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Full Length Research Paper

Screening of systemic fungicides and biochemicals against seed borne mycoflora associated with *Momordica charantia*

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Study of seed borne fungi associated with bitter gourd seeds were conducted under *in vitro* condition in Department of Plant Pathology, Bahauddin Zakariya University, Multan, Pakistan. Two hundred (200) seed samples of *Momordica charantia* (bitter gourd) were collected from southern regions of Punjab province (Multan, Khanewal and Bahawalpur). Six fungal species were isolated out of which *Aspergillus flavus* showed highest percentage that is, 27.3% followed by *Rhizopus stolonifer* 17.98%, *Alternaria alternata* 13.34%, *Aspergillus niger* 5.23%, *Myrothecium roridum* 7.37% and *Fusarium solani* 6.69%. More number of fungi was observed by using blotter paper technique when compared with agar plate method. Of the three systemic fungicides used include ridomil gold MZ, bavistin, and score; and two low cost chemicals such as salicylic acid and boric acid. Ridomil gold MZ gave good results at all concentrations (20, 30 and 40 mg/10 ml) against all the isolated fungi compared with other fungicides. Salicyclic acid gave the best results against isolated fungi compared to boric acid.

Key words: *Myrothecium roridum*, bitter gourd, salicyclic acid, southern Punjab, bavistin, Pakistan.

INTRODUCTION

Cucurbits belong to the family Cucurbitaceae and consist of about 118 genera and 825 species, according to the last taxonomic treatment (Jeffery, 1990). Cucurbits are among the most important plants supplying humans with edible fruits and useful seeds. Plants of this family are very similar in above ground development, but they have high genetic diversity for fruit, shape and other characteristics, resulting in a variety of uses. The most important cultivated genera bitter gourd (*Momordica charantia* L.) is the summer vegetable grown extensively throughout the country and covers an area of about 5697 ha with an annual production of about 52099 tons in the country (Anonymous, 2005) which serve as the main source of nutrition, energy, valuable vitamins and minerals. All the cucurbits grown are subjected to various diseases, among which are fungal diseases such as powdery mildew caused by *Erysiphe cichoracearum* DC., downy mildew by *Pseudoperonospora cubensis* Berk. Curt, Fusarium wilt by *Fusarium oxysporum* f. sp. *Melonis Fom,* (muskmelon) seedling blights by *Pythium* spp. have been reported as some of the common diseases of cucurbits grown in Pakistan (Wahid et al., 1988).

Rath et al. (1990) worked on rotting of bitter gourd (*M. charantia* L) and analyzed 138 samples of fruits collected from the markets of Orissa in 1988 to 1989 out of which 15% showed fungal rots caused by *Aspergillus flavus* Link.ex Fr., *Aspergillus niger* Van.,Tiegh, *F. oxysporum* Schlechtex Fr, *Geotrichum candidum* Link, *Mucor* sp., *Rhizopus arrhizus* Fischer and an unidentified fungus.

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Nair (1982) analyzed the seeds of 8 cucurbitaceous vegetable crops and 11 fungal species were isolated. According to Hafiz (1986), 42 seed samples of summer vegetables, including cucumber, bottle gourd collected from different localities in Pakistan, have shown high percentage of fungal infestation, ranging between 57 and 67.5% and the figures were lower in the case of surface disinfected seed, 19.8 to 31.8%. Naseema et al. (1983) reported externally as well as internally seed borne fungi from the seeds collected from the stores. Fungi such as *A. flavus, A. niger* and *Rhizopus stolonifer* Ehrenb. ex Fr. Link. were the most common fungi associated with seeds of amaranthus, (*Amaranthus gengeticus* L.), okra, (*Abelmoschus esculentus* Moench), aubergine, (*Solanum melongena* L.), bitter gourd, (*M. charantia*), cowpea, (*vigna unguiculata* L.Walp), cucumber, (*Cucumis sativus* L), pumpkin, (*Cucurbita pepo* L), snake gourd, (*Trichosanthes anguina* L) and tomato, (*Lycopersicon lycopersicum* L. Karst). Palodhi and Sen (1983) reported the presence of *F. oxysporum, Fusarium moniliforme* Sheldon and *Fusarium solani* (Mart) App and WR on seeds of muskmelon, pumpkin and cucumber. Ali et al. (1988) from Pakistan isolated *Myrothecium roridurm* Tode ex Fr. up to 2.5% from seeds of bitter gourd and confirmed its pathogencity. Devi and Selvaraj (1994) studied germination of bitter gourd enhanced by soaking its seeds in a number of chemicals like, bavistin, boric acid, calcium hydroxide, calcium oxychloride, sodium dihydrogen phosphate, potassium dihydrogen phosphate and succinic acid. Keeping in view the importance of seed-borne diseases, this study was conducted with the following objectives: (1) to identify the mycoflora associated with bitter gourd seeds; (2) to determine the efficacy of different fungicides and non-hazardous chemicals against the seed borne mycoflora.

MATERIALS AND METHODS

Experimental study was carried out during 2009 to 2010 *in vitro* in the Laboratory Department of Plant Pathology, Bahauddin Zakariya University (BZU), Multan, to determine the number of fungi associated with the seeds of different cucurbit species and to find out impact of seed-borne fungi on seed health followed by their management with different fungicides and chemical methods.

Collection and preservation of seed samples

Seed samples of bitter gourd from three regions of southern Punjab including, Multan, Bahawalpur and Khanewal were collected in sterilized polythene bags and preserved at 4°C for long storage and at room temperature (25°C) for short periods unless processed. Seeds were generally untreated.

Pre-treatment of samples

The experiment was carried out in both the standard blotter and agar plate method. Seed samples, apparently showing higher symptoms of seed-born infection, were selected for this experiment. Seed samples (200 seed) were surface disinfected with (1%) sodium hypochlorite solution for about 2 min at room temperature (Mittal et al., 1999a). The main objective of surface disinfection was to eliminate contaminants and to detect internally seed-borne fungi. Seeds were rinsed thrice with sterilized distilled water before planting. Ten seeds were placed on blotters and in each Petri plate, containing 20 ml potato dextrose agar which were incubated at 25±2°C for 8 days under day and night fluorescent light. The seeds were examined 8 days after planting. The fungi with different characteristics were cultured on PDA medium. Confirmation was made by examining the cultures slides under compound microscope.

Isolation of fungi from seeds

Blotter paper method

Three layers of blotting paper (8 cm, diameter) were cut according to the size of Petri plate, placed at the bottom and moistened with water (ISTA, 1993). Superfluous water was drained off. Two hundred (200) seed samples were surface disinfected with (1%) Sodium hypochlorite solution for about 2 min at room temperature and placed in Petri plates (Mittal et al., 1999a). Plates were incubated at 25±2°C for eight days under alternating cycle of 12 h day and night fluorescent light. The main objective of the surface disinfection is to prevent the seed from saprophytic fungi and to detect internally seed-borne fungi.

Agar plate method

For agar plate method, potato dextrose agar (PDA) medium was prepared as follows: agar-agar (20 g), peeled potatoes (250 g), dextrose (20 g) and water (1000 ml). In each plate of 9 cm diameter, 20 ml of melted-sterilized medium was poured and solidified at room temperature in laminar flow. Ten surface disinfected seeds, after washing were placed in each plate in four replicates. The plates were incubated at 25±2°C for seven days with alternate light and dark 12 h cycle. Colonies of fungi and fungal species were examined regularly.

Effect on germination

Seed samples of bitter gourd from three regions (Multan, Khanewal and Bahawalpur) naturally infected with seed-borne fungi were tested through germination. Two hundred (200) seed samples from each region were placed separately on anchor brand paper (24 x 48 cm) in four rolls, each roll with 50 seeds. Papers were put in polyethylene bags and incubated at 25±2°C for 12 to 15 days. Moisture was provided to keep the papers wet. One set of experiment using health or pathogen which was free from seed-borne pathogen was considered as control and no pre-treatment was given to any seed sample in the case of control. After eight days, the rolled papers were unfolded and seedlings were examined individually for three categories: Normal seedling, non-germinated seeds and rotted seeds.

The fungi were examined under stereomicroscope on germinated and non-germinated seeds basis. Diseased portions of the seedlings was cut and plated on (PDA) to confirm the association of pathogens. Data regarding germination was recorded 16 days after placing. The results were recorded in percentage.

$$\text{Percentage frequency} = \frac{\text{No. of seeds infested}}{\text{Total no of seed plated}} \times 100$$

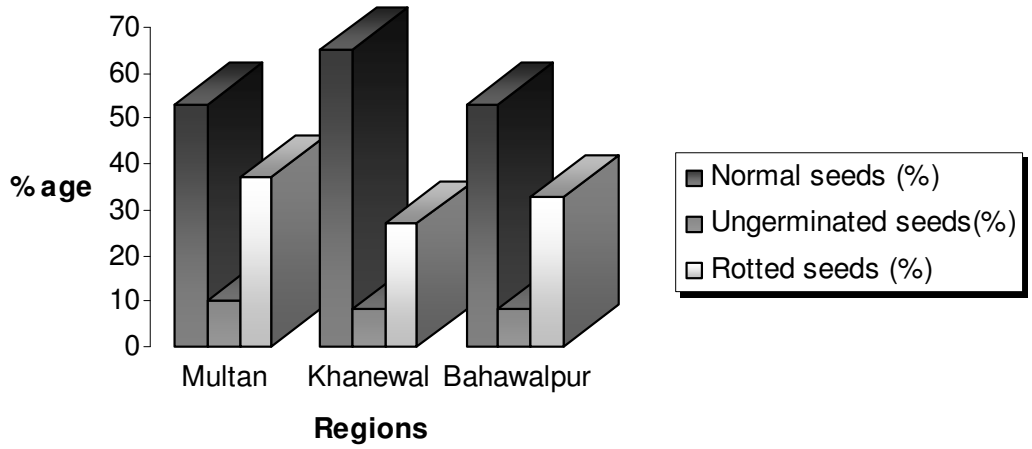

Figure 1. Germination percentage of bitter gourd.

Observation and identification of fungi

All Petri plates in both methods were examined under a stereomicroscope and the number of seeds infested and the fungal colonies developed were recorded as follows:

$$\text{Percentage frequency} = \frac{\text{No. of seeds infested}}{\text{Total no of seed plated}} \times 100$$

The fungi were identified on the basis of their typical structures and basic characters as suggested (Barnett, 1960; Melone and Masket, 1964). The frequency of each fungus was determined in the percentage from the colonies of all fungi developed.

***In vitro* management**

Soaking method

Soaking method following Gangopadyay and Kapoor (1977) was used with some modifications. Two hundred (200) seeds of bitter gourd were soaked in different concentrations of chemicals (Salicylic acid and boric acid) and fungicides (Ridomal gold, bavistin and score) and left for 1 h to enable the seeds to absorb the fungicides. After treatment, seeds were air-dried for 30 min and analyzed for their efficacy against seed-borne mycoflora following standard blotter and agar plate methods.

Chemical seed treatment

Three fungicides namely ridomil gold, bavistin and score and two chemicals salicylic acid and boric acid with different concentrations were used in this experiment. Ridomil gold (metalaxyl+mancozeb) 20-30-40 mg/10 ml, bavistin (Carbendazim) 20-30-40 mg/10 ml, score (difenoconazole) 5-6-7 µl/10 ml, disprin (salicylic acid) 20-30-40 mg/10 ml, and borax (boric acid) 20-30-40 mg/10 ml. A total of 200 seeds of bitter gourd, naturally infected with important seed-borne fungi were treated individually with the fungicides at 20, 30, 40 mg/10 ml and 5, 6, 7 µl/10 ml, respectively and chemicals with 20, 30, 40 mg/10 ml. Ten seeds were plated in each Petri plate.

Experiments were conducted in quadruplicate with 50 seeds in each plate. Standard blotter paper method was used. Incubation of treated seed was carried out at 25±2°C for 8 days. Two hundred (200) seeds were also plated on blotter without any treatment of fungicide to serve as control. Seeds were examined under stereoscopic microscope and fungi were identified based on habit characters on seeds and colony characters on blotter around the seeds. Result was expressed in percentage and data analyzed statistically.

Statistical analysis

All the analysis was done using sigmaplot 12. systat software Inc. and Microsoft Excel 2003.

RESULTS AND DISCUSSION

A total of six fungal species including *A. alternata*, *M. roridum*, *F. solani*, *Rhizopus* sp., *A. niger* and *A. flavus* Link ex Gray, were isolated from the seeds of *M. charantia.* Out of the three regions, Khanewal showed the highest percentage of normal seeds followed by Multan and Bahawalpur (Figure 1). Of the six fungal species isolated, *A. flavus* was the most prevalent fungus (26.39 to 27.31%) followed by *Rhizopus* sp. (16.69 to 17.98%), *A. alternata* Nees, (12.67 to 13.34%), *A. niger* (8.14 to 9.23%), *M. roridum* (5.98 to 7.37%) and *F. solani* (5.31 to 6.69%) on blotter paper (Figure 2). In the case of PDA, the percentage of isolated fungi decreased when compared with the blotter paper method such as *A. flavus* (16.19 to 19.43%) followed by *Rhizopus* sp. (10.39 to 13.69%), *A. alternata* (5.12 to 9.12%), *A. niger* (6.23 to 7.21%), *F. solani* (4.60 to 6.23%) and *M. roridum* (4.65 to 5.96%) (Figure 3). It was concluded that *A. flavus* showed the highest percentage followed by *Rhizopus* spp., *A. alternata, A. niger, M. roridum* and *F. solani* (Figure 4).

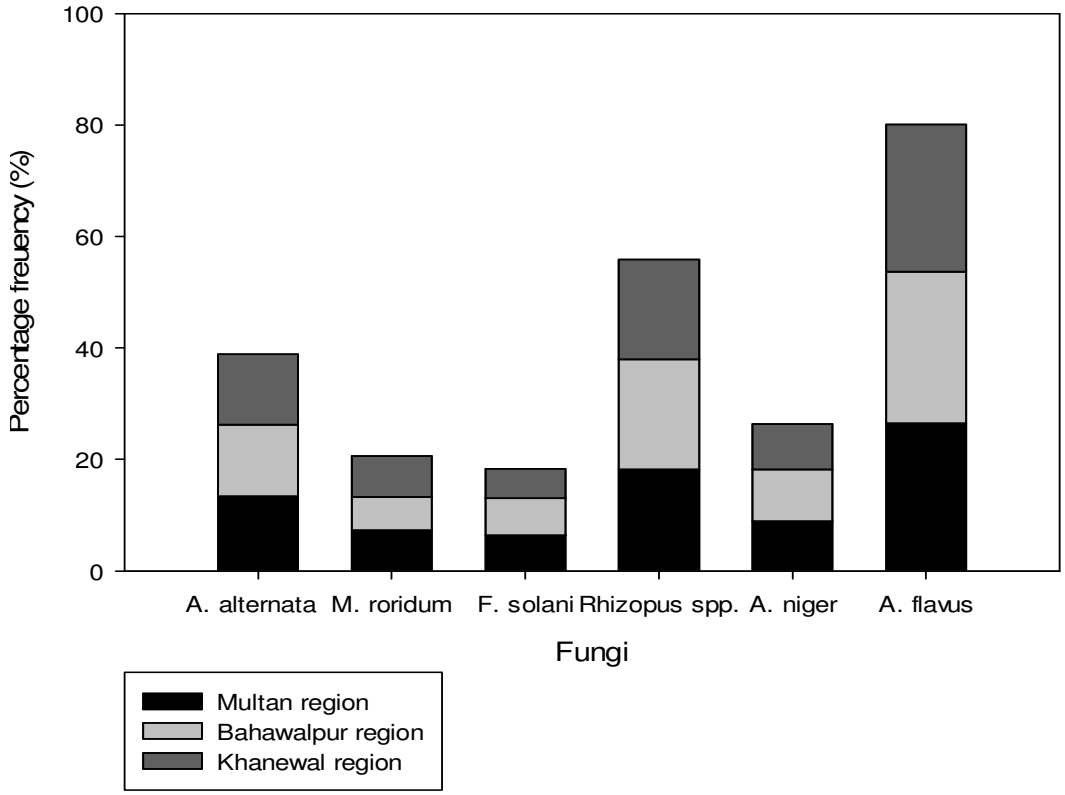

Figure 2. Percentage frequency of seed borne fungi associated with seeds of *M. charantia* by blotter paper method.

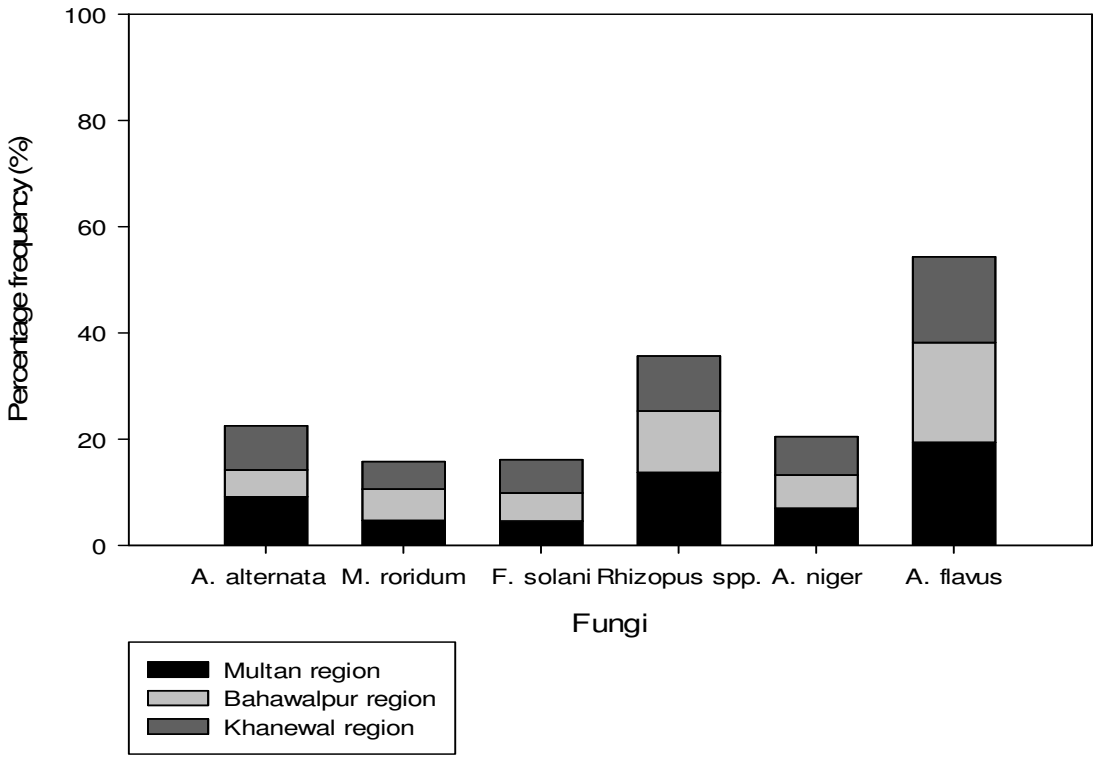

Figure 3. Percentage frequency of seed borne fungi associated with seeds of *M. charantia* by PDA method.

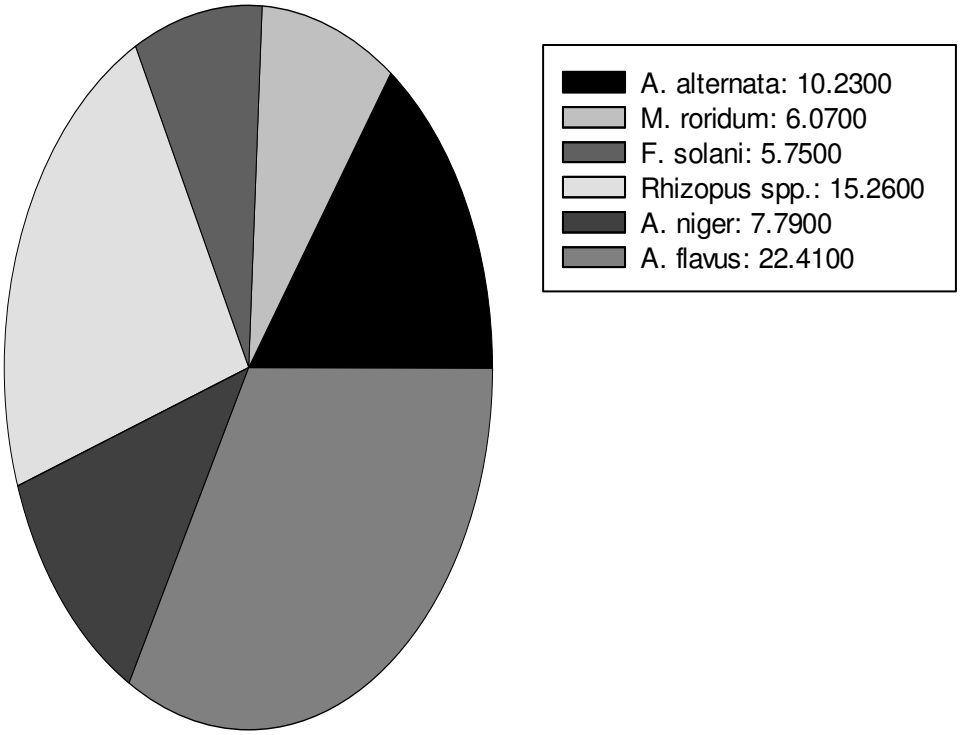

Figure 4. Mean frequency of seed borne fungi associated with *M. charantia* seeds.

High frequencies of *A. flavus* and *A. niger* on bitter gourd seeds results in low germination of bitter gourd seeds that is similar to the findings of Christensen (1967) and Shakir and Mirza (1992). This study showed that *Aspergillus* sp. and *Rhizopus* sp. were isolated from bitter gourd seed and it is similar to the findings of Sultana and Ghaffar (2007). *Myrothecium* was found in close association with non-germinated and rotted seeds (Sultana and Ghaffar, 2007). *M. roridum* found more pathogenic fungi that cause leaf spot (Shauket et al., 1988), blight (Ali et al., 1988) also repor-ted as crown rot pathogen (Chase, 1983) and fruit rot pa-thogen (Sharma and Bharghava, 1978). Sheikh (1990) and Shakir and Mirza (1992) reported the association of *M. roridum* with cucurbits seeds.

In this study, the maximum number of fungi was isolated by using blotter paper method compared with other methods. These results is similar to the findings of Khan et al. (1988) detection of seed borne fungi in cotton and sunflower (Dawar, 1994; Gowdar et al., 2007) and is contrary to the findings of other workers (Neergard, 1977; AlKassim, 1996; AlKassim and Monawar, 2000). Blotter pa-per method was found suitable for the detection of seed borne fungi of cucurbits (Begum and Momin, 2000). It was observed that the incidence of *Aspergillus* sp. and *Rhizopus* sp. are lowered by disinfecting the seeds with 2% $NaOCl_2$ and this is similar to the results of Sultana and Ghaffar (2007). Seeds of Multan region showed the highest percentage of fungi that is why soaking method was applied on seeds of Multan region.

Effect of seed dressing fungicides and chemicals on seed borne fungi of bitter gourd

Bitter gourd seeds were treated with three fungicides that is, ridomil gold, bavistin and score and two chemicals, salicylic acid and boric acid, to determine their effect on seed-borne fungi. The seed sample was naturally infected with six seed-borne fungi such as *A. alternata, M. roridum, F. solani, Rhizopus* spp. *A. niger* and *A. flavus.*

The seeds were treated individually with the fungicides at different doses at 20, 30, 40 mg/10 ml and 5, 6, 7 µl/10 ml of water, respectively and chemicals with 20, 30, 40 mg/10 ml of water. All these fungicides and chemicals significantly reduced the population of all fungi present in naturally infected seed samples at different doses at 20, 30, 40 mg/10 ml and 5, 6, 7 µl/10 ml, respectively. But ridomil gold controlled almost all the pathogens at the dose of 30 and 40 mg/10 ml. Only *Rhizopus* spp. 1% and *A. flavus* 1.5% frequency could survive after treatment at 30 mg/10 ml. But there were no pathogens at 40 mg/10 ml (Table 1). Bavistin controlled all the fungi at 40 mg/10 ml but not at 20 mg/10 ml and 30 mg/10 ml as effectively as ridomil gold and this study is contrary to

Table 1. Effect of seed dressing fungicides and chemicals on seed borne fungi of bitter gourd.

Fungicide	Untreated seed control	%	20 mg or 5 ul/ml*	%	30 mg or 6 ug/ml*	%	40 mg or 7 ug/ml*	%
RidomilGold	*Alternaria alternata*	9.12	*Alternaria alternata*	0	*Alternaria alternata*	0	*Alternaria alternata*	0
	Myrothecium roridum	4.65	*Myrothecium roridum*	2	*Myrothecium roridum*	0	*Myrothecium roridum*	0
	Fasuarim solani	4.6	*Fasuarim solani*	1	*Fasuarim solani*	0	*Fasuarim solani*	0
	Rhizopus spp.	13.69	*Rhizopus spp.*	2	*Rhizopus spp.*	1	*Rhizopus spp.*	0
	Aspergillus niger	6.99	*Aspergillus niger*	0	*Aspergillus niger*	0	*Aspergillus niger*	0
	Aspergillus flavus	19.43	*Aspergillus flavus*	4.5	*Aspergillus flavus*	1.5	*Aspergillus flavus*	0
Bavistin	*Alternaria alternata*	9.12	*Alternaria alternata*	3.5	*Alternaria alternata*	2	*Alternaria alternata*	0
	Myrothecium roridum	4.65	*Myrothecium roridum*	1.5	*Myrothecium roridum*	0	*Myrothecium roridum*	0
	Fasuarim solani	4.6	*Fasuarim solani*	3	*Fasuarim solani*	2	*Fasuarim solani*	0
	Rhizopus spp.	13.69	*Rhizopus spp.*	3.75	*Rhizopus spp.*	2.25	*Rhizopus spp.*	0
	Aspergillus niger	6.99	*Aspergillus niger*	2.75	*Aspergillus niger*	1.75	*Aspergillus niger*	0
	Aspergillus flavus	19.43	*Aspergillus flavus*	8.5	*Aspergillus flavus*	4.5	*Aspergillus flavus*	0
Score* (liquid form)	*Alternaria alternata*	9.12	*Alternaria alternata*	2.3	*Alternaria alternata*	1	*Alternaria alternata*	0
	Myrothecium roridum	4.65	*Myrothecium roridum*	3	*Myrothecium roridum*	0	*Myrothecium roridum*	0
	Fasuarim solani	4.6	*Fasuarim solani*	4	*Fasuarim solani*	1.75	*Fasuarim solani*	0
	Rhizopus spp.	13.69	*Rhizopus spp.*	3.5	*Rhizopus spp.*	2	*Rhizopus spp.*	0
	Aspergillus niger	6.99	*Aspergillus niger*	2	*Aspergillus niger*	0	*Aspergillus niger*	0
	Aspergillus flavus	19.43	*Aspergillus flavus*	7.5	*Aspergillus flavus*	2.5	*Aspergillus flavus*	0
Chemical	**Untreated seed control**	**%**	**20 mg/ml**	**%**	**30 mg/ml**	**%**	**40 mg/ml**	**%**
Salicylic acid	*Alternaria alternata*	9.12	*Alternaria alternata*	3.5	*Alternaria alternata*	2.75	*Alternaria alternata*	0
	Myrothecium roridum	4.65	*Myrothecium roridum*	2.5	*Myrothecium roridum*	2	*Myrothecium roridum*	0
	Fasuarim solani	4.6	*Fasuarim solani*	3.25	*Fasuarim solani*	0	*Fasuarim solani*	0
	Rhizopus spp.	13.69	*Rhizopus* spp.	5	*Rhizopus* spp.	0	*Rhizopus* spp.	0
	Aspergillus niger	6.99	*Aspergillus niger*	3	*Aspergillus niger*	0	*Aspergillus niger*	0
	Aspergillus flavus	19.43	*Aspergillus flavus*	8	*Aspergillus flavus*	3.25	*Aspergillus flavus*	0
Boric acid	*Alternaria alternata*	9.12	*Alternaria alternata*	4	*Alternaria alternata*	3	*Alternaria alternata*	1.3
	Myrothecium roridum	4.65	*Myrothecium roridum*	3.25	*Myrothecium roridum*	0	*Myrothecium roridum*	0
	Fasuarim solani	4.6	*Fasuarim solani*	4	*Fasuarim solani*	2.5	*Fasuarim solani*	1
	Rhizopus spp.	13.69	*Rhizopus* spp.	4	*Rhizopus* spp.	1.25	*Rhizopus* spp.	0
	Aspergillus niger	6.99	*Aspergillus niger*	3.25	*Aspergillus niger*	0	*Aspergillus niger*	0
	Aspergillus flavus	19.43	*Aspergillus flavus*	9	*Aspergillus flavus*	4.5	*Aspergillus flavus*	1.8

the findings of (Ibiam et al., 2000, 2006) on rice seeds. According to Ibiam et al. (2000 and 2006), bavistin was also effective at 30 mg/10 ml. Score ranks second among the fungicides to control fungi; it was also not effective at 20 and 30 mg/10 ml, but control all fungi at 40 mg/10 ml. All the fungicides gave good result at 40 mg/10 ml. *In vitro* rice seed treatment with bavistin decreased the mortality of seedlings and also controlled the seed rot problem (Narmada and Kang, 1992). Ibiam et al. (2006) stated that systemic fungicides either inactivate or killed the pathogen in the seeds or seedlings as the germination of seed starts. The metabolic activities of fungi could not be arrested at lower concentrations of fungicides; it may be due to the fact that fungicides are unable to destroy few fungi at lower concentrations. As the concentration of fungicides increased, the metabolic activities of the fungi were completely destroyed. Many systemic fungicides had been checked for their efficacy to control diseases in safflower that is caused by *F. oxysporum* (Chakarbarti and Basuchaudhary, 1980). Morshed (1995), conducted experiments on *Phaseolus vulgaris* and reported the efficiency of bavistin on seed borne fungi like *Fusarium* sp. and *Alternaria* sp. The findings of this study showed similar observations with respect to the effect of bavistin on the reduction of mycoflora that was given by (Sharma et al., 1996).

In chemical application, salicylic acid controlled almost all the pathogen at dose of 30 and 40 mg/10ml. Only *A. alternata, M. roridum* and *A. flavus* with 2.75, 2 and 3.25%, respectively could survive after treatment with salicyclic acid at 30 mg/10 ml. But boric acid was found less effective at 30 mg/10 ml compared with salicylic acid. There were no pathogens at 40 mg/10 ml. Both the chemicals gave good result at 40 mg/10 ml. Salicyclic acid was found to be more effective in controlling the fungi compared with boric acid. Raskin (1992), considered salicyclic acid as the plant hormone and found it to be helpful in reducing the damages caused by fungi, bacteria and viruses. According to Nie (2006), salicyclic acid is an important factor in systemic acquired resistance against fungi, bacteria and viruses. According to Yalpani et al. (1991) in induced cucumber and tobacco plants, salicylic acid played a critical role in the conventional systemic acquired resistance. Some workers focused on the interactions between plants and virulent or avirulent pathogens while studying the role of salicylic acid (Conti et al., 1996). When salicylic acid was applied on the surface of tobacco plants it result in reduced blue mold disease both in greenhouse assays and in the micro-titer plate assays (Zhang et al., 2002).

Of the three systemic fungicides and two chemicals used, ridomil gold MZ gave the best result and boric acid showed minimum control and it is contrary to the findings of (Somani, 1988; Jalali and Mehta, 1994) that boric acid showed best result compared with the organomercurial compounds and systemic fungicides when applied to potato seed against black scurf of potato. This study show that a combination of different chemicals reduced the seed borne incidence of *Fusarium* sp. and *Alternaria* sp., and is similar to the results given by (Agarwal et al., 1977).

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