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Characterization of the culturable component from rhizosphere zone of wheat and rice fields of Malwa region in Punjab

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Abstract
The present study was undertaken to characterize the dominant culturable fungal and bacterial biodiversity isolated from wheat and rice fields that may further help to study the pathogenic potential of these fungi to cause the yield losses in wheat and rice agro-ecologies. Soil samples were collected from the soil of the rhizosphere zone of rice and wheat fields of the Malwa region (Talwandi Sabo, Faridkot, Sardulgarh) in Punjab from 2018 to 2019 in three Intervals to assess the biodiversity of microorganisms. The microflora was isolated using the soil dilution plating technique. The fungal population obtained from the soil of wheat-rice rhizosphere comprised of *Acremonium* sp., *Aspergillus* spp. viz *A. niger*, *A. flavus*, *A. ochraceus*, and *A. fumigatus*, *Alternaria* spp. *viz. A. alternata*, *A. brassicae*, *Curvularia lunata*, *Cladophialphora bantiana*, *Helminthosporium* sp., *Fusarium* spp. *viz. F. oxysporum*, *F. chalmydosporum*, *Mucor* sp., *Penicillium* spp. viz. *P. chrysogenum*, *P. expansum*, *Rhizpous* spp. More than 30 bacterial strains were also isolated from the three different locations. The bacterial colonies were predominantly gram-positive rod-shaped or filamentous and gram-negative cocci. Among the ten fungal genera the *Aspergillus* spp. *viz. A. niger*, *A. flavus*, and *Penicillium* spp. *viz. P. chrysogenum* and *P. expansum* were dominant on potato dextrose agar (PDA) plates. The frequency of occurrence of common species in wheat soil samples was maximum for genus *Aspergillus* (*A. niger*, *A. flavus*) and *Penicillium* (*P. chrysogenum*) *viz.*, 24.35%, 16.16%, 19.23%, respectively, while *Acremonium* sp., *Aspergillus ochraceus*, and *A. fumigatus* showed the minimum frequency of occurrence *viz.*, 3.84%, 2.56%, and 3.84%, respectively. However, in rice fields, *A. niger*, *A. flavus*, *P. chrysogenum*, and *P. expansum* showed the maximum frequency of occurrence (19.14%, 13.82%, 15.95%, and 14.89%, respectively). The results showed that the genus *Penicillium*. and *Aspergillus* were dominant in rhizosphere soils of Talwandi sabo and Faridkot while genus *Fusarium* was prevalent in the Sarudulgarh region. The dominance of these fungal pathogens may be due to the release of aflatoxins by *Aspergillus* spp. and fumonisins by *Fusarium* spp., which may have an inhibitory effect on the survival of other microbial diversity.

Keywords: fungal diversity, isolation, Malwa region, microflora population, rhizosphere soil

Introduction
The rhizosphere is a narrow region of soil that is under the direct influence of root secretions by plants and associated soil microorganisms. The rhizosphere microorganisms predominantly metabolize the root exudates and comprise living forms such as bacteria, fungi, and actinomycetes. Diverse populations of microorganisms constitute a sustainable soil environment conducive for the maintenance of root health, nutrient uptake, and mitigating environmental stress both biotic and abiotic [18]. Fungal biodiversity is fundamental for soil ecosystem functioning [17], especially in forest and agricultural soils, the fungi play a key role in several essential processes such as organic matter decomposition and release of elements by mineralization. About 1.5 million fungal species are reported to be present in natural ecosystems, however, only 5-10% has been described formally [8]. In agricultural ecosystems, soil-borne plant pathogens are adapted to grow and survive in bulk soil, but the rhizosphere is the infection court where the pathogens encounter the plant and establish a parasitic relationship. Both pathogenic and symbiotic fungi are present in the rhizosphere. The zygomycetes and hyphomycetes establish more readily in the rhizosphere because they can effectively metabolize simple sugars [15].

Pathogens survive in soil as propagules, such as chlamydospores, sclerotia, thick-walled conidia, or hyphae which survive in plant roots and crop residues and remain viable for a long time [4]. When conditions are favorable, fungus protoplasm is stimulated to germinate by root or seed exudates and it chemo tactically grows toward the plant. In the rhizosphere, plants and microorganisms permanently interact in a continuum ranging from deleterious to beneficial and neutral groups [5]. The rhizosphere is a highly dynamic region, which is directly influenced by abiotic factors such as mineral composition and physical properties of the soil. Depending upon physical properties such as water permeability, soil texture, and mineral concentrations rhizosphere soil can vary in concentration from 100-10,000 folds. Additionally, minerals can bind to plant-derived organic compounds by chelating tendency, thereby potentially altering their availability to soil microorganisms. These rhizosphere soil characteristics affect both plant growth and the interfering microbial community [3]. Four main groups of plant pathogens viz, fungi, nematodes, bacteria, and some viruses are found in the rhizosphere [1], but only two of them are major players: (fungi, nematodes, and a few groups of bacteria are considered to be soil-borne, probably non-spore bacteria cannot survive well in the soil for long periods whereas bacteria such as *Agrobacterium tumefaciens* (crown gall) and *Ralstonia solanacearum* causing bacterial wilt of tomato to require wound or natural opening to penetrate plant roots [6][12]. Commonly found pathogens in the soil are *Aspergillus* spp., *Fusarium* spp., *Alternaria* spp., *Pyricularia* sp., *Helminthosporium* sp. and *Rhizoctonia* spp. The present study was conducted to examine the dominant culturable fungal diversity from wheat and rice fields. Documentation of fungal biodiversity may further help to study the pathogenic potential of these fungi to cause the yield losses in wheat and rice agro-ecologies.

Materials and Methods

Collection of soil samples: The investigation was carried out in the Malwa region of Punjab which includes eleven districts extending from 29.30º N to 32.32º N and 73.55º E to 76.50º E. The climate conditions of Malwa regions are arid and semi-arid annual temperatures ranging from 1ºC to 46ºC (min/max). The soils are predominantly calcareous, pH ranging from 7.8 to 8.5, and soil texture is sandy loam to silt. Three rhizosphere soil samples (Table 1) of wheat and rice were collected three times a year during 2018-19 from three locations in the Malwa region of Punjab viz., fields of Guru Kashi University, Talwandi Sabo (Bathinda), Faridkot, and Sardulgarh. The soil samples were collected from the surface area reaching about 10-15 cm depth and near the rhizosphere region of plants. The biological and technical replicates were maintained up to 10 samples per location. The collected soil samples were brought to the laboratory in sterile polythene bags and stored at 4ºC until further analysis. Each sample bag was labeled appropriately by indicating the site of collection, time, date, and place of collection.

Isolation of rhizosphere soil microflora

The soil microbial diversity was isolated following the serial dilution and soil dilution plating technique [16]. Soil dilutions were made by suspending 1g of the soil of each sample in 9ml of sterile distilled water in a test tube and shaking vigorously to obtain 10^{-1} dilution, further 1 ml dilution taken from the first dilution and introduced into another labeled test tube to obtain 10^{-2} dilution. This process serial wise repeated to obtain up to 10^{-9} dilution. Dilutions of 10^{-3}, $10^{-4,}$ and 10^{-5} were used to isolate fungi to avoid over-crowding of fungal species. The pure cultures of fungi were maintained on potato dextrose agar (PDA) slants at 25˚±2ºC. The cultures of bacteria were purified and maintained on the nutrient agar slants at 30˚±2ºC. The colonies established on artificial growth media were subculture by transferring to freshly prepared PDA plates. The pure cultures of soil fungi and bacteria were incubated at room temperatures for 3–5 days.

Characterization of soil microflora

The fungal species were identified based on cultural characteristics. The colony morphology was examined by visual observations. The microscopic examination was done by staining fungal hyphae with lactophenol cotton blue and observed under a compound microscope for conidia, conidiophores, and arrangement of spores [2][7][10][11]. The rhizospheric bacteria isolates were characterized based on their colony characters and response to gram staining as characteristics) described by Bergey's Manual of systematic bacteriology.

Statistical analysis

The frequency of occurrence of fungi in rhizosphere soil of wheat and rice was determined as follows.

$$\text{Frequency of occurrence} = \frac{\text{Total No. of CFU of an individual sp.}}{\text{Total No. of CFU of all spp.}} \times 100$$

*CFU – Colony Forming Unit

Results and Discussion

The study was conducted in the Department of Plant Pathology (Guru Kashi University, Talwandi Sabo, Bathinda) to examine population diversity, morphological characteristics, and percentage diversity of microflora in the rhizosphere of wheat and rice fields. The study revealed that the population diversity of bacterial cultures/gm of soil sample was more in comparison to fungi and actinomycetes. The numbers of fungi ranged from 2 to 8 (per gram of soil sample), the bacterial population was 6 to 14 (per gram of soil sample) whereas, actinomycetes isolated ranged from 0 to 4 (per gram of soil sample) respectively, from all three sampling sites (Table 2). The variations in the population of fungi, bacteria, and actinomycetes in the three locations may be due to variable physio-chemical properties of soil and the seasonal variations. Also, the soil characteristics may have been more conducive for bacteria compared to fungi and actinomycetes. In a micro biome, bacteria are more adapted compared to other eukaryotic microflora due to their simple structure. The complex physiology of the fungi and actinomycetes subjects them to more competition based on synergistic or antagonistic responses. The relative numbers and biomass of bacteria were more in soil compared to another microorganism [9]. In our study, we observed that the fungal and bacterial population thrived well during the monsoon period due to the prevalence of suitable temperature and high moisture content in the rhizosphere of rice in comparison to the rhizosphere of wheat. The isolations from rhizosphere soil of wheat and rice from three fields of three different locations of Punjab *viz*., Bathinda, Faridkot, and Sardulgarh comprised of ten genera (Table 3) based on the morphological and microscopic examination (Fig 1&2). The

prevalent genera included *Acremonium* sp., *Aspergillus* spp. such as *Aspergillus niger*, *A. flavus*, *A. ochraceus*, *A. fumigatus*, *Alternaria* spp. such as *Alternaria alternata*, *A. brassicae*, *Curvularia lunata*, *Cladophialphora bantiana*, *Helminthosporium* sp., *Fusarium* spp. such as *Fusarium oxysporum*, *F. chlamydosporum*, *Mucor* sp., *Penicillium* spp. such as *P. chrysogenum* and *P. expansum*, *Rhizopus sp.* In a similar investigation, various strains of *Aspergillus*, *Fusarium*, and *Alternaria* spp., were isolated from the soil of wheat cultivated area of Uttar Pradesh [14].

In the present investigation, more than 30 bacterial cultures were obtained from the three different locations, however, bacterial isolates-the gram-positive rod-shaped, gram-positive filamentous and gram-negative cocci formed the dominant microflora (Fig 3). The bacterial colonies isolated from different locations revealed a variation in colony color, size, and growth rate. The bacterial population was higher in rice rhizosphere soil as compared to wheat, probably, due to suitable temperature, moisture content and organic root exudates released by the roots of rice plants. The frequency of occurrence of each fungal species in both wheat and rice rhizosphere soil sample were statistically analyzed (Table 4&5). The graphical representation (Fig 4&5) of frequency reveals that the maximum frequency of occurrence of common species of fungal genera *Aspergillus (A. niger*, *A. flavus)* and *Penicillium (P. chrysogenum)* was recorded in wheat soil samples *viz.*, 24.35%, 16.16%, 19.23% respectively, while the minimum frequency of occurrence of genus *Acremonium*, and *Aspergillus* (*A. ochraceus*, and *A. fumigates)* was recorded *viz.*, 3.84%, 2.56%, and 3.84% respectively. In the case of rice maximum frequency of occurrence of fungal species such as *A. niger*, *A. flavus*, *P. chrysogenum*, and *P. expansum* was 19.14%, 13.82%, 15.95%, and 14.89% was observed respectively. The results of related study also revealed the soil microbial diversity of the indigenous community, their distribution and behavior in soil habitats [13]. This study is a modest effort to understand soil microbial diversity in rhizosphere ecosystems in Malwa region of Punjab, with a focus on preliminary assessment and identification of soil microflora of rhizosphere soils of wheat and rice.

Table 1: Collection of rhizosphere soil samples from different locations in the Malwa region of Punjab.

Soil sample	Regions	Isolation I (Jan-2018)	Isolation II (Aug-2018)	Isolation III (Jan-2019)
S_1	Bathinda field (Punjab)	Rhizosphere of wheat	Rhizosphere of rice	Rhizosphere of wheat
S_2	Faridkot field (Punjab)	Rhizosphere of wheat	Rhizosphere of rice	Rhizosphere of wheat
S_3	Sardulgarh field (Punjab)	Rhizosphere of wheat	Rhizosphere of rice	Rhizosphere of wheat

Table 2: Population dynamic of microflora per gram of soil samples

S. No	Isolation of microflora from soil	Bathinda			Faridkot			Sardulgarh		
		F	B	A	F	B	A	F	B	A
1	Isolation-I (Jan-2018) Rhizosphere of wheat	6	6	1	6	10	0	2	8	0
2	Isolation-II (Aug-2018) Rhizosphere of rice	8	9	3	8	14	2	4	12	2
3	Isolation-III (Jan-2019) Rhizosphere of wheat	7	8	3	6	12	4	3	10	3

F- Fungi, **B-** Bacteria, **A-** Actinomycetes

Table 3: Isolated fungal diversity from three different regions of Punjab (Malwa region)

S. No	Fungal isolates	Bathinda	Faridkot	Sardulgarh
1	*Acremonium* sp.	-	-	+
2	*Aspergillus niger*	+++	+++	+++
3	*Aspergillus flavus*	++	-	++
4	*Aspergillus ochraceus*	+	-	-
5	*Aspergillus fumigates*	-	+	-
6	*Alternaria alternate*	+	-	-
7	*Alternaria brassicae*	+	-	-
8	*Curvularia lunata*	+	-	-
9	*Cladophilaphora bantiana*	+	-	-
10	*Fusarium oxysporum*	-	-	++
11	*Fusarium chalmydosporum*	-	-	+
12	*Fusarium* sp.	-	-	+
13	*Helminthosporium* sp.	+	-	-
14	*Mucor* sp.	+	+	-
15	*Penicillium chrysogenum*	+++	+++	+++
16	*Penicillium expansum*	++	-	-
17	*Penicillium* sp.	+	+	-
18	*Rhizopus* sp.	+	+	-
19	Unidentified	+ (1 Isolate)	+ (1 Isolate)	-

+ Fungi observed, - Fungi not observed

Table 4: Frequency of occurrence of mycoflora in rhizosphere soil of wheat

Regions	Total Colonies	The average number of individual colony										
		Ac	Ani	Afl	Ao	Afu	Fo	Fch	Mu	Pch	Pex	Rh
Bathinda	27	0	6	4	2	0	0	0	2	6	5	2
Faridkot	25	0	7	5	0	3	0	0	3	5	0	2
Sardulgarh	26	3	6	4	0	0	5	4	0	4	0	0
Total	78	3	19	13	2	3	5	4	5	15	5	4
% Contribution		3.84	24.35	16.66	2.56	3.84	6.41	5.12	6.41	19.23	6.41	5.12

Ac- *Acromonium* sp., Ani- *A. niger*, Afl- *A. flavus*, Ao- *A. ochraceus*, Afu- *A. fumigatus*, Fo- *F. oxysporum*, Fch- *F. chalmydosporum*, Mu- *Mucor* sp., Pch- *P. chrysogenum*, Pex- *P. expansum*, Rh- *Rhizopus* sp.

Table 5: Frequency of occurrence of mycoflora in rhizosphere soil of rice

Regions	Total Colonies	The average number of individual colony													
		Ac	Ani	Afl	Ao	Afu	Ala	Alb	Clu	Cla	Fo	Fch	Hs	Pch	Pex
Bathinda	36	0	7	4	3	0	3	2	4	2	0	0	2	5	4
Faridkot	25	0	6	4	2	2	0	0	0	0	0	0	0	6	5
Sardulgarh	33	4	5	5	0	0	0	0	0	0	6	4	0	4	5
Total	94	4	18	13	5	2	3	2	4	2	6	4	2	15	14
% Contribution		4.25	19.14	13.82	5.31	2.12	3.19	2.12	4.25	2.12	6.38	4.25	2.12	15.95	14.89

Ac- *Acromonium* sp., Ani- *A. niger*, Afl- *A. flavus*, Ao- *A. ochraceus*, Afu- *A. fumigatus*, Ala- *A. alternata*, Alb- *A. brassicae*, Clu- *C. lunata*, Cla- *C. bantiana*, Fo- *F. oxysporum*, Fch- *F. chalmydosporum*, Hs- *Helminthosporium* sp., Pch- *P. chrysogenum*, Pex- *P. expansum*

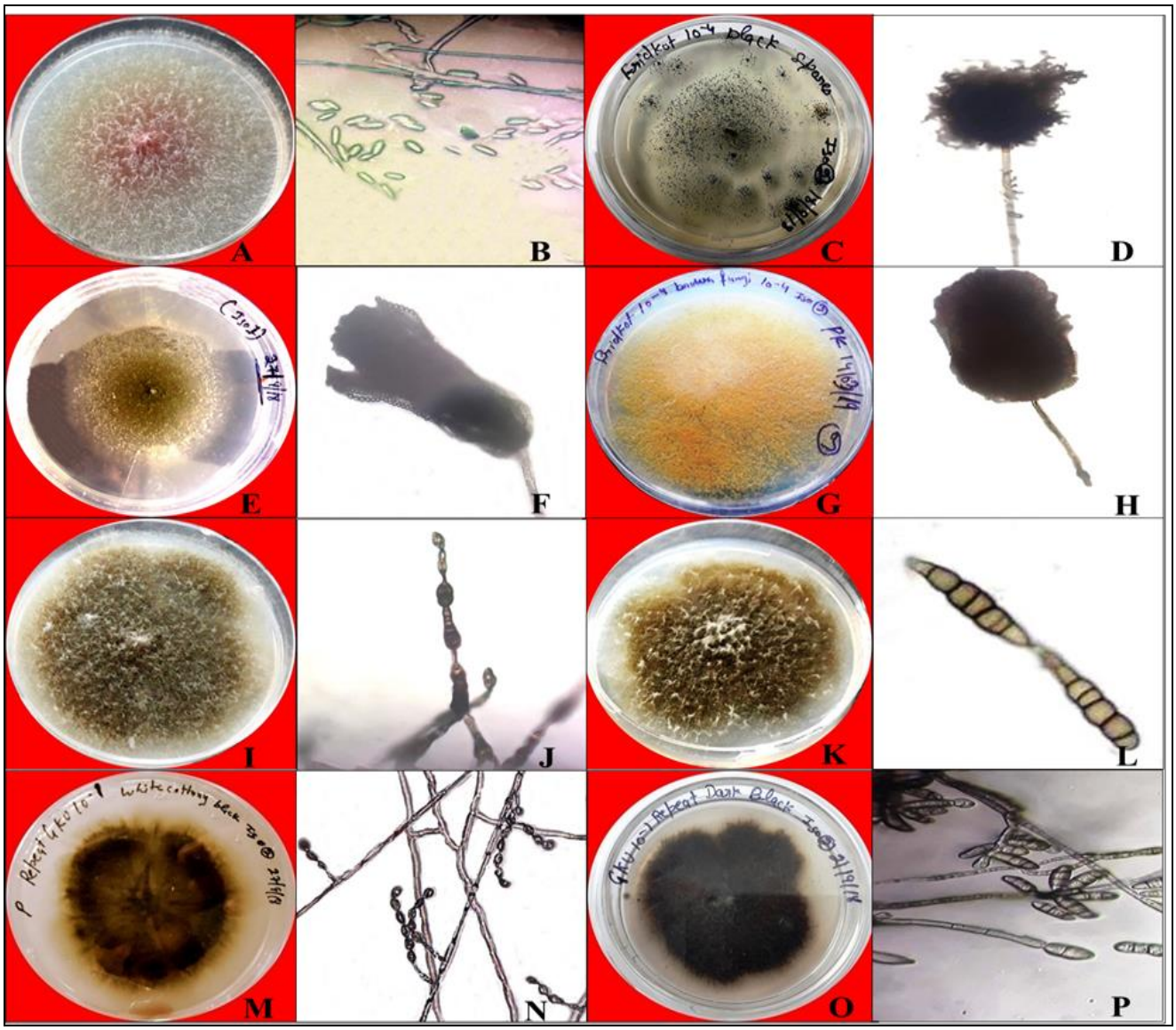

Fig 1: Fungal isolates from rhizosphere soil:- (A-B) *Acremonium* sp., (C-D) *A. niger*, (E-F) *A. flavus*, (G-H) *A. ochraceus*, (I-J) *A. alternata*, (K-L) *A. brassicae*, (M-N) *C. bantiana*, (O-P) *C. lunata*

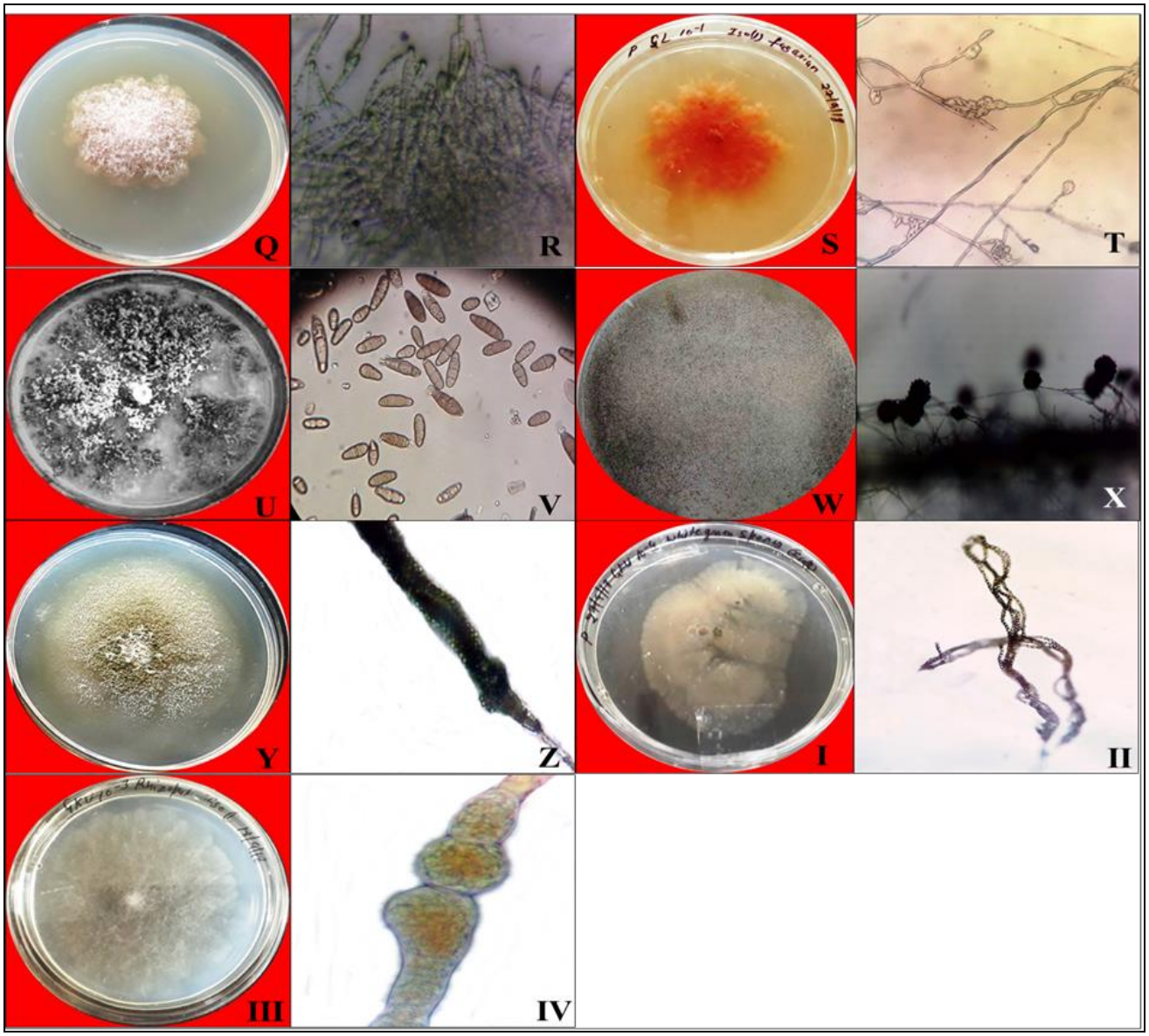

Fig 2: Fungal isolates from rhizosphere soil:- (Q-R) *F. oxysporum*, (S-T) *F. chalmydosporum*, (U-V) *Helminthosporium* sp., (W-X) *Mucor* sp., (Y-Z) *P. chrysogenum*, (I-II) *P. expansum*, (III-IV) *Rhizopus* sp.

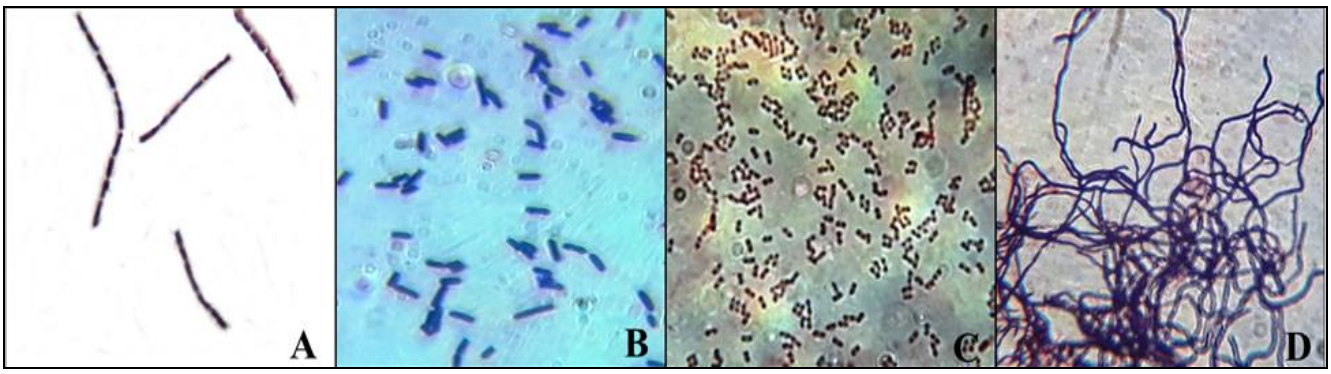

Fig 3: Bacterial isolates:- (A) Gram-positive rods in the chain (B) Gram-positive rod-shaped (C) Gram-negative cocci (D) Gram-positive filamentous

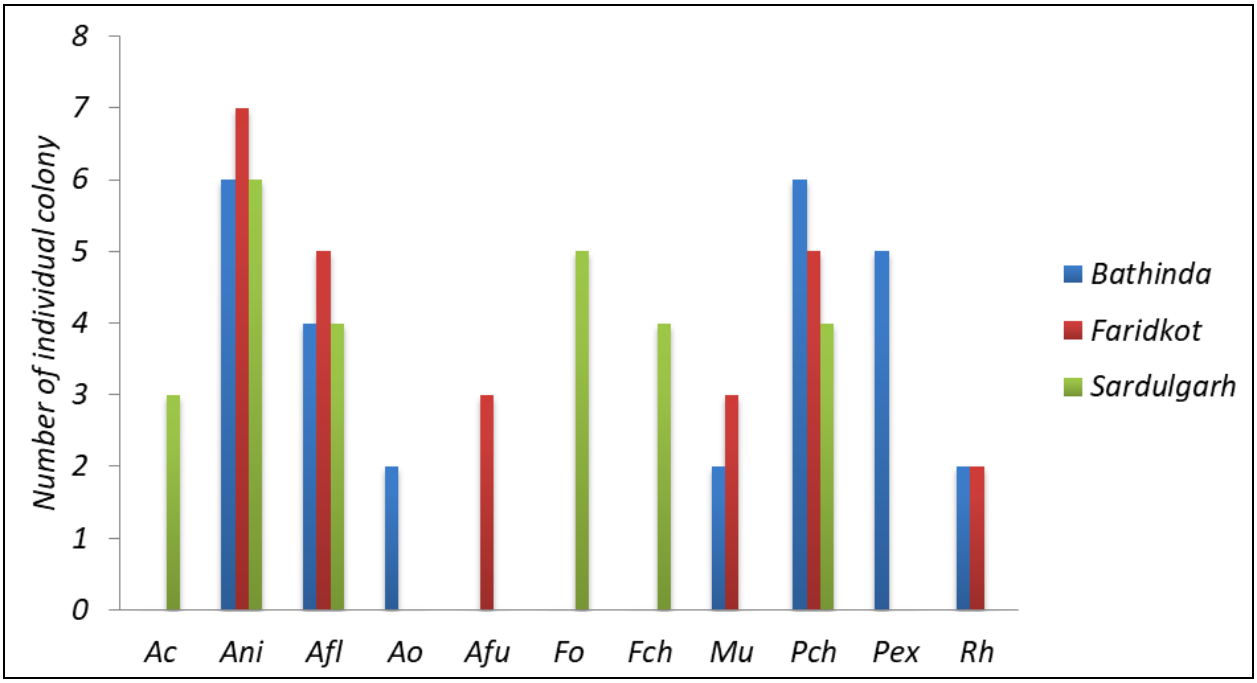

Fig 4: Frequency of mycoflora in wheat rhizosphere soil Ac- *Acromonium* sp., Ani- *A. niger*, Afl- *A. flavus*, Ao- *A. ochraceus*, Afu- *A. fumigatus*, Fo- *F. oxysporum*, Fch- *F. chalmydosporum*, Mu- *Mucor* sp., Pch- *P. chrysogenum*, Pex- *P. expansum*, Rh- *Rhizopus* sp.

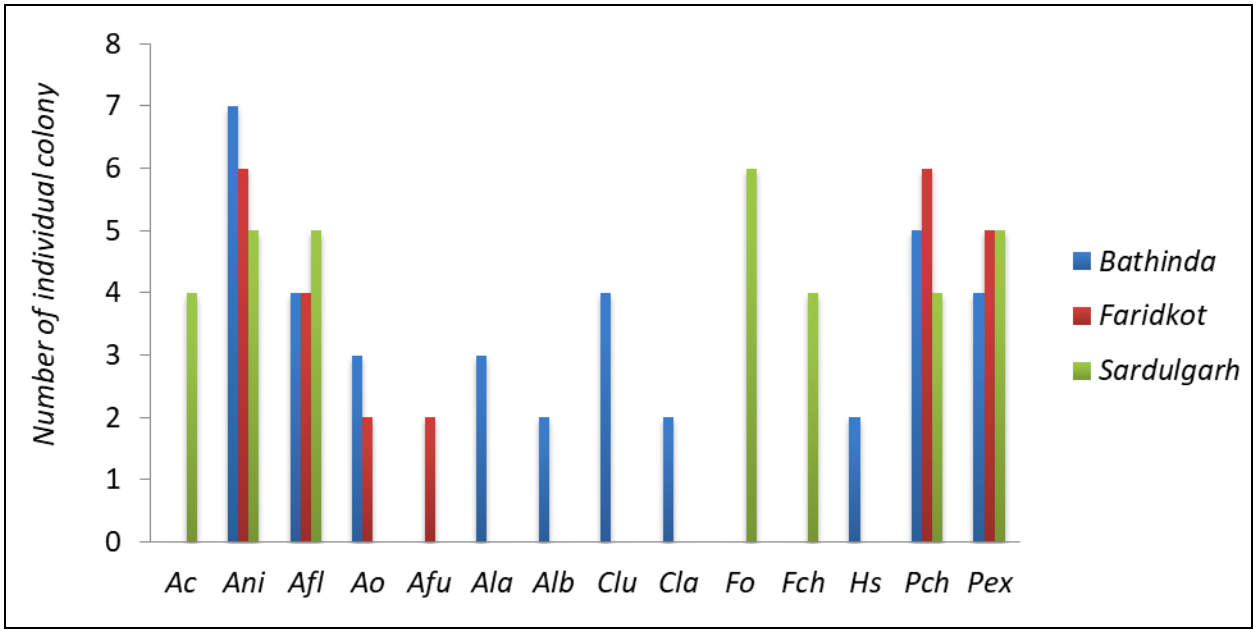

Fig 5: Frequency of mycoflora in rice rhizosphere soil Ac- *Acromonium* sp., Ani- *A. niger*, Afl- *A. flavus*, Ao- *A. ochraceus*, Afu- *A. fumigatus*, Ala- *A. alternata*, Alb- *A. brassicae*, Clu- *C. lunata*, Cla- *C. bantiana*, Fo- *F. oxysporum*, Fch- *F. chalmydosporum*, Hs- *Helminthosporium* sp. Mu- *Mucor* sp. Pch- *P. chrysogenum*, Pex- *P. expansum*, Rh- *Rhizopus* sp.

Conclusion

In our investigation, the some fungus such as *Penicillium* and *Aspergillus* were dominant genera in rhizosphere soils of Talwandi sabo and Faridkot while genus *Fusarium* was prevalent in the Sarudulgarh region. The dominance of these fungal pathogens may be due to the release of aflatoxins by *Aspergillus* spp. and fumonisins by *Fusarium* spp., which may have an inhibitory effect on the survival of others microbial diversity in rhizosphere microbime, while the antibiotic produced by the *Penicillium* spp., may be causing inhibition of the growth and germination of other fungal and bacterial pathogens.

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