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PHYTOHORMONES PRODUCTION BY METAL TOLERANT NON-PATHOGENIC FUSARIUM SOLANI KUSF0301

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ABSTRACT

The present study explores phytohormones production of Fusarium soil isolate and also their production under the condition of heavy metal stress. Fusarium sp. was recovered from an agricultural field of Murshidabad district in West Bengal, India. by soil dilution plate technique on selective pentachloronitrobenzene (PCNB) medium and was later identified by routine morphological studies and molecular analysis. The Fusarium species was proven non-pathogenic based on germination percentage and vigour index values of the tested seed plants. The isolate produced IAA in tryptophan supplemented medium (370 µg/ml) and also produced GA in huge amount (4800 µg/ml). The Fusarium species was found to be quite resistant to the tested heavy metals and its MIC value ranges from as low as 175 ppm (Cd) to as high as 350 ppm (Fe). The isolate was capable of producing both IAA and GA in metal contaminated broth. But, Cd (50 ppm) completely checked both IAA and GA production. Zn (50 ppm) was also toxic to the species in terms of its ability to produce IAA. But GA production continued unabated (3500-4250 µg/ml) in presence of the other heavy metals at 50 ppm concentration. Thus, the soil isolate could be used in metal contaminated agricultural soil to improve yield.

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

1. INTRODUCTION

The microbial synthesis of phytohormones viz., auxin and gibberellin are one of the important factors in soil fertility (Kampert and Strzelczyk 1975). Indole acetic acid (IAA) and Gibberellin (GA) and are secondary metabolites, produced commercially from fungi for the agricultural purpose (Deacon 1984, Bruckner 1992). Fungi also produce compounds that are similar to plant hormones, such as ethylene (ET), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA). These hormones are well described to control plant development and to trigger important plant signalling events during biotic and abiotic stresses (Peleg and Blumwald, 2011; Pozo *et al.*, 2015; Spence and Bais, 2015).

Indole-3-acetic acid (IAA) is a major plant auxin that stimulates cell elongation, cell division and differentiation in plants. Tryptophan is the key precursor for biosynthesis of IAA in plants and microorganisms, and application of exogenous tryptophan increases IAA production. Root exudates are the main sources of tryptophan in soil. Several biosynthetic pathways for IAA production exist, sometimes in parallel in the same organism (Davies, 1995).

GA controls many aspects of plant growth and development including seed germination, seedling emergence, stem and leaf growth, floral induction and sex expression in plants (Crozier et al, 2000). Chemically, all gibberellins are tetracyclic diterpenoid carboxylic acids. There are,

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at least, 136 types of GAs identified from higher plants (128 species), 28 GAs from fungi (7 species), and only four GAs from bacteria (Joo et al., 2005). Detailed characterization at chemical, biochemical and genetic levels for GA_3 biosynthesis in *F. fujikuroi* has been reported (Tudzynski et al., 2002).

Plants produce low amount of GA_3, therefore microorganisms have been utilized for commercial production of GA_3. The fungi are not dependent on GAs for their development but produce and secrete large quantities of the compounds to modify the behavior of their host or as signaling factors towards the host plant (Bhattacharya et al., 2012). Besides *F. fujikuroi*, GA production had also been reported from other species of *Fusarium* such as, *F. solani*, *F. oxysporum*, *F. pallidoroseum F. proliferatum*, *F. sacchari*, *F. semitectum*, *F. subglutinans* and *F. verticillioides* (Srivastava et al., 2003; Zainuddin et al., 2008).

The term 'heavy metals' strictly refers to metallic elements which have a specific mass higher than 5 g/cm^3. Some of these metals, eg., Zn, Cu, Mn, Ni and Co are micronutrients necessary for plant growth, while Cd, Pb, As and Hg have no known biological function (Nies, 1999). Pollution of agronomic soils with heavy metals is one of the serious environmental concerns since the metals are not degraded biologically and persist in the environment indefinitely. There are various sources the metal contamination in soil. These include burning of fossil fuels, mining and smelting of metalliferous ores, application of fertilizers and pesticides, municipal wastes, sewage sludge amendments, effluents from industries like electroplating, leather tanning, wood preservation, pulp processing, steel manufacturing, etc (Congeevaram et al., 2007). Elevated levels of heavy metals not only affect soil microbial activity and crop production, but also threaten human health through transmission via food chain or contamination of ground or surface waters (Mclaughlin et al., 1999).

The present study was undertaken to evaluate IAA and GA productions by the *Fusarium* soil isolate and explore the potential of the isolate to produce phytohormones under metal stressed condition. The isolate could be proven to be of much use in agricultural sectors to increase the crop productivity.

2. MATERIALS AND METHODS

Collection of soil sample: A soil sample was collected from the rhizospheric region of the rice plant located in Rakhaldaspur village of Raninagar block I, Murshidabad district, West Bengal, India. The agricultural field was placed in close vicinity of Padma River near Indo-Bangladesh boarder region and cultivated for several crops throughout the year where no *Fusarium* diseases were reported previously.

Isolation and identification of fungus: The soil was screened for isolation of the fungi by dilution plate technique on selective peptone PCNB agar medium [composition (g/l): peptone 15, KH_2PO_4 1.0, $MgSO_4$, $7H_2O$ 0.5, PCNB 1.0, agar 20, pH 6] supplemented with streptomycin sulphate 1.0 g/l and neomycin sulphate 0.12 g/l. The plates were incubated at 28°C for 5-7 days until visible sign of colony growth occurred. Fungal isolate was identified by observing the colony morphology, sporulation and pigmentation on Czapek's Dox agar (CDA) medium. The isolate was further identified based on rDNA analysis. For this, genomic DNA was extracted and used as template for amplification of the rDNA region using the primer pair LROR (TCCGTAGGTGAACCTGCGG) and LR5 (GCTGCGTTCTTCATCGATGC). The amplified

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product was sequenced and the sequence was analysed using Nucleotide BLAST function at NCBI to find similarity of the sequence with nucleotide database.

Pathogenecity test: Effect of culture filtrate on germination of seeds of six plants such as chickpea (*Cicer arietinum*), black gram (*Vigna mungo*), cucumber (*Cucumis sativus*), chilli (*Capsicum* sp.), mustard (*Brassica* sp.) and paddy (*Oryza sativa*) was evaluated to check whether the isolate was pathogenic or not. The isolate was cultivated in CD broth for 14 days and their culture filtrate was used for the study. Ten seeds were taken for each experimental set up. Seeds were surfaced sterilized in 0.1% $HgCl_2$ solution for 1 min and washed thrice with sterile water. The surface sterilized seeds were then transferred to culture filtrate of the *Fusarium* isolates and kept at 4°C for overnight period. On the subsequent day, the seeds were placed on sterilized pre-soaked blotting paper kept within the petri dish. After five days, percentage of seed germination, length of radical and plumule were recorded. Vigour index was also calculated using following formula:

Vigour Index (VI)= Root length + Shoot length × Germination %

Assay of Indole Acetic Acid (IAA) production: The *Fusarium* isolate was tested for its ability to produce IAA in CD broth amended with L-tryptophan (1000 ppm) and incubated at 28°C for 14 days. 1 ml of culture filtrate and uninoculated broth (control) were mixed with 2 ml of Salkowski's reagent and incubated at room temperature for 25 min. The intensity of pink colour developed by the reaction was measured immediately at 530 nm (Gordon & Weber, 1951). Amount of IAA produced was calculated using the standard curve prepared with known concentration of pure IAA.

Assay of Gibberellin (GA) production: Efficacy of gibberellin production by the *Fusarium* soil isolate was assayed by growing the isolates in Czapek's Dox broth at 28ºC for 14 days and the amount of gibberellin in the culture supernatant was determined by spectrophotometric method using phosphomolybdic acid reagent (Graham & Henderson, 1961). One ml culture filtrate of the *Fusarium* isolate was taken out into 25 ml of volumetric flask, mixed with 15 ml of phosphomolybdic acid reagent and placed in a boiling water bath for 1 hour. After that, the temperature of the flask was reduced to room temperature and then final volume was made to 25 ml with distilled water. The absorbance of blue color developed was measured at 780 nm using distilled water as blank and the concentration was determined using a standard curve prepared from the standard solutions of gibberellins. Mycelial dry weight of the fungal isolate was also determined to make a correlation with the GA production.

Metal tolerance test: The *Fusarium* isolate was tested for its tolerance to the five heavy metals (Cd, Cu, Fe, Ni and Zn) present individually in the broth medium. For this, different sets of potato dextrose broth were prepared amended with increasing concentrations of individual metal at 50 ppm interval. One control broth set without metal was also prepared. Subsequently each broth was inoculated with the equal amount of mycelia disc from 7 days old pure culture of the isolate and incubated at 28°C for two weeks. Growth of the *Fusarium* isolate was monitored by measuring the mycelial dry weights. Tolerance was measured by observing minimum inhibitory concentration (MIC) and tolerance index (Zafer et al., 2007; Ezzouhri, 2009). Minimum inhibitory concentration (MIC) of the metal was considered as the lowest concentration of the metal that inhibited visible growth of the isolate. Tolerance index (TI) of the five metals was

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estimated at three concentrations (50, 100 and 150 ppm) and calculated as the percentage value of the ratio of the dry weight of treated culture to that of untreated culture.

Study of IAA production under heavy metal stress: IAA producing *Fusarium* isolate was tested for the ability to produce IAA in presence of the heavy metals (Cd, Cu, Fe, Ni and Zn). Five sets of CD broths were prepared amended with 50 ppm concentration of the respective heavy metals and 1000 ppm L-tryptophan as precursor. The fungal isolate was inoculated in the broths and incubated at 28ºC for 14 days. IAA production was estimated by Salkowski's regent.

Study of GA production under heavy metal stress: The potential GA producing *Fusarium* isolate was tested for the ability to produce GA in presence of the heavy metals (Cd, Cu, Fe, Ni and Zn). Five sets of CD broths were prepared amended with 50 ppm concentration of the respective heavy metals and the fungal isolate was inoculated in the broths and incubated at 28ºC for 14 days. GA production was estimated by phosphomolybdic acid regent.

3. RESULTS AND DISCUSSION

Isolation and identification of the fungus: On selective peptone PCNB agar medium the fungal isolate with characteristic morphology appeared. The isolate, designated as KUSF0301 was selected for further study. On CDA medium the fungal isolate showed white, circular, compact, smooth, fast growing, light green pigmentation (Fig. 1). It abundantly produced straight to falcate, dorsal side more curved than ventral, medium sized (16.25-26.25 µm X 3.12-3.75 µm) macroconidia having 3-4 septa with slightly curved apical cell and foot shaped basal cell (Fig. 1). Microconidia oval, elliptical; short sized (5.25-10.50 µm X 1.75-2.25 µm) having 0-1 septa. Chlamydospores were lacking. Molecular identification of the fungal isolate KUSF0301 was performed based on rDNA sequence analysis. 550 bp amplicon of rDNA region of the isolate was observed on agarose gel and a stretch of 518 bp had been sequenced. Search for sequence homology through nucleotide BLAST function in NCBI database was performed and maximum identity (100%) was found with the rDNA sequence of *F. solani* TVD (KF494114). Based on all these key specifics, the fungal isolate was identified as *Fusarium solani.* The partial rDNA region of the fungal isolate KUSF0301 had been submitted to the genebank under the accession no. MF136401 (Islam & Datta, 2023).

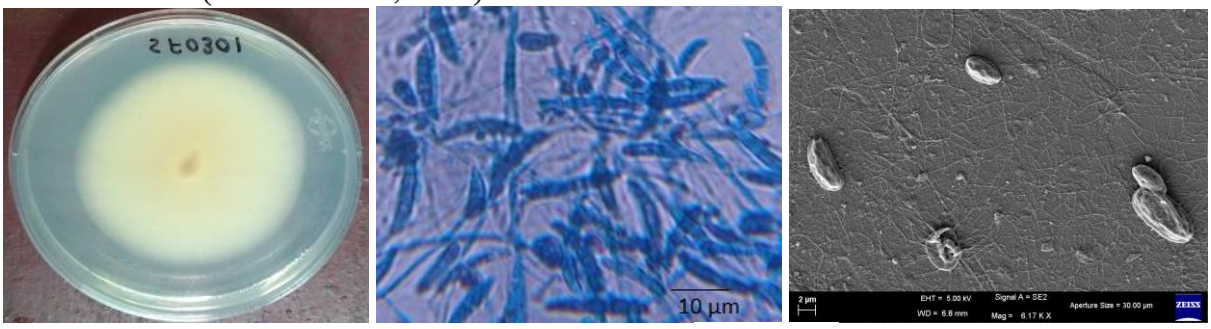

Fig. 1: Reverse side of growth of *Fusarium solani* KUSF0301 on CDA medium after 7 days (left); Microscopic field of *Fusarium solani* KUSF0301 showing macroconidia stained with cotton blue (middle); SEM photograph of a single macroconidium of *Fusarium solni* KUSF0301 (right) [Islam & Datta, 2023]

Pathogenecity test: Effect of culture filtrate on seed germination and vigor index of the tested seed plants have been depicted in table 1. Most of the seeds showed germination although

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percentage of germination varied among plants. The culture filtrate of the particular *Fusarium solani* MF136401 did not show any inhibitory effects on germination of the tested seed plants thus establishing the nonpathogenic nature of the isolate. Moreover, growth stimulatory effects of the fungal culture filtrate on the three plants viz., black gram, chili and paddy were evidenced by the increase in percentage of seed germination and vigor index. Seed germination in all the inoculated treatments was conspicuously comparable to the control (Fig. 2). There was also early onset of seed germination in few inoculated seeds as well.

Table 1. Pathogenecity Test of *Fusarium solani* KUSF0301

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
Fusarium equiseti MF803160	90	42.3	100	38.9	80	150.4	60	46.6	80	66.6	100	64.2
Control	80	23.2	100	28.4	70	119.7	60	40.2	70	38.5	80	16.2

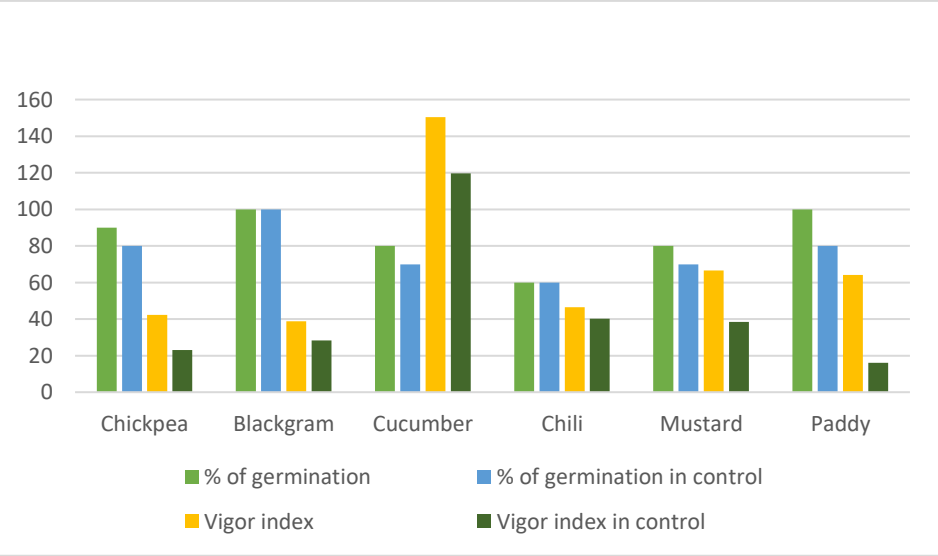

Fig. 2: Effect of culture filtrate of *Fusarium solani* KUSF0301 on germination and vigor index of seed plants

IAA and GA production by the *Fusarium* isolate:

Production of IAA and GA has been presented in table 2. After two weeks of incubation the *Fusarium* species produced 370 µg/ml of IAA and 4800 µg/ml of GA. Thus, the isolate was

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proven to be an efficient phytohormone producer. Interestingly, mycelial biomass production was also found to be improved with the concomitant increase in IAA & GA production.

Table 2: IAA and GA production by *Fusarium solani* KUSF0301

Isolate Number	Mycelial dry wt. (gm)	IAA production (µg/ml)	GA production (µg/ml)
Fusarium solani KUSF0301	0.121	370	4800

Minimum inhibitory concentrations and Tolerance index: The MIC value for cadmium, copper, iron, nickel and zinc of the *Fusarium* isolate was studied by growing them in PD broth with increasing concentration of each metal at 25 ppm interval. The resistance level of the isolate was independent against individual metal ions (Table-3) The isolate showed minimum resistance against Cd (175 ppm) and Zn (225ppm), followed by Ni (250 ppm), Cu (275 ppm) and Fe (350 ppm) with MIC values ranging from 175-350 ppm (Fig. 3).

Table 3.: Minimum inhibitory concentrations (ppm) of heavy metals of *Fusarium solani* KUSF0301

Isolate number	Heavy metal (ppm)				
	Cd	Cu	Fe	Ni	Zn
Fusarium solani KUSF0301	175	275	350	250	225

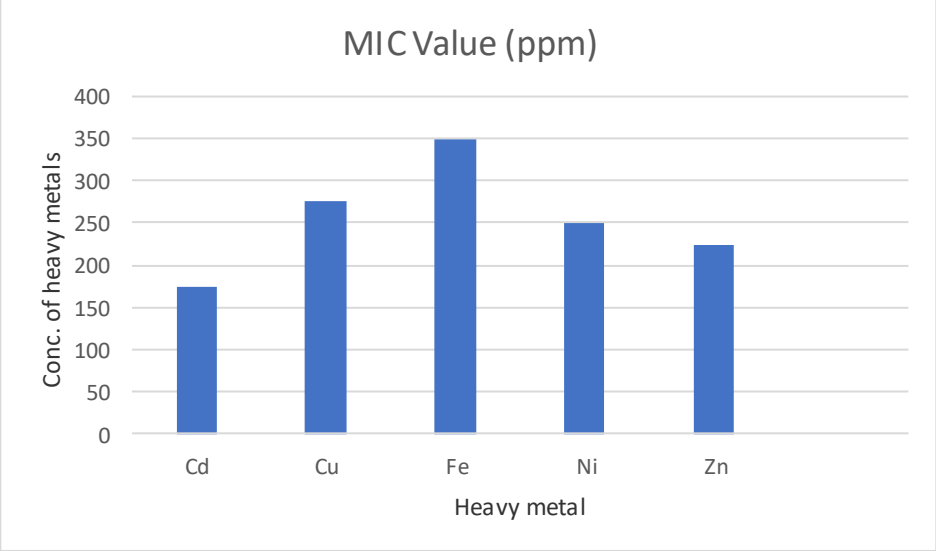

Fig. 3: Metal tolerance of *Fusarium solani* KUSF0301

IAA production under heavy metal stress: IAA producing ability of the *Fusarium* isolate was tested in presence of heavy metal at 50 ppm concentration (Table 4). Although the isolate could grow in Cd and Zn supplemented medium, its IAA production was completely checked under Cd and Zn stress (Fig. 4). In metal contaminated broth, IAA production varied from 110-310 µg/ml. Unaffected IAA production was observed in presence of Ni (310 µg/ml).

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GA production under heavy metal stress: GA production abilities of the *Fusarium* isolate also showed variations in the range of 3500-4250 µg/ml, in presence of the heavy metals at 50 ppm concentration. Although the isolate could grow in presence of Cd, it was unable to produce GA in the condition (Fig. 4). Isolate KUSF0301 also produced GA under heavy metal stress but comparatively in less amounts in presence of Ni (3500 µg/ml) and Zn (3700 µg/ml) than in the control broth. Though, in presence of both Cu and Fe, GA production by SF0301 was found to be on the higher range (Table 4).

Table 4: IAA and GA production by *Fusarium solani* KUSF0301 under heavy metal stress

Sl. No.	Heavy Metal	IAA production (µg/ml)	GA production (µg/ml)
1.	Cd	-	-
2.	Cu	110	4100
3.	Fe	130	4250
4.	Ni	310	3500
5.	Zn	-	3700
6.	Control	370	4800

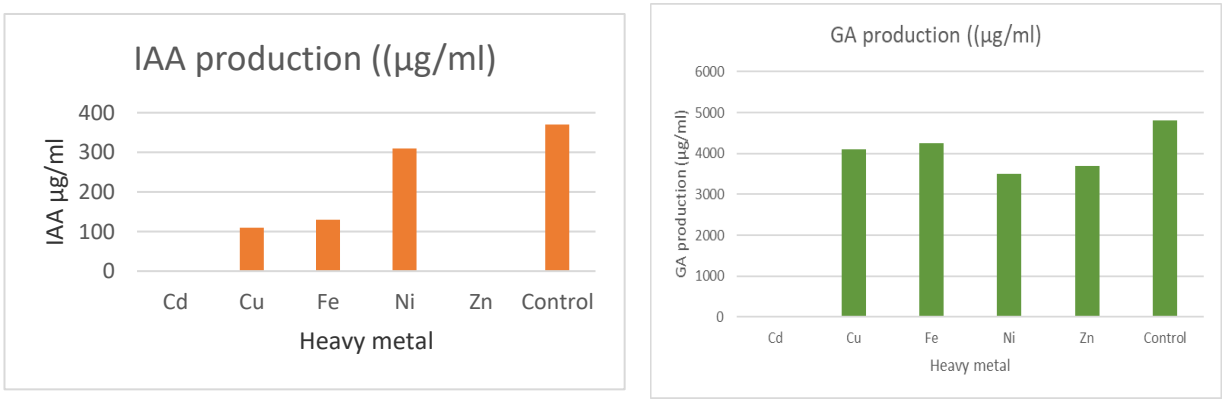

Fig. 4: Effect of heavy metals on phytohormone production by *Fusarium solani* KUSF0301

4. DISCUSSION

The presence of heavy metals in the agricultural soils in Murshidabad district would likely to be explored for isolation of heavy metal resistant fungi. The presence of heavy metals influenced the mycelial growth of the *Fusarium* soil isolates, depending on the type of metals and their concentration.

The order of tolerance of the metals by the isolates was recorded as Fe>Cu>Ni>Zn>Cd (Table 2). Similar results were obtained by Mittra et al. (2004) and Rai et al. (1995) who studied the influence of Pb, Cu, Zn and Cd in the media on the growth of *F. oxysporum*, and the greatest influence on the inhibition of growth was exhibited by Cd, whereas the least influence was exhibited by Zn. Cd can cause cellular damage and also inhibits DNA replication and appears to make it more susceptible to nucleolytic attack (Volesky & Holan, 1995). Isolates belonging to

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the genus *Fusarium* was reported to tolerate Cu up to 12.5 mM (Larini et al., 1997). *Fusarium* sp. was reported to accumulate Fe (51 mg/g dry biomass) but was unable to develop at 3.5 mM Fe (Levinskaite et al., 2009). Isolates of the genera *Aspergillus, Penicillium* and *Fusarium* showed MICs of Zn in the range 15 - 20 mM, 12.5 - 20 mM and 12.5 - 15 mM, respectively (Larini et al., 2009).

The tested *Fusarium* isolate exhibited IAA production at variable amounts in the presence of different heavy metals (Fig. 4; Table 4). But Cd completely checked IAA production at 50 ppm concentration in the tested *Fusarium* isolate. Inhibitory effect of Cd on IAA production was also reported by Singh et al. (2015) and Paul & Datta (2017). Also, Zn exerted toxic effects on IAA production in KUSF0301. *F. delphinoides* isolated from chickpea rhizosphere showed tryptophan dependent IAA production (Kulkarni et al., 2011).

KUSF0301 was tested with respect to their abilities to produce GA in presence of different metals in culture medium (Table 4; Fig. 4). Like the effects on IAA production, Cd could also pose its lethal effects on GA biosynthesis of the tested *Fusarium* isolate. The production of gibberellin was adequately on the higher range in presence of different heavy metals (3500-4250 µg/ml) except Cd. Hasan (2002) also reported gibberellin and auxin production by plant root-fungi and their biosynthesis under salinity-metal interaction.

5. CONCLUSION

The tested *Fusarium* soil isolate was found to be metal tolerant against Fe, Cu and Ni. Minimum inhibitory concentration was found least in case of Cd for the tested *Fusarium* soil isolate. The promising *Fusarium* isolate could exhibit plant growth promoting trait like phytohormone production (IAA and GA) even in metal stressed conditions. The findings of the present investigation highlighted that the soil isolate of *Fusarium* has great potential to enhance plant growth. The non-pathogenic, metal tolerant *Fusarium* soil isolate could be exploited as seed inoculants for improvement of crop yield in metal contaminated agricultural fields. However, this assessment of plant growth promotion by the *Fusarium* isolate needs further study under field conditions to confirm the present findings and also to recommend the isolate as bio-inoculants.

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