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Short Note

Incidence and detection of seed mycoflora of Indian mustard (*Brassica juncea* L) and their deteriorative effect on plant health

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Abstract

Sixteen saprophytic as well as parasitic mycoflora belonging to eight genera were recorded in the blotter and agar plate methods. These were *Alternaria brassicae*, *A. alternata*, *A. brassicicola*, *Aspergillus niger*, *A. flavus*, *A. nidulans*, *A. fumigatus*, *A. ochraceous*, *Curvularia lunata*, *Drechslera tetramera*, *Rhizopus nigricans*, *Mucor* sp., *Penicillum* sp., *Chaetomium* sp., *Metarrhizium* sp. and *Fusarium oxysporum*. The fungal species recorded in potato dextrose agar plate method (PDA) test were common to those observed in blotter test but percent incidence of fungi varied in blotter test and PDA. Sample collected from Alwar showed maximum incidence of mycoflora. Six fungal species belonging to four genera including *A. brassicae* were found pathogenic and adversely affect plant status. *A. brassicae* was found to be highly pathogenic as it showed maximum reduction in seed germination and vigour index with enhanced pre and post-emergence mortality and produced symptomatic seedling with small necrotic lesions on cotyledonary leaves. Pathogenic seed mycoflora including *A. brassicae* were recovered maximum on seed coat followed by endosperm while absent on cotyledon and embryo.

Keywords: *Indian mustard, mycoflora, seed germination, seedling vigour index*

Introduction

Rapeseed-mustard group of crops shares 24.4 % area, 26.8 % production and 33 % vegetable oil production of total oilseeds in the country (Choudhary *et al.*, 2021). *Brassica juncea* (L) Czern & Coss. belongs to family Brassicaceae (Crucifereae) is commonly known as Indian mustard and globally used as oilseed, vegetable and condiments (Saleem *et al.*, 2017). It is the second most important oilseed crop of India after soyabean in terms of area and production. In India, the crop was grown over 8.06 mha area which produced 11.75 mt during 2021-22 (GOI, 2022). In Rajasthan, rapeseed-mustard occupies a prime place amongst all the oilseed crops grown. Rajasthan ranks first both in area and production of rapeseed-mustard in the country. It is grown in the districts namely Alwar, Bharatpur, Ganganagar, Tonk, Hanumangarh, Bikaner, Jodhpur, Sawai Madhopur, Jalore and Jaipur. Rapeseed-mustard comprising an area under cultivation was about 4.16 mha and the production was 7.14 mt in Rajasthan during 2021-22 (Anonymous, 2022).

In agriculture, seeds of many crops are known to carry various types of pathogenic and non-pathogenic fungi which are commonly known as seed mycoflora or seed-borne fungi. Depending upon the presence of fungi either on seed coat or in the seed, it is further called as external seed-borne fungi and internal seed-borne fungi, respectively. Ghosh *et al.* (2018) studied the mycoflora associated with oilseeds that bring several undesirable changes and making them unfit for consumption and sowing. Further, association of mycoflora adversely affects quality and health of seeds. They detected many fungal species in seed samples of oil seed crops and these were *Alternaria* sp., *Curvularia* sp. *Fusarium* sp., *Helminthosporium* sp., *Penicillium* sp., *Mommoniella* sp., *Aspergillus* sp., *Mucor* sp. and *Rhizopus* sp. and among these, *Alternaria* sp. as well as *Aspergillus* sp. were the most destructive pathogen of oilseeds. The seeds of mustard are known to carry several fungal pathogens which alter physio-chemical properties of the seeds during storage, losses of the seed weight, germination potential, medicinal properties and discoloration, causing the losses to the extent of 24 % (Ashraf and Choudhary, 2008). In India, various researchers have studied the incidence of seed borne fungi of several species of *Brassica* under storage environment from various geographical locations (Ghugal and Thakre, 2014). Siddiqui (2013) worked with seed borne mycoflora of mustard namely *Alternaria* sp.,

Rhizoctonia sp., *Aspergillus* sp., *Mucor* sp. and *Rhizopus* sp. etc. He concluded that the full potential of this crop is far from being exploited due to several abiotic and biotic stresses. The infected seeds may fail to germinate, transmit disease from seed to seedling and from seedling to growing plants. A very less information of seed borne microflora was available on rapeseed-mustard specially in Rajasthan conditions. Hence, this study was undertaken to find out the microflora associated with the seed of rapeseed mustard.

Materials and Methods

Isolation of mycoflora

For isolation of external and internal seed mycoflora of mustard, following two incubation methods (ISTA, 1976) were used. Standard blotter method: Ten composite samples of different mustard growing districts of Rajasthan were studied. Four hundred seeds per sample, 200 untreated and 200 surface sterilized with 0.1 % mercuric chloride for 1 minute used throughout the blotter test. Twenty-five seeds were spaced per petri plate containing three well moistened blotters and incubated at 25±1! under 12h of alternating cycles of artificial light from fluorescent tubes and darkness for 7 days. Percentage of seed germination, seed borne mycoflora, symptoms on seedlings and other abnormalities were recorded on 7th day of incubation.

Potato dextrose agar plate method: Two hundred seeds from each composite sample surface sterilized with 0.1 % mercuric chloride for 1 minute were aseptically plated in petri plates (25 seeds/plate) containing 20 ml of potato dextrose agar (PDA). PDA was prepared by autoclaving the mixture of extract of 200 g peeled potatoes, 20 g dextrose and 20 g agar in one litre of distilled water at 15 lb pressure for 20 minutes. Streptomycin (200 ppm) was added to molten PDA just before pouring it into the plates to check the bacterial growth. The plates were incubated for 7 days at 25±1! in 12/12h alternating cycles of artificial day light and darkness. Colonies of fungi on and around the seeds were examined by naked eyes as well as under stereoscopic binocular microscope on the 8th day of incubation. Fungal species which has maximum incidence with rapeseed-mustard seeds was further tested for pathogenicity. All the further studies were conducted with this pathogenic fungus.

Purification of mycoflora

Isolated seed mycoflora was purified either single spore technique or hyphal tip method.

Single spore technique: In case of single spore technique, serial dilutions of spore suspension from 7 days old culture was made in sterilized distilled water until a dilution containing 10-15 spores/ml was achieved. One ml of this diluted spore suspension was poured in petri plate containing solidified 2 % plain agar autoclaved at 15 lb pressure for 20 minutes under aseptic conditions. Spore suspension was evenly distributed by tilting the petri plate in various directions. After few minutes, excess suspension was removed from petri plate. Inoculated petri plates were incubated at 25±1! for 24h. Germinating single spore was located and marked under the microscope with the help of dummy objective and transferred on 2 % PDA slants, aseptically. Inoculated slants were subsequently allowed to grow and sporulate.

Hyphal tip method: This method is same as described earlier except that instead of single spore, hyphal tip was marked and transferred on 2 % PDA slants. The fungi isolated by agar plate method and purified by hyphal tip culture method were maintained by periodical transfer on PDA. Seed mycoflora were identified on the basis of morphological characters.

Identification of mycoflora

Purified seed mycoflora were identified on the basis of their morphological and colony characters. Isolated and identified mycoflora from different samples were kept for further studies. The Identity of the *A. brassicae* obtained on PDA slants was confirmed with standard culture of *A. brassicae* in the laboratory. The culture was maintained and sub-cultured on PDA slants and kept in refrigerator till further use.

Pathogenicity test

Thirteen fungal species belonging to eight genera were isolated from all the ten seed samples by agar plate method and these were *Alternaria brassicae*, *A. alternata*, *A. brassicicola*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus ochraceus*, *Curvularia lunata*, *Drechslera tetramera*, *Metarrhizium* sp., *Penicillium* sp., *Chaetomium* sp. and *Fusarium oxysporum*. As per literature, *Alternaria* sp., *Aspergillus niger, Curvularia lunata* and *Fusarium oxysporum* have been reported to be pathogenic with rapeseed-mustard crop. Among these pathogenic seed mycoflora, maximum incidence was recorded with *A. brassicae*. Therefore, its pathogenicity further proved by soil test.

Soil test

Prior to sowing, plastic pots (9×15 cm) were sterilized with copper sulphate solution and filled with sterilized soil + FYM (soil: FYM = 3:1), sterilized at 15 lb pressure for one hour for three consecutive days. These pots were inoculated with fungus inoculum multiplied on sorghum

grains @ 20 g/ pot. The pots were covered with polythene bags and kept for 24 hours in cage house. Treatments were laid out in the pots in completely randomized design. Seeds were sown in pots and kept under covered wire cages and watered on alternate days. Pre- and post-emergence losses and disease symptoms in plants were recorded. The symptomatic plant parts washed under tap water and dried under shade. Small bits of 5 mm size along with some healthy portion, taken from infected part and exhibiting symptoms were sterilized with 0.1 % mercuric chloride solution for 1 minute, rinsed thrice in sterile distilled water and then transfer to PDA slants and incubated in BOD incubator at 25±1! for 15 days. The culture of the fungus thus obtained was purified by single spore isolation technique. The identification of the fungus obtained on PDA slants was confirmed with standard culture of *A. brassicae* in the laboratory.

Phytopathological studies

Phytopathological studies were carried out using mustard seed sample of Alwar. One hundred apparently healthy surface sterilized seeds were tested for planting value (*i.e.* data on seed germination, seedling symptoms, seedling survival and mortality, vigour index) by standard blotter test and test tube seedling symptom test (ISTA,1976).

Standard blotter test: One hundred apparently healthy surface sterilized seeds (with 0.1% mercuric chloride for 1 minute) were taken. The seeds of every sample were then rolled on 7 day old sporulating culture of each isolated fungus separately, thriving on PDA contained in petri plates. Ten inoculated seeds were plated on moistened blotter paper in each petri plate and incubated in BOD at 25±1! for 15 days. The un-inoculated surface sterilized apparently healthy seeds served as control. Observations on seed germination and pre-emergence mortality were recorded on 7th day while for post-emergence mortality on 15th day of seed plating.

Seedling symptom test: In order to observe symptoms of disease incited by seed borne pathogenic mycoflora in the seedling stage, agar test in test tubes (Agarwal and Sinclair, 1987) was employed for this purpose with slight modification.

Agar test in test tubes: The method is same as described earlier except that instead of petri plate, test tubes with 1 % sterilized plain agar having one seed per test tube, (50 seeds per sample) were used.

Percent mortality and seedling vigour index were calculated by using following formula (Abdul-Baksi and Anderson, 1973):

Seedling Vigour index = Germination (%) × (Root length + Shoot length)

$$\text{Mortality (\%)} = \frac{\text{Mortality with test fungi} - \text{Mortality in check}}{\text{Mortality with test fungi}}$$

Among mycoflora tested, *Alternaria brassicae* showed maximum reduction in germination percent and vigour index (seedling vigour), less than 50 percent of the vigour index observed in control considered as highly pathogenic, so further management studies were carried out with *A. brassiace* only.

Histopathology of infected seeds

Highly contaminated seed sample collected from Alwar district was selected and used for histopathological studies. Presence of the pathogen in different parts of the seeds was tested by component plating method (Mathur *et al*., 1975). Fifty seeds were taken from highly infected mustard seed sample and were washed 4 or 5 times with sterile distilled water. The components were separated with the help of sterilized forceps and a pair of needles under the stereobinocular microscope. The seeds were soaked in sterile distilled water for overnight and seed coat was separated than after that remaining part i.e. endosperm, cotyledon and embryo will be separated. Each component was surface sterilized in aqueous solution of 0.1 % mercuric chloride and placed on PDA in petri plates. The plates were incubated for 7 days at 25±1!. The percent seed part showing presence of mycelial growth was recorded.

Results and Discussion

Phytopathological effects of seed borne fungi

Seed borne infection of various fungi caused an adverse effect on the germination of seed and resulted in poor symptomatic seedlings.

Standard blotter test: It is evident from Table 1 that seeds inoculated with individual pathogenic fungus caused lowest germination, higher pre-emergence and post-emergence mortality and reduced seedling length and vigour index as compared to control. Common fungi that showed higher percent incidence in incubation tests and already reported as pathogenic were used to see their phytopathological effects. Among these, *Aspergillus niger*, *Curvularia lunata*, *Fusarium oxysporum*, *Alternaria brassicicola*, *A. alternata* and *A. brassicae* caused lowest seed germination and high percent of pre- and post- emergence mortality as compared to control. Lowest seed germination (48.0 %) and maximum pre-emergence (36.5 %) and post- emergence mortality (30.0

Table 1: Effect of seed mycoflora on seed germination, pre-emergence and post-emergence mortality and seedling vigour tested by standard blotter method

Mycoflora	Percent germination emergence	Percent mortality		Seedling length (cm)		Vigour index
		Pre emergence	Post length	Radicle length	Plumule	
Alternaria alternata	54	26.0	9.3	3.5	4.0	405
Alternaria brassicae	48	32.0	14.4	2.0	3.5	264
Alternaria brassicicola	56	24.0	08.4	4.5	5.4	552
Aspergillus niger	62	18.0	09.3	4.3	4.5	543
Curvularia lunata	68	10.9	15.0	5.0	5.8	731
Fusarium oxysporum	57	23.0	12.0	4.0	4.3	470
Control	79	0.0	0.0	6.3	8.5	1165
CD (p=0.05)	4.8	2.8	1.6	1.1	1.0	-

%) with lowest seedling length (2.0 cm/ 3.5 cm) and vigour index (264) were observed in seeds inoculated with *A. brassicae* followed by *A. alternata* and *Fusarium oxysporum*. Highest seed germination (68.0 %) and lowest pre and post-emergence mortality (10.9 % and 15.0 %, respectively) were observed in seeds inoculated with *Curvularia lunata*. In the present investigation revealed that among all the fungi tested, *A. brassicae* showed minimum seed germination with maximum pre and post-emergence mortality and reduced vigour index, therefore, *A. brassicae* was recognized as highly pathogenic seed borne fungi. This is in conformity to the findings of Singh *et al*. (2014), Rai *et al*. (2015) and Dhaliwal and Singh (2019), who found that *A. brassicae* is a highly destructive pathogen of rapeseed-mustard crop, affect all growth stages and seed germination.

Test tube seedling symptom test: It is evident from Table 2 that seeds treated with individual fungus caused lowest germination, higher pre and post-emergence mortality and reduced seedling length and vigour index as compared to control. Common fungi that showed higher percent incidence in incubation tests and already reported as pathogenic were used to see their phytopathological effects. Among these, *Aspergillus niger*, *Curvularia lunata*, *Fusarium oxysporum*, *A. alternate*, *A. brassicicola* and *A. brassicae* caused lowest seed germination and high percent of pre and post-emergence mortality as compared to control. Lowest seed germination (45.0 %) and maximum pre-emergence (35.0 %) and post-emergence mortality (16.7 %) with lowest seedling length (3.0 cm/ 2.8 cm) and vigour index (258.7) were observed in seeds inoculated with *A. brassicae* followed by *A. alternata* and *Fusarium oxysporum*. Highest seed germination (75.0 %) and lowest pre and post-emergence mortality (05.0 % and 9.8 %, respectively) with highest seedling length (6.0 cm/ 7.6 cm) and vigour index (1020) were observed in seeds inoculated with *Curvularia lunata*. Seed borne inoculum of the *A. brassicae* produced lesions on the cotyledonary leaves and then in first true leaves which resulted in a light small to heavy necrotic flecking lesion on upper surface of cotyledon. Symptomatic seedling showed rotting, yellowing of leaves, stunting and darkening of root-shoot transition zone. The degree of infection was directly correlated with the amount of fungal growth on seed. Heavy growth of the

Table 2: Effect of seed mycoflora on seed germination, pre-emergence and post-emergence mortality and seedling vigour tested by test tube method

Mycoflora	Percent germination emergence	Percent mortality		Seedling length (cm)		Vigour index
		Pre emergence	Post length	Radicle length	Plumule	
Alternaria alternate	57	23.0	8.6	3.6	4.0	433
Alternaria brassicae	45	35.0	16.6	3.0	2.8	259
Alternaria brassicicola	62	18.0	9.3	4.1	6.5	657
Aspergillus niger	70	10.0	10.5	5.3	6.2	802
Curvularia lunata	75	5.0	9.8	6.0	7.6	1020
Fusarium oxysporum	69	11.0	13.1	4.5	3.8	569
Control	80	0.0	0.0	6.6	7.0	1088
CD (p=0.05)	4.3	2.1	1.1	1.1	1.3	-

fungi resulted in complete failure of seed germination while sparse and moderate growth produced symptomatic seedlings. Moderate fungal growth often arrested seed germination at an early stage, cotyledons failed to emerge out of the seed coat. This finding has been substantiated by the work of Meena *et al*. (2016) wherein, artificial inoculation with both seed and cotyledon method result in successful infection and the appearance of black spot symptoms of the disease.

Histopathology of infected seed

To detect the fungus from various components of mustard seeds *viz*., seed coat, endosperm, cotyledon and embryo, component plating method was used. It was observed (Table 3) that maximum site of infection of pathogenic seed mycoflora was in seed coat. In all these four categories of infected seed components, the infection of pathogenic mycoflora was maximum on seed coat (2.0-12.0 %) and minimum on endosperm (2.0-4.0 %) whereas cotyledons and embryo were free from infection. In cotyledons and embryo of the seeds, the fungal infection was not detected. Among these pathogenic mycoflora the maximum infection in different parts of seeds was shown by *A. brassicae* (0-12.0 %). The greatest infection of *A. brassicae* was in seed coat (12.0 %) followed by endosperm (4.0 %) and it was absent in cotyledon and embryo. The various components of the seed on incubation yielded mycelial growth and sporulation of *A. brassicae*, *A. brassicicola*, *A. alternata*, *Aspergillus niger*, *Aspergillus flavus*, *Curvularia lunata* and *Fusarium oxysporum*. The fungal mycelium as well as conidia was more prone on seed coat wherein, endosperm showed least incidence of mycelium. In cotyledon and embryo, the fungal infection was not detected. This result correlates with findings of Bhatnagar and Balhara (2007) and Prasad *et al*. (2010), who found that infection of *Alternaria* sp. was more on seed coat than cotyledons and embryo. Under compound microscope it was observed that *A. brassicae* showed septate and branched mycelium, muriform conidia with long, cylindrical beak. This finding is line with the work of Singh *et al*. (2014) and Giri *et al*. (2013). By observation of slide under compound microscope, it was found that mycelium of *A. brassicae* was septate and branched. Conidia of the fungus were obclavate to muriform, having transverse and longitudinal septa with cylindrical, long and pale brown beak.

Conclusion

A total of sixteen saprophytic as well as parasitic mycoflora belonging to nine genera were recorded in the blotter and agar plate methods. These were *A. brassicae*, *A. alternata*, *A. brassicicola*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus fumigatus*, *Aspergillus ochraceous*, *Curvularia lunata*, *Drechslera tetramera*, *Rhizopus nigricans*, *Mucor* sp., *Penicillum* sp., *Chaetomium* sp., *Metarrhizium* sp. and *Fusarium oxysporum*. The fungal species recorded in PDA test were common to those observed in Blotter test but percent incidence of fungi varied in blotter test and PDA plate method. Sample collected was from Alwar showed maximum incidence of mycoflora. Phytopathological effects of seed borne fungi were tested in which six fungal species belonging to four genera including *A. brassicae* were found pathogenic and adversely affect plant status. In seedling symptom test, seedlings produced necrotic lesions on the cotyledonary leaves, then in first true leaves which resulted in a light small to heavy necrotic flecking lesion on upper surface of cotyledon and also showed seed and seedling rot, yellowing of leaves, stunting and darkening of root-shoot transition zone. The location of pathogenic seed mycoflora in seed components of mustard seed, pathogenic fungus was present on seed coat than cotyledons and embryo.

Table 3: Detection of pathogenic seed mycoflora by component plating method

Mycoflora	Percent incidence of mycoflora in seed components*			
	Seed coat	Endosperm	Cotyledon	Embryo
Alternaria alteranata	6.0	2.0	0	0
Alternaria brassicae	12.0	4.0	0	0
Alternaria brassicicola	2.0	0	0	0
Aspergillus flavus	4.0	0	0	0
Aspergillus niger	2.0	0	0	0
Curvularia lunata	6.0	0	0	0
Fusarium oxysporum	4.0	0	0	0
Un-identified	6.0	2.0	0	0

*Average of 50 seed components

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