Utilization of *Azadirachta indica* Leaf Extract for Controlling Seed-borne Pathogens of Fenugreek

[1]M. A. Patekar, [2]R. P. Biradar
[1]Assistant Professor, [2]Assistant Professor and Head,
Department of Botany,
Shivaji Mahavidyalaya, Udgir, Dist: Latur (MS), India

Abstract: The present investigation has been undertaken in the *Marathwada* region of Maharashtra during the period from December 2014 to December 2016 to determine the prevalence of fungi in the selected vegetable seeds. Nine fungal genera of Fenugreek (Methi) *viz. Aspergillus* spp., *Alternaria* spp., *Fusarium* spp., *Mucor* spp., *Rhizopus* spp., *Rhizoctonia* spp., *Penicillium* spp. and *Chaetonium* spp. were found to be associated on standard blotter paper method and Agar plate method. Whereas, *Aspergillus* spp., *Fusarium* spp. and *Alternaria* spp. were found to be most dominant. Leaf extracts of various concentrations (2.5%, 5.0%, 7.5% and 10.0%) of *Azadirachta indica* were examined against isolated dominant fungi. The growth inhibition was found to be significantly and gradually increased from 0 to 99.15 % with the increase in concentration of extracts.

Key words **– Leaf extract, seed-borne fungi, vegetable seed**

I. INTRODUCTION

India is the second largest producer of vegetables in the world, next to China. Vegetables provides nutrients vital for health and maintenance of our body. Fenugreek is one of the most important commonly consumed leafy vegetable in India. Fenugreek (*Trigonella foenum – groecum L.*) a minor legume field crop is native of south eastern Europe and Africa. Fenugreek is also commercially cultivated in several countries including India. Seeds of fenugreek have characteristic odour, flavor and taste. Health status of seed is nothing but the absence of insect infestation and fungal infection in or on the seed. Diseases caused by fungus are one of the major constraints to the production of fenugreek in field condition. Most of the diseases are due to seed borne fungi associated with the seeds. Various fungal pathogen causes diseases on Fenugreek such as Cercospora leaf spot due to *Cercospora traversiana*, Charcoal rot due to *Macrophomina phaseolina*, Root rot due to *Rhizoctonia solani* and Fusarium wilt due to *Fusarium oxysporium.* Thus, seed health testing for the presence of seed borne pathogens and their control by various means is an important aspect in management of crop diseases. Management of plant diseases is the most important aspect in achieving higher yields. Medicinal plant extracts have been accorded a lot of importance for crop protection against pest and diseases due to their target specificity and safety. Plant extracts and products have been observed to be effective against large number of pathogens. (Rathod *et al.*, 2012). Rao (2015) observed antifungal potential of twenty medicinal plant leaf extracts at 10.0 and 20.0 %concentration against seedborne fungi like *Alternaria alternata*, *Aspergillus flavus*, *A. niger*, *Curvularia*, *Fusarium moniliformae*, *F. solani*, *Helminthosporium* spp. And *Penecillium* spp.This investigation was carried out for isolation and identification of seed borne fungi and their control by ecofriendly way.

II. RESEARCH METHODOLOGY

2.1 Collection of seed samples (Cultivars)

The method described by Neergaard (1973) has been adopted for the collection of seed samples. Accordingly, two random samples of following seeds (250 gm each) were collected from local farmers and market places of *Marathwada* region of Maharashtra state of India.

Fenugreek (*Trigonella foenum-graecum* L.)

2.1.1Pusa
2.1.2 Methi local

During the course of studies, seed samples were separately collected and stored in plastic containers without any treatment of fungicide / insecticide at laboratory conditions.

2.2 Detection of Seed Mycoflora

The seed mycoflora was isolated by using different methods such as Standard blotter paper method and Agar plate method as recommended by International Seed Testing Association ISTA (1966), De Tempe (1953), Neergaard (1973) and Agrawal (1976).

Observations were recorded in percent incidence of seed borne fungi associated with unsterilized seeds. Fungi appeared on seed were isolated in pure culture for identification and for further study. Two different methods of isolation techniques were used for assessment of seed mycoflora.

2.2.1 Standard blotter paper method

A pair of sterile blotter papers of 8.5 cm diameter were soaked in sterile distilled water and were placed in pre-sterilized petriplates of 9 cm. diameter. Ten seeds of test sample per petriplate were then placed equidistance on moist blotter. The plates were incubated at $28^0 \pm 2^0$C under diurnal conditions.

2.2.2 Agar plate method

Pre-sterilized petriplates were poured with 15 mL of autoclaved Potato Dextrose Agar (PDA). On cooling the medium, ten seeds per plate of the sample to be studied were equidistantly placed aseptically. Incubation and other details of the study were same as described for blotter test method. The various moulds appeared on seeds in blotter paper and Agar plates were isolated and maintained on PDA/ GNA slants.

2.2.3 identification of fungi

Detailed examination of fungal characters was done by compound microscope after seventh day of incubation period and identification was confirmed with the help of identification Manual (Mathur and Kongsdal, 2003) and pictoral atlas of soil and seed fungi. (Watanabe, 2002).

The percentage incidence of mycoflora observed in seed samples were calculated by using following formulas.

$$\text{Percentage incidence of fungus} = \frac{\text{No. of seeds containing particular fungus}}{\text{Total no. of seeds}} \times 100$$

$$\text{Percentage incidence of seed mycoflora} = \frac{\text{No. of seed infected by fungi}}{\text{Total no. of seeds}} \times 100$$

2.3 Bio-control of seed borne pathogens by using plant extracts:

Leaf extracts of various concentrations (2.5%, 5.0%, 7.5% and 10.0%) of *Azadirachta indica* were examined against isolated dominant fungi.

Preparation of aqueous extracts:

Green leaf samples (100gm) were collected and washed very carefully with distilled water. Then plant parts were ground with conventional grinder called '*Mortar and pastel*' which is available and popular in every Indian farmer's house. Then grounded material were dipped in to 100 ml distilled water for 48 hours for complete extraction of the active ingredient from the extracted samples (Ahmed *et al.*, 2013). After that the water and ground material were filtered with the help of muslin cloth. This extract filtered with the help of Whatman's grade filter paper no. 1. Then crude extracts were preserved in glass bottles and kept in refrigerator at 4 +/- 2^0C for further use.

Efficacy of leaf extracts with different concentrations were examined by Food poison technique. (Nene and Thapliyal, 1993). The linear mycelial growth of fungi has been taken after seventh day of incubation.

Linear mycelial growth inhibition was calculated by following formula:

$$\text{Percentage of fungal growth inhibition} = \frac{gC + gT}{gC} \times 100$$

Where,

gC= Mycelial growth of fungus in control plate (cm)

gT= Mycelial growth of fungus in treated plates (cm)

2.4 Data Analysis

Data was analysed by Analysis of Variance (ANNOVA) and LSD was calculated at P=0.05 for significance.

III. EXPERIMENTAL RESULTS AND DISCUSSION

3.1 Fungi associated with Local seeds of Fenugreek

The data given in Table 1 and Plate no. I and II revealed that total 12 fungal species were isolated from seeds *viz. Aspergillus flavus, Aspergillus fumigatus, Aspergillus nidulans, Alternaria alternata, Fusarium oxysporum, Fusarium moniliforme, Mucor mucedo, Rhizopus stolonifer, Rhizopus nigricans, Pencillium chrysogenum* and *Curvularia lunata.*

The average percent fungal incidence was 15.83% under blotter method, in comparison to that observed in agar plate method (16.68%), without any significant difference among them (t= 0.17). In case of all other fungi, except *Aspergillus flavus, Aspergillus niger, Alternaria alternata* and *Fusarium oxysporum* the percent incidence was significantly decreased. Wide variation among the frequencies were observed for various fungi as indicated by the values of coefficient of variation (C.V.).

Table 1: Fungi associated with seeds of Fenugreek (Local Seeds)

Sr. No.	Name of the Fungi	Percentage of incidence	
		Standard Blotter	Nutrient Agar
1	*Aspergillus flavus*	40	33.3
2	*Aspergillus niger*	20	16.7
3	*Aspergillus fumigatus*	13.3	10
4	*Aspergillus nidulans*	16.7	10
5	*Alternaria alternata*	26.7	33.3
6	*Fusarium oxysporum*	33.3	36.7
7	*Fusarium moniliforme*	16.7	20
8	*Mucor mucedo*	13.3	6.7
9	*Rhizopus stolonifer*	10	10
10	*Rhizopus nigricans*	0	10
11	*Penicillium chrysogenum*	0	6.7
12	*Curvularia lunata*	0	6.7
Statistical Analysis			
	Mean	15.8333	16.675
	SD	13.7874	11.4478
	CV	87.0781	68.6522
	SE	3.9800	3.3047
	CD 5%	8.7562	7.2703
	CD 1%	12.3780	10.2776
	t	0.1693	

3.2 Fungi associated with Pusa seeds of Fenugreek

The data given in Table 2 and Plate III and IV showed eleven fungal species *viz. Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Alternaria alternata, Alternaria tenuis, Fusarium oxysporum, Rhizopus stolonifer, Mucor mucedo, Rhizoctonia* spp., *Penicillium chrysogenum* and *Chaetonium* spp. While, last three were only recorded in agar plate method.

In blotter paper method, the fungal incidence recorded at 14.24% which was increased to 22.13% when agar plate method was used. However, the difference in the percentage incidence was statistically non-significant.

Table 2: Fungi associated with seeds of Fenugreek (Pusa Seeds)

Sr. No.	Name of the Fungi	Percentage of incidence	
		Standard Blotter	Nutrient Agar
1	*Aspergillus flavus*	26.7	40
2	*Aspergillus niger*	23.3	30
3	*Aspergillus fumigatus*	10	16.7
4	*Alternaria alternata*	33.3	36.7
5	*Alternaria tenuis*	13.3	16.7
6	*Fusarium oxysporum*	33.3	36.7
7	*Rhizopus stolonifer*	6.7	23.3
8	*Mucor mucedo*	10	13.3
9	*Rhizoctonia* spp.	0	13.3
10	*Penicillium chrysogenum*	0	10
11	*Chaetonium* spp.	0	6.7
Statistical Analysis			
	Mean	14.2364	22.1273
	SD	11.5496	12.9358
	CV	81.1272	58.4608
	SE	3.4823	3.9003
	CD 5%	7.7656	8.6976
	CD 1%	11.0390	12.3639
	t	1.6406	

Dominant fungal pathogens isolated from both seed samples were *Aspergillus flavus*, *Alternaria alternata* and *Fusarium oxysporum.* Leaf extracts of *Azadirachta indica* were screened against dominant fungi.

3.3 Effect of *Azadirachta indica* on percentage growth inhibition of fungi

Table 3 and Fig. 1 explains effect of *Azadirachta indica* A. Juss. on *Aspergillus flavus, Alternaria alternata* and *Fusarium oxysporum* in the form of percent inhibition of their growth. All the concentration of leaf extracts of *Azadirachta indica* A. Juss. showed an antifungal activity against test fungi. An increased inhibitory effect of the mycelial growth was observed with increase in concentration from 2.5% to 10.0%. At 2.5%, 5.0% and 7.5% concentration, the inhibition was 25.7%, 54.28% and 87.09% in *Aspergillus flavus*, 45.16%, 80.64% and 87.09% in *Alternaria alternata* and 29.11%, 58.22% and 77.21% in *Fusarium oxysporum*. Complete growth inhibition was recorded at 10.0% concentration.

The analysis of variance (ANOVA) and the values of variance ratio (F) indicated that there was significant (p=0.01) variation in inhibition of growth due to various concentrations of aqueous extracts. The variation due to the three fungi under investigation was also significant at p=0.05 indicating the response of different fungi differently. The growth inhibition significantly and gradually increased from 0 to 99.15 % with the increase in concentration of extracts.

Table 3: Effect of *Azadirachta indica* A. Juss. on percentage growth inhibition of fungi

Sr. No.	Fungal species	Concentration/Growth inhibition %			
		2.5	**5**	**7.5**	**10**
1	***Aspergillus flavus***	25.71	54.28	80	100
2	***Alternaria alternata***	45.16	80.64	87.09	100
3	***Fusarium oxysporum***	29.11	58.22	77.21	97.46
	Mean	33.33	64.38	81.43	99.15

Table 3a: Analysis of variance (ANOVA)

Source	df	SS	MSS	F	S/NS
Concentration	4	18686.3596	4671.5899	117.9426	S
Fungal species	2	359.4828	179.7414	4.5379	S
Error	8	316.8720	39.6090	-	-
Total	14	19362.7144	-	-	-
SE	5.1387				
CD 5%	11.8703				
CD 1%	17.2660				

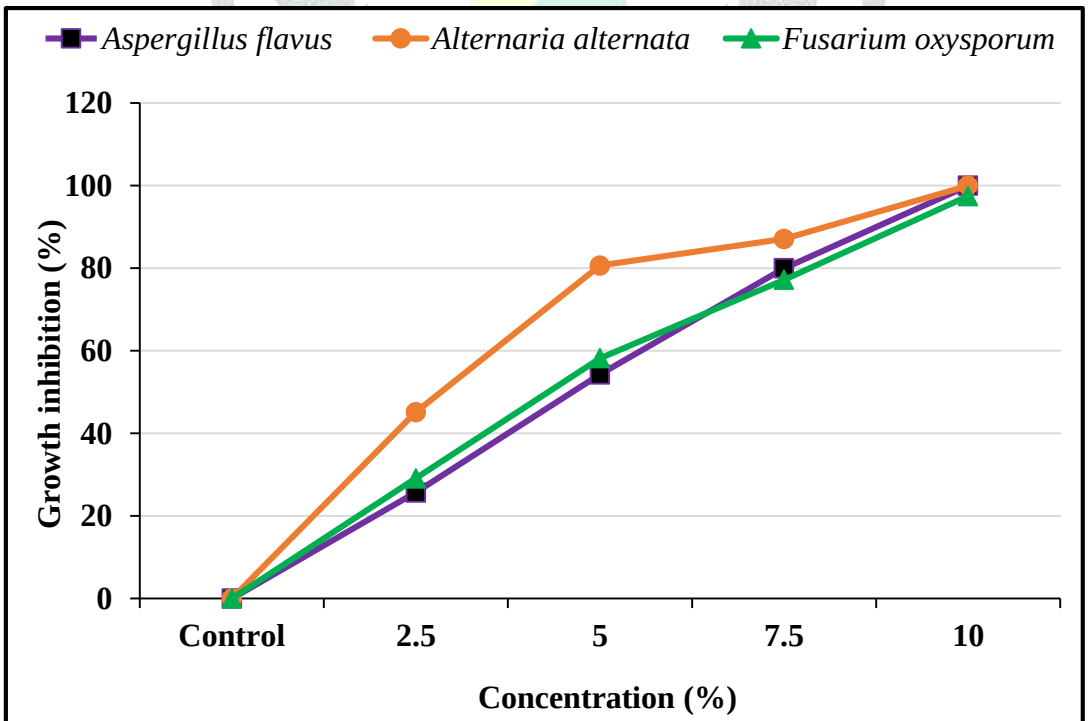


Fig. 1: Effect of *Azadirachta indica* A. Juss. on percentage growth inhibition of fungi

IV. DISCUSSION

Nine fungal genera of Fenugreek (Methi) *viz. Aspergillus* spp., *Alternaria* spp., *Fusarium* spp., *Mucor* spp., *Rhizopus* spp., *Rhizoctonia* spp., *Penicillium* spp., *Chaetonium* spp. and *Curvularia* spp. were found to be associated on standard blotter paper method and Agar plate method. Whereas, *Aspergillus* spp., *Fusarium* spp. and *Alternaria* spp. were found to be most dominant. Similar results were noted by Hedawo *et al.* (2011). In other pathogenicity studies of Fenugreek carried out by El-Nagerabi (2002) on Potato dextrose agar and moist filter paper at 28 ± 2^0c obtained 21 genera of fungi. Among these, *Aspergillus* with fifteen species was most prevalent followed by *Drechslera* spp., *Rhizopus* spp., *Alternaria* spp. and *Fusarium* (six species each), *Emericella* spp., *Cladosporium* spp., *Penicillium* spp., *Chaetonium* spp. and *Curvularia* spp.

Al-Kassium and Monawar (2000) observed identical fungal pathogens on Tomato, Eggplant, Okra, Radish and Jews mallow seeds viz. *Alternaria, Aspergillus* and *Fusarium.* Similarly, Hossain *et al.* (2014) observed the prevalence of eleven different vegetable seeds collected from Mymensingh region of Bangladesh and recorded Ten genera *viz. Alternaria* spp., *Aspergillus flavus, Aspergillus niger, Botrytis cinerea, Chaetomium funicola, Curvularia* spp., *Fusarium* spp., *Penicillium* spp., *Phoma* spp. and *Rhizopus* spp.. The presence of *Aspergillus flavus, Fusarium* spp. and *Alternaria alternata* and their association with seeds were reported by Embaby *et al.* (2006), Ignjatov *et al.* (2012), Islam *et al.* (2012), Rathod *et al.* (2012) and El-Gali (2015).

In the present investigation, antifungal activity of *Azadirachta indica* A. Juss at 2.5%, 5.0%, 7.5% and 10.0% concentration against the mycelial growth of *A. flavus, A. alternata* and *F. oxysporum* was studied. Similar investigation on antifungal activity of plant extracts against seed born mycoflora was reported by other researchers. (Sangoyomi *et al.*, 2010; Ahmad *et al.*, 2016). The good inhibitory effect was observed at 7.5% concentration and best effect was achieved at 10.0% concentration. Similar findings were observed by Hassanein *et al.* (2010) with *Azadirachta indica* A. juss. leaf extract against *Alternaria solani* and *Fusarium oxysporum*, the causal agents of early blight and wilt of tomato plants (*Lycopersicon esculentum* Mill.) respectively. Extracts of leaf, seeds, bark, etc. of various higher plant have been reported to passes antifungal activity under laboratory trails. (Stangarlin *et al.*, 2011; Lakshmeesha *et al.*, 2013; Pawar, 2015).

V. CONCLUSION

The incidence of major mycoflora found statistically similar in company seeds as well as seeds collected from farmers as tested by Standard blotter paper and Agar plate method. The detection of seedborne pathogenic fungi and seed diseases is an important aspect of integral disease management. The given study confirms *in vitro* biological activity of *Azadirachta indica* towards *Aspergillus flavus, Alternaria alternata* and *Fusarium oxysporum* with enhancing the growth of seedling.

REFERENCES

[1] **Agarwal V.K. (1976).** Technique for the detection of seed-borne fungi. *Seed Res.*, 4: 24-31.

[2] **Ahmad L., Pathak N. and Zaidi R.K. (2016).** Antifungal potential of plant extracts against seed-borne fungi isolated from Barley seeds (*Hordeum vulgare L.*). *Journal of Plant Pathology and Microbiology*, 7(5): 350.

[3] **Ahmed M., Hossain M., Hassan K. and Dash C.K. (2013).** Efficacy of different plant extract on reducing seed borne infection and increasing germination of collected Rice seed sample. *Universal Journal of Plant Science*, 1(3): 66-73.

[4] **Al-Kassim M.Y. and Monawar M.N. (2000).** Seed borne fungi of some vegetable seeds in Gazan province and their chemical control. *Saudi Journal of Botanical Science*, 7(2): 179-184.

[5] **De Temp J. (1953).** The blotter method of seed health testing programme. *International Seed Testing Association*, 28: 133-151.

[6] **El-Gali Z.I. (2015).** Seed-borne fungi of bean (*Phaseiolus vulgaris*): detection, pathogenicity and biological control *in vitro*. *International Journal of Nano Corrosion Science and Engineering*, 2(1): 33-41.

[7] **El-Nagerabi A.A.F. (2002).** Determination of seedborne fungi and detection of aflatoxins in Sudanese Fenugreek seeds. *Phytoparasitica*, 30(1): 61-66.

[8] **Embaby E.M. and Abdel-Galil M.M. (2006).** Seed borne fungi and mycotoxins associated with some legume seeds in Egypt. *Journal of Applied Sciences Research*, 2(11): 1064-1071.

[9] **Hassanein N.M., Abou Zeid M.A., Youssef K.A. and Mahmoud D.A. (2010).** Control of tomato early blight and wilt using aqueous extract of neem leaves. *Phytopathol. Mediterr.*, 49: 143-151.

[10] **Hedawoo zg.B. and Chakranarayan R.G. (2011).** Isolation of fungal species from the seeds of some Indian spices. *Biosci. Biotech. Res. Comm.*, 4(2): 208-210.

[11] **Hossain I., Dey P. and Dilruba K. (2014).** Quality of vegetable seeds collected from Mymensingh region in Bangladesh. *International Journal of Applied Sciences and Biotechnology*, 2(1): 103-108.

[12] **Ignjatov M., Milosevic D., Nikolic Z., Gvozdanovic-Varga J., Jovicic D. and Zdjelar G. (2012).** *Fusarium oxysporum* as causal agent of Tomato wilt and fruit rot. *Pestic. Phytomed. (Belgrade)*, 27(1): 25–31.

[13] **ISTA. (1996).** International Rules for Seed Testing. *Seed Science and Technology*, 13: 299-513.

[14] **Islam M.T. and Faruq A.N. (2012).** Effect of some medicinal plant extracts on damping-off disease of winter Vegetable. *World Applied Sciences Journal*, 17(11): 1498-1503.

[15] **Lakshmeesha T.R., Sateesh M.K., Vedashree S. and Mohammad S.S. (2013).** Antifungal activity of some medicinal plants on Soybean seed-borne *Macrophomina phaseolina. Journal of Applied Pharmaceutical Science*, 3(2): 84-87.

[16] Mathur S.B. and Kongsdal O. (2003). Common laboratory seed health testing methods for detecting fungi. Danish Government institute of seed pathology for developing countries, Copenhagen, Denmark. *Published by ISTA, Switzerland.* p. 425.

[17] Neergaard P. (1973). Detection of seed-borne pathogens by culture tests. *Seed Sci. Technol.* 1(1): 217-254.

[18] Nene Y.L. and P.N. Thapliyal (1993). Fungicides in plant diseases control. *Oxford and IPH. Publishing Co. Pvt. Ltd., New Delhi,* p. 531.

[19] Pawar B.T. (2015). Antifungal activity of some seed extracts against seed-borne pathogenic fungi. *International Archive of Applied Sciences and Technology*, 6(2): 44-46.

[20] Rao J.K. (2015). Antifungal potential of some medicinal plants against seed-borne fungi. *International Resource Journal of Science and Engineering*, 3(1): 28-32.

[21] Rathod L.R., Jadhav M.D., Mane S.K., Muley S.M. and Deshmukh P.S. (2012). Seed borne mycoflora of legume seeds. *International Journal of Advanced Biotechnology and Research*, 3(1): 530-532.

[22] Rathod L.R., Jadhav M.D., Pawar P.V. and Muley S.M. (2012). The ultimate natural source: Eco-friendly activity of medicinal plant to control seed borne pathogens of Groundnut. *Bionano frontrier: Eco evolution 2012, Colombo-Srilanka International Journal of Advanced Biotechnology and Research*, 197-199.

[23] Sangoyomi T.E., Asiedu R. and Ekpo E.J.A. (2010). Effect of ten plant extracts on mycelial growth and conidial production of four fungi associated with yam tuber rot. African *Journal of Root and Tuber Crop*, 8(1): 24-30.

[24] Stangarlin J.R., Kuhn O.J., Assi L. and Schwan-Estrada K.R.F. (2011). Control of plant diseases using extracts from medicinal plants and fungi. *Science against microbial pathogens: communicating current research and technological advances; Formatex*, 1033-1042.

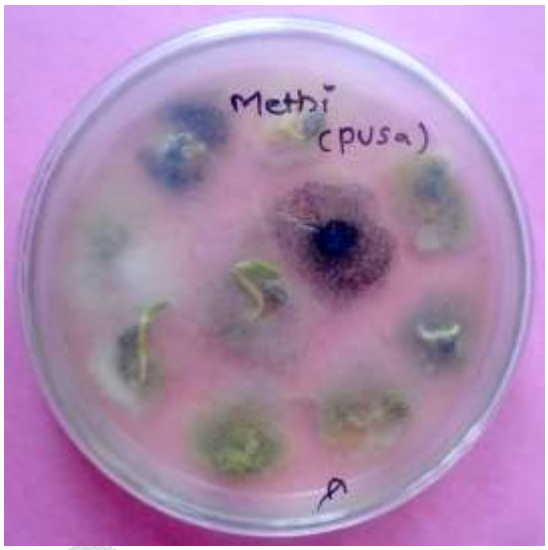
[25] Watanabe T. (2002). Pictoral Atlas of soil and seed fungi: Morphologies of cultured fungi and key to species, Second edition. *CCR Press, Boca Raton, London.* P. 484.

I **II**

PLATE I – Fungi associated with Fenugreek (Local) on blotter paper

PLATE II – Fungi associated with Fenugreek (Local) on Agar plate

III



IV

PLATE III – Fungi associated with Fenugreek (Pusa) on blotter paper

PLATE IV – Fungi associated with Fenugreek (Pusa) on Agar plate