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ROLE OF BIOFERTILIZERS IN ACCUMULATION OF NICKEL IN SUNFLOWER (*HELIANTHUS ANNUUS* L.) AND THE EFFECT OF NICKEL ACCUMULATION ON ENDOGENOUS LEVEL OF HORMONES IN SUNFLOWER

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ABSTRACT:

The objective of the study was to determine the effect of Biofertilizers i.e. Phosphate solublizing bacteria (PSB), Effective Microorganism (EM) and *Rhizobium* in the uptake and accumulation of nickel by different parts of sunflower (*Helianthus annuus* L.). The results revealed that all biofertilizers has increased accumulation of nickel significantly as compare to control during both stages. At different concentrations of nickel that were applied externally to the plants, different fertilizers show different behavior. At lowest external concentration of nickel *Rhizobium* treated plants has maximum accumulation while at highest external concentration of PSB and EM treated plants accumulate maximum nickel. Translocation of nickel in treated plants was also increased as compare to control plants during vegetative stage of development while insignificant during 50% flowering stage. Accumulation of nickel was also increased as the external concentration increased. In addition, effect of nickel accumulation on Gibberellins (GA) and Indole-acetic acid (IAA) contents were also observed in leaves of sunflower at vegetative stage. Both GA and IAA contents were affected by nickel accumulation.

INTRODUCTION:

Toxic substances and heavy metals released from different industries are major sources of environmental pollution. Heavy metals are added in soil by various anthropogenic activities, most common of which are agricultural practices, sewage sludge and industrial effluents. Three aquatic plants such as parrot feather (*Myriophylhum aquaticum*), creeping primrose (*Ludwigina palustris*) and water mint (*Mentha aquatic*) were examined for their ability to remove heavy metals from contaminated water. The plants were obtained from a Solar Aquatic System treating municipal wastewater. All the three plants were able to remove Fe, Zn, Cu, and Hg from the contaminated water. The average removal efficiency for the three plant species was 99.8%, 76.7%, 41.62%, and 33.9% of Hg, Fe, Cu, and Zn, respectively (Kamala, *et. al.,* 2004). The excessive amount of nickel has highly toxic effects, including

dermatitis and respiratory disorders, inhalation may cause lung cancer. It also inhibits the enzyme activity that includes cytochrome oxidase, isocitrate dehydrogenase and maleic dehydrogenase (Mido, *and Satake* 1995).

Phytoremediation is an emerging clean up technology with low tech. and low cost for remediation of contaminated soils, ground water and wastewater. It not only provides low cost technology for clean up soil but also offer containment of leachates, maintenance and improvement of soil structure, fertility and biodiversity (Cunninghan *et. al*., 1995). Sunflower is one of the plant species that can be used in phytoremediation due to its tolerance to heavy metals and also due to its rapid growth and greater biomass production (Salt *et. al*., 1995). Various factors effects accumulation of heavy metals in plants one of them is microbes in rhizosphere. Microbes

in the rhizosphere play vital role in phytoremediation processes (Sorrensom, 1997). Microorganisms can affect trace metals mobility and availability to the plant, they can produce iron chelators and siderophores for ensuring iron availability, reduce soil pH and solubilize metal phosphates (Barber and Lee, 1974; Crowley, *et. al.*, 1991).

Heavy metal accumulation affects various physiological processes in plants. One of them is change in the endogenous level of phytohormones. Phytohormones mediate many diverse plant processes. Within a plant single hormone can regulate many processes and at the same time different hormones can influence a single process. GA plant hormones regulate important processes in the life cycle of plants, like seedling development, plant height and crop yield (Hooley, 1994; Hedden and Kamiya, 1997; Monna, *et. al*., 2002). Plant hormones decreases the toxic effect of heavy metals, as in gibberellic acid treated seeds, toxic effects of barium were reduced while germination and growth of maize seedling were not effected (Iqbal and Ijaz, 2002).

The aims of this study were to investigate the relative performance of plants and microbes for nickel accumulation and to monitor the stage specific effect as well as the pattern of distribution of nickel in plants. In addition changes in the endogenous level of growth promoting hormones viz. Gibberellins and Auxins were determined in the treated plants as a mechanism of controlling plant growth.

MATERIALS AND METHODS:

Sunflower (*Helianthus annuus* L.) seeds (Parsun-1) were obtained from National Agriculture Research Center, Islamabad, Pakistan. Seeds were germinated in earthen pots (30 × 40 cm) having soil contained sand, soil and cow dung (1:1:1) along with chemical fertilizers i.e. Diammonium phosphate and urea. After a week seeds were germinated and at stage of five leaves stage thinning was performed and selected one plant per pot. Plants were watered daily. At five-leaf stage biofertilizers (PSB, *Rhizobium* and EM Bokashi) were applied to the plants and after a week of this treatment different concentrations of nickel were applied to the plants instead of water and given to plants whenever there was a need of water. Accumulation of nickel was determined at two stages i.e. 65 days after treatment of nickel and 80 days after treatment of nickel while the endogenous level of hormones was determined after 65 days of treatment of nickel. Experiment was a randomized complete block designed performed in green house, three replicate plants were used for each treatment in all experiments. Statistical analyses were performed using M-stat program, analysis of variance were performed to see the significant effect of these treatment and DMRT was performed to distinguish difference among different treatments.

Biofertilizers Treatments

Broth culture for PSB strain, 54RB and *Rhizobium leguminosarum* strain, TAL-102 were prepared. Seven days old broth culture of both PSB and *Rhizobium* were applied to the plants at 10 ml /plant.

EM Bokashi

Bokashi was prepared, following method described by Yamada and Xu in 2000. The mixture of 1% EM, 1% molasses, organic matter (rice bran) and water was added until it became 30% moist. It was then left to ferment for one week. A pleasant sweet sour smell indicated the completion of process. EM Bokashi was added to each pot at the rate of 1.9gm at sowing time.

TREATMENTS:

Following treatments were given during the experiment.

C (Control)- Nickel (0.04ppm) applied to soil

PSB- Nickel (0.04ppm) + PSB applied to soil

EM- Nickel (0.04ppm) + EM Bokashi applied to soil; R- Nickel (0.04ppm) + *Rhizobium* applied to soil; C (Control)- Nickel (0.6ppm) applied to soil; PSB- Nickel (0.6ppm) + PSB applied to soil; EM- Nickel (0.6ppm) + EM Bokashi applied to soil; R- Nickel (0.6ppm) + *Rhizobium* applied to soil; C (Control)- Nickel (0.7ppm) applied to soil; PSB-Nickel (0.7ppm) + PSB applied to soil; EM-Nickel (0.7ppm) +EM Bokashi applied to soil; R-Nickel (0.7ppm) + *Rhizobium* applied to soil. **Soil Analysis:** Soils were analyzed for availability of different metal i.e. Mn, Ni, Cr, Zn, Ca, by ammonium acetate EDTA method (Cottenie, *et. al.,* 1982). The soil extract was then analyzed by Shimadzu AA-670 Atomic absorption/flame spectrophotometer.

The extraction from plant tissues was carried out by Perchloric acid digestion method (Allen, 1974). Metal concentration was determined by Shimadzu AA-670 Atomic absorption/flame spectrophotometer.

Hormone Analysis: Hormones were extracted and purified from fresh leaves of sunflower following Kettner and Doerffling, (1995) procedure. The plant leaves (1g) were ground in 80% methanol at 4 ^{0}C with an antioxidant, butylated hydroxyl toluene (BHT). The leaf was extracted at 4 ^{0}C for 72 hr with subsequent change of solvent. The extracted sample was centrifuged and the supernatant was reduced to aqueous phase using rotary thin film evaporator. The pH of aqueous phase was adjusted to 2.5-3.0 and extracted four times with ½ volume of ethyl acetate. The ethyl acetate was dried completely using rotary thin film evaporator (RFE). The dried sample was re-dissolved in 1ml of methanol (100%) and was analyzed on HPLC using UV detector and C-18 column. For identification of hormones, samples filtered through 0.45 millipore filters and were injected into column. Pure IAA and GA were used as standard for identification and quantification of plant hormones. These growth hormones are identified on the basis of retention time and peak area of the standards. Methanol-acetic acid-water (30:1: 70v/v/v) were used as a mobile phase. The wavelength used for the detection of IAA was 280nm (Sarwar *et. al.,* 1992), where as for GA analysis it was detected at 254nm (Li et al., 1994).

RESULTS

Total Accumulation and role of Biofertilizers: In case of lowest concentration (0.04ppm) of nickel, it has been observed that *Rhizobium* has increased total accumulation of nickel in plants significantly during both stages that is 65 days after treatment and 85 days after treatment while non-significant results were obtained in case of PSB and EM at this concentration. At concentration of 0.6 ppm, after 65 days of treatment only significant results were obtained in PSB treated plants while after 80 days of treatment, all microbes treated plant has showed significant results but most significant results were obtained in PSB treated plants. In case of highest concentration of nickel i.e. at 0.7ppm, both EM and PSB treated plants has significantly high accumulation as compare to control irrespective to the number of days of treatment (Table 1).

Distribution in different parts: Bio-fertilizers has increased the translocation of nickel significantly during vegetative stage. At this stage, when 0.04ppm concentration was given, nickel was concentrated in stem whereas roots showed the least accumulation. PSB treatment has no effect but EM treatment significantly decreased the accumulation of nickel in stem and treatment with *Rhizobium* resulted in the increased in translocation of Ni showing significantly higher concentration in stem and leaves (Table 2). With increase in nickel concentration (0.6ppm), leaves showed maximum accumulation irrespective to various treatments. In roots there was no significant difference among various treatments except PSB where root showed decrease in accumulation, as translocation in this case was maximum showing significant high concentration

in stem as well as in leaves (Table 2). At highest external concentration of Ni, translocation has increased and leaves showed maximum accumulation as compared to stem and roots. PSB and EM treated plants showed maximum translocation as maximum accumulation of nickel was observed in leaves (Table 2).

At reproductive phase i.e. at 50% flowering stage, nickel was mostly concentrated in roots irrespective to external concentrations of nickel. At concentration of 0.04ppm, *Rhizobium* treatment showed significantly high accumulation of nickel in roots while PSB showed significant decreased in accumulation in roots. As for as translocation is concern it has been observed that leaves of fertilizer treated plants has high nickel concentration as compare to control (Table 3). At concentration of 0.6ppm, accumulation was significantly high in roots of all microbial treated plants while leaves showed minimum accumulation (Table 3). At the highest external concentration i.e. 0.7ppm of nickel, roots showed maximum while leaves has minimum accumulation, irrespective to different treatments (Table 3).

Effect of nickel accumulation on endogenous level of phytohormones: At concentration of 0.04 ppm of nickel GA contents increased significantly in all microbial treated plants while highest increase was observed in *Rhizobium* treated plants where accumulation was also maximum (Table 4). At concentration of 0.6ppm of nickel GA contents was increased in PSB and *Rhizobium* treated plants while significant decrease in GA contents were observed in EM treated plants (Table 4) where accumulation was also less (Table 3). At external concentration of 0.7ppm GA contents significantly increased in all microbial treated plants but most significant increased was observed in PSB treated plants along with maximum nickel accumulation (Table 4). Results also showed that at concentration of 0.7 ppm of nickel when accumulation was increase significant decrease in GA contents were observed as compare to 0.6ppm of nickel treatment where the accumulation was less as compare to this treatment (Table 4).

In case of endogenous level of IAA it has been observed that IAA contents has also increased in all microbial treated plants as compare to control while EM treated plants showed highest level of IAA contents at external concentration of 0.04ppm of nickel (Table 4). At concentration of 0.6ppm of nickel, same results were obtained while at highest external concentration (0.7ppm) of nickel PSB and *Rhizobium* showed non-significant results as compare to control, only significant increased in IAA contents were observed in EM treated plants (Table 4).

DISCUSSION:

Effect of Ni concentration on accumulation in plant: It is inferred from the present findings that accumulation of nickel increased linearly with increase in the concentration of nickel in soil both at vegetative and at 50% flowering stage. The increase in accumulation with increase of concentration in soil was in accordance with previous literature that showed increase in nickel accumulation in plant as the concentration of nickel increased in hydroponic culture (Robinson, *et. al*., 2003).

Role of biofertilizers in Ni accumulation: All biofertilizers at different concentrations effect positively towards accumulation of nickel in plants. These results are in accordance with reports indicating the positive role of microbes in nickel uptake (Abou-Shanab, *et. al.*, 2003). Sadowsky, *et. al.*, (1999) reported that rhizosphere bacteria growing in polluted soil play vital role in phytoremediation.

Phosphate solublizing bacteria has increased accumulation significantly at both high concentrations during two stages that might be due to its positive effect plant growth that increase

surface area of roots, which ultimately increased accumulation in plants. These results were supported by the previous findings that showed that *Microbacterium arabinogalactanolyticum*, which is an acid producer as well as phosphate solubilizer, significantly increase the accumulation of nickel by *Alyssum murale* (Abou-Shanab *et al.*, 2003).

The results revealed that in case of EM treated plants during both stages significant increase was observed at higher concentrations but not at high concentration that might be due to accumulation of nickel in EM itself. These results were supported by the previous findings that showed that the heavy metal concentration was reduced in soil after EM-treatment (Akbar, 1996).

Rhizobium significantly increases the accumulation just at low concentrations but not at high concentrations of nickel, which showed that at high concentration of nickel, *Rhizobium* not able to survive. As a consequence of this, heavy metals had negative effect on growth and indole-acetic acid production by the *Rhizobium* sp. particularly at high concentration (Rabindanath, 1999).

Distribution in different parts of plant: It is inferred from the present results that the accumulation in various parts of plant differed. These results were supported by the previous workers that plant organs might significantly differ in the accumulation of heavy metals (Santos, *et al.*, 2002). Nickel accumulation has increased in leaves as concentration was increased in soil during vegetative (65 DAT) stage while decrease in accumulation was observed in leaves at 50% flowering (80 DAT) stage showed that the translocation was high during vegetative stage as compare to reproductive stage. Robinson *et. al*., (2003) revealed that the leaves were the primary sink for nickel accumulation in *Berkheya coddii*. Roots showed significantly less accumulation during vegetative phase while accumulation of nickel increase in roots at reproductive stage. Oda and Kawasaki (2001) reported that the accumulation of cadmium was maximum in leaves and stem during vegetative stage, while at reproductive stage accumulation was decreased in stem and leaves, as it was translocated to seeds.

Effect of nickel accumulation and microbes on GA and IAA contents: Results revealed that the GA and IAA contents in leaves during vegetative phase were affected due to accumulation of nickel as well as in leaves of microbes treated plants showed that these hormones might be detoxify the toxic effects of heavy metal. Accumulation and treatment of microbe are interlinked that affects level of phytohormones as microorganisms increase the accumulation of nickel, which effects level of hormones in plants. These are favored by previous findings that the ethylene biosynthesis was affected due to high cadmium concentration (Pezzarossa, *et. al.*, 1991). Plant hormones play vital role in stress conditions, abscisic acid function in conserving water by reducing water loss, in reducing the rate of plant growth and in mediating adaptive responses (Liu *et. al.*, 2003). At concentration of 0.7 ppm of nickel significant decrease in GA contents as compare to 0.6 ppm of external concentration of nickel showed slight toxic effect of nickel accumulation, which has decreased the endogenous level of GA contents. Previous findings also revealed that unfavorable environmental factors lead to sharp changes in the balance of phytohormones associated with not only accumulation of ABA, but also with a decline in the level of growth activating hormones such as IAA and cytokinins (Zholkevich and Pustovoytova, 1993).

CONCLUSION:

- Biofertilizers has ability to increase accumulation of nickel in sunflower.
- Translocation of nickel can be increased by the use of biofertilizers.
- Both the metal concentration and microbial treatment interlinked with the change in endogenous contents of GA and IAA.

Table:- 1 DMRT of mean showing total accumulation of nickel in sunflower

Treatments	Nickel Concentrations					
	0.04ppm		0.6ppm		0.7ppm	
	65 DAT	80 DAT	65 DAT	80 DAT	65 DAT	80 DAT
Control	63.00	70.63	66.83	72.10	78.50	78.20
PSB	64.30	70.70	75.37	88.13	83.37	96.40
EM	57.00	69.00	67.33	85.20	84.60	94.53
R	70.33	76.60	70.33	81.63	80.00	92.20

All such mean values under a category which share a common letter in same column are insignificantly different, otherwise they differ at $p<0.05$

*EM= Effective microorganisms, PSB= Phosphate solublising bacteria, R= *Rhizobium*

Table 2 DMRT of mean showing distribution of nickel (µg/g) in different parts of sunflower treated with different nickel concentrations

Treatments	Nickel Concentrations (65 Days)								
	0.04ppm			0.6ppm			0.7ppm		
	L	S	R	L	S	R	L	S	R
Control	19.0	36.0	10.7	40.7	12.3	14.0	40.5	8.00	29.9
PSB	18.0	36.7	9.77	52.2	18.7	4.86	53.5	7.00	22.7
EM	20.0	25.3	11.7	44.2	6.33	16.8	54.0	9.53	21.3
R	25.0	38.3	6.73	43.3	11.0	16.7	40.0	10.0	30.0

Table 3: DMRT of mean showing distribution of nickel (µg/g) in different parts of sunflower treated with different nickel concentrations

Treatments	Nickel Concentrations (80 Days)								
	0.04ppm			0.6ppm			0.7ppm		
	L	S	R	L	S	R	L	S	R
Control	11.2	27.2	32.6	14.9	25.5	32.0	19.1	26.2	33.0
PSB	16.4	24.1	30.3	14.9	35.5	38.1	25.7	30.1	40.9
EM	14.9	22.2	32.2	20.8	28.2	36.5	25.3	31.3	38.2
R	15.6	26.2	35.0	18.0	28.5	35.4	25.0	30.6	36.6

Table 4: DMRT shows the endogenous level of GA and IAA contents in leaves (µg/g) when applied different concentrations of nickel

Treatments	GA/IAA contents at different nickel concentrations					
	0.04ppm		0.6ppm		0.7ppm	
	GA	IAA	GA	IAA	GA	IAA
Control	8.687	2.902	13.77	3.078	9.996	3.047
PSB	12.44	3.417	18.78	3.588	12.04	3.200
EM	12.65	4.124	13.30	4.148	11.58	3.488
R	16.42	3.262	15.84	3.588	10.24	3.085

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