Pathogenicity assessment of two *Alternaria spp.* isolates on the seeds and seedlings of three cauliflower hybrids

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Abstract

The fungus *Alternaria* is known to be one of the important pathogens of cauliflower crops globally including in Iraq resulting in significant losses in yield by infecting leaves which is considered the main site of photosynthesis to cause reduction both quantitatively and qualitatively depending on the environmental conditions. This study aimed to evaluate the pathogenicity of two isolates of *Alternaria spp*. on seeds and seedlings of three cauliflower hybrids and to identify the most tolerant of infection. A field survey was undertaken to detect the pathogen in some fields of Najaf, Baghdad, Babylon and Wasit governorates in addition to their isolation, purification, morphological and molecular identification. Three imported cauliflower hybrids (Emma Snow, Steady and White Flake) which are widely grown in the study area were assessed under opportunistic conditions to determine the susceptibility to the pathogen. Several experiments were carried out to assess the pathogenic fungi on the seeds and seedlings of the cauliflower hybrids tested. The survey results indicated the appearance of symptoms similar to those of *Alternaria* leaf spot on cauliflower plants in the fields of the studied areas. Two isolates of the pathogenic fungus were obtained from infected leaf samples, and both were purified. Morphological and microscopic examinations confirmed that they belonged to *Alternaria* spp. Molecular identification of the isolate obtained from the research station at the University of Kufa confirmed it as *A. alternata*, which was registered as a new isolate in the National Center for Biotechnology Information (NCBI) under accession number PV124558. The second isolate, obtained from the Jebla area in Babylon governorate, was identified as *A. burnsii* and registered as a new isolate under accession number PV124578. Both fungi were recorded as causal agents of *Alternaria* leaf spot disease in Iraq for the first time. The results of the pathogenicity tests on the three studied cauliflower hybrids revealed variability in the severity of infection caused by the two pathogenic fungi. *A. alternata* produced the highest seed rot percentage of 47.77%, while *A. burnsii* recorded the lowest fresh seedling weight on PDA medium at 0.016 g. Regarding the severity of infection on the hybrids Emma Snow, Steady, and White Flake in the pot experiment, *A. burnsii* scored 2.00%, 4.667%, and 4.333%, respectively, according to the disease severity scale. In comparison, *A. Alternata* registered 4.66%, 3.66% and 3.33%, while the control treatment showed 0.00%. The results indicated that the hybrids Steady and White Flake were the most susceptible to the pathogen in plastic pots and field conditions, respectively, whereas Emma Snow was the least affected among the tested hybrids. The study concludes that Emma Snow hybrid exhibited the lowest sensitivity to the disease and, therefore, could be recommended for cultivation in fields infested with the pathogen.

Keywords: cauliflower, leaf spot, *Alternaria* spp., pathogenicity

ISSN 2072-3857

Introduction

Cauliflower (*Brassica oleracea* L.) belongs to the family Cruciferae (Brassicaceae) and is considered a major common winter vegetable crop, in recent times, consumer interest in cauliflower has increased and it is one of the most-consumed vegetables largely due to its high nutrition. Cauliflower is presumed to have originated from the island of Cyprus before reaching Syria, Turkey, Egypt, Italy, Spain, and northwestern Europe [1]. Cauliflower is a rich source of bioactive phytochemicals, such as indole-3-carbinol and glucosinolates, that possess anticancer activity [2]. There are also important health benefits from those phytochemicals and these benefits will help with preventing cancers, cardiovascular diseases, and diabetes [3].

India has a cultivated area of about 410.9 thousand hectares, making it the largest cauliflower producer in the world, with China the next largest. The total world area planted with cauliflower is almost 1258.1 thousand hectares [4]. In Iraq the cultivated area of cauliflower, was around 5903 dunams, and in the Iraqi governorates, the largest area was in Baghdad, which had 2037 dunams with a production value of some 5583 tons, next was Diyala with 1387 dunams and 3409 tons [5].

Cauliflower is prone to multiple diseases and is sensitive to numerous pathogens, especially, fungi. Among these factors are leaf spot and seed and seedling-associated diseases caused by *Alternaria spp*., which can harm the productivity of all vegetable crops, including cauliflower. They are common pathogenic fungi, capable of causing leaf spot, seed rot, and seedling death. It can reduce germination rates and cause poor early growth. Seeds and seedlings are the most sensitive parts of the plant life cycle, as any fungal influence during these stages can lead to significant result in a substantial loss of both quantity and quality from the crop yield [6,7]. In India, for instance, yield losses related to such diseases can often range from 30–50%, or reach as high as 60% in certain extreme instances [8,9]. The various cauliflower hybrids have different responses to fungal pathogens since some hybrids demonstrated relative resistance in earlier studies and some hybrids were relatively susceptible to fungal pathogens. Therefore, it is important to understand the effects of these fungi on the various hybrids in order to develop integrated disease management strategies that include the use of resistant cultivars, recommended preventative practices and management agricultural practices[10]. Therefore, this study aimed to evaluate the effect of two isolates of *Alternaria spp*. on the seeds and seedlings of three cauliflower hybrids and to assess their susceptibility to infection. thereby contributing to the selection of the most resistant hybrids within integrated disease management strategies.

Materials and Methods

Isolation and Identification of *Alternaria spp*.

Samples of infected cauliflower leaves were collected from several fields cultivated with this crop in the governorates of Najaf, Baghdad, Babylon, and Wasit for the isolation, purification, and identification of the causal pathogens. The infected parts were cut into small pieces (0.5–1 cm), their surfaces were then sterilized using sodium hypochlorite solution (1–2%) for one minute, followed by washing them three times with distilled water to remove traces of sterilization. They were then dried on filter paper and placed on Petri dishes containing PDA medium. The plates were incubated at 25 ± 2 °C for five days. After the fungal colonies had developed, they were purified and identified in the Plant Pathology Laboratory, Department of Plant Protection, College of Agriculture, University of Kufa, based on their characteristics.

Seeds of Cauliflower Hybrids Used in the Study

Based on the survey conducted at the beginning of the study, seeds of three imported cauliflower hybrids (Steady, Emma

ISSN 2072-3857

Snow, and White Flake) were selected. These hybrids were chosen because they are widely cultivated in the study areas.

Morphological Identification of Alternaria spp. isolates

Morphological identification of the *Alternaria spp.* isolates was based upon phenotypic characteristics of the purified fungal colonies such as color, pigmentation, growth pattern, and growth rate. The structures and conidia that were produced by the fungus were also examined for their color, shape, size, length, width, number of cells per spore and sporulation pattern. Identification was made using specialized taxonomic keys [11,12].

Pathogenicity Test of *A. burnsii* and *A. alternata* Isolates on Cauliflower Hybrid Seeds in Petri Dishes

A total of 36 Petri dishes (9 cm in diameter) each with 20 ml of PDA medium were prepared. A 0.5 cm disc taken from the margin of a 7-day-old colony of the pathogenic isolates, *A. burnsii* and *A. alternata*, which had grown separately on PDA, was inoculated in the centre of each plate with three replications for both isolates, respectively. Control treatments were prepared in three replications but without fungal inoculation. After 48 hours, cauliflower hybrid seeds were sterilised with 2% sodium hypochlorite for 2 minutes and rinsed several times with sterile distilled water to remove any disinfectant residues and dried on filter paper. Ten sterilised seeds were sown per plate and arranged in a circle around the growing margins of the fungal colonies. In the control treatment, sterilised seeds were sown on non-inoculated PDA plates with the same number of seeds as in the test treatment. All plates were incubated at 25 ± 2 °C. After 7 days, germination percentage was calculated and after 14 days the percentage of rotten seedlings was calculated according to the method of [13], and according to the following equations:

$$\text{Germination \%} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

$$\text{Seedling rot\%} = \frac{\text{Number of rotted seedlings}}{\text{Total number of emerged seedlings}} \times 100$$

Pathogenicity Test of Fungi Causing Cauliflower Leaf Spot on Seedlings in Plastic Pots.

This experiment was carried out using a mixed soil mixed with a percentage of peat moss and placed in plastic pots (100 g/pot), with three replications for each treatment. They were carefully watered and covered with perforated polyethylene bags. A control treatment was also carried out following the same previous steps. All pots were planted with the three cauliflower hybrid seeds (5 seeds/pot) superficially sterilized with a 1% sodium hypochlorite solution (NaOCl) for 2 minutes and washed with sterile distilled water several times to eliminate the effects of sterilization. They were well watered, taking into account re-watering whenever necessary, and distributed randomly under natural conditions. The percentage of seed germination was calculated 10 days after planting according to the following equations:

$$\text{Germination \%} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

After 20 days of sowing, the seedlings were thinned to three plants per pot. When the plants were 30 days old, they were inoculated with the spore suspension of both isolates. After 7 days measurements were taken, with the aerial part length and the fresh and dry weight of the shoot system. Disease severity was assessed using a four-grade scale described by [14], as follows: 0 = healthy plants; 1 = chlorosis and yellowing of the plant; 2 = temporary wilting; 3 = permanent wilting and death of the plant, and according to the equation:

ISSN 2072-3857

$$\text{Infection severity\%} = \frac{\text{total number of infected} \times \text{degree of infection}}{\text{Total number} \times \text{Maximum value on the severity scale}} \times 100$$

Statistical analysis

The results were statistically analyzed according to the analysis of variance (ANOVA) as per the randomized complete design (CRD), the least significant difference (L.S.D 0.05) test was used to compare and separate the means (Gomez and Gomez, 1984), this is done using statistical analysis software GenStat12.9.

Results and Discussion

Pathogenicity Test of *A. burnsii* and *A. alternata* on Cauliflower Hybrid Seeds on PDA Medium

The data given in Table 1 displayed the impact of the fungal isolates on cauliflower hybrid seeds, sown in petri dishes of PDA medium. The highest percentage of seed rot occurred in the Emma Snow hybrid, treated with *A. alternata* (80%), and was followed by the same hybrid treated with *A. burnsii* (76.66%). In the control treatment, overall germination failure in treatment caused by pathogens was 3.3%. The high percentage can be explained by the ability of both fungal isolates to synthesize compounds, such as toxins and hydrolytic enzymes, which circulate within the host cells to create infection. in addition to the susceptibility of the hybrid. This concurs with the findings[15], who reported that most *Alternaria* species secrete toxins including Tenuazonic acid, Alternariol, Alternariol Monomethyl ether, Altenuene, and Tentoxin, which disrupt the vital functions of the host plant and lead to tissue death. The strong effect of these pathogenic fungi may result from direct seed infection and inhibition of growth or from the secretion of enzymes and toxins that reduce seed viability [9].

In a study conducted by [16] on the seeds of various hybrids of broccoli, *A. brassicicola*, the cause of *Alternaria* leaf spot, completely inhibited seed germination (100%). In the case of the present study, the Steady hybrid had the lowest permill amount of seed rot, that being treated with *A. alternata* (10%) then *A. burnsii* (17%), contrasted by the control, where none of the seeds germinated, with only 3% of the seeds failing to germinate. This less susceptibility may be due to genetic differences and the fact that the Steady hybrid has a resistance gene. The Steady hybrid is a first-generation hybrid not only recognized for it earlier maturing abilities, but also for its high early and total yield, improved curd quality (higher firmness, color, and uniformity), and pest, disease, and adverse environmental condition resistance [17].

ISSN 2072-3857

Table 1. Effect of Pathogenic Fungal Isolates on Germination and Seed Rot Percentage of Cauliflower Hybrids Grown in Petri Dishes 10 Days After Sowing

Fungi	Hybrids			Means
	Emma snow	Steady	White flake	
A. burnsii	76.66	16.66	40.00	44.44
A. alternata	80.00	10.00	53.30	47.77
Control	3.30	3.33	10.00	5.54
Means	53.32	10.00	34.43	
L.S.D $_{0.05}$	Fungi = 16.83, Hybrids = 16.83, Interaction = 29.16			

Each value in the table represents the mean of three replications.

Pathogenicity Test of *A. burnsii* and *A. alternata* on Fresh Seedling Weight of Cauliflower Hybrids on PDA Medium

The results shown in Table 2 show that the Alternaria species impacted the fresh weight of the cauliflower hybrid seedlings. The highest fresh seedling weight was measured in the *A. alternata* treatment at 0.018 g while the lowest was in the *A. burnsii* treatment at 0.016 g in comparison to control seedlings which were 0.023 g. In the same table, results showed significant differences in terms of fresh seedling weight of the cauliflower hybrids used in the study. The Steady hybrid had a significantly heavier seedling weight at 0.024 g, followed by White Flake and Emma Snow at 0.018 g and 0.014 g respectively .

With respect to the interaction treatment, we found the highest mean seedling weight was produced in the treatment with the fungus *A. alternata* with the hybrid Steady, at 0.024 g in contrast recorded 0.027 g in the control treatment, followed by Emma Snow, which resulted in 0.015 g compared to 0.020 g in the control. Conversely, the lowest seedling weight was observed in the treatment with the fungus *A. burnsii* in the hybrid Emma Snow with a mean seedling weight of 0.008 g compared to the control with 0.014 g, followed by White Flake, which produced 0.019 g compared to 0.020 g in the control, respectively.

The present findings showed that both *Alternaria* species had an impact upon the fresh weight of seedlings, but that the extent of that impact differed between hybrids. This is in agreement [18] who reported that the fresh seedling weight of ten cauliflower hybrids differed upon inoculation with *A. brassicae*, in their study the differences in fresh weight were related to a difference in hybrid susceptibility. The impact of the fungi on seedling weight may have related to the parasitic nature of *Alternaria* species, or the production of various toxins and secondary metabolites in the growth medium. According to [19], *Alternaria spp*. produce many different toxins, which enable them to overcome host resistance. They damage plant tissues by creating cell wall-degrading enzymes. Fungal infection also may limit the plant's ability to uptake water and nutrients, in turn reducing growth or wilting seedlings, especially juvenile seedlings [9].

ISSN 2072-3857

Table 2. Effect of Pathogenic Fungal Isolates on Fresh Seedling Weight (g) of Cauliflower Hybrids Grown on PDA Medium in Petri Dishes

Fungi	Hybrids			Means
	White flake	Steady	Emma snow	
A .burnsii	0.019	0.022	0.008	0.016
A.alternata	0.015	0.024	0.015	0.018
Control	0.020	0.027	0.020	0.023
Means	0.018	0.024	0.014	
L.S.D $_{0.05}$	Fungi = 0.00658, Hybrids = 0.00658, Interaction = 0.0114			

Each value in the table represents the mean of three replications.

Pathogenicity Test of *A. burnsii* and *A. alternata* on Cauliflower Hybrid Seedlings Grown in Plastic Pots

Based on the results shown in Table 3, significant differences were observed between the pathogenic isolates, *A. burnsii* and *A. alternata*. *A. alternata* recorded the highest mean disease severity of 3.89%, followed by *A. burnsii*, which recorded a percentage of 3.67% compared to the control treatment, which recorded a percentage of 0.00%. The results of Table (3) indicated that there were no significant differences in disease severity among cauliflower hybrids. As for the interaction between fungal isolates and cauliflower hybrids, it was observed that the Steady hybrid recorded the highest average disease severity of 4.167%, while the disease severity was lower for the type *A. burnsii* on Emma Snow hybrid with an average of 2.00% compared to the control treatment, which recorded an average of 0.00%. Further examination of this data would suggest the fungal pathogen and susceptible hybrid indicated interaction likely had a direct impact on the disease severity on seedlings. Heavy fungal growth on sensitive seedlings and subsequent colony formation contributed to severe weakening or death [20]. The fungus produces millions of spores which can infect seedlings via wounds or natural openings and increased with severity of the infection [21]. While the difference in sensitivity to pathogen infection between different hybrids may be due to genetic reasons such as the thickening of the cuticle layer or the production of antifungals by the plant's defense system as a mechanism against pathogens [22].

Table 3. Effect of *A. burnsii* and *A. alternata* on Cauliflower Hybrid Seedlings (Disease Severity) Grown in Plastic Pots.

Fungi	Hybrids			Means
	Emma snow	Steady	White flake	
A .burnsii	2.000	4.667	4.333	3.67
A.alternata	4.667	3.667	3.333	3.89
Control	0.00	0.00	0.00	0.00
Means	2.22	2.78	2.56	
L.S.D $_{0.05}$	Fungi = 0.632, Hybrids = N.S, Interaction = 1.095			

Each value in the table represents the mean of three replications.

Effect of the Pathogenic Fungi *A. burnsii* and *A. alternata* on Dry Weight of Cauliflower Hybrid Seedlings in Plastic Pots.

ISSN 2072-3857

The results shown in Table (4) showed the effect of the two pathogenic fungi on the dry weight of cauliflower seedling hybrids, where the *A. burnsii* and *A. alternata* treatments recorded the highest average of 0.0062 and 0.0055 g, respectively, compared to the control treatment, which recorded 0.0111 g. The results in the same table showed significant differences between cauliflower hybrids in the dry weight trait of seedlings, as the Steady hybrid outperformed with an average of 0.0084 g, followed by the White flake hybrid with an average of 0.0077 g, then the Emma snow hybrid, which recorded the lowest average of 0.0067. As for the interaction, the highest average dry weight of seedlings

was recorded in the *A. burnsii* fungus treatment in the Steady hybrid, as the average seedling weight recorded 0.0074 g compared to 0.0114 g in the control treatment, followed by the same fungus treatment in the White flake hybrid, which recorded 0.0069 g compared to the control treatment, which recorded 0.0100 g, while it recorded The lowest seedling dry weight was recorded in the *A. alternata* treatment on the Emma snow hybrid at 0.0039 g compared to 0.0119 g in the control treatment. The spots caused by the two pathogenic fungi on the seedlings of inoculated cauliflower hybrids can block or affect the plant's food production due to the inability to photosynthesize. They also affect the uptake of water and other nutrients, which negatively impacts the seedling's nutrient content and consequently reduces its dry weight [8]. Infection with the pathogenic fungus can also stunt growth and affect the seedling's health and vigor, reducing its dry weight [23].

Table 4. Effect of *A. burnsii* and *A. alternata* on Dry Weight (g) of Cauliflower Hybrid Seedlings in Plastic Pots.

Fungi	Hybrids			Means
	Emma snow	Steady	White flake	
A .burnsii	0.0043	0.0074	0.0069	0.0062
A.alternata	0.0039	0.0066	0.0062	0.0055
Control	0.0119	0.0114	0.0100	0.0111
Means	0.0067	0.0084	0.0077	
L.S.D $_{0.05}$	Fungi = 0.0015, Hybrids = 0.0015, Interaction = 0.0025			

Each value in the table represents the mean of three replications.

Conclusion

The results of the study showed that *A. alternata* and *A. burnsii* are predominant causal agents of Alternaria leaf spot of cauliflower and have been reported for the first time in Iraq. The study concluded that there was some variation among the hybrids in disease severity, with the hybrid Emma Snow being the least susceptible and Steady and White Flake being the most. These findings support the possible use of hybrids that are less susceptible as part of an integrated management program and indicate that selections of hybrids should continue in order to improve the selection of resistant varieties.

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ISSN 2072-3857