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Phytoremediation potential of nickel by *Cyperus rotundus* along with its rhizospheric fungi

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The interaction of *Cyperus rotundus* (obtained from the fields of TIFR, Mumbai) and its pathogen *Dreschlera* sp. was analyzed in Nickel contaminated water. Nickel is one of the most commonly found heavy metals in contaminated water and soil. The nickel phytoremediation ability of *Cyperus rotundus* which is maximum at 14mg/l and decreased from 16mg/l, was analysed spectrophotometrically (1.814 and 1.564 respectively at 445nm) and by atomic absorption spectroscopy, was found to be enhanced in the presence of its fungal pathogen *Dreschlera* sp. Infection by *Dreschlera* sp. spores was found to cause a rapid increase in rate of *Cyperus* root and shoot formation and hence increase its phytoremediation potential. This increase appeared to be more on infection of the plant with fungal spores than the fungal exotoxin. The phytoremediation potential of the plant in its place of inhabitation near nickel contaminated water bodies was analyzed and compared to that in standard ion containing Knop's solution and in ion free water. In all the cases, infection by the *Dreschlera* sp. spores and even by the fungal exotoxin caused a marked increase in the phytoremediation capacity of the plant (2.328 in 14mg/l nickel contaminated water in *Dreschlera* infected plant). Methanolic extract of the plant was screened for its total phenolic and flavonoid content which was enhanced upon infection with *Dreschlera* sp. The IC50 value of the *Dreschlera* infected plant extract was found to be more that of the uninfected plant. The variation in the antioxidant property of the infected and uninfected plant in the presence of nickel was analyzed. Results indicated that the remarkable antioxidant property in the presence of 12mg/l nickel (0.382 at 517nm) was comparable to that of Gallic acid which is a standard antioxidant (0.442 at 517nm). The MIC of the methanolic plant extract was analysed against a range of Gram positive and Gram negative bacteria such as *Staphylococcus aureus* and *Escherichia coli* respectively. Further, the antifungal activity of the plant extract was analyzed and it was observed that the antibacterial and antifungal property against fungal pathogens other than *Dreschlera* sp. appeared to be enhanced in case of *Dreschlera* sp. infected *Cyperus* samples. These truly reflect a multi-dynamic consequence of the fungal infestation in *Cyperus rotundus*.

Key words: *Cyperus, Dreschlera,* Knop's solution, nickel, phytoremediation

INTRODUCTION

Due to rapid industrialization and urbanization, the pollution of the environment with toxic heavy metals has increased at an alarming rate, which is a matter of grave concern. The major factors contributing towards such a change can be includes both natural and anthropogenic causes (Subhashini and Swamy, 2014a,b). Various methods are presently being used to overcome this problem but most of them are costly and sometimes do not even result in optimum performance. Developing an economically effective and environmentally friendly techniques and methods for the remediation of soils and wastewaters polluted with such toxic heavy metals is a subject of interest on a global scale (Yadav and Chandra, 2011). Nickel (Ni) is among the various trace heavy metals that pollute the environment. It generally contaminates water bodies and soil. Moreover, the occurrence of Ni in the soil causes various environmental problems such as contamination of water bodies, decrease in crop production and subsequent crop damage and serious health hazards to animals and humans. An integral part of this bioremediation technology includes Phytoremediation. The process takes advantage of the unique and selective uptake capabilities of plant root systems, together with translocation and bioaccumulation abilities of the entire plant body. It is a technique that utilizes the naturally occurring processes by which plants,

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along with their microbial rhizosphere organisms, sequester such pollutants and help to purify the contaminated soil and water ((Subhashini and Swamy, 2013).

The effectiveness of phytoremediation depends greatly on the availability of specific plant species which are able to tolerate and accumulate high concentrations of heavy metals. Leaves of *Cyperus rotundus* were found to accumulate Ni in roots, shoots and leaves (Sivapalan, 2013).

Cyperus rotundus (Family-Cyperaceae), also known as purple nutsedge or nutgrass, is a common perennial weed which can grow upto an average height of about 100cm. The stems grow to about 25 cm tall and the leaves are linear, dark green and grooved on the upper surface. Inflorescences are small, with 2-6 bracts, consisting of tiny flowers with a red-brown husk. *C.rotundus* is native to India, but is now found in tropical, subtropical and temperate regions. The plant grows in abundance on wetlands. The phytoremediation ability of *Cyperus rotundus* was further enhanced by the infection of the fungi *Dreschlera* sp. Primarily, Ni is taken up by the plants by passive diffusion and active transport. Ni, once absorbed by the plant roots, is easily carried to shoots and can also accumulate in the leaves which can be spectrophotometrically determined. It is of immense importance to use the native and naturally occurring plants of the contaminated site for phytoremediation because these plants are better adopted in terms of survival and growth under various environmental stresses than those that are introduced from other environment (Mc Groth,1988; Burken *et al.*2001; Chang and Corapcioglu,2001;Srivastava *et al.* 2014).

MATERIALS AND METHODS

Physical characterisation of the plant sample

The plant leaf was taken and the physical characterization of the plant was carried out on parameters of: Wet weight, dry weight, length, breadth, perimeter, total area of the leaf, area of infected portion, % of infection, EC, DO, pH, temperature (Table 1).

Identification of the plant pathogen

From the infected leaf spot, the plant pathogen isolated was characterized using Lacto phenol cotton blue staining post the growth observation in Nutrient Agar and CzapekDox Agar media. No gram staining was done as no bacterial colonies were observed in the Nutrient Agar media (Table 2).

Estimation of tolerance of Cyperus rotundus to Nickel

The estimation of the tolerance of *Cyperus rotundus* to Nickel was done in a hydroponic solution (Knop's solution). The plants were grown in pots containing the nutrient solution (Knop's solution). The uptake was estimated for 7 days at a broad metal range. In addition to these, control blank sets of pots were also maintained. The tolerance optimum was further found out using a narrower range of Nickel (Table 3).

Effect of fungal toxin on the root and shoot of the plant in presence of different metal concentrations

The effect of the fungal toxin on the root and shoot of *Cyperus rotundus* was assessed by allowing the fungal toxin to infest the plants. Thereafter which, the plants were subjected to growth in Knop's solution under different metal concentrations.

Analysis of Nickel tolerance by Dreschlera sp.

The fungal spores were grown in CzapekDox broth in the presence of different Nickel concentrations. The fresh and the dry weight of the fungal biomass were then calculated after 10 days of growth.

Analysis of phytoremediation potential of the plant when kept under different growth conditions

[Key: Experimentally induced infected: An infection of *Dreschlera* sp. was allowed on the healthy plant, where after which there was a development of the infection spot on the plant, which is in accordance with the Koch's postulate.]

The phytoremediation ability of the plant was assessed when the plant (Healthy, experimentally induced infected, fungal toxin infected) was kept under different growth conditions of Distilled water, Knop's solution, pond water (its natural place of inhabitation) and allowing the plant to grow under different concentrations of Nickel. The Nickel

acquired by the plant was assessed spectrophotometrically at 445 nm.

A spectrophotometric analysis was performed to study the Nickel uptake by the healthy and induced infected plant under varying concentrations of Nickel. The main reagents used were Boric acid, Dimethyl glyoxime and Potassium persulfate. 25g of the plant root/shoot sample was mascerated in 25ml of conc. HNO3. To it, 2.5ml of EDTA was added. To this mixture, 5ml of boric acid was added and the mixture was put in a separating funnel. Now, 1ml of DMG and 5ml of hexane were added and the funnel was shaken well for about 2 minutes. The mixture was then allowed to stand for 2 minutes. The middle layer of the heterogeneous mixture was separated and collected in a sterile test tube. To it, 2ml DMG, 0.5ml of Potassium persulfate and 1.25ml of ammonia were added in order. A blank set was also maintained that contained all the reagents except for the plant sample. The absorbance of the solution was recorded at 445m.

Analysis of retention of the heavy metal during phytoremediation

To demarcate that the heavy metal Nickel is being absorbed by the plant species (healthy and the experimentally induced infected) and is not merely adsorbed onto the surface of the plant, the plants were grown in Knop's solution with the heavy metal for a period of 7 days. Thereafter the plant was removed from the solution and placed in fresh Knop's solution without the heavy metal and kept for two days. The amount of heavy metal which would pass on to the fresh solution was then assessed using spectrophotometric analysis.

Analysis of antioxidant activity of the plant during Dreschlera sp. infection

A set of experiment was carried out to study the antioxidant property of the healthy plant and the experimentally induced infected plant in the presence of the heavy metal against a standard antioxidant (Gallic acid). For this study, the plant samples were first grown in various metal concentrations (12mg/l, 14mg/l and 16mg/l). 2g of the plant root sample was taken and 2ml of methanol was added to it. After 20 minutes, the samples were finely crushed and grinded with the help of a mortar and a pestle. The mixture was filtered and the filtrate was obtained in sterile test tubes. To 2ml of filtrate, 1ml of DPPH was added and the test tubes were kept in dark as DPPH is light sensitive. A positive control was made that contained 1ml DPPH and 2ml methanol. A negative control was prepared that contained 1ml of DPPH and 2ml of Gallic acid. A blank containing only 2ml methanol was also made. All the test tubes were wrapped with aluminum foil or kept in dark and allowed to stand for 10mins. Then the absorbance of the solutions was recorded at 517 nm.

Analysis of antimicrobial activity of the methanolic plant extract

The MIC of the methanolic plant extracts was analysed against a range of Gram positive and Gram negative bacteria. The Gram positive bacteria selected were *Bacillus subtilis* and *Staphylococcus aureus*. The Gram negative bacteria selected were *Escherichia coli* and *Pseudomonas aeruginosa*. Further, the antifungal activity of the plant extracts obtained from the healthy and the experimentally induced infected plant was analyzed against fungal pathogens other than *Dreschlera* sp. The fungal pathogens selected for the study were *Aspergillus* spp and *Alternaria* spp. The inhibitory action on bacteria was observed in the form of a zone of inhibition on the bacterial lawns around the place where the plant extract was applied. The fungal inhibition on the other hand was assessed using the measurement of the weight of fungal mycelia in CzapekDox Broth media in the presence of healthy and *Dreschlera* sp. infested plant extract.

Analysis of polyphenolic and the flavonoid content of the healthy and fungal infected plant

Test for polyphenolic content

100µl of the plant extract was taken in a test tube with the help of micropipette. To it, previously prepared 2ml of 2% sodium carbonate solution was added and kept at incubation for 2 minutes at room temperature. Then to it 100µl of 50% of Folin-Ciocalteau's reagent was added and kept for 30 minutes incubation at room temperature. The color of the solution changed to blue. The blank was prepared by giving water in place of sample extract in the test tube and the rest process is same. The result was observed spectrophotometrically at 720nm.

Test for flavonoid content

50µl of plant extract was taken in a test tube and to it 2450µl of methanol was given with the micropipette so that the sample is 2.5ml in total. To it 150µl of 5% sodium nitrite solution was given and kept for 5 minutes incubation at room temperature. Then to it 1.5ml of 10% aluminum chloride solution was given and kept for 6 minutes incubation at room temperature. The blank was prepared by using water in place of sample in the test tube and the similar steps were repeated thereafter. The results were obtained after spectrophotometric observation at 510nm.

RESULTS AND DISCUSSION

Evaluating the tolerance of nickel by *Cyperus rotundus*, it was observed that concentrations upto 16mg/l can be tolerated by the plant for at least 5 days. A high phytoremediation potential of *Cyperus rotundus* was noted for nickel concentration of 14mg/l. However, the phytoremidiation capacity appeared to be enhanced during infection by the fungal pathogen *Dreschlera* sp. Infection by the spore suspension of *Dreschlera* sp. caused a greater increase in the phytoremediation potential

Table 1: Physical parameters of plant characterisation

Parameters	Observation
Dry weight	0.12g
Wet weight	1.250g
Length	17cm
Breadth	0.9cm
Perimeter	35cm (approx.)
Total area of the leaf	10.44 sq cm
Area of infected portion	6.786 sq cm
% of infection	65%
EC	15.2µS and 40.3µS
DO	9.1ppm and 8.1ppm
pH	5.5
Temperature	19.8°C

of the plant than the fungal toxin. *Dreschlera* sp. infection at the roots of the plant appeared to cause a sudden increase in the root and shoot lengths of the plant and even led to the appearance of new shootlets. This probably led to the greater uptake of nickel by the plant thus increasing its phytoremidiation potential.When the plant was infected by fungal toxin, there was a very minute increase in its phytoremediation potential. This is because the toxin only caused an overall damage

Table 2: Fungal spore characteristics

Fungal isolate	*Drechslera* sp.
Observation of the spore	Isolated, thick walled, incomplete appearing septa (pseudosepta), smooth, cylindrical and elliptical.

to the plant tissues but was not able to stimulate further root and shoot development which would have increased its phytoremediation potential (Table 4 ,5,6,7).

Knop's solution appeared to be most effective hydroponic liquid media for growth of *Cyperus* during phytoremidiation. However, when the phytoremidiation potential assay was carried out in the wet lands at its actual site of inhabitation there was a marked increase in the

Table 3: Key : Estimation of the tolerance of *Cyperus rotundus* to Nickel

+	Plant remains healthy
-	Plant dried
X	Plant died

Table 4: Plant tolerance to Nickel over a period of 7 days

	Metal concentration					
Day of observation	Control	1mg/l	5mg/l	10mg/l	20mg/l	30mg/l
Day 1	+	+	+	+	+	+
Day 2	+	+	+	+	+	-
Day 3	+	+	+	+	+	-
Day 4	+	+	+	+	+	X
Day 5	+	+	+	+	+	X
Day 6	+	+	+	+	-	X
Day 7	+	+	+	-	X	X

Since the plant appears to show tolerance between concentrations of 10mg/l to 20 mg/l, the range was narrowed to find the optimum concentration for metal tolerance.

Table 5: Tolerance of the plant to a narrow range of Nickel concentration

	Metal concentration				
Day of observation	Control	12mg/l	14mg/l	16mg/l	18mg/l
Day 1	+	+	+	+	+
Day 2	+	+	+	+	+
Day 3	+	+	+	+	+
Day 4	+	+	+	+	+
Day 5	+	+	+	+	+
Day 6	+	+	+	-	-
Day 7	+	+	+	-	X

Table 6: The effect of fungal toxin on the root and shoot of the plant at different metal concentration

100µl fungal toxin+ Knop's (50ml)+Ni(conc.)	Initial root length(cm)	Root length change after 7 days(cm)	Other changes observed
12	4.0	5.5	New growth of a 1.5 cm shoot
14	8.5	9.5	New root:3.2cm New shoot:1 cm
16	7.0	7.5	New root: 7cm New shoot:4cm
18	5.0	7.6	New root: 5.4cm New shoot: 5.5cm

Table 7: Fungal biomass of *Dreschlera* sp.

Nickel concentration (mg/l)	Wet weight of biomass (gm) after 10 days	Dry weight of biomass (gm)
Control (0mg/l)	3.5286	3.2678
12	3.0568	2.8970
14	2.1534	2.0897
16	0.8970	0.6788
18	0.2378	0.1788

Table 8: Phytoremediation ability of the plant at different metal concentrations in Knop's solution

Metal concentration (mg/l)	Phytoremidiation potential of the plant(in terms of OD 445)in the presence of:		
	Only Plant	*Dreschlera* sp. spore suspension infected plant	*Dreschlera* sp. toxin infected plant
12	1.6290	1.8770	1.6990
14	1.8977	1.7654	1.9245
16	1.5644	1.3786	1.6670

Table 9: Phytoremediation ability of the plant at different metal concentrations in distilled water(laboratory experimental set up)

Metal concentration (mg/l)	Phytoremidiation potential of the plant(in terms of OD 445)in the presence of:		
	Only plant	*Dreschlera* sp.spore suspension infected Plant	*Dreschlera* sp. toxin infected plant
12	1.4522	1.5280	1.4978
14	1.4899	1.8790	1.5288
16	1.0677	1.4677	1.0320

Table 10: Phytoremediation ability of the plant at different metal concentrations in wet land pond water (actual site of inhabitation)

Metal concentration (mg/l)	Phytoremediation potential of the plant(in terms of OD 445)in the presence of:		
	Only plant	Dreschlera s.p spore suspension infected plant	Dreschlera sp . toxin infected plant
12	1.2356	2.0978	1.3278
14	1.5699	2.6955	2.3428
16	1.0267	1.3658	1.0780

Table 11: Retention of Nickel during phytoremediation in the presence and absence of the fungi

Metal concentration (mg/l)	Retention potential of the plant (grown in Knop's solution) in the presence of:	
	Only plant	*Dreschlera* sp. spore suspension infected plant
12	1.547	1.767
14	1.880	1.977
16	1.421	1.343

Table 12: Anti-oxidant activity of the plant due to *Dreschlera* sp.infection

Sample	Antioxidant activity	Antioxidant activity+ *Dreschlera* sp. infection	IC50 values Healthy plant	With *Dreschlera* infection
Plant	0.323	0.283	62.04	66.74
Plant + Nickel (12mg/l)	0.208	0.184	75.55	78.37
Plant + Nickel (14mg/l)	0.369	0.264	56.66	68.97
Plant + Nickel (16mg/l)	0.765	0.588	10.10	30.90

Positive control: 0.851 (DPPH+Methanol) Negative control: 0.142 (DPPH+ Gallic acid). IC50=100 x $(A_c-A_s)/A_c$
Where A_c=absorbance of the positive control A_s=absorbance of sample

Table 13: Flavonoid and polyphenolic content of the plant samples

Polyphenolic content of PE	Polyphenolic content of experimentally induced infected PE	Flavonoid content of PE	Flavonoid content of experimentally induced infected PE
1.0456	1.8796	0.7688	0.9877

Table 14: Antimicrobial activity of the plant extracts due to *Dreschlera* sp. infection

Fungal sample	Fungal biomass in the presence of plant extract	Fungal biomass in the presence of plant extract with a *Dreschlera* sp. infection
Aspergillus spp.	1.0122	3.5755
Alternaria spp.	1.2100	2.8435
Penicillium spp.	1.1211	2.6150

Table 15: Anti-bacterial activity of the plant extracts

Bacteria	Diameter of zone of inhibition i of plant extract (mm)	Diameter of zone of inhibition in the presence of plant extract and *Dreschlera* sp.
Escherichia coli	12.5	14.7
Bacillus subtilis	13.3	15.0
Staphylococcus aureus	15.1	15.9
Pseudomonas aeruginosa	14.8	15.5

phytoremidiation potential on *Dreschlera* sp. infection. This indicates that in real setups when the plant grows in wetlands and is infested by *Dreschlera* sp. which is one of the most common pathogens of *Cyperus rotundus*, there is an appreciable increase in its phytoremidiation potential, even more than in laboratory experimental setups containing Knop's solution and distilled water. However, uptake of metal by increased root and shoot lengths does not always justify phytoremidiation potential of any plant. Several plants show remarkable metal uptake which remain accumulated near the root tips in contact with the medium only to be released into the medium later. So the retention of nickel after uptake in the roots of the plant was assayed. Maximum retention was observed at 14mg/l after which the retention potential appeared to decrease. This maybe due to the fact that concentrations of 16mg/l and above appears to be toxic for the plant tissues thus hampering their retention ability (Fig.1;Table 8,9,10,11).

The dynamic nature of *Dreschlera* sp. infection on *Cyperus rotundus* was also indicated by the enhancement of the antioxidant potential of *Cyperus rotundus*. The presence of nickel also appeared to contribute to the enhancement in the free radical generating potential of the plant, particularly at a concentration of 14mg/l.The increase in the flavonoid and polyphenolic content of *Cyperus rotundus* during infection by *Dreschlera* sp. was noted. This was in accordance to the results of the previous experiment which indicate an increase in the antioxidant potential of the plant as flavonoids and polyphenols are natural antioxidants of plants. As a defence mechanism against Dreschlera sp. infection, the plant produces significant amounts of polypehnolics and flavonoids thus making it a potent storehouse of antioxidants.Further, it was

noted in *Cyperus rotundus*, that as a defence mechanism to *Dreschlera* sp. infection, certain antimicrobial compounds are generated, which, if exposed to a low density of other microbes, have the ability to eliminate those microbes. The microbes used under study were the fungal *Aspergillus* spp., *Alternaria* spp. and *Penicillium* spp., the Gram positive *Staphylococcus aureus* and *Pseudomonas aeruginosa* and the Gram negative *Bacillus subtilis* and *E.coli*. This was established when the *Dreschlera* sp. infected plant samples were used in a disc diffusion experiment and the zone of inhibition was compared to that when the plant extract was used alone. Thus, it can be said that *Dreschlera* sp. infected *Cyperus rotundus* can be used to control the initial stages of infection of the above organisms(Table 12,13,14,15).

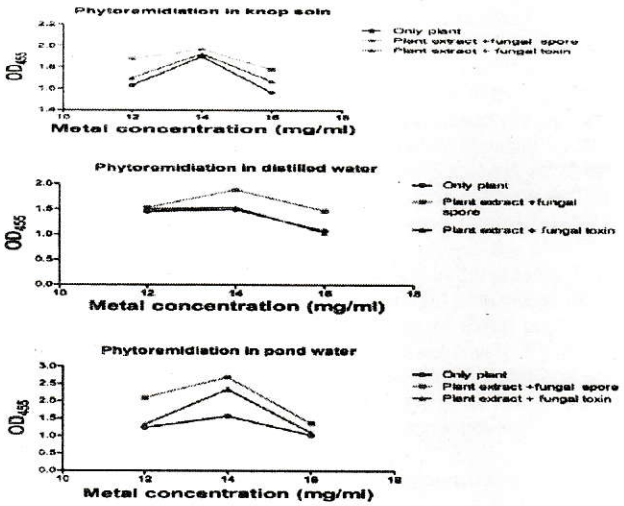

Fig. 1 : Growth of new root and shoots in the plant in the presence of fungal toxin

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