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Seasonal effect on the diversity of soil fungi and screening for arsenic tolerance and their remediation

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ABSTRACT

The seasonal variations were closely linked to climatic factors such as air temperature, rainfall, humidity, and other factors, all of which had a significant impact on soil characteristics, organic matter, and microbial population. The nutritional and physicochemical characteristics of their environment have an impact on soil microbe survival and dissemination. Heavy metal deposition in soil and plants, both edible and non-edible components, is linked to the consumption of heavy metal contaminated foods and the substantial health risks they provide. Seasonal diversity of soil fungi, as well as the screening of arsenic-resistant fungi and their ability to play a substantial role in bioremediation, was investigated in this work. The highest number of fungal species (17) was likewise found in the winter season, while the lowest number of species (11) was found in the summer. There were seven *Aspergillus* species, four *Penicillium* species, two *Alternaria* species, and single species of other fungi found. During the monsoon and winter seasons, the population of *Aspergillus niger* was at its peak. The genus *Penicillium*, on the other hand, reaches its peak number during the summer. Five fungi, *Aspergillus nidulans*, *A. niger*, *Aspergillus* sp. isolate HKK4, *Aspergillus* sp., and *Penicillium* sp., were found as arsenic tolerant. *Aspergillus* sp. isolate HKK4, which was isolated as arsenic tolerant and could tolerate more than 500 ppm of arsenic, outperformed all other fungus in terms of P-solubilization and arsenic removal.

1. INTRODUCTION

The North India's climatic condition shows great variation in seasonal changes. It became cold from November to mid-March, hot from April to June, and monsoon from July to September. These seasonal changes strongly associated with climatic factors such as air temperature, rainfall, and humidity which greatly influenced the soil characteristics, organic matter, and microbial population in soil [1,2]. Soil is the heaven of nutrients for appropriate plant growth and development, and it plays a crucial part in the successful management of profitable agriculture. Nutrients are transferred from the soil to plants and then to animals. River flooding and microbial decomposition of plant and animal residues supply fresh nutrients to the soil. Man-made sources such as manures and fertilizers are also used to keep soil nutrient levels stable [3]. The nutritional and physicochemical features of their habitat, such as organic matter content [4,5], pH [6] water content, and temperature, all influence their survival and dispersal of soil microbes [7]. In particular, exposure to ideal temperature and the frequency and intensity of precipitation affect the activity and diversity of fungi in dry and semiarid habitats; even little precipitation events can affect primary production, enhancing carbon input and hence decomposition [8,9]. Due to natural and anthropogenic activity, heavy metals are deposited in soil, absorbed, and transferred from soil to plants and then animals in the same way as nutrients are. The accumulation of heavy metals in soil and plants, both edible and non-edible portions, is directly linked to the intake of heavy metal contaminated foods and the serious health concerns they offer [10].

Arsenic (As) is a naturally occurring but dangerous element that can be found in rock, soils, water, air, and also deposited in biological tissues. It is one of the poisonous substances that pose a significant risk to a huge number of people [11,12]. The Asia, notably Bangladesh and West Bengal (India), has the most severe arsenic contamination in surface soil and water [13,14]. Humans, cattle, and crops have all suffered as a result of the use of these ground water sources for irrigation and drinking [15,16]. Arsenic-contaminated soils are remediated using soil microorganisms and plants. Soil microorganisms and plants are mostly used in the remediation of arsenic-contaminated soils. Metals are used by these microorganisms to obtain energy. Among the microbes, fungi are very important microbial community in soil's microbial diversity. In fungi, biosorption, bioaccumulation, methylation, and biovolatilization, among other mechanism, are important in arsenic remediation. Under aerobic conditions, As (V) enters the fungal cell through the phosphate transporter, whereas aquaporins and hexose provide a pathway for As (III) species to enter

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the cell transporter [17-20]. Many fungal species such as *Penicillium* sp., *Aspergillus* sp., *Gliocladium* sp., and *Scopulariopsi*s sp. reported for the purpose of arsenic remediation through the processes of precipitation, complexation, redox reactions, and nutrient availability, phosphate solubilizing microorganisms alter arsenic intake as well as stress mitigation [21].

The soil fungus communities in the study site's soil are characterized in this paper. One of the main goals of this research was to see if there were any variations in the occurrence of fungal communities, and another way was to investigate at the screening of arsenic-tolerant fungal communities and their involvement in toxicity mitigation. In this study, seasonal diversity of soil fungus was determined along with the screening of arsenic-tolerant fungi and their ability to play a significant role in bioremediation.

2. MATERIALS AND METHODS

2.1. Sample Collection

The biodiversity of below-ground fungal population was surveyed extensively. Three times every year, samples were collected. The first sampling was place during the monsoon season followed by the winter and summer seasons. Random sampling in a defined region of the field was done with a 1 m quadrat, and at least five samples were taken (four from corner and one from central). After removing 1–2 cm soils into zip-locked sterile poly bags, the sample's above foliage portions were removed, and the rhizospheric soils and roots were collected.

2.2. Physicochemical Properties of Soil

The physicochemical parameters of all collected soil samples were analyzed at the Motilal Nehru Institute of Farmer's Training Institute, Indian Formers Fertilizer Cooperative, Phulpur, Prayagraj. Inductive coupled plasma atomic emission spectroscopy (ICP-AES) was used to investigate the arsenic concentration in soil (Maker-Element XR, Model-Thermo Fisher Scientific, Germany). The sample was rinsed with double-distilled water and dried in a hot air oven (PID controlled) at 80°C for the measurement and quantification of arsenic content in plant and soil. The dry sample was crushed and sieved properly. One gram of sieved sample was placed in a 150 mL Erlenmeyer flask, and 5.0 mL of concentrated HNO_3 and 1.0 mL of H_2SO_4 were added, covered with a watch glass, and left for 24 h. This set up was then heated at 80°C on a hot plate for 1 h and then left to cool, after which 5 mL of $HClO_4$ was added and heated at 80°C until clear content was obtained. The content was diluted and filtered through Whatman filter paper after cooling at ambient temperature. By adding doubly distilled water, the final volume was increased to 10 mL. ICP-AES was used to determine the amount of arsenic in the soil sample (S.A.I.F. Laboratory, IIT Mumbai).

2.3. Determination of Fungi Diversity

Filamentous fungi were isolated using potato dextrose agar (PDA) medium by diluted plate method [22]. Ten grams of freshly collected soil samples were dissolved in 100 mL of double-distilled water and properly mixed. This solution was serially diluted up to 10^{-3} and 10^{-4}. Then, 0.5 mL of diluted solution was taken for the isolation of fungi on PDA. Inoculated Petri plates were incubated at 25 ± 2°C for 48–72 h. Fungal colonies were counted and identified on the basis of micromorphological characteristics using stained slides under high-power compound microscopy. Fungal morphology was studied macroscopically by analyzing colony features (color and texture) and microscopically by staining with fungal stain and observing conidia, conidiophores, and spore arrangement under a compound microscope. The fungus was identified and named using literature and also from the book "A Manual of Soil Fungi." [23].

2.4. Isolation and Characterization of Arsenic-Tolerant Fungi

Enrichment culture was used to isolate arsenic-tolerant filamentous fungus. Ten grams of soils were dissolved in 100 mL of sterilized distilled water and thoroughly shaken. The solution's supernatant was serially diluted up to 10^{-4} dilution, and 1.0 mL was placed on a Petri plate containing PDA medium supplemented with As (III) (0, 25, 50, 100, 200, 300, 400, 500, 1000, and 2000 ppm) individually in the form of $NaAsO_2$. Filter sterilized As (III) solution was added into autoclaved PDA medium. Non-inoculated Petri plate served as the control. Streptomycin (30 mg/liter) was added to the culture medium to suppress bacterial growth. Finally, the fungal colonies that appeared on the PDA containing As (III) (500 ppm) were pure cultured on a slant and stored at 4°C in a deep freezer. The "A Manual of Soil Fungi" was used to identify and name the isolated fungi (Gilman, 1957).

2.5. *In vitro* Arsenic Removal Test of Screened Arsenic-Tolerant Fungi

The ability of tolerant fungus to reduce arsenic was investigated using the shaking flask culture method. PDB medium (100 mL) was placed in seven Erlenmeyer flasks, each containing 15 ppm As (III) in the form of $NaAsO_2$. Six fungi were incubated in six flasks, with one flask left blank to check for any abiotic As (III) to As (V) reduction. A 1.0 cm fungal disc with the same beginning inoculum was collected from the neighboring margin of a 4-day-old pure subculture. All flasks were incubated for 21 days at 26 ± 2°C in an orbital shaker incubator at 120 rpm. The filtrate was centrifuged at 15,000 rpm in an ultracentrifuge after the fungus culture in the flask was filtered using Whatman paper no. 1. Atomic Absorption Spectrometer (iCE 3000 Series, Model 3500 AAS, Thermo Scientific, UK) from C.I.L., Botany Department, University of Allahabad, was utilized to examine the clear filtrate.

2.6. *In vitro* Phosphate Solubilizing Activity of Screened Arsenic-Tolerant Fungi

Phosphate solubilizing capacity of tolerant filamentous fungus was determined by measuring the available monophosphate in the growing conditions. Pikovskaya's (PKV) broth medium was used to investigate the phosphate solubilizing activity. In duplicate, 100 mL PKV broth medium with 5.0 g $Ca_3(PO_4)_2$ was placed in seven 250 mL Erlenmeyer's flasks. Each flask was infected with 1.0 cm diameter fungal discs from a fresh culture of each fungus. The flasks were incubated at 26 ± 2°C for 192 h in an orbital shaker incubator at 80 rpm. Six flasks were filled with fungus, while one flask was left blank as a control.

Whatman No. 1 filter paper was used to filter incubated cultures. A 10 ml chloromolybdic (ammonium molybdate [15 g] was dissolved in warm double-distilled water [400 ml], agitated well, then 10 N HCl [342 ml] was added to it and the final volume was elevated to 1000 ml) and 20 ml double-distilled water were added to a 5 ml aliquot of culture filtrate. Five drops of chlorostannous acid (2.5 g $SnCl_2.2H_2O$ mixed in 10 ml concentrated HCl and added double-distilled water to make the final volume to 100 ml) were added to the mixture after thorough shaking, and the volume was raised to 50 ml. The optical density of the solution was measured using a spectrophotometer at 660 nm, and the concentration of P in the culture filtrate was calculated using a standard

curve made up of P solutions with known concentrations. Hundred ppm KH_2PO_4 stock solutions were used to produce the standard curve (0.4390 g KH_2PO_4 dissolved in double-distilled water and raised volume up to 1000 ml). This solution was used as a stock solution, and different concentrations were made using the stock solution.

3. RESULTS

3.1. Physicochemical Characteristics of the Soil

The physicochemical parameters of soil samples collected from the chosen location. The soil samples' elemental analysis revealed that the soil is moderately alkaline, with a pH of 8.1. The electrical conductivity (0.49) was within the usual range. The soil's nutritional level was quite low. Organic carbon (0.21) and phosphorus (16 kg/ha) levels were both extremely low. The potassium level was moderate (168 kg/ha) as measured in K_2O, whereas Zn was 2.0 kg/ha, Cu was 1.44 kg/ha, Fe was 12.40 kg/ha, and arsenic was 6.68 mg/kg.

3.2. Diversity of Fungi

The diversity of soil fungi shows its maximum population in winter season and lowest in monsoon season. The diversity of fungal population is given in Table 1. Maximum numbers of fungal species (17) were also recorded in winter season and the lowest numbers of species (11) were recorded in summer. *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus* sp., *Penicillium* sp., *Penicillium* sp. 1, and sterile mycelia had 100% frequency [Table 2], while *Alternaria alternata* and *Cunninghamella verticillata* had only 33.33%. Seven *Aspergillus* sp., four *Penicillium* sp., two *Alternaria* sp., and single species of other fungi were found during this survey. *A. niger* population stood maximum during monsoon and winter season. However, in the summer season, genus *Penicillium* shows maximum population.

3.3. Isolation of Arsenic-Tolerant Fungi

Arsenic-tolerant fungi were isolated by enrichment culture method, from the culture medium amended with 500 ppm of sodium arsenite. These arsenic-tolerant fungi were identified as *Aspergillus nidulans, A. niger, Aspergillus* sp. isolate HKK4, *Aspergillus* sp., and *Penicillium* sp. [Figure 1].

3.4. *In vitro* Arsenic Bioremoval Capacity of Isolated Arsenic-Tolerant Fungi

*In vitr*o arsenic removal capacity determination of isolated arsenic-tolerant filamentous fungi in shake flask culture method. Maximum *in vitro* As (III) removal was recorded by *Aspergillus* sp. isolate HKK4 (6.4 ppm) followed by *Aspergillus* sp. (6.1 ppm), *A. niger* (6.0 ppm), *A. nidulans* (5.2 ppm), and *Penicillium* sp. showed minimum (3.2 ppm), however, some amount (1.1 ppm) also removed abiotically during test [Table 3].

3.5. *In vitro* Phosphate Solubilizing Potentiality of Isolated As-tolerant Fungi

In vitro P solubilization (the tricalcium phosphate [$Ca_3(PO_4)2$]) potentiality of arsenic-tolerant filamentous fungi in the PKV medium recorded maximum by *Aspergillus* sp. isolate HKK4 (4.9 ppm) followed by *Aspergillus* sp. (4.4 ppm), *A. niger* (3.42 ppm), *Penicillium* sp. showed minimum (3.11 ppm), and *A. nidulans* (2.9 ppm), respectively, however, some amount (0.26 ppm) solubilized abiotically also during experiment [Table 4].

Table 1: Diversity of soil fungi under different seasonal conditions at study site, Prayagraj

Fungal species	Population of fungi (1 X 10^4 g^{-1} oven dry soil)											
	Monsoon				Winter				Summer			
	I	II	III	Average	I	II	III	Average	I	II	III	Average
Alternaria alternata	-	-	-	-	2.0	2.0	6.0	3.33	-	-	-	-
Alternaria sp.	-	2.0	6.0	2.7	4.0	6.0	2.0	4.0	-	-	-	-
Aspergillus flavus	-	-	-	-	12.0	4.0	2.0	6.0	4.0	-	4.0	2.67
Aspergillus fumigatus	4.0		8.0	4.0	4.0	4.0	4.0	4.0	2.0	4.0	-	2.0
Aspergillus nidulans	-	-	-	-	4.0	6.0	8.0	6.0	6.0	4.0	-	3.33
Aspergillus niger	6.0	8.0	8.0	7.33	10.0	8.0	10.0	9.33	8.0	6.0	6.0	6.67
Aspergillus terreus	2.0	6.0	4.0	4.0	6.0	6.0	2.0	4.67	6.0	4.0	-	3.33
Aspergillus sp. isolate HKK4	6.0	2.0	4.0	4.0	6.0	4.0	4.0	4.67	6.0	10.0	8.0	8.0
Aspergillus sp.	-	4.0	4.0	2.67	8.0	6.0	4.0	6.0	2.0	6.0	4.0	4.0
Cladosporium sp.	-	-	-	-	4.0	2.0	2.0	2.67	2.0	4.0	-	2.0
Cunninghamella verticillata	-	-	-	-	-	2.0	4.0	2.0	2.0	-	-	0.67
Curvularia sp.	-	-	4.0	1.33	4.0	4.0	2.0	3.33	-	-	-	-
Fusarium oxysporum	4.0	2.0	4.0	3.33	4.0	6.0	4.0	4.67	-	-	-	-
Mucor sp.	4.0	4.0	6.0	4.67	4.0	-	2.0	2.0	-	-	-	-
Penicillium chrysogenum	6.0	4.0	8.0	6.0	-	-	-	-	6.0	8.0	12.0	8.67
Penicillium sp.	4.0	6.0	8.0	6.0	8.0	4.0	6.0	6.0	8.0	6.0	8.0	7.33
Penicillium sp. 1	4.0	-	4.0	2.67	4.0	8.0	6.0	6.0	10.0	8.0	4.0	7.33
Trichoderma sp.	-	-	-		2.0	4.0	6.0	4.0	2.0	2.0	-	1.33
Sterile mycelia	-	2.0	4.0	2.0	4.0	4.0	6.0	4.67	2.0	2.0	-	1.33
Total				50.7				83.34				58.66

Table 2: Distribution and frequency of fungi at study site, Prayagraj

Fungi	Distribution			Frequency (%)
	Monsoon	Winter	Summer	
Alternaria alternata	–	+	–	33.33
Alternaria sp.	+	+	–	66.66
Aspergillus flavus	–	+	+	66.66
Aspergillus fumigatus	+	+	+	100
Aspergillus nidulans	–	+	+	66.66
Aspergillus niger	+	+	+	100
Aspergillus terreus	+	+	+	100
Aspergillus sp. isolate HKK4	+	+	+	100
Aspergillus sp.	+	+	–	66.66
Cladosporium sp.	–	+	+	66.66
Cunninghamella verticillata	–	+	–	33.33
Curvularia sp.	+	+	–	66.66
Fusarium oxysporum	+	+	–	66.66
Mucor sp.	+	+	–	66.66
Penicillium chrysogenum	+	–	+	66.66
Penicillium sp.	+	+	+	100
Penicillium sp. 1	+	+	+	100
Trichoderma sp.	–	+	+	66.66
Sterile mycelia	+	+	+	100
Total fungal species	14	17	12	

Table 3: *In vitro* arsenic removal ability of screened fungi

Filamentous fungi	Initial As (III) (ppm)	Residual As (III) (ppm)	As (III) removal (ppm)
Control	15	13.9	1.1
Aspergillus nidulans	15.0	9.8	5.2
Aspergillus niger	15.0	9.0	6.0
Aspergillus sp. isolate HKK4	15.0	7.9	6.4
Aspergillus sp.	15.0	10.9	6.1
Penicillium sp.	15.0	12.3	3.7

Table 4: *P* solubilizing potentiality of arsenic-tolerant fungi

Filamentous fungi strains	Available monophosphate in medium (ppm)
Control	0.26
Aspergillus nidulans	2.9
Aspergillus niger	3.42
Aspergillus sp. isolate HKK4	4.9
Aspergillus sp.	4.4
Penicillium sp.	3.11

4. DISCUSSION

The survey area was in Prayagraj, close to the Ganga River. This location is frequently flooded by Ganges water every year. The Ganges water was regularly mixed with treated waste water from a sewage

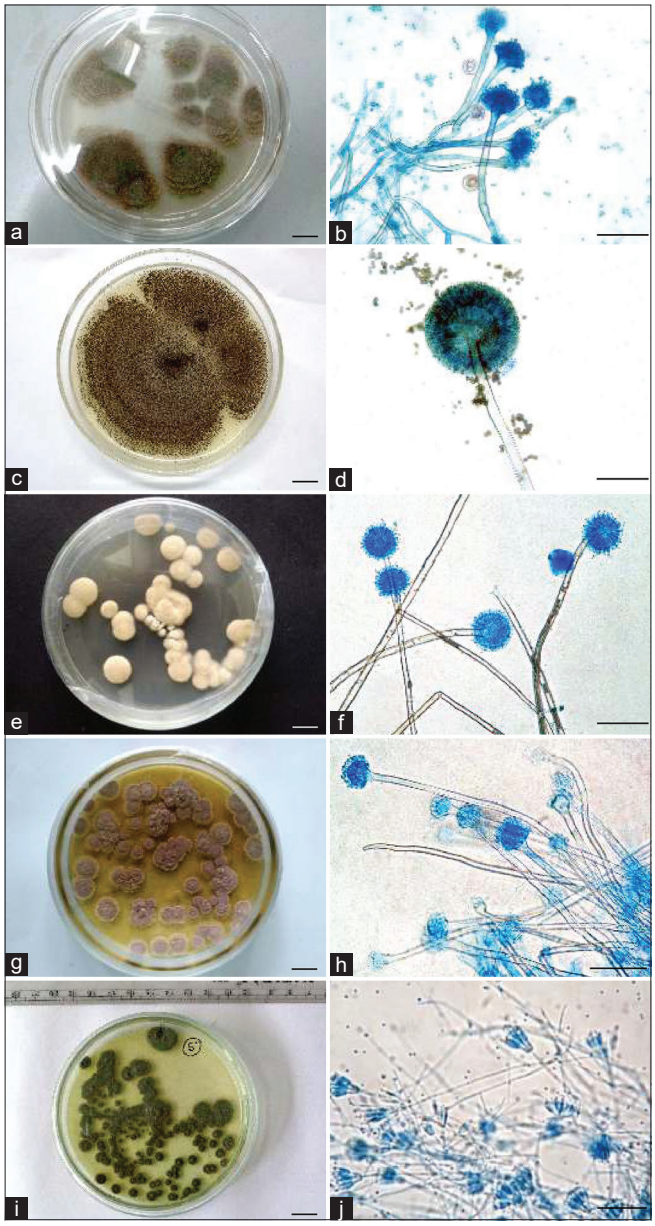

Figure 1: Arsenic-tolerant fungi isolated through enrichment culture method; (a and b) *Aspergillus nidulans,* (c and d) – *A. niger*, (e and f) – *Aspergillus* sp. isolate HKK4, (g and h) – *Aspergillus* sp., (i and j) – *Penicillium* sp. Scale bar (Petri plate) = 1 cm, scale bar (microscopic photos) = 50 µm

treatment plant near Buxi Bandh. The soil at the survey location is from the middle Ganga plain. The electrical conductivity was average, but the carbon concentration was low. Arsenic content may come from a rock weathering aquifer, and this way accumulates in soil every year [24-26].

In this study, seasonal diversity of soil fungal communities was determined. The diversity of filamentous soil fungi population was recorded in the soil of surveyed site. Moderate numbers of fungal species were recorded. Dominating genera were *Aspergillus* and *Penicillium*. The seasonal fluctuations were recorded on population dynamics; maximum population was in winter season. The diversity of filamentous fungi is influenced by optimum temperature, precipitation, nutrient status, and available organic material in soil [8,27,28]. He *et al*. (2017) conducted a comparative study

on soil fungal population in temperate and subtropical zones with respect to seasonal variation and concluded that vegetation and plant diversity, pH, nutrient conditions, etc., are the main factors for soil fungal community composition. Further, they reported that the temperate regional forest soils have more fungal diversity [28]. Voříšková *et al*. (2013) reported the similar results of fungal diversity in relation with seasonal changes and plant phenology [29]. Both bacterial and fungal communities of soil are much influenced by edaphic environmental factors and driven ecosystem process [30]. Various studies also have the similar results and mentioned that in tropical climatic conditions, *Penicillium* and *Aspergillus* were dominated genera [31-33]. In addition, *Aspergillus* and *Penicillium*, both are facultative saprophytes, the wet condition/amount of water in soil helps in sporulation [33,34].

Five filamentous fungi, namely, *Aspergillus nidulans*, *A. niger*, *Aspergillus sp*. isolate HKK4, *Aspergillus sp*., and *Penicillium sp*., were shown to be capable of tolerating up to 500 ppm sodium arsenite in the medium during the investigation. In addition, an *in vitro* arsenic bioremediation test was carried out. *Aspergillus* sp. isolate HKK4 eliminated up to 6.1 ppm of arsenic. The heavy metals are bound and checked by the fungal cell wall, which is made up of chitin and various H^-, N^-, and O^- functional groups found both inside and outside the cell wall [35,36]. Arsenic-resistant fungi absorb and store it in their cells, and their biomethylation is also well recognized, being transformed into various organic molecules such as monomethyl amine (MMA), dimethylarsinic acid (DMA), and trimethylarsine oxide, as well as gaseous arsines being released directly into the atmosphere. The arsenic reductase, GSH glutathione, monomethylarsonic acid reductase, and monomethylarsonic acid transferase enzymes were abundant in the arsenic-resistant fungi [37-40].

In the test of phosphate solubilizing activity *in vitro* conditions, the highest value (4.9 ppm) was found in *Aspergillus* sp. isolate HKK4. Both phosphorus and arsenate are analogs of one another. Phosphate ions in soil interact with arsenate, reducing its absorption and translocation [41]. Phosphorus is also very important for plant growth and reproduction. The availability of "P" for growing plants is increased by "P" solubilizing fungus [42]. Phosphatases [43,44] and organic acids [45] are secreted by various *Aspergillus* species, which facilitate in the breakdown of tricalcium phosphate and make it available for plant growth. Other researchers came to similar results. According to Edvantoro *et al*. (2004), adding methylating fungi to polluted soils (1390 mg As/kg) can boost arsenic volatilization rates 8-fold [46]. According to Čerňanský *et al*. (2009), *Neosartorya fischeri* was more efficient arsenic volatilizes than *Aspergillus clavatus*, while *A. niger* was the least capable of the three species [47]. Hiremath *et al*. (2016) identified *Aspergillus flavipes* SIT-CH-4 from Pavagada Taluk, Tumkur District, Karnataka, India, as one of the fluoride-resistant fungal species [36]. Youssef *et al*. (1987) investigated *Aspergillus flavipes* extracellular phytase activities in the presence of several inhibitors, including sodium arsenate and sodium arsenite (0.01 mg/ml), and found that enzymatic activities were 29.4% and 26.5%, respectively [48].

In heavy metal contaminations, Sharaf and Alharbi (2013) found *Aspergillus* to be the most prevalent genus [49]. Guimarães *et al*. (2019) investigated the capacity of *Penicillium* sp. and *Aspergillus* sp. isolated from paddy soil to produce volatile arsenic species (57.8% and 46.4 percent, respectively) in an *in vitro* trapping system, and found that TMA was the most abundant volatile species, followed by MMA and DMA [50]. Maheswari and Murugesan (2009 and 2011) isolated *Aspergillus nidulans* and *Aspergillus flavus* from arsenic polluted soil and examined their roles in its remediation from soil and aqueous medium [51,52]. Singh *et al*. (2015) isolated and discovered 15 fungal strains that sustained a concentration of 1000 g/l arsenate and studied them further for arsenic remediation [53]. Only seven fungal species, including *Aspergillus oryzae* FNBR L35 and *A. nidulans* FNBR LK1, were shown to be acceptable for plant growth promotion in pot conditions.

5. CONCLUSION

Survey site was a naturally disturbed habitat, with low above-ground and below-ground diversity. Saprophytic fungi were dominant qualitatively as well as quantitatively. In the soil, clay-silt was deposited and soil was highly sandy (entisol) low in phosphorus (P) and organic matter. The genus, *Aspergillus* and *Penicillium,* was the dominant genera among all isolated fungal species. The climatic seasonal changes affect the diversity, occurrence, as well as population density of soil fungi. The maximum fungal species was reported from the winter season. *Aspergillus* sp. isolate HKK4 isolated as arsenic-tolerant which tolerated more than 500 ppm and also performed best among all the screened fungi for P solubilization as well as arsenic removal ability. The fungi can be the efficient tool for the remediation of arsenic contamination from the agricultural fields such as the isolated strain of *Aspergillus* sp. isolate HKK4 shows their significant potency in arsenic remediation and tolerance. *Aspergillus* sp. isolate HKK4 can develop as the potent arsenic bioremediation tool after further studies in the future.

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7. AUTHORS' CONTRIBUTIONS

Dheeraj Pandey and Harbans Kaur Kehri designed the experiment and conducted it. All other authors contributed significantly to participate in the drafting of the paper or critically revised it for key intellectual content material and agreed to submit to the present journal.

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9. CONFLICTS OF INTEREST

The authors declare that they do not have any conflicts of interest.

10. ETHICAL APPROVALS

This experiment does not conduct animal experiments.

11. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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