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Expression of plant growth promoting traits of *Fusarium solani* KUSF0104 under metal stressed conditions

Roushan Islam*[1] and Bejoysekhar Datta[2]

[1]*Department of Botany, Sripat Singh College, Jiaganj-742123, Murshidabad, West Bengal, India*
[2]*Department of Botany, Kalyani University, Kalyani-741235, Nadia, West Bengal, India*

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Corresponding Author: **Roushan Islam** | E-Mail: **(rislammsd@gmail.com)**

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ABSTRACT

The present study explores the plant growth-promoting traits of Fusarium soil isolate and also their estimation under various conditions of metal stress. Fusarium sp. was isolated from an agriculturally important soil of Murshidabad district in West Bengal, India by serial dilution plate technique on selective medium using pentachloronitrobenzene and was later identified by spore morphology and molecular study. The Fusarium sp was found to be non-pathogenic soil isolate based on increased germination percentages and vigour index values of six seed plants viz., chickpea, black gram, cucumber, chilli, mustar and paddy . The fungal isolate produced substantial amount of IAA in tryptophan-supplemented broth (370 µg/ml) and also produced GA in colossal amounts (4800 µg/ml). The Fusarium sp. could resist the tested heavy metals viz., Cd, Cu, Fe, Ni and Zn and MIC values were in the range from as low as 175 ppm (Cd) to as high as 350 ppm (Fe). The isolate produced IAA and GA in metal-contaminated broths. But, Cd at 50 ppm concentration completely inhibited both IAA and GA production. Zn at 50 ppm concentration was also found to be inhibitory to the isolate with regard to its ability to produce IAA. But GA production continued uninhibited (3500-4250 µg/ml) in the presence of the tested heavy metals at minimum (50 ppm) concentrations. Siderophore production by the isolate was found to be on the higher range (400-540 nmole/ml) in presence of Cu, Fe and Zn. Phosphate solubilisation remained unaffected (1400-1800 ppm) by the Fusarium isolate in presence of the tested heavy metals except Cd. Thus, the soil isolate could be of immense use in metal-contaminated agricultural soils to increase yields of agronomically important crops.

Keywords: *Fusarium, non-pathogenic, germination, vigour index, phytohormone, IAA, GA, heavy metal, agriculture etc.*

Introduction

Heavy metals are metallic elements which have specific mass considerably higher than the normal metals in the periodic table (5 g/cm^3). A number of heavy metals acts as essential micronutrients necessary for plant growth, while toxic metals like Cd, Pb, As and Hg have no significant roles in biological systems [1]. Heavy metal pollution in agricultural soils is one of the major environmental threats as the metals are not biodegradable and persist in the soil indefinitely. Filamentous fungi, such as *Aspergillus, Penicillium, Fusarium, Rhizopus* and *Trichoderma* have been reported to exhibit significant tolerance towards these toxic heavy metals, and are now known to be leading micro-organisms in metal polluted sites [2,3].

Akin to several plant growth-promoting rhizobacteria, some non-pathogenic rhizospheric fungi were reported to support plant growth upon root colonisation and were typically designated as 'plant growth-promoting fungi' [4]. They are potentially applied in agriculture as biofertilizer agents. They are reported to synthesise hormones such as auxin (IAA) and gibberellic acid (GA) and transport these in plants [5]. Several PGPF were reported to solubilise inorganic phosphates, making these available to the plant. They were also found to secrete siderophore under iron deficiency to meet their iron requirement and also make the iron available to the plant. Siderophore was also found to be crucial in the control of soil borne plant diseases caused by variety of pathogens with an improper iron uptake system [6].

Auxin (IAA) is one of the major plant hormones that can be synthesised by diverse soil microorganisms including fungi and bacteria. Tryptophan is the necessary precursor molecule for the biosynthesis of IAA in soil microorganisms. Root exudates of the plants release are enough tryptophan in soil. Important genes in biosynthetic pathway e.g., IaaM and IaaH genes have been identified from a number of *Fusarium* species [7].

There are more than 100 types of gibberellins identified in higher plants In fungi, more than 20 GAs have been isolated. But in bacteria, only four GAs were recovered [8]. The fungi do not use GAs for their own development but can produce and supply great quantities of this exogenous GAs to amend the response of their host and act as signalling molecules for the host plant [9]. GA production has been reported in several other species of *Fusarium* by number of investigators [10-12].

Under conditions of iron starvation, microorganisms compete with other iron-requiring microorganisms through hyper activation of iron uptake system. One component of the system is the siderophore which is a Fe^{3+} ion specific chelating agent released extracellularly by certain microorganisms present in the soil [13].

Phosphorus (P) is second most essential macronutrients required for plant growth and development. However, bulk amount of soil phosphorus is present in the insoluble form and becomes inaccessible to the plants [14]. Many soil microorganisms can efficiently release P from inorganic forms through phosphate solubilisation and subsequent mineralisation by secreting a number of organic acids [15].

A variety of organic acids viz., malic acid, fumaric acid and gluconic acids are responsible for solubilising the inorganic phosphates present in soil [16].

The present study is significant considering the exploration of several plant growth-promoting traits of *Fusarium* sp. under conditions of metal stress which can be subsequently proven to be effective in improving the crop yield using the metal-tolerant plant growth-promoting species of *Fusarium*.

Materials and Methods

Collection of soil: The soil was collected from the root region of the plant of an agricultural field located in Bamnabad village of West Bengal, India. The agricultural field was situated on banks of Padma river. The agricultural land was known for cultivation for multiple crops throughout the year. It is worth mentioning that there were no previous reports of any *Fusarium* diseases in the agricultural field.

Isolation and identification of *Fusarium* sp.: The fungus was soil isolated by the serial soil dilution plate technique on selective peptone PCNB agar medium amended with two antibiotics viz., streptomycin (1.0 g/l) and neomycin (0.10 g/l). The plates were put in incubator at 27°C for 6-7 days till a visible colony growth occurred. The *Fusarium* isolate was later identified considering several parameters viz., conidia production, pigment production and morphology of the colony on CDA medium. The identity of the isolate was further confirmed by molecular analysis using sequenced r-DNA. The g-DNA of the isolate was extracted and used as a template for further amplification of the r-DNA region using the primer pair ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) [17]. The amplified DNA product was sequenced and analysed using the Nucleotide BLAST at NCBI to find out homology of the sequenced DNA with the nucleotide database.

Pathogenicity test: Seeds of six different higher plants were surface sterilised using 0.1% $HgCl_2$ solution for 45 sec. and washed three times with sterile water. 10 surface sterilised seeds of each plant were put into the culture filtrate of the *Fusarium* isolate separately and were kept at 8°C for an 12 hours. The seeds were plated on sterile wet blotting paper kept within the petri dish on the following day. After 5-7 days, root-shoot lengths and the % of seed germination were calculated. Vigour index of the individual seed was also considered by using the standard formula.

Metal tolerance test: The tolerance to the five different heavy metals viz., Cd, Cu, Fe, Ni and Zn was tested in the broth medium. Different sets of PD broth were prepared and supplemented with increasing concentrations of individual heavy metals. A control broth without metal was also prepared. Each broth was inoculated with 5mm diameter of mycelia disc from a week old pure culture of the isolate and incubated at 27°C for 14 days. Mycelial dry weights of the isolate were measured after the incubation period. Tolerance was estimated observing the minimum inhibitory concentration (MIC) and tolerance index (TI) [18,19].

Effect of heavy metals on IAA production: The *Fusarium* isolate was evaluated for IAA production in the presence of different heavy metals viz., cadmium (Cd), copper (Cu), iron (Fe), nickel (Ni) and zinc (Zn). Freshly prepared CD broths were supplemented with minimum concentration (50 ppm) of the individual heavy metals and 1000 ppm tryptophan. After inoculation in the broth medium and subsequent incubation at 27ºC for 2 weeks, the production of IAA was estimated by spectrophotometric method using Salkowski's regent.

Effect of heavy metals on GA production: The GA producing *Fusarium* isolate was evaluated with regard to its GA production in the presence of different heavy metals viz., cadmium (Cd), copper (Cu), iron (Fe), nickel (Ni) and zinc (Zn). Freshly prepared CD broths were supplemented with minimum concentration (50 ppm) of the individual heavy metals. After inoculation in the broth medium and subsequent incubation at 27ºC for 2 weeks, the production of GA was estimated by spectrophotometric method using phosphomolybdic acid regent.

Effect of heavy metals on siderophore production: The soil isolate was also tested for production of the iron chelator-siderophore in the presence of different heavy metals viz., cadmium (Cd), copper (Cu), iron (Fe), nickel (Ni) and zinc (Zn). Sets of CD broths were made and supplemented with minimum concentration (50 ppm) of the individual heavy metal. The *Fusarium* isolate were inoculated into the broths and incubated at 27ºC for 2 weeks. Estimation of siderophore produced in different broths was done by spectrophotometric method using the CAS reagent.

Effect of heavy metals on phosphate solubilisation: The Metal-tolerant *Fusarium* isolate was evaluated for its potential to solubilise inorganic phosphate in metal contaminated broth medium. Different sets of Pikovskaya's broths were prepared and supplemented with the minimum concentration (50 ppm) of the individual heavy metals viz., Cd, Cu, Fe, Ni and Zn. The *Fusarium* isolate was inoculated in the broths and incubated at 27ºC for 2 weeks. Amount of soluble phosphate in the filtrate was finally estimated spectrophotometrically using molybdate vanadate reagent.

Ammonia production under heavy metal stress: Freshly grown *Fusarium* culture was inoculated in 25 ml of peptone water supplemented with heavy metals, viz., Cu, Cu, Fe, Ni, Zn at 50 ppm concentration each and incubated at 27°C for 1 week. 10 ml of the culture filtrate was mixed with 0.5 ml Nessler's reagent. Uninoculated peptone water treated with Nessler's reagent was kept as control. Development of yellow to brown colour was considered a positive result for ammonia production [20].

Results and Discussion

Isolation and identification of the *Fusarium* sp.: On PCNB medium, the fungal isolate appeared was definitely *Fusarium*, with all its characteristic growth and morphology of the colony. The isolate was designated as KUSF0104. On CDA medium the soil isolate showed circular, white, smooth, dense and fast-growing colony with ventral surface turned dark brown at later days of incubation (Fig. 1). It produced macroconidia in abundance which were falcate having certain degree of curvature; number of septa varied between 3-5 (Fig. 1). Microconidia were ellipsoid to fusiform; with no. of septa varied between 0-1 (Fig. 1). Chlamydospores were abundant.

They were intercalary or terminal in aerial mycelia, produced singly, in pair, in chains and in clusters, spherical (10-15.5 µm in diameter), verrucose (Fig. 1).

Molecular identification of the fungal isolate KUSF0104 was carried out by sequence homology through nucleotide BLAST in NCBI database. Maximum similarity (99%) was matched with the r-DNA sequence of *F. Solani* Zbf-R4 (99%) (KX079482). The fungal isolate was eventually recognized as *Fusarium solani*. The r-DNA sequence of the *Fusarium* isolate KUSF0104 was deposied to the gene bank under the accession no. Mf136400.

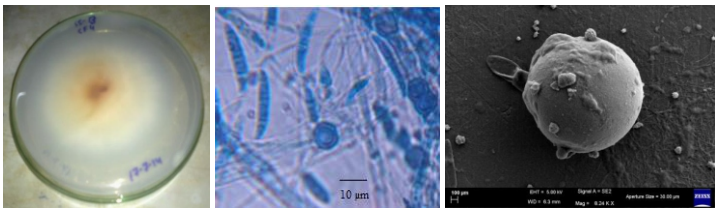


Fig. 1: Fusarium solani KUSF0104 on CDA medium after 7 days (left); Macroconidia and chlamydospores of Fusarium solani KUSF0104 (middle); chlamydospore of Fusarium solani KUSF0104 (right)

Pathogenicity test: Results of the pathogenicity test have been shown in table 1. Most of the seeds germinated and the percentage of seed germination was variable in different plants. Culture filtrate of the *Fusarium* isolate KUSF0104 (MF136400) was not inhibitory with respect to germination of the seeds which eventually proved the fact that the isolate was non-pathogenic. In addition, growth stimulatory effects of the culture filtrate of the *Fusarium* isolate on at least three plants were evident in the results (Table 1, Fig. 2). Early onset of seed germination was also found in some of the inoculated seeds.

Table 1: Pathogenicity test of Fusarium solani KUSF0104

Isolate no.	Chickpea		Blackgram		Cucumber		Chili		Mustard		Paddy	
	% of germination	Vigour index	% of germination	Vigour index	% of germination	Vigour index	% of germination	Vigour index	% of germination	Vigour index	% of germination	Vigour index
*Fusariumequiseti*MF803160	80	21.6	100	30.4	80	128.8	50	38.6	70	60.6	100	55.6
Control	80	23.2	100	28.4	70	119.7	60	40.2	70	38.5	80	16.2

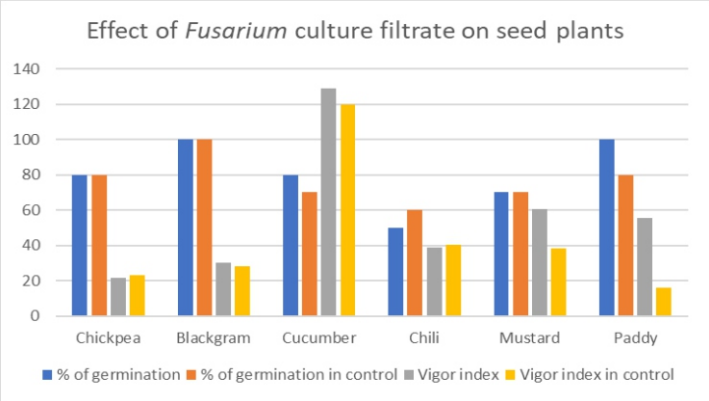


Fig. 2: Germination percentage and vigor index of seed plants inoculated with culture filtrate of Fusarium solani KUSF0104

Metal tolerance of *Fusarium* sp.: Results of metal tolerance of the *Fusarium* isolate considering MIC and TI values against different heavy metals viz., cadmium, copper, iron, nickel and zinc are depicted in Table 3. The isolate showed considerable resistance against all the tested heavy metals.

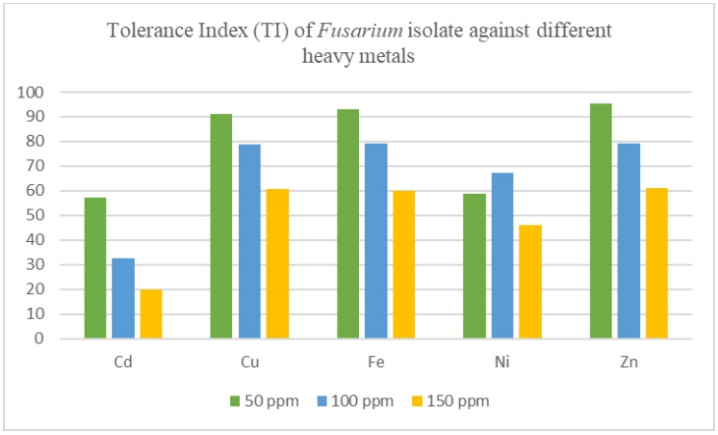
Tolerance indices of the *Fusarium* isolate against the heavy metals at three different concentrations (50, 100 and 150 ppm) were also tested and the results have been shown in Table 2. Except Cd and Ni, high tolerance indices were found in all three different concentrations of the heavy metals (Fig. 3).

Table 2: Tolerance Index (TI) of Fusarium solani KUSF0104 against different concentrations of heavy metals

Sl No.	Heavy metal	Concentration (ppm)	Tolerance index (TI)
1.	Cd	50	57.1
		100	32.4
		150	20.0
2.	Cu	50	91.2
		100	78.8
		150	60.7
3.	Fe	50	93.1
		100	79.1
		150	60.0
4.	Ni	50	58.7
		100	67.1
		150	46.1
5.	Zn	50	95.2
		100	79.1
		150	60.9

Table 3. MIC values (ppm) of heavy metals against Fusarium solani KUSF0104

Fusarium isolate	Cd	Cu	Fe	Ni	Zn
KUSF0104	175	225	225	225	250



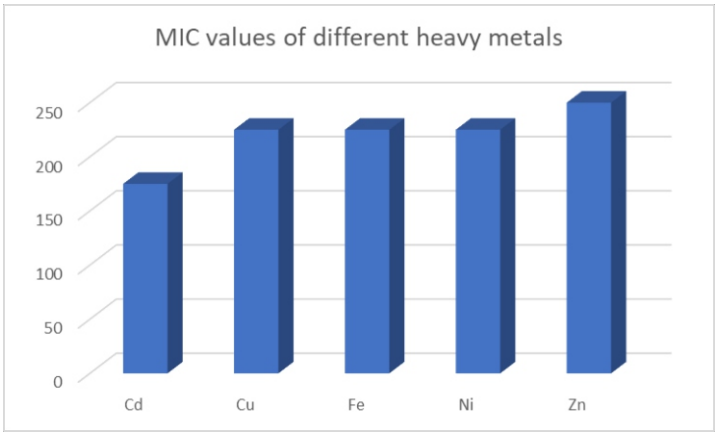


Fig. 3: Tolerance Index (TI) at different concentrations of respective heavy metals (left) and minimum inhibitory concentrations (MIC) of heavy metals of Fusarium solani KUSF0104 (right)

Effect of heavy metals on IAA and GA production of *Fusarium* sp.: IAA production of the isolate was completely inhibited in Cd and Ni supplemented medium. The results are presented in Table 4. Considerable amount of IAA production was noticed in presence of Cu or Fe and Zn (120-170 µg/ml).

No GA production was found in presence of Cd and Ni, although the isolate showed decent growth in medium supplemented with the corresponding heavy metals (Fig. 4; Table 4). Unaffected GA production was observed in presence of Cu or Fe or Zn (2800-3000 µg/ml).

Table 4: Effect of heavy metals on auxin and gibberellin production of the Fusarium isolate

Sl no.	Heavy metal	IAA production (µg/ml)	GA production (µg/ml)
1	Cadmium (Cd)	-	-
2	Copper (Cu)	120	3000
3	Iron (Fe)	170	3000
4	Nickel (Ni)	-	-
5	Zinc (Zn)	150	2800
Control		370	3100

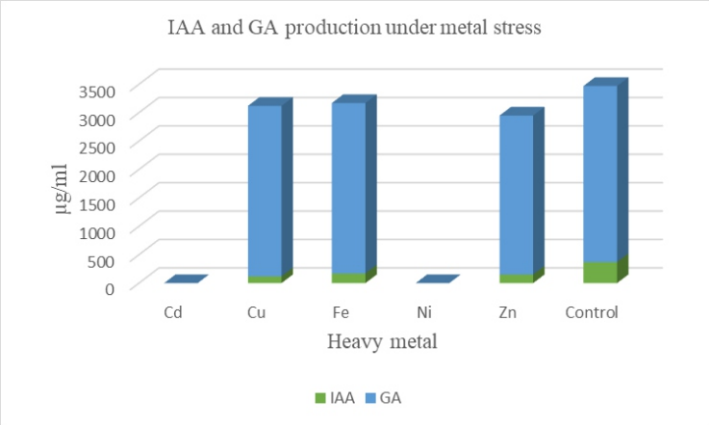


Fig. 4: Auxin and Gibberellin production by Fusarium sp. KUSF0104 under heavy metal stress

Effect of heavy metals on siderophore production of *Fusarium* sp.: In spite of growth in Cd supplemented broth, it did not produce any siderophore in the medium (Fig.5; Table 5). Among the other four heavy metals, Ni affected siderophore production of *Fusarium* isolate to a reasonable extent (80 nmole/ml), but considerable amount of siderophore was produced in presence of Cu, Fe and Zn (400-540 nmole/ml). It is worth mentioning that iron stress in the medium was provided via application of $FeSO_4$ which may not be inhibitory for siderophore production since siderophore can only bind to Fe^{3+} ion, not the Fe^{2+} ion.

Table 5: Effect of heavy metals on siderophore production by Fusarium sp. KUSF0104

Sl. No.	Heavy Metal	Siderophore production (nmole/ml)
1	Cd	-
2	Cu	400
3	Fe	540
4	Ni	80
5	Zn	500
Control		590

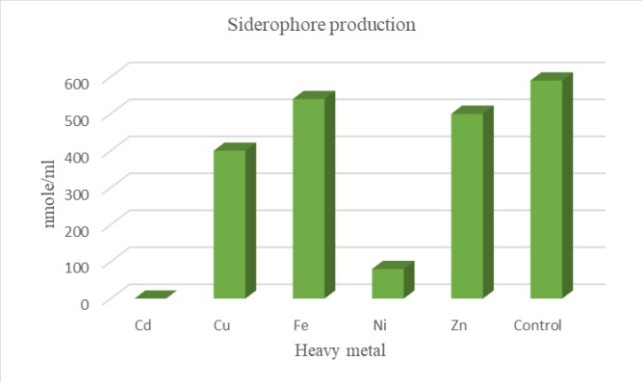


Fig. 5: Siderophore production by Fusarium sp. KUSF0104 under heavy metal stress

Phosphate solubilisation under heavy metal stress: Results of phosphate solubilisation of the isolate is depicted table 6. The isolate failed to exhibit phosphate solubilisation property in presence of Cadmium (Cd). The isolate was able to solubilise inorganic phosphate in presence of Fe, Cu, Ni and Zn to the same extent (Fig. 6).

Table 6: Phosphate solubilisation of Fusarium sp. SF0104 under heavy metal stress

Sl. No.	Heavy metal	Phosphate solubilisation (ppm)
1.	Cd	-
2.	Cu	1590
3.	Fe	1800
4.	Ni	1400
5.	Zn	1620
6.	Control	1800

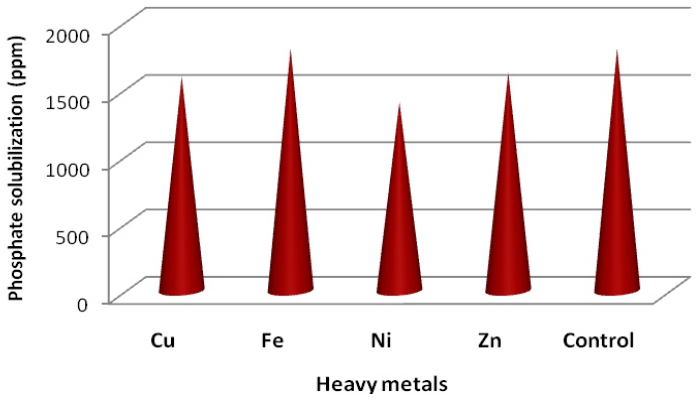


Fig. 6: Effect of heavy metals on phosphate solubilisation by Fusarium sp. Sf0104

Ammonia production: Soil microorganisms secrete ammonia to inhibit growth of other organisms. The *Fusarium* isolate was studied for ammonia production as its peptone water culture turned brown with the addition of Nessler's reagent (Fig. 7;).

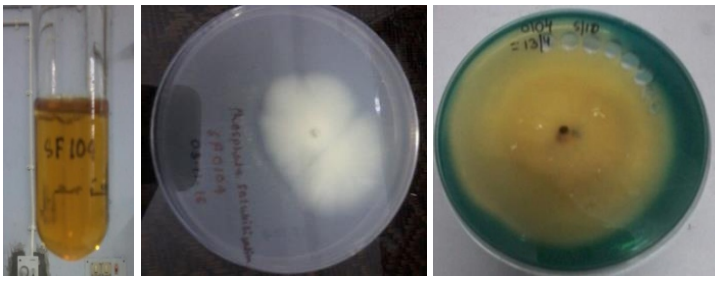


Fig. 7: Production of ammonia (left); solubilisation of calcium phosphate (middle); siderophore production (right)

DISCUSSION

At comparatively lower concentrations of the metals, the tested fungal isolate was found to be resistant and exhibited noticeable growth, normally matching up to the growth of control. Higher metal concentrations caused a slight slower growth reduction. The order of tolerance shown by the isolate against the heavy metals was record as Zn>Fe=Ni=Cu>Cd.

The potential IAA-producing *Fusarium* isolate was characterised further in terms of their IAA production under heavy metal stressed conditions. The *Fusarium* isolate exhibited IAA production in variable amounts when exposed to different heavy metals (Fig. 4; Table 4). But, Cadmium (Cd) completely inhibited IAA production at minimum concentration (50 ppm). Lethal effects of Cadmium (Cd) on auxin production (IAA) was also documented by other investigators [21,22]. IAA production was also found to be completely blocked in the presence of Nickel (Ni). IAA production by the isolate was found in the range 120-170 µg/ml in the presence of heavy metals.

Cadmium (Cd) and nickel (Ni) also posed their detrimental effects on Gibberellic acid (GA) biosynthesis of the *Fusarium* sp. Other heavy metals did not affect GA production.There was hardly any drop in GA synthesis when compared with the control. In presence of Cu, Fe and Zn, the GA production (2800-3000 µg/ml) was found to be matching nearly the control (3100 µg/ml).

The *Fusarium* isolate was also evaluated for its ability to produce the iron chelator metabolite siderophore in presence of wide variety of heavy metals in culture broth (Table 5; Fig. 5). Expectedly, Cd posed toxic effect on siderophore production of the *Fusarium* isolate. Ni was also found to be slightly inhibitory in siderophore production of *F. solani* SF0104 (80 nmole/ml). The other heavy metals did not affect siderophore production. There was no noticeable any drop in siderophore production when compared with the control. In presence of Cu, Fe and Zn, siderophore production (400-540 nmole/ml) was found to be matching with the control the control (590 nmole/ml).

The promising phosphate solubiliser *F. Solani* KUSF0104 was characterised with respect to its potential to solubilise phosphates under heavy metal stress (Table 6; Fig. 6). Cd completely checked the ability of the isolate to solubilize insoluble phosphates. Significantly, in the presence of the rest of the heavy metals, phosphate solubilization was found substantially on the higher range (1400- 1800 ppm). Cd concentration upto 10 µg/ml did not affect siderophore production and phosphate solubilisation ability of *Ralstonia mannitolilytica* KUCd7[23].

Production of ammonia is considered as important plant growth-promoting trait since it shows inhibitory effect to control soil borne pathogens[24]. Moreover, in aqueous medium it forms ammonium salt which at a low concentration (20-200 µM) is absorbed by the plant [25]. The *Fusarium* isolate was found to be a potential ammonia producer in metal-contaminated broth (Fig. 7).

CONCLUSION

The *Fusarium* isolate exhibited in-vitro plant growth-promoting attributes when exposed to different heavy metals such as Copper (Cu), iron (Fe), nickel (Ni) and zinc (Zn). The soil isolate demonstrated considerable auxin (IAA) production in presence of Cu (120 µg/ml), Fe (170 µg/ml), Zn (150 µg/ml); The isolate *Fusarium solani* SF0104 also produced GA in presence of Cu, Fe and Zn substantially. Siderophore production was found to be reasonably on the higher range (80-540 nmole/ml) in presence of wide variety of the heavy metals. Phosphate solubilization was found to be nearly matching with that of control in presence of all the tested heavy metals except cadmium (Cd). In addition, ammonia production by the soil isolate was also satisfactory in the presence of different heavy metals.

Thus, it can be inferred that the *Fusarium* isolate has the potential for growth in a wide range of cultural conditions in the presence of heavy metals and it could be exploited for growth promotion of a number of crop plants in field conditions, as revealed by several plant growth-promoting attributes of the fungus, even when exposed to wide variety of heavy metals.

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