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Original Paper

Seed borne fungal pathogens associated with common bean (*Phaseolus vulgaris* L.) seeds and their impact on germination

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Abstract
Seed-borne fungi of bean are a serious problem worldwide causing damping-off and wilt diseases on common bean (*Phaseolus vulgaris* L.) plants. Several pathogenic fungal were isolated from seed samples collected from commercial markets in Egypt (Pronco, Nebraska, Giza 3, Giza 6 and Tema). *Fusarium oxysporum* and *Fusarium solani* were the common fungi isolated from seeds followed by *Alternaria* sp., *Rhizoctonia solani*, *Helminthosporium* sp. and *Penicillium* spp. Pathogenicity test indicated that, *F. oxysporum* and *R. solani* isolates were the most fungal isolates significantly induced damping off on bean plants. Bean seeds treated with peppermint oil caused a highly reduction in the infection and reduced fungal transmission from seeds to seedlings. Furthermore, vigor of bean seedlings raised from the treated seeds was better than that developed from the untreated ones which was reflected in the improved properties of the plant and increased the crop in the future.
Keywords: Common bean, Essential oil, *Fusarium* spp., Peppermint, Seed-borne fungi.

Introduction:

Common bean (*Phaseolus vulgaris* L.) is one of the most important vegetable crops grown in Egypt, that occupies a great figure in local consumption and export. The area covered by common bean production alone in Egypt is estimated to be more than 196910 Fadden (Agriculture Directorates of Governorates 2010-2011). Seed is the most important input for crop production. Pathogen free healthy seed is urgently needed for desired plant populations and good harvest. About 16% annual crop losses due to plant diseases, at least 10% loss is incurred due to seed-borne diseases (Fakir, 1983). The most common seed borne fungi on dry beans (*P. vulgaries*) were *Alternaria* spp. *Asperagillus* spp., *Fusarium* spp., *Botrytis* spp., *Chaetomium* spp., *Penicillium* spp., *Rhizopus* spp., *Cladosporium* spp. and *Trichothecium* spp. (Domijan *et al.,* 2005).

These fungi are transmitted by seeds and can be preserved as conidia in the coat or as mycelia at the seeds surface (Gargouri *et al*., 2000). In Egypt, the main pathogens responsible for damping-off and wilt incidence of bean are *R. solani* (Kühn) and *F. oxysporum* f. sp. phaseoli, respectively (El-Mougy *et al*., 2007).

Association of seed-borne fungi causing several diseases in seeds was reported in Cucumber (*Macrophomina phaseolina*) (Nasreen *et al*., 2009), Watermelon (*Fusarium* spp.) (Boughalleb and Mahjoub, 2006).

Analysis of seed infection level is a valid investigation tool to foresee the disease development transmitted by seeds (Taylor *et al*., 2001). Few studies were done on the localization of theses pathogens on seeds (Michail *et al*., 2002). Many seed borne fungi were generally managed by synthetic chemicals, which were considered both efficient and effective. Hence in recent time,

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application of plant metabolites for plant disease management has become important viable component of Integrated Pest Management, as plant metabolites are eco-friendly where botanicals place an important role (Sahayaraj *et al*., 2009). During our regular screening, highly significant activity for some essential oils were recorded, since some plants are already known to posses several biological activities.

The objectives of this study were: 1) to isolate the fungi associated with bean seeds, 2) to determine the infection level for each seed lot by fungi, 3) to introduce manual seed cleaning and seed treating with essential oil vapors on the *in vitro* for reducing infestation level.

Materials and Methods:

The present investigation was conducted from 2009-2012 in the Departments of Horticulture and Agricultural Microbiology, Faculty of Agriculture, Sohag University, Egypt. Common Bean seed samples (Pronco, Nebraska, Giza 3, Giza 6 and Tema) were collected from commercial lots used for sowing in farms. Seeds were stored at room temperature (25 ± 2ºC) until using and further they were subjected to initial seed health testing.

Seed infection evaluation:

Whole seed test:

Bean seeds were disinfected by soaking for 1 min in 1 % sodium hypochlorite. Initial seed health testing was conducted by using agar plate method. Seeds were placed at the rate of 10 seeds per Petri plate containing 20 ml of potato dextrose agar (PDA). Plates were incubated for seven days. After seven days of incubation, the fungal colony growth was examined under stereo-binocular microscope (Khare, 1996).

Location of the pathogen in the seed:

The location of the pathogen in the seed was studied by employing component plating technique. Seeds were washed four times with tap water, then surface sterilized in one percent sodium hypochlorite solution as mentioned above. These seeds were washed again with sterile water and soaked in water for 30 min. On the other hand, other seed samples were used without sterilization, just soaked in water for 30 min. Seeds were dissected aseptically using sterile needle and forceps. The separated seed parts viz., seed coat, cotyledon and embryo were plated immediately before drying on plates contained. The plates were incubated at 25± 2^{o}C for seven days. Then, they were examined under stereo-binocular microscope for the presence of the pathogen in different seed parts.

The number of contemned parts was counted every 3 days for period 15 days. The infection level of each part was evaluated according to the following formula: Infection level (%) = total number of infected seeds x 100 / total number of tested seeds. The fungal colonies whose developed around every seed part were replicated on PDA (potato dextrose agar) added with sulphate streptomycin.

Isolates used and their preservation:

Six isolates of *F. oxysporum* were obtained from examined seeds. The different isolates were coded and maintained on PDA at 8°C.

Production of inoculum and pathogenicity test:

The different of fungal isolates were grown in shaked culture for 10 days at 25°C. The culture suspension was filtered through one layer of cheese cloth. The concentration of spores was determined by hemacytometer and adjusted with sterilized water to 1×10^6 CFU (colony forming units) ml^{-1}. The spore suspensions were used as inoculum for pathogenicity test in two ways. The first: bean seeds were sown in a steamed soil mixture (peat, compost and clay 60: 20: 20 vol/vol, respectively) in plug trays and maintained at 25°C, with 12 h/day of fluorescent light. After appearance the first true leaf, root of plantlets were dipped for 10 min in the pathogen spore suspension prepared as described above. Inoculated plants were then transplanted into steamed soil in pots (1.5 L volume). Control plants were prepared similarly but soaked in sterilized water. Six replicates were used. Each replicate consisted of five plants. The experiment was carried under glasshouse conditions with the minimum temperature ranging between 30 to 34°C. Typical

symptoms of wilt started to be visible 10 days after artificial inoculation. Plants were checked for disease development and wilted plants were counted. The data are expressed as percent of dead plants 30 days after the artificial inoculation.

The second: bean seeds were surface sterilized by soaking for 2 min in 1 % sodium hypochlorite. After washing with sterilized water, seeds were soaked for 5 min in the pathogen spore suspension prepared as described above. Seeds were air dried then cultivated into steamed soil in pots. Seeds were sown at rate 6 seeds/pot and ten pots as a replicates were used. Control seeds were soaked in sterilized water instead of spore suspension. Data were calculated as percentage of pre- and post-emergence damping-off 15, 40 days after sowing, respectively. The number of survival plants was recorded at the end of the experiment.

Identification of fungi:

The identification of fungi was done based on the spore morphology and colony characters of the fungus by referring to the 'Illustrated genera of Imperfect fungi' (Barnett and Hunter, 1972) and 'Dematacеous hyphomycetes' (Ellis, 1971). The cultures of *Alternaria*, *Helminthosporium* and *Rhizoctonia* isolated from the infected seeds were sent to Assiut University Mycology Centre (AUMC) for the confirmation of species. These cultures were further purified by following single spore isolation technique for *A. alternata, F. oxysporium* and hyphal tip method for *R. solani* (Tuite, 1969). Thus, obtained pure cultures were maintained on Potato Dextrose Agar slants. Such culture tubes were preserved in a refrigerator at 5^{o}C and renewed once in a month for further studies.

Paper towel method (Rolled towel method):

This method was employed to know the effect of seed-borne inoculum on seed quality parameters of bean i.e. to carry out germination and vigor tests. Also to see the effect of different seed treatments on seed-borne inoculum as the International Seed Testing Association Rules (Anon, 1996). Randomly, 100 seeds were selected and placed on two layers of moist blotting papers, which were placed on a polythene page, at ten seeds per row. Then, these seeds were covered with another moist paper and rolled carefully to avoid any excess pressure on seeds. These towels were incubated at 25^{o}C for ten days. The first count of germination was taken on fourth day and final count was taken on tenth day. All the morphologically normal seedlings were counted and germination percentage was determined. To find out the seedling vigour, ten normal seedlings were randomly taken from the germinated test and shoot and root length was measured Mean root length was expressed in cm. The mean shoot length was expressed in cm. Vigour index was calculated by the following formula.

Vigor index = Seed germination (%) x Seedling Length (Shoot + Root Length (cm).

Overcoming seed-borne fungi of bean:

In each treatment, seeds soaked for 5 min in the pathogen spore suspension prepared as described above then used.

***In vitro* evaluation of peppermint oil by rolled towel method:**

Seeds were soaked in 100 ml of oil emulsions (1, 2 and 3%) for 15 min and then dried in shade for 1 h. Seeds soaked in sterile distilled water served as control. The treated seeds were tested in four replicates of 100 seeds by employing rolled paper towel method, and then incubated at 25± 2^{o}C for ten days. Percent of infection and germination were recorded after ten days of incubation. Seedling vigor was also calculated as stated earlier.

***In vitro* evaluation of peppermint oil from vapor phase:**

Doses of peppermint oil (5, 10 and 15 µl) applied to a filter paper (100 mm diameter). The filter paper was mounted on the inverted lid in a Petri dish (140 mm x 23 mm, which offers 400 ml air space). Hundred of the tested seeds were placed in each dish. The dishes were sealed with vinyl tape and left for 48 h at 30 ºC. Seeds not exposed to oil vapors were used as control. The treated seeds were sown in a steamed soil in pots and maintained at 25°C. Seeds were sown at rate 6 seeds/pot. Ten pots were used as a replicates for each

treatment. Data were calculated as percentage of pre- and post-emergence damping-off 15, 40 days after sowing, respectively. The number of plant survival was recorded at the end of the experiment. The disease ratios were determined by recording the number of non-emerged seeds (pre-emergence damping-off), while post-emergence damping-off and surviving plants were recorded 40 days after sowing. The equations described by Khalifa (1987) were used as follow: Pre-emergence (%) damping-off = No. of non emerged seeds x 100 / No. of sown seeds. Post-emergence (%) damping-off = No. of killed seedlings x 100 / No. of sown seeds. Surviving plants (%) = No. of surviving plants x 100 / No. of sown seeds.

Results:

Evaluation of seed health testing methods:

Seed samples of bean were used for evaluation of seed health testing. The results are presented in the Table 1. PDA medium was found to be suitable for isolation of *R. solani*, *F. oxysporum*, *A. niger*, *H. oryza*, and *Rhizopus* sp. Totally, five fungi genera including both saprophytic as well as pathogenic were recorded. The results of this study indicated the dominance of *R. solani* (28.2%) followed by *F. oxysporum* (12%) in Giza 6. Other saprophytic fungi were isolated. Ten days of incubation permitted to detect the highest number of contaminated seeds.

Common Bean varieties	Occurrence of fungi (%)				
	R. solani	***A. niger***	***H. oryza***	***F. oxysporum***	***Rhizopus* sp.**
Pronco	12.3	1.2	1.1	6.2	0.0
Nibraska	17.0	2.3	3.2	5.3	2.1
Giza 3	11.4	1.2	4.1	8.2	0.0
Giza 6	28.2	1.3	0.0	12.0	0.0
Tema	19.0	0.0	2.0	7.4	3.5
Mean	17.6	1.2	2.1	7.8	1.1

Table (1): Percentage occurrence of seed borne fungi in varieties of bean seed samples by PDA medium.

Location of the pathogen in the seed:

The incubation of seeds without coats allowed to development of fungi internally located. The location of the pathogen in the seed was studied by employing component plating technique and the results are presented in the Table 2. Seed-borne pathogens were noticed in highly percentage both in the seed coat and cotyledon. Most of the fungi were located on seed coat and a lower degree of infection was observed in cotyledon and embryo. Only *F. oxysporum* was recorded for embryo of seeds at low frequency (Table 2).

Seed disinfection	Seed component		
	Seed coat	**Cotyledon**	**Embryo**
Fusarium oxysporium	26	4.1	0.10
***Rhizoctonia* solani**	7	1.3	0.00

Table (2): Location of *F. oxysporium* and *R. solani* percentage in the different seed parts of tested bean seeds vr.Giza 6

Pathogencity test:

Final observations after ten days showed that minimum germination was recorded in *R. solani* treated pots (0.0%) as compared to non-treated pots (86.7%), followed by *F. oxysporum* (11.6%) (Table, 3). In all fungal pathogens, specially *F. oxysporium* was found to transmit to the germinating seeds causing post emergence death. The rate of transmission of the seed pathogens from seeds to seedlings causing post emergence death were always lower than that of transmission to seedling infection or seedling mortality. The highest percentage of pre emergence death or seed rot (96.7%) was occurred by *R. solani*. On the other hand, post emergence death (15%) and seedling infection were recorded from the seedlings transmitted from *F. oxysporium* infected seeds (Table, 3). *F. oxysporium* and *R. solani* isolates collected from seed revealed to be pathogenic to bean seeds (figure, 1A and B). These inoculated fungi were re-isolated from dead seedlings and rotted seeds from each pot to confirm their pathogenic role.

Fungal tested	On seeds				On plants
	Pre-emergence damping off (%)	Post-emergence damping off (%)	Survived plants (%)	Germina-tion (%)	Dead plants (%)
R. solani	96.7	3.3	0.0	0.0	0.00
F. oxysporium	53.3	15.0	31.7	11.6	56.7
A. niger	26.7	0.0	73.3	73.3	0.00
Rhizopus sp	18.3	0.0	81.7	81.7	0.00
H. oryza	13.3	8.3	48.3	80.7	0.00
Negative control	0.0	0.0	1.6	86.7	0.0

Table (3): Pathogenicity testing of some seed-borne pathogenic fungi of Common bean under greenhouse conditions.

Figure (1): Pathogenicity test by using isolated fungi, rotted seeds show covered with mycelia (right) compared with control one (left).

Evaluation of peppermint oil for overcoming seed borne infections

1- *In vitro* evaluation by rolled towel method

Rolled towel method was employed to know the effect of seed borne inoculum on seed-quality parameters of bean *i.e* to carry out germination and vigour test of apparently healthy and infested bean seed. The results of the experiment (Table, 4) indicated that, there was reduction in germination with the increase in the seed infection by *R. solani*. The highly infected seed samples percentage (83.36) showed lower germination (42.34%). They also exhibited the poor vigour index (88.9). Infected seeds were failed to germinate. Apparently treated seed samples by 1, 2 and 3% peppermint oil, (had per cent seed infection of 20.43, 16.35 and 11.41, respectively) showed higher germination percentage (72.18, 76.95 and 83.74%, respectively) with higher seedling vigour index (252.6, 323.2 and 468.9, respectively).

2- *In vitro* evaluation by vapor phase technique

The effect of seed infection by *R. solani* on germination and emergence was high on untreated seeds (pre-emergence damping off (68.3%). Seeds failed to germinate, were found to be covered with fungal mycelia. Emerged seedlings were found rotted and blackened. Seedling mortality was significantly high (15% on post-emergence damping off). Symptoms in seedling were wilting, blackening of hypocotyls and radical axis and brownish discoloration in root. Highly significant reduction was noticed on pre-emergence damping off when seeds were evaporated with oil (10 and 15 µl/400ml air) then sowed on autoclaved soil (Table, 5). On the other hand, the same treatment completely inhibited post-emergence damping off. Survival plants were increased from 16.7 in control to 81.7 and 86.7% after 10 and 15 µl oil treatment, respectively (Table, 6).

Oil concentration (%)	Infection (%)	Germination (%)	Vigour index (%)
1	20.43 a	72.18 a	252.6 a
2	16.35 a	76.95 a	323.2 b
3	11.41 a	83.74 a	468.9 c
Control	83.36 b	42.34 b	88.9 d

Table (4): Effect of seed treatment with peppermint oil on seed-borne infection of *R. solani* and other seed quality parameters of bean by rolled towel method.

Oil concentration µl/400 ml air	Pre-emergence damping off (%)	Post-emergence damping off (%)	Survived plants (%)
5 µl	28.3	3.3	73.3
10 µl	18.3	0.0	81.7
15 µl	13.3	0.0	86.7
Control	68.3	15.0	16.7

Table (5): Effect of seed treatment with peppermint oil on seed borne infection of *R. solani* with vapor phase.

Discussion

The sustenance of healthy seed means that good seed in good yields. To increase the production of bean qualitatively and quantitatively, farmer requires healthy quality seeds with high percentage of germination and purity. Hence, it is imperative that the seeds must be tested before they are sown in the field. This study indicated that, the highest occurrence of fungi on bean seed varieties differed greatly. Maximum fungal association was recorded with *R.* solani and *F. oxysporium* and the minimum with *Rhizopus* sp., *H. oryzae* and *A. niger*. Many important diseases of plants caused by fungi are reported to be seed borne (Neergaard 1997). A seed borne pathogen present externally, internally or associated with the seed as contaminant may cause seed abortion, seed rot, seed necrosis, reduction or elimination of germination capacity as well as seedling damage resulting in development of disease at later stages of plant growth by systemic or local infection (Khanzada *et al*., 2002). Results in this study showed that bean seeds were strongly infested by *F. oxysporum* and *R. solani* both located at the surface and internal of seeds especially cotyledon. *Macrophomina phaseolina* have been recovered from testa, tegmen and embryo of sponge gourd seed (Shakir *et al*., 1995). In the present study, aqueous and vapors of peppermint oil was tested for its efficacy as a antifungal for seed protectant. The results were highly promising as many of the seed mycoflora, whereas fungal inhibition without affecting the seed germination compared to control was found. The results indicating the possible application of this essential oil with already available fungicides to bring down the usage of synthetic fungicides and indirectly its side effects on several biological system. Amaresh (2000) reported that, among plant extracts, neem leaf extract (5%), *Ocimum canum* L. leaf extracts (5%) and *Bougainivillea* sp. leaf extracts were found to be effective in controlling both *Alternaria* blight and rust. John Sudhakar (2002). reported the efficacy of garlic clove extract, bitter gourd leaf extract, turmeric rhizome extract and onion bulb extract, in inhibiting mycelial growth of *M. phaseolina* of maize.

High percentage of above mentioned potential pathogens having both internal and external mode of infestation and adverse effect on seed germination urge that seed should be treated before sowing to obtain good germination and healthy crop. For the production of healthy and certified quality seeds, seed health certification programme has to be followed and seed must be tested and treated with suitable seed dressing fungicides.

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الملخص العربى

مسببات الأمراض الفطرية المنقولة ببذور الفاصوليا وتأثيرها على إنباتها

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تعتبر الفطريات التي تنتقل عن طريق بذور الفاصوليا من المشاكل الخطيرة التى تواجة الزراع سواء بمصر أو في جميع أنحاء العالم حيث تسبب عنها عدة أمراض مثل سقوط البادرات والذبول. وقد تم عزل عدة فطريات من البذور التي تم جمعها من الأسواق التجارية في مصر (برونكو، نبراسكا، الجيزة ٣، الجيزة ٦ وتيما). كانت فطريات فيوزاريوم أوكسيسبورم وفيوزاريوم سولاني المعزولة من بذور الفاصوليا أكثرها شيوعا تليها فطريات الألترناريا، رايزوكتونيا سولاني، هيلمينسوسبوريوم ثم فطر البنسليوم. أشار اختبار القدره المرضية أن فطر فيوزاريوم أوكسيسبورم وفطر رايزوكتونيا سولاني من أهم العزلات الفطرية التي تسبب بشكل كبير عرض سقوط البادرات على نباتات الفاصوليا. معاملة بذور الفاصوليا بأستخدام زيت النعناع أدت الى الحد من انتقال العدوى الفطرية من بذور الفاصوليا الى النباتات الناتجة عنها. علاوة على ذلك، كانت قوة شتلات الفاصوليا الناتجه من البذور المعالجة أفضل من تلك غير المعالجة مما ينعكس على تحسين خصائص النبات وزيادة المحصول في المستقبل.