Isolation of Cd tolerant fungi and their role in plant growth promotion.

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Abstract

Heavy metal contaminated soil is a serious environmental concern that has a negative impact on a agriculture and ecosystem.This study was conducted to investigate the tolerance of some resistant fungal strains from soils contaminated with heavy metals. Fungi genera including, *Aspergillus* spp., *Penicilliuum* spp. and *Curvularia* spp. resistant to heavy metal cd were isolated after screening soil samples from Tapi river(Surat, Gujarat).The objective of soil sample screening was to investigate the status of heavy metal Cd and to identify the heavy metal tolerant fungi and also their role in plant growth promotion(PGP). The results were revealed that all three isolates were resistant to Cd.Among the isolated fungi, *Curvularia* spp. was the most tolerant against Cd with 0.96mg/ml concentration, which makes them attractive potential candidates as bioremediation agent.One of the interesting features of present study is that the heavy metal tolerant fungi showed promising plant growth promoting properties.

Key words: 1.PGP 2.fungi 3.cadmium 4.tolerance.

Introduction

Metal resistance is distinct as the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to metal species concerned. Heavy metals are indicated to be harmful pollutants in soil negatively affecting the species composition and function of the indigenous microorganisms, including fungi. Heavy metal can exert harmful effects in many ways, depending on environmental factors and metal species (Shazia Iram *et al*., 2013). Metals can variously influence soil fungi by changing fungal morphology and physiological activity and affect the growth rate, reproduction process, enzyme production etc. (Levinskaite L., 2001). Heavy metal pollution of water and soil is one of the great consequences of industrialization in the sector of mining, petroleum refining, automobiles, paints etc. (Iram *et al*., 2012)

In the spite of such ecosystem uniqueness of the Tapi river it is least explored for studying of heavy metal tolerant fungi for the plants adapted to this eco system. Fungi caused asymptomatic infection in living plant tissue and are widely studied for Plant Growth Promoting (PGP) traits they possess (Ahmad *et al*., 2008). The term PGPR was introduced by Kloepper and colleagues in 1978. Cultivable rhizobacterial isolates can be screened for obtaining potent PGPR by assessing their PGP traits using *in vitro* methods. A hypothesis for such a screening involves biochemical estimation for phosphate solubilization, Indole 3 acetic acid (IAA), siderophore, potassium solubalization. Soluble phosphate support plant growth as they act as macro nutrients where as phytohormone IAA accelerates root growth. Bacterial siderophore produced in the rhizosphere can indirectly support plant growth by suppressing hazardous effects of biotic stresses (Aeron *et al*., 2011). Only few researchers have reported that microbes are isolated from river soil sample (Chowdhury *et al*., 2009). However their role in plant growth promotion and adaptation to these extreme environments has not been studied in details. The study of microbial population associated with plants growth in such unique environment may thus provide valuable information on microbial distribution and their role in plant establishment and development. Pesticides can also act in a positive manner in combination with entomo-pathogens. Thiamethoxam is a neonicotinoid insecticide and belongs to a new insecticides group. It has a similar mode of action of nicotine (Abbink 1991).

This study was carried out to isolate Cd tolerant fungi and the role they play in plant growth promotion by determining some of the plant growth promoting traits.

Materials and Methods:-

Study area and sample collection

The main purpose of the present study was to observe the tolerance of isolated fungi (*Aspergillus spp*., *Penicillium spp*. and *Curvularia spp*.) towards heavy metal (Cd), so contaminated soil samples were collected from the Tapi river(Latitude 21° 05’ 60.00” N and Longitude 72° 40’ 59.99” E)Surat city (Gujarat, India). The water and soil of the Tapi river is contaminated by industrial effluents, sewage and contaminated heavy metals and toxic chemicals.

Preparation of media

Potato dextrose agar (PDA) media was used for the isolation of fungi. For the preparation of potato dextrose agar (PDA), potatoes were peeled, sliced and boiled and then sieved through a clean muslin cloth to get a broth (with distilled water) to which dextrose sugar and agar was added. The prepared media was then autoclaved at 15 PSI and 121°C.

After autoclaving 30mg/l streptomycin was added in the medium to suppress the bacterial growth. The media was then poured into the petri plate and allow it to solidify. Once the agar solidified, the plates were placed in inverted position at room temperature.

Isolation and identification of fungal isolates

The soil samples were collected and dilutions prepared with distilled water, from the dilutions a loop full of sample was taken and streaked on the PDA plate. The plates were incubated for 3-5 days at room temperature. After the incubation, distinct colonies were collected and identified.

The cultures were identified on the basis of macroscopic (colonial morphology, color, texture, shape, diameter and appearance of the colony) and microscopic characteristics (septation in mycelium, presence of specific reproductive structures, shape and structure of conidia and presence of mycelium). Three fungi were selected and identified as *Aspergillus spp., Penicillium spp.* and *Curvularia spp.*

Heavy metal tolerance test experiment for fungi:-

Isolated fungal colonies of *Aspergillus spp*., *Penicillium spp*. and *Curvularia spp*. were tested for their tolerance to different concentrations of heavy metal [$Cd(No_3)_2$]

Potato dextrose agar media was used for heavy metal resistant experiment. The different concentrations of heavy metal were used for the selection of fungi. The PDA media was prepared and amended with various concentrations (0.032, 0.064, 0.096 g/15ml) of heavy metal. The pH was maintained at 5.6.

Media was then autoclaved at 121°C and 15 psi. After autoclaving, the medium plates were poured and then allow it to solidify the pure culture of fungus was inoculated in the center with the help of wire loop. All the plates were then incubated at room temperature for 3-5 days. The growth of fungi was monitored from the point of inoculation or the center of the colony. The diameter of fungi was measured in mm.

Screening of the isolates for Plant Growth Promoting Properties.

1. Indole Acetic Acid Production

Culture supernatants were used for the detection of IAA. For this the fungal cultures were grown in 25ml Potato dextrose broth amended with 50µg/ml tryptophan, followed by incubation at 28°C for 48 hours. The cultures were then centrifuged at 10,000rpm for 15 min and 3 ml of supernatant was taken for each culture and 2-3 drops of O-phosphoric acid was added. Then 4 ml of Salkowski reagent (1 ml of 0.5 M $Fecl_3$ in 50 ml of 35% $HClO_4$) was added to each aliquot. The samples were then incubated for 25 minutes at room temperature. The absorbance was then read at 530 nm.

2. Phosphate Solubilization Assay

Sterile plates were prepared with the Pikovskaya's medium. The small piece of fungi was placed on the plates and incubated in an incubator at the plates at 28°C for 7 days. After the incubation, the plates were then examined. Visual detection of phosphate solubilizing ability was observed (Rodriguez and Fraga., 1999)

3. Potassium Solubilization Assay

The plates were prepared with PDA medium and mica powder. 0.6mm plug from an actively growing colony was placed on the plate and incubated in an incubator at 28°C for 7 days. The plates were examined. Visual detection of potassium solubilizing ability was observed. (Rodrigues and Fraga., 1999)

4. Siderophore Production

Siderophore (iron chelator) production of the isolates was determined using chromazurol S(CAS) method developed by Schwyn and Neilands (1937). The nutrient agar medium was supplemented with CAS dye 60.5 g in 50 ml, Iron III solution (1 mM $Fecl_3.H_2O$ and 10 mM Hcl in 10 ml) and Hexa-Decyl Trimethyl Ammonium Bromide (HDTMA) (72.9 mg in 40 ml). Plates were kept for 2-4 days incubation and orange color zone surrounding the colonies were observed.

Screening of isolates for Pesticide (Thiamethoxam) Tolerant Activity

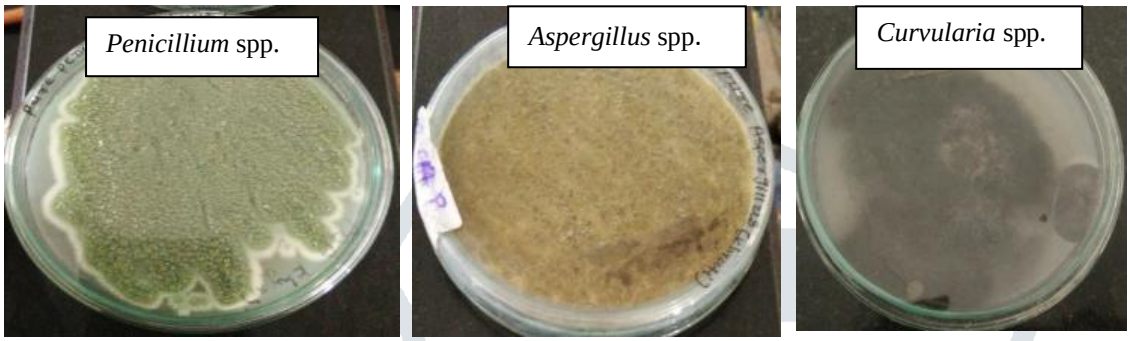
PDA medium amended with various concentrations of Thiamethoxam was prepared. 50ml of PDA was taken in flask of 250ml capacity and sterilized in an autoclave. Two different concentrations of the pesticide 0.01mg/ml and 0.02mg/ml were prepared in distilled water. 50ml of a concentration was aseptically transferred to the flask containing 50ml PDA. The mixture was then poured

into petri dish. Two different concentrations of Thiamethoxam were taken for three different fungi. After the medium solidification, the fungi *Aspergillus spp*., *Penicillium spp*. and *Curvularia spp*. were transferred to the medium containing the Thiamethoxam with the help of plugs of 6mm were taken in an actively growing colonies of fungi & were transferred to the media amended with Thiamethoxam. PDA plates without a fungicide but inoculated with the fungi served as a control. The inoculated plates were incubated at room temperature for 5-7 days. After incubation the radial growth of the colony in each treatment was measured.

Results and Discussion:-

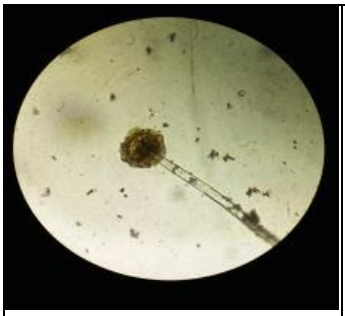
Isolation and identification of the fungi:-A total three isolates were recovered from the collected soil sample (n=3). The isolates were identified on the basis of their morphological characteristics on potato dextrose agar (PDA) and microscopic examination of isolates using picric acid as mounting medium was carried out. They were identified as *Aspergillus spp*., *Penicillium spp*. and *Curvularia spp*. The morphological features and microscopic pictures of the isolates are presented through figure.

Figure 1-Isolates grown on PDA agar plates



On PDA plates, *Aspergillus spp*. produced initially yellowish brown colonies. *Penicillium spp*. produces green color colonies and *Curvularia spp*. produces shiny velvety-black colored, fluffy growth on PDA. On microscopic examination, *Aspergillus sp*. appear to have radiating conidial heads while the conidiophores will appear rough and flask shaped phialides, conidiophores simple or branched and conidia unicellular in case of *Penicillium sp*. In case of *Curvularia sp*. appears as septate, brown hyphae, brown conidiophores, and conidia are visualized. Conidia are straight or pyriform, brown, multi septate and have dark basal protuberant hila.

Figure 2- Microscopic observation of selected isolates.



Microscopic observation of *Aspergillus spp.*

Microscopic observation:-

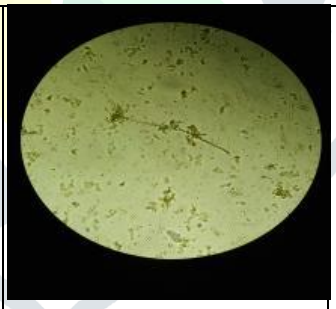
Aspergillus spp.

→Radiating conidial head attach to vegetative hyphae

→Conidiophore rough

→Dark brown color conidia

→Filamentous fungi



Microscopic observation of *Penicillium spp.*

Microscopic observation:-

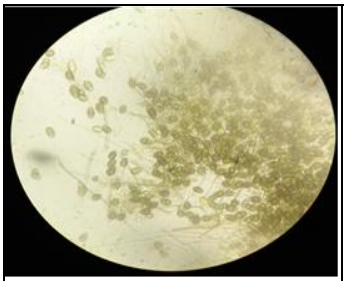
Penicillium spp.

→Thin hyphae

→Conidiophore simple or branched

→Septate hyphae

→mycelia branched



Microscopic observation of *Curvularia spp.*

Microscopic observation:-

Curvularia spp.

→Septate

→Brown conidiophore

→Brown hyphae

→Conidiophore simple or branched

To Screen and Select potential Plant Growth Promoting Heavy Metal Tolerant Fungi

1. Screening of Heavy Metal Tolerance

Total 3 isolates were collected and screened for heavy metal tolerance properties. All 3 samples were showed tolerance to cadmium nitrate heavy metal salt. P*enicillium spp*. tolerated up to 0.64mg/ml of $Cd(No_3)_2.4H_2O$ and *Aspergillus spp*. and *Curvularia spp*. were shown high tolerance with maximum 0.96mg/ml of $Cd(No_3)_2.4H_2O$ concentrations. All three isolates showed metal tolerance capacity.

Figure 3- Screening of metal tolerance by selected fungi on Potato dextrose agar (PDA) plate.

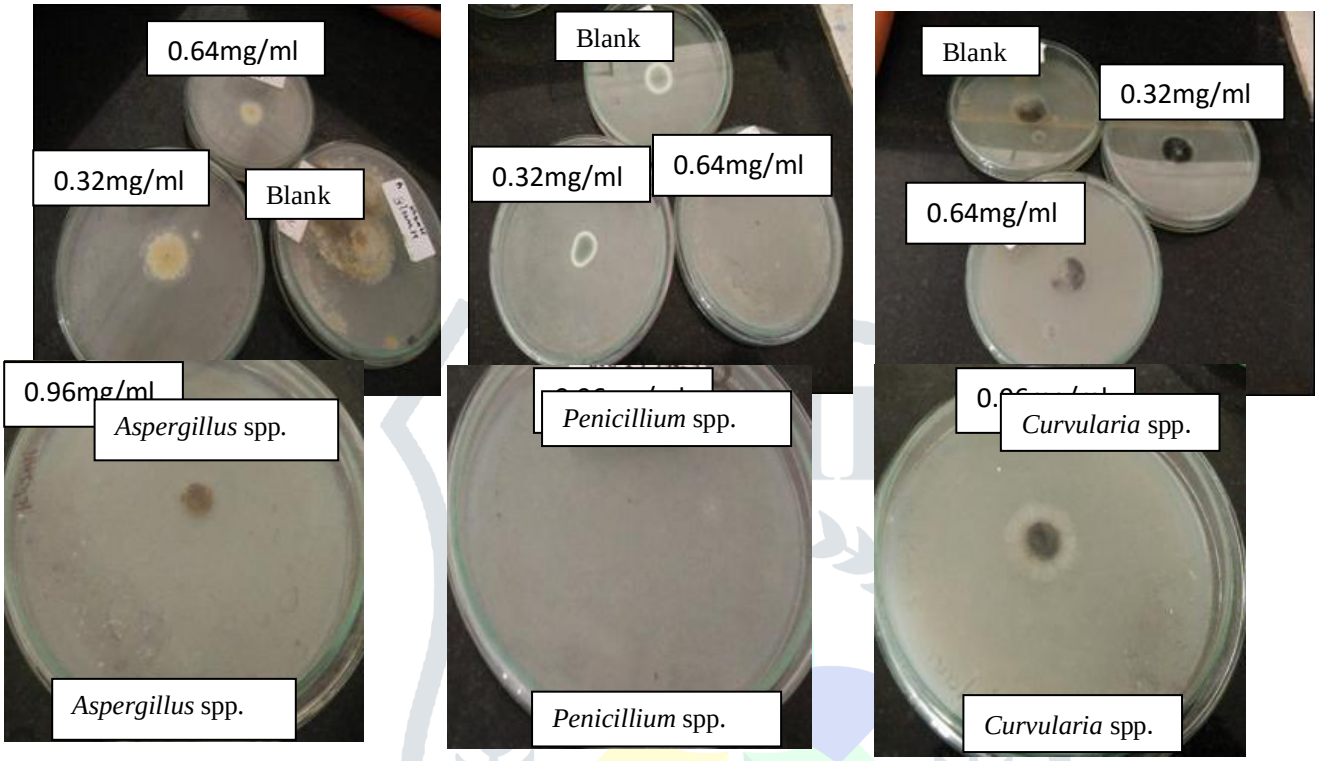


Table 1-Results of heavy metal tolerance by fungi.

Concentrations of Heavy Metal	*Aspergillus spp.*			*Penicillium spp.*			*Curvularia spp.*		
	120 hours	144 hours	168 hours	120 hours	144 hours	168 hours	120 hours	144 hours	168 hours
Blank	31mm	45mm	46mm	17mm	25mm	32mm	23mm	32mm	31mm
0.32 mg/ml	13mm	22mm	26mm	14mm	17mm	24mm	21mm	27mm	31mm
0.64 mg/ml	12mm	17mm	21mm	-	-	-	19mm	25mm	30mm
0.96 mg/ml	10mm	13mm	19mm	-	-	-	16mm	21mm	27mm

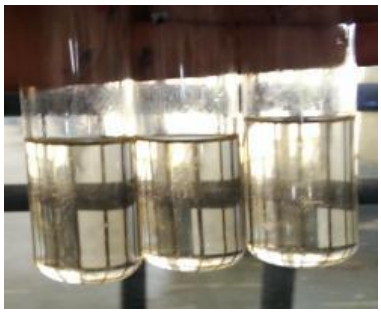
2. Indole Acetic Acid Production

IAA production was checked with use of Salkowski reagent. Color development was first visible at the highest IAA concentration within minutes and continued to increase in intensity for a period of 30 min.

Table 2- Results of IAA production by selected isolates

Isolates	Results
Aspergillus spp.	-
Penicillium spp.	-
Curvularia spp.	-

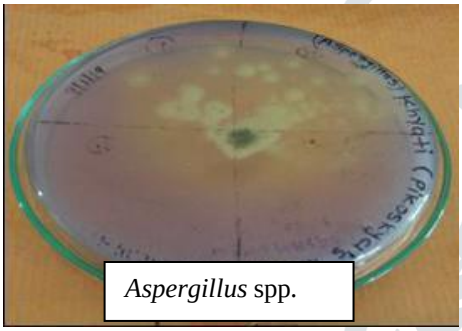
Figure 4-IAA production by selected isolates



3. Phosphate Solubilization test

All three selected isolates showed positive results for phosphate solubilizing test. *Aspergillus sp.* gave the highest capacity for phosphate solubilization showing larger zones.

Figure 5-Phosphate Solubilization by selected isolates



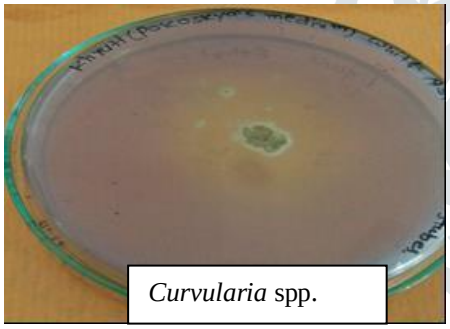


Table 3– Results of phosphate solubilization

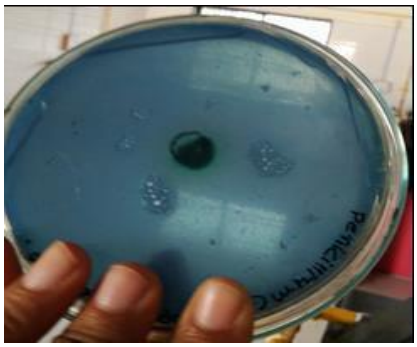
Isolates	Results
Aspergillus spp.	+
Penicillium spp.	+
Curvularia spp.	+

4. Siderophore production

Aspergillus spp. shows positive results while *Penicillium spp.* and *Curvularia spp.* not produce Siderophore production in CAS agar plate. Development of orange zone surrounding colony was considered as positive for Siderophore production as shown in figure

Figure 6- Siderophore production by selected isolates

Aspergillus spp.



Penicillium spp.

Curvularia spp.

Table 4- Results showing Siderophore producing fungi.

Isolates	Results
Aspergillus spp.	+
Penicillium spp.	–
Curvularia spp.	–

5. Potassium Solubilization Assay

All three selected isolates were shown positive results for the potassium solubilizing test. *Aspergillus spp*. have highest potassium solubilizing capacity and *Curvularia spp*. shown negative results towards potassium solubilizing ability.

Figure 7- Potassium solubilization by selected fungi

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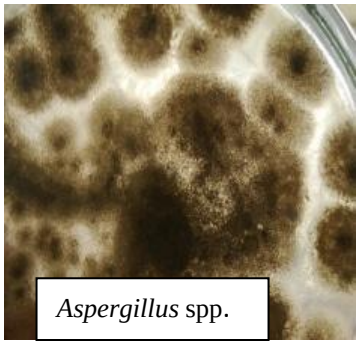
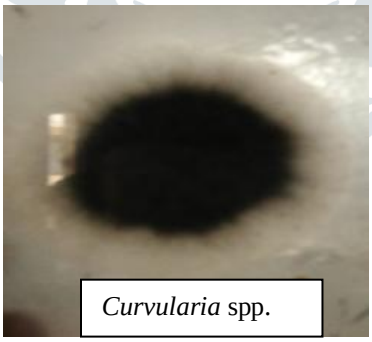
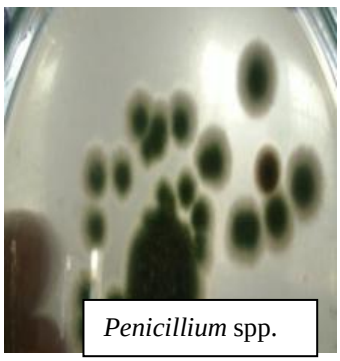


Aspergillus spp.



Curvularia spp.



Penicillium spp.

Table 5– Result showing Potassium solubilization

Isolates	Results
Aspergillus spp.	+
Penicillium spp.	+
Curvularia spp.	–

6. Effect of Thiamethoxam on selected isolates

All the selected isolates had the capacity to grow in the presence of pesticide (Thiamethoxam). *Aspergillus spp*. showed luxuriant growth in control plates without pesticide, but colony diameter 21mm and 19mm was observed in plates with pesticide containing 0.1mg/ml & 0.2mg/ml respectively. With compared to Aspergillus spp. and Curvularia spp. *Penicillium spp*. have the lowest capacity to grow in the presence of pesticide with the diameter of 11mm and 8mm in 0.1mg/ml and 0.2mg/ml concentration respectively.

Figure 8- Thiamethoxam tolerant fungi

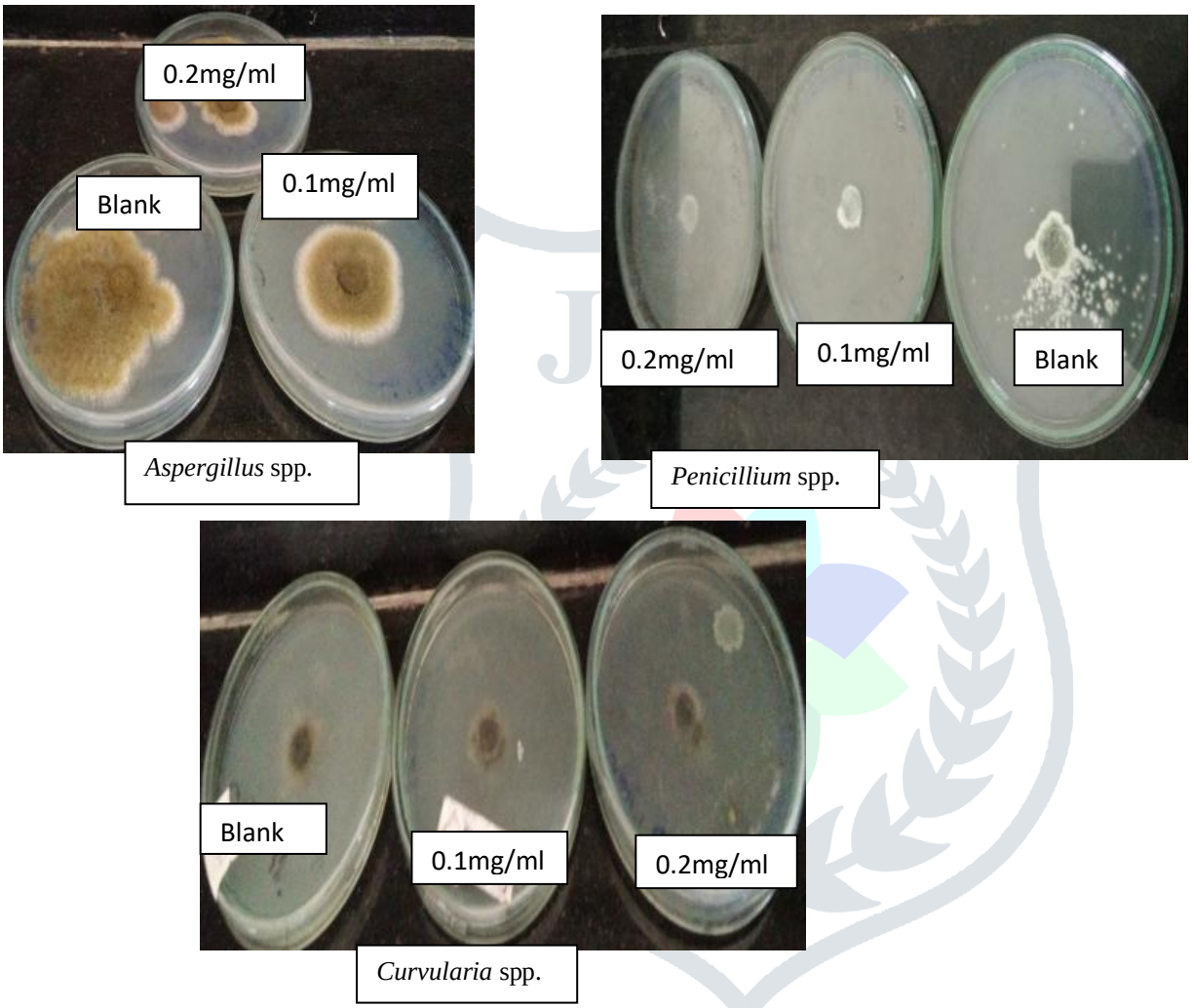


Table 6- Results of Thiamethoxam tolerance capacity of selected isolates.

Concentrations	*Aspergillus spp.*	*Penicillium spp.*	*Curvularia spp.*
Blank	46mm	13mm	31mm
0.1mg/ml	21mm	11mm	19mm
0.2mg/ml	19mm	8mm	16mm

Figure 9- Tolerance indices of the fungal isolates (*Aspergillus spp*.) to cadmium.

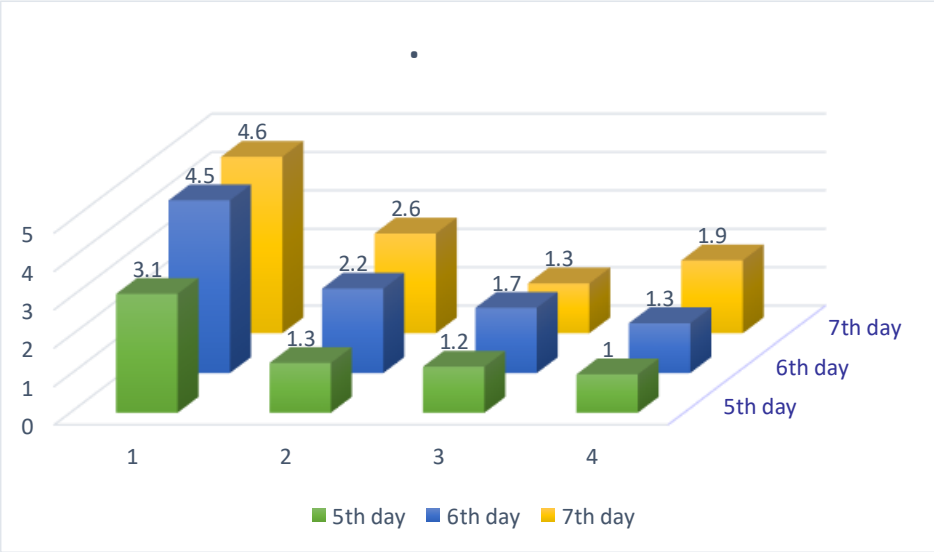


Figure 10-Tolerance indices of the fungal isolates (*Penicillium spp*.) to cadmium

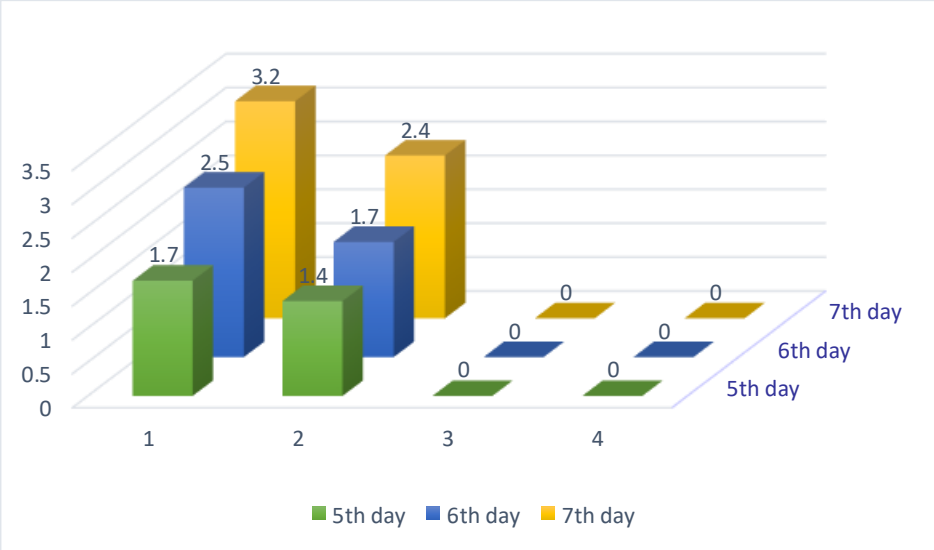


Figure 11-Tolerance indices of the fungal isolates (*Curvularia spp*.) to cadmium

Figure 12-Tolerance indices of the fungal isolates to Thiamethoxam pesticide at concentration 0.1mg/ml

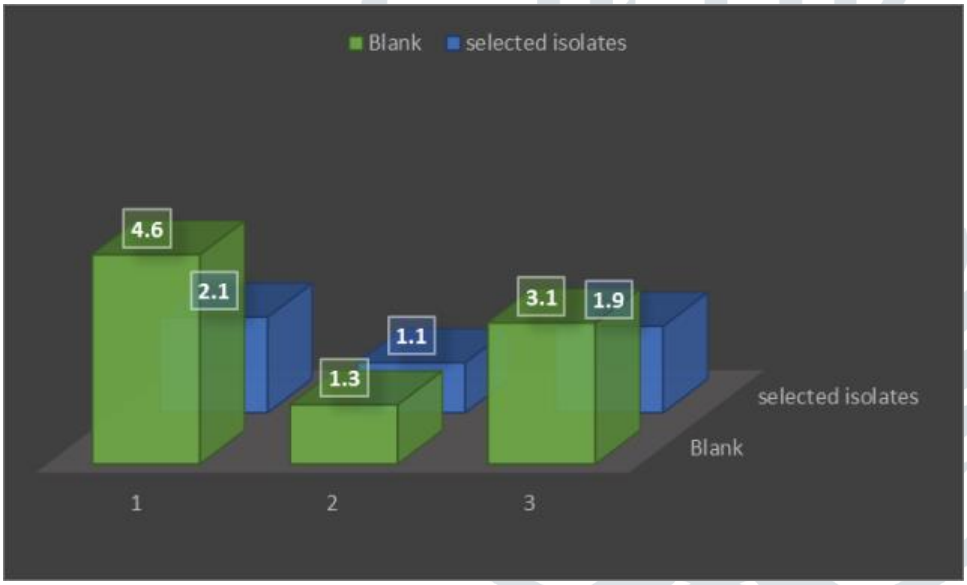
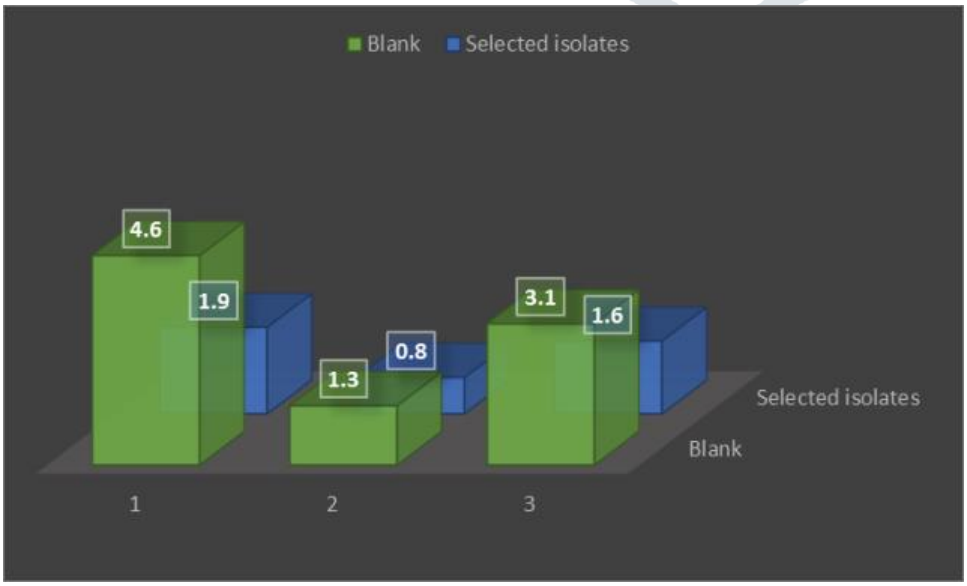


Figure 13-Tolerance indices of the fungal isolates to Thiamethoxam pesticide of concentration 0.2mg/ml



Discussion

In heavy metal assay, Figure 3 shows *Penicillium* spp. had tolerated limit of 0.32mg/ml on Cd salt concentration but in case of *Aspergillus* spp. & *Curvularia* spp. they tolerated up to 0.96mg/ml of Cd salt concentration with the diameter of 19mm and 27mm respectively. Table 1 shows the colony diameter of respective isolate at different metal concentrations. Rao *et al*, also observed that with the increasing metal concentration, fungi can increase the rate of heavy metal removal by saturation adsorbents concentration by increasing mobilization of metal ions. Their toxicity may differ, depending on the isolate and its site of isolation (Rao *et al*., 2005). Kumar *et al*, observed that *Penicillium spp., Aspergillus spp. and Cunninghamella spp*. had tolerance limits of 6-14mM for each Cd and Zn.

One of the interesting features of present study is that the heavy metal tolerant fungi showed promising plant growth promoting properties. The most metal resistant fungi could also be analysed in terms of metal distribution in the cell which can reveal the relative importance of intracellular and extracellular mechanisms that help to cope with metal stress. Cd distribution in selected isolates can be investigated (Lima *et al*, 2006). Furthermore the isolated fungi also shown the pesticide (Thiamethoxam) resistance capacity. All three heavy metal tolerate fungi gave negative result in IAA production as shown in figure 4 but *Aspergillus spp.* & *Penicillium spp*. have capacity to solubilize potassium. *Aspergillus* spp. have highest capacity to solubilize phosphate and potassium. In siderophore production assay only *Aspergillus* spp. gave positive result with orange color zone surrounding the colony in CAS agar plate. All three isolates also have the capacity to grow in the presence of Thiamethoxam pesticide. Table 6 shows the colony diameter of the selected isolates.

Conclusion

Soils contaminated with heavy metals pose serious threat to agroecosystems and consequently to human health. Different approaches employs to clean up metal polluted sites, adequate success has not been achieved so far. In order to achieve greater success, efforts are needed to create public awareness about metal toxicity and help from government body to carefully monitor and regulate the discharge of properly treated by-products of different industries. Microorganisms have been shown to possess an ability to survive by adapting or mutating at high concentrations of toxic heavy metals. The research was aimed to study the diversity and plant growth promotion abilities of fungi in the Tapi river soil sample and its utilization for farming. The fungi were isolated and were characterized according to their heavy metal tolerance and PGP properties. The isolates were identified on the basis of morphological characteristics and microscopic examination. All the isolates were tolerant to Cd heavy metal salt. The highest tolerance level was observed in case of *Curvularia spp*. 0.96mg/ml. Isolates were screened for PGP properties and the isolates were further used for screening of pesticide (Thiamethoxam) tolerant capacity.

Acknowledgement

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References

1. Abbink, J. (1991). The biochemistry of imidacloprid. *Pflanzenschutz-Nachrichten Bayer (Germany, FR)*.
2. Aeron, A., Kumar, S., Pandey, P., & Maheshwari, D. K. (2011). Emerging role of plant growth promoting rhizobacteria in agrobiology. In *Bacteria in agrobiology: Crop ecosystems* (pp. 1-36). Springer, Berlin, Heidelberg.
3. Ahmad, F., Ahmad, I., & Khan, M. S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological research*, *163*(2), 173-181.
4. Chowdhury, A. R. (2009). Recent advances in heavy metals induced effect on male reproductive function-A retrospective. *Al Ameen J Med Sci*, *2*(2), 37-42.
5. Iram, S., Parveen, K., Usman, J., Nasir, K., Akhtar, N., Arouj, S., & Ahmad, I. (2012). Heavy metal tolerance of filamentous fungal strains isolated from soil irrigated with industrial wastewater. *Biologija*, *58*(3).
6. Iram, S., Zaman, A., Iqbal, Z., & Shabbir, R. (2013). Heavy Metal Tolerance of Fungus Isolated from Soil Contaminated with Sewage and Industrial Wastewater. *Polish Journal of Environmental Studies*, *22*(3).
7. Kumar, R., Sharma, A. K., Singh, P., Dhir, B., & Mehta, D. (2014). Potential of some fungal and bacterial species in bioremediation of heavy metals.
8. Levinskaite, L. (2001). Effect of heavy metals on the individual development of two fungi from the genus Penicillium. *Biologija*, *1*, 25-30.
9. Rao, K. R., Rashmi, K., Latha, J., & Mohan, P. M. (2005). Bioremediation of toxic metal ions using biomass of Aspergillus fumigatus from fermentative waste.
10. Rodríguez, H., & Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology advances*, *17*(4-5), 319-339.
11. Severino, L. S., Lima, R. D. L. S., & BELTRÃO, N. D. M. (2006). Composição química de onze materiais orgânicos utilizados em substratos para produção de mudas. *Embrapa Algodão-Comunicado Técnico (INFOTECA-E)*.