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Nickel toxicity to photosynthetic attributes in the leaves of lentil (*Lens culnaris* Medic. Masar)

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Abstract
Nickel is an essential micronutrient for plant growth but beyond optimum limits it becomes highly toxic. The excess concentration of nickel in the soil deleteriously effect the plant metabolism particularly photosynthesis. Lentil (*Lens culinaris Medic.*) is an important legume crop. The objective of this study was to investigate the effects of nickel on main photosynthetic pigment, Hill activity and activity of key enzyme of photosynthesis i.e. Rubisco. Along with this changes in the morphological parameter related with this process were also taken into consideration i.e. number and size of stomata. In the sand culture experiment lentil plants were analyzed on the 30th day after sowing in the sand amended with various levels of Nickel (1mM, 4mM, and 6mM). Nickel at all levels tested decreased the number and size of stomata in the leaves, chlorophyll a, chlorophyll b and total chlorophyll content, Hill activity and Rubisco activity as compared to control plants.

Keywords: Lentil, *Lens culinaris*, cadmium, nickel, hill activity, Rubisco, stomata

Introduction
The amount of heavy metals in the environment is increasing continuously as a consequence of the increased environmental pollution from industrial, agricultural and municipal sources (Adrino, 1986) [1]. Nickel is one such ubiquitous transition metal which is emitted into environment from both natural and anthropogenic sources (WHO 1991). The concentration of nickel is increasing in soils by human activities such as mining works, emission of smelters, burning of coal and oil, sewage, phosphate fertilizers, bad watering pratices in agricultural land and excessive and non judicious use of pesticides (Gimeno-Gracia *et al*. 1996; Bell *et al*., 2001, Parsariello *et al*., 2002) [14, 5, 21]. Although Nickel is a heavy metal, but it is one of the essential microelement for plants, animals and humans, but toxic at higher concentrations exceeding optimum intake values. Beyond its (Seregin and Kozhevenikova, 2006; Chan *et al*. 2009) [23, 8] permissible limits Ni^{2+} in soils causes various physiological alterations and toxicity symptoms like chlorosis and necrosis in different plant species (Pandey and Sharma 2002; Madhava Rao and Sresty, 2000) [21, 20].
Heavy metals are known to cause non-specific inhibition of photosynthesis by several direct and indirect means. The slow rate of photosynthesis is related to disrupted chloroplast structure, inhibited activities of Calvin cycle enzymes and CO_2 deficiency caused by stomatal closur e (Sergin and Ivanov 2001). Excessive Ni is known to inhibit seed germination, plant growth, mitotic activities (Madhava Rao and Sresty, 2000), [20] induce chlorophyll degradation, chlorosis, and interfere with photosystems I and II activity (Leon *et al*. 2005) [18].
The purpose of the present study is to contribute to a better understanding of biochemical and physiological responses of lentil *(Lens culinaris Medic.)* subjected to nickel stress. Our work focuses on the study of the effects of different concentrations of nickel on size and number of stomata, chlorophyll content, Hill activity and enzyme Rubisco in the leaves of Ni^{2+} treated 30 days old Lentil plants grown in sand culture.

Material and Method
Seeds of lentil (*Lens culinaris var. Masar* 9-12) used for the present investigation were procurred from the market.

Seeds of uniform size and shape were selected for the study. To avoid any fungal growth during germination, seeds were surface sterilized with 0.1% mercuric chloride solution for about one minute and then washed thoroughly with distilled water. Seedlings of lentil were raised in acid free sand as supporting medium in half strength Hoagland nutrient medium supplied with increasing concentrations of $NiCl_2$ (1, 4 and 6mM) (Hoagland and Arnon, 1983) [16] in polythene bags under field conditions in the month of November. 2 kg sand was taken in each polythene bag and three replicates were maintained for each treatment. The Hoagland solution was supplied at 15 days interval. Analysis of different biochemical components was studied in 30 days old leaves.

Extraction and Estimation of Chlorophyll

Chlorophyll in leaves was estimated by Anderson and Boardman (1964) [2] of sand cultured plants at 30 days. 100mg of leaves were homogenised in 5ml of 80 percent acetone in a pestle and mortar. The extract was centrifuged at 3000 rpm for 10 min. The supernatant was retained and the residue again crushed in 3ml of 80 percent acetone. The two supernatants were pooled and the final volume was adjusted to 10ml with acetone. The absorbance was recorded at 645 nm and 663 nm.

$$\text{Chlorophyll a} = a = \left[12.7(A_{663}) - 2.69(A_{645})\right] \frac{V}{1000 \times W}$$

$$\text{Chlorophyll b} = b = \left[22.9(A_{645}) - 4.86(A_{663})\right] \frac{V}{1000 \times W}$$

$$\text{Total Chlorophyll} = \left[20.2(A_{645}) + 8.02(A_{663})\right] \frac{V}{1000 \times W}$$

Where,

V= total volume of extract (ml)

W= Fresh weight of sample (g)

A_{663} = Absorbance at 663 nm

A_{645} = Absorbance at 645 nm

The chlorophyll content was expressed as mg $Chlg^{-1}FW$.

Estimation of Hill Activity

The Hill activity in leaves of 30 days stage was estimated by Larer and Bar-Akiva (1979) [19] method. For preparation of chloroplasts, 200mg leaf segments were macerated in 5ml of Hepes grinding medium (Cockburn *et al*., 1968). The suspension was filtered and centrifuged at 2000 rpm for 5min. The supernatant was collected and centrifuged at 1500rpm for 30 min. The precipitation was dissolved in 5ml of Hepes suspension medium.

To assay Hill activity, 0.2ml of chloroplast suspension and 5ml of 2,6-di-chlorophenol-indophenol (dissolved in phosphate buffer, pH 6.5 and diluted appropriately to absorbance nearly 0.5) were mixed. Changes in absorbance were recorded at 620 nm before and after illumination of the reaction mixture for five minute. Chlorophyll determination from the chloroplast suspension was done by acetone extraction according to Arnon (1949) [3] method. Hill activity was expressed as change in absorbance mg^{-1} total chlorophyll min^{-1}.

Stomatal frequency and Size

The stomatal size and frequency were measured from the lower surface of 30 days old control and Ni treated leaves. The leaf epidermis was separated with the help of forceps and strips were mounted in glycerine. The frequency and size of stomata was recorded at 400 magnification under the microscope. The size of stomata as measured with the help of an ocular micrometer. Ten counts were made from each segment. The area of stomata was calculated by multiplying its length with breadth and then with 0.785 (i.e. a common factor meant for elliptical structure).

Assay of enzyme Ribulose-1,5-bisphosophate Carboxylase (RuBP)

The method of Downston and Slatyer (1971) [11] was used. Reaction mixture for this enzyme in a total volume of 3ml contained bicine 30 µmoles, $MgCl_2$ 30 mmoles. 2-mercaptoethanol 15 µmoles; $NaHCO_3$ 150 µmoles, ATP 10 µmoles; NADP 0.25 µmoles; Glycerofaldehyde phosphate dehydrogenase 4 units. After adjusting the pH of reaction mixture to 8.7 the reaction was initiated by adding RuBP 0.2 µmoles and change in O.D. was followed at 340 nm at 30 seconds intervals up to a period of 3 min. The enzyme activity was expressed as µmoles min^{-1} mg^{-1} protein.

Results:

Chlorophyll content: Increasing concentrations of Nickel decreased the total chlorophyll content in leaves (Table 1). The suppression by 1mm and 6mm level of Nickel was 15 and 30 percent as compared to control. With nickel treatment not much change was observed in content of Chl a, but the chlorophyll b content decreased significantly. The ratio of Chl a/Chl b also affected. Compared to control Chl a/b ratio increased with increasing concentration of Nickel.

Table 1: Effect of Nickel on the Chlorophyll Content ($mg.g^{-1}$ FW) and Hill Activity (Δ O.D. min^{-1} mg^{-1} FW) in the leaves of lentil at 30 Days

Treatment	Chla	Chlb	Total Chl.	Chla/b	Hill Activity
Control	0.717	0.818	1.535	0.876	0.144
1mM Ni	0.723	0.578	1.301	1.250	0.117
4mM Ni	0.702	0.421	1.123	1.660	0.091
6mM Ni	0.687	0.380	1.067	1.800	0.070

Number and Size of Stomata:

The number and size of stomata were recorded on the leaves of 30 day old plants in a fixed microscopic area (Table 2). Different treatments with Nickel reduced the number as well as size of stomata. At l and 4mm leaves of Nickel the number of stomata decreased by 29 and 41 percent respectively. Stomatal size also reduced by 10 and 24 percent in 1mm and 4mm concentration respectively.

Table 2: Effect of Nickel on Number and Size of Stomata in Leaves of lentil at 30 days after sowing

Treatment	No. of Stomata/unit microscopic area	Stomatal area (μm^2)
Control	17.00 ± 0.91	48.47 ± 1.06
1mMni	12.00 ± 0.62*	43.52 ± 0.98*
4mMni	10.00 ± 0.68*	36.41 ± 0.84*

* significant ($P \leq 0.05$)

Hill Activity:

Nickel treatment inhibited the Hill activity in the leaves (Table 2). At lower concentration of Ni (1mm) the inhibition in Hill activity was 18 percent and at higher concentration of Nickel (6mm) decrease in Hill activity was up to 50 percent.

Ribulose bis-phosphate carboxylase (RuBPc)

Activity of RuBP carboxylase decreased significantly with increasing concentrations of nickel. With 1 and 4mM Ni the percent inhibition was 40 and 70% respectively as compared to control.

Table 3: Effect of different concentrations of Nickel on the activity of Ribulose-bis-Phosphate carboxylase in the leaves of 30 days old lentil leaves

	mole/min/mg Protein	
Control	17.00 ± 0.91	% inhibition
1mMni	0.287	40
4mMni	0.14	70

Discussion

Heavy metals are known to cause non-specific inhibition of photosynthesis by several direct or indirect means. The reduced rate of photosynthesis by heavy metals is because of disruption of chloroplast structure, blocked chlorophyll synthesis, inhibition of calvin cycle enzymes and CO_2 deficiency caused by stomatal closure (Seregin and Ivanov 2001).

The reduction in amount of chlorophyll affects the Hill reaction. We have observed that Ni^{2+} treated leaves show less Hill activity as compared to control plants. Our results are in agreement with Fateham *et al*. (2012) [13] who reported that rate of Hill reaction as an ability of chlorophyll a in the reaction centre of $PSII_{680}$ to split water decreased by increasing Ni^{2+} concentration in Maize.

Our results also indicate that the leaves of Ni^{2+} treated plants had reduced amount of chlorophyll. The content of chlorophyll b was more reduced which resulted in an increase in chl a/chl b ratio. Baszynski *et al*. (1980) [4] have shown that heavy metal Cadmium also lowered the chlorophyll content in tomato plants before affecting photosynthesis. A reduction in chlorophyll content in the leaves of Ni^{2+} treated plants has been observed by (Ewais, 1997; Piccini and Malavolta, 1992; Leon *et al*. 2005). Our results are in agreement with the earlier studies revealing reduction in chlorophyll content in the leaves of Ni^{+2} treated plants.

The photosynthetic efficiency of leaves is also known to decrease as a result of deceased rate of CO_2 diffusion due to changes in stomatal conductance. Carlson *et al*. (1975) reported a direct effect of heavy metals on in vitro stomatal regulation. Inhibition of photosynthesis also arises from reductions in stomatal size and number in leaves of Nickel stressed plants that limit plant CO_2 uptake (Sheroran *et al*. 1999; Bishnoi et al. 1993) [6]. We have same observations in 30 day old lentil leaves treated with Ni^{2+}. The decreased in stomatal area might be responsible for reduced carbon dioxide exchange.

Heavy metals are also reported to inhibit photosynthesis by exerting their effect at the level of dark reaction (Weigel, 1985). Activity pattern of RuBP carboxylase enzyme in Nickel treated leaves have shown that there was drastic inhibition of activity of this enzyme. Same reduction in the activity of Rubisco has been observed by Sheoran *et al*. (1999) in C. *Cajan* leaves. The inhibition in Calvin cycle reaction would lead to accumulation of ATP and NADPH, thus blocks PSII and inhibiting overall photosynthesis (Krupa and Baszynski, 1995) [17].

Though plants cannot complete their life cycle without adequate supply of this metal, excess of Ni toxicity is illustrated by inhibition of photosynthesis, mineral nutrition, enzymatic activities (Sreekanth *et al*., 2013).

Conclusion

Though nickel is an essential metal and plays an important role in plant metabolism, but its toxicity becomes a particular apprehension, due to growing industrial use. The results of present study have shown that nickel treatment was inhibitory to chlorophyll content, number of and size of stomata Hill activity and the activity of enzyme RuBP carboxylase. The decreased chlorophyll content of lentil leaves might be due to active involvement of Ni in chlorophyll biosynthesis. In addition, the decrease in number of stomata per unit area as well as stomatal size observed in lentil leaves with Ni decreased the rate of CO_2 diffusion and thus resulted in decrease in Hill activity. Ni also inhibited activity of key enzyme of dark reaction i.e. RuBP carboxylase activity is reduced so overall photosynthesis get reduced by Ni treatment.

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