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Review Article

Toxins and Metabolites of Seed Borne Fungi Alters Seed Germination in Soybean

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ABSTRACT:

Cultivars of soybean viz. MAUS 2 yielded *Rhizopus stolonifer, Mucor mucedo, Aspergillus niger, A. flavus, Penicillium chrysogenum, Fusarium oxysporum, Rhizoctonia leguminicola, Cephalosporium aregatium*. MAUS 1 yielded *Absidia sp., Aspergillus fumigatus, Alternaria tenuis, Cladosporium herbarum* beside those isolated from MAUS 2. MAUS 32 yielded all the above reported fungi from the two other varieties.

Seed borne fungi deteriorate the seeds by secretion of toxins. These toxins kill the living cells. Toxins are synthesized by fungi in liquid medium as a metabolite. So, selected seed borne fungi from respective oilseeds were grown in liquid medium. The culture filtrate as well as spore suspension of fungi were assessed for the effect on the seeds. The seeds were inoculated with the spore suspension of respective fungi and also treated with culture filtrate from these fungi. With increase in treatment time of the seed there was reduction in seed germination in all the cases. Toxin secretes by these fungi inhibits the seed germination is evident from these results.

[I] INTRODUCTION

The oilseed crops like soybean (*Glycine max* L.) is one of the important cash crops grown in Marathwada region of Maharashtra. It is well established fact that, the seeds of these crops are reported to carry many moulds both in field and in storage. The associations of moulds adversely affect the health of seeds and seedlings resulting into seed rotting and seedling diseases. The common seed moulds found to be associated are mainly species of *Aspergillus, Macrophomina, Fusarium, Rhizopus, Penicillium, Alternaria, Cladosporium, Curvularia, Trichoderma, Sclerotium, Chaetomium, Helminthosporium* etc.

In the process of seed bio-deterioration the moulds have been found to cause quantitative and qualitative changes in chemical composition of the seeds and poison food and feeds making them unsuitable for human and animal consumption. Production of enzymes and toxins by the moulds has been found to be correlated with the degree of bio deterioration. Therefore, in the present investigation emphasis has been given on the following aspects.

Seed germination due to association of seed moulds has been found a common fact in course of many crops. Shrotri *et al.* (1983) confirmed that association of *A. flavus, A. niger, Alternaria alternata, Botrytis cinerea, Curvularia pallescens, C. tuberculata* and *Fusarium* sp. produced varying degree of germination loss. Mahajan and More (1990) studied the infection of *A. niger, A. Fumigatus, A. niduluns*, which reduces the percentage germination in sunflower [1,2]. Kumar *et al.* (1993) reported 78%

reduction in seed germination due to *Sclerotinia sclerotiorum* [3].

The culture filtrate of seed-borne fungi on seeds germination and seeding growth has been studied by several workers. Toxins are important metabolites of mycoflora involved in the process of seed deterioration [4, 5]. The detailed information about toxin is richly available for leaf spot causing fungi while, the testing of culture filtrates of seed-borne fungi against seed germination have been worked out by several workers in the area of seed pathology.

Culture filtrate of different seed-borne fungi affect greatly the process of germination in oil seeds. Among Jowar seed mycoflora the species of *Alternaria, Fusarium* and *Cladosporium.* Christensen and Kaufman 1969 observed seed mycoflora in jowar they isolated species of *Alternaria, Fusarium* and *Cladosporium* [5]. Bhale *et al.*, (1982) observed effect of culture filtrate in *Curvularia* sp. *Trichoconidle* sp. on seed germination Deshpande and Kulkarni (1990) in safflower due to *A. flavus, A. niger* and *R. Stolnifer* [6, 7]. Sandikar *et al.*, (1993) found variable toxin effect of *A. Carthami* and *A. flavus* in different varities of safflower, various seed borne fungi seed borne fungal toxin as sesame [8,9].

MATERIAL AND METHODS

Collection of Seed Samples

The methods described by Neergaard (1973) have been adopted for the collection of seed samples. Accordingly, seed samples (half kg each) were collected from field, store houses and market places. A composite sample was prepared by mixing the individual samples together, preserved in jute bags at room temperature during the studies. Seed of following of Soybean (*Glycine max* L.) and their cultivars Cv. MAUS 2, MAUS 1 and MAUS 32 were used in the study [10].

Assessment of Seed Mycoflora

The seed-borne fungi of oilseeds were detected by agar plate and blotter test methods as recommended by International Seed Testing Association (1966) and Neergaard (1973). The procedure of agar plate and blotter test methods [10, 11].

Agar plate method: In this method, pre-sterilized borosil glass petriplates of 10 cm diameter were poured with 25 ml of autoclaved PDA (Potato dextrose agar) or Casein agar medium of pH 5.6. On solidification, ten seeds per plate were equispaced placed aseptically. Four hundred seeds were used in every experiment. The plates were incubated at room temperature (27±2°C) for eight days. On 8th day the seeds were examined under stereoscopic microscope for the preliminary determination of fungal growth. The fungi occurring on each and every seed were isolated and identified. Pure cultures of the identified seed-borne fungi were prepared and maintained on PDA slants.

Seeds were pre-treated with 0.1% solution of $HgCl_2$ for two minutes were used to isolate only internal seed mycoflora. Subsequently the seeds were washed thoroughly with distilled water and placed on agar plates. Seeds without such pre-treatment were employed for the study of total (Internal + external) seed mycoflora.

Blotter Test Method: White blotter papers of 8.5 cm diameter were soaked in sterile distilled water, placed in pre-sterilised borosil glass petri-plates of 10cm diameter. Ten seeds per plate were placed at equidistance aseptically on the moist blotters. Four hundred seeds were used in each experiment. The conditions and other detail remains the same.

Identification and Isolation of Seed-Borne Fungi

The fungi occurring on seed plated on ***Potato Dextrose Agar (PDA)*** plates and moist blotter plates were preliminary identified on the basis of sporulation characters. Detailed examination of fungal characters was done by using compound microscope and identification was confirmed with the help of manuals. Pure cultures of the

identified fungi were prepared and maintained on PDA Slants.

Spore suspension

Spore suspension was prepared by adding 10ml sterile water to a 8-day-old PDA slant culture and 5ml of this was used as inoculum in all experiments unless and otherwise stated. In every case the spore suspension was standardized to have 0.8 O.D. (optical density). All experiments were conducted in the triplicate and results are presented. Cultures were incubated at 27±2ºC in the laboratory and variation in temperature was recorded during the period of study. In all the experiments the cultures were incubated for 7-10 days unless and otherwise stated.

Toxin Production

Production of toxin was studied by growing some common and dominant seed-borne fungi of oilseeds like *Alternaria niger, Aspergillus flavus*, and *Fusarium oxysporum* on liquid GN medium of pH 5.6. Twenty five ml of the medium was poured in 100 ml borosil glass conical flasks, autoclaved and inoculated separately with 2 ml spore suspension of the test seed-borne fungi which were maintained on PDA slants for seven days. The flasks were incubated at room temperature (27±2°C) for ten days. After incubation, the culture filtrates were collected in pre-sterilised culture bottles from the flasks by filtering the contents through Whatman filter paper No.1 and treated it as crude toxin preparation.

Spore suspension was prepared from heavily sporulating slant. One hundred seeds of each variety of oilseeds were soaked separately in the spore suspension (CF) for 1 hour. The soaked seeds were then placed on moist blotters in sterilised borosil glass petriplates (10 cm diameter). The plates were incubated for ten days at room temperature (27±2°C). After incubation percent seed germination were recorded. Ten germinated seeds were equidistantly spaced on moist blotter previously dipped in fungal metabolites.

RESULTS:

In the preliminary and earlier studies, it was recorded growth of some notable fungi on agar plates were found to be suppressed due to increased surface mycoflora. This indicates that the pathogenic fungi might be weak competitors in comparison to the surface mycoflora (*Chaetomium globosum, Aspergillus flavus, Trichoderma virides* and *Rhizopus stolonifer* etc.).

The seeds of soybean MAUS 2, MAUS 1 and MAUS 32 showed varied susceptibility for infection, as they yielded different amount of seed mycoflora including many saprophytic fungi including species of *Mucor, Rhizopus, Fusarium, and Aspergilli.*

Metabolites of seed borne fungi have different effects on germination. In majority of cases they affected germination adversely. Experiments were set to study this aspect in detail. Seeds were observed further for development of seedlings and those which did not develop have been marked with '*' in the tables.

Effect of Aspergillus niger on seed germination soybean seeds

Effect of spores of *Aspergillus niger* and its metabolites on the seed germination of *Glycine max* L. Cv. MAUS 2was studied. Percentage germination was found to decrease with the increase in period of treatment with spore suspension or metabolite. In spore suspension percentage dropped from 40 (1 hr.) to 0 (12 hr.). Percentage of germination considerable recovery was evident when observation were made after 48 hours. Percentage germination in metabolites dropped from 50 (1 hr.) to Zero (10 hr.). It was neutralized after 48 hours of incubation. Seeds receiving 12 hour soak in spore suspension and those receiving 10 hours treatment of metabolites did not develop healthy seedlings. Seed germination was 100 % in control.

The period of treatment of either the spore suspension or metabolite of *Aspergillus niger* percentage seed germination of *Glycine max* L.

Cv. MAUS 1 decreased. In spore suspension percentage germination dropped from 60 (1 hr.) to 0 (12 hr.). Slightly increase was recorded in the percentage of germination after 48 hours.

Percentage recovery was 30 to 40. The metabolite treatment spore germination was reduced from 50 to Zero (1 hr. and 12 hr.). At the 6 hrs response to suspension and metabolite was similar. Treatment resulted in decrease in the percentage germination. Seeds treated with suspension for 12 hours failed to produce healthy seedlings as the conidial heads emerged from the radicle. Seeds with 10 to 12 hours metabolite treatment failed to germinate (Table 1).

Spore suspension and metabolite were found to affect adversely seed germination of *Glycine max* L. Cv. MAUS 32. With the increased period of treatment of suspension and metabolite percentage germination decreased by 80 % after 24 hours of germination percentage germination remained unchanged even after 48 hours. Seed with 12 hours treatment did not develop in to healthy seedlings.

Table 1: Effect of spore suspension and metabolites of *Aspergillus niger* on seed germination soybean

Period of treatment (hrs.)	Cv. MAUS 2				Cv. MAUS 1				Cv. MAUS 32			
	% Germination after				% Germination after				% Germination after			
	24-48 hrs. Spore suspension		24-48 hrs. Metabolite		24-48 hrs. Spore suspension		24-48 hrs. Metabolite		24-48 hrs. Spore suspension		24-48 hrs. Metabolite	
1	40	90	50	100	60	80	50	80	100	100	100	100
2	40	90	40	100	60	80	50	70	90	100	100	100
4	40	90	40	100	60	80	30	50	80	90	90	90
6	30	80	40	90	40	50	20	50	90	90	80	90
8	30	80	30	60	40	40	20	50	70	80	70	80
10	20	70	00	60*	30	40*	00	00	40	40	40	40
12	00	70	00	30*	00	40*	00	00	20	20	20	20*

Germination in control 100. * damaged seedlings.

Effect of Aspergillus flavus on seed germination soybean seeds

Effects of spores Aspergillus flavus and its metabolites on the germination of *Glycine max* L. Cv. MAUS 2 was studied. Percentage germination was found to decrease with the increase in period of treatment with spore suspension or metabolite. In spore suspension percentage dropped from 40 (1 hr.) to Zero (12 hrs.). It was observed after 48 hours in all treatments except those involving 10 to 12 hrs soak. Percentage germination in metabolites dropped from 50 (1hr.) to Zero (12 hrs.). The inhibitory effect was neutralized after 48 hours of incubation. Fungal metabolite was found to be more inhibitory than the spore suspension. Seeds with 12 hrs metabolite treatment failed to develop healthy seedlings (Table 2). Both the spore suspension and metabolites of *Aspergillus flavus* were found to inhibit seed germination, in *Glycine max* L.Cv. MAUS 1, progressively with increase in period of treatment. Percentage dropped from 60 (1 hr.) to Zero (12 hrs.) in spore suspension. In the percentage of germination was observed after 48 hours of germination was observed after 48 hours of germination. Percentage of germination decreased from 60 % (1 hr.) to Zero (12 hr.) with the treatment of metabolite. Considerable recovery was noticed after 48 hours of incubation. 12 hours treatment in case of spore suspension and 10 to 12 hrs treatment with metabolite was found to be injurious and resulted in Browning of radicle in case of metabolite and the formation of conidial heads with the spore suspension treatment.

Metabolite and spore suspension *Aspergillus flavus* inhibitory affect on percentage germination of *Glycine max* L. Cv. MAUS 32 seeds. In suspension percentage germination decreased from 100 (1 hour) to 20 (12 hr.). Slight increase in the percentage of germination was observed after 48 hours of incubation. Percentage

germination dropped from 100 to 20 (1 hour to 12 hours) in the presence of metabolites. The recovery in percentage germination after 48 hours of germination was comparatively negligible. Seeds with 12 hours treatment of metabolite failed to develop healthy seedlings.

Table 2: Effect of spore suspension and metabolites of *Aspergillus flavus* on seed germination soybean

Period of treatment (hrs.)	Cv. MAUS 2				Cv. MAUS 1				Cv. MAUS 32			
	% Germination after				% Germination after				% Germination after			
	24-48 hrs. Spore suspension		24-48 hrs. Metabolite		24-48 hrs. Spore suspension		24-48 hrs. Metabolite		24-48 hrs. Spore suspension		24-48 hrs. Metabolite	
1	40	90	50	100	60	80	50	80	100	100	100	100
2	40	90	50	100	60	80	50	80	90	100	100	100
4	40	90	40	100	60	80	40	60	90	100	90	90
6	30	80	40	90	40	50	30	50	80	90	90	90
8	30	80	40	90	40	40	20	50	90	90	80	90
10	30	80	30	60	30	40*	20	50	70	80	70	80
12	00	70	00	30*	00	40*	00	00	20	20*	20	20*

Germination in control 100. * damaged seedlings.

Effect of Fusarium oxysporum on seed germination soybean seeds

Effects of spores *Fusarium oxysporum* and its metabolites on the seed germination of *Glycine max* L. Cv. MAUS 2 was studied. Spore suspension percentage dropped from 40(1 hr.) to Zero (12 hr.). Considerably recovery in percentage germination was evident when observations were made after 48 hours. Percentage germination in metabolites dropped from 50 (1 hr.) to 30 (10 hrs.). Here also inhibitory effect was neutralized after 48 hours of incubation. Seeds receiving 12 hours soak in spore suspension and those receiving 10 hours treatment of metabolites did not develop healthy seedlings. Germination was cent percent in control (Table 3). Both the spore suspension and metabolite of *Fusarium oxysporum* were found the inhibit seed germination in *Glycine max* L. Cv. MAUS 1 progressively with increase in period of treatment. Percentage dropped from 60 (1 hr.) to Zero (12 hours). Slight increase was recorded in the percentage of germination after 48 hours. Percentage recovery was 20 to 30. With the metabolic treatment spore germination was reduced from 60 to 30 (1 hr. and 10 hr.) At 6 hrs response to suspension and metabolites was similar. Treatment resulted in rapid decrease in the percentage germination. Seeds treated with suspension for 10 to 12 hours failed to produce healthy seedlings as the conidial heads emerged from the radicle. Seeds with 10 to 12 hrs metabolite treatment failed to germinate.

Spore suspension and metabolite of *Fusarium oxysporum* Cv. MAUS 32 found to effect adversely. With the increased period of treatment of suspension and metabolite, percentage germination decreased by 80 % after 24 hours of germination. Percentage germination remained unchanged even after 48 hours. However recovery of germination after 48 hours was still evident.

Table 3: Effect of spore suspension and metabolites of *Fusarium oxysporum* on seed germination soybean

Period of treatment (hrs.)	Cv. MAUS 2				Cv. MAUS 1				Cv. MAUS 32			
	% Germination after				% Germination after				% Germination after			
	24-48 hrs. Spore suspension		24-48 hrs. Metabolite		24-48 hrs. Spore suspension		24-48 hrs. Metabolite		24-48 hrs. Spore suspension		24-48 hrs. Metabolite	
1	40	90	50	100	60	80	50	80	100	100	100	100
2	40	90	50	100	60	80	50	70	90	100	100	100
4	30	90	40	100	50	70	50	70	80	90	90	90
6	30	80	40	90	50	70	30	50	80	80	80	90
8	30	80	30	60	40	50	20	50	70	80	70	80
10	20	70	30	60*	30	40*	20	50	40	40	40	40
12	00	70	00	30*	00	40*	00	00	20	20*	20	20*

Germination in control 100. * damaged seedlings.

DISCUSSION

Studies were undertaken on selected oil seed crops viz. soybean, sesame and groundnut the major oil seed crops of Marathwada region. The work mainly was concentrated on seedling emergence, association of fungi and mortality rate was studied. The effect of physical presence of the fungi and their metabolites like and toxins in the process of bio-deterioration was also investigated.

Seeds of three varieties of soybean, sesame and groundnut were screened for the composition of seed mycoflora using blotter and Agar plate methods [11]. It is evident from the studies on isolation of seed mycoflora that mycoflora was found to be recovered more on agar plate than on blotter.

The result indicates that the compounds which are known to be inhibitory for various metabolic activities also proved to be inhibitory for toxic Production. Among various such chemical, respiratory inhibitors showed maximum inhibition for toxin production in the fungi. This clearly suggests the relation of toxin production with the process of active respiration in the fungi.

Role of fungal toxins in damaging seed health and seedling vigour is now and established fact [6]. Similar studies have bean carried out in the present investigation and emphasis had been given on production and the properties of toxin produced by seed-borne fungi of safflower. The toxin in the culture filtrates of fungi was tested against seed germination, detached leaves and cut shoots of different plants.

Among different behaviours of culture filtrates results suggest that culture filtrate of *Fusarium oxysporum* must be having a non-host specific toxin, as its effect on seed germination of all the three oilseeds. At the same time it was interesting and clear that the culture filtrates of *Aspergilli* might be having a broad host range, as its culture filtrate was found to be equally toxic to hosts as well as non host crops. Similar type activities were also recorded in case of seeds mycoflora of legumes [12].

Regarding the effect of culture filtrates in different varieties of safflower it can be concluded that the toxic effect of culture filtrate cannot be equally destructive to all the varieties of host, as the culture, filtrate of seed borne fungi of safflower caused inhibition of seed germination at different levels in different varieties. This type of varietals response of toxin was also recorded earlier by Khairnar (1987) in seed borne fungi of bajra and Bhikane (1988) in seed borne fungi of legume [12, 13].

It is clear from the results regarding production of aflatoxin by different isolates of *A. flavus* that though majority of isolates of *A. flavus* showed positive test for aflatoxigenic nature, they also varied in their degree of aflatoxin production. This clearly suggests existence of aflatoxigenic strains in *A. flavus*. The results regarding the aflatoxin production on different substrates gives an idea that *A. flavus* might be very much specific for quality of substrate for aflatoxin production, as seed meal of groundnut and maize supported maximum aflatoxin production while, that of sesamum proved inferior. These compounds are known to inhibit germination of seeds and probably do not allow seed borne fungi to develop colonies.

Experiments designed to make out whether the seed borne fungi and their metabolites individually had differential effect on seed germination indicated that metabolites were more inhibitory. Groundnut seeds treated with metabolites of *A. niger* gave reduced germination. In sore suspension percentage germination was recovered after 48 hours. Slight stimulation was also evident. Stimulation of germination in spore suspension indicates synergestic action of lipase by the fungus and the seed. Such stimulation has been reported earlier also by Roy & Pandey, (1971) and in experiments conducted further fatty acids or fungal metabolites were found to stimulate germination of excised embryos [14].

Twelve hour treatment of culture filtrates however resulted in collapse of seedlings. It indicated that germination is less sensitive to the imbibed toxin than seedling development and post-germination development probably is blocked by imbibed toxin. Reduction in Percentage germination is not the only criteria to be considered in determining toxicity and pathogenicity and hence post-germination development also should be taken into consideration in any seed pathological study.

When we consider increased germination through change in sequence of seed borne fungi and improved germination through fungicide treatment together we find the former treatment many times superior than the chemical. If properly manipulated this may become an useful tool in increasing viability.

Stimulation of seedling growth *A. flavus*, *A. niger* produce lipases and cell wall degrading enzymes and also toxins and these are probably responsible for all these effects.

In general there was loss in seed weight following treatment by metabolites of *A. niger*, *R. stolonifer*, *A. flavus*, and *F. oxysporum*. This is as expected because metabolites of seed borne pathogens contain toxic substances and enzymes which alter permeability and enhance dissolution of reserve food [15].

In case of *Verticillium albo-atrum* there is an increase in seed weight following treatment of metabolites. It has been reported that *Verticillium albo-atrum* stimulates growth [16].

A comparison of seed weight in groundnut reveals different patterns. Seed weight exhibit reverse relationship in *A. hypogea* seeds treated with metabolites of *Fusarium oxysporum A. niger* or *A. flavus*. We find change in seed weight for the first 24 hours whereas after 48 hours seed weight decreased.

The aspects studied in this work revealed that both germination and post germination are integral part of the studies of seed pathology. The sensitivity of seed germination seedling emergence and further growth solely depends upon the mycoflora associated. The dominance of any fungus is influence due to presence of the substrate and specific nutrient in the seed. The ability of any seed borne fungus also depends on its efficiency to utilize the protein and oil content in the seeds subsequently the lipase synthesizing ability. The dominant fungi inhibit seed germination through secretion of toxin or metabolites inhibitory to the seed germination or seedling growth. The possibility of synergistic activity of enzymes and toxin in inhibiting seed germination and seedling growth cannot be denied. However to overcome this problem fungicides treatment may provide and better alternate.

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