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Determining the Effectiveness of Mycorrhizal Inoculation on Increasing the Resistance of Vetch against Different Doses of Nickel

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ABSRACT

In the present study, the effect of different doses of Ni application on certain growth parameters and nickel accumulation was investigated in vetch (*Vicia sativa*) plant. In the study, which was carried out as a pot experiment under greenhouse conditions, Ni was applied to vetch plant at doses of 0-25-50 mg Ni kg^{-1}. In the study, it was also investigated whether mycorrhizal inoculation, which is known to be effective in increasing the resistance of plants against heavy metal toxicity, had an effect on increasing the resistance of vetch.For this purpose, *Glomus mosseae* type mycorrhiza in the form of mycorrhiza (+) and mycorrhiza (-) was inoculated as 500 spores per pot during sowing. In the experiment that was carried out under controlled conditions in sand culture, Hoagland solution was regularly given to the plants. Plant height measurements were taken for 4 weeks during the experiment. Furthermore, chlorophyll content, plant shoot and root weight (g), single root weight (g), root length (m g^{-1} root), leaf relative water content (%), the amount of CO_2 released from the growth medium, inoculation rate in the plant roots and hypha (%), vesicle and arbuscle numbers were determined in the plants that were harvested at the beginning of flowering. According to the variance analyses conducted on the data obtained as the result of the study, it was found out that mycorrhizal inoculation did not have an effect on the given parameters, nickel negatively affected plant development but this state was not found to be statistically significant.

1. Introduction

Vesicular-arbuscular mycorrhiza fungi are associated with the majority at the terrestrial plants (Quilambo 2003).Heavy metals(HM) are among the most toxic inorganic substances which have contaminated large area of land due to use of sludge, pesticides, fertilizers, and emissions from municipal waste incinerators, car exhausts, residues from metalliferous mines, and smelting industries. Optimum concentrations of the some metal ions such as Cu, Zn, Fe, Mn and Ni are taking part in redox reactions, electron transfers, a multitude of enzyme catalyzed reactions in various cellular metabolism. But the toxic concentration of the some essential metals or the nonessential ions of Cd, Hg, Pb, Ag, As, Al, etc. are strongly poisonous to metal-sensitive enzymes, resulting in growth inhibition and death of the organism (Upadhyaya et al. 2010).

Mycorrhiza is the mutualistic symbiotic association (non-pathogenic) of a specific group of soil-borne fungi with the roots of higher plants (Sieverding 1991). Plant receives support from AM fungi, with the help of its symbiotic association, in the aspect of uptake of phosphorus and other nutrients, enhancement of growth hormones, increase of protein content, increase of lipid, sugars, amino acid levels, increase of tolerance to heavy metals, increase of salinity tolerance, and resistance to root-borne pathogens (Salt et al. 1995). Mycorrhizae have also been reported in plants growing on HM-contaminated sites indicating that these fungi have evolved a HM-tolerance and that they may play a role in the phytoremediation of the site.

Phytoremediation, a sustainable and inexpensive technology based on the removal of pollutants including heavy metal from the environment by plants. However, phytoremediation is a slow process (Ouziad et al. 2005).

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AM fungi can also act as a filtration barrier against transfer of heavy metals to plant shoots. The protection and enhanced capability of uptake of minerals result in greater biomass production, which is an important criteria for successful remediation. Indigenous AM isolates existing naturally in heavy metal-polluted soils are more tolerant than isolates from nonpolluted soils, and are reported to efficiently colonize plant roots in heavy metal-stressed environments (Hrishikesh et al. 2010).

It will be important in future to include the AM symbiosis in the design of both research plans and applications, with the ultimate goal of increasing the efficiency of phytoremediation. Galli et al. (1994) suggested that mycorrhizae can play a crucial role in protecting plants roots from heavy metals. Various authors have reported isolating spores of arbuscular mycorrhizal fungal texa such as *Glomus* and *Gigaspora* associated with most of the plants growing in heavy metal polluted habitats (Raman et al. 1993; Raman and Sambandan 1998; Chaudry et al. 1999). Weissenhorn and Leyval (1995) isolated only *Glomus mosseae* and Duek et al. (1986) isolated *Glomus fasciculatum* alone from the heavy metal polluted soils. Pawlowska et al. (1996) surveyed a calamine spoil mound rich in Cd, Pb and Zn in Poland and recovered spores of *Glomus aggregatum, G. fasciculatum* and *Entrophospora* spp. from the mycorrhizospheres of the plants growing on spoil.

Nickel hyperaccumulation was found by Minguzzi and Vergano (1948) in *Alyssum bertolonii* from serpentine soils in Italy, with up to 10,000 µg Ni/g DW (1%). Shallari (1997) was investigated the availability and the potential of hyper accumulator *Alyssum murale* to extract nickel from pools in the soil that are not available to plant. The occurrence of arbuscular mycorrhiza (AM) in nickel-(Ni)-hyperaccumulating plants of the Asteraceae family growing on Ni-enriched ultramafic soils in South Africa was surveyed. All plants were found to be consistently colonised by AM fungi, with the abundant formation of arbuscules. Inoculation of *B. coddii* with *Glomus intraradices* (BEG) was successful, but only plants with abundantly developed arbuscules showed increased yield. In other cases, shoot biomass was similar to noninoculated plants (Turnau et al. 2003).

Jamal et al. (2002) indicate that mycorrhize can be used as effective tools to supply sufficient Zn in generally Zn-deficient Pakistani soils and to ameliorate the toxicity of trace metals in polluted soils. The contents of Ni in mycorrhizal soybean plant tissues were higher than those in the mycorrhizal lentil plant tissues. Ker and Charest (2010) have reported that the AM colonization significantly increased the glutamine synthetase (GS) activity in roots, this being likely an indicator of an enhanced Ni tolerance. These findings support the hypothesis that AM symbiosis contributes to an enhanced Ni plant uptake and tolerance and should be considered as part of phytoremediation strategies.

Vivas *et al.* (2006) were researched co-inoculation of *Trifolium repens* L. (white clover) with an indigenous Ni-adapted AM strain of *Glomus mosseae* and a Ni-adapted bacterium (*Brevibacillus* sp.). These results suggest that selected bacterial inoculation improved the mycorrhizal benefit in nutrients uptake and in decreasing Ni toxicity.

It is primarily necessary to select the suitable types of plants in order to decontaminate heavy metal-polluted soils through phytoremediation. Furthermore, as mentioned in several studies, certain types of mycorrhizae help plants to develop resistance against heavy metal toxicity. Taking all these into consideration, the effect of *G.mosseae* spore type mycorrhizal inoculation on the germination, growth and development of vetch (*Vicia sativa*) plants in different doses Ni were investigated in the present study.

2. Material and Methods

2.1. Media preparation

In this study sand was used as growing media. According to the randomized plots experimental design with three replications. After weighing the sand and filling the pots, the pots were inoculated with mycorrhiza spores at a depth of 5 cm from the surface. Ni was applied to vetch plant at doses of 0-25-50 mg Ni kg^{-1}.Experiment were set up using vetch seed (*Vicia sativa* L.; Tamkoc-2000 type) in a pot experiment under greenhouse conditions.During the period of plant growing the plants was watered with Hoagland solution. The general analysis of the sand was carried by KLD Analytical Laboratories (Table 1).

Table 1
The analysis results of sand media used in the experiment

Sand Properties	
pH (1:2.5)	7.46
EC (1:5 µS cm^{-1})	479
Organic matter (%)	0.125
$CaCO_3$ (%)	10.05
Ca (mg kg^{-1})	4185
P (mg kg^{-1})	3.00
Na (mg kg^{-1})	45.08
K_2O (meq 100 g^{-1})	0.174
Mg (mg kg^{-1})	216.7
Fe (mg kg^{-1})	5.87
Cu (mg kg^{-1})	0.345
Mn (mg kg^{-1})	8.677
Zn (mg kg^{-1})	0.216

2.2. Mycorrhizal inoculation and glasshouse experiment

The *Glomus mosseae* type mycorrhiza spore used in the experiment. The added of *Mycorrhiza* spores into the each of the pots as 50 spore 10 gr $soil^{-1}$.

2.3. Plant height (cm):

Each plant was measured from the soil surface to top of the plant.

2.4. Plant weight and root weight (g):

All plants harvested, cleaned and cut from the roots were weigthed. The avarege value was found by taking the arithmetic mean.

2.5. Root lengt (m g^{-1})

In order to estimating the total root length we have used grind intercepts methods and using Tennant's formula (Tennant 1965).

2.6. Leaf relative water content (RWC) (%)

The content was determined according to Barrs and Weatherley (1962).

2.7. Chlorophyll content

A chlorophyll meter SPAD-502 also popularly known as spadmeter was used (Minolta Corporation, Japan).

2.8.The rate of soil respiration (mgCO_2 100g soil 24h^{-1})

Soil respiration was measured as CO2 evolution after 24 h of incubation period at 25°C, in darkness, with moisture content adjusted at 50% of water holding capacity, according to Isermeyer (1952). Data were expressed as mg CO_2 100 g^{-1} dry soil.

2.9. Determination of VAM infection and counting spor

After harvesting, roots were collected and root samples were washed carefully with deionizer water, and then preserved in a mixture of ethanol, glacial acetic acid and formalin with a proportion of 92:2:6 (v/v). Root clearing and staining was done according to the method described by Koske and Gemma (1989). Entire, cleared with KOH (10%) and stained with Trypan blue (0.05%) with the lactophenol being changed to lactoglycerol. Naturally dark pigmented roots were cleared with 10% hydrogen peroxide for one hour before the staining with Trypan blue.

The percentage of root colonization was calculated by the gridline intersect method and, when the amount of roots was low, by the slide method. The percentage of AM colonisation was calculated as the number of segments infected out of 100 segments that were examined under a stereo microscope at 40X magnification (Giovannetti and Mosse 1980).

2.10. Spore extraction and quantification

After harvesting each soil sample (10 g fresh mass) in pots was sieved according to the sieving and decanting procedure of Gerdemann and Nicolson (1963). The sieving was centrifuged at 3500 rpm (10 g) for 10 min. The pellet was resuspended in 50% sucrose and centrifuged again at 3500 rpm (10 g) for 1 min. After the centrifuging process, the supernatant was poured on to a 50-100 µm sieve, washed thoroughly with deionized water and then placed in a 9 cm petri-dish for examination under a stereomicroscope (x40).Spores and debris were collected on 50-100 µm sieves with tap water. Some spores were tightly grouped in sporocarps and it was difficult to count the number of spores per sporocarps, so, to simplify the procedure, we referred to a sporocarp as one spore (Zhao et. al. 2003).

2.11. Sterilization

The sand were used as growing media was autoclaved for sterilization at 121°C during 120 min. Also vetch seed was sterilized with NaClO 0.1 %.

2.12. Statistical analysis

The data obtained through the measurements were statistically analyzed using Minitab and Mstat software.

3. Results and Discussions

The present study was conducted in order to examine the effects of applications with and without mycorrhiza inoculation with three different levels of Ni on common vetch plants in sand media. In the study, significant differences were observed both between the VAM(+) and VAM(-) applications and among Ni levels regarding plant height, leaf chlorophyll content, plant shoot weight, root weight, root heigh, leaf relative water content, rate of soil respiration, mycorrhizal vesicule-arbuscule and hypha rates in plant roots ($P<0.01$ and $P<0.05$). The applications of VAM(+) and VAM(-) and different levels of Ni didn't effected soil respiration rate of (mg CO_2 100g soil 24 h^{-1}) growing vetch plant(Table 2,3a,3b).

Mycorrhizal vesicule rate, root heigh, plant shoot weight, root weight and leaf relative water content of vetch increased in the third of Ni doses and second (+VAM) growth medium compared to the other doses and media.The first doses of Ni(Ni_0) was more effective on mycorrhizal hypha rates, leaf chlorophyll contents and single root weight than other Ni doses,Nevertheless these parameters were found to be +VAM higher than -VAM .

The highest value of medium plant height (29.94 cm) and soil respiration rate (3.46 mg CO_2 100g soil 24 h^{-1}) in vetch were obtained at second dose of Ni. The highest values of root height were obtained at Ni_3 doses and second media (-VAM), respectively 508.38 m g^{-1} and 464.63 m g^{-1}.Also it can be seen that the highest values in vetch were generally obtained in the first media (+VAM) and third of Ni doses (Table 2).

Table 2

The effect of mycorrhizae and different Ni doses on the growth of vetch

Doses of Ni (mg Ni kg^{-1})	Vesicule rate (%)	Arbuscule rate (%)	Hypha rate (%)	Root length (m g^{-1})
Ni_0 (0)	11.12	0	6.67	388.34
Ni_1 (25)	10.01	0	0.00	389.89
Ni_2 (50)	13.34	0	0.00	508.38
Growing media				
VAM+	22.97	0	4.45	393.11
VAM-	0	0	0.00	464.63
Ni applications	ns	Ns	Ns	ns
Mycorrrhiza	**	Ns	Ns	ns
Ni*Mycorrrhiza Interaction	ns	Ns	Ns	ns

Table 3a

The effect of mycorrhiza and different Ni doses on some plant properties of vetch

Doses of Ni (mg Ni kg^{-1})	Plant length I	Plant length II	Plant length III	Plant length IV	Leaf chlor. Content	Plant shoot weight (g)
Ni_0 (0)	22.86	27.58	33.06	33.50	11.05	1.77 b
Ni_1(25)	23.56	28.34	33.09	34.78	9.52	1.75 b
Ni_2(50)	23.42	28.39	32.58	34.00	9.13	2.10 a
LSD P<0.05	ns	ns	ns	ns	ns	*
Growing media						
VAM+	23.50	27.48	32.22	34.11	11.54	1.93
VAM-	23.05	28.72	33.59	34.07	8.26	1.82

Doses of Ni (mg Ni kg^{-1})	Root weight (g)	Single Root weight (g)	Root length (m/g)	Leaf relative water content (%)	Soil respiration rate (mg CO_2 100g soil 24 h^{-1})
Ni_0 (0)	1.78	0.26	63.50	75.35	2.02
Ni_1(25)	1.82	0.13	47.00	75.21	3.46
Ni_2(50)	2.14	0.11	54.17	77.06	2.97
LSD P<0.05	ns	ns	ns	ns	ns
Growing media					
VAM+	1.98	0.20	54.33	77.18	3.42
VAM-	1.84	0.13	55.44	74.57	2.21

Table 3b

The effect of mycorrhiza and different Ni doses on some plant properties of vetch

Mycorrhiza	Doses of Ni	Leaf chl. content	Plant shoot weight (g)	Root weight (g)	Single Root weight (g)	Root length (m/g)
VAM+	Ni_0	12.59	1.92	1.96	0.36	60.33
	Ni_1	11.47	1.68	1.71	0.11	47.67
	Ni_2	10.57	2.18	2.28	0.13	55.00
LSD P<0.05		ns	*	*	ns	ns
VAM-	Ni_0	9.51	1.61	1.60	0.16	66.67
	Ni_1	7.57	1.82	1.92	0.14	46.33
	Ni_2	7.70	2.02	1.99	0.10	53.33
LSD P<0.05		ns	ns	ns	ns	ns

Mycorrhiza	Doses of Ni	Leaf relative water content (%)	Soil resp. rate ($mgCO_2$100g soil 24 h^{-1})	Vesic. rate (%)	Arb. rate (%)	Hypha rate (%)
VAM+	Ni_0	79.01	2.61	22.23	0	13.34
	Ni_1	77.53	3.22	20.01	0	0
	Ni_2	75.00	4.43	26.68	0	0
LSD P<0.05		ns	ns	ns	ns	ns
VAM-	Ni_0	71.69	1.42	22.23	0	0
	Ni_1	72.90	3.70	20.01	0	0
	Ni_2	79.12	1.51	26.68	0	0
LSD P<0.05		ns	ns	ns	ns	ns

The highest values for the third of Ni doses and in the first media were obtained (the number of mycorrhizal vesicule rate %13.34, plant height 2.10 g, shoot weight 2.14 g and leaf relative water content %77.06). As you can be seen that the highest plant parameters (except plant high) in this study were obtained from VAM + application and Ni_2 dose(Table 3a,3b).

Therefore, we can be recommended that this plant can be used for phytoremediators of Ni stress in contaminated soils. Although the mechanisms that enable mycorrhizal plants to tolerate of heavy metal toxicity are not well known, it seems that the mycorrhizal fungus Glomus mossea decreases the translocation of Ni to various structures of the plant. Thereby at the tolerance mecanism of vetch inoculated with *Glomus mosseae* toward Ni toxicity can be increase.

Plant stress caused by excessive concentration of heavy metals is alleviated by mycorrhizal fungi (Rivera-Becerril et al. 2002, Shen et al. 2006). Mycorrhizal fungi may increase the tolerance of plants to heavy metals through the following mechanisms: immobilization of heavy metals by compounds secreted by the fungus, precipitation in polyphosphate granules in the soil, adsorption in fungal cell walls, and chelating of metals inside the fungus (Gother and Paszkowski 2006).

In this study is striking aspected that rate of vesicule, arbuscule and hyphe were differently effected on Ni contaminated. For example; the highest rate of mycorrhizal vesicule (13.34%) was obtained by the highest dose of nickel and also in the other doses were obtained rate of mycorrhizal vesicule (in first dose was obtained 11.12%, in the second dose was obtained 10.01%). Unlike the mycorrhizal arbuscule didn't obtain (0%) any of the doses of Nickel. The formations of hyphae were obtained only the first dose (6.67%) of Nickel. Because, arbuscular mycorrhizal fungi propagate by spores, hyphae, and colonized root fragment, and the relative importance of these different propagules varies depending on the environmental conditions (Smith and Read 1997).

In addition to this may be due to higher electrical conductivity in contaminated soils resulting in greater availability of soluble metals, leading to increase AM fungalmediated uptake by plants (Raju et al. 1990; Liao et al. 2003), and the effect of heavy metals on mycorrhizal occurrence and infectivity seems to be more of a function of the available rather than total content in the soil (El-Kherbawy et al. 1989).

It was found that mycorrhizal inoculation was more successful in contaminated soil with Ni. Becauase at Ni_3 dose has been obtained the highest infection rate of mycorrhiza.

Furthermore, no sign of toxicity was observed in vetch plant. More detailed further studies are needed on vetch to investigate the use of this plant in decontaminating with Ni polluted soils through VAM inoculation.

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