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Optimizing growth conditions for *Fusarium humuli*, the cause of pod rot in black gram (*Vigna mungo* L. hepper): A study on media, pH, and temperature effects

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Abstract
Black gram (*Vigna mungo* L. Hepper), a vital crop in South Asia, plays a crucial role in traditional cuisines and sustainable farming practices. Despite its significance, black gram faces challenges from various diseases, with pod rot caused by *Fusarium* spp. emerging as a recent concern. This study aims to identify the *Fusarium* species responsible for pod rot in Pantnagar, Uttarakhand, India, through morphological and molecular assays, and to investigate its optimal conditions.The mycelial growth and sporulation of the identified pathogen were examined across eight different culture media, with pH levels ranging from 4.0 to 8.0, and varying temperature levels from 0°C to 40°C. A *Fusarium* isolate was obtained from symptomatic black gram pods and identified as a member of the *Fusarium incarnatum-equiseti* species complex. Subsequent molecular analysis confirmed its similarity to *F. humuli*. Among the culture media tested, Potato Dextrose Agar proved to be the most effective. pH studies revealed that the pathogen thrived best at pH 6.5 and 7.0, while temperatures of 28°C were optimal for both growth and sporulation. These findings provide insights into the environmental factors influencing *Fusarium* spp. pathogenicity, thereby guiding future studies and disease management strategies.

Keywords: Black gram, *Fusarium* spp., India, optimal conditions, pH, pod rot, temperature

Introduction
Black gram (*Vigna mungo* L. Hepper), commonly known as urad bean, holds significant agricultural and culinary importance across various regions worldwide, particularly in South Asia. Its role in traditional cuisines and agricultural practices is profound, and it serves as a cornerstone of sustainable farming. As a rotation crop, black gram enhances soil fertility by harnessing atmospheric nitrogen through a symbiotic relationship with nitrogen-fixing rhizobia bacteria (Senaratne and Ratnasinghe, 1993; Lengwati *et al*., 2020) [14, 11]. This enriches the soil, benefiting subsequent crops. Nonetheless, similar to any agricultural crop, black gram cultivation confronts challenges such as pest invasions and diseases.

Reports from around the world have documented approximately eight diseases affecting black gram, including anthracnose (*Colletotrichum lindemuthianum* or *C. gloeosporioides*), brown leaf spot (*Alternaria alternata*), dry root rot and stem canker (*Macrophomina phaseolina*), leaf crinkle disease (urdbean leaf crinkle virus), Cercospora leaf spot (*Cercospora canescens* or *C. cruenta*), powdery mildew (*Erysiphe polygoni* or *Podosphaera fusca*), rust (*Uromyces appendiculatus*), web blight (*Rhizoctonia solani*), and yellow mosaic disease (Mungbean yellow mosaic virus) (Parthasarathy *et al*., 2020) [13]. Recently, a novel disease, pod rot caused by *Fusarium* spp., has emerged in Indian black gram crops (Verma *et al*., 2023) [18]. This fungal pathogenic invader particularly targets black gram cultivated during the *Kharif* season, resulting in significant yield losses or even complete crop failure (with disease incidences ranging from 80 to 92%). Affected pods exhibit the emergence of white to light pink fungal growth at the pod tips, gradually extending to envelop the entire pod surface.

Fusarium spp. have a broad geographical presence, colonizing soils, plants, and the atmosphere (Burgess *et al*., 1994) [2].

However, the prevalence of *Fusarium* in soil and its subsequent infestation of plants are subject to diverse influencing factors. Key among these factors are soil pH, temperature, and water activity, all of which wield direct influence on the development, survival, and propagation of *Fusarium* species, as well as the resulting crop damage caused by these pathogens (Yadav *et al*., 2014) [20]. Additionally, variations in carbon and nitrogen sources play a pivotal role in influencing pathogen growth (Dubey, 2016) [6]. Furthermore, environmental variables like temperature, moisture, and pH profoundly impact the production of toxins by different fungal species, with these elements proving crucial in understanding mycotoxicosis epidemiology (Jimenez *et al*., 1996) [9]. Identifying the preferable and non-preferable nutrient sources, pH ranges, and temperatures will significantly aid researchers in future studies and the formulation of effective disease management strategies within the agricultural field.

Recent findings have reported five distinct *Fusarium* species involved in causing pod rot in black gram crops in India (Verma *et al*., 2023; Verma *et al*., 2024) [18, 19]. With this information, our primary objective was to identify the exact *Fusarium* spp responsible for causing pod rot in black gram cultivated in Pantnagar, Uttarakhand. Subsequently, we conducted experiments to ascertain the optimal culture medium, pH levels, and temperature conditions conducive to the mycelial growth and sporulation of the identified pathogen.

Material and Methods

Pathogen isolation, identification and pathogenicity test

Diseased black gram pods, exhibiting typical pod rot symptoms, were collected from the Crop Research Center at Govind Ballabh Pant University of Agriculture and Technology in Pantnagar, Uttarakhand, India (29.0222° N, 79.4908° E, and 243.84 m). The collected symptomatic pods were washed, cut into pieces along with some healthy portions (0.5 × 0.5 mm), surface-disinfested with 1% sodium hypochlorite (NaOCl) for 30 seconds, followed by three consecutive rinses with sterile distilled water. Subsequently, they were dried on sterilized filter paper, aseptically placed on potato dextrose agar (PDA) amended with 30 mg/liter streptomycin sulfate, and then incubated at 25±2°C for 5-6 days or until visible mycelial growth was observed. The resulting fungal culture was purified using the hyphal tip technique. The isolated pathogen was then inoculated onto potted 45-day-old black gram plants (Pant urd-19) to ensure pathogenicity. Five healthy plants were sprayed with 50 µl of a conidial suspension of the isolate (10^7 conidia ml^{-1}, 10 ml/plant), while five plants sprayed with sterile distilled water were treated as controls. The inoculated plants were covered with sterilized plastic bags to maintain humidity and were kept in a greenhouse. The appearance and progression of symptoms were observed on a daily basis. The pathogen was later reisolated from the inoculated pods and confirmed through morphological characterization and molecular assays.

Isolates were cultured on carnation leaf agar at 25±2°C for morphological studies. Colony colors were observed following an RGB color code chart. Micro-morphological features were examined using a compound microscope with a digital camera at 40X and 100X magnifications. For identification, references like 'The Fusarium Laboratory Manual' by Leslie and Summerell (2006) [12] were consulted. For further confirmation, molecular identification was carried out. Fungal DNA (Deoxyribonucleic acid) was extracted using the method outlined in (Zhang *et al.*, 1998) [21] Amplification of the internal transcribed spacer (ITS) region and the translation elongation factor-1 alpha (tef1) genes was performed using primers ITS-1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), EF-1 (5′-ATGGGTAAGGARGACAAGAC-3′) and EF-2 (5′-GGARGTACCAGTSATCATGTT-3′) and fRPB2-5F (5′-GAYGAYMGVGATCAYTT-3′) fRPB2-5R (5′-CCCATRGCTTGYTTRCCCAT-3′). The obtained amplicons were purified and outsourced to HiMedia Laboratories Pvt. Ltd (Delhi, India) for sequencing. The resulting sequences were deposited in GenBank and subjected to blast analysis in NCBI as well as Polyphasic identification in the FUSARIOID database tool. Phylogenetic analysis was carried out using MEGA X and FUSARIOID.

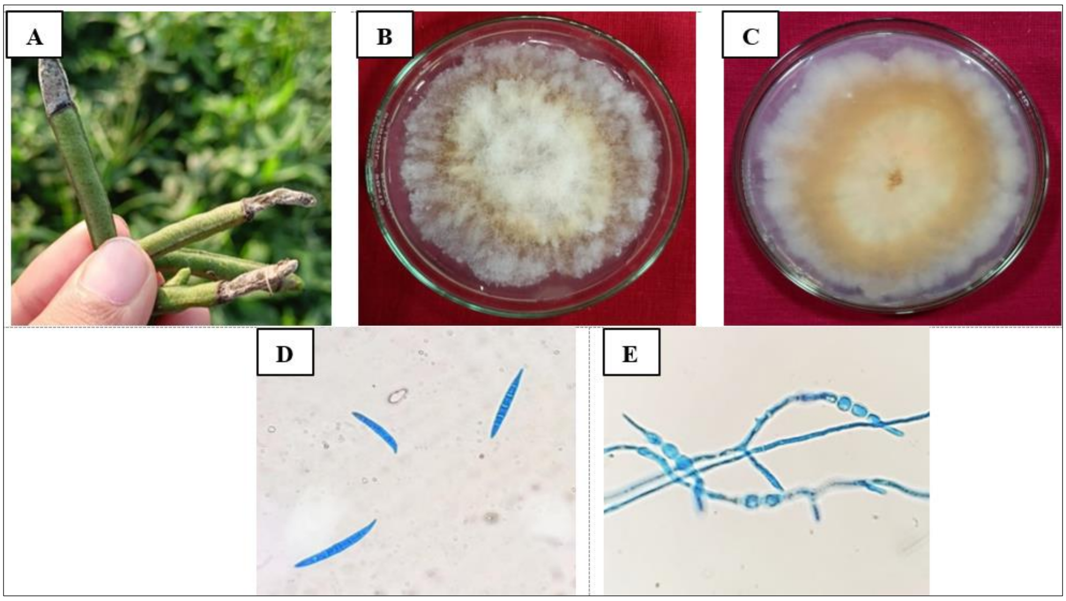

Fig 1: A. Pod rot of black gram B. upper view of *Fusarium humuli* colony C. Reverse view of colony D. Macroconidia (100x) E. Chlamydospores (100x).

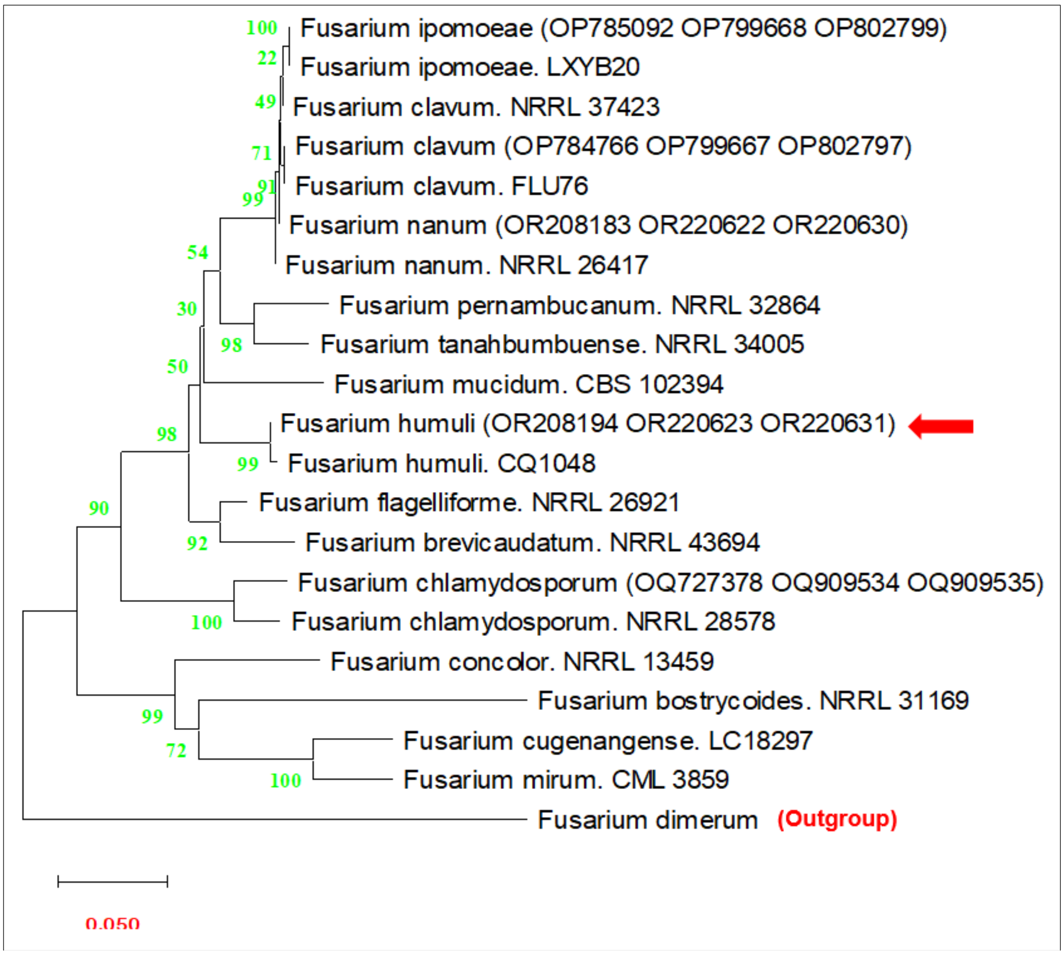

Fig 2: Phylogenetic analysis of isolates using the Maximum Likelihood statistical method. The resulting tree is proportionally scaled, with branch lengths represented in units identical to the evolutionary distances.

Effect of culture media

Eight different culture media were utilized to determine the most optimal one for promoting mycelial growth and sporulation of pathogen (Table 1). Each culture medium underwent preparation in 1000 ml of water, followed by autoclaving at 121.6°C and 15 psi for 20 minutes. After cooling to 45°C, the mediums were poured into 90 mm Petri dishes for solidification. For the inoculation process, a 5 mm disc from a 7-day-old pathogen culture was placed at the center of each plate and incubated at 25±2°C until full growth was achieved on any plate. The observations encompassed parameters, including colony diameter and sporulation (Microconidia, macroconidia, and chlamydospores). For spore quantification, a loopful suspension was introduced to 20 ml of distilled water, and spore counting was conducted using a haemocytometer, following the method outlined by Tyagi and Pudel (2014) [17]. The most effective medium was subsequently employed for further experiments.

Effect of pH

To assess the impact of pH, a range of ten pH levels from 4.0 to 8.0 (in 0.5 increments) were incorporated into the medium. Using a pH meter, adjustments were made by adding either 0.1N HCl or NaOH before autoclaving the medium. Sterilized Petri plates containing the adjusted medium were inoculated with 5mm mycelium discs and subsequently incubated at 25±1°C. Data concerning growth and sporulation were logged upon achieving full growth on any of the plates.

Table 1: Different culture media and their composition

Sl. No.	Culture medium	Composition
1.	Asthana & Hawker's medium	D-Glucose 5g, Potassium nitrate 3.50g, Potassium dihydrogen Phosphate 1.75g, Magnesium sulphate 0.75g, Agar-agar 20g
2.	Corn Meal agar	Corn Meal Infusion 2.0 g, Tween80 7.0 ml, Agar-agar 20g
3.	Czapeks Dox agar	Sodium nitrate 2g, Di potassium hydrogen phosphate 1g, Magnesium sulphate 0.5g, Potassium chloride 0.5g, Ferrous sulphate 0.01g, Sucrose 30g, Agar-agar 20g
4.	Host extract agar	Healthy black gram pods (green) 200 g, Agar-agar 20g
5.	Malt agar	Malt extract (powdered) 20.0 g, Glucose 20.0 g, Peptone 1 g, Agar-agar 20g
6.	Potato Carrot agar	Peeled and sliced potato and carrot (200 g and 25.0 g respectively), Agar-agar 20g
7.	Potato Dextrose agar (PDA)	Peeled and sliced potato 200g, Dextrose 20g, Agar-agar 20g
8.	Richards's agar	Potassium nitrate 10g, Potassium monobasic phosphate 5g, Magnesium sulphate 2.5g, Ferric chloride 0.02g, Sucrose 50g, Agar-agar 20g

Effect of Temperature on Pathogen Growth

The culture medium was prepared, sterilized, poured, and then inoculated with 5 mm pathogen culture discs. These inoculated plates were incubated at varying temperature levels, 0, 5, 10, 15, 20, 25, 28, 30, 35, and 40°C. Similarly to the previous process, data regarding growth and sporulation were collected once full growth was observed on any of the plates.

Statistical analysis

Each treatment was replicated three times in a completely randomized design. For statistical analysis, the data were subjected to Duncan's Multiple Range Test at P = 0.05 by using R Studio Software (version: 2023.12.0+369).

Results

Pathogen isolation, identification and pathogenicity test

A single isolate was obtained from collected symptomatic black gram pods. The cultured isolate displayed a pale white appearance with abundant floccose aerial mycelia that gradually transitioned to a beige hue after 7-8 days. The reverse side of the culture initially exhibited a light pink shade, which later transformed into a brownish-yellow color with an undulating margin. Notably, the macroconidia were slender, sickle-shaped, and either straight or moderately curved. They were hyaline, possessed a hooked apical cell, and featured 3-6 septa. The measurements of these macroconidia were observed to be 22.4-30.6×2.2-2.9 µm (n=30). Chlamydospores, on the other hand, were intercalary in arrangement, globose in shape, and characterized by their thick walls, often forming chains (Fig 1). The microconidia were conspicuously absent. Utilizing morphological characteristics, the isolate was identified as a member of the *Fusarium incarnatum-equiseti species complex* (FIESC), in accordance with the details outlined by Leslie and Summerell (2006) [12].

Subsequently, the obtained sequences were assembled, leading to the creation of a consensus sequence. Initial BLAST analysis at the NCBI did not yield definitive identifications. However, employing a polyphasic identification within the FUSARIOID database revealed a substantial similarity ranging from 98.9% to 99.6% with *F. humuli*. The sequences were submitted to the NCBI GenBank and were assigned the following accession numbers: ITS: OR231129, *tef*: OR239424, *rpb2*: OR239428. To gain a broader perspective, a phylogenetic tree was constructed featuring the isolated fungal pathogen, along with reference sequences (retrieved from NCBI database). For comprehensive evolutionary analyses was done in FUSARIOID database using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method (Fig 2).

Effect of culture media

The investigation into the growth patterns of *F. humuli* involved the utilization of a diverse range of eight media. Remarkably, on the eighth day of incubation, the fungus exhibited full plate coverage (9.0 cm) when cultivated on PDA. Notably, Czapek's Dox agar medium displayed the next highest growth at 8.9 cm, closely followed by corn meal agar and potato carrot agar, both registering growths of 8.8 cm. The fungus sporulated excellently in PDA and Asthana & Hawker's medium (Table 2) (Fig 3). Based on these findings, PDA was identified as the most effective medium and was subsequently utilized in further experiments. These results are in concurrence with the observations documented by Anjaneya (2002) [1], Ingole (1995) [8], and Khan *et al.* (2011) [10], all of whom reported predominant *Fusarium* spp. growth on PDA

Table 2: Effect of different media on the growth and sporulation of *F. humuli*

Culture media	Growth (in cm)	Sporulation (macroconidia ml^{-1})
Asthana and Hawker's medium	8.7^{a}	1.67×10^{6}
Corn meal agar	8.8^{a}	0.89×10^{6}
Czapek's Dox	8.9^{a}	1.24×10^{6}
Host extract agar	8.6^{b}	1.30×10^{6}
Malt Extract Agar	8.5^{c}	1.56×10^{6}
Potato carrot agar	8.8^{a}	0.78×10^{6}
Potato Dextrose Agar	9.0^{a}	1.67×10^{6}
Richards synthetic agar	8.6^{b}	0.78×10^{6}

The values following the same letter are not significantly different according to Duncan's Multiple Range Test (DMRT) at P= 0.05

Effect of pH

The growth and sporulation of the pathogen were observed to be at their maximum at pH 6.5, followed by pH 6.0, 7.0, and 5.5, while at pH 8.5, both growth and sporulation were minimized (Table 3) (Fig 4). The data align with the understanding that excess acidity and basicity are unfavorable for the pathogen's growth and sporulation, as noted by Singh and Kumar (2016) [16]. These findings are consistent with Buttar *et al.* (2022) [3], who reported an optimum pH of 6.5 for the growth of *F. equiseti* and *F. chlamydosporum.* Consequently, the present study unequivocally indicates that the most suitable pH levels for the growth of the test fungus are 6.5 and 7.0. Sharma *et al.* (2005) [15] explored the impact of pH on *F. oxysporum* f. sp. *lini* and found that the tested *Fusarium* spp. could sporulate and thrive at pH 5.5. This diversity in optimal pH conditions underscores the species-specific nature of pH preferences among *Fusarium* pathogens.

Table 3: Effect of different pH on the growth and sporulation of *F. humuli*

pH	Growth (in cm)	Sporulation (macroconidia ml^{-1})
4.0	5.4^{h}	0.62 x 10^{6}
4.5	5.6^{f}	0.66 x 10^{6}
5.0	5.8^{e}	0.83 x 10^{6}
5.5	6.2^{d}	1.32 x 10^{6}
6.0	6.6^{b}	1.54 x 10^{6}
6.5	9.0^{a}	1.54 x 10^{6}
7.0	6.4^{c}	1.46 x 10^{6}
7.5	5.5^{g}	0.70 x 10^{6}
8.0	5.2^{i}	0.42 x 10^{6}
8.5	5.2^{i}	0.40 x 10^{6}

The values following the same letter are not significantly different according to Duncan's Multiple Range Test (DMRT) at P= 0.05

Effect of temperature

Each pathogen has an optimal temperature for survival and host plant infection. In this study, we observed that *F. humuli* exhibited maximum mycelial growth and sporulation at 28°C. However, temperatures below 15°C and above 35°C had a detrimental effect on fungal growth, as indicated in Table 4 and Fig 5. Gupta *et al.* (2010) [7] found that radial development of *F. oxysporum* f. sp. *psidii* and *F. solani* peaked at 28°C. Desai *et al.* (2016) [5] reported that *F. udum* achieved maximum growth and sporulation at 28°C on PDA. Daami-Remadi *et al.* (2006) [4] discovered that the optimal temperature for maximal mycelial development of *F. oxysporum* f. sp. *tuberose* was between 25 and 30°C, while it was 30°C for *F. solani*. The consistent findings across these studies affirm that 28°C is favorable for the development and sporulation of *Fusarium*.

Table 4: Effect of different temperature on the growth and sporulation of *F. humuli*

Temperature	Growth	Sporulation (macroconidia ml^{-1})
0°C	0.0^{h}	-
5°C	0.0^{h}	-
10°C	0.0^{h}	-
15°C	2.4^{f}	0.32 x 10^{6}
20°C	4.1^{e}	1.21 x 10^{6}
25°C	5.5^{b}	1.52 x 10^{6}
28°C	9.0^{a}	1.66 x 10^{6}
30°C	5.2^{c}	1.50 x 10^{6}
35°C	5.1^{d}	1.48 x 10^{6}
40°C	2.1^{g}	0.30 x 10^{6}

The values following the same letter are not significantly different according to Duncan's Multiple Range Test (DMRT) at P= 0.05

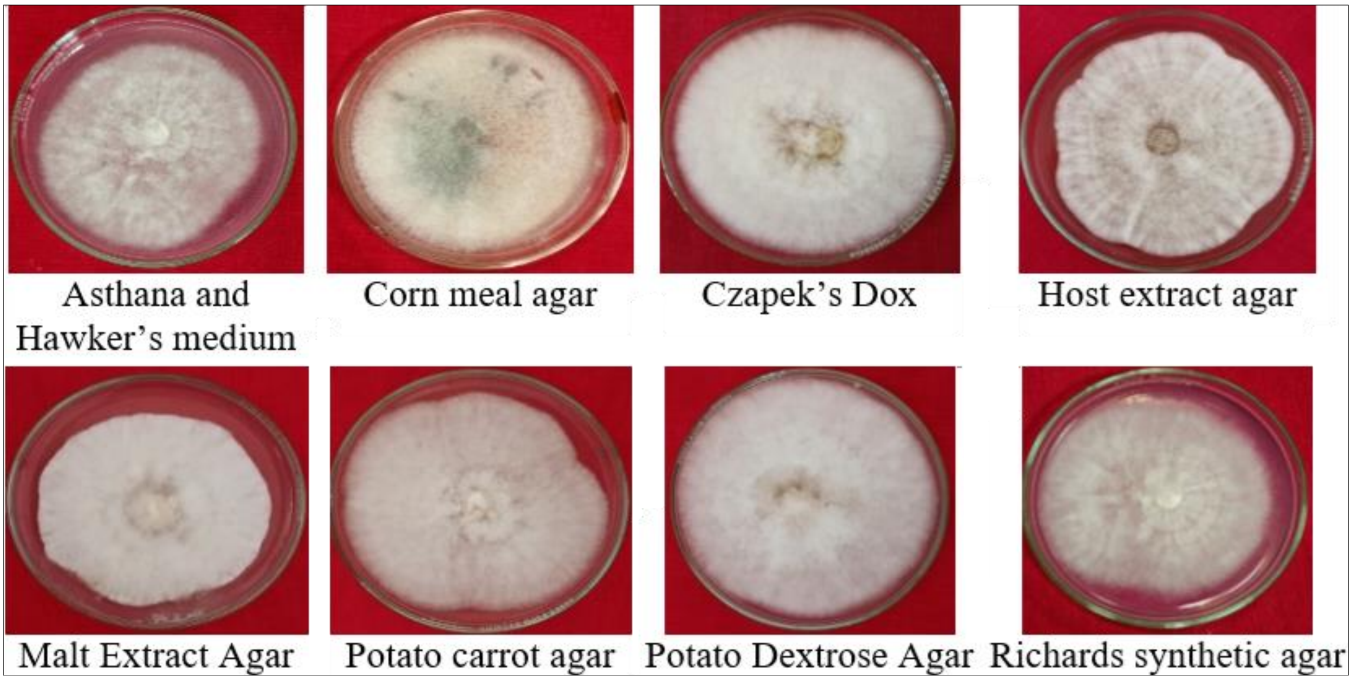

Fig 3: Growth of *F. humuli* on different media

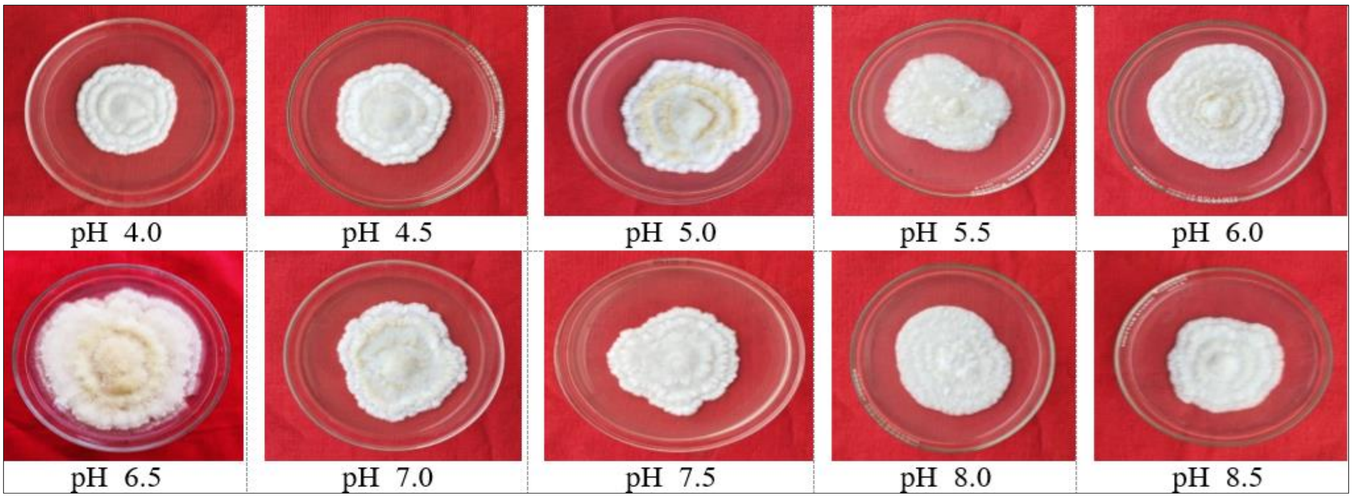

Fig 4: Growth of *F. humuli* on different pH

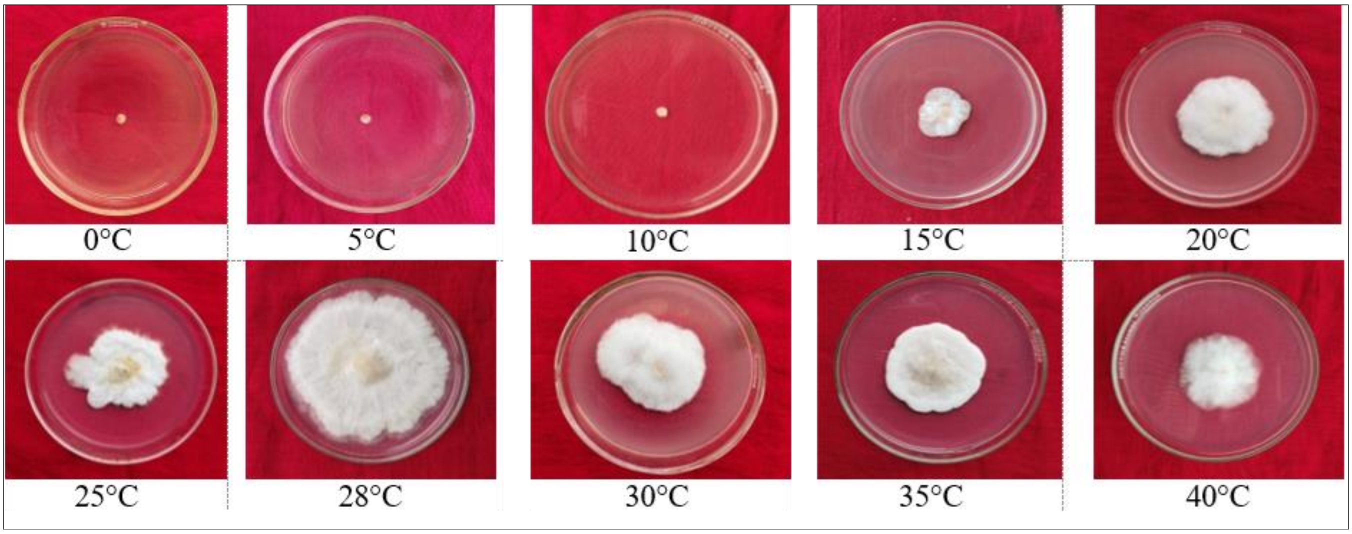

Fig 5: Growth of *F. humuli* on different temperature

Conclusion

Understanding the optimal conditions for growth and sporulation is crucial for creating an environment that maximally supports the development of plant pathogens. This knowledge is fundamental for laboratory research. Moreover, it is essential for developing effective disease management strategies by manipulating factors such as media composition, pH, and temperature. Researchers can explore methods to control or mitigate the impact of these pathogens on crops. The influence of environmental factors, such as temperature and pH, on the prevalence and severity of plant diseases is significant. Studying these effects aids in predicting and managing disease outbreaks, providing insights into how these organisms interact with their environment and host plants. This understanding contributes to the development of targeted interventions. Farmers and agricultural researchers can leverage this information to implement measures that reduce the risk of plant diseases, ultimately enhancing crop yields and overall agricultural productivity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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