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MICROBIOLOGY

Diversity of Arbuscular Mycorrhiza Associated with Long Term Wastewater Irrigation in the Peri-urban Soil of Varanasi

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ABSTRACT

Heavy metals introduced into soil by irrigating with sewage effluent can influence the soil's microflora and, in particular, the profusion, miscellany, and activity of arbuscular mycorrhizal fungi. This study focused on the spore density, species abundance and diversity of arbuscular mycorrhizal fungi in the heavy metal affected soil in the peri-urban areas of Varanasi, on inceptisol after long-term irrigation. Identification through spore morphology showed existence of three species in the rhizosphere of fifteen crop species widely grown in the region**.** The physico-chemical analysis of the native soils revealed that they were neutral to alkali pH ranging from 7.3 to 8.9 and habituated three different species of AM fungi including *Glomus mosseae, Glomus fasciculatum* and *Glomus intraradices*. Spore density in samples ranged from 56 to 330 spores 100 g-1 soil. Species richness of AMF ranged from 2 to 3. Shannon–Weiner diversity index ranged from 0.497 to 1.053.

Highlights

- Heavy metal alters the composition and activity of mycorrhizal communities.
- The richness and microbial diversity decreased with increasing concentration of heavy metal.

Keywords: Waste water irrigation, AM spore, heavy metal, Species richness, Shannon–Weiner diversity index

Water demand has exceeded the reliable supply of surface water and renewable ground water due to rapid growth in municipal and industrial use in the most *intensively* farmed zone of the country. The agricultural sector is the foremost consumer of water in using two-thirds of accessible resources. The emergent struggle for scarce water resources, coupled with laws restrictive ground water pumping, has led to deployment of low quality water in irrigated agriculture in the Indogangetic plains (Kumar and Rakshit 2012). However, applying waste water to arable lands also involves convinced environmental and agricultural risks (Sharma *et al.* 2008). Waste water varies from fresh water with elevated contents of electrolytes, dissolved organic matter, suspended solids, and biochemical and chemical oxygen demand. These diverse constituents in the applied water can affect soil physico-chemical and biological properties. Increased the amount of heavy metals could be toxic for soil microorganisms. Although the concentration of heavy metals in waste water are low, long-term use of these waste waters on agricultural lands often results in the build-up of elevated levels of these metals in soils. Among soil microorganisms Arbuscular Mycorrhizal Fungi (AMF), an important biotic component of agricultural soils are known to play a key role in the mobilization and immobilization of metal cations (Rai *et al.* 2013; Pal *et al.* 2015; Pal *et al.* 2016; Rakshit and Ghosh, 2009), thereby changing their availability to plants by effectively enlarging the

rhizosphere. AMF occur in almost all habitats and climates , including in disturbed soils such as those derived from mine activities, but soil degradation usually produces changes in the diversity and abundance of AMF populations (Rakshit *et al.* 2016). AMF populations are decisive during and after soil disturbance because of their role in the establishment and survival of plants. Thus, changes in the diversity of their population produced by the application of high amounts of metal are expected to interfere with the possible beneficial effects of this symbiotic association, since reestablishment of AMF populations is slow.

However, only a small number of studies have been carried out involving interactions between AMF and metals as a basis of soil disturbance. The majority of the results already obtained derive from laboratory and pot experiments, with metal salts used as the source of heavy metals, which are not very representative of natural field conditions, under which metals usually accumulate in a less-available chemical form. Heavy metals can delay, reduce, and even completely eliminate AM colonization and AMF spore germination in the field.

On the other hand, crops raised on the metal-contaminated soils may accumulate metals in sufficient quantities to cause clinical problems both to animals and human beings consuming these metal rich plants. To our knowledge, no studies have been reported on the long-term effects of increasing concentrations of waste water on the diversity of mycorrhiza. In this context, the present study was undertaken to assess how AM fungal diversity is influenced by the addition of waste water for an extended period.

MATERIALS AND METHODS

The experiment was conducted at an urban fringe of subtropical area of Varanasi city (Fig. 1), situated in the eastern gangetic plain (25 °18′ N latitude and 83 °01′E longitude and 76 m above the sea level) of northern India with an average annual rainfall of 1100 mm and a mean annual temperature ranges between 20-42°C and 9-28°C respectively. This field site has been contaminated by surface application of sewage sludge and surface irrigation with wastewaters generated from domestic sewage, effluents discharged from small scale fabric, plastic, battery industries, dyeing, metal plating, bicycle tires and heavy agricultural equipments located in the urban areas of Varanasi since the 1990s.

Soil samples were collected in triplicate from rhizosphere of fifteen crop species at a depth of 0–30 cm and combined to give approximately 500 g soil .Roots were separated and then 100 g air-dried soil was employed for extraction of *Arbuscular mycorrhizal* fungal spores. Rest were air dried, crushed, and passed through 2-mm-mesh sieve and stored at ambient temperature before analysis of soil properties and concentrations of heavy metals by standard soil analysis technique. The plant available Cd and Ni were extracted by DTPA solution and analysed in atomic absorption spectrophotometer. The physicochemical parameters are analysed using statndard procedures as detailed in Jackson (1973). Plant available Cd and Ni were extracted by DTPA solution and analysed in atomic absorption spectrophotometer. Metal concentrations and the selected soil properties are showed in Table 1. According to the Indian National Standards Institution (Awasthi, 2000), this soil is seriously contaminated with Cd as described in previous studies (Sharma *et al.* 2008).

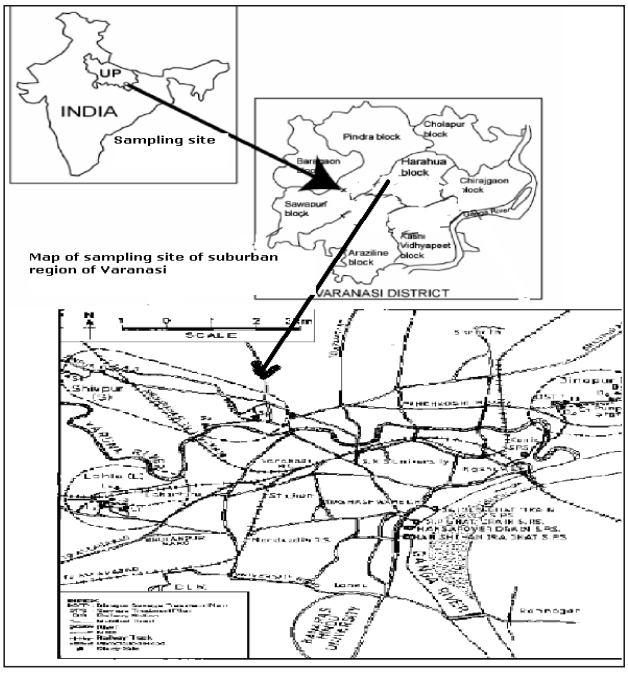

Fig. 1: Map of study sites in suburban region of Varanasi

Arbuscular mycorrhiza spore extraction, identification and spore count

AMF spores were isolated from 100 g of soil by the wet sieving using two sieves with aperture

sizes of 425 and 63 µm and decanting method (Gerdemann & Nicolson 1963), followed by sucrose centrifugation using a 1.17 M sucrose solution at a speed of 2000 rpm for 5 min. After centrifugation, the supernatant was poured through 50-mm-pore-size mesh and quickly rinsed with tap water. Spores were counted with a Don caster dish under the dissecting microscope and grouped according to morphological characteristics. Intact AM fungal spores were examined and counted under stereo microscope and identifications were made by observing diagnostic characteristics such as spore wall, colour, size and type of hyphal attachment with the help of (Schenck and Perez 1990) and were compared to the morphological descriptions of species presented on the International Culture Collection of Vesicular-Arbuscular Mycorrhizal Fungi (INVAM) webpage (http://invam.caf.wvu.edu).

Species richness and Diversity index

Number of individuals spore for each species was counted from the sample in the Microscopic field. Species richness is calculated by Number of species found in a 100 g soil sample. AM fungi diversity was evaluated using the Shannon-Weiner diversity index which has two main components, evenness and number of species (Shannon & Weiner, 1963). The Shannon–Weiner index (*H'*) was calculated according to the formula $H' = -\Sigma(ni/N) \log2(ni/N)$, where *ni* represents individuals of a species and *N* represents the total number of species.

All the data are presented in terms of means and standard error of triplicates. Data on AMF spore count in response to different soil properties were subjected to one sample t test.

RESULTS AND DISCUSSION

Soils of the experimental sites were neutral to alkaline with a pH value higher than 7.5 (Table 1). Experimental sites recorded OM 0.77 to 3.52 g kg^{-1} and total N of 0.11-0.15% respectively (Table 1). The soil is low in organic carbon, medium to high in phosphorus and medium to low in potassium content The 2:1 extract of soil exhibited low EC. Continuous application of waste water to the soil led to higher concentrations of heavy metals (mg kg^{-1}) in the soil with DTPA (diethylenetriaminepentaacetic acid) -extractable Ni and Cd ranged between 16.50-20.85 and 5.7-6.75 respectively. Among the two heavy metals studied Cd in the soil were above the permissible limits of Indian and EU standards.

In total, 1899 AM fungi spores were derived using wet sieving and sucrose gradient centrifugation methods from fifteen different crops rhizosphere soil. Spore density in samples ranged from 56 to 330 spores 100 g^{-1} soil (Table 1). Maximum spore density was observed in marigold rhizosphere and minimum in cauliflower rhizosphere. The soil appear to have accumulated large quantities of heavy metals as a result of the irrigation so that concentrations have increased and now approach the thresholds for healthy soil with reference to Cd. The differences between soil samples were comparatively small. The lowe values in cauliflower may be due to the presence of fungitoxic compounds in root cortical tissue or in root exudates that may reduce susceptibility of plants to mycorrhization (Tester *et al.* 1987). The total number of AMF spores strongly decreased with increasing amounts of heavy metals, but the AMF propagules never disappeared completely in soils irrigated with waste water for the last twenty five years, suggesting a

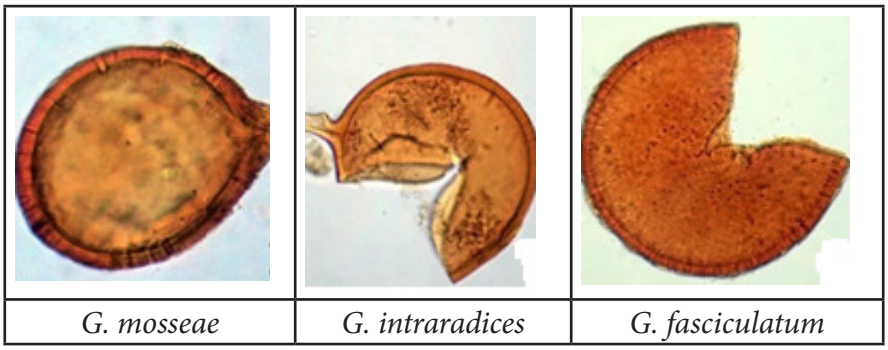

Fig. 1: Dominant arbuscular mycorrhizal fungal species isolated rhizosphere of different crops

Table 1 : Physicochemical and microbiological properties of rhizosphere soil.

		pH 1:2.5	EC µSm^{-1}	OM g kg^{-1}	Total–N %	Av P mgkg^{-1}	Av K mgkg^{-1}	DTPA extractable Cd* (mg kg^{-1})	DTPA extractable Ni* (mg kg^{-1})	Spore density				Species richness	H index
											Glomus mosseae	Glomus fasciculatum	Glomus intraradices		
Cereals	Rice	8.1	0.31	0.77	0.15	8.9	77	6.75	20.85	94	70	10	14	3	0.603
	Sorghum	7.7	0.23	0.85	0.12	8.2	80.1	5.9	17.6	163	95	39	29	3	0.641
	Barley	7.8	0.21	0.89	0.11	8.1	79.8	6.1	18.1	160	90	50	20	3	0.664
Vegetables	Bajra	7.9	0.2	0.82	0.12	8.3	80.1	6.1	19.1	151	85	34	32	3	0.739
	Palak	8.2	0.21	2.15	0.14	8.4	76	6.3	19.4	85	52	-	43	2	0.710
	Papaya	8.2	0.25	3.25	0.11	8.1	82.3	5.9	17.3	166	105	24	37	3	0.497
	Turmeric	7.9	0.1	3.12	0.14	8.2	75	6.4	19.3	84	40	-	44	2	0.746
	Raddish	8.98	0.316	1.25	0.14	8.9	71	6.5	19.6	71	35	25	11	3	0.871
	Okra	7.9	0.15	1.2	0.13	8.3	80	6.1	19	154	80	38	36	3	1.053
	Cauliflower	7.8	0.21	1.1	0.15	8.9	62.5	6.75	20.15	56	34	-	22	2	0.669
Flowers	Marigold	7.73	0.072	1.25	0.11	8.1	82.1	5.7	16.5	330	175	50	105	3	0.140
	Hibiscus	7.73	0.09	2.16	0.15	8.5	74	5.9	17.1	62	24	12	26	3	0.832
	Jasmine	7.8	0.09	1.78	0.14	8.1	69	5.8	17.3	105	70	18	17	3	0.914
	Rose	8.1	0.11	1.56	0.15	8.1	65.3	5.8	17.2	73	24	21	28	3	0.917
	Bela	7.8	0.08	2.1	0.13	8.6	78.5	6	17	145	85	39	21	3	0.738
	Range	7.73-8.98	0.072-0.316	0.77-3.52	0.11-0.15%	8.1-8.9	62.5-82.3	5.7-6.75	16.50-20.85	56-330	24-175	10-50	11-105	2-3	0.140-1.053
	Mean	8.0	0.2	1.6	0.1	8.4	75.5	6.1	18.4	125.28	69.21	27.92	28.85	3	0.746
	SD	0.3	0.1	0.8	0.0	0.3	5.9	0.3	1.3	71.72	41.49	12.61	23.52	0.425	0.229

**The standard values of heavy metals i.e., Cd and Ni are 0.8 and 50 mg kg-1 respectively for soils intended for agricultural uses with pH ranging from 6.5 to 7.5 based on Indian National Standard .*

certain adaptation of these indigenous AMF to such environmental stress. The rhizosphere of flower crops contained more spores than the cereals and vegetables. Three dominant morphospecies of AM fungi were identified using spore characteristics (Fig. 1). Based on spore size and color, wall structure, and hyphal attachment following species which were dominant as follows *G. fasciculatum, G. intraradices, G. mosseae* and several other unidentified species. The composition of the AM fungal population in the various host plants' rhizospheres with different soil heavy metal level is recorded and at higher rates of metal content in soil. Species richness of AMF ranged from 2 to 3. *Glomus* occurred most frequently and, overall, were the most prevalent, containing 3 species namely, *Glomus mosseae, Glomus fasciculatum*, *Glomus intraradices*. In this study, *Glomus mosseae was* the most widely distributed species. The next most widely distributed taxon was *Glomus intraradices* followed by *Glomus fasciculatum*. Among the different broader crop category *Glomus intraradices* was dominant in flowers while *Glomus fasciculatum* was less dominant in vegetables. Other least frequently distributed glomus species were *Gigaspora* sp. and *Scutellospora*. It was evident that the three ecotypes were frequently found in the soils, showing consistent differences with regard to their tolerance to the presence of heavy metals. Total AMF spore numbers decreased with increasing amounts of heavy metals in the soil.

However, species richness and diversity as measured by the Shannon-Wiener index increased in soils having intermediate amount of heavy metal but decreased in soils having higher amount of heavy-metal (Table 2). It can be inferred based on the results of this study that size and diversity of AMF populations were modified in metal-polluted soils. Heavy metals introduced into soil by irrigating with sewage effluent can affect the soil's microflora and, in particular, the abundance of *Arbuscular mycorrhizal* fungi. AM fungal spore varied greatly between plant species. AM fungal species belonging to the genus *Glomus* were found in rhizosphere samples of different crop species. The ability of *Glomus* to dominate soil rhizosphere indicated that *Glomus* has a broad host range and the spores of Glomus species have different temperature and pH preferences for germination (Wang *et al.* 1997). Host plant also had a significant effect on the total AMF spores produced in the rhizosphere, *Sorghum bicolor* being the trap plant that produced AMF spores most effectively possibly because of the higher root growth rate of this plant species, which can facilitate further contact with most AM fungi present in the soil. Host plants also exerted a differential effect on AMF diversity, with *T. erecta* and *S. bicolor* promoting significantly higher number as well as levels of diversity in their rhizospheres than those produced by cauliflower and raddish. Therefore, the selection of plant rotation on *Arbuscular mycorrhizae* is significant and should be considered in field management. Variation in spore density and AMF colonization in relation to host plants can be linked to factors such as plant phenology, dependency on mycorrhiza, changes in the soil micro-environment, or unknown host characteristics. Total AMF spore number decreased significantly with increasing amounts of heavy metals in soil, from 330 spores (per 100 g of dry soil) in marigold with a heavy metal content mg kg^{-1} to 56 spores in cauliflower with a heavy metal content mg kg^{-1}.

Species diversity was calculated using Shannon–Weiner diversity index ranged from 0.497 to 1.053 (Table 1). The highest occurred in okra and the lowest in papaya. A negative correlation was shown between the total number of AMF spores and soil heavy metal content (Ni and Cd) corroborating its sensitivity to the presence of heavy metals (Table 2). Correlation coefficient was higher for the concentration of free cations (Cd^{2+}) in the

Table 2: Pearson's correlation coefficient (r) between *Arbuscular mycorrhizal* spore density, H index and heavy metal content

Parameters	Spore density(number of AMF spores / 100 g of dry soil)	H index
DTPA(diethylenetriaminepentaacetic acid) -extractable Cd (mg kg^{-1})	-0.56*	0.109
DTPA(diethylenetriaminepentaacetic acid) -extractable Ni (mg kg^{-1})	-0.52*	-0.283

** $p < 0.05$, ** $p < 0.01$*

soil solution compared to Ni as it is toxic to soil microbes. This fungitoxic effect of metal can cause inability by certain AMF species' to colonize the root system and/or to multiply in the rhizosphere. Shannon index values was negatively correlated with the heavy metal content of the soil implicating the role of heavy metals on biodiversity markers. Only AMF species better adapted to the disturbance produced by the addition of metals would overcome the stress situation and complete their life cycles.

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