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GENETIC DIVERSITY OF *ALTERNARIA BRASSICICOLA*, THE LEAF SPOT PATHOGEN OF CABBAGE (*BRASSICA OLERACEA* L.)

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SUMMARY

Alternaria brassicicola is a major necrotrophic pathogen causing black leaf spot in cabbage and other cruciferous crops. This study aimed to characterize 19 pathogenic isolates of *Alternaria* spp. collected from three cabbage-growing regions in Egypt based on morphology, pathogenicity and genetic diversity. Molecular identification based on ITS sequence clustered the collected isolates along with *A. brassicicola* confirming their identity. Deep morphological analysis delineated the variations in conidial size and septation among the isolates. Pathogenicity test was performed on detached and attached leaves, showing wide variations in lesion size and disease severity among isolates. Genetic diversity analysis used five ISSR primers, generating 46 bands with 72.61% polymorphism. Cluster analysis grouped the isolates into two main clusters, although no clear correlation appeared between genetic patterns, geographic origin, or virulence. Antifungal in vitro assays showed differential sensitivity to difenoconazole, azoxystrobin, mancozeb, and copper oxychloride, with difenoconazole being the most effective at the recommended doses per each. The combined variation in morphology, virulence, genetic sequences, and fungicide sensitivity suggests high adaptive potential in local *A. brassicicola* population. These findings highlight the need for integrated disease management using diverse resistance sources and fungicide rotation strategies for sustainable control.

Keywords: *Alternaria brassicicola,* genetic characterization, leaf spot disease, ITS sequencing, molecular markers, disease severity assessment, cluster analysis, resistance breeding

Key findings: High genetic, pathogenic, and fungicide-response variability among *Alternaria brassicicola* isolates underscores their capacity for rapid adaptation in cabbage fields. Accordingly, resistance-breeding programs should pyramid multiple resistance sources and screen candidate cultivars against a broad, representative panel of isolates to secure durable, wide-spectrum control of the leaf-spot disease.

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INTRODUCTION

Alternaria brassicicola (Schwein.) Wiltshire is a necrotrophic ascomycete responsible for black-leaf-spot disease in a broad array of cruciferous hosts, with cabbage (*Brassica oleracea* var. *capitata*) among the most seriously affected crops worldwide (Deep *et al*., 2014). Characteristic dark lesions develop on leaves, stems, and siliques; under warm, humid conditions, these lesions readily coalesce, triggering premature defoliation, head discoloration, and pronounced yield and quality losses. Primary inoculum consists of wind- and rain-dispersed conidia, yet the pathogen also persists between seasons, on seed coats and in crop debris, permitting long-distance dissemination and perennial re-establishment in successive plantings (Linde *et al*., 2010; Kreis *et al*., 2016).

Cabbage ranks among Egypt's most economically important vegetables, cultivated in both commercial fields and smallholder systems. As foliage constitutes the marketable product, even moderate black-spot infection renders heads unfit for sale. Recent surveys consistently list *A. brassicicola* as the dominant causal agent of cabbage leaf spot across major production governorates, and growers rely heavily on fungicidal sprays for protection (Galal *et al*., 2018). Such dependence is unsustainable: heavy chemical inputs elevate production costs, raise environmental concerns, and exert strong selection pressure that can foster fungicide resistance. An integrated strategy grounded in host resistance and informed by local pathogen biology is therefore essential.

Although this fungal pathogen is traditionally regarded as strictly asexual, multilocus population studies have revealed unexpectedly high genotypic diversity and evidence of cryptic recombination (Bock *et al*., 2005; Linde *et al*., 2010). High diversity enhances adaptive potential, enabling populations to overcome single-gene host resistances, tolerate environmental stresses, and, in some cases, evolve to reduced sensitivity to fungicides. Understanding the genetic and pathogenic structure of regional populations is, thus, a prerequisite for breeding programs that seek durable resistance and for devising evidence-based chemical-control schemes.

DNA-based molecular markers have become indispensable for dissecting fungal population biology. Techniques, such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and, more recently, inter-simple sequence repeat (ISSR) analysis, have uncovered substantial intraspecific polymorphism in *Alternaria* spp. from numerous hosts (Gherbawy, 2005; Adss *et al*., 2016). ISSR markers are particularly attractive to routine diagnostics because they are inexpensive, technically straightforward, and highly polymorphic, even among closely related isolates. Studies from India (Deep *et al*., 2014) and Iran (Nasr Esfahani, 2018) confirmed their utility for *A. brassicicola* diversity assessments, while ISSR-based surveys of *A. solani* on tomato in Egypt demonstrated the method's suitability under local lab conditions (Adss *et al*., 2016). Nevertheless, comparable data for Egyptian *A. brassicicola* populations remain scarce.

Despite the global importance of *A. brassicicola*, little is known about the genetic diversity and pathogenic behavior of its populations in Egypt. Addressing this gap is essential for developing resistant cultivars and designing effective, locally adapted management strategies. This study characterized 19 pathogenic isolates of *A. brassicicola* collected from cabbage plants showing leaf spot symptoms. Morphological and molecular analyses confirmed species identity, and pathogenicity assays revealed varying levels of virulence. ISSR-based genotyping uncovered substantial genetic diversity, while fungicide sensitivity tests demonstrated differential responses among isolates. These findings underscore the need for integrated disease management approaches that combine resistance breeding with informed fungicide rotation. Altogether, the study provides a foundation for sustainable, long-term control of cabbage leaf spot disease.

Table 1. Geographic origin, isolate codes, and disease levels of cabbage leaf spot in El-Sharkia Governorate, Egypt.

Location	GPS coordinates		Isolates	Disease incidence %	Disease severity %
	Latitude	Longitude			
Al-Husseiniya	30°47′55.0″N	32°04′16.9″E	C-Y1 to C-Y7	95	47.22
Hehya	30°39′50.9″N	31°35′37.2″E	C-H1 to C-H6	92	37.22
Belbeis	30°23′22.8″N	31°32′31.7″E	C-B1 to C-B6	98	53.78

MATERIALS AND METHODS

Disease survey and sample collection

During the 2023–2024 winter-growing season (from November to February), the survey of cabbage leaf spot commenced in nine fields (100 plants/field) planted with the commercial variety 'OS Cross F1' across three districts of El-Sharkia, Egypt: Al-Husseiniya, Hehya, and Belbeis (Table 1). Disease incidence (DI) recording was the proportion of plants bearing lesions. Disease severity (DS) rating on each plant used a visual 0–9 scale (Sharma *et al*., 2004) that distinguishes four symptom classes: 0 = healthy; 1–3 = minor flecking (≤15 pinpoint spots); 4–6 = moderate lesion development; and 7–9 = extensive coalescence and tissue collapse. Scores' conversion to percentage DS employed the formula DS (%) = [Σ(n × v) / (N × V)] × 100, where n is the number of plants in each severity category, v is the severity value, N is the total number of assessed plants, and V is the maximum value on the scale. Symptomatic leaves showing brown necrotic lesions with yellow halos entailed collection and being photographed for further analysis. Sample sortation into paper bags preceded their transport to the laboratory in an icebox containing cooling blocks.

Isolation and purification

Cabbage leaf samples succeeded their cutting into ~5 mm pieces containing both diseased and healthy tissues, rinsing in sterile distilled water (5 min), surface sterilizing in 0.5% sodium hypochlorite (2 min), re-rinsing (5 min), and drying on sterile filter paper. Surface-sterilized pieces' placement on water agar in 9 cm Petri dishes received incubation at 28 °C for three days. Emerging fungal colonies sustained subculturing on a potato dextrose agar (PDA) and incurred purification using hyphal tip and single-spore techniques (Choi *et al*., 1999). A 5-mm plug from each pure culture entailed transferring to fresh PDA before incubation at 28 °C with a 12 h light/12 h dark cycle for nine days.

Morphological characterization

Isolates' identification relied on colony appearance on the PDA after nine days of inoculation, conidial shape, and septation patterns (Simmons, 2007). Recording colony color, texture, and growth pattern also transpired. The length and width of conidia, along with the length of transverse and longitudinal septa, reached measurements for 50 conidia per isolate with an ocular-microscope micrometer. The calculation of mean values and standard deviations (SD) also occurred.

Pathogenicity test

Pathogenicity assessment sequentially used detached and attached leaf assays (Bock *et al*., 2005). The detached-leaf assay first proceeded on all 74 *Alternaria* isolates grown on PDA for 7–10 days at 28 °C under a 12 h light/dark cycle. Agar plugs' (1–2 mm) placement ensued on third–fourth true leaves detached from 45–50-day-old 'OS Cross F1' cabbage plants (three plugs per leaf, three leaves per isolate) in Petri dishes lined with moist filter paper. Inoculated leaves gained incubation at 25 °C ± 2 °C for 3–5 days. Lesion number, diameter, and total infected area measurements engaged ImageJ, with the pathogen re-isolated to fulfill Koch's postulates. Based on these results, 19 pathogenic isolates succeeded in their selection

for further evaluation using an attached-leaf assay in the greenhouse. Cabbage plants of the same age obtained spraying with conidial suspensions (1×10^6 conidia/mL) containing 0.05% Tween 20 until runoff. Control plants received sterile water spraying. Plants remained under high humidity (>85% RH) for 24 h, maintaining temperature at 25 °C ± 2 °C. Each treatment achieved replications on three plants. Lesion assessment 14 days post-inoculation continued, with disease severity rating using the 0–9 scale (Sharma *et al*., 2004) and converted to percentage severity. Koch's postulates incurred confirmation by re-isolation of inoculated pathogens.

DNA extraction

Genomic DNA extraction followed Atallah *et al*. (2022) with modifications. Each isolate grown on the PDA at 28 °C for nine days had 50 mg of fresh mycelium scraped into a 1.5 mL tube. Then, the tube received 200 µL of CTAB lysis buffer (2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 1% PVP, and 7 µL β-mercaptoethanol), with the mycelium ground using a sterile micropestle. Next, 500 µL of CTAB buffer and 7 µL of proteinase K succeeded in adding, vortexing, and incubating at 65 °C for 5 min. After centrifugation at 13,000 g for 5 min, the transfer of 200 µL of supernatant to a new tube progressed. The DNA precipitation used 600 µL of ice-cold 100% ethanol and 150 µL of 5 M NaCl, incubated at −20 °C for 1 h, and centrifuged at 13,200 g for 10 min. The pellet washing with 1000 µL of 70% ethanol followed, then centrifugation at 10,000 g for 5 min continued, before air-drying, resuspending in 50 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA), and storing at −20 °C.

Molecular identification and phylogenetic analysis

In confirming the species identity of *Alternaria* spp., six representative *Alternaria* isolates (two per location) became choices for the internal transcribed spacer (ITS)-based identification. DNA extraction continued, and the ITS region's amplification used universal primers ITS5 and ITS4 (White *et al*., 1990) in a 25-µL PCR reaction. It consisted of 12.5 µL of master mix (2X *ama*R OnePCR™), 1 µL of DNA sample (~20 ng), 1 µL of ITS5 primer (10 pmol/µL), 1 µL of ITS4 primer (10 pmol/µL), and 9.5 µL of double-distilled (nuclease-free) water. Amplification conditions included an initial denaturation at 94 °C for 4 min, followed by 35 cycles (94 °C for 60 s, 56 °C for 45 s, and 72 °C for 60 s), and a final extension at 72 °C for 5 min. Amplicons (~600 bp) reached visualization on agarose gel, with purifying and sequencing by Macrogen (South Korea) before submission to GenBank. Confirming species identity and retrieving reference sequences entailed the isolates' first comparison against entries in the NCBI GenBank database using the BLASTn tool (https://www.ncbi.nlm.nih.gov/BLAST). Reference sequences of *A. brassicicola*, as well as closely related species, such as *A. alternata* and *A. tenuis*, acquired inclusion for comparative purposes, while *Stemphylium vesicarium* served as an outgroup to root the tree. The accession numbers of all sequences used in the analysis, including those of this study, appear in Figure 2. Multiple sequence alignment took place using the ClustalW algorithm in MEGA11 (Tamura *et al*., 2021), with alignments manually inspected and trimmed to remove poorly aligned ends. The phylogenetic tree construction utilized the neighbor-joining (NJ) method based on the Kimura 2-parameter model, with the robustness of branches evaluated using 1,000 bootstrap replications, with values ≥70% considered to indicate strong support.

ISSR-PCR amplification and electrophoresis

Ten ISSR primers from the University of British Columbia (UBC825, UBC811, UBC808, UBC901, UBC814, UBC826, UBC827, UBC835, UBC868, and UBC840) reached initial screening to evaluate their amplification efficiency (Igwe *et al*., 2017). Five primers (Table 2) producing clear, reproducible bands were options for analyzing the genetic diversity of pathogenic *A. brassicicola* isolates. PCR proceeded in 20 µL reactions containing 10 µL of 2X amaR OnePCR™, 1 µL of primer (10 pmol), 1 µL of

Table 2. ISSR primer banding patterns: Monomorphic, polymorphic, and unique bands with polymorphism percentage and PIC values.

Codes	Number of scored bands	Monomorphic bands	Polymorphic bands	Unique bands	Polymorphism (%)	PIC
UBC811	7	3	4	1	57.14	0.13
UBC808	11	1	10	2	90.91	0.25
UBC901	6	4	2	1	33.33	0.05
UBC826	12	1	11	1	91.67	0.3
UBC827	10	1	9	2	90.00	0.27
Average	9.20	2.00	7.20	1.40	72.61	0.20
Total	46	10	36	7	-	-

ISSR: Inter simple sequence repeat; PIC: polymorphic information content.

DNA (~20 ng), and nuclease-free water. Amplification took place in a MyGene™ Peltier Thermal Cycler under the conditions 95 °C for 7 min, 35 cycles of 95 °C for 1 min, primer-specific annealing for 1 min, 72 °C for 1.5 min, final extension at 72 °C for 10 min, and holding at 4 °C. PCR products gained separation on 1.5% agarose gels using a 1 kb ladder (GeneDireX), then staining and visualization (Noran *et al*., 2024).

Genetic diversity analysis

Genetic diversity among the 19 *A. brassicicola* isolates collected from cabbage plants in three different locations (Al-Husseiniya, Hehya, and Belbeis) underwent analysis using ISSR markers. Banding patterns obtained from ISSR profiles received scoring as binary data (presence = 1, absence = 0), with a binary matrix generated. Jaccard's coefficient helped calculate genetic similarity between isolates. A dendrogram construction utilized the unweighted pair group method with arithmetic mean (UPGMA) to visualize the clustering pattern among the isolates. The clustering analysis and dendrogram generation continued using NTSYSpc software (version 2.2) to evaluate the genetic relationships among isolates.

Antifungal sensitivity to commercial fungicides

The antifungal activity of four commercial fungicides at the concentrations recommended by the manufacturers—Mancozeb (2.0 mg/mL), azoxystrobin (0.25 mg/mL), difenoconazole (0.25 mg/mL), and copper oxychloride (2.4 mg/mL)—achieved evaluation in vitro against *A. brassicicola* using the poisoned food technique (Grover and Moore, 1962). Fungicides succeeded their mixing into PDA at ~50 °C before pouring into Petri dishes, having each plate inoculated in the center with a 5-mm mycelial disc cut from the margin of a 5-day-old actively growing *A. brassicicola* colony, mycelium side down. Control plates lacked fungicides. All plates attained incubation at 28 °C ± 2 °C until the fungal colony in the control treatment fully covered the surface of the Petri dish (90 mm in diameter). The percentage of mycelial growth reduction involved calculating as ([C − T]/C) × 100, where C is the colony diameter in the control and T is the colony diameter in the fungicide treatment. Each treatment had five replicates.

Statistical analysis

All assays followed a randomized complete block design. Pathogenicity metrics (lesion diameter, infected area, disease severity; n = 3 leaves/isolate) and fungicide inhibition data (n = 5 plates/isolate) underwent ANOVA in Statistix 10, with means separated using Tukey's HSD ($P \leq 0.05$). Pathogenicity boxplot generation used GraphPad Prism 9.3.1. ISSR polymorphism calculation was the proportion of polymorphic to total bands, and primer informativeness assessment was by $PIC_i = 2f_i(1 - f_i)$, where PIC_i is the polymorphic

information content of marker 'i' and f_i is the frequency of band presence (Roldán-Ruiz *et al*., 2000).

RESULTS

Survey of cabbage leaf spot disease

Observation of the cabbage leaf spot symptoms occurred during the 2023–2024 growing season in three major production districts in El-Sharkia Governorate. Among these locations, the highest DS (53.78%) and DI (98%) recordings resulted in Belbeis, followed by Al-Husseiniya and Hehya (Table 1). Leaf samples collected from these locations exhibited typical symptoms of leaf spot disease, including circular to irregular brown necrotic lesions surrounded by yellow halos (Figure 1).

Fungal isolation and identification

Fungal isolates totaling 74 (found to be *Alternaria* spp.) were notable from cabbage leaf spot samples, of which identifying 19 as *Alternaria brassicicola* based on morphological traits consistent with Simmons (2007). Conidial measurements revealed variation among isolates in size, beak dimensions, and septation, with conidial length ranging from 28.4 to 32.8 µm, width from 8.8 to 12.9 µm, beak length from 3.8 to 6.7 µm, and beak width from 2.2 to 5.5 µm. The number of transverse septa ranged from 3.0 to 8.2, while longitudinal septa varied between 1.1 and 3.3 per conidium. Confirming the identity, ITS sequencing for six representative isolates ensued. Phylogenetic analysis (Figure 2) grouped them within the *A. brassicicola* clade, supporting the morphological classification.

Pathogenicity test

Out of 74 *Alternaria* isolates, 19 succeeded in their confirmation as pathogenic based on their ability to induce lesions in both detached- and attached-leaf assays (Figure 3). In detached leaves, necrotic lesions were visible, with lesion diameters ranging from 0.61 to 2.41 cm and infected areas from 0.88 to 13.69 cm². The most virulent isolates (C-B6, C-H2, and C-B2) produced the largest lesions, while C-Y2 and C-H3 showed minimal symptoms.

Figure 1. Field symptoms of cabbage leaf spot disease observed in El-Sharkia Governorate, Egypt. (A–C) Collected from Al-Husseiniya; (D–F) from Hehya; (G–J) from Belbeis. (A–I) Symptoms include circular to irregular brown necrotic lesions, often with chlorotic halos. Lesions may coalesce, causing extensive yellowing and tissue collapse. (J) It shows severe head infection with tissue decay.

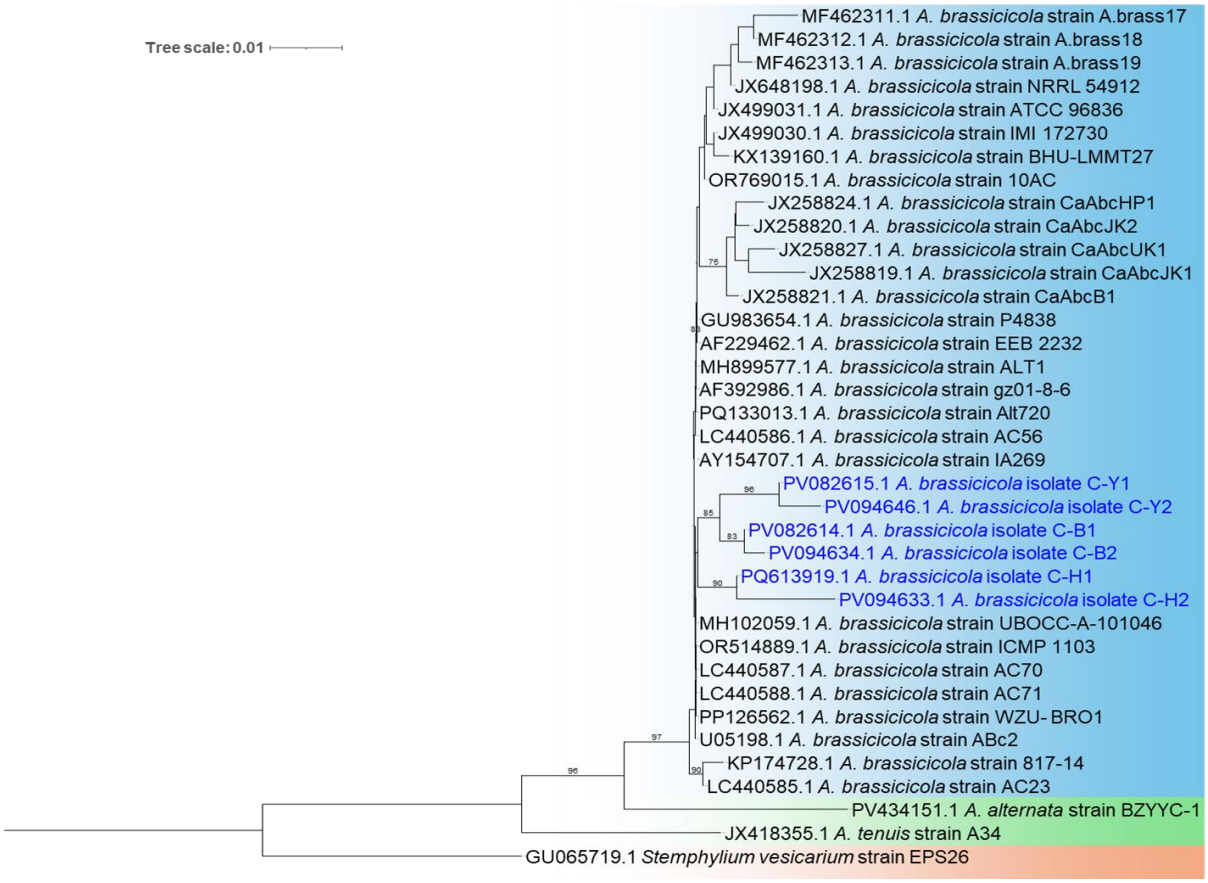

Figure 2. Phylogenetic tree of *A. brassicicola* isolates based on ITS region sequences. The neighbor-joining tree construction used the MEGA11 software based on the Kimura 2-parameter model with 1000 bootstrap replications. The dataset included six isolates obtained in this study (highlighted in blue) and reference strains retrieved from the GenBank, representing major lineages of *A. brassicicola* and closely related species. The alignment, manually trimmed, sought to remove ambiguous regions. The use of *Stemphylium vesicarium* strain EPS26 helped root the tree. Bootstrap support values ≥70% are shown at the corresponding nodes.

In the attached leaf assay, disease severity ranged from 14.53% to 84.80%, following a similar trend. Highly virulent isolates (C-B6, C-H2, and C-B2) caused the most severe symptoms, whereas C-Y2 and C-H3 caused the least. Controls and non-pathogenic isolates showed no symptoms. Koch's postulates reached fulfillment by re-isolation, and the recovered isolates exhibited morphological characteristics consistent with those initially inoculated.

Based on their pathogenicity results on attached leaves and using the 0–9 disease severity scale of Sharma *et al*. (2004), the 19 pathogenic isolates achieved grouping into three categories: low virulent (scores 1–3), moderately virulent (scores 4–6), and highly virulent (scores 7–9). The virulence levels varied among isolates, with most falling into the moderately virulent group (nine isolates), while six isolates exhibited low virulence, with four categorized as highly virulent. These categories are present alongside the dendrogram in Figure 4 to explore whether any relationship exists between virulence levels and the clustering patterns derived from the ISSR marker analysis.

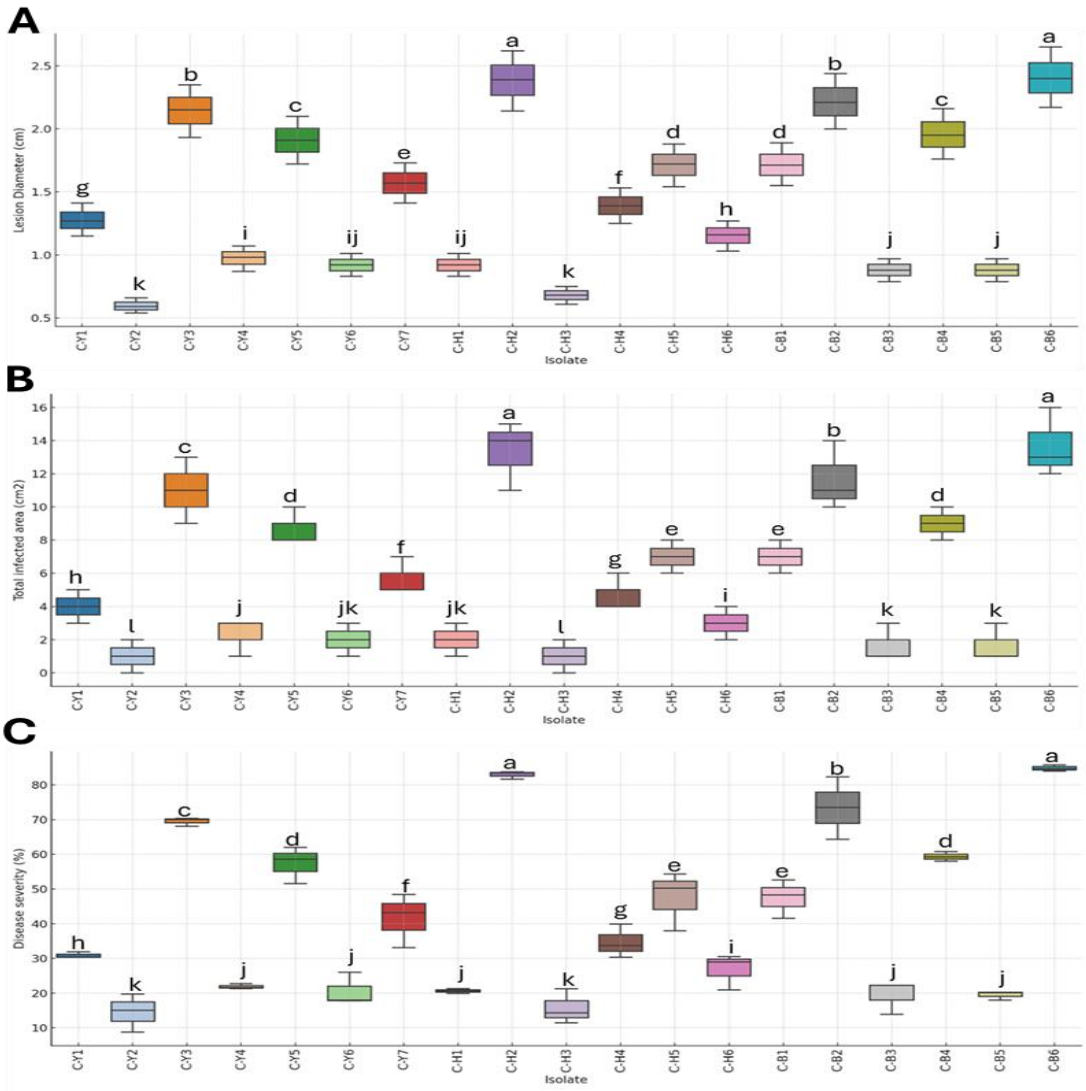

Figure 3. Pathogenicity assessment of 19 *A. brassicicola* isolates on cabbage plants. (A) Lesion diameter (cm) and (B) total infected area (cm^2) measured on detached leaves. (C) Disease severity (%) evaluated on whole plants. Different letters above the boxes indicate statistically significant differences among isolates based on Tukey's HSD test at $p \leq 0.05$.

ISSR analysis

ISSR analysis of the 19 *A. brassicicola* isolates using five effective primers (out of 10 tested) produced 46 bands, including 36 polymorphic, 10 monomorphic, and seven unique bands (Table 2). The percentage of polymorphism ranged from 33.33% (UBC901) to 91.67% (UBC826), with an average of 72.61%. PIC values varied between 0.05 and 0.30, with UBC826 having the highest discriminatory power (PIC = 0.30).

Cluster analysis

Cluster analysis of the 19 *A. brassicicola* isolates based on ISSR markers and UPGMA dendrogram (Figure 4) revealed two main clusters at 0.57 similarity. The first included four isolates from Al-Husseiniya (C-Y4 to C-

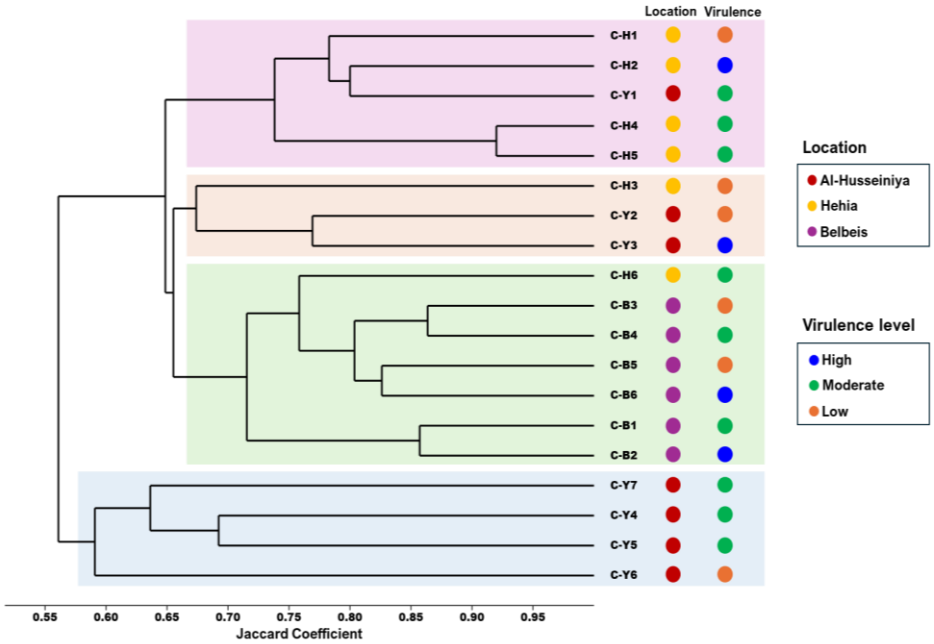

Figure 4. UPGMA dendrogram showing genetic relationships among 19 *A. brassicicola* isolates from cabbage based on ISSR marker profiles using Jaccard's similarity coefficient. Left-colored points indicate the geographic origin of each isolate, while right-colored points represent the virulence level. Shaded areas indicate major genetic clusters.

Y7). The second cluster, encompassing the remaining isolates, further broke at 0.66 similarity into three sub-clusters: (1) four Hehya isolates (C-H1, C-H2, C-H4, and C-H5) and one from Al-Husseiniya (C-Y1), (2) one Hehya isolate (C-H3) with two from Al-Husseiniya (C-Y2 and C-Y3), and (3) all six Belbeis isolates (C-B1 to C-B6) plus one from Hehya (C-H6).

Fungicide sensitivity

The radial growth inhibition of 19 *A. brassicicola* isolates attained evaluation against four fungicides at recommended concentrations by the manufacturers (Table 3). Difenoconazole exhibited the highest efficacy, with inhibition ranging from 17.04% (C-H2) to 88.13% (C-Y6), while azoxystrobin ranged from 20.60% (C-Y3) to 83.78% (C-H4). Mancozeb and copper oxychloride showed lower and more variable effects, with inhibition ranging from 12.00% to 73.64% and 7.20% to 62.98%, respectively. Difenoconazole had the highest median inhibition, followed by azoxystrobin and mancozeb, whereas copper oxychloride was the least effective. A wide range of sensitivity was noticeable among isolates for all treatments. Detailed inhibition values and statistical differences among isolates appear in Table 3.

DISCUSSION

Despite many global reports on *Alternaria brassicicola* in brassicas, data on Egyptian populations have been scarce. Our survey of three key districts in El-Sharkia clearly demonstrated the pathogen's prevalence and severity, with Belbeis showing the highest incidence (98%) and severity (53.78%). The variation in disease levels among locations may be due to differences in local environmental conditions, cultivation practices, or pathogen distribution, although no consistent correlation emerged. Typical necrotic-halo lesions matched earlier

Table 3. Radial growth inhibition (%) of 19 *A. brassicicola* isolates treated with difenoconazole (0.25 mg a.i./mL), azoxystrobin (0.25 mg a.i./mL), mancozeb (2.0 mg a.i./mL), and copper oxychloride (2.4 mg a.i./mL).

Isolate	Difenoconazole (0.25 mg a.i./mL)	Azoxystrobin (0.25 mg a.i./mL)	Mancozeb (2.0 mg a.i./mL)	Copper oxychloride (2.4 mg a.i./mL)
C-Y1	43.04^{ef}	23.58^{jk}	48.29^{de}	36.20^{f}
C-Y2	19.42^{g}	47.64^{e}	45.42^{e}	26.07^{g}
C-Y3	21.89^{g}	20.60^{k}	29.62^{h}	49.78^{e}
C-Y4	54.24^{d}	30.29^{hi}	73.64^{a}	58.69^{bc}
C-Y5	75.22^{c}	36.84^{fg}	72.91^{a}	35.09^{f}
C-Y6	88.13^{a}	59.93^{d}	12.00^{j}	15.04^{h}
C-Y7	85.71^{ab}	30.33^{hi}	59.36^{c}	46.49^{e}
C-H1	40.60^{f}	38.64^{fg}	70.84^{ab}	49.04^{e}
C-H2	17.04^{g}	35.07^{gh}	47.89^{e}	47.76^{e}
C-H3	81.69^{b}	71.11^{c}	46.87^{e}	7.20^{i}
C-H4	74.93^{c}	83.78^{a}	38.47^{f}	49.04^{e}
C-H5	46.27^{e}	46.09^{e}	52.73^{d}	15.00^{h}
C-H6	86.18^{ab}	40.16^{f}	67.04^{b}	54.22^{d}
C-B1	87.91^{a}	68.40^{c}	34.98^{fg}	26.07^{g}
C-B2	21.22^{g}	20.69^{k}	14.78^{j}	62.98^{a}
C-B3	46.91^{e}	55.78^{d}	25.04^{i}	48.49^{e}
C-B4	18.44^{g}	38.27^{fg}	31.20^{gh}	57.91^{cd}
C-B5	86.47^{ab}	76.09^{b}	39.53^{f}	46.93^{e}
C-B6	57.00^{d}	26.40^{ij}	70.33^{ab}	62.49^{ab}
Control	0.00^{h}	0.00^{l}	0.00^{k}	0.00^{j}

Values represent the mean percent reduction in mycelial growth compared with the untreated control. Different letters within each column indicate statistically significant differences among isolates according to Tukey's Honestly Significant Difference (HSD) test at $p < 0.05$.

descriptions of *A. brassicicola* on cabbage (Sharma *et al*., 2004; Chauhan *et al*., 2024) and reflect its necrotrophic lifestyle and toxin production (Macioszek *et al*., 2018).

Morphological screening of 74 isolates yielded 19 that conformed to Simmons' (2007) diagnostic criteria for *A. brassicicola*, exhibiting colony colors from gray-white to dark brown and conidia with 3–8 transverse and 1–3 longitudinal septa. ITS sequencing of six representatives placed them firmly within the *A. brassicicola* clade alongside global references, confirming species identity and underscoring the reliability of ITS for *Alternaria*-level discrimination (Blagojević *et al*., 2020).

Pathogenicity assays revealed substantial aggressiveness variation: isolates C-B6, C-H2, and C-B2 produced the largest detached-leaf lesions (up to 13.69 cm²) and the highest whole-plant severity (up to 84.8%), whereas C-Y2 and C-H3 were mildly virulent. Such variability parallels earlier studies showing wide pathogenic ranges in *A. brassicicola* (Kumar *et al*., 2020) and likely reflects genetic differences that influence toxin or enzyme production as well as micro-environmental preferences (Balamurugan *et al*., 2025).

ISSR profiling with five informative primers generated 46 bands (36 polymorphic; 72.6% mean polymorphism), confirming high intraspecific diversity in line with reports for other *Alternaria* spp. (Kale *et al*., 2012; Adss *et al*., 2016). PIC values ranged from 0.05 to 0.30, with UBC826 (= 0.30) as most informative, in agreement with Mohammadi and Bahramikia's (2019) threshold of PIC > 0.25 for useful discrimination. UPGMA clustering split the isolates into two principal clusters and three subclusters, yet geography and virulence proved poorly correlated—consistent with earlier findings of random regional distribution in *A. brassicicola* and related species (Nasr Esfahani, 2018). This pattern suggests frequent long-distance

dispersal or seed exchange and indicates that ISSR similarity alone does not predict aggressiveness, as also noted by Adhikari *et al*. (2021).

Fungicide assays further highlighted population heterogeneity: difenoconazole gave the highest median inhibition, whereas copper oxychloride was least effective, and individual responses spanned 7%–88% inhibition (Table 3). No clear correlation was evident between isolate virulence and fungicide sensitivity. Similar variability, reported by Kreis *et al*. (2016) for azoxystrobin, underscores the risk of relying on a single active ingredient. Taken together, the combined genetic, pathogenic, and chemical-response diversity observed here advocates integrated management: breeding programs should use diverse pathogen panels, and field control should rotate fungicides with different modes of action to slow resistance development.

CONCLUSIONS

Alternaria brassicicola isolates showed wide morphological, pathogenic, and ISSR-based genetic diversity, even among fields within a single district. ISSR profiles separated isolates clearly, yet genotype, geographic origin, and virulence appeared unlinked, implying additional functional or epigenetic factors govern aggressiveness. Isolates also differed markedly in sensitivity to four fungicides, signaling a risk of reduced chemical efficacy if relying on single modes of action. Consequently, durable control will require breeding programs that screen resistance against diverse, highly virulent isolates and field management that rotates fungicides with contrasting modes of action. Genomic tools targeting virulence-related genes are now vital to refine surveillance and accelerate the development of sustainable brassica leaf-spot control.

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