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STUDIES ON FUNGI ASSOCIATED WITH PEA SEEDS AND THEIR EFFECT ON GERMINATION AND SOME SEED CHARACTERS

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ABSTRACT: Seed borne fungi of 45 pea seed samples of Master-B cultivar were examined. These samples were collected from main production area of Egypt. A total number of 28 species, representing 19 genera of fungi was isolated and identified from pea seeds. The agar plate method was more accurate for detection of the most associated seed borne fungi compared with blotter method. Test tube agar method of 50 seeds from each of Master-B, Entsar 1, Entsar 2 and Sugar gum cultivars revealed incidence of 10 species, representing 8 fungi genera. These fungi were isolated from different seedling parts. Scanning electron microscopy (SEM) was used to examine seed borne fungi in dry seed surface of two pea cultivars Cambados (curly) and Oregon sugar (smooth).The curly cultivar hosted more fungi than the smooth one. Six categories of discoloration pea seeds were investigated using agar plate method. A total of 27 species, representing 19 genera were isolated and identified from pea seeds with different color categories. The effect of discoloration on seed characters and germination were examined. Discoloration of deteriorated seeds was associated with decreased total protein, total phenols, weight of 1000 dry seeds and seed germination percentages comparing with healthy ones. On the contrary, moisture contents in healthy seeds recorded lower percent compared with all discoloration seed categories and insect infection.

Key words: Pea-seed borne fungi, blotter method, test tube agar method, pea seed discoloration, scanning electron microscopy (SEM).

INTRODUCTION

Pea (*Pisum sativum* L.) belongs to leguminoceae family, which has an important ecological advantage for its contributes to the developments of low-input farming systems by fixing atmospheric nitrogen and it serves as a break crop which further minimizes the need for external inputs. Legumes constitute the third largest family of flowering plants, comprising more than 650 genera and 18.000 species **(Lewis *et al.,* 2005)**.

Peas are grown all over the world for its fresh use, preservation, high level of digestibility, which is more than the most of the legumes. Dried peas have been found to contain 23.5% crude protein, 1.7% ether extract and 2.9% ash **(Igbasan *et al.,* 1997).** In Egypt, pea pods are harvested for human consumption as a fresh vegetables or freezing. Legumes also accumulate natural products (secondary metabolites) such as isoflavonoids that are considered beneficial to human health through anticancer and other health-promoting activities **(Dixon and Sumner, 2003). T**here are several factors which are responsible for their low production, among them diseases which played an important role (**Nine 1986; Pal 1996**).

Sonawane *et al.* (2004) reported that seed samples of some pea cultivars collected from India were analyzed by agar plate or blotter method for the presence of seed borne fungi. *Aspergillus flavus, Aspergillus niger, Fusarium moniliforme* (*Gibberella moniliformi*s),

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F. roseum, Macrophomina phaseolina, Mucor globosus, Helminthosporium tetramera (*Cochliobolus spicifer*) and *Rhizopus nigricans* (*R. stolonifer*) were dominant. The agar plate method yielded a higher number of fungi than the standard blotter method.

Ali *et al.* (1982) tested 214 samples of commercial field pea seeds using a modified agar plate method. They found that 90% of samples were infected with *Ascochyta pinodes* (Berk and Blox) Jones, with levels of infection ranging from 1-45%, making it as the most important seed borne pathogen. Seventy two percent of seed samples were infected with *Macrophomina phaseolina* (Tassi) Goidanich with levels of infection ranging from 1-35%. Thirty-one and 24% of seed samples were infected with *Phoma medicaginis* (Jones) Boerema and *Fusarium oxysporum* Schl. f.sp. *pisi,* respectively. Only 10% of samples were free from infection. **Michall *et al.* (1998)** showed that, pea seeds considered an important source of Ascochyta blight in Egypt. The level of Ascochyta seed borne infection had an impact on disease severity of the growing plants. Seed samples with a high level of Ascochyta infection (34 and 32%) sown in cultivated soil resulted in significant blight and seed infection as well as significant reduction in seed yield of the new crop. **Ozgonen and Merve (2011)** reported that the seed mycoflora of pea were changed according to seed groups with or without surface sterilization. The most common isolated fungi were *Fusarium* spp., *Alternaria* spp., *Macrophomina phaseolina*, *Phytophthora megasperma, Rhizoctonia solani* and *Sclerotium rolfsii.* Also **Wilman (2014)** suggested that *Alternaria* spp. were the most common fungi associated with pea seed in Poland, followed by *Fusarium* spp., *Stemphylium* spp., *Ulocladium* spp., *Botrytis cinerea*, *Epicoccum nigrum* and *Phoma pinodella*. There was variation in association of fungi in different cultivars and in different season. The fodder cultivar displayed a lower infection level than edible cultivar. They concluded that *Alternaria* spp. were the most frequent fungi present in pea seeds and *Fusarium* spp. were likely the most dangerous, having in mind their established mycotoxigenic ability.

El-Wakil *et al.* (2011) used Scanning Electron Microscopy (SEM) to study fungi colonization, infection and establishment on different sesame seed parts infected with *Macrophomina phaseolina.* They clearly detected successful colonization of *M. phaseolina* to seed tissues associated with different forms of pycnidial shapes were observed.

Seed borne pathogens might cause seed abortion, seed rot, seed necrosis, reduction or elimination of germination capacity as well as seedling damage resulting in development of diseases at later stages of plant growth by systemic or local infection **(Bateman and Kwasna, 1999)**. Losses in seed quality occur during field weathering, harvesting and storage. Several factors contribute to the susceptibility for seed deterioration. The basic causes are temperature, relative humidity and seed moisture content. Invasion and tissues damage caused by microorganisms or insects. The rate of deterioration fluctuates critically from one species to another and also among varieties of the same species **(Jatoi *et al.,* 2001)**. Seed borne fungi affect adversely to nutritive value of pulses. Biodegradation of protein content of pulses by their common and dominant seed borne fungi like *Aspergillus flavus, A.fumigatus, A.niger, Drechslera tetramera*, *Fusarium moniliforme*, *Rhizopus stolonifer etc*. has been reported through artificial infestation of the pulses like green gram, black gram, chickpea and pigeon pea. Results reveal considerable degradation in protein content of the test pulses **(Kandhare, 2014)**.

The present study aims to isolate and identify the seed borne fungi associated with pea (*Pisum sativum* L.) grown under Egyptian condition, using various different methods of seed health testing. Scanning electron microscopy (SEM) was also used to study the relation between pea seed surface and contaminated fungi. The effect of natural infection by fungi in field on seeds component and germination was also studied.

MATERIALS AND METHODS

Seed Samples

Seed samples of different cultivars were collected from the major pea growing fields from Egypt Governorates including Ismailia (5 samples), Sharkia (11), Kalubiya (4), Dakahliay

(4), Dimyata (5), Behera (7) and Beni-Swef (7). In addition, other seed samples were also obtained from Horticulture Research Institute (4) and Central Administration of Seed Production (2) during 2011-2016 were included in this study. The weight of each sample was 350 gram. The samples stored in sterilized paper bags and transferred directly to the laboratory at ambient storage temperature of 28±2 for further studies.

Seed Health Testing

Detection of seed borne fungi was carried out using the following procedures which published by the International Seed Testing Association **(ISTA, 1999 and 2008)**. Seeds investigation was carried out using blotter, agar plate and test tube agar methods.

Blotter method

Forty five pea seed samples of master-B cultivar were investigated using blotter method. Eight hundred seeds of each sample were tested and divided into two groups; first group was undisinfested seeds and the second one was surface disinfected in 1% (available chlorine) sodium hypochlorite solution for 2 min followed by 3 successive rinses in sterile water. The excess water was removed by placing the treated seeds between two sterilized tissue paper until dryness. Replicates of ten seeds were plated in three moistened blotters with distilled water in 9- cm diameter sterile Petri dishes. The plates were incubated at 20 ± 2ºC for 7 days under12 hours cool white fluorescent light with alternating cycles of 12 hours darkness.

Agar plate method

The preparations and procedures were the same as for blotter method except that the medium in Petri dishes was potato dextrose agar.

Test tube agar method

In this method, symptoms can easily studied being visible on roots as well as green parts. Fifty seeds of each variety Master-B, Entsar 1, Entsar 2 and Sugar gum obtained from Horticulture Research Institute were tested for detection of seed borne fungi using test tube agar described by **Khare *et al.* (1977)**.

Isolation, Purification and Identification of Seed Borne Fungi

After seven days incubation of each previously three used method, incubated seeds were examined under a stereoscopic microscope (6-50 X magnification) to detect seed borne fungi and study their morphological characteristics. Whenever necessary, a compound microscope was used to confirm the identification. In consultation with Commonwealth Mycological Institute description sheets, Danish Government Institute of Seed Pathology publication, the fusarium laboratory manual and research work of **Gilman (1957), Barnet and Hunter (1972), Nelson *et al.* (1983), Booth (1985), Burgess *et al.* (1988), Singh *et al.* (1991) and Tadja *et al.* (2009)**were used to confirm the obtained results.

Scanning Electron Microscopy (SEM) Applied to Seed Borne Fungi Examination in Blotter Method

Techniques of fungal observation in infected seeds with light microscope and stereomicroscopy can be supplemented by alternative methods with greater precision using Scanning Electron Microscopy as a complementary methodology to identify seed borne fungi using SEM according to **Alves *et al.* (2013).**

Two experiments were carried out in the first one, dry seeds of two pea cultivars *i.e.* Cambados (curly) and Oregon sugar (Smooth) collected from greenhouse experimental were surface scanning. In the second test 400 seeds of Master-B seeds were submitted to blotter method, and then examined using (SEM -JEOL JSM 6510 IV) at Scanning Electron Microscopy Center, Fac. Agric., El-Mansoura University, Egypt. The digital images were generated, filtrated and recorded using the computer.

Percentage Incidence of Seed Borne Fungi in Six Discoloration Categories of Natural Infection Pea Seeds

Pea seed samples Master-B were visually inspected and graded into six categories according to seed discoloration: 1) Apparently healthy seeds; 2) Seeds with yellow and brown spots; 3) Seeds with untypical spots; 4) Small and malformed seeds; 5) Insect infection appearance; 6) Mechanical broken seed coat. Randomly 400 seeds were taken from each

category of discolored pea seeds and investigated for percentage incidence of seed borne fungi using potato dextrose agar method.

Effect of Seed Discoloration on Seed Germination and Characters

Total protein estimation was done by Lowry's method according to **Wadje and Baig (2006)** and total phenolic compounds were determined using the Folin-ciocalteau method **(Singleton *et al.*, 1999)** in healthy and discolored pea seed category samples. Also the germinability tests were carried out according to the international rule of seed testing **ISTA (1993).** Moisture content of seeds was determined according to the method of **AOAC (1980).** Finally the weight of 1000 seeds was recorded.

Data Analysis

The data were statistically analyzed by using Completely Randomized Design (CRD) as suggested by **Gomez and Gomez (1984)**.

RESULTS AND DISCUSSION

Blotter Method

Results in Table 1 show that a total number of 28species, representing 19 genera of fungi were isolated from pea seeds by blotter method test. The highest percentages incidence of isolated fungi from disinfested seeds were *Alternaria alternata* (1.91%), *Fusarium oxysporum* (0.93%), *Alternaria tenuis* (0.84%), *Fusarium poae* (0.69%), *Aspergillus niger* (0.36%), *Aspergillus tmarii* (0.31%), *Fusarium solani* (0.29%), *Rhizoctonia solani* (0.28%), *Acromoinum strictum* (0.27%), and *Stemphylium botryosum* (0.27%). The moderately incidence percentage of isolated fungi were *Penicillium* sp. (0.23%), *Aspergillus flavus* (0.22%), *Cladosporium herbarum* (0.22%), *Chaetomium globosum* (0.21%), *Fusarium equiseti* (0.21%) and *Sclerotinia sclerotiorum* (0.21%) while the lowest incidence percentage of isolated fungi from disinfested seeds were *Botrytis cinerea* (0.10%), *Rhizopus stolonifer* (0.09%), *Trichoderma harazinum* (0.09%), *Myrothecium* sp. (0.06%) and *Fusarium avenaceum* (0.04%). On the other hand, some fungi were not detected in disinfested seeds as *Botryodiplodia theobromae, Mucor hiemalis* and *Trichothecium* sp. The results indicated that these fungi are externally infested seeds.

Results in Table 1 also show that the highest incidence percentages of isolated fungi from undisinfested seeds were *Alternaria alternata* (Fr.)Keissler (4.02%), *Alternaria tenuis* Auct. (1.63%), *Fusarium poae* (Peck) Woll. (0.88%), *Cladosporium herbarum* (Pers.: Fr.) Link (0.87%), *Aspergillus niger* Tieg. (0.83%), *Penicillium* sp. (0.83%), *Aspergillus flavus* (Link) *Fr.* (0.79%) and *Stemphylium botryosum* Wallr.(0.68%).The moderately incidence percentage of isolated fungi were *Botryodiplodia thiobromae* (0.20%) and *Myrothecium* sp. (0.24%) while, the lowest incidence percentages of isolated fungi were *Fusarium avenaceum* (0.02%), *Fusarium equiseti* (0.04%), *Trichothecium* sp. (0.06%) and *Fusarium moniliforme* (0.06%), respectively. Similar results of isolated pea seed borne fungi were obtained by **Ali *et al*. (1982), Abdel-Hafez (1984), Czyzewska (1993), Marcinkowska (1997), Saber *et al.* (1998), Begum *et al*. (2004) and Narayan** and **Ayodhya (2013).**

Agar Plate Method

Results in Table 2 show that the highest incidence percentages of isolated fungi from disinfested seeds were *Fusarium oxysporum* (1.28%), *Alternaria alternata* (1.10%), *Trichoderma harazinum* (0.72%), *Acromoinum strictum* (0.53 %) and *Nigrospora* sp. (0.53%). The moderately incidence percentages of isolated fungi were *Alternaria tenuis* (0.49%), *Fusarium poae* (0.49%), *Epicoccum purpurascens* (0.41%), *Rhizoctonia solani* (0.39%), *Penicillium* sp. (0.36%), *Aspergillus flavus* (0.32%), respectively.

The results also showed that some fungi were not detected in disinfested seeds as *Botryodiplodia theobromae, Chaetomium globosum, Fusarium pogonea, Mucor hiemalis, Myrothecium* sp. and *Trichothecium* sp. Also results in Table 2 observed that the highest incidence percentages of isolated fungi from undisinfected seeds in agar plate method were *Alternaria alternata* (2.13%), *Rhizopus stolonifer* (1.06%), *Acromoinum strictum* (0.98%), *Trichoderma*

Table 1. Incidence of seed borne fungi in 45 seed samples of pea using blotter method

Isolatd fungi	Disinfested seeds				Undisinfested seeds			
	NSI	Occurrence (%)	Infection (%)	Range of infection	NSI	Occurrence (%)	Infection (%)	Range of infection
Acromoinum strictum	6	13.33	0.27	1-5	10	22.22	0.60	1-7
Alternaria alternata	26	57.77	1.91	1-9.5	32	71.11	4.02	3.75-15.5
Alternaria tenuis	23	51.11	0.84	1-2	24	53.33	1.63	2.75-3.5
Aspergillus flavus	5	11.11	0.22	1.5-2.75	15	33.33	0.79	0.5-4.5
Aspergillus niger	11	24.44	0.36	0.5-3	24	53.33	0.83	1-2
Aspergillus tmarii	12	26.67	0.31	0.5- 3	15	33.33	0.62	0.5-4.5
Botryodiplodia theobromae	0	0.0	0.0	0.0	3	6.67	0.20	2-4
Botrytis cinerea	5	11.11	0.10	0.75-1.5	10	22.22	0.55	0.5-5
Chaetomium globosum	8	17.78	0.21	0.5-1.5	13	28.89	0.34	0.5-2.5
Cladosporium herbarum	9	20.00	0.22	1-1.5	19	42.22	0.87	1-3
Epicoccum purpurascens	5	11.11	0.18	1-3	8	17.78	0.45	0.5-6
Fusarium avenaceum	2	4.44	0.04	1.0	1	2.22	0.02	1.0
Fusarium equiseti	8	17.78	0.21	0.5-3.25	2	4.44	0.04	0.5-1.5
Fusarium moniliforme	7	15.56	0.16	0.5-1.5	3	6.67	0.06	0.5-1.75
Fusarium oxysporum	13	28.89	0.93	1-4.5	12	26.67	0.39	1-2.25
Fusarium poae	11	24.44	0.69	1-5.25	16	35.55	0.88	0.5-5.5
Fusarium pogonea	5	11.11	0.11	0.25-1.5	10	22.22	0.29	0.5-2.25
Fusarium solani	9	20.00	0.29	0.5-3	8	17.78	0.33	0.75- 3
Mucor hiemalis	0	0.0	0.0	0.0	5	11.11	0.51	4-5
***Myrothecium* sp.**	3	6.67	0.06	0.5-1	6	13.33	0.24	0.75-2.75
***Nigrospora* sp.**	5	11.11	0.18	0.5-3	10	22.22	0.36	0.75-3.5
***Penicillium* sp.**	7	15.56	0.23	1-4	16	35.55	0.83	1-7
Rhizoctonia solani	9	20.00	0.28	0.5-2.75	15	33.33	0.47	0.5-3
Rhizopus stolonifer	3	6.67	0.09	1-2	10	22.22	0.81	1.25-12.5
Sclerotinia sclerotiorum	7	15.56	0.21	0.75-3	7	15.56	0.29	1-4
Stemphylium botryosum	12	26.67	0.27	0.25-4	14	31.11	0.68	0.75-6
Trichoderma harazinum	5	11.11	0.09	0.5-1	5	11.11	0.53	1.25-8
***Trichothecium* sp.**	0	0.00	0.0	0.0	4	8.89	0.06	0.5-1
LSD at 0.05%			0.08				0.12	

Total number of samples=45 NSI= Number sample infected

Occurrence (%) = Number sample infected×100/45 Infection (%) = Total of infected seeds /400×100

Table 2. Incidence of seed borne fungi in 45 seed samples of pea using agar plate method

Isolated fungi	Disinfected seeds				Undisinfested seeds			
	NSI	Occurrence (%)	Infection (%)	Range of infection	NSI	Occurrence (%)	Infection (%)	Range of infection
Acromoinum strictum	8	17.78	0.53	2- 6	9	20.00	0.98	2.5-9
Alternaria alternata	18	40.00	1.10	1- 7	28	62.22	2.13	1-8.75
Alternaria tenuis	19	42.22	0.49	0.75-1.5	23	51.11	0.86	0.75-2.75
Aspergillus flavus	7	15.56	0.32	0.75-4	9	20.00	0.58	0.75-6
Aspergillus niger	11	24.44	0.32	0.5-3	22	48.89	0.53	0.5-2
Aspergillus tmarii	5	11.11	0.18	0.25-4	13	28.89	0.67	0.5-3.75
Botryodiplodia theobromae	0	0.0	0.0	0.0	8	17.78	0.15	0.25-3
Botrytis cinerea	4	8.89	0.18	1-3	9	20.00	0.61	1.75-4
Chaetomium globosum	0	0.0	0.0	0.0	5	11.11	0.10	0.5-1.5
Cladosporium herbarum	3	6.67	0.09	1-1.5	14	31.11	0.53	1-4
Epicoccum purpurascens	8	17.78	0.41	0.5- 9	5	11.11	0.37	1.75-6
Fusarium avenaceum	4	8.89	0.07	0.5- 1	2	4.44	0.03	0.5-1
Fusarium equiseti	6	13.33	0.12	0.5-1	1	2.22	0.01	0.5
Fusarium moniliforme	3	6.67	0.06	0.5 -1.75	3	6.67	0.15	1- 3
Fusarium oxysporum	17	37.78	1.28	0.5 -7	13	28.89	0.75	1.5-5
Fusarium poae	10	22.22	0.49	1 -5.25	8	17.78	0.20	0.5-4
Fusarium pogonea	0	0.0	0.0	0.0	2	4.44	0.06	0.5-2
Fusarium solani	5	11.11	0.10	0.5-1	3	6.67	0.09	1.25-2
Mucor hiemalis	0	0.0	0.0	0.0	3	6.67	0.78	5-25
Myrothecium **sp.**	0	0.0	0.0	0.0	3	6.67	0.07	1.0
Nigrospora **sp.**	10	22.22	0.53	0.5-7	11	24.44	0.57	1.5-4.5
Penicillium **spp.**	8	17.78	0.36	0.5-5	15	33.33	0.81	0.75-8
Rhizoctonia solani	14	31.11	0.39	0.5-2.5	15	33.33	0.34	0.5-3
Rhizopus stolonifer	4	8.89	0.22	2.5	8	17.78	1.06	2.5-12.5
Sclerotinia sclerotiorum	7	15.56	0.26	0.5-6	7	15.56	0.48	1.5-8
Stemphylium botryosum	7	15.56	0.29	0.5-6	11	24.44	0.77	1- 5
Trichoderma harazinum	5	11.11	0.72	3 - 8	6	13.33	0.91	1-10
Trichothecium **spp.**	0	0.0	0.0	0.0	7	15.56	0.21	0.5-2.75
LSD at o.05%			0.06				0.17	

Total number of samples=45 NSI= Number sample infected

Occurrence (%) = Number sample infected×100/45 Infection (%) = Total of infected seeds /400×100

harazinum (0.91%), *Alternaria tenuis* (0.86%), *Penicillium* sp., (0.81%), *Mucor hiemalis* (0.78%), *Stemphylium botryosum* (0.77%), *Fusarium oxysporum* (0.75%), *Aspergillus tmarii* (0.67%), *Botrytis cinerea* (0.61%), *Aspergillus flavus* (0.58%), *Nigrospora* sp. (0.57%), *Aspergillus niger* (0.53%) and *Cladosporium herbarum* (0.53%) while, the lowest incidence percentages of the isolated fungi from undisinfested seeds in agar plate method were *Fusarium equiseti* (0.01%), *Fusarium avenaceum* (0.03%), *Fusarium pogonea* (0.06%), *Myrothecium* sp. (0.07). Similar results of isolated pea seed borne fungi were obtained by **Sonawane *et al.* (2004)**.

In general results in Tables 1 and 2 show that blotter and agar plate method revealed the same fungal species, 28 species, representing 19 genera of fungi isolated from pea seeds.

In addition the agar plate method was more accurate for detection of most isolated seed borne fungi percentages on pea seeds comparing with blotter one. These results are in agreement with the findings of **Gill *et al.* (1983)** on some Nigerian leguminous seeds, **Abdel-Al (1994)** on alfalfa seeds, **Shakir and Mirza (1994)** on chickpea seed, **Solanke *et al.* (1997)** on soybean seeds, **Godika *et al.* (1999)** on sunflower, **Sonawane *et al.* (2004)** on pea and **Shaker *et al.* (2010)** who suggested that nutrients from the media might play an important role in initiation of growth of fungi on pulses. We suggested that seed leaching as a removal substances from seeds in blotter method such as sugars, amino acids and other chemicals played a very important roles in encourage or suppression the fungus growth mycelium and spore germination. Also **Singh *et al.* (2017)** reported that legumes are a good source of bioactive phenolic compounds which played significant roles in many physiological, as well as, metabolic processes. Phenolic acid, flavonoids and condensed tannins are the primary phenolic compounds that are present in legume seeds which affected on recovery fungi. On the contrary, other research workers reported that blotter method test was found superior in isolation of more number of fungal colonies over agar plate one. These results are in the same trend of **Dawar (2005)** on chickpea seeds, **Tariq *et al.* (2005) and Venugopal *et al.* (2015)** on soybean.

Blotter method was preferable for detecting some seed borne fungi with the percentage of infection in disinfested and undisinfested seeds, respectively such as *Alternaria alternata* (1.91-4.02), *Alternaria tenuis* (0.84 -1.63), *Aspergillus niger* (0.36-0.83), *Chaetomium globosum* (0.21-0.34), *Cladosporium herbarum* (0.22-0.87), *Fusarium poae* (0.69-0.88), *Fusarium solani* (0.29-0.33), *Myrothecium* sp. (0.06-0.24) respectively. Also, the obtained results showed that blotter and agar plate tests could not be used singly for seed borne fungi detection but more than one method must be used. Results of this study were in accordance with those obtained on soybean and four other crops by **Agarwal *et al.* (1972),** on lucerne **(Singh and Gupta, 1984)**, on soybean **(El-Gantiry 1985),** on alfalfa **(Abdel-Al, 1994)** and **Rathod *et al.* (2012)** on groundnut seeds.

The most characterized feature observed is that total count of fungi, and number of infected samples was slightly higher in undisinfested samples than disinfested samples in both tested methods and are in agreement with findings of **Ozgonen and Merve (2011) and Dawar *et al.* (2015).**They reported that seed mycoflora were changed according to seed groups, with or without surface sterilization. On the other hand, *Fusarium* spp. were detected in disinfested seeds of pea in both tested methods at the highest rate compared with undisinfested seeds. The removal of externally seed borne fungi by surface disinfestation with 1% (available chlorine) sodium hypochlorite solution for 2 min proved to be suitable method for isolating the internally seed borne fungi of pea. These results are in agreement with finding of **Perveen Shahida and Abdul Ghaffar (1995)** who proved that surface disinfestation provides a chance for the internally seed borne fungi to appear in greater number, also with the recommended by **(ISTA, 1993)**.The recovery of most isolated fungi in blotter and agar plate method even after surface sterilization of seeds indicated the presence of these fungi inside as well as on the surface of the seeds. The results are in agreement with findings of **Ozgonen and Merve (2011) and Ramesh *et al.* (2013)**.

Test Tube Agar Method

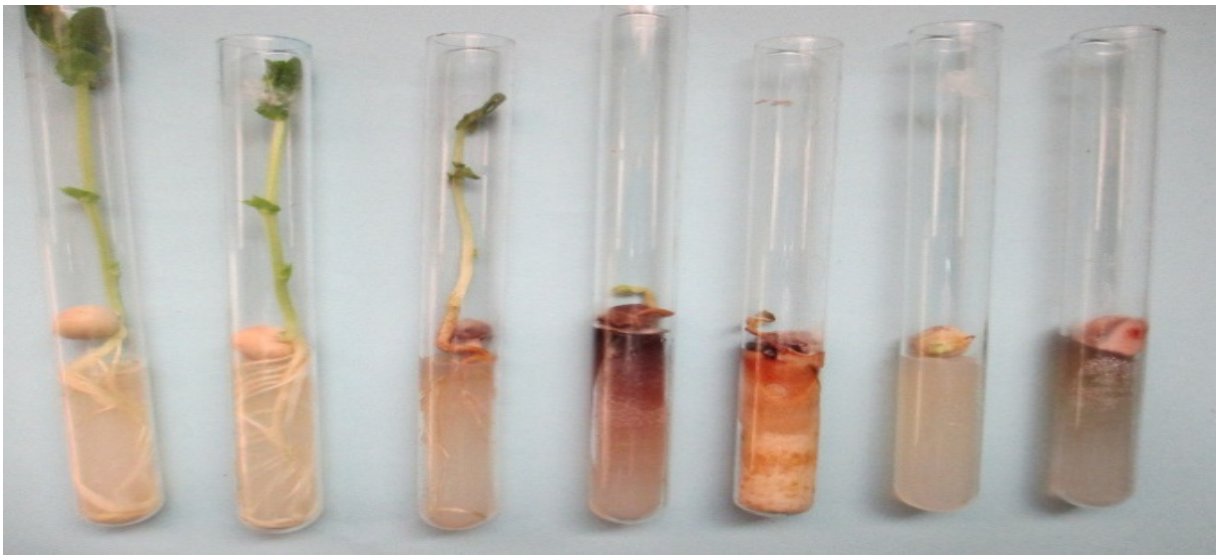
Results in Table 3 show that 10 species, representing 8 genera of fungi, were isolated from

Table 3. Isolated fungi from undisinfested pea seed cultivars as healthy seeds, seeds rot and seedling blight using test tube agar method after 14 days

Cultivar and isolated fungi	Healthy-looking seedlings (%)	Seed rot (%)	Seedling blight (%)	Recovery fungi (%)		Percentage of recovery fungi
				Seed rot	Seedling blight	
Master-B	80	14	6	-	-	-
Alternaria alternata	-	-	-	4	2	6
Stemphylium botryosum	-	-	-	2	0	2
Aspergillus flavus	-	-	-	6	0	6
***Cladosporium* sp**	-	-	-	0	2	2
***Epicoccum* sp.**	-	-	-	2	0	2
Fusarium solani	-	-	-	4	0	4
Entsar 1	90	6	4	-	-	-
Alternaria alternata		-	-	2	2	4
***Cladosporium* sp**	-	-	-	0	2	2
Aspergillus niger	-	-	-	6	0	6
***Nigrospora* sp.**	-	-	-	2	0	2
Entsar 2	96	4	2	-	-	-
Alternaria alternata	-	-	-	4	2	6
***Cladosporium* sp**	-	-	-	0	2	2
Rhizctonia solani	-	-	-	6	0	6
Sugar gum	88	10	2	-	-	-
Alternaria alternata	-	-	-	0	2	2
***Cladosporium* sp**	-	-	-	0	2	2
Aspergillus niger	-	-	-	4	0	4
Fusarium oxysporum	-	-	-	6	0	6
Fusarium solani	-	-	-	2	0	2
Total percentage	-	-	-	50	16	66

different seedling parts of four pea cultivars, on water agar medium. The total recovery fungi percentage from rotted seeds were (50%) while from seedling blight were (16%).The rotted seeds percentages in Master-B, Entsar1, Entsar2 and Sugar gum were 14,6,4 and 10% while seedling blight percentages were 6, 4, 2 and 2%, respectively.

The results shown in (Fig.1-a,b) show healthy seedling developed from healthy seeds. Meanwhile, natural infected seedling might escape and survival (Fig. 1-c) and heavily infected seeds with *Fusarium* spp. and *Rhizoctonia* sp. were recovered and germinate (Fig. 1-d,e,f) and the seedling died at the early stage of the plant growth also Fig. (1-g) show



1. a 1. b 1. c 1. d 1. e 1. f 1. g

Fig. 1a-g. Pea seedling (14 days old) developing in test tube agar method showing different symptoms of diseases resulting from naturally infected seeds

heavily infected seed that covered with the *Fusarium* spp. growth and failed to germinate. These results are agreement partialy with those obtained by **El-Gantiry (1985)** and **Abdel-Al (1994).** In test tube agar method seed germination and seedling development under controlled conditions both of infected seed and seedling may develop symptoms comparable to those developed under field conditions and provide valuable information pertaining to field performance of the sowing seeds.

Scanning Electron Microscopy (SEM) Applied to Examine Seed Surface and its Relation to Infected Fungi

Two experiments were carried out using SEM. In the first one, dry seeds of two pea cultivars Cambados (curly) and Oregon sugar (Smooth) were scanned to investigate the seed surface. Fig. 2-a of smooth pea seed Oregon sugar cultivar show less loads of fungal spores and fragments of fungal mycelia compared with curly pea seed Cambados cultivar (Fig. 2-b). Although the Oregon sugar seeds have cracks and ruptures that might be have more number of fungal fragments and spores. These results might be contributed in primary indicator about seed contaminated surface with fungus fragments and spores in addition smooth seeded peas do not exude as much carbohydrate and inorganic salts as do wrinkled seed peas and are thus less susceptible to seed and seedling infection **Kraft (1991)**. We suggest that scanning electron microscopy of dry seeds might be more provide when investigating seeds for biotrophic fungi as rust, powdery mildew and downy mildew spores density. These results are in agreement with finding of **Machado (2002).** He reported that the groups of fungi are biotrophic and necrotrophic and specific or more selective methods are required for their reliable detection in routine analysis of various formae specialis of fungi such as *Fusarium* spp., *Colletotrichum* spp., *Phomopsis* spp. Thus, considering the advantages of SEM related characteristics such as increase, fast image digitalization and acquisition, easiness of preparation and operation of samples, as well as relatively accessible costs, this approach might became a viable contribution to decision support in routine seed health analysis.

In the second test, 400 pea seeds of Master-B cultivar submitted to blotter method. Seeds were subject to conditions that enable pathogen growth and expression and then prepared and observed with SEM. The images of some recovering fungi were generated as Fig. 2-c *Fusarium* sp., presenting macroconidia, heads and

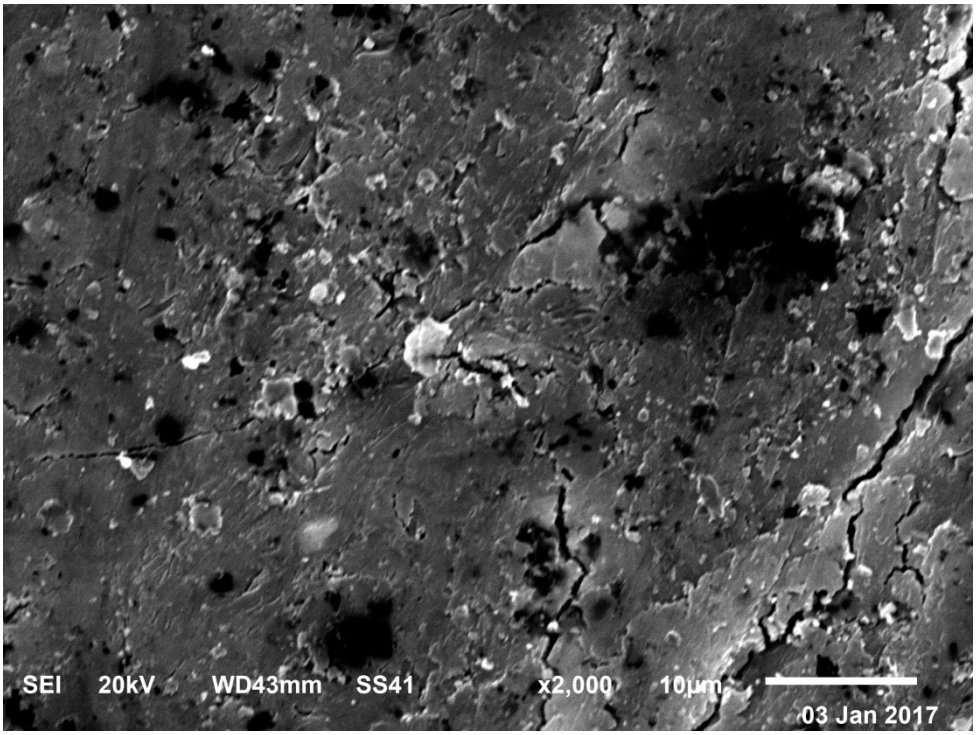


Fig. 2-a. Spherical and smooth pea seed surface (Oregon cultivar) 2000X

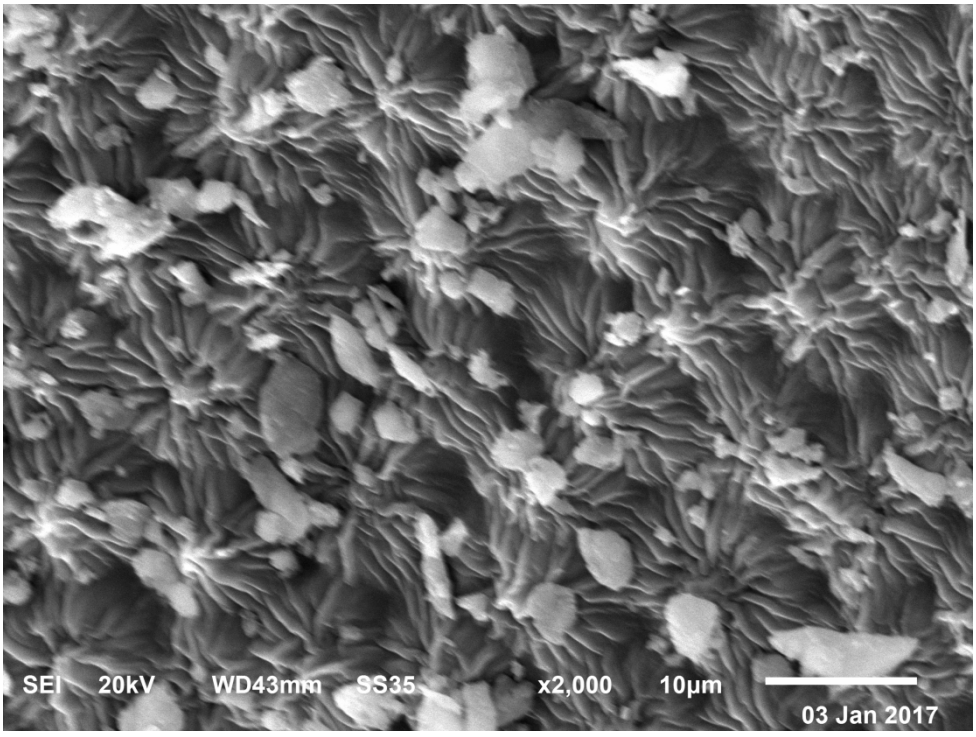


Fig. 2-b. Rough and curved pea seed surface (Cambados cultivar) 2000X

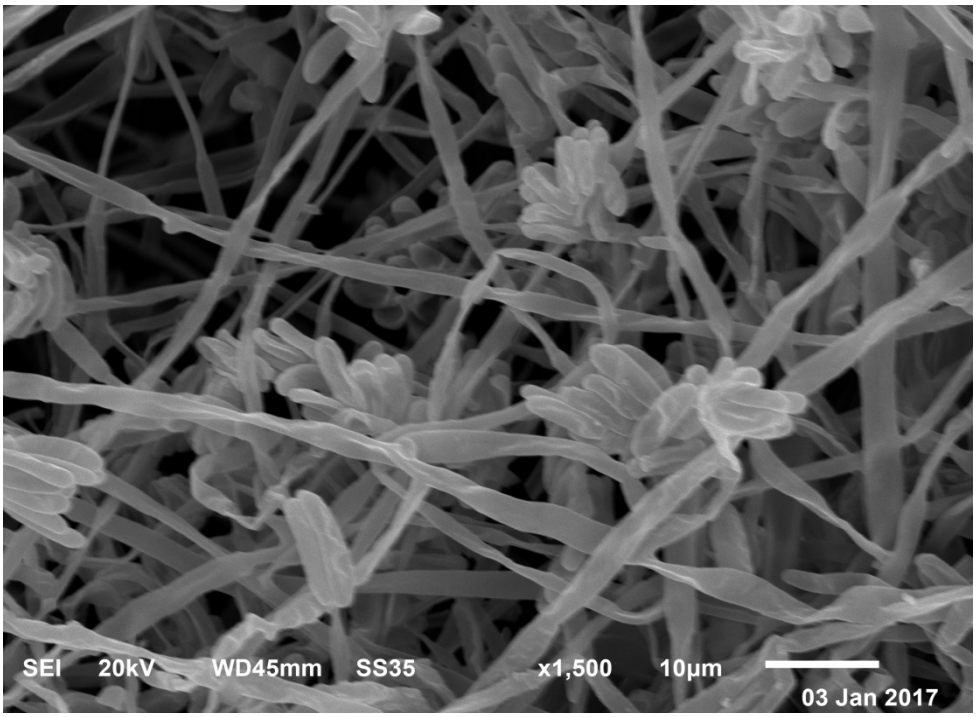


Fig. 2-c. Scanning electron photograph of pea seed surface used in laboratory diagnosis of *Fusarium* sp., presenting macroconidia, heads and conidium at the phialide apex (1500X)

conidium at the phialide apex. (Fig. 2-d) *Botryodiplodia theobromae*, presenting mycelium and pycnido spores (Fig. 2-e) *Myrothecium* sp. presenting colony and mycelium. Similar results were obtained on seeds of cotton, common bean and maize by **Alves and Pozza (2009) and Alves *et al.* (2013)**.

The Incidence of Seed Borne Fungi on Different Discoloration Categories of Pea Seeds

Results in Table 4 indicate that a total of 27 species, representing 19 genera were isolated from pea seeds with different color (Fig. 3). The isolated fungi could be arranged according to percentages of frequency from healthy seeds as follows; both *Alternaria alternata* and *Fusarium oxysporum* (5.0%), *Aspergillu flavus* (3.0%), *Alternaria tenuis* (2.0%), *Fusarium solani* (1.25%), both *Cladosporium herbarum, Fusarium equiseti* and *Fusarium poae* (1.0%), *Trichoderma harazinum* (0.75%) and *Nigrospora* sp. (0.25%). The most prevalent in seeds with dark brown and yellow spots were *Alternaria alternata* (8.0%), *Alternaria tenuis* (6.5%), *Sclerotinia sclerotiorum* (5.50%), *Fusarium oxysporum* (3.5%) and *Chaetomium globosum* (3.0%). The most prevalent in seeds with untypical spots were *Alternaria alternata* (15.0%), *Alternaria tenuis* (10.0%), *Sclerotinia sclerotiorum* (5.25%), both *Fusarium moniliforme* and *Fusarium oxysporum* (3.5%).The most prevalent in seeds with Small and malformed seeds were *Fusarium oxysporum* (7.0%), *Nigrospora* sp. (4.0%), *Alternaria alternata* (3.5%), both *Acromoinum* sp. and *Fusarium solani* (3.0%), *Alternaria tenuis* (2.75%), both *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (2.5%). The most prevalent in seeds with insect infection were *Alternaria alternata* (7.5%), *Aspergillu flavus* (7.0%), *Sclerotinia sclerotiorum* (5.0%), *Alternaria tenuis* (4.75%), *Nigrospora* sp. (3.0%), and *Rhizopus stolonifer* (2.50%).

The most prevalent associated fungi, in seeds with mechanical broken seed coat, were *Aspergillu flavus* (5.50%), both *Alternaria tenuis* and *Sclerotinia sclerotiorum* (5.0%), *Alternaria alternata* (4.5%), *Fusarium oxysporum* (3.75%), *Aspergillus niger* (2.75%) and *Rhizopus stolonifer* (2.25%). Similar results have also been reported by **Czyzewska (1983)**. He stated that *Ascochyta* spp. produce distinctive

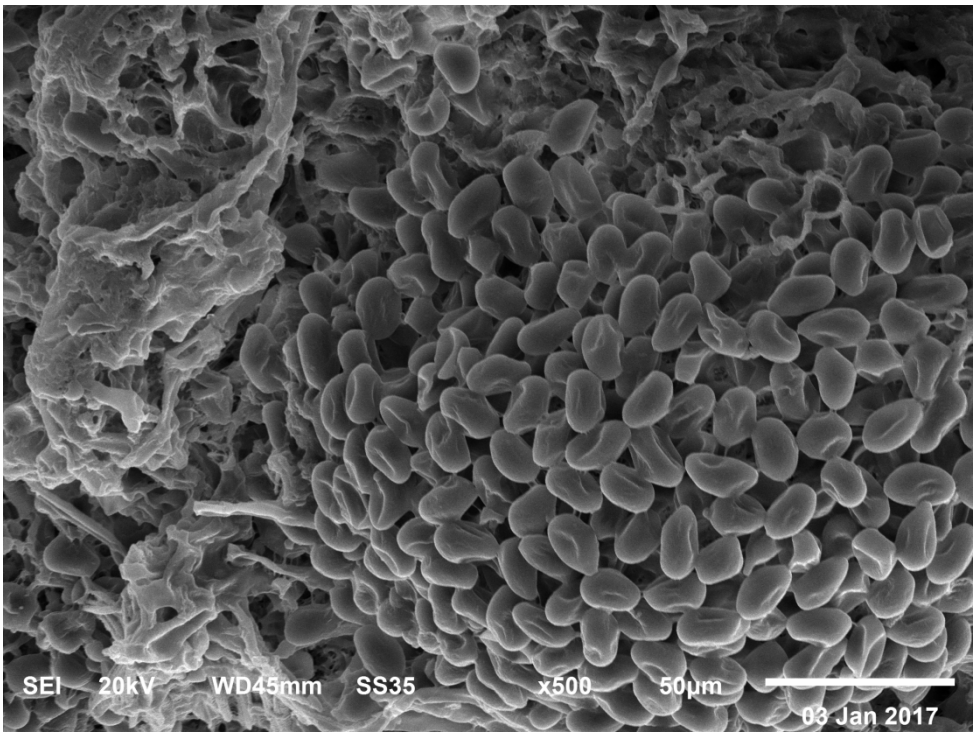


Fig. 2-d. Scanning electron photograph of pea seed surface used in laboratory diagnosis of *Botryodiplodia theobromae* presenting mycelium and pycnido spores (500X)

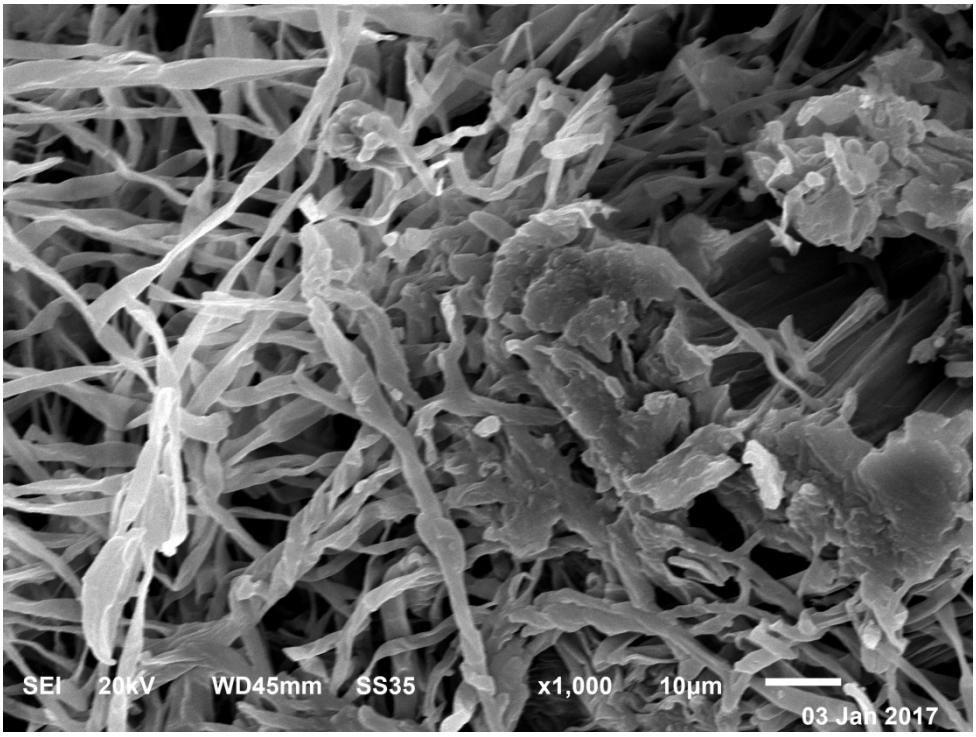


Fig. 2-e. Scanning electron photograph of pea seed surface used in laboratory diagnosis of *Myrothecium* sp. presenting colony and mycelium (1000X)

Fig. 3. Categories of discoloration pea seeds (6x)

Table 4. Percentage of seed borne fungi isolated from healthy and discolored seeds of pea Master-B cultivar using potato dextrose agar plate method

Isolated fungi	Seeds color / (%) of the isolated fungi					
	Healthy seeds	Dark brown and yellow spots	Untypical spots	Small and malformed seeds	Insect infection	Mechanical broken seed coat
Acromoinum sp.	0.00	0.75	1.25	3.00	2.00	1.50
Alternaria alternata	5.00	8.00	15.00	3.50	7.50	4.50
Alternaria tenuis	2.00	6.50	10.00	2.75	4.75	5.00
Aspergillu flavus	3.00	0.75	1.25	0.00	7.00	5.50
Aspergillus niger	0.00	1.50	0.75	0.00	2.50	2.75
Aspergillus fumigatus	0.00	0.50	1.00	0.50	2.50	1.00
Botryodiplodia theobromae	0.00	0.50	0.50	0.00	0.50	0.50
Botrytis cinerea	0.00	1.50	1.25	0.75	1.00	0.00
Chaetomium globosum	0.00	3.00	2.50	0.25	0.00	0.00
Cladosporium herbarum	1.00	2.25	1.00	0.50	1.50	1.00
Epicoccum sp.	0.00	2.25	1.75	0.00	1.25	1.25
Fusarium equiseti	1.00	1.00	1.75	2.00	0.00	1.00
Fusarium moniliforme	0.00	2.5 0	3.50	0.75	1.50	1.25
Fusarium oxysporum	5.00	3.50	3.50	7.00	0.00	3.75
Fusarium poae	1.00	0.50	0.75	0.75	0.00	0.00
Fusarium pogonea	0.00	0.25	0.00	0.25	0.25	0.25
Fusarium solani	1.25	1.2 5	1.00	3.00	0.50	0.50
Mucor hiemalis	0.00	2.00	1.50	0.00	5.00	0.00
Myrothecium sp.	0.00	0.50	0.25	0.00	0.50	0.25
Nigrospora sp.	0.25	1.50	2.25	4.00	3.00	1.00
Penicillium sp.	0.00	1.25	0.25	0.00	2.00	0.50
Rhizoctonia solani	0.00	1.50	1.25	2.50	0.7 5	1.50
Rhizopus stolonifer	0.00	1.25	1.00	1.00	2.50	2.25
Sclerotinia sclerotiorum	0.00	5.50	5.25	2.50	5.00	5.00
Stemphylium botryosum	0.00	1.00	0.75	0.00	1.50	0.00
Trichoderma harazinum	0.75	2.00	0.00	0.00	0.00	0.00
Trichothecium sp.	0.00	1.50	0.25	1.25	0.00	0.25
LSD at 0.05%	0.07	0.22	0.59	0.29	0.14	0.44

spots on the seeds. *Alternaria tenuis* produce untypical spots, while, *Fusarium, Botrytis, Sclerotinia, Rhizoctonia* fungi do not produce spots in pea seeds.

Results also showed that damaged seeds are more infected with saprophytic fungi than other seeds categories. These results are in agreement with finding of **Kochler (1957).** He observed that damaged seeds were much more susceptible to saprophytes and pathogens, than normal seeds during both storage and under field conditions.

Effect of Discoloration on Seed Characters and Germination

The results in Table 5 show losses in total protein, total phenols, weight of 1000 dry seeds and seed germination percentages in all discoloration seeds categories comparing with healthy seeds. On the contrary, moisture contents in healthy seeds record the lowest percent comparing with all seeds discoloration categories. Also, Fig. 4 show the effect of seed infection by pathogenic fungi in field and its effects on discoloration and morphology. These results are in agreement with **Quenton *et al.* (2003)** and **Castillo *et al.* (2004).** They explained that the biodeterioration of seeds due to many fungi which parasites on seeds during primordial, maturing and stored. Invasion of seeds can resulted in various damage including, reduce yields of seed, in both quantitatively and qualitatively, discolorations, decreases germinibility, mycotoxin production and total decay.

Table 5. Effect of infection and discolored pea seeds on some quality and component characteristics

Seeds color of Pea	Total protein (%)	Total phenols (mg/g)	Weight of 1000 dry seeds (g)	Moisture content (%)	Seed germination (%)
Apparently health seeds	33.20	1.50	335.8	13.8	100
Dark brown and yellow spots	25.25	0.71	310.4	14.5	60
Seeds with untypical spots	20.45	0.93	296.7	14.3	65
Small and malformed seeds	25.75	1.42	135.5	14.0	12
Insect infection	0.16	0.33	260.3	14.8	25
Mechanical broken seed coat	0.26	0.67	293.7	14.4	5
LSD at 0.05	1.67	0.09	7.42	0.58	3.71

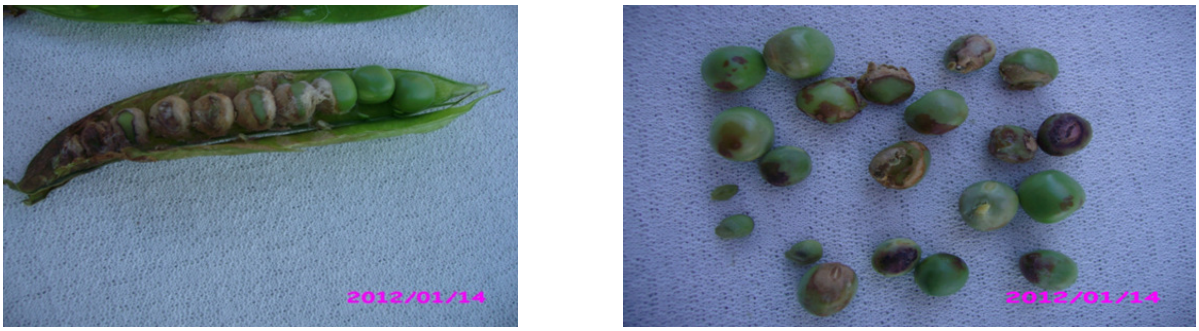


Fig. 4. Discolored pea seeds with different natural fungal infection collected from felids (6x)

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دراســـات على الفطـــريات المصـــاحبـة لبـذور البسلة وتأثيـرها على إنبـات البـذور وبعض صفاتهـا

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هدفت الدراسة إلى تحديد الفطريات المصاحبة لبذور البسلة فى مصر وتأثيرها على حيوية البذور وصفاتها بأستخدام طريقتى أوراق الترشيح المبللة وأطباق الأجارلبيئة مستخلص البطاطس والدكستروز تم فحص ٤٥ عينه من بذور البسلة صنف ماستر-بى تم تجميعها من المناطق الأكثر زراعة فى الوجة البحرى فى مصر، تم فى كل طريقة فحص وأختبار ٨٠٠ بذرة من كل عينة وتم تعقيم نصفها بهيبوكلوريت الصوديوم ١% لمدة دقيقتين، والنصف الآخر لم يتم تعقيمه، تم تعريف ٢٨ نوعا من الفطريات المعزولة ووجد أنها تابعة إلى ١٩ جنس من الفطريات، ووجد أن طريقة أطباق الآجار أعطت نسبة مئوية أعلى للفطريات المعزولة عن طريقة أوراق الترشيح المبللة، إلا أن طريقة أوراق الترشيح كانت أكثر كفاءة فى بعض الفطريات مثل الترناريا الترناتا والترناريا تينس وأسبرجلس نيجر وكوتوميوم قلبوسم وفيوزاريوم بيوا وفيوزريوم سولانى وميروسيسيوم، وبأستخدام طريقة الآجار فى الأنابيب تم أختبار وفحص ٥٠ بذرة من كل صنف من الأصناف (ماستر-بى، انتصار ١، انتصار ٢، وشجر جم)، وتم عزل عشرة انواع من الفطريات تابعة لثمانيه أجناس فطرية من جميع أجزاء البادرات، تم أستخدام الميكرسكوب الألكترونى لفحص السطح الخارجى لصنفين من أصناف بذور البسلة الجافة، ووجد أن البذور المجعدة للصنف كومبادوس تحمل جراثيم وقطع ميسليوم أكثر من البذور الملساء للصنف أورجينو، كذلك تم فحص البذور بعد تحضينها بطريقة ورق الترشيح المبللة وتصويرها على الكمبيوتر الملحق بالميكرسكوب الألكترونى، أيضاً تم فحص ٦ أقسام من البذور الملونة بعد فحصها ظاهريا بأستخدام طريقة أطباق الآجار، وقد تم عزل ٢٧ نوعا من الفطريات تابعة لتسعه عشر جنسا فطرياً، أيضا تم دراسة تأثير الإصابة بهذه الفطريات على إنبات البذور وبعض صفاتها ووزن الألف بذرة، حيث وجد أن جميع أقسام البذور المصابة والملونة قد أنخفضت نسبة الإنبات بها وكذلك محتواها من البروتين والفينولات عن البذور السليمة، وعلى العكس من ذلك زادت نسبة الرطوبة بها عن البذور السليمة.

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