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Antifungal activity exhibited bacterial strains isolated from wheat-rice rhizosphere soils and their antagonistic nature toward various fungal isolates

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Abstract
The rhizobacteria with antagonistic activity toward plant pathogens play an essential role in plant growth and development. But, the role of growth-promoting rhizobacteria (PGPR) in the adaptation of plants in extreme environments is yet not completely understood. To analyze the effect of rhizobacteria on plant pathogens, the microbial biodiversity was assessed from the rhizosphere soil of wheat and rice collected from the Malwa region (Talwandi Sabo, Faridkot, Sardulgarh) of Punjab during January 2018 to January 2019 in three Intervals. Nine fungal genera were obtained from the soil of the wheat-rice rhizosphere and identified as follows:- *Acremonium* sp., *Aspergillus* spp., *Alternaria* spp., *Curvularia lunata*, *Cladophialphora bantiana*, *Helminthosporium* sp., *Fusarium* spp., *Mucor* sp., *Penicillium* spp. More than 30 bacterial isolates were obtained from the three different areas and further screened against isolated fungal genera. Out of thirty, two bacterial isolates *Bacillus subtilis* 2B10 and 2C5 were found to capability in growth inhibition range from 10 to 30 mm and 8 to 27.5 mm against *Penicillium* spp., *Aspergillus* spp., *Curvularia lunata*, *Fusarium* spp., and several other fungal isolates respectively. The *B. subtilis* 2B10 and 2C5 exhibit maximum growth inhibition 30 mm and 27.5 mm of *P. chrysogenum* and *Helminthosporium* sp., respectively. The present study identified specific characters in the isolated rhizobacteria, which make them good candidates as antagonists and might contribute to plant adaptation to pathogenic microbes. The application of such results in agricultural fields may improve and enhance plant growth in rhizosphere soils.

Keywords: Rhizosphere, microflora, antagonistic, suppressive soil

Introduction
The rhizosphere is a complex ecosystem that contains a wide range of microbial diversity compares to others ecosystem and it is an environment that plant itself creates and responsible for constituting of the microbial diversity which influential force on growth and health of plant (Lynch 1990) [6]. The rhizosphere a hot spot of microbial interaction as root exudates released by plant roots are the food source for microorganisms and driving a force on their population density and activities (Raaijmakers 2009) [13]. The diverse group of microflora is essential to a sustainable biosphere that enables many functions in the rhizosphere such as maintenance of root health, nutrient uptake, tolerance of environmental stress, and protection from pathogenic microbes (Zake *et al.*, 2011) [17]. Both deleterious and beneficial microflora are attracted to the rhizosphere zone and establish a parasitic relationship with the roots of plants. The plant growth promotion is the result of a parasitic relationship between beneficial microbes and plants, while the microorganisms that adversely affect plant growth and health are the pathogenic microbes. A large amount of carbon source (i.e. rhizodeposits) is released by the plant roots into the rhizosphere that utilizes by the complex food web microbial community. The population density of microbial diversity directly related to the quality and quantity of rhizodeposits.

The rhizosphere is an infection court that harbors a wide range of pathogens such as *Fusarium oxysporum* (Wilt disease), *Ralstonia solanacearum* (Bacterial wilt of tomato), *Agrobacterium tumefaciens* (Crown gall) (Genin and Boucher 2004; Nester *et al*. 2005) [3, 11], *Helminthosporium oryzae* (Brown spot of rice), *Rhizoctonia solani* (Sheath blight of rice) (Singh *et al*. 2014) [15], directly influences the growth and health of the plant, and establishes the parasitic relationship. The soil-borne pathogens that adversely affect the yield of vegetable crops are *Pythium* spp., (Damping off seedling), *Phytophthora* spp., (Late blight of potato), *Streptomyces scabies* (Potato scab), and some of the storage fungi, *Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp., and *Mucor* spp. that can grow and survive, on the stored

food grains. In the complex rhizosphere, both microfauna, and microflora can suppress the growth and development of pathogens and influence the outcome of infection. A wide range of rhizosphere beneficial bacteria such as *Bacillus*, *Pseudomonas* can compete for nutrients and space with plant pathogens and proved to be potential for the production of bio fungicides that attack the phytopathogenic fungal cell wall. These properties exhibited by beneficial rhizosphere microbes against fungal phytopathogens, not give us a better understanding of environmental and ecological benefits, but also their impact as an attractive alternative for chemical use in agriculture. The aim of the investigation is the evaluation of the natural suppression of various fungal genera by the rhizosphere bacterial isolates.

Material and Method

Site description and location: Punjab is located in northwestern India has an area of 50,362 km^2. It extends from the latitudes 29.30º North to 32.32º North and longitudes 73.55º East to 76.50º East. Malwa is a region of the Punjab south to the river Sutlej, this area makes up the majority of the Punjab region consisting of 11 districts. Punjab climate is characterized by extreme hot and extreme cold conditions. Annual temperatures in Punjab ranges from 1ºC to 46ºC (min/max) but can reach 49 ºC in summer and 0ºC in winter. Malwa region receives less rainfall and experiences higher temperatures. The soil is predominately calcareous, developed under hot and arid to semi-arid conditions. The pH values range from 7.8 to 8.5 which shows that the soil is normal in reaction. The texture of the soil of the Bathinda and Faridkot is sandy loam to silt.

Collection of rhizosphere soil samples: Three rhizosphere soil samples of wheat and rice were taken in three intervals (Jan-2018 to Jan-2019) from three areas (1) (Bathinda) (2) Faridkot (3) Sardulgarh of Punjab (Malwa region).

Isolation and culture maintenance of rhizosphere soil microflora: The soil-borne microflora was isolated following the soil dilution plating technique (Waksman,1992) at 10^{-2}, 10^{-3}, 10^{-4} dilution. Each fungal culture was purified and further maintained by sub-culturing regularly on PDA slants at 27ºC, while each bacterial culture was purified and further maintained on the NA slants at 32ºC and stored at 4ºC before use in experiments.

Characterization and Identification of soil microflora: Purified fungal morphology was studied macroscopically by observing colony features (Colour and Texture) in the center plate technique and microscopically observed under a compound microscope for conidia, conidiophores, and arrangement of spores (Aneja, 2001) [1]. The fungi were identified with the help of literature Mukadam (1997) [8], Gilman (1957) [4], and Nagamani (2006) [10]. The rhizospheric bacteria isolates were identified by studied their colony morphology and Gram staining (microscopic morphology) described by Bergey's Manual of systematic bacteriology.

Extraction of bacterial bioactive metabolites: The nutrient broth was used for the growth as well as the high production of bioactive metabolites for bacteria strain. A loopful purified bacteria was transferred in a 250 ml Erlenmeyer flask containing 50 ml of nutrient broth (NB). The cultures were incubated at 32ºC with 180 rpm shaking at the rotator shaker for 2 days. After shaking10-20 ml of each sample was harvested and centrifuged for 15 min at 10,000 rpm for separation of supernatant. Then the supernatant was filtered through a bacterial filter (Millipore filter 0.45µm) to get cell-free samples.

***In vitro* screening of bacterial isolates for antifungal activity**

Microbiological screening of different bacterial isolates was evaluated by the agar well diffusion method (Murray *et al*., 1995, later modified by Olurinola, 1996) [9, 12].

Agar well diffusion method. Agar well diffusion method is a modification of the disc diffusion method used to determine the antimicrobial activity. After solidification of poured PDA in petri plates, wells of 5 mm in diameter were made using sterile cork borer, and then the cell-free bacterial filtrates (100 µl) were added separately in wells by pipette and fungal phytopathogens were spread on the surface of the cultivated PDA. The Petri dishes were incubated at 25-28 ºC for 48-72 hours.

Primary screening. The primary screening was used for determination of the capability of the bacterial isolates to produce antifungal activity without giving a significant idea about the production or yield potential of the organism.

Secondary screening. The active bacterial isolates selected from primary screening were further subjected to secondary screening, against various selected other phytopathogenic fungi. Antifungal activity of each cell-free extract was determined using a well diffusion assay and 50 to 100µl of bacterial extract having optical density OD 1.1 was loaded and tested. The filtrate was transferred aseptically into conical flasks and stored at 4ºC for further use.

Determination of antifungal activity (Zone of inhibition)

After incubation of petri plates, the diameter of the zone of inhibition (mm) around the wells was measured to evaluate the antifungal activity of bacterial isolates.

Results and Discussion

The experiments were conducted in the department of plant pathology (Guru Kashi University, Talwandi Sabo, Bathinda) to determine the antagonistic properties of rhizosphere bacterial isolates against various fungal genera.

Isolated microflora: Nine genera of mycoflora were obtained from the rhizosphere soil of wheat and rice of three different areas of the Malwa region (Punjab) and identified as follows:- *Acremonium* sp., *Aspergillus* spp. such as *A. niger* isolate 1, *A. niger* isolate 2, *A. niger* isolate 3, *A. flavus*, *A. ochraceus*, *A. fumigatus*, *Alternaria* spp. such as *A. alternata*, *A. brassicae*, *Curvularia lunata*, *Cladophialphora bantiana*, *Helminthosporium* sp., *Fusarium* spp. such as *F. oxysporum*, *F. chlamydosporum*, *Mucor* sp., *Penicillium* spp. such as *P. chrysogenum*, *P. expansum*. Seth *et al.*, (2016) conducted similar studies and isolated various strains of *Aspergillus*, *Fusarium*, *and Alternaria* from the soil of wheat cultivated area of Uttar Pradesh. More than 30 bacterial isolates were obtained from the three different areas and maximum strains of obtained bacterial isolates were gram-positive rod-shaped and gram-negative rod-shaped identified as *Bacillus* spp. and *Pseudomonas* spp., respectively.

***In vitro* screening of bacterial isolates for antifungal activity**

Primary screening: In the primary screening of antifungal activity, 30 bacterial isolates were tested against isolate

Fusarium sp. Out of 30 bacterial isolates, two bacterial isolates 2B10 and 2C5 were obtained from the soil samples of rice rhizosphere soil of Sardulgarh and Faridkot respectively, showed good inhibitory effect against *Fusarium* sp. Both antifungal efficient bacterial isolates were selected for further secondary screening against other fungal genera by agar well diffusion assay. Kapur *et al.,* (2018) [5] were recently investigated a study on primary screening of actinomycetes against *Fusarium oxysporum* and other phytopathogens.

Identification of bioactive producing bacterial isolates: Based on morphological characteristics (Colony feature), staining (Gram staining), and biochemical test (Starch hydrolysis test), both antifungal compounds producing bacterial isolates 2B10 and 2C5 were identified as *Bacillus subtilis* strains (Fig 2). Similar studies were conducted by (Zhenxiang *et al.,* 2018) [18] on the morphological, biochemical characteristics of novel *Bacillus subtilis.*

Secondary screening: Both the selected bacterial isolates with noticeable antifungal activities were further tested against 17 fungal isolates, out of which 13 were found to have inhibition zone formed by *Bacillus subtilis* 2B10 and 2C5 respectively as shown in (Table & Fig 1). The *B. subtilis* 2B10 and 2C5 were more effective against *Penicillium* spp., *Aspergillus* spp. with a maximum zone of inhibition ranges from 10 to 30 mm and least effective against *Fusarium* spp. with the zone of inhibition ranges from 8 to 13 mm. The *B. subtilis* 2B10 was not much effective against *A. brassicae, A. alternata, Aspergillus* sp., *Fusarium* sp., and *Rhizopus* sp., while *Bacillus subtilis* 2C5 was effective against the *A. alternata.* The *B. subtilis* 2C5 formed the maximum zone of inhibition (27.5) against *Helminthosporium* sp., as compared to *B. subtilis* 2B10 (Fig 3&4). Both *B. subtilis* 2B10 and 2C5 were active against *C. lunata* with the zone of inhibition 10 mm and 13 mm respectively. Similarly the antagonistic and inhibitory effect of *Bacillus subtilis* against *Fusarium* spp., *Alternaria* spp., *Aspergillus* spp., and *Helminthosporium* spp. was previously studied by (Matar *et al.,* 2009; Bharose *et al.,* 2018) [7, 2].

The observed data revealed that the rhizospheric soil of rice was more favorable niches for the growth of soil microflora during the summer period (June to August) and fields of the Sardulgarh and Faridkot regions contain antagonistic microorganisms as compared to Bathinda. These antagonist bacteria play an important role in the natural suppression of major soil-borne and post-harvest fungi. The results of the study indicate that the rhizospheric soil of the Talwandi sabo region contains fewer numbers of antagonist microbes, can be conducive soils, while Sardulgarh and Faridkot region contain antagonistic bacterial strains along with pathogens could be suppressive soils.

Table 1: Average mean of inhibition zone formation by *Bacillus subtilis* 2B10 and 2C5 against various selected fungal genera

S. No.	Fungal isolates	Inhibition zone by *Bacillus subtilis* (2B10) in (mm)	Inhibition zone by *Bacillus subtilis* (2C5) in (mm)
1	*Aspergillus niger* (1)*	12.2	10.5
2	*Aspergillus niger* (2)**	14.2	9
3	*Aspergillus flavus*	18	10
4	*Aspergillus ochraceus*	12	0
5	*Aspergillus* niger (3)***	11	9
6	*Alternaria alternate*	0	11
7	*Alternaria brassicae*	0	0
8	*Curvularia lunata*	10	13
9	*Fusarium oxysporum*	9	8
10	*Fusarium chlamydosporum*	13	12
11	*Fusarium* sp.	11	0
12	*Helminthosporum* sp.	14	27.5
13	*Penicillium chrysogenum*	30	16.7
14	*Penicillium expansum*	18	10
15	*Penicillium* sp.	27	21
16	*Rhizopus* sp.	0	0
17	*Ustilaginoidea virens*	11	12.5

*Isolate 1st ** Isolate 2nd, *** Isolate 3rd

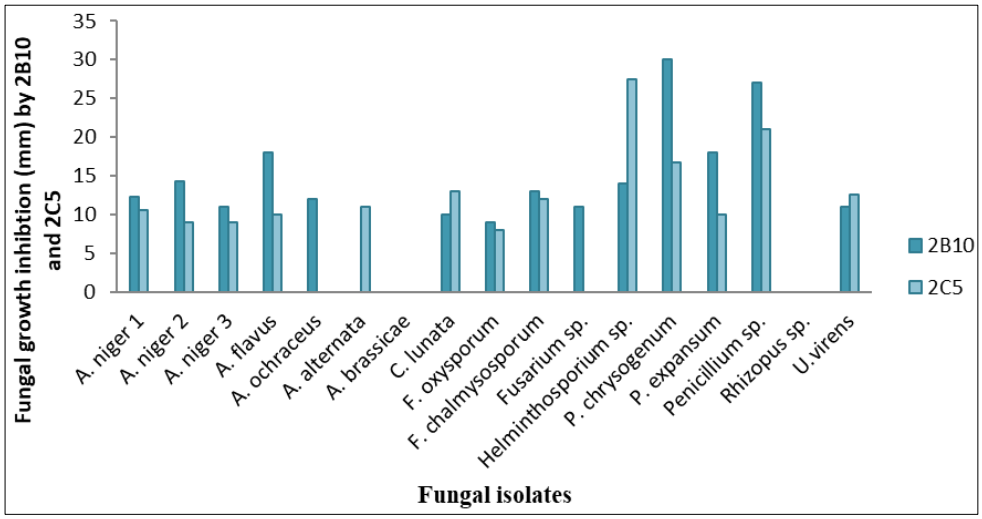
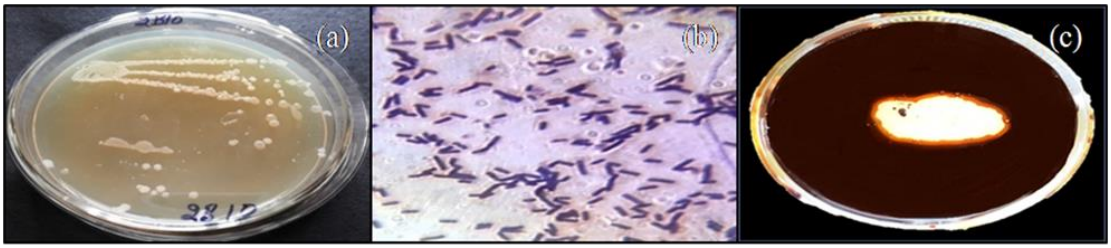
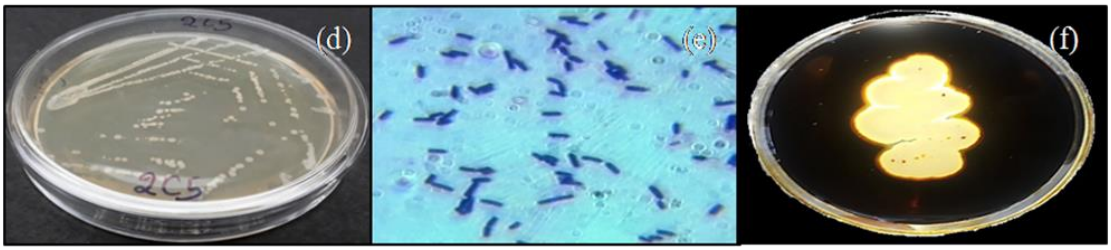


Fig 1: Graphical representation of fungal growth inhibition by *Bacillus subtilis* 2B10 and2C5 against selected fungal isolates



(a) Colony morphology of *Bacillus subtilis* (2B10) (b) Bacterial shape (c) Strach hydrolysis test



(d) Colony morphology of *Bacillus subtilis* (2C5) (e) Bacterial shape (f) Strach hydrolysis test

Fig 2: Colony morphology of active bacterial isolates, their shape, and starch hydrolysis test

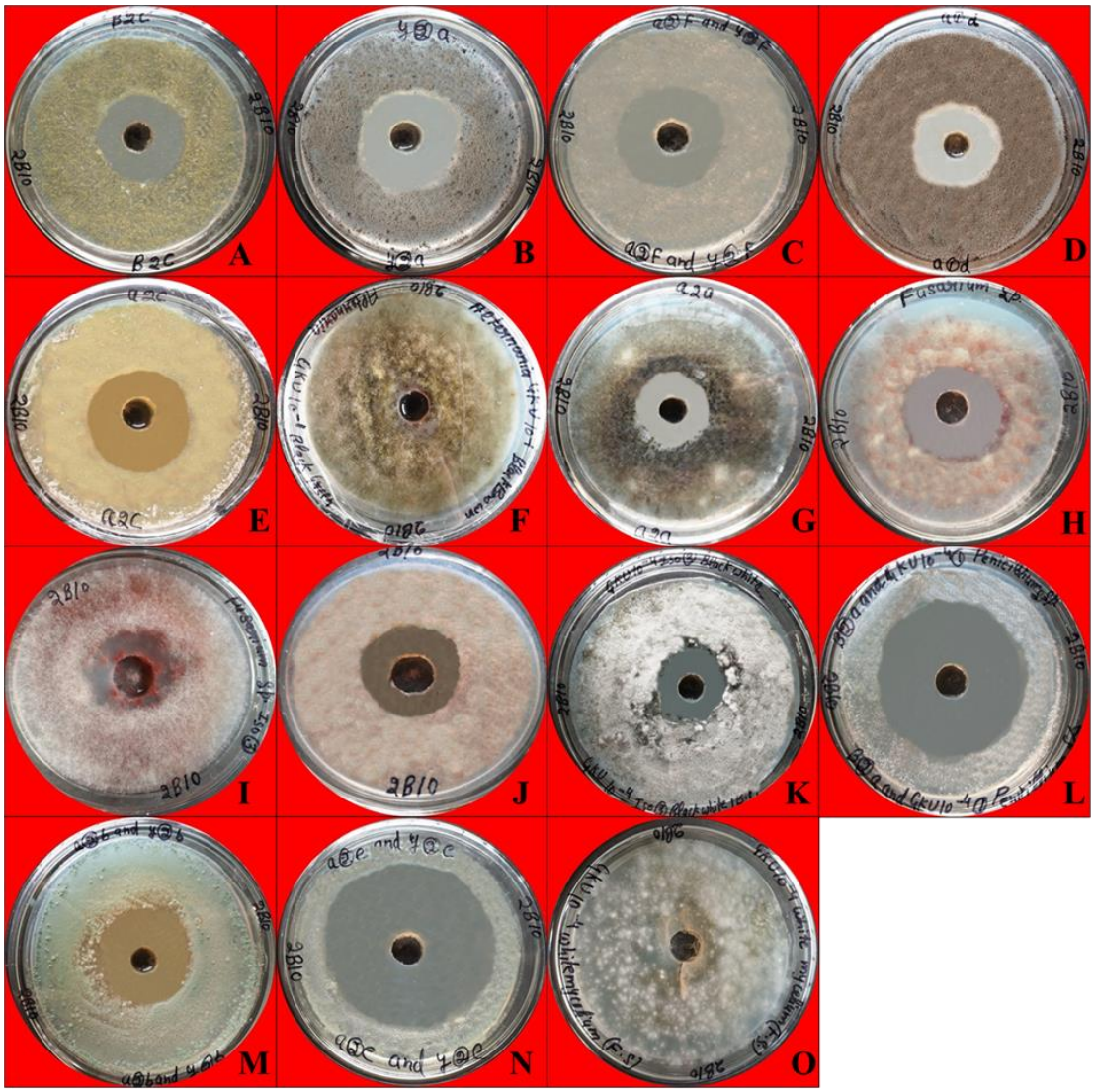


Fig 3: (A) *A. flavus* (B) *A. niger* 1 (C) *A. niger* 2 (D) *A. niger* 3 (E) *A.ochraceus* (F) *A. brassicae* (G) *C. lunata* (H) *F. chalmydosporum* (I) *F. oxysporum* (J) *Fusarium* sp. (K) *Helminthosporium* sp. (L) *P. chrysogenum* (M) *P. expansum* (N) *Penicillium* sp. (O) *U. virens*

Fig 3: Antagonistic activity (zone of Inhibition) of *Bacillus subtilis* (2B10) against selected fungal isolates

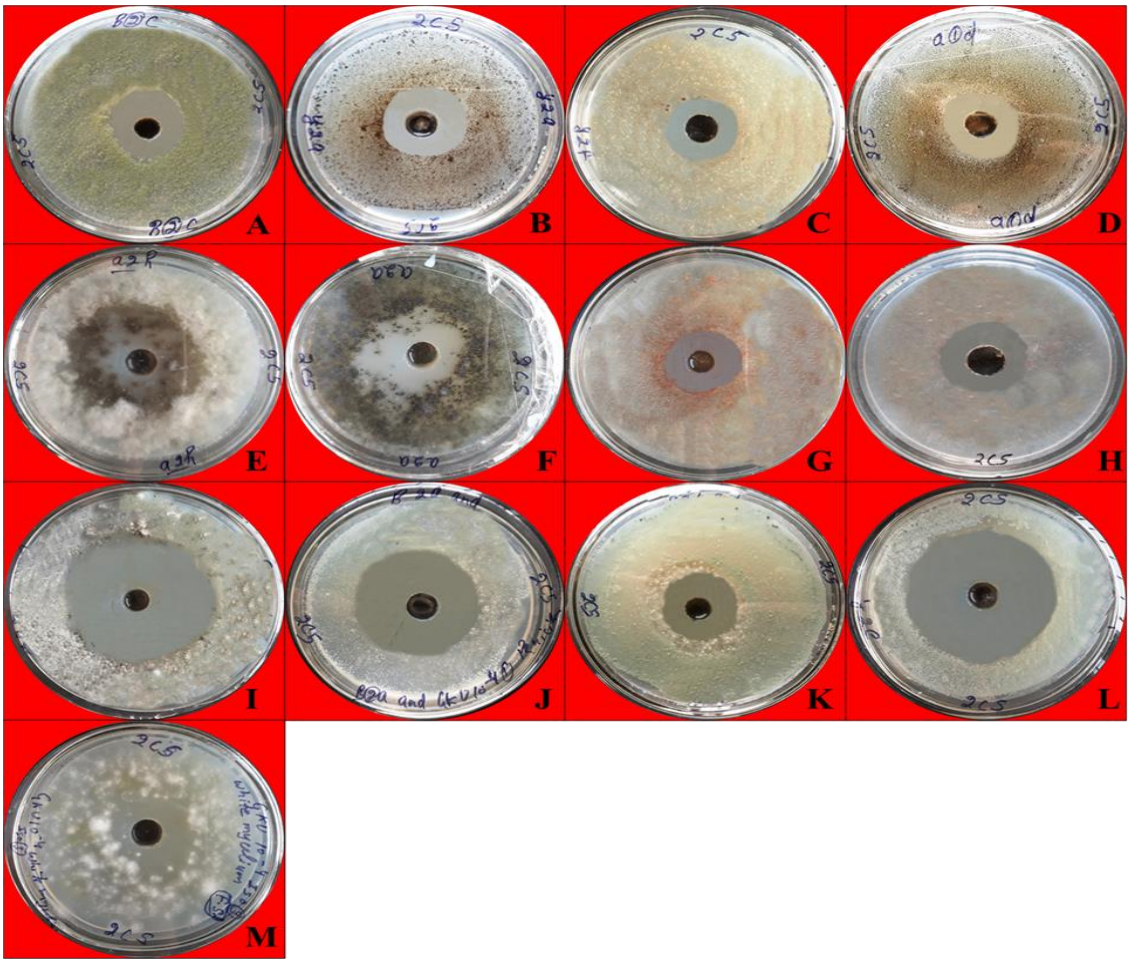


Fig 4: (A) *A. flavus* (B) *A. niger* 1 (C) *A. niger* 2 (D) *A. niger* 3 (E) *A. alternata* (F) *C. lunata* (G) *F. oxysporum* (H) *F. chalmydosporum* (I) *Helminthosporium* sp. (J) *P. chrysogenum* (K) *P. expansum* (L) *Penicillium* sp. (M) *U. virens*

Fig 4: Antagonistic activity (zone of Inhibition) of *Bacillus subtilis* (2C5) against selected fungal isolates

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