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Pathogenicity, Phytotoxicity and Enzymatic Potential of Sorghum Grain Mold Pathogens

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A B S T R A C T

Sorghum grain mold disease is a serious threat for sorghum cultivation. Being a complex disease involving many fungi, basic understanding of pathogenicity is essential. In the present investigation, 14 sorghum seed samples were tested by dry seed examination and blotter method for the presence of fungi. The results revealed prominent infection of *Alternaria* spp. (21%) and *Fusarium* spp. (23%). In seedling pathogenicity test *Drechslera* sp. reduced vigour index significantly by reducing shoot and root lengths. Phytotoxicity of selected fungi in detached leaf bioassay revealed toxicity to monocot leaves. The *Curvularia* sp., *Drechslera* sp., and *Fusarium* sp. induced necrosis in rice, sorghum and sugarcane leaves. Enzymatic potential of selected fungi was realised in plate assay. The *Drechslera* sp. and *Fusarium* sp. are prominent producers of enzymes essential for pathogenicity. The present work demonstrated wide occurrence of grain mold pathogens in sorghum seed samples and also assessed their pathogenicity. The detached leaf bioassay and enzyme plate assays were useful in the detection of virulence in fungal isolates.

Introduction

The sorghum [*Sorghum bicolour* (L.) Moench] an important cereal crop being used as food for humans and fodder for animals. This crop is cultivated in semi-arid regions of the world. It serves as a major food crop in India (Forbes *et al.,* 1992; Thakur *et al.,* 2006b; Selle *et al.,* 2018; Corallo *et al.,* 2023). Grain mold in sorghum is a disease complex caused by several fungi. This disease is most prevalent in sorghum growing areas of the world resulting in serious damage (Rychlik *et al.,* 2016; Ssepuuya *et al.,* 2018; Vanara *et al.,* 2018).

The grain mold pathogens are known to cause different symptoms like grain discoloration, decrease in grain nutritive value and mycotoxin production resulting in overall reduction of seed quality (Thakur *et al.,* 2006b; Ojuri *et al.,* 2018; Park *et al.,* 2018). Many of the fungi are seed-borne and some of them are seed transmitted. Most sorghum genotypes are susceptible to grain mold disease (Ambekar *et al.,* 2011; Little *et al.,* 2012; Prom *et al.,* 2014; Prom and Cuevas, 2023).

Different varieties of sorghum show differential resistance to grain mold pathogens (Esele *et al.,* 1993; Prom *et al.,* 2011; Prom *et al.,* 2014; Prom and Cuevas, 2023). The fungal pathogens involved may reduce germinability of seeds and also the seedling vigour (Prom, 2004; Yago *et al.,* 2011). Diverse fungi are involved in disease complex and hence, it would be interesting to study their impact on sorghum. In view of this, tests were made to detect grain molds in sorghum seeds. Additionally, pathogenicity and enzyme producing potential of these fungi have been reported.

Materials and Methods

Sample collection

The collection of sorghum seed samples was done from different places in Davangere and Dharwad districts of Karnataka, India. The place of cultivation and date of collection of the samples were also noted.

Seed health testing

The seed health testing for the collected seed samples was performed through dry seed examination and standard blotter method. The seed samples collected were differentiated based on their morphological features, extent of concomitant contamination and dry weights in dry seed examination. Seeds in each sorghum sample were differentiated into healthy, shrivelled, discoloured, damaged or broken seeds, other crop seeds and inert matter (Neergaard, 2005).

The grain mold pathogens were detected through standard blotter method, by placing 25 sorghum seeds per plate equidistantly on wet blotter layers in triplicates. The incubation of these plates was done for five days at room temperature. Each seed was observed through stereo-binocular microscope (Magnüs MS 24).

The observed fungi were tease mounted by staining with cotton blue in lactophenol and observed through binocular light microscope (Olympus CH20i). The seed-borne fungi were identified with the help of standard identification manuals (Ellis, 1971; Barnett and Hunter, 1972; Navi *et al.,* 1999). The incidence of each fungus was recorded in percentage. The pure cultures of grain mold pathogens were established in Potato Dextrose Agar (PDA) medium and maintained in slants.

Pathogenicity of selected fungi on sorghum seed germination and seedling vigour

The effect of seed-borne pathogens isolated from sorghum seeds on the seed germination and seedling vigour were tested using Paper towel method (ISTA, 1985). The seed treatment of sorghum seeds was done separately for the four selected fungi. These seeds were placed equidistantly in each set. An incubation period of seven days was provided for the seeds with the conditions such as normal light and laboratory temperature of 25±5℃. The first count was made on the fourth day by considering the number of normal seedlings. For seedling vigour evaluation, the shoot length and root length of normal seedlings were measured using centimetre scale on the first count. The second count was made on the tenth day by considering only normal seedlings for seed germination. The seeds without any treatment were served as control (Haque *et al.,* 2007; Ora *et al.,* 2011). Germination percentage of seed samples were calculated. The Vigour Index (VI) of each treatment was calculated by following the formula (Haque *et al.,* 2007).

Vigour Index (VI) = (Average shoot length + Average root length) × Seed germination (%)

The results for all the parameters were considered to calculate standard error.

Detached leaf bioassay for phytotoxicity

The capacity of seed-borne fungi to produce phytotoxic metabolites was tested through detached leaf bioassay (Vidhyasekaran *et al.,* 1986; Duarte and Archer, 2003). Leaves of three monocot species (Sorghum, Sugarcane, and Rice) and three dicot species (Cucumber, Ridge gourd and Cluster bean) were considered in detached leaf bioassay. The incubation was done for the different fungal species inoculated in Potato Dextrose Broth (PDB), at room temperature (25±5℃). After five days of incubation, the fungal culture filtrate was obtained by filtration and centrifugation at 5000 rpm for 10 minutes.

Designated areas on leaf surface were scratched mildly with a fine needle. The culture filtrate of 50µl from the selected fungi were placed on the scratched area of the leaf surface. The leaf area placed with distilled water and sterile PDB separately served as the controls. The toxicity was assessed based on changes occurring in the

spotted region at 24 hours regular intervals for up to 72 hours.

Plate assay for fungal enzymes

The fungal isolates capable of secreting enzymes responsible for hydrolyzing plant cell wall constituents were determined through enzyme plate assay using modified Czapek Dox Agar (CZA) medium. Medium composition was altered by replacing sucrose with soluble starch, carboxymethyl cellulose (CMC) and pectin as sole carbon source for the detection of amylase, cellulase and pectinase respectively in separate trails (Tuppad and Shishupala, 2014). The growth of fungal isolates was observed after 24 and 48 hours of incubation periods. For each fungus/enzyme three replicates were maintained. The zone of clearance of respective substrate was observed by flooding the plates with 3% iodine solution (Kasana *et al.,* 2008). The Enzyme Index (EI) for both 24 and 48 hours of incubation periods were separately calculated for each isolate.

$$\text{Enzyme Index (EI)} = \frac{\text{Zone of Clearance}}{\text{Colony Diameter}}$$

Results and Discussion

Seed health testing

In dry seeds, discolorations were observed in the form of yellowing, browning, and blackening of the seeds. The mean dry weights of all the seed components in each sample were tabulated (Table 1). A total of seven sorghum samples were apparently healthy. Among them, six samples were not containing any shrivelled seeds. The Sample-2 had more shrivelled seeds and more discoloured seeds. Maximum number of damaged seeds was found in the Sample-13. Other crop seeds and shrivelled seeds were not presented in the Sample-4. Less number of damaged seeds were found in the Sample-4 and the Sample-14.

Different fungal species growth was observed on seeds kept in blotter method. The seed-borne fungal isolates obtained from sorghum by blotter method were belonged to nine different genera. They were, *Alternaria* spp., *Aspergillus* spp., *Colletotrichum* spp., *Curvularia* spp., *Drechslera* spp., *Fusarium* spp., *Gloeocercospora* spp., *Penicillium* spp., and *Rhizopus* spp. Among the grain mold pathogens of sorghum, *Fusarium* spp. (23%), *Alternaria* spp. (21%), and *Drechslera* spp. (10%) were the prominent genera whereas *Colletotrichum* spp., and *Curvularia* spp. were found to be the least occurrence. The incidence of *Aspergillus* spp. was more among the samples, accounting for 28% (Fig.1).

Pathogenicity of selected fungi on sorghum seed germination and seedling vigour

Pathogenicity of grain mold pathogens were tested by considering their impact on germination of sorghum seeds and seedling vigour. All the fungi tested did not reduce the sorghum seed germination.

Significant reduction in shoot length was induced by *Drechslera* sp. and hence, contributing for reduced seedling vigour. The *Curvularia* sp. induced significant reduction in root length and also, reduced VI. However, both *Alternaria* sp. and *Fusarium* sp. induced moderate increase in VI owing to increased root and shoot length (Table 2).

Detached leaf bioassay for phytotoxicity

The selected fungi showed various levels of phytotoxicity against target plant leaves. The phytotoxicity is shown by the fungal isolates on different leaves through detached leaf bioassay at 72 hours (Fig. 2). The toxic effects of fungal culture filtrates were more pronounced on monocot plants. On Rice leaves and Sugarcane leaves, the *Curvularia* sp., *Drechslera* sp., and *Fusarium* sp. induced necrotic spots (Fig. 2.1. and 2.3.) whereas, *Drechslera* sp. and *Fusarium* sp. induced toxic symptoms on Sorghum leaves in the form of brownish necrotic patch with yellow halo (Fig. 2.2). These fungal culture filtrates were not able to induce any symptoms on dicot leaves (Fig. 2.4., 2.5. and 2.6).

Plate assay for fungal enzymes

Amylase enzyme assay

Selected fungi showed a range of amylase activity. The *Alternaria* sp. was not able to utilize starch at the first 24 hours of incubation. The *Curvularia* sp. showed higher amylase activity at the 24 hours of incubation and this was followed by *Drechslera* sp. and *Fusarium* sp. All the four fungal species tested were able to utilize starch as sole source of carbon during the 48 hours of incubation period (Fig. 3).

Table.1 Dry weight of different components associated with sorghum seed samples in dry seed examination.

Sorghum Samples	Place of Collection	Dry weight of the material (g)						
		Total Seeds	Healthy Seeds	Shrivelled Seeds	Discolored Seeds	Damaged/ Broken Seeds	Other Crop Seeds	Inert Matter
1.	APMC, Davangere	17.56 ± 0.84	15.38 ± 1.19	0.28 ± 0.16	1.08 ± 0.24	0.18 ± 0.06	0.48 ± 0.06	0.16 ± 0.07
2.	APMC, Davangere	17.27 ± 2.97	13.85 ± 2.90	0.85 ± 0.23	1.50 ± 0.51	0.80 ± 0.10	0.15 ± 0.04	0.12 ± 0.04
3.	APMC, Davangere	28.60 ± 0.27	28.09 ± 0.29	0 ±0	0.21 ± 0.04	0.17 ± 0.05	0.05 ± 0.03	0.08±0
4.	Gopanal, Davangere	23.74 ± 0.45	23.30 ± 0.43	0±0	0.26 ± 0.04	0.10±0	0±0	0.08 ± 0.02
5.	Gopanal, Davangere	25.84 ± 0.26	25.08 ± 0.48	0.08 ± 0.05	0.20 ± 0.05	0.24 ± 0.07	0.09 ± 0.02	0.15 ± 0.06
6.	Gopanal, Davangere	27.80 ± 0.18	27.04 ± 0.32	0±0	0.26 ± 0.03	0.23 ± 0.10	0.15 ± 0.04	0.12 ± 0.07
7.	Gopanal, Davangere	29.36 ± 0.18	28.39 ± 0.25	0.02 ± 0.01	0.38 ± 0.06	0.42 ± 0.06	0.07 ± 0.04	0.08±0
8.	Harihar, Davangere	33.43 ± 0.78	32.93 ± 0.74	0±0	0.19 ± 0.07	0.2 ± 0.01	0.07±0	0.04 ± 0.01
9.	Harihar, Davangere	28.98 ± 0.38	27.88 ± 0.37	0.05 ± 0.03	0.51 ± 0.06	0.27 ± 0.03	0.09 ± 0.01	0.18 ± 0.06
10.	Tholhunase, Davangere	21.46 ± 0.14	20.34 ± 0.12	0.06 ± 0.03	0.41 ± 0.11	0.44 ± 0.06	0.10 ± 0.02	0.11 ± 0.05
11.	Tholhunase, Davangere	24.31 ± 0.23	23.16 ± 0.36	0.14 ± 0.04	0.49 ± 0.06	0.37 ± 0.10	0.06 ± 0.02	0.09 ± 0.01
12.	Tholhunase, Davangere	30.22 ± 0.65	29.6 ± 0.64	0±0	0.12 ± 0.05	0.43 ± 0.07	0.04 ± 0.03	0.03 ± 0.03
13.	Hubli, Dharwad	27.98 ± 0.19	26.38 ± 0.06	0.03 ± 0.01	0.09 ± 0.01	1.41 ± 0.19	0.05 ± 0.05	0.02 ± 0.02
14.	Hubli, Dharwad	37.10 ± 0.95	36.07 ± 0.82	0±0	0.72 ± 0.15	0.11 ± 0.02	0.18 ± 0.14	0.02 ± 0.02

APMC- Agricultural Produce Market Committee.

Figure.1 Incidence of seed-borne fungi in sorghum seed samples.

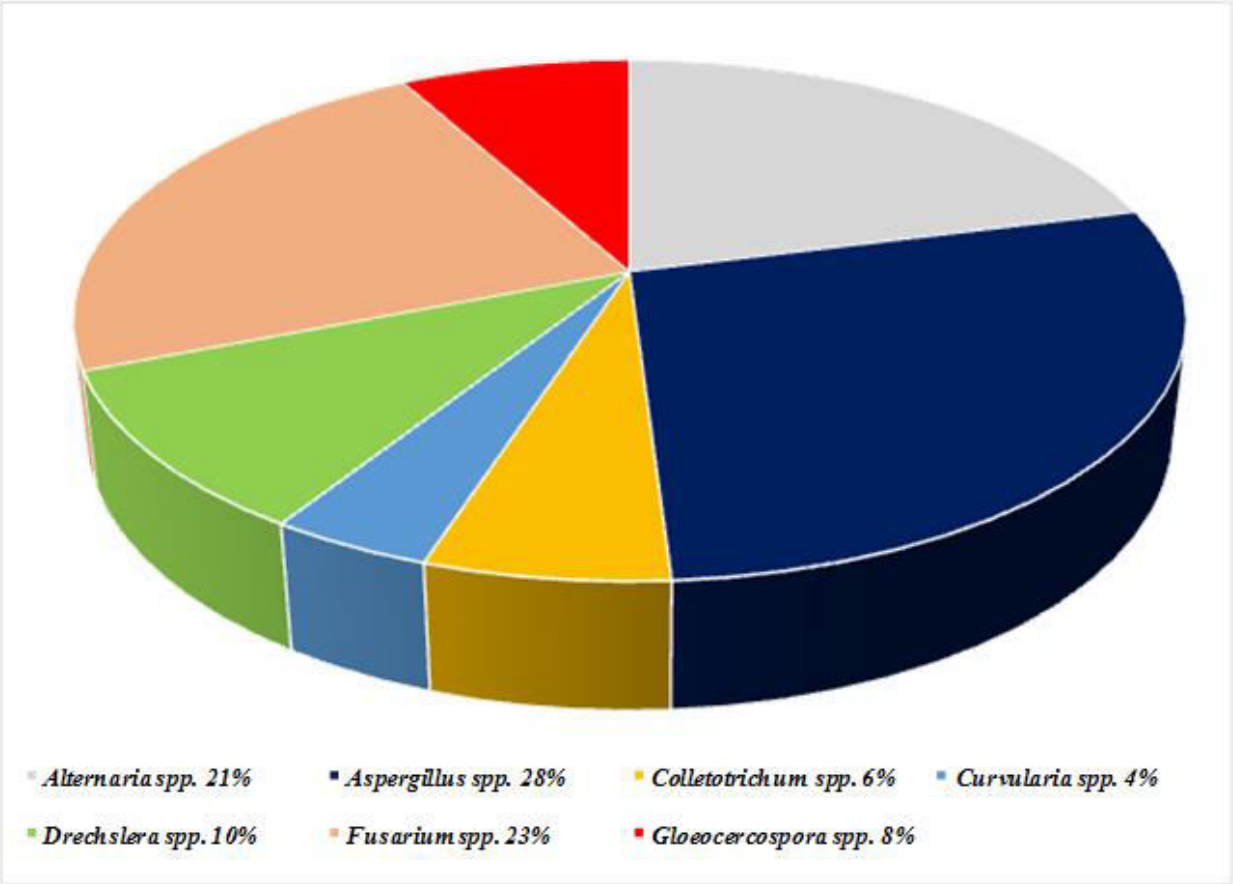
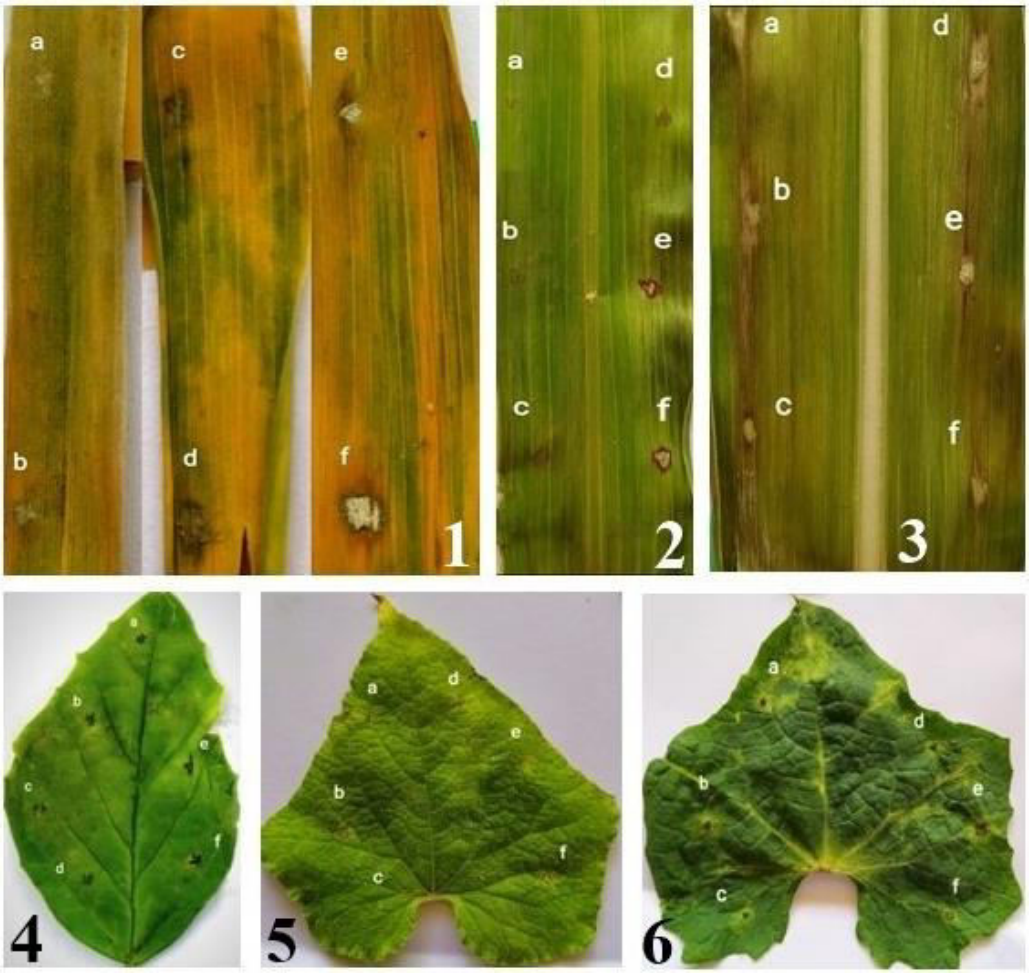


Table.2 Effect of grain mold pathogens on sorghum seed germination and seedling vigour**.**

Treatment	Seed germination (%)	ASL± SE	ARL±SE	Vigour Index ± SE
Control (untreated)	98	6.71 ± 0.25	8.23 ± 0.36	1464±61
***Alternaria* sp.**	94	7.33 ± 0.20	9.02 ± 0.58	1536±61
***Curvularia* sp.**	96	6.80 ± 0.18	5.99 ± 0.20	1227±61
***Drechslera* sp.**	97	4.26 ± 0.13	6.93 ± 0.18	1085±61
***Fusarium* sp.**	97	7.56 ± 0.22	8.46 ± 0.51	1553±61

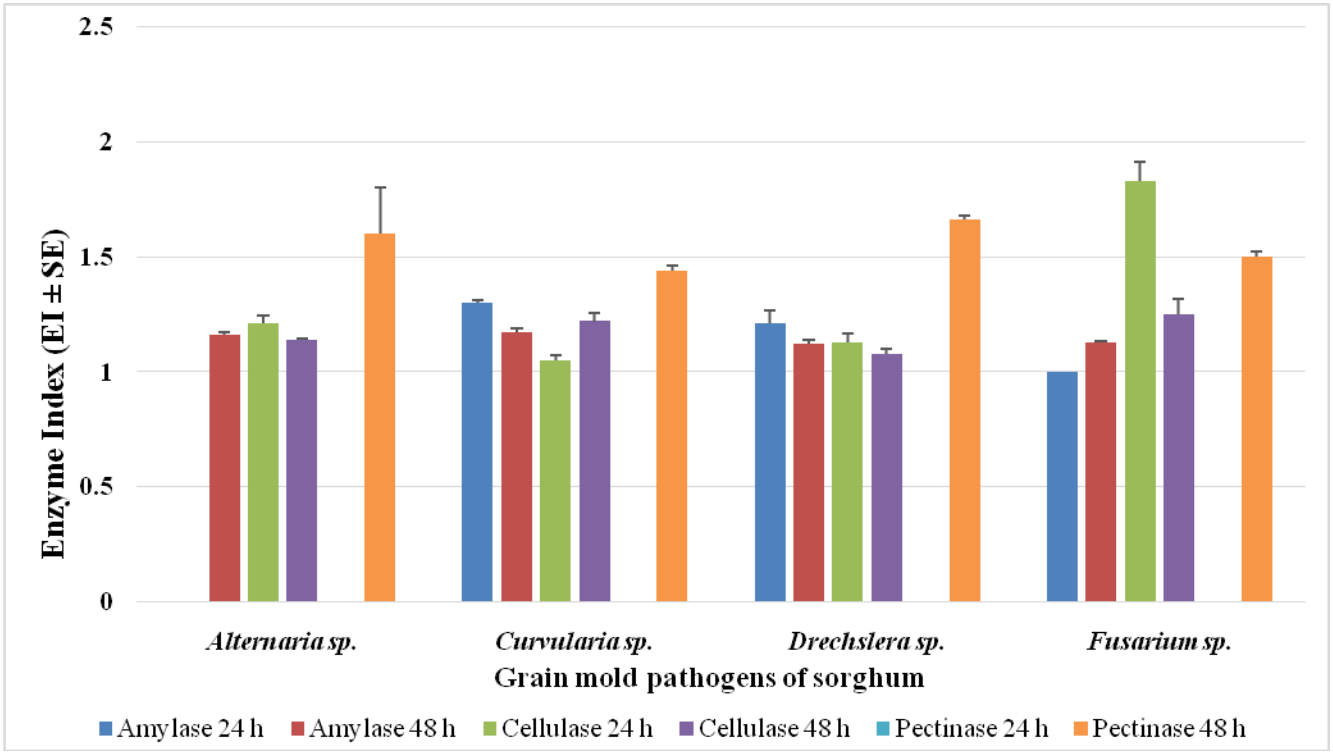
ASL- Average Shoot Length (cm), ARL- Average Root Length (cm), SE- Standard Error, Vigour Index: (ASL+ARL) × Seed germination.

Figure.2 Effect of fungal culture filtrates on monocot and dicot leaves at 72 hours of incubation in detached leaf bioassay.



2.1-Rice
2.2-Sorghum
2.3-Sugarcane
2.4-Cluster Bean
2.5-Cucumber
2.6-Ridge Gourd
a-Distilled water
b-Sterile Potato Dextrose Broth
c-*Alternaria* sp.
d-*Curvularia* sp.
e-*Drechslera* sp.
f-*Fusarium* sp.

Figure.3 Enzyme activity at different time intervals shown by grain mold pathogens of sorghum in enzyme plate assay.



$$\text{Enzyme Index (EI)} = \frac{\text{Zone of Clearance}}{\text{Colony Diameter}}$$

Cellulase enzyme assay

The *Fusarium* sp. showed the highest EI for cellulase at 24 hours of incubation. The moderate capacity of cellulose utilization was observed in the remaining fungi tested separately for both 24 and 48 hours of incubation periods (Fig. 3).

Pectinase enzyme assay

None of the fungi were able to utilize the pectin at first 24 hours of incubation. The *Drechslera* sp. showed higher EI at 48 hours of incubation compared to *Fusarium* sp. and *Curvularia* sp. The *Alternaria* sp. was not able to utilise pectin during 48 hours incubation period (Fig. 3).

Various sorghum samples collected from different places showed levels of contaminating fungi. Grain mold pathogens were also encountered in different samples indicating the disease is prevalent in Karnataka. Wide occurrence of these fungi in seed samples is of great concern (Thakur *et al.,* 2006a; Prom *et al.,* 2021).

Most effective seedling vigour reducer was *Drechslera* sp. followed by *Curvularia* sp. indicating their pathogenic potential. The fungi do cause serious damage to the crop at early stages of growth (Forbes *et al.,* 1992; Prom, 2023). Higher incidence of these fungi is matter of concern for farmers. Detached leaf bioassay was found to be useful to determine the phytotoxicity of fungi and their host specificity (Vidhyasekaran *et al.,* 1986).

Grain mold pathogens tested in the present work proved to produce toxic metabolites. Hence, this test can be used for rapid screening of virulent isolates or resistance of the host genotypes. Such toxin contaminated grains may be dangerous for human consumption. High incidence of *Fusarium* sp. needs to be taken into consideration in view of human health (Ambekar *et al.,* 2011; Ssepuuya *et al.,* 2018; Mielniczuk and Skwarylo-Bednarz, 2020; Corallo *et al.,* 2023).

Production of enzymes associated with pathogenicity revealed significant amount of various enzymes produced by these fungi. Such fungi have both toxins and enzymes for pathogenicity (Agrios, 2005). Hence, it is important to use laboratory assays to determine virulence of fungal isolates based on enzymatic potential.

The grain mold incidence, toxin and enzyme production clearly demonstrated the importance of these fungi in sorghum health. Strategic management efforts are required for keeping the grain mold at bay. It may be interesting to determine virulence of fungal strains and resistance of sorghum varieties using *in-vitro* assays.

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Author Contribution

Medha Timmanna Bhat: Investigation, formal analysis, writing—original draft. S. Shishupala: Validation, methodology, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Conflict of Interest: The authors declare no competing interests.

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