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ESSENTIAL OILS SHOW ANTIFUNGAL ACTIVITY AGAINST SEED-BORNE MYCOFLORA ASSOCIATED WITH OKRA SEEDS

Uzma Sitara[1]*, Ali Akbar[2], Muhammad Abid[3] and Akhlaq Ahmad[1]

[1]*Food Quality & Safety Research Institute, Southern Zone Agricultural Research Centre, Karachi-75270, Pakistan.*
[2]*Department of Microbiology, Faculty of Life Science, University of Baluchistan Quetta, Pakistan*
[3]*Dr. A. G. Lab of Aerobiology and Plant Pathology, Department of botany, Federal Urdu University of Arts, Science & Technology, Gulshan-e- Iqbal Campus, Karachi*
Correspondence author's email: uzmasitara@yahoo.com

ABSTRACT

Few essential oils were extracted from the seed of black cumin (*Nigella sativa*). Asafoetida (*Ferula asafoetida*) and garlic (*Allium sativum*) to determine their antifungal activity against 13 seed borne fungal species *Aspergillus niger, A. flavus, A. called, A. wentii, Alternaria alternata, A. solani, Drechslera hawaiiensis, D. tetramera, Fusarium moniliforme, F. semitectum, F. solani* and *Rhizoctonia* spp. found associated with okra seeds. The fungi were isolated by standard blotter and potato dextrose agar (PDA) methods by ISTA techniques from three different okra seed varieties viz., sabzpari, shazadi (Pakistani) and green star (Indian). Essential oils were extracted from black cumin (*Nigella sativa*). Asafoetida (*Ferula asafoetida*) and garlic (*Allium sativum*) seeds were tested for their antifungal activity at concentrations 0.15%, 0.25% and 0.35% against the seed borne fungi of okra seeds. Here, we demonstrate that asafoetida oil @ 0.25% and 0.35 % significantly inhibited the growth of all fungi. It was revealed that the Pakistani variety (shazadi) shown less number of fungi followed by green star and sabzpari. This study will help to identify and select okra varieties showing defence against seed borne diseases.

Key words: seed borne fungi, antifungal activity, okra varieties, essential oils

INTRODUCTION

Okra (*Abelmoschus esculentus* L. (Moench.) is an important vegetable summer crop. The average annual yield of okra is comparatively lower in Pakistan than in many other countries. During 2013-2014 the total yield of okra was 14147 million hector (Anon., 2013-14). There are several reasons of low yield of okra, such as soil type, time and method of sowing, bad seed quality, and irregularities in irrigation and fertilizer applications. In addition, Fungi are one of the most important and prevalent pathogens of okra crop. For instance, seed-borne fungi *Macrophomina phaseolina, Colletotrichum dematium, Fusarium oxysporum* and *F. moniliforme* have been found to cause seed rot and seedling blight (Fakir *et al*., 1976; 1977). *Fusarium oxysporum*, *Colletotrichum dematium, Cercospora abelmoschi, Corynespora cassiicola* and *Macrophomina phaseolina* are known to cause foot and root rot, Anthracnose and die back, Cercospora leaf spot, Corynespora leaf spot and seed blight, respectively in okra crop (Anam *et al*., 2002; Gilmar *et al*., 2009; Zahoor *et al*., 2012). Kassam and Munnawar (2000) have isolated *Alternaria, Fusarium* and *Aspergillus* spp., from okra seed. Similarly, Nutsugah *et al*. (2004) have also reported the association of fungal species *Fusarium pallidoroseum* and *F. moniliforme* on okra seeds. In a survey, *Sharma et al*. (2013) found eleven major fungi viz., *Aspergillus flavus, A. niger, A. nidulans, A. fumigatus, Alternaria alternate, Curvularia lunata, Rhizopus nigricans, Cladosporium oxysporum, Penicillium chrysogenum, P. citrinum, Stachybotrys atra, Chaetomium globosum, C. murorum, Rhizoctonia bataticola* on okra fruit. Because of widespread association of fungal pathogens and the damage these pathogens cause to okra crops there have been need to protect this crop from fungal infestation. Few inorganic chemicals have been found effective against fungal pathogens infesting okra, however showed significant negative effects on okra crops. Because of the undesirable effects of these chemicals; there has been a demand to find alternative tools to control fungal pathogens. Natural antifungal products such as, plant parts, plant extracts and nonpathogenic microbes have shown promising results against fungal pathogens (Momin and Nasir, 2001; Mashooda *et al*., 2009; Akbar and Anal, 2013).

The essential oil and their constituents have been shown to possess antibacterial and antifungal activities (Sridhar *et al*., 2003). Zangoie *et al*., (2013) have reported antifungal activity in *Asafoetida* against *Bipolaris sorokiniana, Verticillium spp, Fusarium graminearum, F. solani* and *Aspergillus niger*. In other study, Thyagaraja and Honso (1996) have found inhibitory effects of *Asafoetida* on *Rhizopus sporus, Mucor dimorphosphorous, Penicillium commune* and *Fusarium solani*. Asafoetida oil has also shown antifungal activity against *Candida albicans* (Kunwar *et al*., 2010; Mishra *et al*., 2010). According to Sitara and Hassan (2011) *Nigella sativa* and

Asafoetida powder at concentration of 0.25% showed strong fungicidal activity towards *Aspergillus niger, A. fumigatus, A. candidus, Alternaria alternata, A. tenuissima, Drechslera hawaiiensis, Trichoderma* sp., *Phoma beta* and *Fusarium moniliforme*. The oil extract of *Nigella sativa* has been reported to show antimicrobial effect against *Candida albicans* in both *in vitro* and *in vivo* tests (Khan *et al*., 2003; Mashhadian and Rakhshandeh, 2005; Erdogrul *et al*., 2009; Haloci *et al*., 2012). Whereas garlic oil possesess fungistatic activity against *Candidus utilis* (Chung *et al*., 2007). The garlic extract has also been found effective antifungal agent against *Curvularia lunata* (Gujar *et al*. 2012). Bodankar and Patil (2011) reported the antifungal activity of garlic towards *Candida albicans, Aspergillus niger* and *Cryptococcus neoformans*. In the present study, we examined three varieties of okra seeds for the presence of fungal pathogens. We also extracted essential oils from garlic, black cumin and *Asafoetida* and tested these extracts to determine their antifungal activity against seed borne fungi of okra.

MATERIALS AND METHODS

Seed varieties: Three varieties of okra seeds viz., shazadi, sabzpari (Pakistani varieties) and green star (An Indian variety) were obtained from Pest Management Research Institute of Pakistan Agriculture Research Council, Karachi, Pakistan.

Isolation of fungal specimens from okra seeds: For the identification of seed borne fungi associated with okra seeds; ISTA techniques were used (ANON., 1993). The following Standard blotter and agar plate methods were used to analyze okra seeds.

Standard blotter and Potato Dextrose Agar methods: In all 200 okra seeds for each variety viz, shazadi, sabz pari and green star were analyzed. For blotter paper method 10 seeds were transferred onto a three layered of moist blotting papers placed in a 9 cm glass autoclaved Petri plates. Thus for 200 seeds, 20 such plates were used. The Petri plates containing test seeds were incubated at 28 ± 2 °C in alternate cycle of 12 hours light and 12 hours dark for 5- 7 days. Similarly, 200 seeds were analyzed by Potato Dextrose Agar (PDA) method. In this protocol, 10 seed were transferred onto a 9 cm glass autoclaved Petri plates containing PDA and incubated at 28 ± 2 °C in alternate cycle of 12 hours light and 12 hours dark for 5- 7 days.

Oil Extraction: Three different essential oils from garlic (*Allium sativum*), black cumin (*Nigella sativa*) and asafoetida (*Ferula asafoetida*) were extracted with solvent (n-hexane) (B.P 60-80ºC) by Soxhlet apparatus for eight hours. These oils were obtained removing n-hexane from the extract on rotary evaporator under reduced pressure.

Agar diffusion method: Agar diffusion method (Nene and Thapliyal, 1979) was used to determine the antifungal activity of essential oils. Required amount of essential oils were dissolved in pure acetone and thoroughly mixed with melted potato dextrose agar to obtain 0.15, 0.25 and 0.35% concentrations. The approximately 10 mL of treated or untreated medium were poured onto Petri plates. The untreated medium was used as control. Ten okra seeds per/ plate were placed in each Petri plate aseptically. There were 3 replicates for each experiment. Petri plates were incubated at 28 ± 2 °C and growth of fungi was recorded after 7 days of incubation. Data were analyzed statistically to determine the difference among various treatments. The fungal species were identified by Barnet and Hunter (1972) and Nelson *et al*., (1983).

RESULTS AND DISCUSSION

We isolated 13 species of fungi including *Aspergillus niger, A. flavus, A. fumigatus, A. called, A. wentii, Alternaria alternata, A. solani, Drechslera hawaiiensis, D. tetramera, Fusarium moniliforme, F. semitectum, F. solani*, and *Rhizopus* sp., from three varieties of okra seeds. We found *Aspergillus* species dominated on okra seed varieties sabzpari and shazadi (Table 1). In a similar study, Kassam and Munnawar (2000) and Odofin (2010) have isolated *Alternaria alternata, Aspergillus niger, Curvularia lunata, Fusarium oxysporum, F. solani, F. moniliforme, Macrophomina phaseolina, Rhizoctonia* sp., *Stemphylium botryosum, Penicillium digitatum* and *Phythium aphanidermatum* from okra seed varieties sabzpari and shazadi. Their results are in agreement with our results. Rahim and Dawar (2015) isolated 75 species belonging to 31 fungal genera from eighteen seed samples of okra. We found a high rate of fungal growth on sabzpari and green star varieties on blotter paper, and reduced growth on PDA, however PDA was found to be more favorable for fungal growth on shazadi (Pakistani) variety.

Essential oils viz., garlic (*Allium sativum*), black cumin (*Nigella sativa*) and asafoetida (*Ferula asafoetida*) at concentration of 0.15, 0.25 and 0.35% were tested for their antifungal activities against all fungi that were isolated from three varieties of okra seeds. We found a significant reduction in the growth of all seed-borne fungi. The rate of growth reduction was directly proportional to the concentration of essential oil in the PDA medium. We found that *Nigella sativa* oil and asafoetida oil at concentrations 0.25% and 0.35% were effective against seed-borne fungi isolated from okra seeds sabzpari variety. Sitara *et al*. (2008) have shown that black cumin (*Nigella sativa*) and

asafoetida (*Ferula asafoetida*) oil used at concentrations of 0.5, 0.15 and 0.25% were effective in inhibiting the growth of eight seed-borne fungi viz., *Aspergillus niger, A. flavus, Fusarium oxysporum, F. semitectum, Drechslera hawaiiensis* and *Alternaria alternata. Nigella sativa* at 0.35 % completely controlled the growth of *Alternaria alternata* and *Aspergillus fumigatus*, whereas *A. flavus* and *F. moniliforme* showed only 1% growth at 0.25% oil concentration (Fig. 1). Garlic oil was not as effective as other oils tested; the oil showed antifungal activity at 0.35% against *F. moniliforme, F. solanai*, and *Alternaria alternata*. Khodavandi *et al.*, (2011) have suggested that garlic extracts can be an effective antifungal agent against *Candida albicans* and *Trichophyton* spp. Tedeschi *et al.*, 2007 obtained significant activity of garlic extract against *Aspergillus, Penicillium, Fusarium* and *Trichoderma* species. We found that *asafoetida* oil used at 0.25 and 0.35% completely inhibited the growth of all fungi on sabzpari variety, whereas *F. moniliforme* showed only 2% growth at 0.25% concentration. The fungus species *F. moniliforme* and *F. solani* were completely controlled at all concentration used, however garlic oil at 0.35% concentration did not suppress the growth of *Aspergillus niger, A. flavus* and *Alternaria alternata* (Fig. 2).

Table 1. Isolation of fungi from different varieties of okra by blotter and PDA method.

No.	**Name of fungi**	**Sabzpari**				**Shazadi**				**Green star**			
		NST.B	**ST.B**	**NST.PDA**	**ST.PDA**	**NST.B**	**ST.B**	**NST.PDA**	**ST.PDA**	**NST.B**	**ST.B**	**NST.PDA**	**ST.PDA**
1	*Aspergillus niger*	93	39	39	9	46	18	92	70	23	13	13	10
2	*A. flavus*	15	10	24	11	12.3	8	98	22	64.5	16.5	8	3
3	*A. fumigatus*	25	11	31.8	13.5	11.8	8.5	60.5	29.8	8	3.5	25	11
4	*A. called*	15	6.9	18	12	14	8	71	30	10	7	31	14.5
5	*A. wentii*	24	18	28	15.6	21	12	20	11	15	8	15	7
6	*Alternaria alternata*	35	16	40	30	31	12.5	26	18	15	7	23	7
7	*Alternaria solani*	0	0	15	6.8	0	0	18	8	0	0	10	4
8	*Drechslera hawaiiensis*	12	4	18	9.5	23.8	7	15	9.5	9.5	3	6	4
9	*Drechslera tetramera*	0	0	0	0	0	0	0	0	0	0	5	2
10	*F. moniliforme*	35	18	8	5	0	0	0	0	10	3	15	6
11	*F. semitectum*	18	6	12	4	0	0	0	0	0	0	0	0
12	*F. solani*	26.5	19	4	3	0	0	0	0	6	2	0	0
13	*Rhizopus* sp.	77	29	46	31	24	10	10	6	20.5	17.5	12	5

NST.B = Non-sterilized (seed) blotter (method), ST.B = Sterilized (seed) blotter (method), NST.PDA = Non-sterilized (seed) PDA (method), ST.PDA = Sterilized (seed) PDA (method)

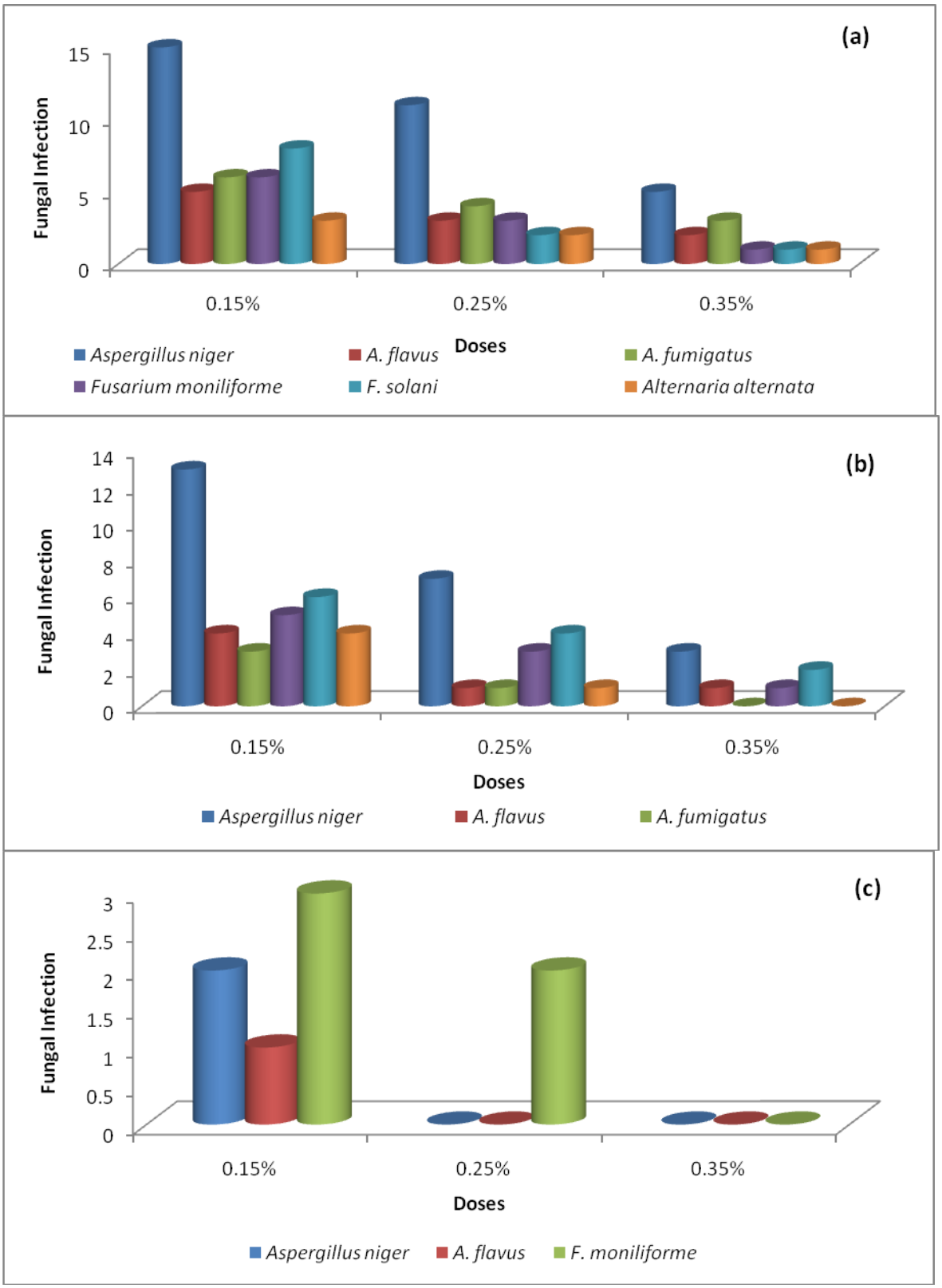

Fig. 1. Control of sabzpari variety okra seed fungi by using different essential oils, (a) = Garlic oil, (b) = Black cumin oil, (c) = Asafoetida oil

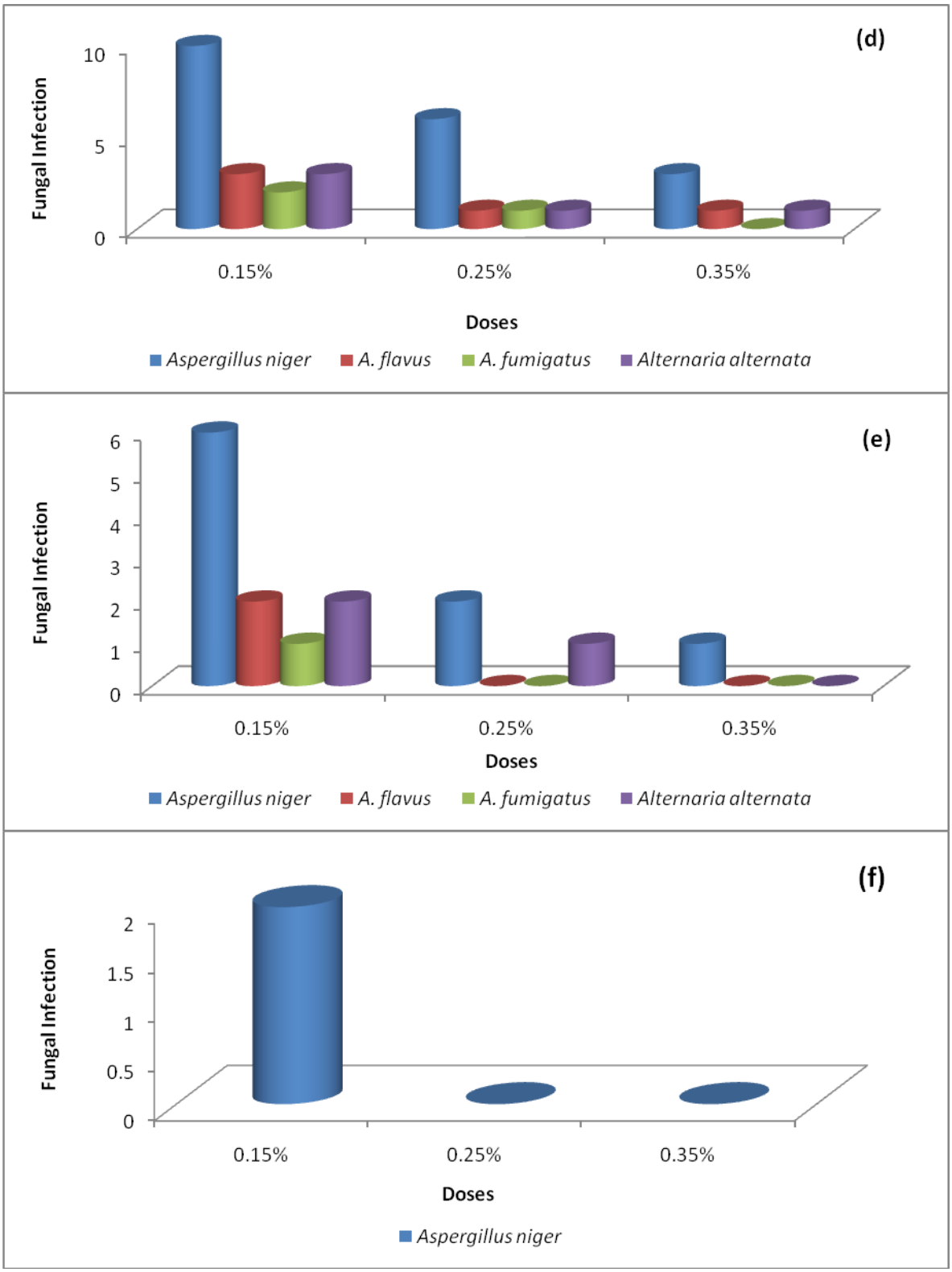

Fig. 2. Control of shazadi variety okra seed fungi by using different essential oils, (d) = Garlic oil, (e) = Black cumin oil, (f) = Asafoetida oil

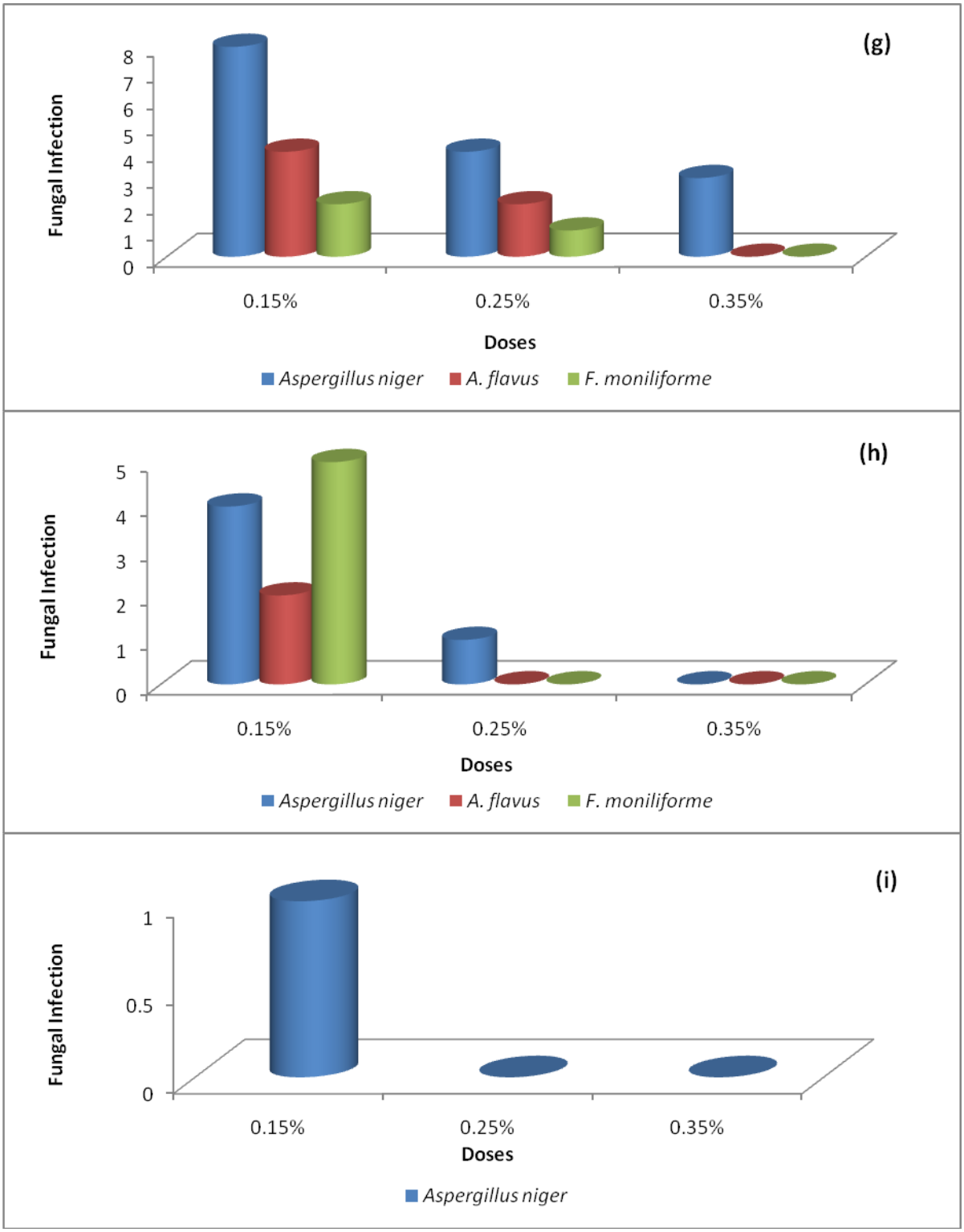

Fig. 3. Control of green star variety okra seed fungi by using different oils, (g) = Garlic oil, (h) = Black cumin oil, (i) = Asafoetida oil

Table 2. Analysis of variance of three varieties of okra.

Varieties	Source of variation	Sum of squares	Degrees of freedom	Mean squares	F value	P value
Sabzpari	Between	367.7	4	91.93	31.7	0.0001
	Error	768.2	265	2.899		
	Total	113	269			
Shazadi	Between	108.3	4	27.06	20.63	0.0001
	Error	229.5	175	1.312		
	Total	337.8	179			
Green star	between	110.1	4	27.53	24.43	0.0001
	Error	146.5	130	1.127		
	Total	256.6	134			

Nigella sativa oil was found to be effective at 0.25 and 0.35% concentration on Shazadi variety. *Nigella sativa* (0.25%) completely controlled the growth of all fungi except *A. niger* and *Alternaria alternata* which showed only 2% and 1% growth respectively; nevertheless at 0.35% concentration this oil suppressed the growth of all fungi but *A. niger* which showed 1% growth only. Among all three oils tested, asafoetida oil showed great antifungal activity against all fungi; all of its doses completely inhibited the growth of all fungi tested, however *A. niger* from shazadi variety showed 2% growth at 0.15% concentration. Asafoetida oil showed inhibitory effect on fungal growth on green star (Indian) variety; only *A. niger* showed little growth (Fig. 3). Olusanmi and Amadi (2010) evaluated the antimicrobial properties of garlic extract on three species of fungi viz, *A. flavus, Curvularia lunata* and *F. moniliforme* . *Nigella sativa* oil at 0.35% concentration also inhibited the growth of all fungi on green star variety. Shohayeb and Halawani (2012) reported the antifungal activity of *Nigella sativa* oil against *Penicillium notatum* and *Aspergillus niger*. Asafoetida oil at 0.25% and 0.35% concentration completely suppressed the growth of all fungi infesting okra seed of green star variety.

Analysis of variance to compare varieties viz., sabzpari, shazadi and green star and essential oil at 0.01 level of significance; showed significant differences (Table 2) at all concentrations i.e., 0.15 ($p<0.01$), 0.25 ($p<0.01$) and 0.35% ($p<0.01$). The result showed that in all varieties less number of fungi was isolated from shazadi variety compared to green star & sabzpari varieties concentration for all varieties. All three okra seed varieties tested were contaminated with various fungal species. These fungi are known to produce a toxin, aflatoxin which can also be a source of food poisoning for consumers. The essential oil tested against fungal species that were found associated with okra seeds were found to be effective at different concentrations against different species. The use of essential oil and perhaps other plant products can control crop seeds fungal contaminations. These practices can help increasing the agriculture productively, as well as reduction of health risk in consumers.

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