Research Article

Isolation, Pathogenicity and Control Evaluation of Safflower Associated Pathogenic Fungi from Al-Qadisiyah Governorate, Iraq

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Abstract | Safflower (*Carthamus tinctorius* L.) is one of the important oilseed crops in Iraq. However, its production is hampered by the incidence of many seed-borne pathogenic fungi including *Fusarium*. This study aimed to collected about 15 seed samples of the safflower crop from 12 safflower cultivating areas of Al-Qudsia Governorate of Iraq., which were collected from different regions of Iraq. The pathogenicity potential of the fungal isolates of *Fusarium* was assessed on the germination of seeds, seedlings and adult plants. According to the results, the most frequent fungal isolates associated with safflower were *Fusarium solani, F. musarum, F. verticelloides, F. oxysporum* and *F. chlamydosporum* with first four isolates being reported for the first time in Iraq. All these fungal isolates were able to cause infection, while the two species *F. oxysporum* and *F. chlamydosporum* were not infective. Moreover, the application of synthetic fungicide Beltanol (8-hydroxyquinoline sulfate) and the alcoholic extract of the basidiomycete fungus, *Clavitia pychderuma*, was most effective in reducing the *Fusarium* infection in pre- and post-emergence infection of safflower.

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Introduction

Safflower (*Carthamus tinctorius* L.) is one of the most important oilseed crops, as its seed oil contains about 35–40% and protein 25%. It is a crop rich in vitamins such as vitamin B12 and carbohydrates (Nazir *et al.*, 2020). Safflower plants are usually attacked by many pests including pathogenic fungi that cause leaf spot, wilt and rust which are transmitted through seeds, causing loss of vitality and thus producing weak plants with reduced quality and quantity of crop produce (Kaya and Ergin, 2023). Many types of fungi have been isolated from safflower seeds, and are characterized for their high pathogenicity. Most of the fungal isolates associated with safflower seeds or plants belonged to the genera *Aspergillus, Fusarium, Cladosporium, Stemphylium* and *Macrophomina* (Ghosh *et al.*, 2018).

For instance, Idris (2003) isolated the fungal species of the genus *Fusarium* from 12 safflower seed samples from different areas of Egypt, which were selected using the paper-blotting method. The most abundant fungus was *Fusarium solani*, with a frequency of 30–63%, while the two species were isolated at a lower frequency of 1–4%. Similarly, Al-

Nafei and Mohammad (2021) and Allen *et al.* (1998) mentioned a group of fungi that are transmitted through safflower seeds, namely *Alternaria sesame* and *Macrophomina phaseolina*.

Many studies have dealt with chemical control of seed-borne pathogenic fungi, whether in treating the soil through drenching or via seed dressing to protect the seeds and seedlings (Jadon *et al.*, 2015; Gupta and Kumar, 2020). Although use of synthetic fungicides is the most effective strategy to eliminate plant pathogens, their long-term negative impacts on the environment through increased pesticidal pollution and human and animal health hazzards cannot be overlooked (Fiedler and Sosnowska, 2017; Kenarova and Boteva, 2023). Similarly, certain phytopathogenic fungi have attained considerable resistance to certain types of synthetic fungicides (Islam *et al.*, 2024), suggesting to look for alternate fungicidal treatments which can be more environment freidly and sustainable such as biolgoical control of fungal diseases by myco-fungicides (de Faria and Wraight, 2007).

Recent research has been intensified in detecting and finding biological factors capable of eliminating pathogens or including them in the integrated resistance program, which limits the presence of agricultural pests and reduces the disease (Beyyavas and Haliloglu, 2021). Research that dealt with plants or their wastes in evaluating their effectiveness against plant-pathogenic soil fungi has also been given attention (Attia *et al.*, 2022; Al-Hazmi and Naguib, 2024). They produce directly or indirectly pathogenic compound, called as allelopathic compounds, which are of utmost importance and vitality in the environment (Fernandes *et al.*, 2024).

Given the importance of safflower crop and because of the lack of studies its seed-borne fungal pathogens of this crop and their sustainable management, this research aimed to identify the pathogens associated with and transmitted by seeds and their effect on seed and plant germination and to evaluate the efficiency of some treatments in preserving seeds and seedlings by these pathogenic fungi.

Materials and Methods

Isolation and identification of seed-borne fungi

Fifteen safflower samples were collected from 2022 crop harvest from different areas of Al-Qudsia Governorate of Iraq and were brought and stored in a refrigerator in tightly sealed polyethylene bags at 40˚C throughout the examination period (Table 1). The presence of fungi was detected using the paper blotting method (Narayanasamy, 2010).

Table 1: *Geographical distribution of safflower samples collected from Iraq and evaluated in this study.*

Sequence / Sample number	Severity of pathogenic injury
0	Healthy plants
1	1.0% of the plant affected by the disease
2	2.0% of the plant affected by the disease
3	5.0% of the plant affected by the disease
4	10% of the plant affected by the disease
5	25% of the plant affected by the disease
6	50% of the plant affected by the disease

About 400 safflower seeds were used from each sample and were planted in Petri dishes with a diameter of 9 cm, as each dish contained three sterile blot/filter papers previously immersed in water containing 0.1% of 2.4D (a selective herbicide) to the saturation limit for the purpose of slowing down seed germination, growth and seedlings to facilitate the examination process.

Petri dishes with 25 seeds per dish were placed in an incubator at a temperature of 24˚C and 12:12 hour light: dark photoperiod. After seven days of incubation, the dishes were extracted and the seeds in each dish were examined with a light microscope. The fungi that grew on the seeds were identified to the level of genus and species. The fungi were purified using the single spore method and the species were identified phenotypically in the dishes based on the colony color and shape, the type of spores, and the sexual and meiotic structures using the taxonomic keys (Ellis, 1976; Crous *et al.*, 2021).

Efficacy of fusarium isolates of safflower seeds

Safflower seeds were planted after surface sterilization with a 2% sodium hypochlorite solution for 5 min in a 1:2 v/v mixture of soil and peat moss. Ten seeds were sown in a pot with a diameter of 15 cm containing 1.0 kg of soil sterilized with methyl bromide gas at a rate of 0.5 kg/m^3 at about 15 days before planting. After the germination process was completed for seven days, these plants were thinned up to 5 homogeneous

seedlings per pot. After 30 days of germination, the seedlings were sprayed with hand-atomizer with a suspension of spores of each fungus, then the fungal isolates, that were purified by the single spore method, were planted using water agar leaf medium, which is one of the best stimulating media for fungal sprouting. After 7 days of incubation, the spores were removed using a sterilized glass rod. It was filtered through two layers of gauze, with the concentration set at 1 ×10 spores mL^{-1} of water containing 0.1% gelatin as a sticking agent, and the spore concentration was determined using a Haemocytometer. The pot was covered with perforated plastic for two days and placed in the greenhouse at temperature of 24–28°C. Experiment was maintained following a completely randomized design with four replicates for each treatment. In control treatment, all replicates were sprayed with sterile water as a comparison. After 25 days of inoculation, the percentage of severity of infection was estimated in 10 randomly selected leaves per plant by following the pathological guide (Al-Abbad, 2020).

Effect of fusarium sp. on seed and seedling germination

The inoculum of *Fusariaum* isolates were prepared using the same method as described above in the previous experiment, using the same spore concentration. Safflower seeds were soaked in the conidial suspension of each *Fusarium* isolate for about 30 min. For the control treatment, seeds were soaked in distilled water only and then were planted in 15 cm diameter pots containing a mixture of soil and peat moss at the same ratio as in the previous experiment. The pots were planted at a rate of 20 seeds per pot, with five pots used as replicates for each treatment. Then, they were covered with perforated plastic and placed in the greenhouse at a temperature of 24–28°C. Experiment was according to the completely randomized design with four replications for each treatment. After two days, plastic bags were removed and returned to them after 7 days to calculate the germination percentage. To calculate the germination percentage for the purpose of estimating the seed or seedling mortality rate before emergence and after 25 days, the mortality rate after emergence was calculated and the fungi present on the non-germinating seeds and dead seedlings were isolated again to confirm the pathogen type.

Determination of the effectiveness of some treatments against F. solani

This experiment contained four treatments, *i.e.*, i) the pathogenic fungus alone (*F. solani*) (Fs), ii) *Pseudomonas fluorescens* (Pf) + Fs isolated from safflower-grown soil, and its antagonistic activity was tested, iii) Fs + *Calvatia pachyderma* fungus extract 2 g/kg of seeds, iv) Fs + Beltanol® (8-hydroxyquinoline sulfate) fungicide and Control (sterilized seeds planted in sterilized soil). The soil mixed with peat moss (in 1:2 ratio) was sterilized and then filled in plastic pots with a diameter of 15 cm at a rate of 1 kg per pot. Then the pathogenic fungus inoculum (Fs) was added, loaded on the sesame seeds at a rate of 0.5% (w/w) for treatments (1– 4). A bacterial inoculum (Pf) was also added to the second treatment, loaded on the sesame seeds at the same rate used and the same time as the pathogenic fungus. The soil was moistened and the pots were covered with perforated nylon bags for two days. Two days later, Beltanol fungicide was added to the soil at a concentration of 0.25 L^{-1} for the fourth treatment, and the alcoholic extract of the basidiomycete fungus *C. pachyderma* for the third treatment. The extraction was carried out according to the method mentioned by Pedram (2012).80 ml was used/pot. The pots were left for 3 days, after which they were planted with yellow seeds at a rate of 20 seeds/pot after sterilizing them superficially and washing them with sterile water. Watering continued for all pots after the planting process placed in perforated nylon bags for a period of three days and were placed in the plastic house. After 7 days, the germination percentage was calculated by estimating the percentage of seed death before emergence, and after 25 days, the percentage of death after emergence was estimated. The causative agent of seed or seedlings death was confirmed by re-isolating and identified the pathogens from the dead seedlings.

Results and Discussion

Isolation and identification of fungi transmitted by safflower seeds

According to the results, there was the presence of more than 20 species of fungi associated with the seeds of safflower (Table 2). The most frequent of all the fungal isolate was the fungus *Fusarium*, which was isolated from all samples with a high frequency as compared to the rest of the fungal strains. A total of five species of this fungus were identified in the study, *i.e., Fusarium solani, F. oxysporum, F. verticelloides, F. musarum* and *F. chlamydosporum*. These results are in line with the report of Srivastava *et al.* (2021). Moreover, the last three species (*F. verticelloides, F.*

musarum and *F. chlamydosporum*) have been first time reported from Al-Qudsia Governate of Iraq.

Table 2: *Fungal isolates associated with the safflower seeds collected from Iraq.*

Fungal species	Sample number	Contaminated seeds	
		Highest (%)	Average (%)
Fusarium solani	12,11,9,6,5,1	8.0	2.6
Fusarium musarum	8,5,4,	3.5	1.3
Fusarium verticillioides	7-6	4.0	2.5
Fusarium oxysporum	12,11,7-5	2.5	1.8
Fusarium chlamydosporum	3,1	0.5	0.5
Fusarium ssp.	9-6,3,1	7.5	4.1
Aspergillus niger	10	0.5	0.5
Aspergillus terrus	12,3	0.5	0.5
Aspergillus japonicas	10,3	1.0	1.0
Mycelia sterilia	11,9,6,1	1.0	1.0
Trichoderma viride	6	0.5	0.5
Nigrospora shaerica	4,2	3.0	1.3
Ulocladium atrum	11,10,5	1.0	1.0
Alternaria alternata	12,3,1	0.5	0.5
Alternaria longissima	8,5,4	3.0	1.3
Alternaria raphani	7,8	5.2	3.2
Alternaria sesamicola	12,11,7-5	7.1	4.0
Alternaria tenuissima	3,1	2.5	1.8
Chaetomium globosum	8,2	1.0	1.0
Cladosporium sp.	7	1.0	1.0
Curvularia lunata	10-8	3.0	1.3
Cylindrocarpon magnusianum	9	1.0	1.0

Leslie *et al.* (2007) stated that these species live as parasites on many types of plants and seeds, including oilseeds such as sesame, safflower and sunflower, as these species were highly frequent in many samples, particularly the species *F. musarum*. Also, approximately 13 fungal species were isolated from one to four samples, and with different repetitions according to the type of fungus and the region from which the sample was taken, as all the fungi that were isolated for the first time do not have any reference or local source indicating their isolation before. Also, the diagnosis is made for a group of isolates in four samples through several attempts and urging them to germinate using different media and exposing them to light and darkness (Liu *et al.*, 2016).

Table 3: *Pathogenicity potential of five isolates of fusarium sp. on 30-days-old safflower plants.*

Treatments	Infestation (%)
Control	0.0
F. solani	70.0
F. musarum	32.5
F. verticillioides	53.0
F. oxysporum	0.0
F. chlamydosporum	0.0
LSD (@ 0.5% probability level)	11.6

It was diagnosed based on the morphology of the colonies, microscopic examination, and the colors that appeared in the culture media. There is also a group of isolates that did not as per taxonomic keys of phytopathogenic fungi and that belong to the fungus *Fusarium* sp. and it is possible that these fungal strains may be previously unidentified species yet because of the high adaptability potential and global distribution pattern of *Fusarium* fungi (Leslie *et al.*, 2007; Alkhalifah *et al.*, 2023).

In addition, three *Fusarium* isolates from the five ones (*i.e. F. verticelloides, F. musarum* and *F. solani*) exhibited their ability to infect safflower plants at the age of 30 days with the severity of infection in their treatments as 30.7, 55.9 and 72.0%, respectively Table 3. It differed significantly from the control treatment in which the infection appeared on all plants. However, the two isolates *F. oxysporum* and *F. chlamydosporum* did not show any disease symptoms on the treated plants, while the species *F. solani* showed white, intertwined tissue protrusions in the form of medium and dense circular spots on the leaves and their blades (Yao *et al.*, 2022). As the infection progresses, these spots reached 1.5–2 cm in diameter and got surrounded by light pink color. It is noted that these circular protrusions unite with each other as the infection progresses and continues until it reaches the stems, as these fungal structures or threads become sunken spots in the stems in which the fruiting boxes and their spores are found (Doohan, 2011). These disease symptoms were consistent with what was reported by Motamedi *et al.* (2016) and Benyan (2024).

As for *F. musarum*, the pathogenic fungus showed a distinctive way of infection in the crop, as the leaves turn yellow, while the transverse sections of the stem showed the discoloration of the internal tissues in a reddish-brown color, starting with the base of the

stem and the discoloration moves upwards, as this fungus begins to grow in the transport vessels inside the plant, which hinders the movement of water and elements in the plant (Marin Galarza, 2013). This infection may result into wilting of the plant, and this is consistent with what was stated by Chung *et al*. (2001) and Motamedi *et al*. (2016) who attributed this infection to the difference in environmental factors that are suitable for the occurrence of the disease and the development of the infection, such as temperature and humidity, which play a major role in the development of symptoms (Doohan *et al*., 2003; Ekwomadu and Mwanza, 2023).

Regarding fungal isolate *F. verticillioides*, it is one of the fungi that causes root rot, which appears in the form of a white mold tending towards pink on the seeds. Then the infection develops into brown lines that take a radial pattern (Tom and Patel, 2021). What distinguishes them from other *Fusarium* pathogens is that they take the form of rotten cones inside the seeds and move to the top of the plant. This depends on the conditions that are suitable for causing the infection (Saldanha *et al*., 2022). These results are consistent with Czarnota *et al*. (2003), who stated that this fungus has a distinctive way of causing infection, and the most important feature is the presence of cones in the place of infection and in the plate surrounding the root, and when the infection develops, the obstacle becomes great in drawing water to the tops of the plant. Al-Nafei and Mohammad (2021) also stated that the presence of this fungus on the seeds leads to their infection and the entry of fungal tissues into the seed and the presence of sporangia and cones. Inside the seeds, these fungal strains grow vigorously, causing the seed to die before it emerges on the soil. In some plants, this fungus has been observed to infect plants after the vegetative growth has occurred through its toxins which persist for a long time and have a profound effect on the plant's structure (Chen *et al*., 2021).

Moreover, it is noticeable that when the nylon bags were removed two days after treatment, it led to a decrease in the humidity level, and thus some fungal species that were dependent on humidity did not complete their penetration into the host. The isolation was repeated, and the same fungi that caused the infection appeared (Golzarfar *et al*., 2012).

The effectiveness of Fusarium isolates on seed and seedling germination

Results as shown in Table 4 exhibited that all fungal isolates significantly increased the pre-emergence mortality rate as compared to the control treatment. The pre-emergence seed mortality rate in these treatments ranged from 10 to 45%. The least effective was *F. chlamydosporum* (10%), and the most effective was *F. oxysporum* (45%). While only three species showed a significant effect on post-emergence mortality rates, namely *F. solani, F. musarum* and *F. verticelloides*. The percentage of seedling death after emergence in these treatments was 12.5, 35.7 and 36.3%, respectively, while the isolates of two species *F. oxysporum* and *F. clamydosporum* did not cause death of the seedlings after emergence. The results of this experiment were identical to the previous experiment in the inability of the two species *F. verticelloides* and *F. chlamydosporum* to infect the plants after emergence.

Table 4: *The effectiveness of five Fusarium sp. isolated from the seeds on safflower seeds and seedling germination.*

Treatments	Seedling germination	
	Before emergence	After emergence
Control	0.0	0.0
F. salani	20.0	12.5
F. musarum	30.0	35.7
F. verticelloides	25.0	0.0
F. oxysporum	45.0	36.3
F. chlamydosporum	10.0	0.0
LSD (@ 0.5% probability level)	5.5	7.4

This result may be due to the weaker ability of the two species to secrete specialized enzymes or toxins that help the fungus to penetrate the host and cause infection. These results are consistent with what was found by Chung *et al*. (2001) and Ataikiru *et al*. (2019) regarding the ability of *F. solani* and *F. musarum* to increase the infection rate and the death rate before and after seedling emergence, and in a noticeable way. Compared to the less effective *F. verticelloides* species, it was observed that the infection was caused by the same fungi during re-isolation.

Evaluation of treatments against F. solani infestation in safflower seeds or seeldings

There was a clear indication of the effectiveness of all fungicidal treatments in reducing the infection and the death rate before and after emergence, in a significant way as compared to the control treatment, which was represented by the presence of the fungus alone (Table 5). The Beltanol pesticide was superior, as the seed or seedling death rate in this treatment before and after emergence was 5.0 and 0.0%, respectively, which

was similar in its effect significantly to the treatment of the extract of the fungus *Clavitia pychderuma*. The mortality rate in this treatment before emergence was 10% and after emergence was 0.0%. The least effective treatment was *Pseudomonas flourecence*. This treatment was effective in reducing the mortality rate before and after emergence as compared to the treatment of the pathogenic fungus alone, as it led to a reduction in the mortality rate of about 20–40%, respectively (Table 5).

Table 5: *Comparative evaluation of the efficiency of some treatments in protecting safflower seeds from infection with the fungus F. solani.*

Treatments	Seed or Seedlings death (%)	
	Before emergence	After emergence
F. solani	40.0	33.3
F. musarum	20.0	10.0
F. solani + *Pseudomonas florescence*	20.0	10.0
F. solani + *Clavitia pychderuma*	5.0	0.0
F. solani + Beltanol	5.0	0.0
Control (sterilized seeds planted in sterile soil)	0.0	0.0
LSD (@ 0.5% probability level)	10.6	

The results obtained are encouraging for the use of the alcoholic extract of the basidiomycete fungus *Calvatia pachyderma* in seed treatment to protect seeds from soil-borne fungi. A review of previous research has demonstrated the effectiveness of this fungus against plant-pathogenic soil fungi such as *F. semitectum, Pythium aphanidermatum, Rhizoctonia solani, Alternaria raphani, Alternaria alternata,* and *Alternaria tenuissima*. In laboratory experiments, under greenhouse and field conditions, and perhaps in combination with *P. flourecence* bacteria, it gave better results, and was safer for the environment, and falls within the modern trends of combating diseases caused by plant pathogens (Sousa *et al.*, 2014).

Conclusions and Recommendations

Based on overall study results, it is concluded that out of five *Fusarium* fungal isolates obtained from safflower samples collected from Iraq, three strains (*F. solani, F. F. verticelloides* and *F. musarum*) were first time reported and were able to cause infection in safflower seeds and seedlings, while the two species (*F. oxysporum* and *F. chlamydosporum*) were not infective. The effect of synthetic fungicide Beltanol® and the alcoholic extract of the basidiomycete fungus, *C. pychderuma*, was superior as compared to the control treatments.

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Novelty Statement

This study reported for the first time five pathogenic isolates of *Fusarium* associated with the seeds of safflower with three of these isolates *i.e. F. solani, F. verticelloides* and *F. musarum* as the most pathogenic and virulent against safflower as pre- and post-emergence pathogens. Moreover, the extract of basidiomycete fungus, *C. pychderuma* was most effective in reducing pre- and post-mergence disease infection and death of seeds and seedlings.

Generative AI or AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have no conflict of interest.

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