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Seed Health Testing and Evaluation of Testing Methods in Different Genotypes of Mustard

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A B S T R A C T

Fungal infections in mustard seeds (*Brassica juncea*), a significant oilseed crop, can significantly impact their quality while decreasing their yield. A research study was carried out to examine seed-borne mycoflora associated with mustard seeds and also to compare various methods used in detecting these harmful fungi. These included the Standard Blotter Method, Deep Freezing Blotter Method, 2,4-D Blotter Method, Water Agar Method and Potato Dextrose Agar Method. Six fungal species, including *Aspergillus flavus*, *Aspergillus niger*, *Rhizopus* spp., *Penicillium* spp., *Chaetomium* spp., and *Fusarium* spp., were identified. The Standard Blotter Method proved to be the most suitable for detection of most fungi while the PDA Method was effective in separating *Fusarium* spp.

Introduction

Oilseeds constitute a significant portion of the agricultural commodity, being the backbone of the vegetable oil industry and also play a crucial role in the global economy. Oilseeds are among the most important crops in the world in terms of meeting the nutritional needs of an ever-growing population, providing edible oil, protein-rich by-products, and as raw materials for industries and manufacturing. In India, oilseeds are significantly important for the agricultural sector, with most of the area under cultivation being occupied by them. Among such varied oilseeds available in India, rapeseed mustard finds a special place for their ability to adapt and survive in moderately low temperatures coupled with economic value, and also versatility in use.

Indian mustard (*Brassica juncea* L. Czern & Coss.), belonging to the Brassicaceae family, is a versatile crop used for oil extraction, vegetables, and condiments (Saleem *et al.,* 2017). In India, it ranks as the second most important oilseed crop after soybean, with a

cultivated area of 7.99 million hectares and a production of 11.96 million tonnes and productivity of 1497 kg/ha during 2022-23 (Anonymous, 2022). Presently, in Karnataka, mustard is cultivated over an area of 0.40 thousand hectares with a production of 0.09 thousand tonnes and productivity of 225 kg/ha (Anonymous, 2022).

Mustard seeds generally contain 30 to 40 per cent oil, making it a significant source of edible oil in Indian households. Other than oil, this condiment is widely consumed in various preparations of dishes. Mustard oil is recognized for its medicinal value. It is anti-inflammatory, antifungal and antibacterial, while its by-product, mustard cake, is very protein-rich animal feed. It offers sustainable agriculture through improved crop rotation and soil fertility.

Seed health is paramount in maximizing yield potentials and utility. Seed-borne fungi, known as seed mycoflora, are commonly found in agricultural crops like mustard. These fungi, either present externally on the seed surface or internally within the seed, are categorized as external or internal seed-borne fungi, respectively. Studies by Ghosh *et al.,* (2018) revealed that seed-associated fungi in oilseeds can cause significant quality deterioration, making seeds unsuitable for planting or consumption. Commonly identified fungi include *Alternaria* sp., *Curvularia* sp., *Fusarium* sp., *Helminthosporium* sp., *Penicillium* sp., *Mommoniella* sp., *Aspergillus* sp., *Mucor* sp., and *Rhizopus* sp., with *Alternaria sp.* and *Aspergillus sp.* being particularly destructive.

In mustard seeds, fungal infections can lead to adverse changes in physical and chemical properties during storage. This results in weight loss, reduced germination rates, discoloration and diminished medicinal value, contributing to yield losses of up to 24% (Ashraf and Choudhary, 2008). Seed health testing is a very important procedure in ascertaining the quality and safety of seeds by detecting and identifying pathogens transmitted through seeds. Research, including work by Ghugal and Thakre (2014) and Siddiqui (2013), has highlighted the prevalence of fungi like *Alternaria* sp., *Rhizoctonia* sp., *Aspergillus* sp., *Mucor* sp., and *Rhizopus* sp. in stored mustard seeds, affecting seed quality and facilitating disease transmission to plants.

Despite its economic significance, there is limited research on the seed-borne fungi affecting rapeseed-mustard under Karnataka's specific conditions. Seed health testing is extremely crucial in a crop like mustard that has vast economic & nutritional significance. Besides, semi-arid areas of Karnataka, mainly Dharwad, suit mustard growth but also favour the seed-borne pathogen proliferation and demand stringent seed health testing protocols to assure disease outbreaks and productivity enhancement. In addition to conducting seed health testing, it is essential to evaluate different seed health testing techniques to determine the most effective approach for detecting seed-borne pathogens in mustard. This study focuses on identifying the fungal microflora associated with rapeseed-mustard seeds in the region and aims to establish the most reliable seed health testing method.

Materials and Methods

Source of seeds

Mustard seed samples from various genotypes were collected from the All India Coordinated Research Project on mustard. The seeds collected were stored in sterilized polythene bags with appropriate labelling. These were then bought to the laboratory of Department of Plant Pathology, College of Agriculture, University of Agricultural Sciences, Dharwad, Karnataka. Then the seeds were dried under shade and stored at room temperature. The details of the collected seed samples are given below:

Isolation method for identification of seed borne fungi

The seed samples collected were initially tested for seed health using the Standard blotter method to identify the probable seed-borne pathogens associated with the seed (Anon, 1999). This method was actually developed by Lucie Doyer in 1938 and in 2014, it was included in the International Seed Testing Association Rules (Anon, 2014). In the current study, four hundred seeds of each variety were tested in three replications. Blotting papers were cut in the size of 90mm, then the papers were dipped in distilled water. After removing the excess water, the papers were placed in sterilized Petri plates. In each Petri plate, 25 seeds which were not treated were kept at an equal distance. Then the plates were labelled with a marker and kept for incubation at room temperature (25 ± 2º C) under alternate cycles of light and darkness for eight days. Later, the fungal growth on the surface of seeds was observed by placing the petri

plate under stereoscopic binocular microscope. The observed colony characters were compared with the standard habit and colony characters and the fungi were confirmed (Anon, 1999). The percentage of seed germination and percentage of seed infection were calculated using the following formulae.

$$\text{Percentage of seed germination} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100$$

$$\text{Percentage of seed infection} = \frac{\text{Number of seeds infected}}{\text{Total number of seeds}} \times 100$$

Evaluation of different seed health testing techniques

There is a need for evaluating the efficiency of different seed health testing methods to find out the method which can effectively detect seed mycoflora in mustard. For this purpose, the mustard cultivar Varuna was used and the five routine methods tested were Standard blotter, Deep freezing blotter, 2,4-D Blotter, Water agar and Agar plate with potato dextrose agar. The percentage of seed infection was calculated using the formula mentioned in 2.2.

Standard blotter method

The seeds were placed at a rate of 10 seeds per Petri plate on moistened layers of blotters and incubated for seven days as described under the standard blotter method.

Deep freezing blotter method

After placing the moistened blotting papers in petri plates, 10 seeds were placed in each plate. Then on the first day, the plates were incubated at room temperatures for 24 hours under alternate cycles of light and darkness. On the second day, the plates were incubated in dark room. Then from the third day, they were kept in light and dark conditions similar to standard blotter method.

2,4-D Blotter method

In this method, the blotting papers were dipped in 0.2 per cent sodium salt of 2, 4 – dichlorophenoxyacetic acid. Then after placing the blotting papers in the petri plates, seeds were kept at a rate of 10 per plate and incubated at room conditions for seven days.

Water agar method

In this method, instead of placing the seeds on moistened blotting papers, they were kept on water agar. First water agar was prepared by dissolving 2 grams of agar in 100ml of distilled water. Then it is autoclaved and poured in sterilized petri plates at the rate of 20ml per petri plate. After the media gets solidified, seeds were placed with the help of sterilized forceps on the petri plate containing water agar medium and kept for incubation for 7 days.

Agar plate method with potato dextrose agar

In this method, potato dextrose agar was used as a medium for incubating seeds. Potato dextrose agar was prepared by dissolving 39 grams of potato dextrose agar in 1000 ml water by heating. Then it is autoclaved and 20ml is poured in each petri plate. After surface sterilizing the seeds in one per cent sodium hypochlorite solution for 1-2 min, they were placed at a rate of 10 seeds per Petri plate and incubated for seven days.

Identification of fungi

After incubation for seven days, the fungal mycelium developed on seeds from different techniques were examined using a stereoscopic binocular microscope. Fungi were identified based on spore morphology and colony characteristics as described by Ellis (1971) and Barnett and Hunter (1972).

Results and Discussion

Seed health testing of mustard seeds using standard blotter method

The collected mustard seed samples were tested for seed health testing using the standard blotter method to identify seed-borne fungi. The results revealed the presence of six fungal species, comprising of saprophytic fungi and pathogenic fungi and they were *Aspergillus flavus*, *Aspergillus niger*, *Chaetomium* spp., *Rhizopus* spp., *Penicillium* spp. and *Fusarium* spp. The identification of these fungi was based on their morphological features and colony characteristics (Ellis, 1971 and Barnett and Hunter, 1972).

Among the fungi detected, *Aspergillus flavus* (37.6%) was the most prevalent, followed by *Aspergillus niger* (34.00%), *Rhizopus* spp. (16.40%), *Chaetomium* spp. (16.40%), *Penicillium* spp. (14.00%) and *Fusarium* spp. (6.80%).

Among all the seed samples tested, Mali MS 90 showed the highest germination percentage (92.60%), followed by ORM 2019-15 (81.20%) and DRMRHT 13-13-5-4 showed lowest germination percentage (62.50%). Regarding seed infection, DRMRCI-141 recorded the highest infection rate (44.67%), followed by NPJ 249 (28.00%) while KMR (E) 21-1 showed the lowest infection rate (12.00%). These variations in germination and infection rates across seed samples suggest potential differences in seed quality and viability (Table 1 and fig. 1).

The high occurrence of *Aspergillus flavus* and *Aspergillus niger* suggests its significant presence as a seed-borne pathogen in mustard seeds. This finding is of concern since *Aspergillus* species are known to produce aflatoxins, which are extremely toxic and can pose risks to human health and animal health. Similar results were obtained by Bhajbhuje (2014) who reported *Aspergillus* as the predominant genus in mustard seeds using the standard blotter method. *Penicillium* spp., *Chaetomium* spp., *Rhizopus* spp. and *Fusarium* spp. were also detected in slightly lower proportions. These fungi may contribute to the deterioration of seed quality and potential seedling infections. Meena (2019) also identified different genera *viz*., *Aspergillus* sp., *Rhizopus* sp., *Penicillium* sp., *Fusarium oxysporum* and *Chaetomium* sp. by using standard blotter method.

Evaluation of seed health testing techniques

The mustard cultivar Varuna was used to evaluate five seed health testing techniques namely Standard blotter, Deep freezing blotter, 2,4-D blotter, Potato dextrose agar and Water agar. The results obtained are presented in Table 2 and fig. 2. Among the methods tested, Standard blotter method proved to be the most effective in detecting the overall seed-borne infections at a rate of 16.33%, followed by Deep freezing blotter method (13.67%).

The standard blotter method was particularly effective for identifying *Aspergillus flavus* (19.67%), *Aspergillus niger* (18.33%), *Penicillium* spp. (14.00%) and *Chaetomium* spp. (15.67%). The Potato Dextrose Agar (PDA) method was most effective for detecting *Fusarium* spp. (26%), followed by the Deep freezing blotter method (20.67%). The Water agar method was best for identifying *Rhizopus nigricans* (24.00%) infection. The 2,4-D blotter method was the least effective in detecting seed-borne infections in mustard seeds.

These findings align with Debbarma and Banik (2021) who identified five different fungal species using PDA, Standard blotter and water agar methods. Similarly, Yekini *et al.,* (2022) reported the presence of *Aspergillus niger, Aspergillus flavus, Penicillium* spp*., Alternaria* spp*., Rhizopus stolonifer* and *Fusarium oxysporum* in mustard seeds using Standard blotter and PDA methods.

The findings from the current study highlights the seed-borne mycoflora associated with mustard seeds with *Aspergillus flavus* and *A. niger* being the most dominant. These two fungi adversely affect seed quality and germination. The Standard Blotter Method was the best for such detection of a larger group of seed-borne fungi, whereas the Potato Dextrose Agar method was beneficial for testing specific pathogens like *Fusarium* spp. In conclusion, the results of these studies emphasize the implementation of stringent protocols for seed health testing, as those conditions in Karnataka favour fungal proliferation. These practices will certainly promote crop productivity and safeguard economic and nutritional interests in addition to promoting sustainable mustard agriculture.

Table.1 Details of Genotypes

S. No.	Genotypes	S. No.	Genotypes
1	DRMRCI 141	6	NPJ 249
2	KMR (E) 21-2	7	ORM 2019-15
3	PHR 8425	8	Mali MS 90
4	DRMRHT 13-13-5-4	9	NPJ 248
	KMR (E) 21-1	10	HUJM (E) 20-4

Table.2 Seed health testing of mustard seed samples by standard blotter method

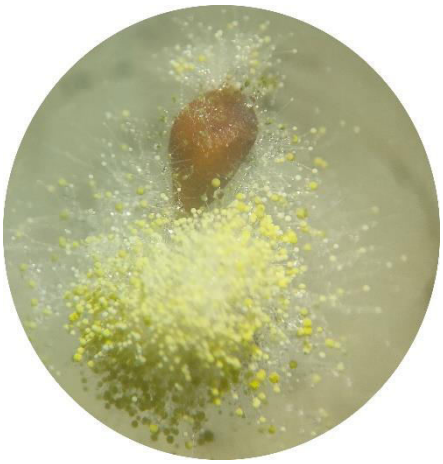
Sl. No.	Genotype	Percentage of germination	Percentage of seed infection						Mean
			Aspergillus flavus	*Aspergillus niger*	*Chaetomium* spp.	*Rhizopus* spp.	*Penicillium* spp.	*Fusarium* spp.	
1	DRMRCI 141	45.00	64.00	56.00	48.00	40.00	44.00	16.00	44.67
2	KMR (E) 21-2	70.40	28.00	16.00	16.00	0.00	12.00	4.00	12.67
3	PHR 8425	72.00	12.00	28.00	24.00	12.00	16.00	0.00	15.33
4	DRMRHT 13-13-5-4	62.50	20.00	16.00	20.00	28.00	20.00	8.00	18.67
5	KMR (E) 21-1	73.00	36.00	20.00	0.00	8.00	4.00	4.00	12.00
6	NPJ 249	66.70	52.00	40.00	28.00	24.00	12.00	12.00	28.00
7	ORM 2019-15	81.20	56.00	48.00	16.00	12.00	20.00	0.00	25.33
8	Mali MS 90	92.60	48.00	56.00	0.00	0.00	8.00	8.00	20.00
9	NPJ 248	72.70	28.00	20.00	8.00	20.00	0.00	16.00	15.33
10	HUJM (E) 20-4	76.70	32.00	40.00	4.00	20.00	4.00	0.00	16.67
Total			376.00	340.00	164.00	164.00	140.00	68.00	1252.00
Mean			37.60	34.00	16.40	16.40	14.00	6.80	125.20
Frequency			30.03	27.16	13.10	13.10	11.18	5.43	-

Table.3 Evaluation of seed health testing methods in detecting seed microflora in mustard

Sl. No.	Treatment	Percentage of seed infection						Mean
		Aspergillus flavus	***Aspergillus niger***	***Penicillium* spp.**	***Rhizopus nigricans***	***Chaetomium* spp.**	***Fusarium* spp.**	
1	Standard blotter method	19.67 (26.32)*	18.33 (25.34)	14.00 (21.96)	21.00 (27.26)	15.67 (23.31)	9.33 (17.79)	16.33 (23.84)
2	Deep freezing blotter method	13.00 (21.12)	15.00 (22.78)	10.33 (18.74)	12.67 (20.84)	10.33 (18.74)	20.67 (27.03)	13.67 (21.70)
3	2,4-D Blotter method	14.33 (22.23)	11.67 (19.97)	11.67 (19.97)	15.33 (23.0)	6.67 (14.97)	6.33 (14.56)	11.00 (19.37)
4	Potato dextrose agar method	7.00 (15.33)	8.33 (16.77)	13.33 (21.40)	17.67 (24.85)	5.33 (13.34)	26.00 (30.64)	12.94 (21.09)
5	Water agar method	10.33 (18.74)	9.67 (18.11)	11.67 (19.97)	24.00 (29.32)	8.00 (16.43)	16.67 (24.09)	13.39 (21.46)
Mean		12.87 (21.02)	12.60 (20.79)	12.20 (20.44)	18.13 (25.20)	9.20 (17.66)	15.80 (23.42)	13.47 (21.53)
S. Em. ±		0.42	0.41	0.44	0.53	0.33	0.53	-
C.D at 1%		1.34	1.32	1.40	1.69	1.06	1.70	-

*Figures in parentheses indicate arc sine values

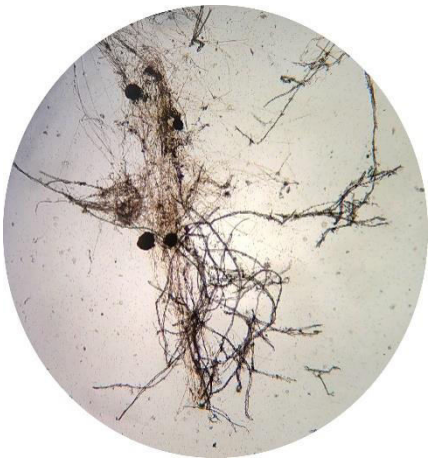
Figure.1 Evaluation of seed health testing methods



Aspergillus flavus

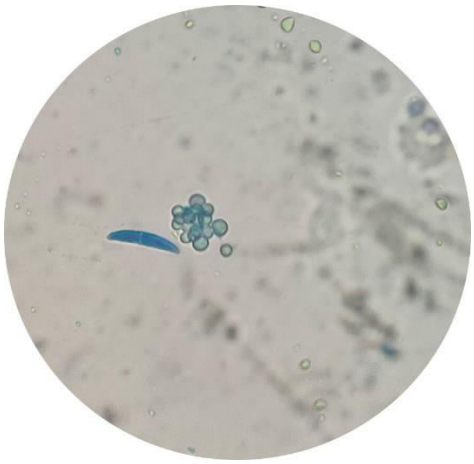
Aspergillus niger

***Chaetomium* spp.**



***Rhizopus* spp.**

***Penicillium* spp.**



***Fusarium* spp.**

Figure.2 Seed borne fungi in mustard identified by Standard blotter method

Standard blotter method

Deep freezing blotter method

2, 4-D blotter method

Potato dextrose agar method

Water agar method

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Author Contributions

D. V. N. D. Sanjana Veni: Investigation, formal analysis, writing – original draft.; Dr. M. S. L. Rao: Formal analysis, writing – review and editing.; Dr. V. R. Kulkarni: Validation, formal analysis, writing – reviewing.; Dr. B. R. Patil: Validation, formal analysis, 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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