02a	0.19 ± 0.015a
50	79.1	1.6 ± 0.08b	3.6 ± 0.29a	0.19 ± 0.03a	0.18 ± 0.013a
100	77.5	1.2 ± 0.16b	3.4 ± 0.30a	0.23 ± 0.01a	0.17 ± 0.013ab
200	72.5	0.7 ± 0.06c	2.4 ± 0.40b	0.14 ± 0.009b	0.14 ± 0.007c
400	65.1	0.7 ± 0.02c	2.1 ± 0.07b	0.13 ± 0.003b	0.10 ± 0.007d

Treatments in each column not sharing the same letter are significantly different at p-level of 0.05.

Chlorophylls and beta carotene

The results for the measurement of chlorophylls and beta carotene are given in Table 2. Photosynthetic pigment chlorophyll 'a' of *Lens culinaris* leaves and cotyledons decreased significantly (p at the most 0.05)(except at 50 ppm Ni) by exposure to different concentrations of nickel compared to controls.

On the other hand, photosynthetic pigment chlorophyll 'b' content of *Lens culinaris* decreased by exposure to nickel at all concentration compared to controls (Table 2). Total chlorophyll content, likewise decreased significantly at all concentrations of Ni (p at the most 0.05) relative to controls. The beta carotene content of seedlings declined significantly at all concentrations of Ni over the controls (p at the most 0.05) (Table 2). These results accord well with the findings of Jagetiya *et al*. (2013) who obtained marked decrease in chlorophylls as well as beta carotene content. Thus, due to nickel reduction in photosynthetic pigments content there is a parallel decrease in photosynthetic products such as sugars (Jagetiya *et al,*2013). Khan and Khan (2010) recorded substantial decrease in chlorophyll content following exposure to nickel. Selvaraj (2018) also reported reduction in chlorophylls and carotenoids in response to application of high Ni concentrations. The immediate and major consequence of reduction in Chlorophylls a and b and the accessory pigment beta carotene is the reduction in photosynthesis. Disruption of carbohydrate and nitrogen metabolism eventually causes reduction in free amino acids and total soluble proteins in leaf due to reduction in photosynthesis. (Selvaraj, 2018), eventually leading to growth reduction.

Table 2. Effect of treatment on chlorophyll 'a', chlorophyll 'b',total chlorophylls and beta carotene.

Treatment Ni concentrations ppm	chlorophyll 'a' mg/g	chlorophyll 'b' mg/g	Total chlorophyll mg/g	Beta Carotene mg/g
0 Control	0.265 ± 0.033a	0.120 ± 0.005a	0.385 ± 0.025a	0.42 ± 0.12a
50 ppm	0.276 ± 0.021a	0.064 ± 0.002b	0.340 ± 0.012b	0.35 ± 0.005b
100 ppm	0.209 ± 0.023b	0.074 ± 0.014bc	0.283 ± 0.013c	0.30 ± 0.004c
200 ppm	0.216 ± 0.027b	0.080 ± 0.012c	0.301 ± 0.008c	0.31 ± 0.006c
400ppm	0.200 ± 0.022b	0.074 ± 0.004d	0.276 ± 0.007d	0.32 ± 0.003c

Treatments in each column not sharing the same letter are significantly different at p-level of 0.05.

Effect ofdifferent concentration of Nickel on amylase activity

The results of the effect of Ni on Amylase activity as expressed by clear zone diameter on starch-agar plates, are given in Table 3, it is evident that amylase activity increased significantly (p at the most 0.05) from 48h to 72h during seedling growth. Further, it is shown that in *Lens culinaris*germinating seeds amylase activity was significantly (p at the most 0.05) retarded at both the observation periods (48 and 72h) at all the nickel concentrations 50 to400ppm.periodsbut was more prominently inhibited at 200 and 400 ppm compared to controls. Ashraf *et al*., (2011) also reported significant reduction in amylase activity due to various Nickel concentration in sunflower (*Helianthus annus* L.). In addition, Ashraf *et al.*,(2011) found inhibitory activity of Ni for a number of enzymes. Moreno *et al*.,(2003) measured activities of urease, phosphatase, β-glucosidase and protease-BAA in soil containing Ni concentrations, and recorded inhibition of enzymatic activities due to nickel various Ni concentrations.

Table 3. Effect of Ni treatment on amylase activity of germinating *Lens culinaris* seeds, in terms of clear zone diameter (cm). Standard errors are given against means.

Treatments Ni Conc. Ppm	Hours of imbibition 48 h	Hours of imbibition 72 h
0 Control	1.17 ± 0.125a	1.36 ± 0.091a
50	1.02 ± 0.104b	1.15 ± 0.094b
100	1.07 ± 0.094b	1.16 ± 0.096b
200	1.00 ± 0.061c	1.14 ± 0.112b
400	0.97 ± 0.042c	1.04 ± 0.018c

Treatments in columns not sharing the same letter are significantly different at p-level of 0.05.
$LSD_{0.05}$ (Time)=0.025; . $LSD_{0.05}$ (Conc.)= 0.041

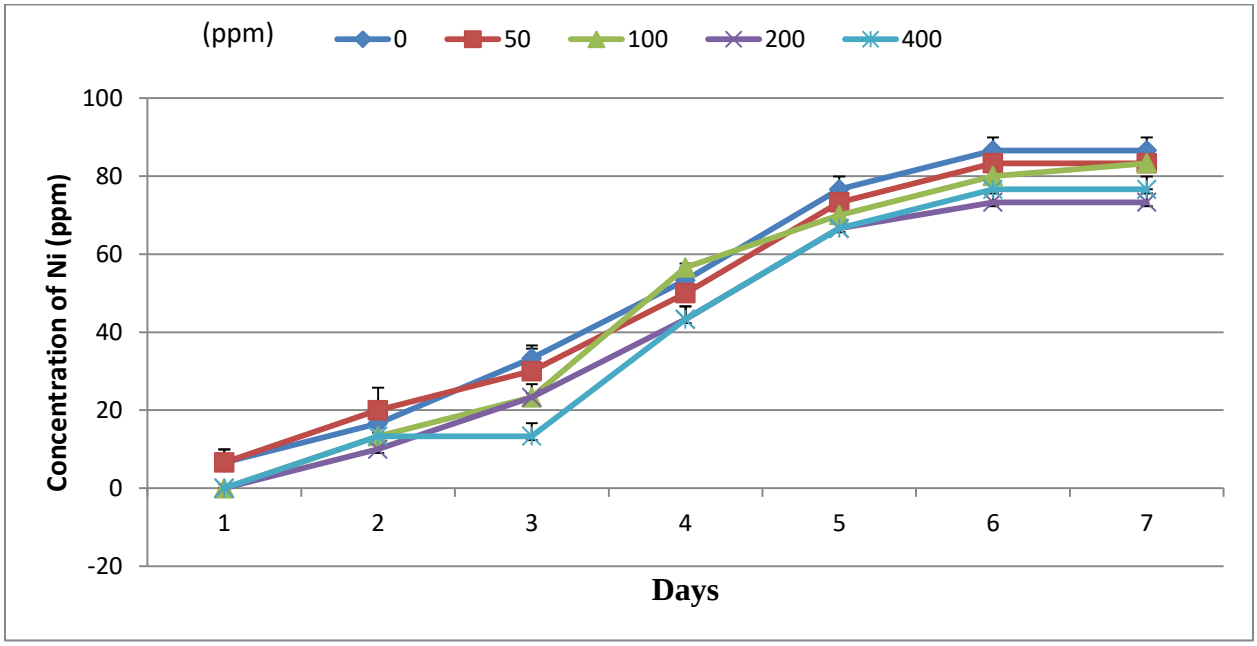

Fig.1. Progress of germination percentage with time (days) of control and Ni treated seeds of *Lens culinaris*

Conclusions

In essence, the study disclosed that Ni is toxic to plant growth and metabolism in concentrations beyond limits required as micronutrients. In this study, Ni in the range (200-400 ppm) remarkably reduced the speed and final seed germination percentage of *Lens culinaris*, adversely affected seedling development and growth, suppressed the synthesis of pigments like chlorophylls a and b and beta carotene and inhibited the amylase activity remarkably which thereby would cause reduced mobility of reducing sugars that are essential for seedling growth and development.

To analyse germination data at various Ni concentrations and time (days) the Repeated Measures Design (RMD) was employed which is an appropriate method for this type of data (Howell, 2002) where successive observations are dependent while the popularly used ANOVA is invalid in this context as its underlying assumptions are not fulfilled since the successive observations are dependent rather being independent. It is suggested that RMD should be employed in future for germination data recorded at various time intervals. RMD is provided by commonly used statistical packages and we recommend the investigators to routinely employ RMD in their research work (Keselman *et al*., 2001) wherever it is appropriate instead of the commonly used ANOVA (that may be invalid in certain situations).

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