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Original Research Paper

Assessing resistance against *Alternaria* leaf spot in marigold genotypes (*Tagetes* spp.): A comprehensive *in vitro* and *in vivo* study

Sharma S.[1], Das A.[2], Bharti[3], Singh K.P.[1], Panwar S.[1], Talukdar A.[4], Arzoo E.[1], Singh A.[2], Bisht P.[2] and Bhatia R.[1*]

[1] Division of Floriculture and Landscaping, [2]Division of Plant Pathology,
[4]Division of Genetics, ICAR-Indian Institute of Agricultural Research, New Delhi - 110 012, India
[3]Division of Sample Surveys, ICAR-Indian Agricultural Statistics Research Institute, New Delhi - 110 012, India
Corresponding author Email: reetaiari@yahoo.com

ABSTRACT

Marigold (*Tagetes* spp.) a member of the Asteraceae family, is a globally acclaimed flower crop owing to its significant industrial value. The present study devised an effective screening method utilising a dual approach study, both *in vitro* and *in vivo*, for identifying potentially resistant genotypes of marigold. Pathogen isolation from infected plants revealed that 7 out of 10 isolates belong to *Alternaria*, confirming its prevalent association with leaf spots in marigold. Pathogenicity assessment revealed isolate I-3 as the most virulent isolate, and hence, it was subsequently used for the dual screening programme. Molecular characterization of the virulent isolate based on Internal Transcribed Spacer (ITS) region sequencing revealed its 100% homology with previously reported sequences of *Alternaria alternata*. Potato dextrose agar and Host-extract media (with dextrose) supported rapid mycelial growth and abundant sporulation. Among 60 genotypes of marigold screened under *in vitro* and *in vivo* conditions, AMS-48, AMS-123, Pusa Parv, Pusa Utsav and MGO-3 exhibited moderate resistance. At the same time, Pusa Basanti Gainda, Pusa Deep and *Tagetes minuta* selections were found to be highly susceptible. These findings were consistent under both conditions, validating the screening methods. The moderately resistant genotypes can be pivotal in marigold breeding programs for developing resilient marigold varieties.

Keywords: *Alternaria alternata*, *in vitro* and *in vivo* screening, ITS, marigold, pathogenicity

INTRODUCTION

Marigold (*Tagetes* spp.), a prominent annual in the Asteraceae family, originated from Mexico and spread globally in the early 16th century (Kaplan, 1960). Initially, 33 species were reported in the *Tagetes* genus (Rydberg, 1915), but later on 55 species were recognised, primarily found distributed in Central and South America (Turner & Nesom, 1993). Its rapid adoption in India can be attributed to features such as prolific flowering, adaptability to diverse soil and climatic conditions, short growth cycle, and ease of cultivation. Marigold flowers are extensively used for garlands and decorative purposes in homes, temples and gardens. Marigold flowers contain carotenoids, making them valuable as colorants, while flavonoids and monoterpenoids contribute to their use in food additives and medicinal applications. Lutein, a carotenoid found in marigold, possesses antioxidant properties and is vital for preventing age-related macular degeneration and supporting infant brain development (Mario et al., 2020).

Among major diseases, leaf spots and flower blight caused by *Alternaria* spp. led to significant economic losses (Meena et al., 2017). The infection initially appears as dark brown to black sunken spots on the lower leaves. It gradually spreads to upper leaves, petioles, and flower buds, causing 50–60% crop loss and reducing the ornamental value of the flowers (Shinde et al., 2018). Optimal conditions for disease development include temperatures of 25°-28°C and prolonged leaf wetness, with specific meteorological factors influencing fungal growth. Currently, no effective fungicides exist to prevent *Alternaria* infections completely; however, reliance on chemical treatments raises environmental concerns (Yang, 2015). The lack of resistant marigold varieties presents

a significant challenge in disease management. Thus, screening for disease-resistant marigold genotypes is essential for developing resistant cultivars, reducing reliance on fungicides, and supporting sustainable organic production. The present research addresses this gap by conducting a comprehensive screening for *Alternaria* infection in marigold crop under both *in vitro* and *in vivo* conditions, a robust methodology that is among the first of its kind. Additionally, molecular identification of the virulent strain further strengthens the reliability and relevance of this study for advancing disease-resistant marigold breeding programs.

MATERIAL AND METHODS

Experimental site and genotypes used

The current study was conducted at the Division of Floriculture and Landscaping, ICAR-Indian Agricultural Research Institute, New Delhi geographically located at 28° 64' N latitude and 77° 15' E, altitude 228 m above MSL, during 2023 to 2024. Sixty marigold genotypes belonging to three different *Tagetes* species, specifically *T. erecta* L., *T. patula* L. and *T. minuta* L., were utilized for screening against *Alternaria* disease.

Acquisition and sustenance of fungal cultures

Marigold samples exhibiting pronounced leaf spot disease were collected from the rainy season crop at the research farm of the Division of Floriculture and Landscaping, ICAR- Agricultural Research Institute, New Delhi. Plants demonstrating characteristic leaf spot and blight symptoms associated with *Alternaria* spp. were meticulously selected. Diseased leaf samples were excised along with the healthy green tissues and subsequently surface sterilized using a 1.0% sodium hypochlorite solution for 1 min, followed by three washings with sterilized distilled water. After drying, samples were inoculated in sterilized 100 mm Petri dishes containing potato dextrose agar (PDA). Fungal cultures were incubated at 26±1°C under alternate light and dark conditions of 12 hours. Emerging fungal colonies were transferred to fresh PDA plates and incubated for an additional 7 days. Identification of *Alternaria* isolates was performed based on morphological characteristics observed microscopically and referenced literature.

To purify fungal cultures, the single spore isolation method as described by Zhu et al. (2011) was followed. Spores formed on a mycelial plate were transferred to 10 mL of sterilized distilled water to create a spore suspension adjusted to 15-20 spores. A loopful of this suspension was added to 20 mL of 1.0% melted agar, mixed and poured into sterilized Petri plates, which were kept inverted after solidification at 26±1°C. A single germinating spore was then transferred to a new PDA tube to establish a monoconidial culture. The pure cultures of *Alternaria* isolates were subsequently stored at 4-5°C for future use.

Determining pathogenic potential of isolates

The pathogenicity test was performed to assess the virulence of isolates and select the most virulent, utilizing a highly susceptible genotype 'AMS-110'and following the Detached-leaf method. Mycelial-cum-spore suspensions from 7-day-old cultures of all isolates were prepared. To prepare the spore suspension, 50 mL of sterile distilled water containing 1.0% Tween 20 (as a surfactant) was used to gently scrape spores from the surface of actively growing culture plates. The spore concentration was adjusted to 2×10^5 spores/mL using a haemocytometer. Healthy leaves were collected, thoroughly washed, and surface-sterilized using 70% ethanol. Each leaf was then wounded at multiple points using a sterile needle, and the petiole ends were sealed with sterile cotton plugs to prevent desiccation. The leaves were uniformly sprayed with the prepared spore suspension and then incubated under controlled conditions at 26±1°C for 10 days. Disease development was monitored daily, and symptoms were recorded systematically. The cotton-plugged petiole ends were kept moist using sterile distilled water to prevent desiccation. Percent Disease Index (PDI) was documented on the 5^{th}, 7^{th} and 10^{th} day post-inoculation to gauge the virulence of *Alternaria* isolates.

Disease severity was assessed using the scoring system established by Hotchkiss & Baxter (Table 1). PDI was calculated using the following formula (McKinney, 1923):

$$\text{Percent Disease Index (\%)} = \frac{\text{Sum of disease ratings}}{\text{No. of disease ratings} \times \text{Maximum rating}} \times 100$$

Table 1 : Disease Scoring Scale (Hotchkiss & Baxter, 1983)

Disease rating	Symptoms/Disease severity
0	No symptoms on leaves
1	Spots covering 1-10% of the leaf area
2	Spots covering 11-20% of the leaf area
3	Spots covering 21-30% of the leaf area
4	Spots covering 31-40% of the leaf area
5	Spots covering >40% of the leaf area

The isolate demonstrating the highest PDI was considered to be the most virulent, hence it was selected for subsequent screening. To confirm Koch's postulates, the pathogen was re-isolated from the artificially infected leaves, establishing the correlation between the inoculated pathogen and the observed symptoms.

Morphological and cultural characterisation of virulent isolate

Microscopic examination for conidial characteristics (length, width, beak length and septations) and investigation of cultural variability of the virulent isolate were performed on six different media, *viz.,* Host-extract medium (with dextrose), sabouraud dextrose agar (SDA), potato dextrose agar (PDA), corn meal agar (CMA), Czapek's agar and Host-extract medium (without dextrose) for characters like colony growth pattern, sporulation, zonation etc.

Molecular confirmation of virulent isolate

DNA extraction from the fungal mycelium was conducted following the CTAB (Cetyl trimethyl ammonium bromide) method outlined by Doyle & Doyle (1987) with slight modifications. The fungus was cultivated in 150 mL of potato dextrose broth for 7 days at 26±1°C. The fungal mycelium was harvested through filtration, and approximately 200 µg (dry weight) was ground to a fine powder using liquid nitrogen in a pre-chilled mortar and pestle. The ground tissue was transferred into 2 mL microcentrifuge tubes containing CTAB extraction buffer (2% (w/v) CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0). This mixture was incubated at 65°C in a water bath for 30 minutes, followed by the addition of an equal volume of chloroform: isoamyl alcohol (24:1 v/v). The samples were centrifuged at 10,000 rpm for 20 minutes at room temperature. The aqueous phase was carefully transferred to a new tube, and DNA was precipitated by adding 600 µL of pre-chilled isopropanol and 100 µL of 3 M sodium acetate. Subsequently, the DNA pellet was washed with 70% ethanol, air-dried, and resuspended in Tris-EDTA (TE) buffer overnight at 4°C. DNA concentration was quantified using a NanoDrop 2000 spectrophotometer.

For identification purposes, ITS region was sequenced using forward primer ITS1 (5'-TCCGTAGGTGAA CCTGCGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR conditions were set as follows: initial denaturation at 96°C for 1 min; 2 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 1 min; 28 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min; final elongation at 72°C for 3 min (Park et al., 2024). The PCR products were observed through Agarose gel electrophoresis stained with ethidium bromide using a 100 bp ladder, and distinct DNA bands were visualized using a Bio-Rad Gel Doc system. Sequencing of PCR products was outsourced to Biologia Research India Pvt Ltd. and resultant ITS sequences were compared for homology with those available in GenBank (https://blast.ncbi.nlm.nih.gov). The sequence for the strain was submitted to GenBank as *Alternaria alternata* strain (Accession No.: PQ219571) and phylogenetic analysis was performed using MEGA software, Version 11.

Comparative screening of marigold genotypes in laboratory and field conditions

Sixty marigold genotypes were evaluated for resistance against *Alternaria* through both *in vitro* and *in vivo* screening. For *in vitro* assessment, the detached leaf method was employed using healthy, disease-free leaves collected from 45-day-old seedlings. The spore suspension was prepared following the same protocol as used in the pathogenicity test. Leaves were surface sterilized with 70% ethanol, wounded with a sterile needle, and uniformly sprayed with the spore suspension. The samples were then incubated at 26±1°C under a 12-hour light/dark cycle for 10 days. Disease severity was recorded by assessing the per cent disease index (PDI) on the 5th, 7th, and 10th days post-inoculation.

While for screening under natural settings (*in vivo*), seeds were sown in plug trays containing a mixture of cocopeat, perlite, and vermiculite (3:1:1). 25-day-old seedlings were transplanted into 4-inch pots, and

screening commenced when the seedlings reached 45 days of age. Five leaves per plant were selected for pathogen inoculation, during which the leaves were pricked and sprayed with a spore suspension calibrated to 2×10^5 spores/mL, the same as per the *in vitro* screening procedure (500 µL of spore suspension was applied to each leaf). A temperature of 25-27°C and 80-90% was maintained by covering pots with a poly sheet. PDI was recorded after the 7th, 14th and 21st day after inoculation. Genotypes were classified into six categories *viz*., immune (PDI = 0%), resistant (0.1-20.0%), moderately resistant (20.1-40.0%), moderately susceptible (40.1-60.0%), susceptible (60.1-80.0%), and highly susceptible (80.1-100%) (Sharma et al., 2002).

Statistical analysis

Completely Randomized Design (CRD) was followed for screening experiment with three replications each. Arcsine transformation of PDI (%) was done and analysis of treatment means was done using LSD post-doc test at 5% significance in R software. Three replications for each treatment, whether in laboratory studies or screening, were employed during this study.

RESULTS AND DISCUSSION

Isolation and identification of *Alternaria* isolates

"Among the ten isolates examined, seven were microscopically identified as Alternaria, highlighting predominant association of the fungus with leaf spot disease of marigold. Recent reports have also established prevalent association of Alternaria and leaf spot disease in this crop (Gurjar et al., 2019 & Marchande et al., 2020). Microscopic observations have revealed that conidia were small, oblong to obpyriform and muriform, with a colour range from mid to dark brown or olivaceous. They typically formed chains of 5-8 or more, with or without beak. Similar growth and sporulation pattern of Alternaria spp. on microscopic observations have been noticed by Oviedo et al., (2013) & Park et al. (2024). Fungal colonies appeared velvety black when cultured on PDA (Fig. 1).

Unveiling virulence in *Alternaria* isolates by pathogenicity assay

Based on the results of the pathogenicity test, isolate 'I-3' was identified as the most virulent *Alternaria* isolate, exhibiting a disease severity of 100% PDI (per cent disease index) within 10 days of inoculation.

Fig. 1 : Isolation of the pathogen using the conventional tissue isolation method

The remaining isolates showed PDI values ranging from 53.33% to 73.33% (Fig. 2). The small spots started to appear by the third day of post-inoculation, with rapid intensification from the fifth day onward. Symptoms became most severe quickly, leading to complete blight and indicating rapid disease progression. Re-isolation of the pathogen from inoculated leaves confirmed Koch's postulates and the pathogenicity of *Alternaria alternata*, conforming to reports by Shingne et al. (2020). Subsequently, isolate I-3 was selected for further screening of marigold genotypes for resistance studies. The isolate has been deposited in the Indian Type Culture Collection (ITCC) at ICAR-Indian Institute of Horticultural Research, New Delhi, under Accession No. 9390.

Morphological and cultural characterisation of virulent isolate 'I-3'

Microscopic analysis of the I-3 isolate revealed that conidia (30 spores) measured an average length of 29.69 ± 0.68 µm, with a range of 26.39 to 37.01 µm. Their width ranged from 8.04 to 11.45 µm, with a mean of 9.76 ± 0.31 µm. The beak length varied between from 4.00 to 7.00 µm, with an average of 5.53 ± 0.24 µm. The conidia typically exhibited 5–7 transverse and 0–3 longitudinal septa.

Cultural observations over 7 days showed the most rapid and statistically comparable radial mycelial growth on PDA (0.55 mm/h) and host-extract media with dextrose (0.54 mm/h), while the slowest growth was recorded on SDA (0.40 mm/h). Sporulation was rated as excellent on PDA and host-extract media with dextrose, good on host-extract media without dextrose, moderate on SDA and Czapek's agar, and scanty on CMA. All media displayed a distinct zonation pattern, and sporulation began 48 hours after inoculation on most media, except SDA and Czapek's agar, where it started after 96 hours (Table 2, Fig. 3). The recent findings aligned with the results of Ramjegathesh & Ebenezar (2012).

Table 2 : Radial mycelial growth of the virulent *Alternaria* isolate (I-3) on different media

Media	Mycelium growth period (days)						Mean mycelium growth rate (mm/h)	Colony colour and Growth pattern	Sporulation time (hrs)	Sporulation Degree	Zonation
	2nd	3rd	4th	5th	6th	7th					
Host-extract media (with Dextrose)	2.54^{a}	3.96^{ab}	5.44^{ab}	6.83^{a}	8.00^{a}	9.25^{a}	0.54^{a}	Dark green to blackish, submerged mycelium with concentric rings	48	Excellent	Present
SDA	1.75^{d}	2.95^{d}	3.80^{d}	5.06^{c}	5.97^{c}	6.91^{c}	0.40^{c}	White surface with greenish base, sub-aerial, irregular, moderately raised mycelium (fluffy), small concentric rings	96	Moderate	Present
PDA	2.62^{a}	4.28^{a}	5.92^{a}	7.19^{a}	8.10^{a}	9.28^{a}	0.55^{a}	Olive green to blackish, submerged mycelium, small concentric rings and regular margins	48	Excellent	Present
CMA	2.10^{c}	3.36^{cd}	4.74^{c}	6.15^{b}	7.48^{b}	8.27^{b}	0.48^{b}	Indigo to dark brown, submerged mycelium, medium concentric rings	48	Scanty	Present
Czapek's Agar	2.37^{ab}	3.62^{bc}	5.08^{bc}	6.30^{b}	7.55^{b}	8.29^{b}	0.48^{b}	Dull white to indigo, sub-aerial, moderately raised mycelium, medium concentric rings	96	Moderate	Present
Host-extract media (without Dextrose)	2.14^{bc}	3.38^{cd}	4.68^{c}	6.10^{b}	7.39^{b}	8.32^{b}	0.49^{b}	Dark green to blackish, submerged mycelium with concentric rings	48	Good	Present
SE $_{(d)}$	0.07	0.12	0.14	0.14	0.12	0.11	0.01				
CD $_{0.05}$	0.15	0.26	0.30	0.30	0.26	0.24	0.02				
CV	3.88	4.14	3.41	2.66	2.03	1.66	-				

*Represents mean radial mycelium observed on respective days and average mycelial growth rate (mm/h), SDA: Sabouraud Dextrose Agar, PDA: Potato Dextrose Agar, CMA: Corn Meal Agar; Sporulation categorization based on Ginoya and Gohel (2015): Excellent (>30), Good (20-30), Moderate (10-20), Scanty (<10) and Nil (0); no. of spores measured at 40X

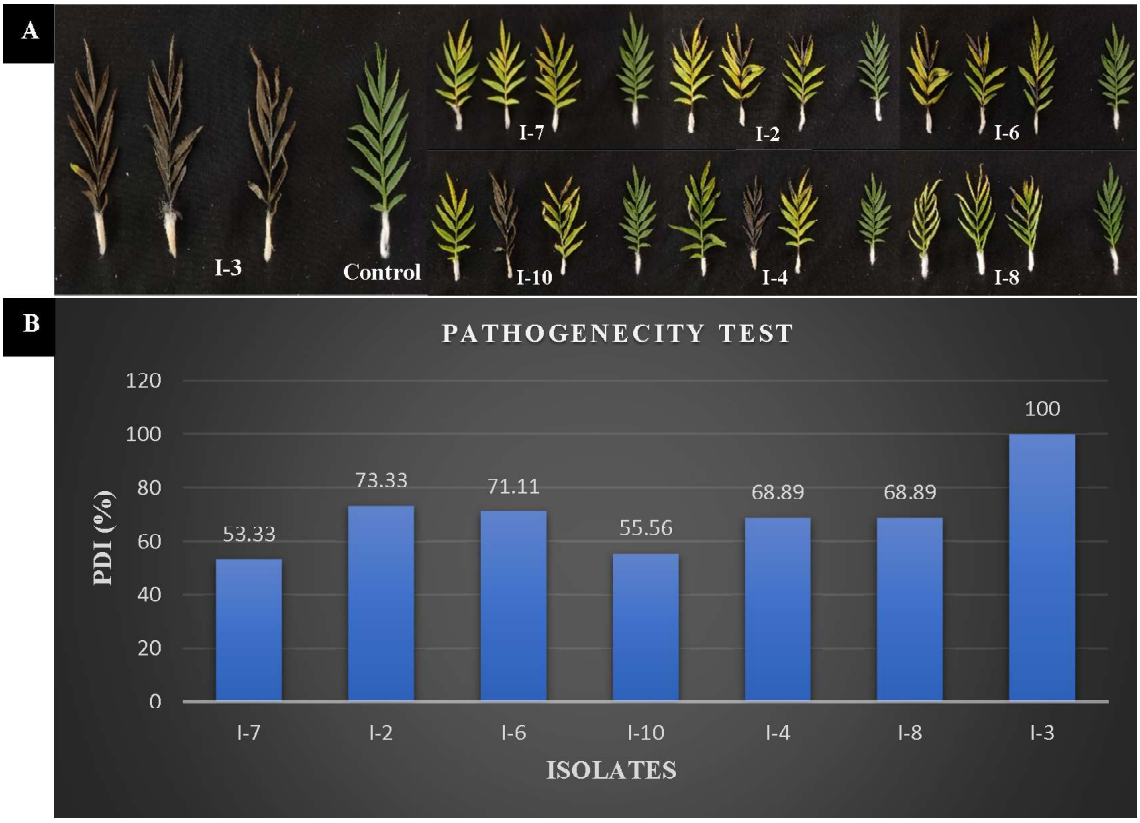

Fig. 2 : Pathogenicity test for assessing virulence of isolates on susceptible host 'AMS-110';
A: Disease reaction of different isolates after 10 days post-inoculation, B: Graphical representation of PDI (%)

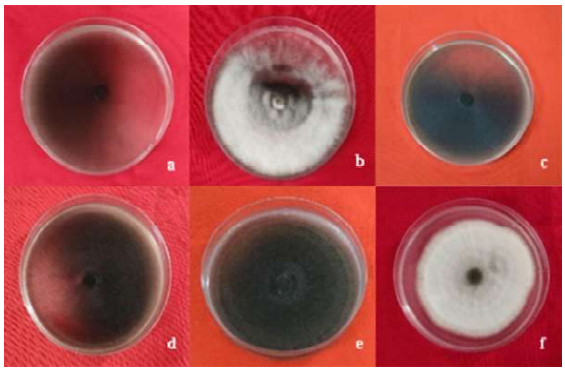

Fig. 3 : Colony characters of isolate I-3 on different media; a: Corn Meal Agar (CMA), b: Czapek's media, c: Host-extract media (without dextrose), d: Host extract media (with dextrose), e: Potato Dextrose Agar (PDA) and f: Sabouraud Dextrose Agar (SDA)

Phylogenetic analysis and molecular identification of I-3 isolate

Polymerase chain reaction (PCR) is a highly sensitive and specific technique that enables the detection of low concentrations of fungal DNA, making it particularly useful for identifying pathogens at early infection stages or in mixed infections. The Internal Transcribed Spacer (ITS) region, known for its high variability among fungal species, serves as a reliable genetic marker for differentiating closely related taxa (White et al., 1990). This molecular approach has significantly enhanced the accuracy of fungal diagnostics and contributed to a deeper understanding of fungal diversity and taxonomy (Oviedo et al., 2013). Genomic DNA with I-3 isolate was extracted using the CTAB method, and successful amplification of the ITS region was confirmed through agarose gel electrophoresis. A distinct DNA band of approximately 550 bp was observed, visualized using a 100 bp ladder stained with ethidium bromide. The amplified ITS sequence was submitted to NCBI GenBank under the accession number PQ21957. Sequence analysis using the NCBI BLAST tool (http://blast.ncbi.nlm.nih.gov) and subsequent phylogenetic analysis using the neighbour-joining method—employing *Aspergillus niger* as an outgroup—confirmed that the ITS sequence of the I-3 isolate showed 100% identity with previously reported *Alternaria alternata* sequences. This molecular identification validated the morphological and pathogenicity-based classification of the isolate (Fig. 4).

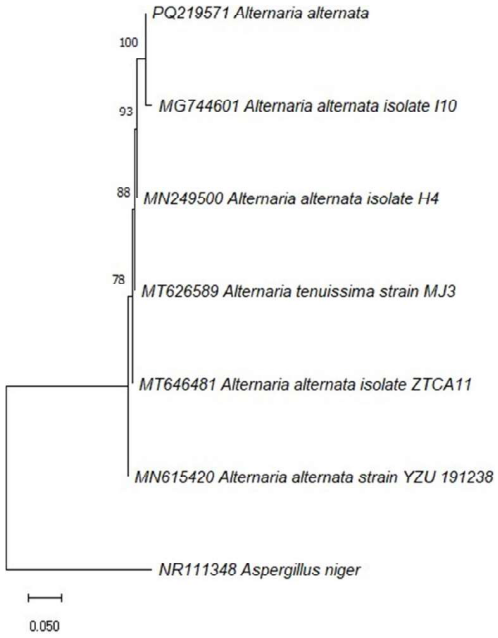

Fig. 4 : A Neighbour-Joining phylogenetic tree of *Alternaria alternata* was constructed based on the ITS gene region, with the tree rooted to *Aspergillus niger*, using MEGA 11. The tree was generated using a Bootstrap value of 1000, and the isolates labelled as REF correspond to previously documented sequences from NCBI GenBank, selected as reference sequences

In vitro and *in vivo* assessment of marigold genotypes for resistance to *Alternaria alternata*

Under *in vitro* screening, symptoms typically emerged on detached leaves 72-120 hours after spraying, more consistently on the 5th day onwards. Initially, spots exhibited a characteristic target-board pattern, but later coalesced to develop blight symptoms. Only five genotypes, namely, AMS-48 (35.56%), AMS-123 (37.78%), Pusa Parv (37.78%), Pusa Utsav (33.33%) and MGO-3 (40.00%), exhibited moderately resistant responses. In contrast, 52 genotypes were classified as highly susceptible, and three exhibited a susceptible reaction, based on PDI on the 10th day of inoculation. None of the genotypes showed a resistant or immune reaction. The relevance of the detached-leaf method was emphasized by Vishwanath et al. (1999), who identified it as an effective approach for assessing resistance to *Alternaria brassicae* in rapeseed and mustard. This technique minimises interference from local growing conditions, eliminates variations in developmental stages at the time of pathogen exposure, and accounts for the presence of other strains or pathogens (Michereff et al., 2012).

Table 3 : Per cent disease index and disease reaction of marigold genotypes screened against *Alternaria alternata* under *in vitro* and *in vivo* conditions

Genotype	*In vitro* Per cent Disease Index (%)			Disease Reaction	*In vivo* Per cent Disease Index (%)			Disease Reaction
	5th	7th	10th		7th	14th	21st	
DAMH-1	48.89^{hijk} (44.36)	100.00^{a} (90.00)	100.00^{a} (90.00)	HS	37.33^{c-k} (37.66)	56.00^{j-p} (48.45)	66.67^{k-o} (54.79)	S
DAMH-4	24.44^{c-g} (29.58)	51.11^{i-q} (45.68)	100.00^{a} (90.00)	HS	25.33^{m-r} (30.20)	62.67^{g-n} (52.44)	78.67^{f-k} (62.64)	S
DAMH-7	26.67^{hijk} (30.79)	62.22^{g-o} (52.09)	100.00^{a} (90.00)	HS	28.00^{l-q} (31.91)	65.33^{d-k} (54.24)	86.67^{b-h} (68.91)	HS
DAMH-12	26.67^{hijk} (30.97)	46.67^{k-q} (43.08)	75.56^{i} (60.42)	S	25.33^{m-r} (30.12)	66.67^{d-k} (54.77)	90.67^{abc} (75.38)	HS
DAMH-16	11.11^{klm} (19.26)	51.11^{j-q} (45.64)	93.33^{b-e} (77.87)	HS	33.33^{g-n} (35.26)	50.67^{m-r} (45.38)	72.00^{b-k} (58.18)	S
DAMH-20	66.67^{c} (54.80)	100.00^{a} (90.00)	100.00^{a} (90.00)	HS	40.00^{d-i} (39.22)	52.00^{l-q} (46.15)	60.00^{mnop} (50.78)	MS
DAMH-24	60.00^{c} (50.92)	93.33^{abcd} (77.87)	97.78^{ab} (85.01)	HS	30.67^{j-o} (33.62)	61.33^{h-o} (51.55)	72.00^{j-n} (58.09)	S
DAMH-28	95.56^{a} (82.86)	100.00^{a} (90.00)	100.00^{a} (90.00)	HS	30.67^{j-o} (33.59)	78.67^{abcd} (62.64)	89.33^{a-f} (71.01)	HS
DAMH-31	20.00^{i-m} (26.03)	46.67^{k-q} (43.59)	95.56^{abc} (82.86)	HS	22.67^{opqr} (28.29)	54.67^{k-p} (47.67)	60.00^{mnop} (50.81)	MS
DAMH-32	28.89^{hij} (32.07)	42.22^{m-q} (40.47)	80.00^{hi} (63.97)	S	22.67^{opqr} (28.29)	48.00^{f-n} (43.84)	53.33^{opq} (46.92)	MS
DAMH-36	60.00^{cd} (50.80)	75.56^{c-j} (60.93)	100.00^{a} (90.00)	HS	28.00^{l-q} (31.91)	52.00^{l-q} (46.15)	58.67^{nop} (50.01)	MS
DAMH-39	24.44^{c-g} (29.07)	84.44^{bcde} (71.94)	100.00^{a} (90.00)	HS	44.00^{b-h} (41.52)	69.33^{b-i} (56.44)	78.67^{f-k} (62.82)	S
DAMH-44	40.00^{d-h} (38.97)	53.33^{h-q} (47.08)	82.22^{fghi} (65.36)	HS	29.33^{k-p} (32.63)	60.00^{j-o} (50.78)	73.33^{i-m} (59.20)	S
DAMH-53	26.67^{hijk} (30.97)	46.67^{k-q} (43.08)	97.78^{ab} (85.01)	HS	25.33^{m-r} (30.12)	66.67^{d-k} (55.23)	77.33^{g-k} (61.64)	S
DAMH-55	57.78^{cd} (49.75)	93.33^{abc} (81.14)	97.78^{ab} (85.01)	HS	22.67^{o-r} (28.36)	37.33^{rst} (37.64)	42.67^{qr} (40.77)	MS
DAMH-63	31.11^{g-j} (33.58)	46.67^{k-q} (43.08)	100.00^{a} (90.00)	HS	21.33^{pqr} (27.18)	64.00^{f-m} (53.15)	86.67^{b-h} (69.27)	HS
DAMH-64	22.22^{h-l} (28.07)	33.33^{pq} (34.63)	91.11^{a-e} (79.64)	HS	32.00^{f-l} (36.82)	72.00^{a-i} (58.09)	85.33^{c-i} (67.81)	S
DAMH-65	86.67^{b} (69.02)	100.00^{a} (90.00)	100.00^{a} (90.00)	HS	36.00^{i-n} (34.36)	52.00^{l-q} (46.28)	77.33^{g-k} (62.10)	HS
AMS-39	53.33^{hijk} (46.97)	82.22^{a-i} (65.79)	100.00^{a} (90.00)	HS	29.33^{k-p} (32.74)	73.33^{a-h} (57.08)	90.67^{a-e} (72.82)	HS
AMS-48	22.22^{h-l} (28.07)	31.11^{q} (33.69)	35.56^{j} (36.59)	MR	10.67^{t} (18.99)	20.00^{u} (26.49)	22.67^{s} (28.36)	MR
AMS-101	23.33^{hijk} (30.97)	53.33^{h-i} (46.92)	91.11^{b-g} (75.72)	HS	26.67^{m-r} (31.08)	66.67^{d-k} (54.80)	80.00^{f-k} (63.43)	S
AMS-102	53.33^{cde} (47.08)	93.33^{abcd} (77.87)	100.00^{a} (90.00)	HS	49.33^{cd} (44.62)	80.00^{ab} (64.10)	90.67^{a-e} (72.64)	HS
AMS-103	28.89^{hij} (32.36)	82.22^{b-f} (69.39)	100.00^{a} (90.00)	HS	42.67^{defg} (40.77)	76.00^{a-f} (60.81)	81.33^{c-j} (64.43)	HS
AMS-104	37.78^{c-i} (37.58)	75.56^{d-i} (62.24)	100.00^{a} (90.00)	HS	36.00^{f-l} (36.85)	65.33^{c-l} (53.98)	84.00^{c-j} (66.53)	HS
AMS-106	22.22^{h-l} (28.07)	71.11^{c-l} (57.70)	100.00^{a} (90.00)	HS	44.00^{def} (41.55)	70.67^{b-i} (57.26)	86.67^{b-h} (68.91)	HS
AMS-107	26.67^{hijk} (30.79)	51.11^{j-q} (45.64)	88.89^{d-i} (70.73)	HS	49.33^{cd} (44.62)	76.00^{a-f} (60.88)	85.33^{c-i} (67.52)	HS
AMS-109	33.33^{f-j} (35.01)	73.33^{c-k} (59.03)	95.56^{abcd} (77.84)	HS	29.33^{k-p} (32.74)	76.00^{a-f} (60.72)	84.00^{d-j} (66.42)	HS
AMS-110	31.11^{g-j} (33.69)	100.00^{a} (90.00)	100.00^{a} (90.00)	HS	37.33^{c-k} (37.64)	82.67^{a} (65.53)	94.67^{ab} (76.83)	HS
AMS-111	20.00^{i-m} (26.56)	71.11^{c-l} (57.70)	97.78^{ab} (85.01)	HS	30.67^{j-o} (33.62)	70.67^{b-i} (57.26)	82.67^{d-j} (65.84)	HS
AMS-112	28.89^{g-j} (32.48)	44.44^{l-q} (41.80)	82.22^{ghi} (65.15)	HS	45.33^{cdef} (42.32)	74.67^{a-g} (60.01)	92.00^{abcd} (73.92)	HS
AMS-116	20.00^{i-m} (26.36)	46.67^{k-q} (43.26)	100.00^{a} (90.00)	HS	24.00^{n-r} (29.28)	42.67^{g-n} (40.71)	49.33^{pq} (44.62)	MS
AMS-123	24.45^{hijk} (29.58)	31.11^{q} (33.87)	37.78^{j} (37.87)	MR	10.67^{t} (18.99)	28.00^{stu} (31.91)	33.33^{rs} (35.26)	MR
AMS-129	24.44^{hijk} (29.46)	64.44^{f-n} (55.01)	100.00^{a} (90.00)	HS	20.00^{rs} (26.31)	72.00^{a-i} (58.09)	84.00^{c-j} (66.53)	HS
AMS-132	22.22^{h-l} (28.07)	44.44^{l-q} (41.79)	86.67^{c-i} (63.97)	HS	36.00^{f-l} (36.80)	69.33^{b-i} (56.45)	85.33^{a-f} (71.49)	HS
AMS-134	20.00^{i-m} (26.56)	60.00^{g-p} (50.92)	91.00^{b-f} (76.16)	HS	26.67^{m-r} (30.99)	60.00^{i-o} (50.78)	72.00^{j-n} (58.09)	S
AMS-135	28.89^{hij} (32.64)	53.33^{h-q} (47.08)	75.56^{i} (61.79)	S	40.00^{d-i} (39.22)	72.00^{a-i} (58.09)	80.00^{f-j} (63.69)	S
AMS-140	93.33^{a} (81.14)	100.00^{a} (90.00)	100.00^{a} (90.00)	HS	54.67^{bc} (47.71)	70.67^{b-i} (57.64)	78.67^{c-j} (62.02)	S
AMS-146	20.00^{i-m} (26.56)	40.00^{nopq} (39.19)	91.11^{b-g} (75.72)	HS	25.33^{m-r} (30.20)	38.67^{qrs} (38.39)	61.33^{l-p} (51.55)	S
AMS-151	33.33^{f-j} (35.01)	51.11^{j-q} (45.53)	100.00^{a} (90.00)	HS	32.00^{i-n} (34.36)	64.00^{f-m} (53.18)	80.00^{a-i} (63.74)	S
AMS-155	53.33^{cde} (46.97)	95.56^{abc} (80.02)	100.00^{a} (90.00)	HS	46.67^{cde} (43.08)	72.00^{a-i} (58.51)	82.67^{f-j} (65.61)	HS
AMS-157	22.22^{h-l} (28.07)	46.67^{k-q} (43.08)	95.55^{abcd} (80.02)	HS	42.67^{defg} (40.77)	77.33^{a-e} (61.71)	85.33^{c-i} (67.81)	HS
AMS-158	20.00^{i-m} (26.36)	75.56^{c-j} (60.93)	100.00^{a} (90.00)	HS	22.67^{o-r} (28.29)	49.33^{n-r} (44.64)	76.00^{hijk} (60.88)	S
AMS-161	35.56^{c-j} (36.64)	75.56^{c-g} (65.27)	100.00^{a} (90.00)	HS	20.00^{qrs} (26.49)	54.67^{k-p} (47.71)	74.67^{ijkl} (60.01)	S
AMS-172	20.00^{i-m} (26.36)	40.00^{nopq} (39.19)	95.56^{abc} (82.86)	HS	25.33^{m-r} (30.12)	50.67^{m-r} (45.38)	78.67^{f-k} (62.64)	S
AMS-174	22.22^{h-l} (28.07)	37.78^{opq} (37.91)	97.78^{ab} (85.01)	HS	25.33^{m-r} (30.20)	48.00^{opqr} (43.84)	76.00^{hijk} (60.88)	S
AMS-175	57.78^{cd} (49.64)	82.22^{c-g} (65.15)	97.78^{ab} (85.01)	HS	32.00^{i-n} (34.28)	60.00^{i-o} (50.84)	78.67^{f-k} (63.26)	S
AMS-176	51.11^{cdef} (45.64)	68.89^{c-m} (56.86)	93.33^{b-e} (77.87)	HS	38.67^{c-j} (38.41)	73.33^{a-h} (59.01)	94.67^{a} (79.05)	HS
AMS-177	57.78^{cd} (49.64)	95.56^{ab} (82.86)	100.00^{a} (90.00)	HS	21.33^{pqr} (27.49)	48.00^{opqr} (43.85)	54.67^{opq} (47.71)	MS
AMS-178	24.44^{hijk} (29.58)	40.00^{nopq} (39.19)	88.89^{c-h} (77.01)	HS	32.00^{i-n} (34.36)	68.00^{c-j} (55.64)	88.00^{b-h} (69.73)	HS
AMS-179	60.00^{c} (52.45)	73.33^{b-g} (66.49)	100.00^{a} (90.00)	HS	41.33^{d-h} (40.00)	68.00^{c-j} (55.64)	80.00^{c-j} (64.10)	HS
MS5	31.11^{g-j} (33.58)	68.89^{c-m} (56.74)	97.78^{ab} (85.01)	HS	32.00^{h-n} (34.45)	61.33^{h-o} (51.61)	81.33^{c-j} (64.43)	HS
MS8	24.44^{hijk} (29.07)	48.89^{i-q} (44.36)	97.78^{ab} (85.01)	HS	26.67^{m-r} (31.08)	49.33^{n-r} (44.59)	84.00^{c-j} (66.71)	HS
Pusa Basanti Gainda	26.67^{hijk} (30.79)	88.89^{b-f} (70.73)	100.00^{a} (90.00)	HS	29.33^{k-p} (32.72)	66.67^{d-k} (55.04)	77.33^{g-k} (61.71)	S
Pusa Parv	17.78^{jklm} (24.85)	33.33^{pq} (35.26)	37.78^{j} (37.91)	MR	13.33st (21.20)	21.31^{u} (27.42)	26.67^{s} (31.08)	MR
Pusa Utsav	6.67^{m} (14.96)	23.33^{q} (30.97)	33.33^{j} (35.20)	MR	17.33^{t} (20.10)	20.00^{u} (26.56)	25.33^{s} (30.15)	MR
MGO3	8.89^{lm} (17.11)	28.89^{n} (32.36)	40.00^{j} (39.15)	MR	13.33st (31.08)	25.33^{tu} (30.16)	30.67^{rs} (35.62)	MR
Pusa Deep	37.78^{c-i} (37.53)	71.11^{d-h} (62.61)	100.00^{a} (90.00)	HS	26.67^{m-r} (32.78)	37.33^{rst} (37.66)	46.67^{pqr} (43.09)	MS
TM-1	100^{a} (90.00)	100^{a} (90.00)	100.00^{a} (90.00)	HS	64.00^{ab} (53.13)	80.00^{abc} (63.52)	85.33^{c-i} (67.81)	HS
TM-2	100^{a} (90.00)	100^{a} (90.00)	100.00^{a} (90.00)	HS	66.67^{a} (54.85)	74.67^{a-g} (60.00)	88.00^{b-g} (69.91)	HS
TM-3	100^{a} (90.00)	100^{a} (90.00)	100.00^{a} (90.00)	HS	61.33^{ab} (51.57)	76.00^{a-f} (60.67)	90.67^{a-e} (72.64)	HS
$SE_{(d)}$	6.03	8.38	5.52		2.82	3.99	4.48	
$CD_{0.05}$	11.94	16.58	10.94		5.99	7.89	8.88	

*values in parenthesis ( ) are arcsine-transformed values

In vivo screening of the same genotypes was performed to validate *in vitro* results and assess the virulence of *Alternaria alternata* in a natural environment. Symptoms began to develop 5-6 days after spraying, with typical circular, brown to tan spots having concentric rings. Disease severity increased significantly between the 14th and 21st day post-inculation. Individual spots were easily figured out to be associated with *Alternaria* infection and were confirmed by re-isolation of the pathogen from inoculated leaves. AMS-48 (22.67%), AMS-123 (27.11%), Pusa Parv (26.67%), Pusa Utsav (25.33%), and MGO-3 (30.67%), were again classified as moderately resistant. The pathogenicity of *Alternaria alternata* in marigold was previously reported by Aktar & Shamsi (2014). In the present study, most genotypes exhibited higher PDI values under laboratory (*in vitro*) conditions compared to natural (*in vivo*) settings. This disparity can be attributed to the higher moisture retention on inoculated leaf surfaces and prolonged pathogen exposure under controlled *in vitro* conditions, which are more conducive to infection. These findings suggest a direct correlation between humidity and disease severity as reported by Hotchkiss & Baxter (1983). This also explains the observation that some genotypes classified as moderately susceptible under *in vivo* conditions showed higher disease severity *in vitro*.

The results from both screening approaches were consistent and complementary, each offering unique advantages. The detached-leaf (*in vitro*) method provided reliable and reproducible results, while *in vivo* screening allowed for a more comprehensive understanding of disease progression under natural conditions (Doullah et al., 2006). Among the evaluated genotypes, those belonging to *Tagetes erecta*, such as Pusa Basanti Gainda, AMS-101, AMS-103, and AMS-151, were more susceptible to *Alternaria* infection than *Tagetes patula* (French marigold) genotypes like Pusa Parv and Pusa Utsav. These observations are in agreement with the findings of Akoijam & Chandel (2010). Notably, Pusa Deep exhibited a high PDI, likely due to the high virulence of the I-3 isolate used for inoculation. Conversely, two African marigold genotypes, AMS-48 and AMS-123, showed moderate resistance to infection (Table 3, Fig. 5).

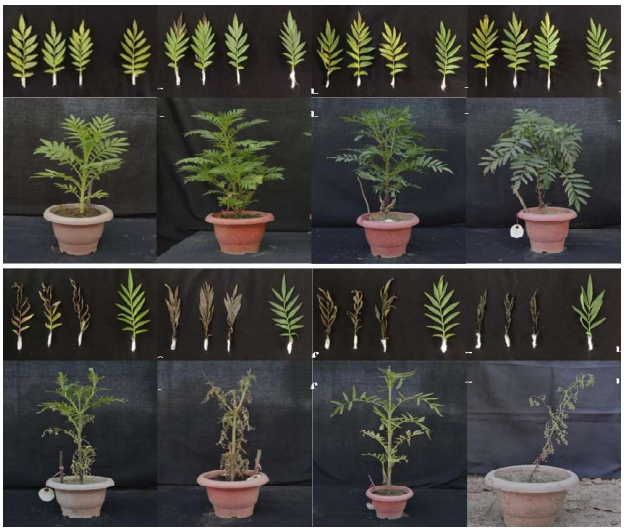

Fig. 5 : Marigold genotypes exhibiting variable severity to *Alternaria* infection under *in vitro* and *in vivo* conditions; a: AMS-48, b: MGO-3, c: Pusa Parv, d: Pusa Utsav, e: AMS-109, f: AMS-110, g: Pusa Basanti Gainda and h: TM-1

CONCLUSION

This study highlighted prominence of Alternaria leaf spot in marigold. ITS-based molecular characterization of a virulent strain of Alternaria alternata was done, which was utilized for screening for resistance. PDA and Host-extract media (from marigold leaves) were found to be promising for culturing because of abundant sporulation and rapid mycelial growth. A dual screening approach (in vitro and in vivo) validated the resistance levels of sixty genotypes, of which five were found to be moderately resistant. These results provided a standardized and more reliable dual screening protocol compared to field screening alone, and the identified genotypes can serve as a valuable foundation for resistance breeding programs in marigold."

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