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Original Research Paper

Morphological and molecular characterization of *Alternaria* spp. causing leaf spot and flower blight of marigold (*Tagetes* spp.)

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ABSTRACT

Globally, leaf spot and flower blight disease, caused by *Alternaria* spp., is the most devastating disease of marigold, especially during rainy season when moderate temperature and high relative humidity prevails. Disease is characterized by appearance of irregular black sunken lesions on the surface of lower leaves which enlarge and progress to stems, buds and flowers causing the death of plants under congenial environment conditions. The pathogen infecting *Tagetes* spp. was identified as *Alternaria tagetica* based on the symptomatology and conidial morphology. This finding was further confirmed by sequencing the nuclear ribosomal internal transcribed spacer regions (ITS) of the fungus which showed ~100% homology with the earlier reported *Alternaria tagetica* isolates affecting marigold across China, Netherlands and USA. Further, ten different growing media were evaluated to study the fungal growth patterns including sporulation to aid large-scale phenotyping of germplasm under controlled conditions. The radial colony and other colony parameters were largely influenced by the type of growing media used. Potato dextrose agar, oat meal agar and V8-juice agar showed desirable colony parameters and showed maximum sporulation of *A. tagetica*.

Keywords: *Alternaria tagetica*, colony parameters, marigold, molecular characterization, nutrient media, sporulation

INTRODUCTION

Alternaria sp. is an imperfect fungi of the phylum Deuteromycetes, owing to lack of sexual stage and reproduces asexually through large multicellular conidia having both longitudinal and transverse septa and an apical beak. The conidia may be produced either solitary or in chains (Simmons, 2007). *Alternaria* fungi comprises both saprophytic, endophytic and/or pathogenic species which cause severe damage to cereals, oilseeds and horticultural crops producing symptoms of blight, leaf spot, stem rot, fruit rot and moldy cores (Elfar et al., 2018). In marigold, the pathogen causes leaf spot and flower blight causing the death of plants especially during the rainy season with prevalence of moderate temperature and high humidity. The disease initially appears as a dark brownish-black irregular-shaped sunken lesion on the lower leaves and gradually progresses toward the upper leaves, petioles and flower buds, causing up to 50-60% crop loss and reducing aesthethic beauty of flowers (Shinde et al., 2018).

Due to their ubiquitous nature, several *Alternaria* species, *A. dianthi, A. zinniae, A. gypsophillae, A. tenuisimma, A. patula, A. porri, A. alternata, A. jacinthicola* and *A. tagetica* are known to cause leaf spots in marigold. Consequently, accurate species identification becomes crucial for its management and germplasm screening for identification of resistant source. Environment and nutrient conditions can influence the spore production in *Alternaria*, thus standardized media source is essential for mass multiplication of spores for high-throughput phenotyping. Manipulation of growing media *viz*., nutrient-rich media or nutrition-depleted media, and addition of supplements are the commonly followed methods to intensify conidia production (Braun et al., 2011). Therefore, there is a need to standardize the suitable media for mass multiplication of conidia. Hence, the present experiment was carried out to identify the *Alternaria* spp. causing leaf spot and flower blight of marigold through morphological and molecular techniques and subsequent identification of a suitable medium for optimum fungal growth and mass production of conidia.

MATERIALS AND METHODS

Isolation and purification of *Alternaria* species

The present study was carried out at ICAR-Indian Institute of Horticultural Research, Bangalore, India. The symptoms were visible during the rainy season in mid June 2022 and infected leaves were then collected from infected marigold plants in July 2022 when the severity of disease had increased. Leaves were surface sterilized using sodium hypochlorite solution (1% v/w) and were inoculated on the PDA supplemented with 0.05 g/L streptomycin in petri-plates which were further maintained in BOD incubator at 28±2°C for 7-10 days. The growing colonies were sub-cultured twice on the same medium to obtain pure cultures.

Morphological and molecular characterization of *Alternaria* species

Conidia from the isolates were collected by flooding the plate with sterile water followed by filtration and were used for morphological characterization as per Zhang (2003). Observation like length and breadth of the conidia, number of transverse and longitudinal septa and beak length were recorded by observing under 20X magnification in a light microscope (ZEISS AXIO Imager, A2, Germany). For further confirmation, genomic DNA was extracted from the mycelial mat following CTAB method (Doyle & Doyle, 1990) and was amplified using ITS1 and ITS4 primers (White et al., 1990) adopting standard PCR protocol (Sonavane et al., 2023). Amplicons were checked on agarose gel (1.2% v/w) and were purified and sequenced by Sanger method in automated DNA sequencer (Eurofins Genomics India Pvt. Ltd., Bangalore).

The ITS sequence of the isolates (deposited in the NCBI Gene bank) was subjected to BLAST in the NCBI database (www.ncbi.nlm.nih.gov). Phylogenetic tree was constructed using the representative nucleotide sequences from NCBI of *Alternaria* spp. infecting marigold using MEGA-X software.

Pathogenicity test

The same isolates used for morphological and molecular analysis were used for the pathogenicity test. Conidia of the tested isolates cultured on PDA were used to obtain conidial suspensions, which were prepared by scrapping the mycelium from the surface of plate with sterile water, which was then filtered through cheesecloth to remove mycelium fragments. The concentration of the collected spore suspension was adjusted to 1×10^6 conidia/mL and Tween 20 (Merck, Modderfotin) was added. Pathogenicity test was conducted by spraying the conidial suspension on month-old plants post-transplantation maintained in controlled conditions with temperature of 25-3°C and relative humidity of 60-80%. Control plants were sprayed with sterile distilled water.

Table 1 : Media compositions used for studying fungal growth and sporulation

Nutrient media	Composition	Concentration of the premix media (g/L)
Broad bean agar (BBA)	Broad bean: 250 g, agar: 20 g	270
Corn meal agar (CMA)	Corn meal: 50 g, agar: 15 g	17
Czapek's dox agar (CDA)	Sucrose: 30 g, $NaNO_3$: 2 g, K_2HPO_4 : 1 g, $MgSO_4$: 0.5 g, KCl: 0.5 g, $FeSO_4$: 0.01 g and agar: 15 g	49.01
Malt extract agar base (MEAB)	Malt extract: 20 g, peptone: 1 g, dextrose: 20 g, agar: 20 g	61
Oat meal agar (OMA)	Oat meal: 60 g, agar: 12.5 g	72.5
Richard's synthetic agar (RSA)	$FeCl_3$: 0.02 g, sucrose: 50 g, agar: 15 g	82.5
Synthetic media (S-media)	Sucrose: 20 g, $CaCO_3$: 30 g, agar: 20 g	70
V-8 juice agar (V8)	V-8 juice: 8.3 g, L-asparagine: 10 g, yeast extract: 2 g, $CaCO_3$: 2 g, glucose: 2 g, agar: 20 g	44.3
Water agar (WA)	Agar: 20 g	20
Potato dextrose agar (PDA)	Peeled potato: 250 g, dextrose: 20 g, agar: 20 g	270

Standardization of growing media for fungal growth and sporulation

Different growing media were prepared using standard protocols and evaluated (Table 1) to identify the best medium for optimum fungal growth. Approximately 15-20 mL media supplemented with streptomycin were poured into sterile petri-plates where mycelial discs (5-6 mm diameter) from 5-day-old actively growing culture were inoculated. The growth pattern of the fungi in different growing media was evaluated in a completely randomized design with three replications and potato dextrose agar (PDA) was considered as control.

The petri-plates were maintained at 28±2°C in BOD incubator and observations *viz*., average days required to cover the maximum area, colony diameter and colony parameters *viz*., colony shape (circular, irregular, filamentous, rhizoid), margin (appearance of the edge: entire, undulate, filiform, curled, lobate), elevation (alleviation: raised, umbonate, convex, crateriform, flat), mycelial density (abundant, moderate, low, extremely low), sporulation, colour of culture, presence/absence of concentric rings and texture (cottony, velvety) were recorded.

Statistical analysis

The observations for average days required to cover maximum area and radial colony of the fungi grown on different media were subjected to one-way ANOVA using SAS v.9.3.

RESULTS AND DISCUSSION

Morphological characterization of the *Alternaria* spp.

In mid-June, a few closely circular or oblong black spots with 1-5 mm diameter were observed on the lower leaves without any symptoms on stems and buds. At the end of July, when the temperature ranged between 19.65 - 29.34°C with a relative humidity of ~80%. Later, the lesions became larger and coalesced, turned black and in severe conditions the lesions also progressed towards the stems, buds and flowers causing death of plants (Fig. 1). The fungal isolates cultured on PDA had typical *Alternaria* colony growth patterns *viz.,* raised, circular shaped, olivaceous green mycelia with cottony texture. The conidia were smooth and light brown with medium to long beak ranging between 9.93 - 69.85 µm and were straight and solitary (Fig. 1). The length and breadth of the conidia ranged between 19.65- 44.61 µm and 10.00-31.73 µm, respectively with the longitudinal (0-3) and transverse (4-5) septa. Based on these morphological observation, the isolate was identified as *Alternaria tagetica.* Similar observations were recorded by Cheng et al. (2019) with conidial length (42-140 µm), width (17-35 µm) and beak length (30-100 µm), with 0-7 longitudinal and 3-8 transverse septa. Higher variability was observed in conidial length in the present study, might be due to the change in nutrient.

Fig. 1 : Characteristics of *Alternaria tagetica* isolates: lesions on leaves (a), stems (b), flower buds (c), flowers (d), conidia 40X (e) and 100X (f)

Molecular characterization of *Alternaria* spp.

The ITS region of the two *Alternaria* isolates *viz*., IIHR-1 and IIHR-2 had amplicon size of 531 bp and 532 bp, respectively. The nucleotide sequences of these isolates were deposited to NCBI database as and the accession numbers OR775568 and OR775573, respectively were obtained. The individual BLASTn searches revealed that the ITS sequences of IIHR-1 and IIHR-2 isolates were 99-100% identical to those of *A. tagetica* isolates infecting marigold reported from the Netherlands, China and Mexico. For phylogenetic analysis representative ITS sequences of *Alternaria* species infecting marigold *i.e. A. tagetica*, *A. tenuissima*, *A. sp* and *A. porri* were used. Phylogenetic analysis represented clustering of IIHR-1 and IIHR-2 isolates together with *A. tagetica* representative isolates infecting marigold (Fig. 2). Cheng et al. (2019), also confirmed *A. tagetica* as the predominant species in Beijing, China infecting marigold and recommended crop rotation as a major strategy for disease management. Thus, based on conidial morphology and molecular phylogeny, it can be concluded that these isolates causing leaf spot and flower blight of marigold belong to *A. tagetica*.

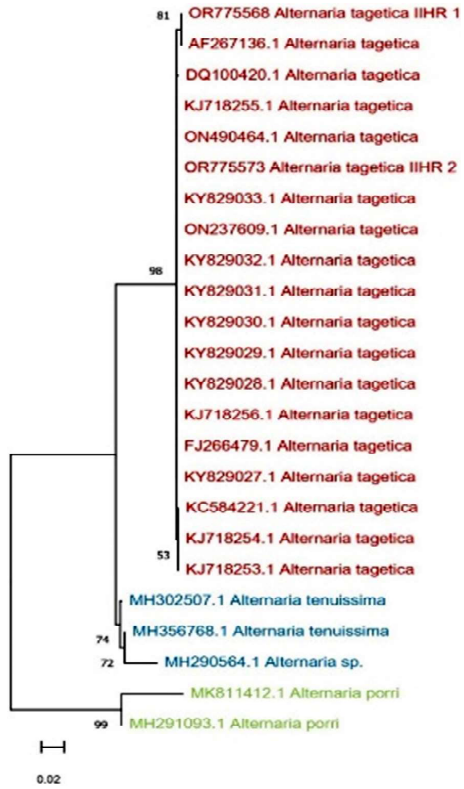

Fig. 2 : Maximum-likelihood phylogenetic tree derived from ITS rDNA gene sequences of *Alternaria* species

Pathogenicity test

Following the morphological and molecular identification, the pathogenicity test confirmed the virulence of the isolate. All the isolates tested caused leaf blight on the plants, without any significant difference between the isolates. Modified disease severity scale 0 to 5 was used to determine the severity of the *Alternaria* isolates (Fig. 3). The typical irregularly shaped necrotic lesion was observed on the surface of the lower leaves, which under favorable conditions progressed toward petioles, stems and flowers. Control plants were symptomless. The fungus recovered from the diseased leaves was identical to its inoculants based on conidial morphology.

Standardization of growing media for fungal growth and sporulation

The quantitative and qualitative parameters of the fungal colony varied significantly across media (Table 2). Maximum days required to cover maximum area ranged between 5.67 (control) to 12.67 (CMA). The diameter of mycelial mat ranged between 4.71 cm

Fig. 3 : Disease rating used in pathogenicity test of *Alternaria tagetica* recorded from marigold. The rating scale for lesion development on leaves from left to right: (a) 0= no lesion development, (b) 1= 1-5% leaf infection, (c) 2= 6-25% leaf infection, (d) 3= 26-50% leaf infection, (e) 4= 51-75% leaf infection, (f) 5= > 75% leaf infection

Table 2 : Mycelial growth, colony parameters and sporulation pattern of *Alternaria* spp. on different media under evaluation

Media	Maximum growth	Colony diameter	Colony parameter								
			Mycelial density	Sporulation	Colony shape	Margin	Elevation	Concentric rings	Culture colour	Reverse colour	Mycelial texture
BBA	6.33^{e}	9.00^{a}	+ + +	+ +	Circular	Filiform	Raised	Present	Olivaceous greyish green	Black	Cottony
CMA	12.67^{a}	4.72^{d}	+ +	+	Circular	Filiform	Umbonate	Present	Dark olive green	Periphery: light brown center: black	Cottony
CDA	9.00^{cd}	6.47^{c}	+ +	+	Irregular	Filiform	Umbonate	Present	Olivaceous green	Periphery: light brown center: black	Cottony
MEAB	11.00^{b}	7.43^{abc}	+ +	+	Irregular	Filiform	Raised	Present	Periphery: whitish grey centre: olivaceous green	Periphery: white centre: black	Velvety
OMA	8.33^{cd}	7.35^{abc}	+ + +	+ + +	Irregular	Filiform	Raised	Present	Greyish green	Periphery: brown centre: black	Velvety
RSA	8.33^{cd}	6.93^{bc}	+ +	+	Circular	Filiform	Umbonate	Present	Periphery: light pink centre: olive green	Periphery: orangish pink centre: black	Cottony
S-Media	11.33^{b}	7.23^{abc}	+	+ +	Filamentous	Filiform	Umbonate	Present	Greyish green	Olivaceous green	Cottony
V8	9.33^{c}	6.67^{c}	+ + +	+ + +	Irregular	Filiform	Raised	Present	Olivaceous green	Periphery: white centre: black	Velvety
WA	7.67^{d}	8.57^{ab}	-	+ +	Filamentous	Filiform	Flat	Present	Olivaceous green	Olivaceous green	Cottony
PDA	5.67^{e}	9.00^{a}	+ + +	+ + +	Circular	Filiform	Raised	Present	Olivaceous greyish green	black	Cottony
S.E(m)±	0.43	0.58									
S.E(d)±	0.61	0.82									
C.D (5%)	1.28	1.7									
C.V (%)	8.40	13.54									

*mycelial density: '+++': abundant; '++': moderate to abundant; '+': low to moderate; '-' : low

*Sporulation per microscopic view: '+++': > 50 conidia; '++': 10- 50 conidia; '+' : <10 conidia

(CMA) to 9.00 cm (control). Moderate to abundant growth of mycelia was observed in PDA, BBA, OMA, V8, while, it was low to moderate in CMA, CDA, RSA, S-Media, MEAB and extremely low in WA. Further, it was observed that carbohydrate-rich media promoted abundant mycelial density with slow radial growth.

Amongst the assessed media, variation for sporulation were observed. OMA, PDA, V8 media produced abundant conidia (>50 conidia/microscopic view) which might be because of differences in carbon source and mineral compositions. Depletion of carbohydrates after sufficient mycelial growth might have produced more conidia thus the number of condidial product is directly proportional to the mycelial growth and radial colony diameter. Further, in V8 juice agar presence of other components might also have stimulated sporulation, larlier reports suggested increased sporulation in $CaCO_3$-supplemented media in several *Alternaria* species (Masangkay et al., 2000). Despite the presence of $CaCO_3$ in S-media, lesser conidia production was observed suggesting the complementing role of carbohydrate and $CaCO_3$.

Significant variation was observed for shape, margin, elevation, texture and colour of the colony across media (Fig. 4). The fungal mat on BBA, CMA, PDA, RSA had circular colony, while, irregular colony was observed on CDA, MEAB, OMA, V8-Juice Agar and filamentous colony was observed on S-media, WA. A filiform margin was doserved in the fungal colony across media types. Mycelial growth with umbonate elevation was observed in CMA, CDA, S-Media, RSA, while, it was flat in WA and raised in BBA, MEAB, OMA, PDA, V8. These results are in line with the findings reported by Farhood & Hadian (2012) for *Alternaria* spp.

In the present study, the culture of *A. tagetica* had visible concentric rings across media. The surface of fungal culture across media had different shades of olive green colour. While in the reverse side, certain media (CMA, CDA, MEAB, OMA, RSA, V8) produced dual colour. The growth and surface colour of mycelia is affected by the temperature, micronutrient content and pH of the medium (Mendes et al., 2008) and the repetitive metabolite accumulation and exhaustion in presence of light leads to production of distinct hyphal bands (Edelstein & Segel, 1983). Farhood & Hadian (2012) also observed greyish mycelia of *Alternaria* on PDA. The fungal colony grown on all the media had cottony texture, except in OMA and V8 where it was velvety indicating the role of nutrient availability in determining texture of colony.

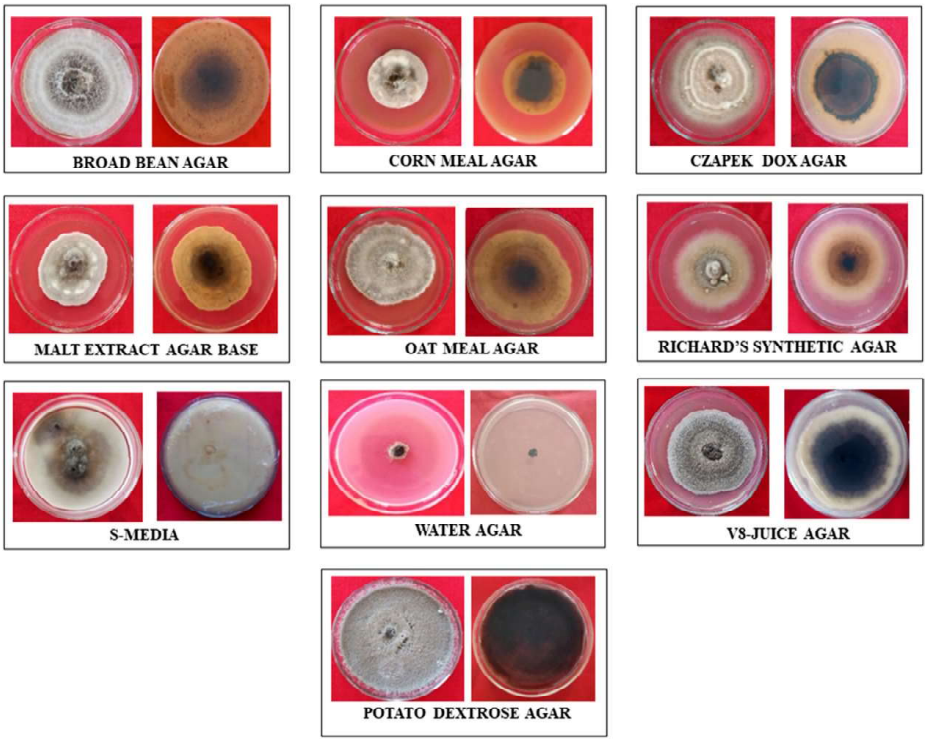

Fig. 4 : Mycelial growth parameter of *Alternaria* spp. on different growing media

CONCLUSION

Marigold is an important annual flower with commercial utility, however production is affected by several *Alternaria* species that uschibit similar symptoms Thus, identification of the fungal species causing the disease is of utmost importance for effective management and future resistance breeding programs. The fungus infecting marigold was identified as *Alternaria tagetica* based on the conidial morphology and molecular phylogeny. The study further demostrated that the growth the fungus varied across the ten nutrient media ivaluated. Among them, potato desctrose agar, Oat mealagar, and V8 juice agar exchibited superior performance in supporting colony development and sporulation.

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