Effect of fungal filtrates on germination of wheat grains and the biological control of these fungi using black pepper extract

Fuzia Elfituri Muftah Eltariki^{a,b}, Abdulmajeed Bashir Melitan^b, Seok Mui Wang^c, Mohammed Abdelfatah Alhoot^{d*}

^aPost Graduate Centre (PGC), Management & Science University (MSU), Shah Alam, Selangor, Malaysia

^bDepartment of Botany, Faculty of Science, Misurata University, Misurata, Libya

^cInstitute for Pathology, Laboratory and Forensic Medicine (I-PPerForM), University Teknologi MARA, Sungai Buloh Campus, Selangor, Malaysia

^dInternational Medical School (IMS), Management & Science University (MSU), Shah Alam, Selangor, Malaysia

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Abstract. Wheat is one of the world's most abundant and essential food crops. It covers a significant area of the earth's surface, higher than any other plant, and tends to be among the top strategic crops. Wheat contamination with fungi leads to rapid deterioration of quantity and quality of wheat products. Many of those fungi are potential mycotoxins producers. This study aimed to isolate and identify fungi that contaminating the wheat grains from the Misurata Agricultural Research Center area and the South Region of Libya. Fungi contaminating wheat grains were isolated on Potato Dextrose Agar (PDA) and identified by culture characteristics and microscopically. Fungal filtrates of two fungal isolates, *Aspergillus niger* and *Rhizopus* sp., were tested for their effects on the germination and seedlings of wheat grains. Furthermore, the effect of acetic extracts of Black pepper (*Piper nigrum*) on the growth of the isolated fungi was also investigated. Ten types of fungi belonging to four genera were isolated and identified. The germination rate of wheat grains irrigated with the filtrate of *A. niger* and *Rhizopus* sp. was 20% and 80% respectively, compared with 100% of the control grains, which were irrigated with water. The culture filtrates of both *A. niger* and *Rhizopus* sp. affect not the only percentage of grains germination but also the morphology of wheat seedlings. It adversely affected the length of the radicles and coleoptiles. The acetone extract of *P. nigrum* showed inhibitory effect ($85.7\% \pm 3.7$ and $44.0\% \pm 3.1$) on the germination of *A. niger* and *Rhizopus* sp. respectively. This study concludes that fungal secretions have pathogenic effects on plant growth, which can lead to potential health risks for the human population. Biological control such as *Piper nigrum* extracts can be an alternative to chemical pesticides for controlling fungal pathogens and their secretions.

Keywords: wheat grains, *Aspergillus*, *Rhizopus*, *Piper nigrum*, germination, biological control

INTRODUCTION

Wheat is one of the most important food crops in the world. It contains carbohydrate (70%), protein (12%) and fat (29%), providing about 20% of the calories in human food worldwide (Putnam *et al.*, 2002). Wheat grains are exposed to

various fungal contaminations starting from the time of physiological maturity until they are used for either agriculture or consumption. More than 100 fungal species have been found contaminating the grains. Some of these fungi

*Author for correspondence: Mohammed Abdelfatah Alhoot, International Medical School (IMS), Management & Science University (MSU), Shah Alam, Selangor, Malaysia. Email – malhoot@hotmail.com

have potential risks to plants grown from these grains, while others can cause problems of decay and release of toxins during storage that has a potential threat to the consumers' health (Kabak *et al.*, 2006; Kent & Evers, 1994). The safety of grains is essential for its use in agriculture, storage, and consumption. Hence, wheat grains should be stored in a proper condition which contains suitable moisture (not exceed 12%), absent of contaminants, residues or harmful substances, grain fracture percentage is not more than 2%, and should be of the same size, color, and smell. The storage conditions should be as appropriate as possible to maintain good grain quality (Lopez-Garcia & Park, 1998).

Wheat grains show the symptoms of deformation, discoloration, or black spots caused by some fungi in the field when the humidity is high (>90%). The most common fungi associated with these symptoms include *Alternaria* sp., *Fusarium* sp., *Cladosporium* sp., *Aspergillus* sp., *Penicillium* sp., and *Rhizopus* sp. (Bhale *et al.*, 2001). Fungal contamination of soybean seeds with *Aspergillus*, *Penicillium*, and *Rhizopus* has shown to cause reduction of germination rate and defected of seedling during field plantation (Anwar, 1995; Ibrahim, 2015).

Black pepper (*Piper nigrum*) is one of the well-known medicinal plants that have prominent roles in pharmaceutical, industrial and agricultural production. It contains active antimicrobial substances, aromatic oils, phenols, aldehydes, and alkaloids. These substances are used in food maintenance, formulation of pharmaceuticals and alternatives to medicines and natural remedies (Jafeer & Kheirallah, 2017; Ody, 2017). *P. nigrum* is a flowering vine of the *Piperaceae*. It is cultivated for its fruit which is usually dried and used as a spice and seasoning that are known as a peppercorn. When fresh and fully mature, it is dark red and contains a single seed. Peppercorns and the ground pepper derived from them may be described just like pepper, or more precisely as black pepper (cooked and dried unripe fruit), green pepper (dried unripe fruit), and white pepper (ripe fruit seeds). Black pepper is native to Kerala in Southwestern India (Hajeski, 2016; Sen, 2004) and is extensively cultivated there and elsewhere in tropical regions. Vietnam is the world's largest producer and exporter of pepper,

producing 34% of the world's *P. nigrum* crop as of 2013 (Yogesh & Mokshapathy, 2013).

Fungi of the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Rhizoctonia* are known to produce toxic metabolites which are known as mycotoxins (Eltariki *et al.*, 2018). These mycotoxins had been reported to degrade seed quality and reduce their viability (Magan & Aldred, 2007; Mohamed-Yasseen *et al.*, 1994). The fungistatic or fungicidal effect of spices is due to the inhibitory action of natural products, and the mechanisms involved are cytoplasm granulation, cytoplasmic membrane rupture, and inactivation and/or inhibition of intracellular and extracellular enzymes. These biological events could take place separately or concomitantly culminating with mycelium germination inhibition and it is also reported that plant lytic enzyme act in the fungal cell wall causing breakage of β -1,3 glycan, β -1,6, glycan, and chitin polymer (Pundir & Jain, 2010). Historically, it has been thought to cure many illnesses, such as cancer, malaria, cholera, nausea, fever, migraine headaches, poor digestion, strep throat, and even coma. Piperine is an alkaloid found naturally in plants belonging to the pyridine group of family *Piperaceae*, such as *P. nigrum* and contains some of the antimicrobial components such as Terpinene, α -pinene, β -pinene, Linaleol and Terpeneol, Piperine, pipene, piperamide and piperamine it has to possess diverse pharmacological activities. Piperine has been found to enhance the therapeutic efficacy of many drugs, vaccines, and nutrients by increasing oral bioavailability by inhibiting various metabolizing enzymes. There is preclinical evidence that it may have modest immune system enhancing properties (Kaho *et al.*, 2019).

Culture filtrates of *Aspergillus* sp. have reported in causing a reduction in seed germination and root-shoot elongation (Jalander & Gachande, 2012). Seed-borne diseases play a significant role in the quantity and quality of the agricultural product. Seed rot, seedling blight, Bipolaris leaf spot, and Curvularia leaf spot are etiologically caused by *Penicillium* sp., *Fusarium oxysporium*, *Aspergillus* sp., *Bipolaris maydis*, and *Curvularia lunata* respectively (Debnath *et al.*, 2012).

This study aimed to identify the fungal species contaminating the wheat grains in Libya and to investigate the effect of isolated fungi filtrates on

grains germination rate and seedling development. Furthermore, the inhibition effect of *P. nigrum* (black pepper) extract on the growth of isolated toxigenic and pathogenic fungi will also be evaluated.

MATERIALS AND METHOD

Sample collection

Wheat grain samples were collected from Misurata Agricultural Research Center area and South Region of Libya. Three samples for each type of wheat, *Triticum aestivum* cv. and the solid wheat cultivar *Triticum durum* cv., were collected randomly from stored grains in each area and transported to laboratory using properly sealed sterile polythene bags. The samples were kept at 4°C until fungal cultivation, isolation, and identification were carried out as described previously (Fente *et al.*, 2001; Sekar *et al.*, 2008).

Isolation and identification of fungi on wheat grains

Fungi carried on wheat grains were isolated on Potato Dextrose Agar (PDA) as previously described (Fente *et al.*, 2001; Sekar *et al.*, 2008). The wheat grains used for culture was randomly selected from the samples and picked up using sterile forceps. Grain seeds were washed aseptically with 10 mL of sterile distilled water. PDA media were prepared, and chloramphenicol (500 mg/L) was added to inhibit bacterial growth. Five grains of wheat were inoculated on each culture media plate with equal distribution. The grains were immersed in the agar to ensure that the grain surface is directly contacted with the nutrient in the medium. Three replicates were maintained for each sample. After inoculation, all dishes were incubated in the incubator at a temperature of 25°C for seven days. Culture plates were checked daily for growth and sporulation. After seven days of incubation, the different fungal colonies were sub-cultured into fresh PDA plates. The isolated fungi were identified based on colony morphology and microscopic characteristics using an optical microscope according to the following references (Domsch *et al.*, 2007; Ebrahim, 1998; Nelson *et al.*, 1983).

Effect of fungal filtrates on germination of wheat grains and seedling growth

Potato Dextrose Broth (PDB) was prepared under septic conditions to prepare the fungal filtrates of isolated fungi from wheat grains were prepared under septic conditions using Potato Dextrose Broth (PDB). These filtrates were prepared from 3 - 4 days sub-cultured *A. niger* and *Rhizopus* sp. on PDA. A piece of 9 mm in diameter of PDA for each fungal isolate was inoculated in a flask containing 25 mL PDB. Three sets of three flasks were assigned for *A. niger*, *Rhizopus* sp., and control (Garuba *et al.*, 2014; Hajieghrari, 2010) (Figure 1). The control flasks were inoculated with PDA only. After the inoculation, all flasks were incubated in the incubator at 25°C. Ten days later, the flasks were removed from the incubator, and the media was separated from the growth by filtration using sterile filter paper (Whatman glass microfiber filter paper Grade GF/F Retention 0.7 Micron).

Figure 1. Growth of *A. niger* and *Rhizopus* sp. on Potato Dextrose Broth.

In order to investigate the effect of fungal filtrates on the germination and growth of seedling of wheat grains, several clean and sterilized Petri dishes were prepared with sterilized filter paper. In each dish, five grains of wheat were sterilized with 0.1% hydrogen peroxide solution, and the grains were irrigation with 10 mL of the filtration. Three dishes containing the same number of wheat grains were used for each type of the filtrations. Two control plates were included in which one was irrigated with PDB, and another one was irrigated with sterile distilled water. All dishes were incubated in the incubator at 25°C. All plates were examined for the growth of grains on day three, five, seven, and ten. The percentage of germination was calculated according to the following equation:

$$\text{Inhibition ratio (\%)} = \frac{\text{Untreated colony diameter} - \text{Treated colony diameter}}{\text{Untreated colony diameter}} \times 100$$

Statistical analysis

Statistical analysis was done using Graph Pad Prism version 5.01 (Graph Pad Software, USA). P values of <0.05 were considered significant. Error bars were expressed in the graphs as $\pm$ SD.

RESULTS AND DISCUSSION

Isolation and identification of fungi on wheat grains

It emerged from the current study that wheat grains obtained from the different production area were found to be contaminated with many types of fungi. A total of 319 colonies were isolated from wheat grains, of which 213 colonies were isolated from the Misurata Agricultural Research Center area, and 106 colonies were from the South Region of Libya. Table 1 shows that the number of colonies isolated from wheat grains from Misurata Agricultural Research Center area is more than the number of colonies isolated from the South Region of Libya. This variation in the number of colonies may be due to the Misurata Agricultural Research Center area has a moderate and humid climate during the sapling time, allowing the moisture-loving fungus and temperate fungi to have a more extended period of growing and reproduction for several generations, and thus producing a large number of germs. In contrary, the South Region of Libya is known for its relatively dry hot climate which could provide a short period of fungi on the cultivated grains to grow and reproduce during the time of the spikes production and consequently resulting in the decrease of production of germs. The results showed that *Fusarium* was predominant in wheat grains from southern Libya, which could be because the desert soils containing more *Fusarium* (Zeller *et al.*, 2003). This fungus also can tolerate adverse conditions such as high temperatures and drought for long periods in the form of chlamydospores (Agrios, 2012; Cook, 1981). Furthermore, its dense presence in the desert soils, especially when cultivating sensitive species, contributes and increases the seriousness of the injury of plants at

different stages which has negatively affects the quality and quantity of the crop (Zeller *et al.*, 2003).

Table 1. The number of fungal colonies isolated from wheat grains obtained from Misurata Agricultural Research Center area and South Region of Libya.

Isolated Fungi	Misurata Agricultural Research Center area	South Region of Libya
<i>Aspergillus</i> sp.	10	-
<i>Aspergillus</i> sp1	2	-
<i>Aspergillus</i> sp2	6	-
<i>Aspergillus</i> sp3	4	-
<i>Aspergillus niger</i>	10	-
<i>Rhizopus</i> sp.	50	-
<i>Fusarium</i> sp.	-	60
<i>Fusarium</i> sp1	43	45
<i>Fusarium oxysporum</i>	41	-
<i>Penicillium</i> sp1	35	1
<i>Penicillium chrysogenum</i>	12	-
Total number of colonies	213	106
Total number of species	10	3
Total number of genera	4	2

Morphological identification of fungal species was made using macroscopic and microscopic characterization. For fungi isolated from Misurata Agricultural Research Center area, ten species belonged to four genera, which are *Aspergillus niger*, *Aspergillus* sp., *Aspergillus* sp1, *Aspergillus* sp2, *Aspergillus* sp3, *Rhizopus* sp, *Fusarium oxysporum*, *Fusarium* sp1, *Penicillium chrysogenum*, and *Penicillium* sp1. Three fungal species belonged to two different genera which are *Penicillium* sp1, *Fusarium* sp1, and *Fusarium* sp were isolated from the South Region of Libya. *Aspergillus niger* and *Rhizopus* sp. (Figure 2) were the most common species isolated from wheat grains in both geographical areas. Thus, these genera were selected to investigate the effect of fungal filtrates on germination of wheat grains and seedling growth. These fungi were isolated from the grains and identified in Assiut University, Egypt.

Effect of fungal filtrates on germination rate of wheat grains and seedling growth

Wheat grains could be contaminated with fungi either in the field or during storage and exhibition in the markets. These fungi are likely to grow and produce toxins that may affect the vitality of the wheat grains. The role of fungal secretion on germination rates and development of seedlings of wheat grains has run out in this study. *A. niger* and *Rhizopus* sp. filtrates reduced the rate of germination after ten days to 20% and 80% respectively compared to germination rate of 100% for grains irrigated with distilled water as shown in Table 2 and Figure 3. This result was supported by a previous study, (Ibraheem *et al.*, 1987) which found that *A. niger*, *A. flavus*, and *Alternaria alternata* had an inhibitory effect of reducing seed germination. These results are also consistent with many previous studies. Khokhar *et al.* reported that *P. chrysogenum* decreased the percentage of seed germination by 20.33%. This fungus had shown a poisoning effect on the seedling of cereal as evidenced in inhibition of the seed germination percentage of wheat at a higher percentage of 90% (Khokhar *et al.*, 2013). The inhibition may indicate that the tested fungi produce toxic metabolites in the broth which they were cultured. *A. niger* and *P. chrysogenum* produced metabolites which are known to reduce germination and seedling development (Haikal, 2008). *A. niger* can produce mycotoxins such as oxalic acid crystals, kojic acid, and malformins depending on the growth condition and the strain of the organism (Garuba *et al.*, 2014). This effect varies with different fungi.

Grains irrigated with *A. niger* filtrate showed a significant reduction of the radicle lengths ($98.3\% \pm 0.6$, $97.8\% \pm 0.5$, $97.8\% \pm 0.2$ and $97.8\% \pm 0.0$) after three, five, seven and ten days respectively.

This reduction rate is statistically significant when compared with irrigated grains with water (Two-Way ANOVA with Bonferroni post-test, $P < 0.001$), as shown in Figure 4. Again, grains irrigated with *Rhizopus* sp. filtrate showed a reduction of the radicle lengths ($63.4\% \pm 3.7$, $64.6\% \pm 4.6$, $66.3\% \pm 4.2$ and $67.4\% \pm 10.8$) after three, five, seven and ten days respectively. This reduction rate is statistically significant when compared with irrigated grains with water (Two-Way ANOVA with Bonferroni post-test, $P < 0.001$) as shown in Figure 4. Similarly, the coleoptile lengths of grains irrigated with *A. niger* and *Rhizopus* sp. filtrates were also significantly shorter compared to grains irrigated with water (Two-Way ANOVA with Bonferroni post-test, $P < 0.001$) as shown in Figure 5. Grains irrigated with *A. niger* filtrate showed ($93.9\% \pm 1.5$, $95.5\% \pm 0.6$, $95.3\% \pm 0.3$ and $94.7\% \pm 1.9$) reduction of the coleoptile lengths after three, five, seven and ten days respectively. Also, grains irrigated with *Rhizopus* sp. filtrate showed ($43.5\% \pm 19.9$, $43.2\% \pm 1.5$, $36.0\% \pm 4.4$ and $29.9\% \pm 4.7$) reduction of the coleoptile lengths after three, five, seven and ten days respectively. These results are consistent with the finding of the previous study (Garuba *et al.*, 2014). This study showed 65.33% and 79.67% germination ratio of corn seeds due to the effect of *A. niger* and *Penicillium chrysogenum* filtrates, respectively, when compared with control.

Table 2. Effect of fungal on wheat grain germination.

Fungal filtrates	Germination rate after 10 days
<i>A. niger</i>	20%
<i>Rhizopus</i> sp.	80%
Control (PDA)	98%
Control (Water)	100%

Figure 2. Microphotographs of fungal species isolated from wheat grains. (A) *A. niger*, (B) *Rhizopus* sp.

Figure 3. Effect of fungi filtrates on the wheat grains germination and growth of seedlings after 10 days.

Figure 4. Effect of *A. niger* and *Rhizopus* sp. filtrates on the length of wheat radicles. A significant reduction of radicle lengths was observed when compared with grains irrigated with PDB and water (Two-Way ANOVA with Bonferroni post-test, $P < 0.001$). Results are expressed as mean $\pm$ SD from a representative experiment performed in triplicate.

Figure 5. Effect of *A. niger* and *Rhizopus* sp. filtrates on the length of wheat coleoptiles. A significant reduction of coleoptile lengths was observed when compared with grains irrigated with PDB and water (Two-Way ANOVA with Bonferroni post-test, $P < 0.001$). Results are expressed as mean $\pm$ SD from a representative experiment performed in triplicate.

Results reported that *A. niger* filtrate has a more adverse effect on the germination rate of wheat grains and the development of their seedlings. It could be due to the ability of the fungus to produce Aflatoxins. These findings are consistent with previous findings (Garuba *et al.*, 2014; Jeswal, 1987; Tewfik *et al.*, 1974) that concluded that Aflatoxins prevent or reduce the rate of germination of many crop seeds. It reduces the growth of the crop seedling as well as the development of the radicles and coleoptiles produced from irrigated grains.

Results also showed that *Rhizopus* sp. has a mild deleterious effect on wheat grain germination, seedling development, and evolution of the radicles and coleoptiles compared to *A. niger* which can be referred due to the differences in substrates and chemical quality from the toxins produced by *A. niger*. These toxins can also inhibit the naturally occurring Gibberellins produced by the embryo during germination leading to a shortening of the radicles and coleoptiles (Jones & Armstrong, 1971; Sinha, 1996).

Evaluation of the effect of *P. nigrum* (black pepper) extract on the growth of isolated fungi. The acetonic extract of *P. nigrum* showed significant inhibition effect on the growth of isolated fungi, *A. niger* and *Rhizopus* sp., from wheat grains, as shown in Figure 6 and Figure 7. The Inhibition rate of the treated fungal cultures with extract of *P. nigrum* were $85.7\% \pm 3.7$ and $44.0\% \pm 3.1$ for *A. niger*, and *Rhizopus* sp.

respectively compared to untreated control (Paired t-tests with two-tailed, $P < 0.001$). The inhibition effect of *P. nigrum* extract on *A. niger* and *Rhizopus* sp. may be due to it contains active compounds such as saponins, phenols, and alkaloids. It is known that these compounds have a powerful effect on many pathogens (Agrios, 2012; Gülçin, 2005; Pundir & Jain, 2010). The result of this study is consistent with previously published results (Abdel Mohsen, 2011). Many plant extracts, including black pepper, showed protection of sunflower plant from decay by *Macrophomina phaseolina*. These plants reduce the fungal infection rate by 53.1%. This inhibition by chili extract could be due to the presence of alkaloids capsaicin, dihydrocapsaicin, and capsiacin. This inhibition is also consistent with another study which showed the inhibition of the growth of pathogenic fungi, namely *Cochliobolus migabeanus*, *Pyricularia oryzae*, and *Rhizoctonia solani* in rice (Tewari & Nayak, 1991).

Figure 6. Effect of *P. nigrum* extract on *A. niger* growth. (A) shows significant inhibition effect on the growth of isolated *A. niger* (Paired t-tests with two-tailed, $P < 0.001$). Results are expressed as mean $\pm$ SD from a representative experiment performed in triplicate. The asterisk denotes the level of statistically significant differences from the untreated control. (B) shows colony morphology of *A. niger* treated with *P. nigrum*. (C) shows colony morphology of *A. niger* untreated with *P. nigrum*.

Figure 7. Effect of *P. nigrum* extracts on *Rhizopus* sp. growth. (A) shows significant inhibition effect on the growth of isolated *Rhizopus* sp. (Paired t-tests with two-tailed, $P < 0.001$). Results are expressed as mean $\pm$ SD from a representative experiment performed in triplicate. The asterisk denotes the level of statistically significant differences from the untreated control. (B) shows colony morphology of *Rhizopus* sp. treated with *P. nigrum*. (C) shows colony morphology of *Rhizopus* sp. untreated with *P. nigrum*.

CONCLUSION

The metabolic products of *A. niger* and *Rhizopus* sp impair the germination and growth rate of wheat and therefore diminishing its quality, especially the stored wheat grains. Hence necessity should be placed on these fungi and their metabolic processes due to their adverse effects on agriculture, health, and industry. Further studies on the biological control of fungi should be encouraged using other types of plants. The active compounds in these plants have the potential to be an effective antifungal agent.

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