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ISOLATION OF SEED-BORNE AND SEED ASSOCIATED FUNGI OF *Lablab purpureus* (L.) SWEET AND THEIR BIOLOGICAL CONTROL

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ABSTRACT

Lablab purpureus (L.) Sweet is one of the most important and nutritious vegetables as well as pulse in Bangladesh and is grown extensively all over the country. It suffers from various fungal infections, which reduce greatly its quality and quantity. Seed-borne and seed associated fungi are one of the factors of substantial damages of the bean genotypes. Seed- borne and seed associated fungi of eleven genotypes of *L. purpureus* (L.) Sweet and their various controlling methods were studied. From eleven bean seed samples, seven types of fungal pathogens were isolated and identified. The most predominant fungi species were *Aspergillus* spp. followed by *Fusarium* sp. and *Rhizopus* sp. Comparatively less frequent fungi were *Penicillium* sp., *Curvularia* sp., *Colletotrichum* sp. and *Alternaria* sp. Plant extracts (*Lawsonia inermis*, *Azadirachta indica* and *Allium sativum*), cow urine, hot water and chemical fungicides (Bavistin, redomil and dithane M-45) were used to observe the efficacy of them against different species of fungi and their effect on germination rate and vigour index of bean seeds. Among the controlling measures, considering fungal infection controlling capacity, germination rate, vigour index as well as cheap, easy, environment friendly, easily available and easily applicable controlling measures, *Azadirachta indica* leaf extract was the best. Genotype GBLB-6, GBLB-11 and GBLB-13 showed more better performance through all the controlling measures and these genotypes may be used in the breeding program for their higher germination rate, higher vigour index and comparatively lower susceptibility to fungal pathogen.

Keywords: *L. purpureus* (L.) Sweet , fungal pathogen , plant extract, biological control

INTRODUCTION

Bangladesh is an agricultural country where 85% of the total population depends upon agriculture. The vegetables and pulses are important crop in our country. *L. purpureus* (L.) Sweet is one of the most important and nutritious vegetables as well as pulse in Bangladesh and is grown extensively all over the country. *L. purpureus* (L.) Sweet belongs to the family Fabaceae (formerly Leguminosae). Commonly it is called Country bean, Lablab bean, Hyacinth bean, Butter bean, Dolichos bean, Sim bean etc. Currently Lablab is one of the major leguminous forage and green manure crop in this area of the world (**Cameron, 1988**). *L. purpureus* (L.) Sweet has been widely distributed to many tropical and sub-tropical countries where it has become naturalized. In South and Central America, East and West Indies, Asia, China and India Lablab is grown as an annual or short-lived perennial vegetables. Country bean contains 20-30% protein on a dry seed, which is nearly three times than in most cereals. Besides this bean contain vitamin A, vitamin C, riboflavin and mineral like magnesium, calcium, phosphorus, potassium, iron, sulphur and sodium (**Jukanti *et al*, 2012**, **Deka and Sarkar, 1990**). Apart from *L. purpureus* (L.) Sweet provide significant nutritional and health benefits, and are known to reduce several non-communicable diseases such as colon cancer and cardiovascular diseases (**Yude *et al*,1993; Jukanti *et al*,2012**). In addition to, the use of phosphate solubilizing bacteria for instance, *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate (P) solubilizers, as inoculants simultaneously increases P uptake by the plant and thus crop yields (**Khan *et al*, 2009**). For this, the richness of legumes in N and P makes them attractive for insect pests and diseases **(Sinclair and Vadez, 2012)**. More over the natural action of converting atmospheric nitrogen into forms available for the plant-animal-soil system improves productivity is an inexpensive, environmentally friendly manner. Usually 15-40 kg nitrogen is fixed for each 1000 kg dry matter of shoots grown (**Humphreys, 1995**).

Seed is the basic unit in crop production. Seeds play a vital role in associating microorganisms, which prove hazardous for the seed or the new plant product from it. The associated microorganisms may be pathogenic, weak parasite or saprophytes, they may be associated internally or externally with the seed or as concomitant contamination as sclerotia, galls, fungal bodies, infected plant parts, soil particles etc. are mixed with the seed. The seed-borne as well as seed associated fungal infection can be effectively reduced if the seeds are treated by fungicide before sowing. But the indiscriminate use of the fungicide has been cautioned as some of these are found to have various harmful residual effects on the surrounding environment (**Beye, 1978**). Further due to the development of new physiological race of pathogen, many of the synthetic fungicides are gradually becoming ineffective (**Wellman, 1977**).

In recent year, some research on the fungal toxicity of extracts of various parts of higher plant have indicated the possibility of their exploitation as natural fungal toxicant for controlling plant diseases (**Misra and Dixit, 1977**; **Naidu and John, 1981**; **Agrawal and Rai, 1984; Anwar *et al*. 1994**). Plant extracts are cheap, can be easily prepared and used whenever required. In fact some workers have already demonstrated the successful use of crop plant (**Singh *et al*. 1990; Gupta, 1997**). *Lawsonia inermis*, *Azadirachta indica* and *Allium sativum* may hinder the pathogenic growth, which has been claimed by some worker. Some scientist reported the antimicrobial, antibacterial and anti-fungal properties of leaf extracts of *Lawsonia inermis* (**Malekzadeh and Shabestari, 1989**). Animal byproduct such as cow urine (**Basak and Lee 2001b**) and physical treatment such as dipping the seeds into hot water are also used to protect seed-borne and seed associated microorganisms (**Lambat *et al*. 1974**).

The aim of our study was to isolate the seed-borne and seed associated fungi of Country bean and evaluate the efficacy of some plant extracts with some treatments like cow urine treatment and hot water treatment as fungal toxicants against the existing fungi and to assess the extent of phytotoxicity if any of them with good fungitoxic effect on germination and vigour index.

MATERIAL AND METHODS

Sample collection

In this study, eleven genotypes of *L. purpureus* (L.) Sweet were collected from different districts from northern region in Bangladesh. Then the seed samples were labeled properly and named as (Genetics and breeding laboratory-2), GBLB-2, GBLB-3, GBLB-5, GBLB-6, GBLB-7, GBLB-9, GBLB-11, GBLB-12, GBLB-13, GBLG-14 and GBLB-15 with date for future reference.

Seed test

For seed test, dry examination, blotter method and agar plate method were applied for seed health testing.

Identification of fungal colony

Identification of fungal colony was made microscopically. The generic identification of each colony was recorded and identification up to species level was tried wherever possible with the help of standard Mycological book and manual (**Gilman, 1971; Ellis, 1971 and Alexopoulos, 1979**).

Plant extracts preparation for treatment

Extractions of plant bulbs and leaf tissues in water and alcohol used as fungicides were done the method with some modifications which are described by **Mohadevan and Sridhar (1982)**. At first five gram tissues were cut into pieces and immediately plunged in water in a beaker and allowed to boil for 5 minutes using 5-10 ml of alcohol for every gram tissue. Then the extraction was done on top of a stem bath. The extraction was cooled in a pan of cold water. After that, the tissues were crushed thoroughly in a motor with a pestle and then passed through two layer of cheese cloth and re-extracted the ground tissues for 3 minutes in distilled water, using 2-3 ml of water for every gm of tissue. The second extraction ensured complete removal of alcohol soluble substance. Cooled and passed through cheese cloth. Cooled both extracts and filtered through Whatman's No 1 filter paper. The volume of the extract was evaporated on a stem bath to dryness. Two hundred ml distilled water was added for 5 gm of tissue and prepared 2.5 % plant extracts. Similarly 1.5% and 2.0% of plant extracts were made to test the efficacy in different concentration. Evaluation of different percentage of plant extracts was done in the laboratory by the blotter method. The seeds were dipped 5 minutes in different concentrations of plant extracts for treatment (**Alam *et al*. 2002**).

Cow urine treatment

Different concentrations of cow urine solution in combination with sterile distilled water were (30:70, 45:55, 60:40) used for the control of seed-borne as well as seed associated fungi on different genotypes of country bean. 30, 45, and 60 ml of cow urine (P^H 9.20) diluted with sterilized distilled water for the preparation of 30:70, 45:55, 60:40 solutions according to **Basak and Lee (2001b**). The seeds were dipped 1 hour in different concentrations of cow urine.

Hot water treatment

For hot water treatment, seeds were surface sterilized by distilled water and this were taken in sterilized test tube with three replicated. Then test tubes were immersed in water bath of 50º C and treated at different time (15 min., 30 min., 45 min. and 60 min.) with control. The treated seeds were removed from the water bath and placed in autoclaved petridishes, which were made moisture chamber by using blotting paper soaked with sterilized distilled water. After 7 days of incubation in 25 ºC temperature all the petridishes were examined, is there any growth of fungi or not.

Chemical treatment

Three chemical fungicides Bavistin DF (Methyl-2-benzimidazole carbamate), Redomil MZ [(Complex of methyl-D) L-N (2, 6-Dimethyl Phenyl) -N (2-Methobis-acetyl alaninite, Zinc salt and polymeric Manganese ethylene bis (Di-thiocarbamate)] and Dithane M-45 (Manganese ethylene bisdithio carbamate plus zinc) were used. The seeds were treated with these chemicals at the rate of 0.25 percent according to **Bkar (1988**) and Jain and **Khare (1972**). Seeds were taken in 100 ml conical flask and required amount of fungicide was added to it. The mixture was then shaken for 15 min. in a mechanical shaker. After 5 min. of treatment, the treated seeds were used for studying the efficacy of the fungicides.

RESULTS AND DISCUSSION

In this experiment, seed borne and seed associated fungal pathogen of eleven genotypes of *L. purpureus* (L.) Sweet was studied and their various controlling methods were applied. From eleven bean seed samples seven types of fungal pathogens were isolated and identified. These were *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Curvularia* sp., *Rhizopus* sp., *Colletotrichum* sp. and *Alternaria* sp. The most predominant fungi species was *Aspergillus* sp. followed by *Fusarium* sp. and *Rhizopus* sp. Results are shown in the Tab 1, figure-A, B and C.

Saprophytic invasion of species *Aspergillus* and *Penicillium* have great potentiality to destroy embryos of the seed and thus reduce seed germination. So, it is not always the pathogenic seed borne fungi, which is responsible to reduce seed germination. Similar observations were followed by (**Neergaard, 1980; Reddy and Khare, 1978; Khare *et al*. 1988; Christensen, 1973**).

Plant extracts, Cow urine, hot water and chemical fungicides were applied to observe the efficacy of them against different species of fungi. The influence of seed germination rate and vigour index was also studied through treatment. In the three plant extracts (mehndi leaf, neem leaf and garlic bulb) treatments among the three concentrations the 2.5% concentration was the best because in this concentration the fungal infections were controlled significantly and expected seed germination rate as well as vigour index were observed higher. Considering germination rate, vigor index and percentage of fungal infection altogether in three concentrations of the three plant extracts, genotype GBLB-6, GBLB-11 and GBLB-13 showed better performance than others.

In Mehendi, Neem leaf and garlic bulb extract treatment it was found that treated seed in 2.5% (99.5±0.4; 98.5±0.7; 97.9±0.7) on the other hand 2.0% (99.2±4; 98.9±0.5; 99.6±0.2) concentration showed good result on seed germination respectively. The 2.5% concentration showed successful control of fungal infection (0.00-9.90%; 0.00-6.60%; 0.00-9.90%) with higher vigour index (1196.8±74.2; 1602.4±131.2; 1243.7±103.0) respectivly (Tab-2, 4). Some scientist reported the antimicrobial, antibacterial and anti-fungal properties of leaf extracts of Mehendi (**Malekzadeh and Shabestari, 1989 and Rosenberg, 1999**). **Khan and Kumar (1990)** conducted an experiment to find the antifungal activity of leaf extracts of Neem (*A. indica*) on wheat seeds mycoflora, using different dilutions. They reported that, treatment caused a marked reduction in seed mycoflora and enhanced seed germination. In Bangladesh **Hasan *et al*. (2005)** conducted a research on antifungal effects of ten plant extracts on wheat seed germination and among all of them Neem (*A. indica*) leaf extracts showed best result which one similar to our result**.** Moreover, **Suratuzzaman (1995)** performed an experiment to control seed-borne *Collectotrichum dematium var. truncatum*, *Macrophomina phaseolina* and *Cercospora kikuchii* of Soybean seed and Garlic and Ginger extracts, gave excellent control of the pathogens.

Among different concentrations in Cow urine treatment (Table-2) the 45% concentration showed reduction of fungal infection (6.60-23.10%) with higher average vigour index (1342.8±96.3) (Table-4). In 60% concentration though the fungal infection (3.30±9.90) was controlled successfully, the average germination rate (68.7±1.7) and average vigour index (295.0±32.6) were reduced remarkably (Table-4). **Basak and Lee (200lb)** performed an experiment by using cow urine for controlling Fusarium wilt caused by *Fusarium oxysporum*. **Sankaranarayanan *et al*. 1994** found that Tamarind seeds have a hard seed coat that causes slow and poor germination. Soaking the seeds in 10% cow urine or in cowdung solution (500 g in 10 litres of water) for 24 h increased the germination percentage from 37% (untreated controls) to 72.6 and 82.8%, respectively.

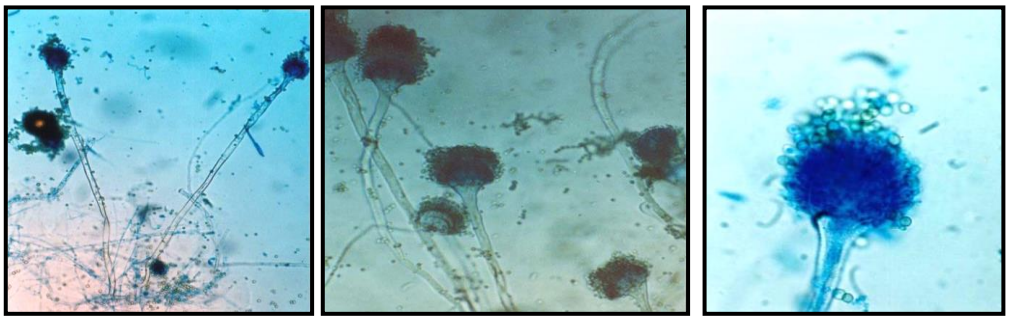
Table 1 Abundance of external and internal seed associated fungi of eleven genotypes of Country bean.

Name of the genotype	Types of observation	Number of seeds	Number of fungal colony	Name and colony number of isolated fungi							% Of abundance
				Aspergillus	*Penicillium*	Fusarium	*Curvularia*	*Rhizopus*	*Colletotrichum*	*Alternaria*	
GBLB-2	External	03	10	3	1	2	0	2	1	1	333.33
	Internal	03	14	5	2	3	1	2	1	0	466.66
GBLB-3	External	03	7	3	0	2	0	2	0	0	233.33
	Internal	03	16	6	2	4	1	3	0	0	533.33
GBLB-5	External	03	10	3	2	2	1	2	0	0	333.33
	Internal	03	12	3	0	4	0	2	1	2	400.00
GBLB-6	External	03	7	2	0	0	0	4	1	0	233.33
	Internal	03	8	1	0	2	0	4	0	1	266.66
GBLB-7	External	03	7	2	2	1	2	0	0	0	233.33
	Internal	03	15	5	2	3	2	0	2	0	500.00
GBLB-9	External	03	14	4	3	3	1	1	2	0	466.66
	Internal	03	13	3	1	3	2	2	0	2	433.33
GBLB-11	External	03	5	0	2	2	0	0	1	0	166.66
	Internal	03	6	1	0	1	0	2	1	1	200.00
GBLB-12	External	03	6	2	1	1	1	0	0	1	200.00
	Internal	03	12	4	3	2	0	2	0	1	400.00
GBLB-13	External	03	4	2	0	1	0	1	0	0	133.33
	Internal	03	12	3	0	2	2	1	1	3	400.00
GBLB-14	External	03	14	5	1	2	2	3	0	1	466.66
	Internal	03	16	6	0	4	2	2	2	0	533.33
GBLB-15	External	03	8	0	0	3	0	3	0	2	266.66
	Internal	03	13	7	2	0	2	0	1	1	433.33
	Total	**66**	**228**	**70**	**24**	**47**	**19**	**38**	**14**	**16**	-

Table 2 Percentage of fungi infected seed after different treatment with control of eleven genotypes of Country bean

Genotypes	Control	Treatment					
		Plant Extracts			Animal byproduct	Physical treatment	Chemical treatment
		Mehendi Leaf	Neem leaf	Garlic bulb			
GBLB-2	62.70	3.30	3.30	6.60	23.10	9.90	0.00
GBLB-3	62.70	6.60	6.60	0.00	19.80	9.90	0.00
GBLB-5	56.10	3.30	6.60	6.60	19.80	9.90	0.00
GBLB-6	29.70	0.00	6.60	0.00	13.20	13.20	0.00
GBLB-7	46.20	9.90	3.30	3.30	13.20	6.60	0.00
GBLB-9	33.00	3.30	3.30	3.30	19.80	13.20	0.00
GBLB-11	49.50	3.30	0.00	0.00	16.50	9.90	0.00
GBLB-12	62.70	6.60	6.60	3.30	9.90	3.30	0.00
GBLB-13	33.00	0.00	0.00	0.00	6.60	3.30	0.00
GBLB-14	33.00	3.30	3.30	9.90	9.90	9.90	0.00
GBLB-15	56.10	3.30	3.30	0.00	9.90	13.20	0.00

Legend: GBLB– Genetics and breeding laboratory, Chemical treatment (Bavistin, dithane M-45, redomil), Physical treatment (Hot water), Animal byproduct(cow urine)



Ai **A ii** **Aiii**

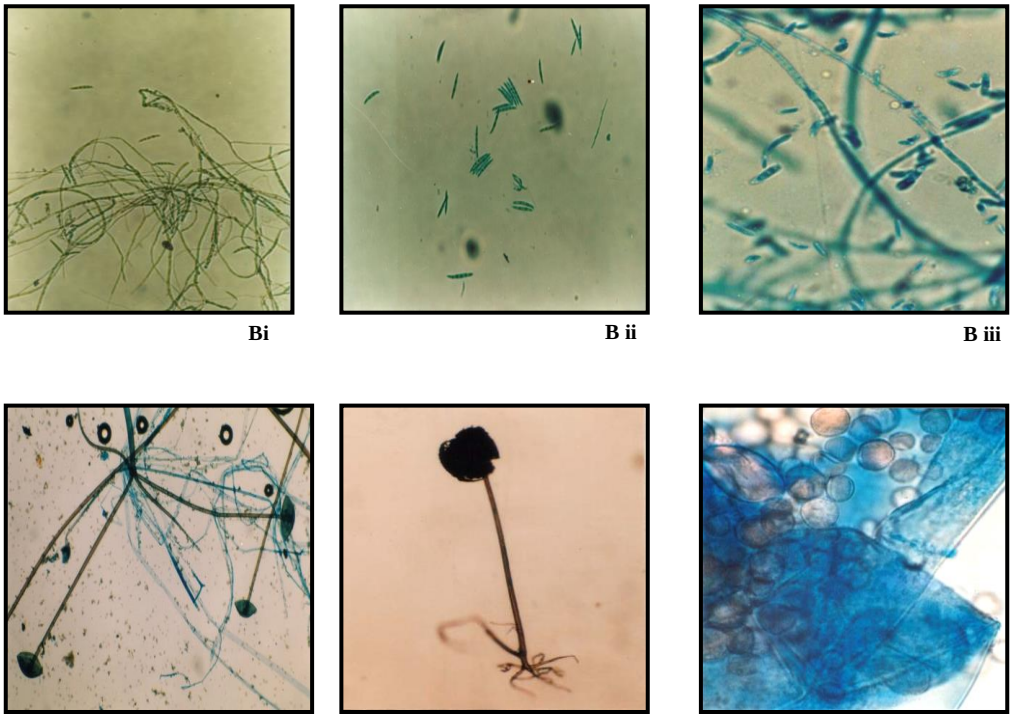
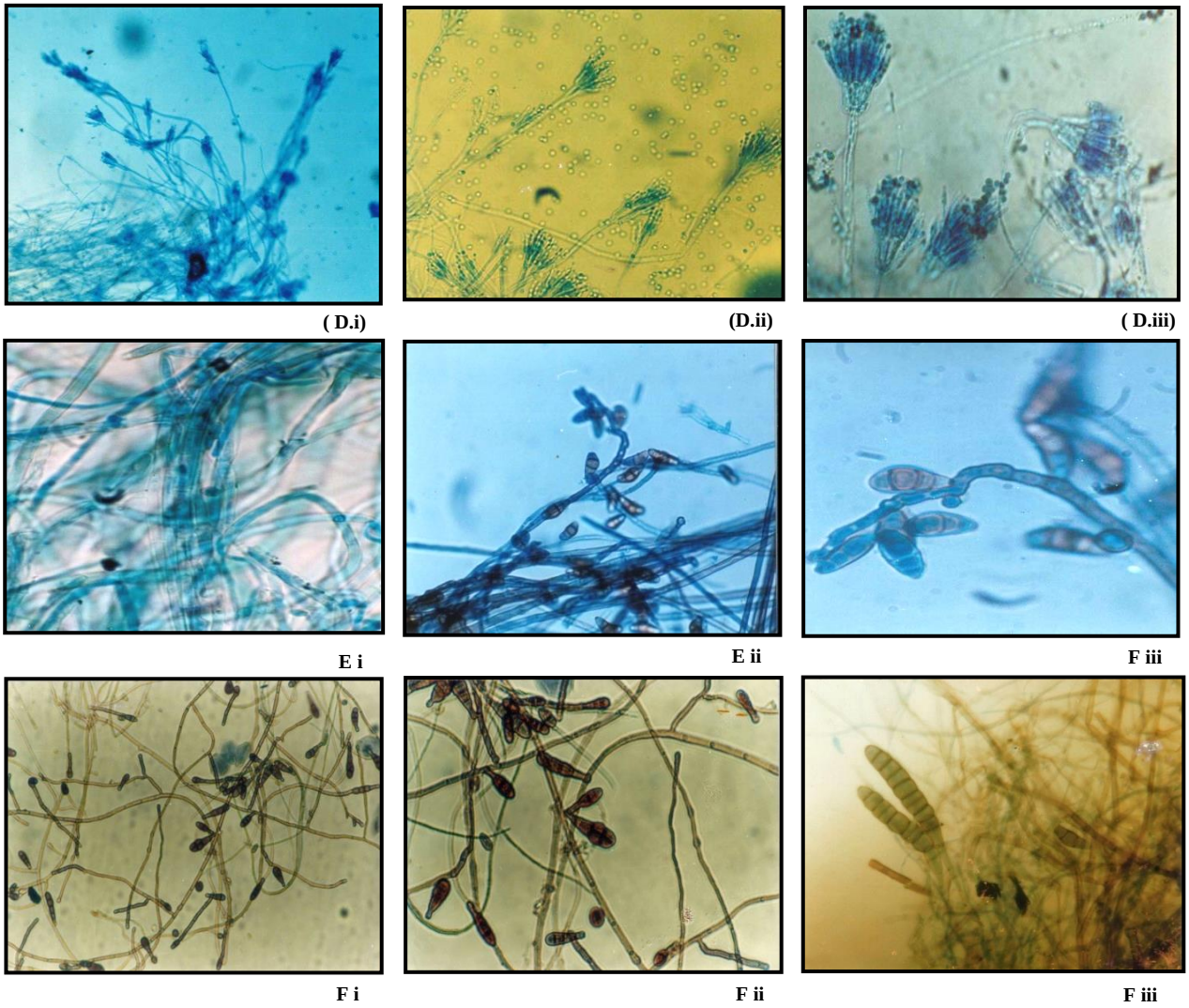
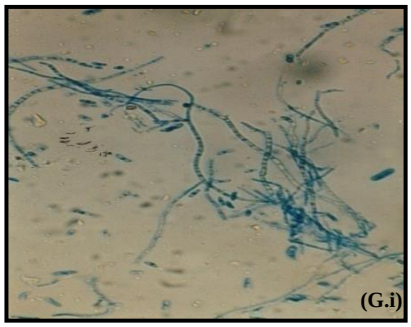
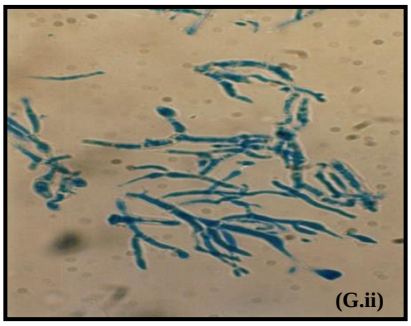


Figure-A. Different stages of *Aspergillus* sp conidiophores with conidia in chain. (i) (2.5×20X) (ii) (2.5×40X) (iii) (2.5×100X) **Figure-B**. Different stages of *Fusarium* sp conidiophores with conidia (i) (2.5×20X) (ii) Conidia (2.5×40X) (iii) (2.5×100X) **Figure-C**. Different stages of *Rhizopus* sp. Sporangiophore with sporangium (i) (2.5×20X) (ii) (2.5×20X) (iii) Exposed sporangium with spores (2.5×100X).







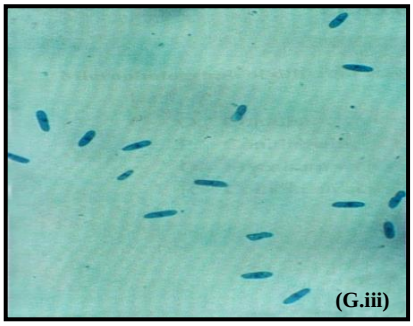


Fig-D. Different stages of *Penicillium* sp. Conidiophores with conidia in chain (D.i) (2.5×20X) (D.ii) (2.5×40X) (D.iii) (2.5×100X) **Fig-E. Different stages of *Curvularia* sp.**Conidiophores with conidia (E.i) (2.5×20X) (E.ii) (2.5×40X) (E.iii) (2.5×100X) **Fig-F. Different stages of *Alternaria* sp.** Conidiophores with conidia(F.i) (2.5×20X) (F.ii) (2.5×40X) (F.iii) (2.5×100X) **Fig-G. Different stages of *Colletotrichum* sp.**Conidiophores with conidia (G.i) (2.5×20X) (G.ii) (2.5×40X) (G.iii)(2.5×40X)

In hot water treatment (50^0C) in (Tab-2) it was observed that treated seeds in 30 min. (98.645±0.555) and 45min. (97.172±0.728) immersion period showed good result of average seed germination as well as successful control of fungal infection (3.30-13.20% and 0.00-9.90% respectively) (Tab-2).

On the other hand seed treatment in 60 min. immersion period controlled the fungal infections completely but it reduced seed germination remarkably (17.400±1.206) (Table-2). **Lambat *et al*. (1974)** reported eradication of Phomabetae in sugar beet seed by hot water treatment at 50^0C for 30 min.

Treatment of seeds by chemical fungicides it was observed that, in case of Redomil and Dithane M-45 treatment the fungal infection were controlled completely and the germination rate of seed was (99.3±0.4) and (98.0±0.7) respectively (Table-3). In Bavistin, the fungal pathogen was controlled successfully (0.00-6.60%) (Table-2) but not completely and the germination rate of seed was 90.600±0495. The values of vigour index were 1131.5±117.2, 1234.0±108.2 and 1056.9±84.3in the treatment of Bavistin, Redomil and Dithane M-45 respectively (Table-4). **Singh and Singh (1986)** reported similar result on seed treatment of Broad bean (Vicia feba) with Dithane M-45, Ceresan dry, Bavistin and Vitavax was evaluated for their reducing seed mycoflora.

Table 3 Seed germination rate of *L. purpureus* (L.) Sweet after different treatment

Genotypes	Control	Treatment					
		Plant Extracts			Cow urine	Hot water treatment	Average value of three chemical treatments
		Mehendi leaf	Neem leaf	Garlic bulb			
GBLB-2	79.20	94.6±2.4	95.7±2.7	97.1±2.0	88.0±7.0	75.3±17.1	97.4±2.0
GBLB-3	69.30	94.9±2.6	96.0±2.8	94.6±2.4	83.6±10.0	74.8±18.9	96.0±2.8
GBLB-5	72.60	95.7±2.8	95.7±1.6	95.7±2.	88.3±9.1	73.5±18.5	96.0±2.8
GBLB-6	82.50	96.0±2.8	96.4±2.97	96.4±3.0	92.0±6.5	77.0±16.6	97.1±1.9
GBLB-7	75.90	97.5±2.1	94.6±2.4	95.7±1.6	86.9±5.9	74.3±16.8	95.7±2.7
GBLB-9	79.20	97.1±1.9	97.1±1.95	96.0±2.8	85.8±6.8	75.9±15.3	94.6±2.4
GBLB-11	85.80	96.4±3.00	97.1±1.95	97.5±2.7	91.6±6.4	78.0±16.9	98.6±1.1
GBLB-12	79.20	96.4±3.0	92.4±1.56	96.0±1.8	84.7±10.4	77.2±16.7	97.1±1.9
GBLB-13	82.50	96.0±2.8	96.4±3.0	96.0±2.8	88.3±9.1	76.9±17.5	96.0±2.8
GBLB-14	69.30	97.5±2.1	96.8±1.8	94.6±1.8	84.7±6.3	75.0±16.0	97.4±2.7
GBLB-15	75.90	96.4±2.9	97.47±2.1	96.4±4.0	86.9±6.3	75.9±16.2	96.0±2.8
Mean±SE	76.8±1.4	96.2±0.3	96.0±0.4	96.0±0.3	87.4±0.8	75.8±0.4	96.5±0.2

Legend: GBLB – Genetics and breeding laboratory, Chemical treatment (Bavistin, dithane M-45, redomil)

Table 4 Vigour index of *L. purpureus* (L.) Sweet after different treatments .

Genotypes	Control	Treatment					
		Plant Extracts			Cow urine	Hot water treatment	Average Value of three chemical treatments
		Mehendi leaf	Neem leaf	Garlic bulb			
GBLB-2	150.45	730.7±301.2	1458.1±331.9	1046.1±142.7	933.1±283.2	842.7±244.2	1123.1±108.3
GBLB-3	227.70	691.3±222.5	1440.1±81.8	775.7±106.8	841.0±254.1	742.4±217.3	1119.8±143.0
GBLB-5	138.60	490.1±146.0	1131.1±40.7	1052.6±30.6	812.0±220.1	693.3±196.7	990.1±65.6
GBLB-6	509.02	1051.3±239.4	1812.6±193.0	1311.0±107.2	1194.1±309.4	1116.5±323.8	1667.2±74.4
GBLB-7	346.50	684.7±175.8	945.0±80.2	905.8±75.7	745.3±220.4	1084.7±298.9	974.7±29.5
GBLB-9	415.87	858.8±173.8	1059.9±55.5	873.2±41.6	814.3±199.6	992.6±292.7	1057.8±182.4
GBLB-11	316.80	1096.0±152.4	1895.3±185.5	1578.4±218.6	1204.9±325.9	1095.9±326.3	1547.7±238.2
GBLB-12	217.80	584.6±160.8	1700.9±46.5	1161.0±96.9	850.8±313.7	986.1±267.9	1087.5±68.3
GBLB-13	206.25	1154.2±127.9	1720.7±181.5	1418.7±158.6	1347.2±398.5	1101.3±326.4	1559.0±53.9
GBLB-14	519.75	971.3±132.5	1081.3±36.4	936.9±68.5	940.5±228.5	1066.6±313.3	685.4±72.4
GBLB-15	254.10	720.6±211.3	1260.6±185.7	1104.4±93.5	988.4±332.6	789.4±217.8	735.9±35.3
Mean±SE	300.26 ±38.47	821.2±63.2	1409.6±96.6	1105.8±67.7	970.1±56.1	955.6±45.7	1140.8±89.1

Legend: GBLB – Genetics and breeding laboratory, Chemical treatment (Bavistin, Dithane M-45,Redomil).

CONCLUSION

Chemical fungicide showed complete control of fungi**.** But chemical fungicides are costly and harmful to health and environment. Now a days the scientists demand cheap, easy and environment friendly controlling measures. Taking all these things into consideration the Plant extracts and Hot water treatment are the suitable controlling measures. In both cases though the fungal pathogens were not controlled completely but successfully. In the three plants extract treatment (Mehendi, Neem and Garlic) the 2.5 % concentration showed the best result in controlling fungal infection and increased seed germination rate as well as vigour index. By giving emphasis on germination rate, vigour index and fungal infection controlling capacity; cheap, *A. indica* environment friendly, easily available and easily applicable controlling measure; *A. indica* leaf extract was the best among the three plant extracts.

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