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Harshita
Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India

UK Tripathi
Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India

JB Khan
Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India

Ved Ratan
Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India

Alka Kushwaha
Department of Botany, D.A.V College, Kanpur, Uttar Pradesh, India

Udit Narain
Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India

Neetu Trivedi
Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India

YK Srivastava
Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India

Corresponding Author:
Harshita
Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India

Characterization of *Fusarium oxysporum* f.sp. *ciceri* and *Rhizoctonia bataticola* isolates causing wilt complex in Chickpea

Harshita, UK Tripathi, JB Khan, Ved Ratan, Alka Kushwaha, Udit Narain, Neetu Trivedi and YK Srivastava

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Abstract
Fusarium wilt (*Fusarium oxisporum* f.sp. *ciceri*) is a major constraint to chickpea production worldwide and under favorable conditions, it is known to cause up to 100% loss. Another important disease emerging as a potential threat to chickpea cultivation in post- flowering stage is dry root rot (*Rhizoctonia bataticola*) because of the extraordinary host range, geographical distribution and environmental adaptability of this pathogen. Six isolates of *F. oxysporum* f.sp. *ciceri* (HF-1, HF-2, HF-3, HF-4, HF-5, HF-6) and two isolates of *R. bataticola* (HR-1, HR-2) were isolated from the infected chickpea root samples collected from different chickpea growing districts of U.P. All the isolates of both the test pathogens exhibited variability in cultural characteristics and pathogenicity. However, these did not show much variation with respect to shape and colour of mycelium, micro conidia, macro conidia and chlamydospores or sclerotia. HF-4 and HR-2 isolates were found to be the most pathogenic isolates.

Keywords: *Fusarium oxisporum* f.sp. *ciceri*, *Rhizoctonia bataticola*, cultural characteristics, morphology, pathogenicity

Introduction
Chickpea (*Cicer arietinum* L.), grown in over forty countries globally, is the world's third most vital grain legume after common bean and pea (Anwar *et al*., 2009). Being vulnerable to a number of fungal pathogens from seedling to maturity stage, chickpea's total production and productivity are quite low despite of the large area under its cultivation (Pande *et al*., 2006) [23]. *Fusarium oxysporum* f. sp. *ciceri* is the most aggressive pathogens of chickpea causing severe economic losses up to 100% under favorable conditions (Halila and Strange, 1997) [30] and in the absence of susceptible host it can survive in soil by producing resting spores (Leslie and Summerell, 2006) [29]. At present, dry root rot caused by *Rhizoctonia bataticola* is also a destructive constraint to chickpea productivity and production in most of the regions of India. *R. bataticola* is a soil and seed borne necrotrophic fungal pathogen that has a global distribution infecting more than 284 plant species throughout the world. Considering the severity and loss caused by these two pathogens, it was thought necessary to initiate comprehensive investigation on the cultural, morphological and pathogenic variation of *Fusarium oxysporum* f. sp. *ciceri* and *R. bataticola* isolates.

Materials and Methods
1. Collection of disease samples
Chickpea plants showing Fusarium wilt and dry root rot symptoms were collected from the fields of different districts of Uttar Pradesh. Each sample was shade dried, wrapped in old news paper, kept in poly propylene bag and clearly marked with sample number, crop, block, district and date of collection. Thereafter, these were brought to the laboratory of Department of Plant pathology, CSAUA&T, Kanpur for further investigation.

2. Isolation of test pathogens
The root samples of chickpea plants showing characteristic symptoms were used for the isolation of test pathogens by following standard tissue isolation method.

The infected chickpea root samples were thoroughly cleaned by washing in sterilized distilled water and dried with sterilized blotting paper. Thereafter, these roots were cut into small pieces, surface sterilized with 1% Sodium hypochlorite solution for 30 seconds and then rinsed in sterilized water for three times after which these cut pieces were dried between folds of sterilized tissue paper. The pieces were placed onto the Petri plates containing solidified PDA under aseptic conditions of Laminar Air Flow cabinet. These inoculated plates were then incubated in a BOD incubator at 25 ± 2 ºC. As soon as the growth of pathogen occurred, a hyphal bit was taken from the periphery of the growing fungal colony with the help of a sterilized needle and was aseptically sub cultured on a PDA slant for preparing the pure cultures of each fungal pathogen isolate separately.

3. Identification of test pathogen

For identification of different isolates of pathogens, their colonies growing on potato dextrose agar medium were examined under Light microscope (Olympus). Based on colony colour, growth pattern, type of mycelium, chlamydospores or sclerotia and the spores produced, tentatively the colonies of different pathogens were separated. Later on the slides of the pathogens having dark colour colonies were prepared in lactophenol only and of those having cottony white colonies were prepared with lactophenol-cotton blue stain. The *Fusarium* cultures were separated by comparing the cultural and morphological characters of the fungus with those as described by Booth (1971) [4]. Likewise, *R. bataticola* cultures were identified using the descriptions given by C.M.I (1970) [6]. Few (most virulent) cultures were also sent to NFCCI (National Fungal Culture Collection of India), Pune, Maharashtra for further reconfirmation of identification of fungal pathogens.

4. Cultural and morphological characterization of pathogens

Cultural and morphological characters of all the isolates of both the fungal pathogens were observed on PDA medium. To study the cultural characters, pathogen culture was grown on PDA, photographed using digital camera and colony colour, substrate colour colony texture, mean colony diameter at 7 DAI (day after inoculation), aerial mycelium (presence or absence) were noted. For morphological studies, the slides of all the isolates having dark coloured colonies were prepared in lactophenol only and of those having cottony white or light coloured colonies were prepared with lactophenol-cotton blue stain. The slides thus prepared were examined under Light microscope (Olympus) and conidial size was measured using ocular micrometer (calibrated using stage micrometer). The features were micro photographed digitally at 40X magnification and mycelium colour, branching pattern, septation and width were noted along with shape, colour, size, septation of microconidia, macroconidia and chlamydospores or sclerotia.

5. Pathogenicity test of the pathogens

The Pathogenicity test was conducted for both pathogens by using sick pot technique with all essential four steps of Koch postulates.

5.1 Preparation of pathogen inoculum

The inoculum of the each isolate was multiplied on sorghum seeds. Sorghum grains were soaked for overnight, excess water was drained out, soaked grains were filled in autoclavable PP (Poly Propylene) bags @100 g/bag and plugged with non-absorbent cotton plugs which were then autoclaved at 15psi or 30 min. After autoclaving the bags were left for proper cooling. Hyphal bits from the growing colonies of *Fusarium oxysporum* f.sp. *ciceri* and *Rhizoctonia bataticola* isolates were cut with a sterile cork borer of 5-mm diameter, transferred to separate PP bags (properly marked with specific isolate's code) under sterilized condition of Laminar Air Flow(LAF) and incubated at 25 ±1ºC in a BOD incubator.

5.2 Preparation of Sick Pots

Plastic pots were thoroughly washed with laboline detergent and water. Soil was cleaned, pulverized and then sterilized in autoclave. The sterilized soil was inoculated separately with each isolate's inoculum @ 5% w/w and filled in separate plastic pots (properly marked with specific isolate's code) which were then regularly sprinkled with a little water and left for ten days in order to build up the inoculum load in the soil. For each isolate separate pot was used and for each isolate 3 replications were maintained.

5.3 Pathogenicity test

In each of the sick pots, 5 chickpea seeds of highly susceptible variety (JG-62) were sown and regular observations were made for the appearance of the disease. Wilted seedlings from these pots were collected in respect of each isolate. These plants were used for re-isolation of the pathogen isolate. The isolates obtained from these plants were compared with the original isolate with which these were inoculated in order to confirm the Koch's postulates. Observations for wilt incidence were recorded at 30 DAS.

Result and Discussion

1. Isolation, purification and identification of pathogens

Six isolates of *Fusarium oxysporum* f.sp. *ciceri* (HF-1, HF-2, HF-3, HF-4, HF-5, HF-6) and two isolates of *Rhizoctonia bataticola* (HR-1, HR-2) were isolated from the infected chickpea root samples collected from different chickpea growing districts of U.P (Table 1 & Fig.1). HF-4 and HR-2 isolates were re-identified as *Fusarium oxysporum* f. sp. *ciceri* (NFCCI 4792) and *Rhizoctonia bataticola* (NFCCI 4791) respectively on basis of identification report of NFCCI (National Fungal Culture Collection of India), Pune, Maharashtra. Similar methodology was followed by Rangaswamy and Mahadevan (1999) [31] for isolation of the pathogen from wilt infected chickpea plants.

Table 1: Test pathogens isolated from diseased plant samples of different districts of U.P.

S. No.	Isolate code	Pathogens	Village/District	GPS Coordinates
1.	HF-1	*Fusarium oxysporum* f. sp. *ciceri*	Barua Kalinjar, Banda	25°6'37.4544"N 80°29'35.0916"E
2.	HF-2	*Fusarium oxysporum* f. sp. *ciceri*	Atra, Hamirpur	25°47'48.876"N 79°29'4.4952"E
3.	HF-3	*Fusarium oxysporum* f. sp. *ciceri*	Nadigaon, Jalaun	26°6'28.476"N 79°0'58.878"E
4.	HF-4	*Fusarium oxysporum* f. sp. *ciceri*	Poonch, Jhansi	25°49'16.1004"N 79°2'34.5804"E
5.	HF-5	*Fusarium oxysporum* f. sp. *ciceri*	Rania, Kanpur	26°24'41.6988"N 80°6'21.8556"E
6.	HF-6	*Fusarium oxysporum* f. sp. *ciceri*	Leta, Mahoba	25°22'49.9944"N 79°34'21.1188"E
7.	HR-1	*Rhizoctonia bataticola*	Ramgarh, Pratapgarh	25°49'54.1992"N 81°44'25.206"E
8.	HR-2	*Rhizoctonia bataticola*	Shah Patan, Banda	25°7'40.3824"N 80°27'50.5764"E

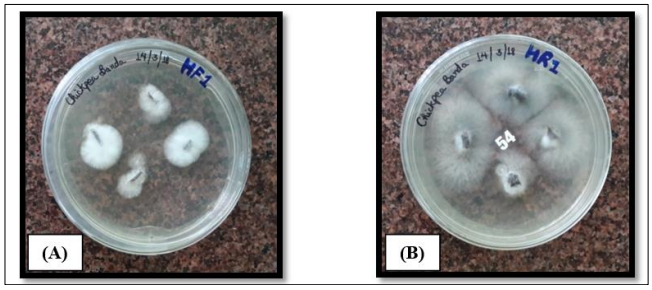

Fig 1: Isolation of Test pathogens from diseased chickpea root samples (A) *Fusarium oxysporum* f. sp. *ciceri* (B) *Rhizoctonia bataticola*

2. Cultural characterization of test pathogens

All the six isolates of *Fusarium oxysporum* f. sp. *ciceri* exhibited variability in cultural characteristics (Table 2 & Fig.2). Three isolates (HF-1, HF-5 and HF-6) exhibited fluffy growth; two isolates (HF-2 and HF-4) had semi-appressed growth while one isolate (HF-3) showed appressed growth. Variation in colony colour was observed in all the isolates on PDA medium. Initially, the colour of all isolates was white, which changed gradually with time showing different shades like dull white (HF-1), purplish white (HF-2), creamy white (HF-3), milky white (HF-4) and cottony white (HF-5 and HF-6). Variation in pigmentation *viz*., brownish, light yellow and violet within the isolates have been reported by several workers (Gupta *et al.*, 1986; Agrawal and Gupta, 2006; Groenewald *et al.*, 2006 and Patel and Anahosur, 2001) [12, 2, 11, 24]. According to Dubey *et al.* (2010) [9], Mandhare *et al.* (2011) [19] and Rosa *et al.* (2011) [32], Fusarium wilt isolates were highly variable in their colony growth pattern, size of colony and pigmentation, which are in conformity with present investigation. Singh *et al.* (2010) [9] also observed dull white to pinkish white, thin and flat hairy to fluffy growth with irregular margins. Similarly, Burgess *et al.* (1989) [5] reported that the *Fusarium oxysporum* was extensively variable in cultural and morphological diversity. Paulkar and Raut (2004) [28] also reported such variations in mycelial growth pattern. Honnareddy and Dubey (2007) [14] found differences in respect of their colony colour, pigmentation of substrate, growth rate, presence of macro conidia and virulence on susceptible variety L 550. The two isolates of *Rhizoctonia bataticola* exhibited significant variability in cultural characteristics (Table 2 & Fig.2). Black and appressed colony was observed in isolate HR-1 while black and fluffy colony with grey aerial mycelium was developed in isolate HR-2. The substratum colour of mature colony in both the isolates was black. Similar findings have been reported by Aghakhani and Dubey (2009) [1], Manjunatha and Naik (2011) [20] and Gupta *et al.* (2012) [12] who analyzed the cultural and morphological diversity in isolates of *R. bataticola* causing dry root rot of chickpea.

Table 2: Cultural characteristics of test pathogen isolates on PDA medium

S. No.	Isolate Code	Colony Colour	Substrate Colour	Colony Texture	Aerial mycelium	Mean Colony Diameter (mm)
1.	HF-1	Dull white	Dark creamish	Fluffy	Present	41.33
2.	HF-2	Purplish white	Purple	Semi-appressed	Present	50.65
3.	HF-3	Creamy white with cream coloured ring delimiting the central zone	Creamish	Appressed	Absent	54.09
4.	HF-4	Milky white	Creamish	Semi-appressed	Present	85.67
5.	HF-5	Cottony white turning dull white at places	Brown	Fluffy	Present	58.47
6.	HF-6	Cottony white	Creamy white	Fluffy	Present	74.91
7.	HR-1	Black	Black	Appressed	Absent	62.62
8.	HR-2	Black with grey aerial mycelium	Black	Fluffy	Present	90.00

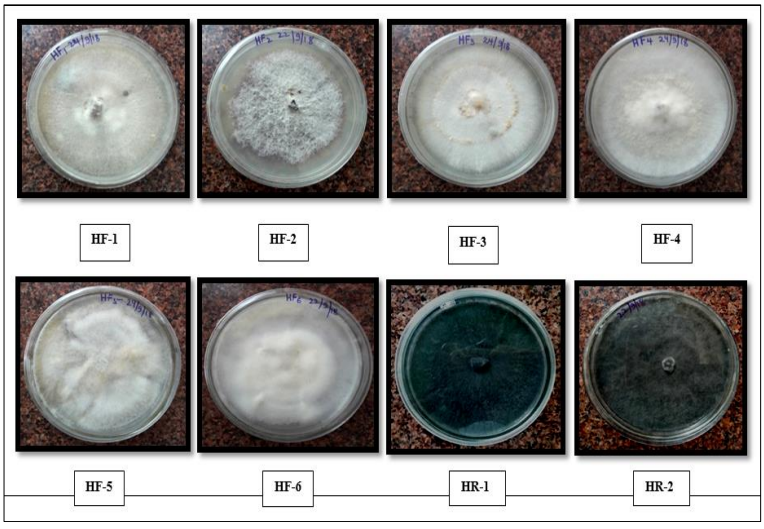

Fig 2: Cultural variability of *F. oxysporum* f. sp. *ciceri* (HF-1, HF-2, HF-3, HF-4, HF-5, HF-6) & *R. bataticola* isolates (HR-1, HR-2)

3. Morphological characterization of test pathogens

All the isolates of *Fusarium oxysporum* f. sp. *ciceri* did not show much variation with respect to shape and colour of mycelium, micro conidia, macro conidia and chlamydospores, where the mycelium was hyaline, cylindrical, profusely branched and septate; microconidia were hyaline, ovoid to ellipsoidal in shape, single celled and 0-1 septate while macroconidia were hyaline, fusiform or sickle shaped, pointed or blunt at both ends with 2-4 septa (Table 3 & Fig.3). Chlamydospores formed in all the isolates were single celled, oval or globose, terminal, intercalary or in chains having 5.58-9.12 µm average diameter. The average size of micro-conidia

measured 4.26-8.25 × 2.64-4.58 µm while that of macro conidia measured 9.58-19.75 × 3.25-5.43 µm. This has been supported by Patil *et al.* (2005) [25] who revealed that the isolates of *F. oxysporum* f. sp. *ciceri* had variation in number and size of macro and microconidia, cultural characters, growth pattern, pigmentation and sporulation. The results also coincided with earlier workers like, Gupta *et al.* (1986) [12], Desai *et al.* (1994) [7]. Dubey *et al.* (2010) [9] documented one hundred and twelve isolates by twelve categories, among which micro conidia size varied from 5.1-12.8 x 2.5-5.0 µm and macro conidia ranged 16.5-37.9 x 4.0-5.9 µm with 1-5 septations. Kaur *et al.* (2015) [16] reported that twenty four isolates of *Fusarium oxysporum* f. sp. *ciceris* produced significant variation in size of micro (8.9-16.9 x 3.1-6.3 µm) and macro (21.7-64.9 x 2.7-10.0 µm) conidia. Both the isolates of *Rhizoctonia bataticola* produced grey coloured septate hyphae that later became darker at maturity. Average hyphal width of HR-1 isolate measured 5.36 µm while that of HR-2 isolate measured 6.49 µm. The isolate HR-1 produced round, grey to black coloured sclerotia whose average size measured 114.45 × 111.28 µm. While in HR-2 isolate, sclerotia were black, irregular in shape having an average size of 126.32 × 112.56 µm (Table 4 & Fig.3). The above observations were in accordance with the descriptions given by Short and Wyllie (1978) [33]. Devi and Singh (1998) [8] observed bigger sclerotia in isolate MP - 2 (400 x 280 µm) while working on *Macrophomina phaseolina* isolates in mungbean. He also observed typical right angled branching of mycelium in one of the isolate and acute to right angle branching in certain isolates.

Table 3: Morphological variability of *Fusarium oxysporum* f. sp. *ciceri* isolates

S. No.	Isolate Code	Micro conidia			Macro conidia			Chlamydospore Diameter (µm)*
		Length (µm)*	Width (µm)*	No. of Septa*	Length (µm)*	Width (µm)*	No. of Septa*	
1.	HF-1	4.26	2.83	0	9.58	3.60	2.2	5.81
2.	HF-2	7.57	3.61	0	13.08	3.25	2.0	9.12
3.	HF-3	5.81	2.64	0.1	12.63	3.56	2.6	5.58
4.	HF-4	7.85	4.02	0	13.26	4.92	2.4	6.02
5.	HF-5	5.33	2.94	0.4	12.39	3.34	3.6	7.41
6.	HF-6	8.25	4.58	0.2	19.75	5.43	1.8	6.96

*Average of 10 observations

Table 4: Morphological variability of *Rhizoctonia bataticola* isolates

S. No.	Isolate Code	Sclerotia			Hyphal Width (µm)*
		Length (µm)*	Width (µm)*	Shape	
1.	HR-1	114.45	111.28	Round	5.36
2.	HR-2	126.32	112.56	Irregular	6.49

*Average of 10 observations

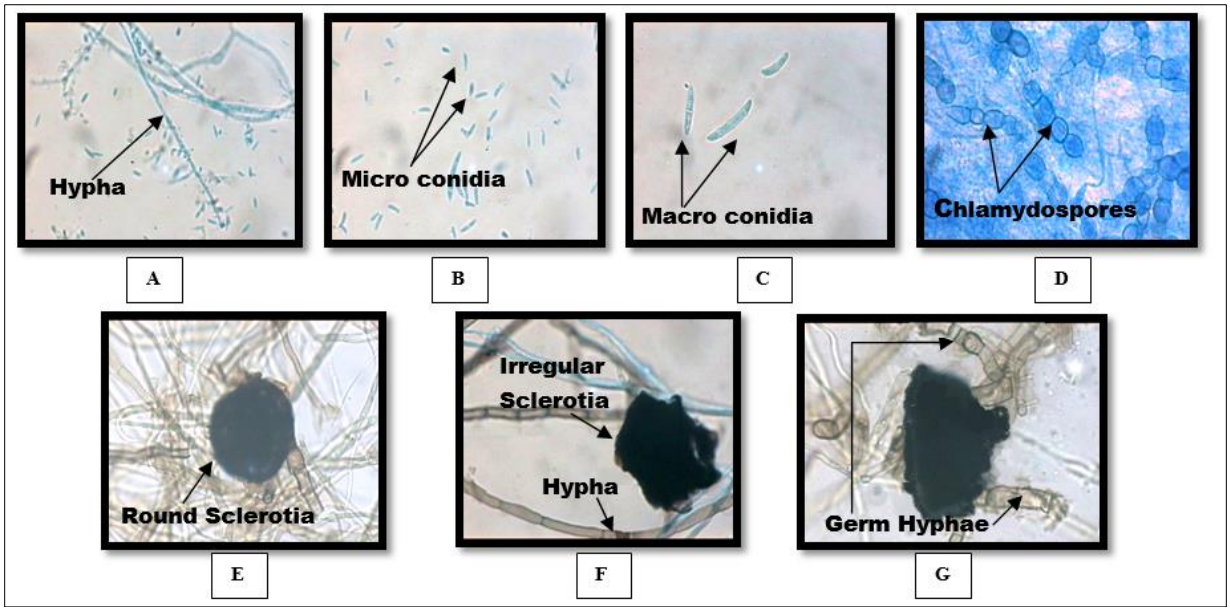

Fig 3: Morphological features of *F. oxysporum* f. sp. *ciceri* [(A) Hypha (B) Micro conidia (C) Macro conidia (D) Chlamydospores] & *R. bataticola* [(E) Round Sclerotia (F) Hypha and Irregular Sclerotia (G) Germinating Sclerotia]

4. Pathogenicity test of the pathogens

Six *Fusarium oxysporum* f.sp. *ciceri* and two *Rhizoctonia bataticola* isolates were subjected to pathogenicity test in pots using highly susceptible variety (JG-62). Based on the mean disease severity (MDS) at 30 DAS, the virulence of each isolate was recorded as low (MDS: 50%), moderate (MDS: 25–50%) or high (MDS: > 50%) as denoted by Nirmaladevi *et al.* (2016) [22]. Isolates HF-4 (*Fusarium oxysporum* f.sp. *ciceri*) and HR-2 (*Rhizoctonia bataticola*) were found to be highly pathogenic recording a disease incidence of 100 per cent (Table 5 & Fig.4). Further studies were conducted with highly pathogenic isolate.

Table 5: Pathogenicity test of *Fusarium oxysporum* f.sp. *ciceri* and *Rhizoctonia bataticola*

S. No.	Treatment detail	No. of seeds sown/ pot*	No. of plants emerged/ pot*	No. of plants wilted/ pot*	Disease incidence* (%)	Virulence
1.	Soil inoculated with HF-1	5	4.67	1.33	30	Moderate
2.	Soil inoculated with HF-2	5	4.33	2	46.67	Moderate
3.	Soil inoculated with HF-3	5	5	2.33	46.67	Moderate
4.	Soil inoculated with HF-4	5	5	5	100	High
5.	Soil inoculated with HF-5	5	3	1.67	36.67	Moderate
6.	Soil inoculated with HF-6	5	5	4	80	High
7.	Soil inoculated with HR-1	5	4	1.67	47.22	Moderate
8.	Soil inoculated with HR-2	5	5	5	100	High

* Mean of three replications

The results obtained are in agreement with the findings of Paulkar *et al.* (2002) [27], Meki *et al.* (2008) [21], Shinde (2003) [35], Khilare *et al*. (2007) [18], Patil *et al.* (2017) [26] who proved the pathogenicity of *F. oxysporum* f. sp. *ciceri* isolates and Katariya *et al.* (2007) [17], Jayalakshmi *et al.* (2008) [15], Veena *et al.* (2014) [36], Gadekar *et al.* (2018) [10] who proved the pathogenicity of *Rhizoctonia bataticola.*

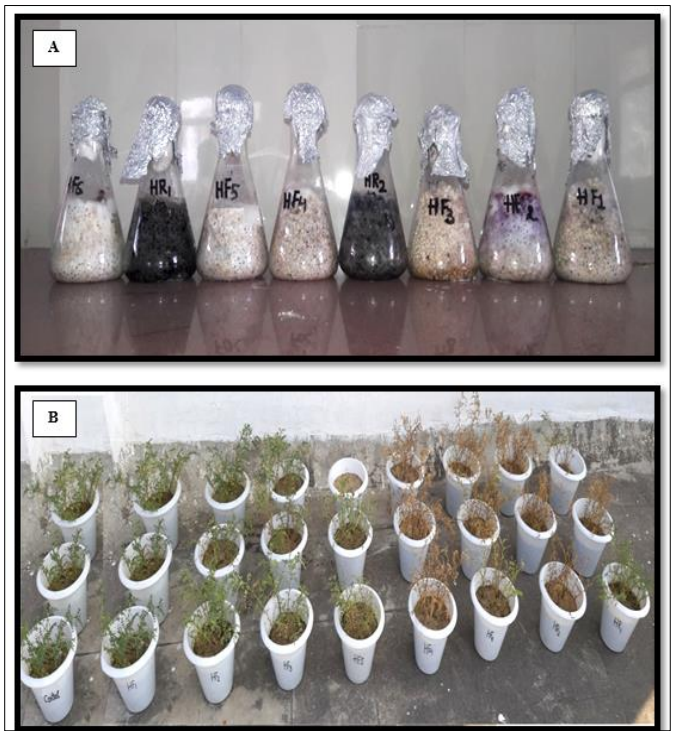

Fig 4: Pathogenicity Test of pathogens (A) Inoculum of pathogen isolates on sorghum grains (B) Pathogenicity Test in soil inoculated conditions

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