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Determination of Toxicity of some Fungal Metabolites on Seed Germination and Pigment Leaching

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Keywords

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Abstract

In present investigation, eighteen fungi were isolated on Potato Dextrose Agar and Rose Bengal Agar medium from abnormal soybean seeds. Out of these eighteen fungi, ten dominant fungi were selected to study the toxic potentialities of culture filtrate of these ten fungi on oilseeds germination, pigment leaching i.e. chlorophyll of spinach and betalain of beet root. Maximum loss of chlorophylls was caused due to toxins of *Aspergillus niger, Aspergillus flavus* and *Penicillium notatum. Fusarium oxysporum* caused minimum loss of chlorophylls. *Aspergillus niger* and *Alternaria dianthicola* was responsible for maximum leaching of betalain pigment while *Curvularia lunata* causes minimum leaching of pigment.

1. Introduction

The production and supply of high quality grain remains of prime importance. Oilseeds must thus be protected in the field against disease and in store after harvest against fungal attack. Attack by fungi not only reduces the quality of the grain but some species of moulds can produce highly toxic chemicals or secondary metabolites known as mycotoxins. Macroscopic observations show that aflatoxins affect certain plants by inhibition of seed germination (Schoental and White, 1965), elongation of the hypocotyls or roots of developing seedlings, or both (El-Khadem, *et al.* 1966; Reiss,1971) and by interference with chlorophyll synthesis (Slowatizky, et. al.1969).

Many fungi are serious parasites of seed primordia, maturing and stored seeds and grains and their invasion can resulted various abnormalities including, reduce yields of seed in both quantitatively and qualititatively, discolourations decrease germinability, mycotoxin production and total decay (Quenton *et al, 2003*; Castillo *et al.*, 2004). One of the most important effects of post harvest decays of fruits and vegetables and especially of seed and feed deterioration by fungi is the induction of mycotoxicoses. Consumption of feeds and foods contaminated by fungi that produce toxic substances called mycotoxins which are secondary metabolites produced by filamentous fungi, contaminate food, feeds or raw materials used in producing them. and causes disease to animals and humans (Agrios, 1978; Moss, 1989). Some common mycotoxicoses caused by common and widespread storage fungi such as *Aspergillus, Penicillium, Fusarium* and *Alternaria* result in severe illness and death. *Aspergillus* and *Penicillium* produce their toxins mostly in stored seeds, hay or commercially processed foods and feeds although infection of seeds usually takes place in the field. Adams (1977) has reported that storage fungi especially *Aspergillus, Penicillium, Rhizopus* and *Mucor* species infect grains after harvest and can grow on them during storage. The genera of mycotoxigenic fungi are mainly represented by *Aspergillus, Penicillium* and *Fusarium* but *Alternaria* among others are also important as food contaminants or pathogens of plants. *Alternaria* produces mycotoxins in grains such as rice and maize. Many *Aspergillus, Penicillium* and *Cladosporium* species are known to produce mycotoxins. Aflatoxin is about the most popular and widespread mycotoxin. Its name derives from the fact that it was originally found to be produced by *Aspergillus flavus* (Agrios, 1978), but is now known to be produced by other species of *Aspergillus*. Aflatoxin B1 is produced by *Aspergillus terreus,* though it may also be produced by *Aspergillus flavus* as well as *Aspergillus oryzae.* It is the most toxic, carcinogenic and most prevalent of the different aflatoxins. Generally, mycotoxins have been implicated as causative agents of different human and animal health disorders (Ciegler and Bennett, 1980). Both the toxigenic fungi and the mycotoxins they produced are potential problems from both health and economic perspectives. Fumes from burning molded hay may also affect animals and man and handling of such hay by farm workers causes in them a toxic dermatitis, conjunctivitis etc. During their metabolic process fungi secrete their metabolites in medium in which it grows. These toxic products formed by several fungal species which readily colonize crops in the field or after

harvest under special conditions of moisture and temperature.

Fungal toxins are injurious to human and animal health. Even at low level they can reduce livestock productivity. The present study therefore, undertaken to evaluate the effect of toxic metabolites of storage fungi on percentage of seed germination, chlorophyll content and betalain pigment leaching of beet root. Culture filtrates of *Aspergillus niger, Aspergillus flavus, Alternaria dianthicola, Curvularia lunata, Fusarium oxysporum, Fusarium equiseti, Macrophomina phaseolina, Rhizopus nigricans, Penicillium notatum, Penicillium chrysogenum* were tested on oilseed germination of groundnut (*Arachis hypogea*), soybean (*Glycin max* L.), sunflower (*Helianthus annus* L.), safflower (*Carthamus tinctorus* L.) and mustard (*Brassica sp.*). Findings will serve the purpose of alerting consumers on the dangers of toxic effect of fungi on poorly stored grains.

2. Materials and Methods

Isolation of storage fungi

Abnormal oilseeds of soybean variety i.e. JS-335 and Eagle were collected from store houses of Nanded district of Marathwada region of Maharashtra state. These oilseeds were then packed in presterilized plastic zip-lock bags. To study the fungi associated with abnormal seeds Potato Dextrose Agar (PDA) and Rose Bengal Agar (RBA) media were used. Storage fungi (*Aspergillus niger, Aspergillus flavus, Alternaria dianthicola, Curvularia lunata, Fusarium oxysporum, Fusarium equiseti, Macrophomina phaseolina, Rhizopus nigricans, Penicillum notatum, Penicillium chrysogenum*) were isolated from soybean seeds and maintained on PDA media.

Culture filtrate preparation

A disc (0.5cm diameter) of mycelia and spores taken from the periphery of 7-day-old cultures of fungus grown on PDA medium was inoculated into 250ml conical flasks, each containing 100 ml of glucose nitrate broth. The broth contains (g/l): glucose 10g, potassium nitrate 2.5g, potassium dihydrogen ortho-phosphate 1g, magnesium phosphate 0.5g. The flasks were allowed to incubate at room temperature for 15 days. Three flasks were used for each fungus per incubation period. The fungal filtrates were obtained by passing the culture through sterile Whatman No. 1 filter paper to obtain a cell-free filtrate.

Effect of fungal filtrate on seed germination

Seeds of groundnut, soybean, sunflower, safflower and mustard were surface sterilized with 1% Mercuric chloride solution for 1 min and rinsed several times in sterile distilled water. All these five oilseeds were then allowed to presoak in fungal culture filtrate for 3h. At the end of presoaking period, the seeds were removed from the filtrates and transferred into the petriplates containing two layered blotter papers soaked with sterile distilled water. About 20 seeds were sown per dish and it was then allowed to incubate for two days at room temperature. Germination counts were made after incubation period of 48h and 72h.

Effect of fungal filtrate on chlorophyll content of spinach

Leaves of spinach were washed with sterile distilled water and later on treated with fungal filtrate of all the storage fungi with 30min, 60min, 90min, 120min, 150min time period. After that O.D. (Chl- a at 645nm; Chl-b at 663nm) of each sample was taken and chlorophyll content was estimated.

Effect of fungal filtrate on betalain pigment content of beet root

3cm cylinders were made with the help of 1cm diameter borer. Cylinders were continuously washed with distilled water until water becomes colorless. These cylinders were then treated with fungal filtrates of storage fungi for specific time. Treated cylinders were then soaked in distilled water for 5min. Finally, O.D. of colored samples was taken at 525nm.

3. Results and Discussions

Isolation of oilseed mycoflora

Seed mycoflora of two different varieties viz. JS-335 and Eagle of soybean cultivated in Marathwada region of Maharashtra state was isolated by using PDA and RBA and the results are given in table 1. *Alternaria dianthicola* showed association with all categories of JS-335 and on Dc category its quantitative dominance was observed. *Alternaria alternata* restricted its growth on Rot category. *Aspergillus niger* showed its quantitative dominance on all categories of JS-335, especially on the Rot category on RBA. *Aspergillus flavus* showed its maximum occurrence on all categories of the JS-335 wherein, Rot category showed quantitative dominance. *Curvularia lunata* showed its quantitative dominance on Dc category of variety JS-335 on PDA while, it occurred on all categories of Eagle except Sh category. On PDA, Rot category of Js-335 showed maximum occurrence of *Curvularia pellescens*. JS-335 on all its categories and both media showed maximum occurrence of *Fusarium oxysporum,* and *Fusarium equiseti* as compared to Eagle variety. All categories of JS-335 on PDA and all categories of Eagle on RBA showed occurrence of *Macrophomina phaseolina. Rhizopus stolonifer* showed its quantitative dominance on Rot category of JS-335 on RBA. Quantitative dominance of *Penicillium notatum* was observed on all categories of JS-335

variety. Dc category of JS-335 showed maximum occurrence of *Penicillium chrysogenum. Trichoderma viridae* showed its occurrence on both the varieties. Out of seventeen fungi isolated, *Aspergillus niger, Aspergillus flavus, Alternaria dianthicola, Curvularia lunata, Fusarium oxysporum, Fusarium equiseti, Macrophomina phaseolina, Penicillium notatum* and *Penicillium chrysogenum* showed their quantitative dominance. Therefore, these ten fungi were selected to study their lipase activity. All the ten dominant fungi were able to metabolize to varying grades of different types of physical factors to lipase production.

Effect of presoaking (3h) of oilseeds in fungal culture filtrate after incubation period of 15 days of storage fungi on percentage of seed germination after 48h was studied (Table 2). It showed significant reduction in seed germination as compared to control. Percentage of seed germination is different according to different culture filtrate. *Aspergillus niger* hampered germination in all the oilseeds, followed by *Macrophomina phaseolina*. Germination percentage of groundnut seed is greatly hampered by storage fungi while, mustard seeds showed highest germination, followed by safflower and soybean seeds after 48h. Metabolites of *Aspergillus niger* and *Aspergillus flavus* are highly toxic for the groundnut and sunflower and it significantly inhibited germination. Culture filtrate of *Fusarium oxysporum* and *Fusarium equiseti* hampered the germination of groundnut and sunflower. Culture filtrate of *Rhizopus stolonifer*, *Macrophomina phaseolina* and *Penicillium notatum* and *Penicillium chrysogenum* significantly hampered the seed germination of groundnut. Mustard seed germination is reduced due to fungal filtrate of *Aspergillus niger*, *Penicillium chrysogenum* and *Macrophomina phaseolina* lowered the seed germination of safflower after 48h.

It is clear from the table 3 that filtrate of *Aspergillus niger, Penicillum chrysogenum* and *Macrophomina phaseolina* inhibited seed germination, which means that metabolites are discharged by the tested fungi in the media in which they were grown. These toxic metabolites can inhibit and reduce percentage of germination.

Germination of oilseeds after incubation period of 72h was studied and results were summarized in table 3. It was observed that culture filtrate of *Aspergillus niger* and *Aspergillus flavus* was toxic for germination of groundnut and sunflower. Culture filtrate of *Fusarium oxysporum* and *Fusarium equiseti* was observed to be toxic for germination of groundnut and sunflower. Seed germination of safflower and mustard was stimulated by culture filtrate of *Macrophomina phaseolina*. Sunflower and mustard seed germination increased in fungal metabolites of *Curvularia lunata*. Soybean seed germination was lowered in toxic metabolites of *Penicillium notatum*. Percentage seed germination was found to be increased in *Penicillium chrysogenum*.

Effect of Culture filtrate of storage fungi on chlorophyll content (Chl-a and Chl-b) was estimated by the method suggested by Arnon (1949). It is clear from the table 4 that c.f. of *Aspergillus niger* was found to be responsible for maximum leaching of Chl-a and Chl-b irrespective of soaking period and it was maximum at 150 min. Chl-a showed maximum leaching when soaked in c.f. of *Aspergillus flavus* and *Penicillium notatum* for 120 min. Leaching of Chl-a and Chl-b was found to maximum at 150 min when soaked in c. f. of all the selected fungi accept *Aspergillus flavus* and *Penicillium notatum*. Maximum leaching of Chl-a was observed in c.f. of *Aspergillus niger, Penicillium notatum, Aspergillus flavus* and *Curvularia lunata*. At 30 min as compared to control c.f. of *Penicillium chrysogenum* and c.f. of *Alternaria dianthicola* caused minimum leaching of Chl-a and Chl-b respectively. At 60 min of soaking as compared to control c.f. *Fusarium oxysporum* and c.f. of *Aspergillus flavus* were found to be responsible for the minimum leaching of Chl-a and Chl-b respectively. *Fusarium oxysporum* showed minimum leaching of Chl-a and Chl-b.

Effect of Culture filtrate of storage fungi on total chlorophyll content was studies and results are given table 5. As compared to control, at 30 min and 60 min c.f. of *Aspergillus niger* caused maximum leaching while, c.f. *Penicillium chrysogenum* showed minimum leaching of total chlorophyll. At 90 min of soaking period c.f. of *Alternaria dianthicola*, at 120 min soaking period c.f. of *Aspergillus flavus* and at 150 min soaking period c.f. of *Fusarium oxysporum* was found to be responsible for the maximum leaching of total chlorophyll. *Penicillium chrysogenum* at 30 min and 60 min, *Fusarium oxysporum* at 120 min and *Rhizopus stolonifer* at 150 min caused minimum leaching of total chlorophyll.

Effect of Culture filtrate of storage fungi on betalain pigment leaching of *Beta vulgaris* was studied at different soaking periods and results are summarized in table 6. At 10 min, 20 min and 40 min of soaking period c.f. of *Alternaria dianthicola*; at 30 min and 50 min of soaking period c.f. *Aspergillus niger* caused maximum leaching of betalain pigment. At 10 min, 20 min and 40 min of soaking period c.f. of *Rhizopus stolonifer* and *Penicillium chrysogenum* and at 30 min and 50 min of soaking period c.f. of *Curvularia lunata* showed minimum leaching of betalain pigment of *Beta vulgaris*.

Sinha et al. (1993) observed seed germination and seedling growth as well as chlorophyll and carotenoid contents of mustard and gram seeds was inhibited or reduced significantly due to different concentrations of Aflatoxin B_1 of *Aspergillus flavus*. Aflatoxin B_1 significantly inhibited seed

germination of maize and chlorophyll and carotenoid of seedling (Prasad et al. 1996). Sinha Kumari (1990) found that at various concentrations of afatoxin B_1 seed germination, seeding growth, nucleic acid and chlorophyll content was reduced. Since storage fungi decreased the chlorophyll content which means that these fungi may utilizes chlorophyll as a substrate. Since *Aspergillus niger* and *Alternaria dianthicola* gave maximum O.D. as compared to other fungi, which means that their filtrates are more toxic. Betalain pigments of beet root are present in vacuole and are highly unstable, fungal toxins might have interact with it causes severe-leaching.

Table 1 Abnormal oilseeds mycoflora (%) incidence on soybean on PDA and RBA

Fungi	Varieties											
	JS-335						Eagle					
	PDA			RBA			PDA			RBA		
	DC	Sh	Rot	DC	Sh	Rot	DC	Sh	Rot	DC	Sh	Rot
Alternaria dianthicola	20	20	-	10	-	20	30	-	-	20	20	10
Alternaria alternata	-	-	10	-	-	-	-	-	10	-	-	-
Aspergillus niger	40	20	10	20	-	60	40	-	40	20	10	-
Aspergillus flavus	30	10	20	10	-	30	50	30	-	40	20	-
Aspergillus fumigatus	-	-	20	30	-	-	-	-	20	30	-	-
Aspergillus terreus	-	10	-	-	-	-	20	-	-	-	-	-
Curvularia lunata	30	-	10	-	10	-	40	-	10	20	-	-
Curvularia pellescens	20	-	50	-	-	40	10	20	30	20	-	20
Colletotrichum sp.	-	-	-	20	-	-	20	-	-	30	-	-
Fusarium oxysporum	50	20	40	20	80	50	-	20	-	-	10	30
Fusarium equiseti	20	10	40	20	-	60	40	-	30	20	-	30
Macrophomina phaseolina	60	30	40	40	10	-	20	50	-	40	20	40
Rhizopus stolonifer	30	-	40	20	-	30	20	30	-	10	30	50
Penicillium notatum	40	10	10	20	-	50	-	-	50	30	-	-
Penicillium chrysogenum	50	-	30	40	-	20	10	-	20	40	10	30
Verticillium sp.	-	-	-	10	-	-	10	-	-	30	-	-
Helminthosporium sp.	10	-	-	-	-	-	10	-	-	-	-	10
Trichoderma viridae	10	20	10	-	-	-	30	20	-	40	10	-

DC- Discoloured; Sh-Shrunkened; Rot- Rotted

Table 2. Effect of presoaking (3h) of oilseeds in fungal culture filtrate after incubation period of 15 days of storage fungi on percentage of seed germination after 48h

Fungi	Oilseeds				
	Groundnut	Soybean	Sunflower	Safflower	Mustard
Germination% after 48h					
Aspergillus niger	20	25	15	55	30
Aspergillus flavus	20	65	25	45	60
Fusarium oxysporum	25	60	30	35	65
Fusarium equiseti	30	55	35	45	50
Rhizopus stolonifer	25	70	45	40	45
Alternaria dianthicola	40	35	50	40	50
Macrophomina phaseolina	25	35	55	30	40
Curvularia lunata	50	55	45	55	65
Penicillium notatum	35	25	40	40	35
Penicillium chrysogenum	30	20	40	65	35
Control	90	95	95	95	95

Table 3. Effect of presoaking (3h) of oilseeds in fungal culture filtrate after incubation period of 15 days of storage fungi on percentage of seed germination after 72h

Fungi	Oilseeds				
	Groundnut	Soybean	Sunflower	Safflower	Mustard
	Germination% after 72h				
Aspergillus niger	35	70	20	85	90
Aspergillus flavus	30	70	35	45	80
Fusarium oxysporum	45	75	45	55	65
Fusarium equiseti	30	85	35	45	55
Rhizopus stolonifer	45	75	45	55	45
Alternaria dianthicola	40	45	50	45	50
Macrophomina phaseolina	60	55	40	90	85
Curvularia lunata	50	55	75	55	65
Penicillium notatum	45	35	45	40	45
Penicillium chrysogenum	40	40	45	75	80
Control	90	95	95	95	95

Table 4 Effect of culture filtrate of storage fungi on chlorophyll content (Chl-a and Chl-b)

Fungi	30 min		60 min		90 min		120 min		150 min	
	Chl-a	Chl-b	Chl-a	Chl-b	Chl-a	Chl-b	Chl-a	Chl-b	Chl-a	Chl-b
Aspergillus niger	0.112	0.153	0.081	0.094	0.077	0.083	0.066	0.080	0.043	0.067
Aspergillus flavus	0.170	0.190	0.160	0.180	0.101	0.111	0.073	0.082	0.085	0.074
Fusarium oxysporum	0.221	0.193	0.204	0.143	0.158	0.168	0.142	0.129	0.137	0.098
Fusarium equiseti	0.182	0.178	0.161	0.169	0.113	0.101	0.104	0.053	0.092	0.047
Rhizopus stolonifer	0.173	0.168	0.166	0.173	0.157	0.117	0.133	0.088	0.103	0.081
Alternaria dianthicola	0.203	0.243	0.133	0.136	0.104	0.125	0.085	0.102	0.071	0.082
Macrophomina phaseolina	0.219	0.239	0.127	0.139	0.101	0.121	0.091	0.097	0.077	0.079
Curvularia lunata	0.217	0.205	0.133	0.164	0.122	0.130	0.114	0.110	0.096	0.078
Penicillium notatum	0.164	0.178	0.143	0.155	0.097	0.112	0.081	0.061	0.099	0.070
Penicillium chrysogenum	0.246	0.238	0.183	0.134	0.102	0.126	0.087	0.105	0.074	0.104
Control	0.265	0.247	0.207	0.218	0.180	0.180	0.163	0.120	0.143	0.106

Values are expressed in O.D.

Table 5 Effect of culture filtrate of storage fungi on total chlorophyll content

Fungi	30 min	60 min	90 min	120 min	150 min
Aspergillus niger	0.261	0.171	0.193	0.170	0.150
Aspergillus flavus	0.360	0.310	0.220	0.140	0.141
Fusarium oxysporum	0.410	0.350	0.311	0.260	0.170
Fusarium equiseti	0.350	0.330	0.214	0.154	0.131
Rhizopus stolonifer	0.338	0.300	0.274	0.221	0.183
Alternaria dianthicola	0.456	0.269	0.185	0.180	0.153
Macrophomina phaseolina	0.478	0.266	0.222	0.184	0.152
Curvularia lunata	0.422	0.297	0.252	0.224	0.166
Penicillium notatum	0.342	0.298	0.209	0.172	0.129
Penicillium chrysogenum	0.484	0.371	0.228	0.192	0.178
Control	0.512	0.425	0.360	0.283	0.249

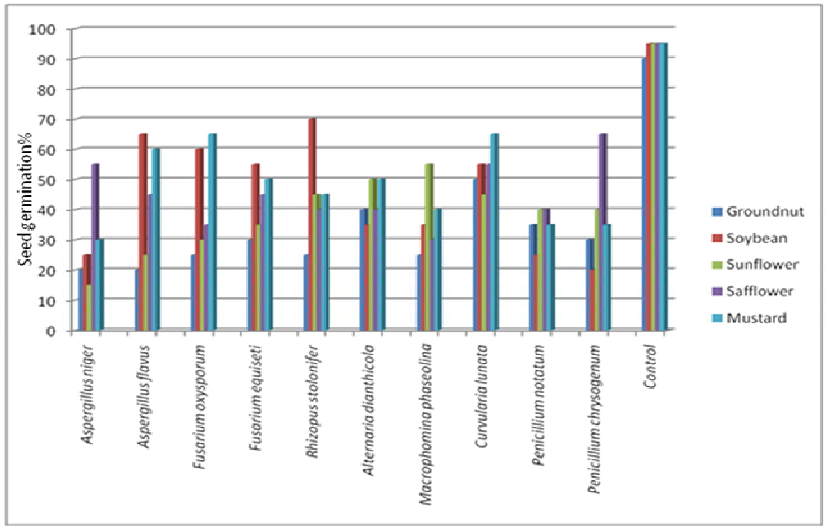
Values are expressed in O.D.

Table 6 Effect of culture filtrate of storage fungi on pigment leaching of *Beta vulgaris*

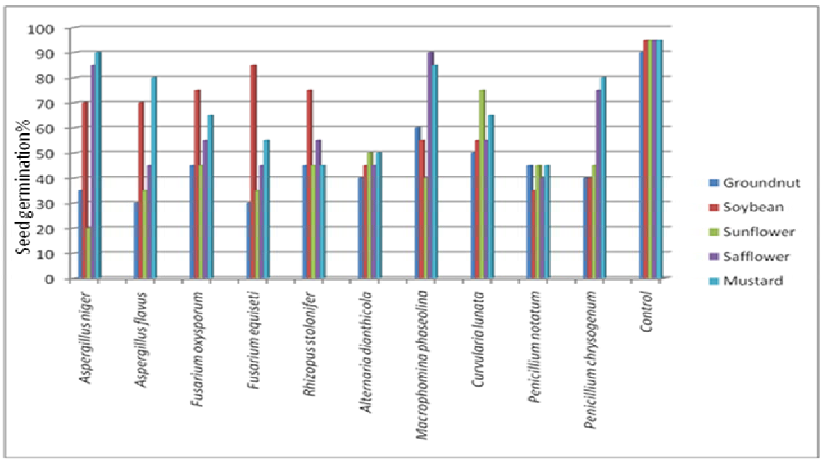
Fungi	10 min	20 min	30 min	40 min	50 min
Aspergillus niger	0.503	0.535	0.798	0.630	0.834
Aspergillus flavus	0.301	0.362	0.412	0.621	0.800
Fusarium oxysporum	0.120	0.173	0.290	0.378	0.510
Fusarium equiseti	0.105	0.198	0.253	0.321	0.420
Rhizopus stolonifer	0.037	0.090	0.150	0.191	0.410
Alternaria dianthicola	0.618	0.656	0.710	0.749	0.810
Macrophomina phaseolina	0.215	0.284	0.320	0.400	0.470
Curvularia lunata	0.125	0.171	0.130	0.281	0.335
Penicillium notatum	0.256	0.288	0.349	0.482	0.518
Penicillium chrysogenum	0.037	0.100	0.168	0.293	0.410

Values are expressed in O.D.

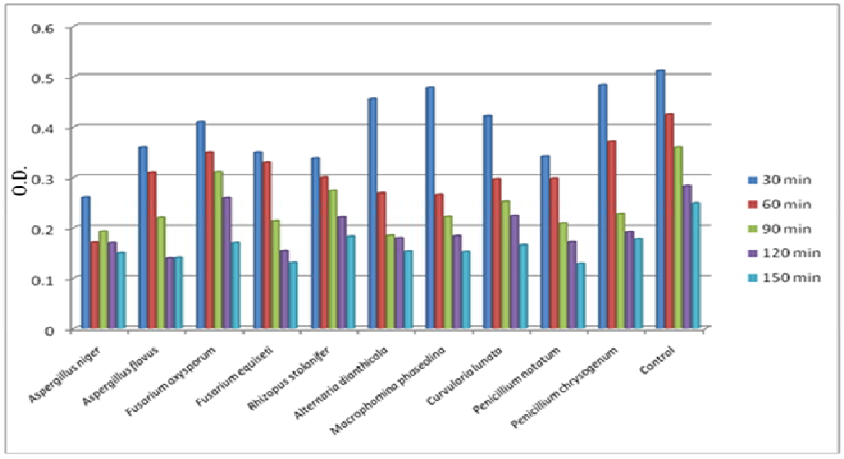
Graph 1 Effect of presoaking (3h) of oilseeds in fungal culture filtrate after incubation period of 15 days of storage fungi on percentage of seed germination after 48h



Graph 2 Effect of presoaking (3h) of oilseeds in fungal culture filtrate after incubation period of 15 days of storage fungi on percentage of seed germination after 72h



Graph 3 Effect of culture filtrate of storage fungi on total chlorophyll content



Graph 4 Effect of culture filtrate of storage fungi on pigment leaching of *Beta vulgaris*

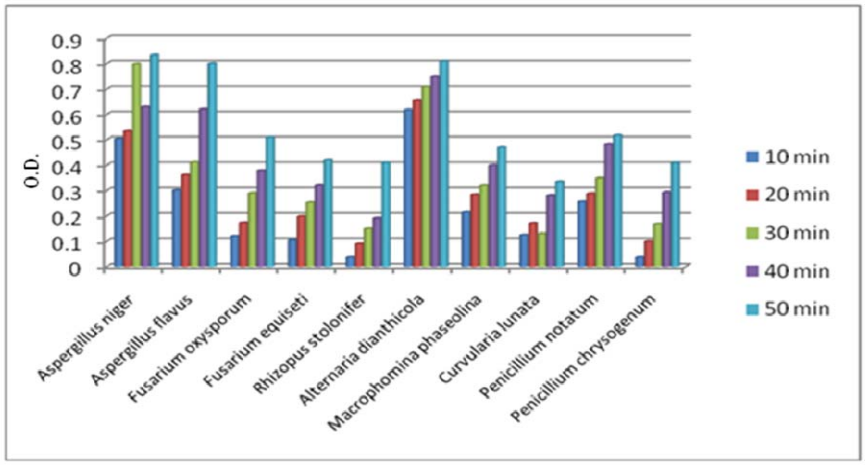


Fig1: Estimation of Chlorophyll from Spinach

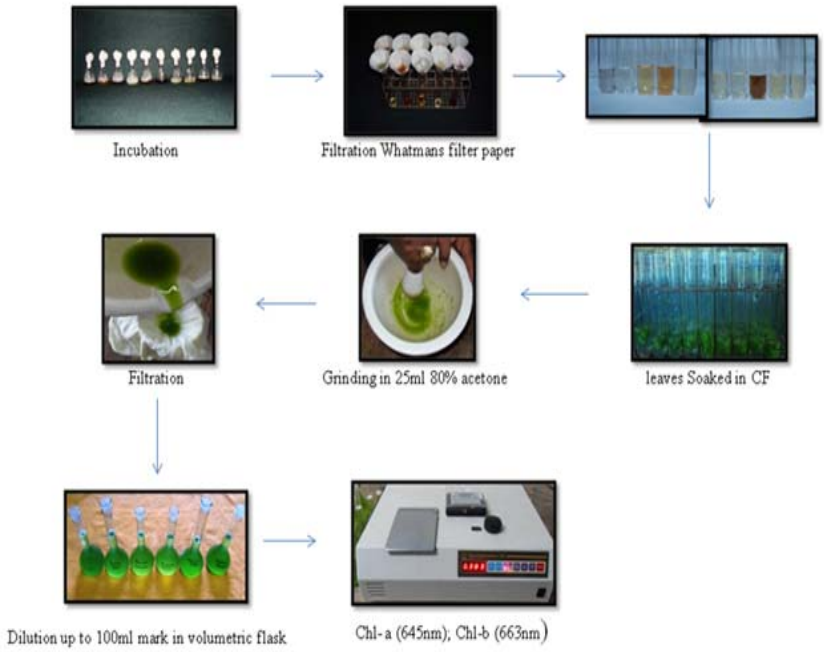
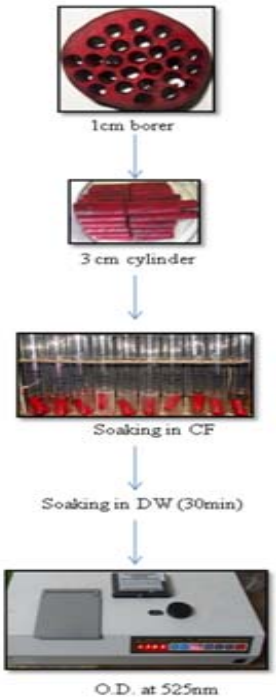


Fig. 2: Effect of culture filtrate of storage fungi on pigment leaching of *Beta vulgaris*



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