Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.7227

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **6 (6):** 1286-1291 (2018)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Molecular Characterization of *Alternaria alternata* (Fr.) Keissler Causing Leaf Blight Disease of Marigold

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Received: 27.10.2018 | Revised: 30.11.2018 | Accepted: 7.12.2018

ABSTRACT

Leaf blight caused by Alternaria sp. is one of the most important and destructive disease of marigold causing severe yield loss in all marigold growing areas of Southern Tamil Nadu. The pathogen Alternaria sp. causing leaf blight disease was isolated from disease infected leaves by tissue segment method and the pure culture was obtained through single hyphal tip method. The isolates proved its pathogenicity on the marigold hybrid tall yellow. Molecular characterization of the isolate at genus level using the ITS primers resulted in amplification of a 528bp fragment and rDNA-ITS region was sequenced. Use of Alternaria species specific primers AaF and AaR confirmed that this isolate as Alternaria alternata at species level. To know the diversity of this isolate, phylogenetic tree analysis was carried out and the results showed that this A. alternata is distinct from the other Alternaria isolate reported earlier from different host plants. In the present study the pathogen causing leaf blight in marigold was isolated and identified as A. alternata through molecular approach at species level.

Key words: *Alternaria sp., rDNA-ITS region, Leaf blight, Marigold*

INTRODUCTION

Marigold (*Tagetes erecta*) is an important commercial flower crop, belonging to family *Asteraceae*. China, India and Peru are the leading countries in production and cultivation of marigold flowers. Marigold occupies an area of 66.13 thousand hectares with an annual production of 603.18 thousand metric tons in India (Horticultural Statistics at a Glance 2017). Marigold is used for various purposes i.e., cut flower, loose flower, pot plant and as bedding plant. It shows pharmacological properties *viz*., anti-microbial activity, insecticidal activity, anti-bacterial activity, nematicidal activity, wound healing activity, mosquitocidal activity, analgesic activity, anti-oxidant and larvicidal activity. Among the biotic stresses, leaf blight caused by *Alternaria sp*., is one of the most destructive diseases which causes heavy yield lose in almost all marigold growing areas of southern Tamil Nadu[3].

Cite this article: Prathima, P., Thiruvudainambi, S., Kalpana, K., Molecular Characterization of *Alternaria alternata* (Fr.) Keissler Causing Leaf Blight Disease of Marigold, *Int. J. Pure App. Biosci.* **6(6):** 1286-1291 (2018). doi: http://dx.doi.org/10.18782/2320-7051.7227

The symptoms of the disease were noticed on leaves, stem and flowers. The disease initiates as circular to oblong brownish spot, later turned dark brown to blackish, enlarged and coalesce to cover almost the entire leaf and part of branches made the plant a burnt appearance[2,4]. On blooming, the inflorescence axis and flower heads were attacked severely and turned the original yellow colour of the flowers to deep brown or black[11].

MATERIAL AND METHODS

Collection and isolation of *Alternaria* sp

A survey was carried out in major marigold growing areas of Southern districts of Tamil Nadu *viz*., Madurai, Tirunelveli, Dindigul, Theni and Virudhunagar districts to assess the incidence and severity of leaf blight disease. The survey was conducted when the crop was at seedling stage to flowering stage in farmers field. The leaf blight pathogen was isolated from disease infected leaf of marigold by tissue segment method. Infected leaf tissue with some adjacent healthy tissues (0.5cm×0.5cm) were cut into small bits and surface sterilized in 70% ethanol for three minutes subsequently washed three times with sterile distilled water. The leaf bits were transformed to sterile filter paper in order to absorb excess moisture and finally the leaf bits were plated onto Petri dishes containing sterile PDA media and incubated at 27 ± 1^0C for seven days. Seven days after incubation, the isolated fugus was identified as *Alternaria sp.*[14] purified and maintained on PDA slants at 4°C for further study.

Pathogenicity test

The pathogen isolated from diseased leaves after purification was tested for its pathogenicity for proving Koch's postulates. Pathogenicity test was carried out using the marigold hybrid tall yellow. Marigold hybrid (tall yellow) seeds were in pots containing pot mixture and grown in the glasshouse. About 40 days old marigold plants were artificially inoculated with the conidial suspension of $2x10^6$ cfu/ml *Alternaria* sp. and covered with polythene bag for 24 hours to maintain the humidity. The plants inoculated with sterile distilled water served as control. After seven days of inoculation, these plants were observed for the of disease symptoms and re-isolation of pathogen was carried from the infected plants[6,12].

DNA Extraction

Genomic DNA of the *Alternaria* sp. was extracted by using the method of Kumar *et al*.[5] the quality and quantity of isolated DNA was checked on 1.0% agarose gels stained with ethidium bromide.

PCR amplification of ITS region and sequencing of rDNA-ITS region

The leaf blight pathogen tentatively identified as *Alternaria* sp. based on morphological characterization was further characterized at molecular level using universal ITS primers[8]. The 18S rDNA and ITS regions from the fungal strain *Alternaria* sp. were amplified using PCR with a final reaction mixture volume of 10 µl (7µl PCR master mix (Taq DNA polymerase, AMPLICON Company), 1 µl of free nuclease water, 0.5 µl of forward and reverse primer (ITS1 and ITS4) and 1µl of DNA template). Thermo cycling procedure were 2 minutes for initial denaturation (95 °C), followed by 38 cycles of denaturation (95 °C) for 1 minute, annealing at 55 °C for 30 seconds, 1minute extension at 72 °C and final extension for 10 minutes at 72 °C. Amplified PCR products were analyzed using 1% agarose gel in TAE buffer solution at 80V for 40 minutes at 25°C. Sequencing of PCR amplified products was carried out using the commercial service (Eurofins Genomics India Pvt Ltd).

Molecular confirmation of *Alternaria alternata* with species specific primer

To further characterize the *Alternaria* sp. at species level the species specific primers AaF (5' GTGCCTTCCCCCAAGGTCTCCG 3') and AaR (5' CGGAAACGAGGTGGTTCAG GTC 3') [9] were used to identify the leaf blight pathogen using PCR. The PCR conditions followed were initial denaturation for 2 min at 95°C followed by 35 cycles of denaturation for 1min at 95°C, annealing for 45sec at 62°C, extension for 30sec at 72°C and final extension for 10min at 72°C. The presence of

amplicons was visualized electrophoretically on 2% agarose gel stained with ethidium bromide.

Genetic data analysis

Sequence identity matrix of *Alternaria* sp. was generated using BioEdit Sequence Alignment Editor (version 7.0.5.3). After multiple alignment, phylogenetic tree analysis was performed with MEGA7 (version 7.0.26) software package.

RESULT AND DISCUSSION

Isolation of the fungus and morphological identification

Leaves showing the blight symptoms consistently yielded *Alternaria* isolates on the PDA plates. The fungus produced abundant brownish mycelia. Conidiophores were simple, olive- brown, septate, branched, which were solitary or in short chains. Matured conidia measure about 96.22 to 49.59 µm in length and 26.53 to 15.90 µm in breadth. Generally, all the isolates produced beaked and beakless conidia. The beak length of conidia varied from 28.89 to 11.58 µm. Conidial characteristics of the isolated pathogen were similar to the conidia isolated from the infected plants. Based on the morphological characters, the organism was identified as *Alternaria sp.* (Fig. 1).

The Pathogenicity

The isolated pathogen was proved to be pathogenic on marigold hybrid tall yellow and identical disease symptoms were observed similar to the field symptoms on leaves and young flower buds which includes dry rotting, necrotic discoloration of the sepals and peduncle. The adjacent spots later coalesced with one another to form large irregular patches. The leaves with severe coalescing of spots drooped and withered leading to death of the entire plant. No symptoms were observed on the water inoculated control plants. Among the different isolates tested, isolate IS-A4 was more virulent in inducing symptoms on inoculated marigold plants than other isolates hence the virulent isolate IS-A4 was used for future studies (Fig 2).

Molecular detection of *Alternaria* sp. with ITS primer

Molecular detection of *Alternaria* sp. at genus level was done by using universal ITS primer pairs ITS1 and ITS4. Agarose gelelectrophoresis of PCR amplified products resulted in amplification of 528bp in all the six isolates tested. Our results are in close agreement with Xie *et al.*[15] who observed all the sequences of ITS rDNA from *Alternaria alternata* samples amplified the expected specific amplicon size of 528 bp (Fig. 3).

Sequencing of rDNA-ITS Region

To confirm the molecular identity of *Alternaria sp.* (IS-A4), the rDNA-ITS region of the present isolate was amplified and sequenced[10]. The rDNA sequence was deposited in the GenBank database under the accession number MH536678. BLAST analysis showed 99 per cent similarity with *A. alternata* sequences reported from other host from India. To assess the relationships with other *Alternaria* isolates the corresponding genomic regions of 10 *Alternaria* sp. showing 99% - 100% sequence identity with the present isolate were obtained from GenBank and a phylogenetic tree was constructed. Phylogenetic analysis results revealed that the present *Alternaria alternata* infecting marigold crop grouped separately from other *Alternaria* spp. reported from other host species (Fig 4).

The rDNA-ITS analyses performed on genomic DNA of *A. alternata* isolate revealed the presence of high level of genetic diversity with other *Alternaria* isolates infecting other host. The rDNA-ITS has the unique potential for providing information across an entire genome. The isolates showed strong genetic similarity within a range of 99% to 100%, indicating high level of identity among them irrespective of hosts. In the phylogenetic tree, the present isolate grouped separately from the other isolates from different hosts indicating *A. alternata* infecting marigold is distinct from other *Alternaria sp*. reported from different host.

Molecular confirmation of *Alternaria* sp. with species specific primer

To further confirm the isolated pathogen *(Alternaria sp*) at species level, PCR was done with species specific primer pairs AaF and AaR[1]. Gel Electrophoresis of PCR amplified produced yielded a 184bp of DNA fragments (Fig.5). The results obtained from the present study are in close agreement with Kordalewska *et al.*[9], who reported that *Alternaria alternata* samples amplified the expected specific amplicon size of 184bp.

Molecular characterization of *Alternaria alternata* (Fr.) Keissler causing leaf blight disease of marigold

Fig. 1. Morphological characters of *Alternaria alternata*, a) Mycelial growth of the *Alternaria alternata* isolate IS-A4, b) Mass of Conidia and c) Microscopic view of single conidia

a b c

Fig. 2. Pathogenicity of different isolates of *Alternaria alternata* in pot culture, PDI= Percent disease incidence, IS-A1=Isolate from Melakkal, IS-A2= Isolate from Nilakottai, IS-A3= Isolate from Ammapatty, IS-A4= Isolate from Azhakunatchiyapuram, IS-A5= Isolate from Sivagiri, IS-A6 = Isolate from Samsikapuram

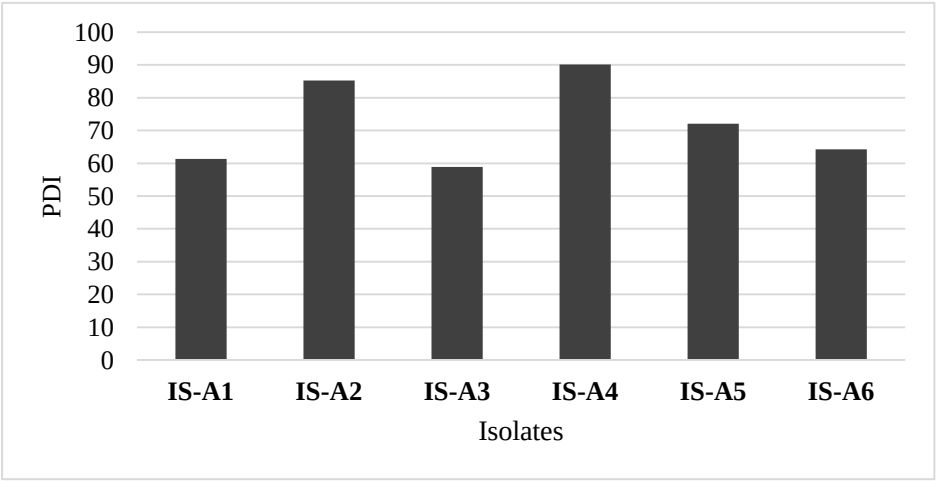


Fig. 3. PCR amplification of *Alternaria* with ITS primer, L=100 bp ladder, 1 to 6 Alternaria isolates from different locations.

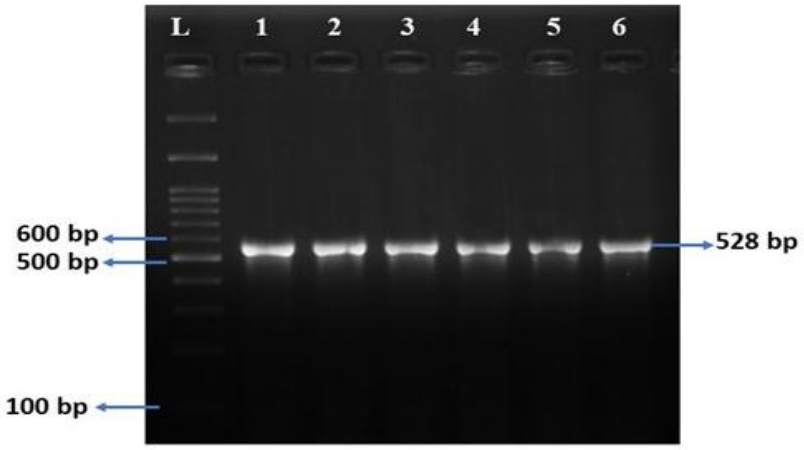


Fig. 4. Phylogenetic tree showing relationship among the related *Alternaria* sp with (●) *Alternaria alternata.*

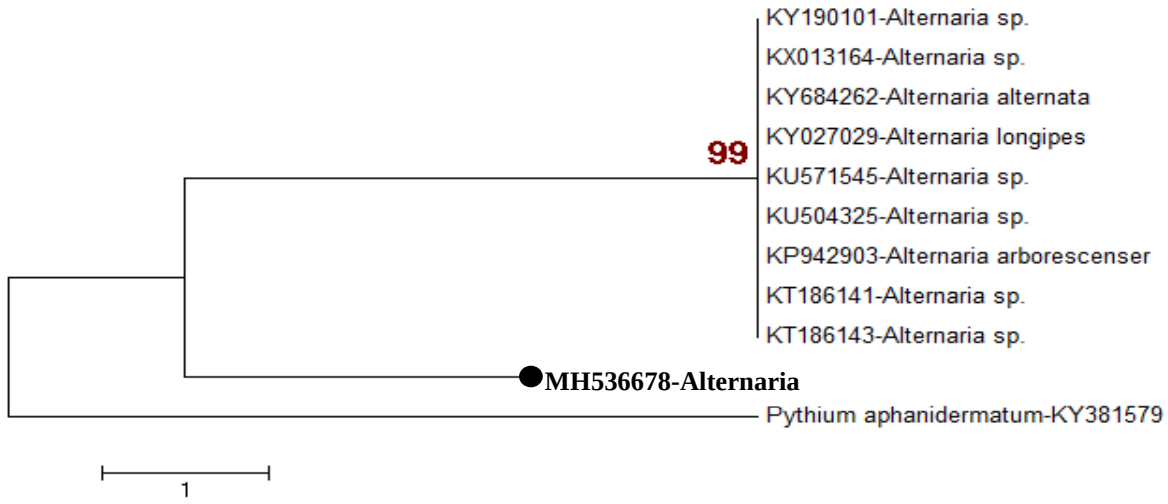
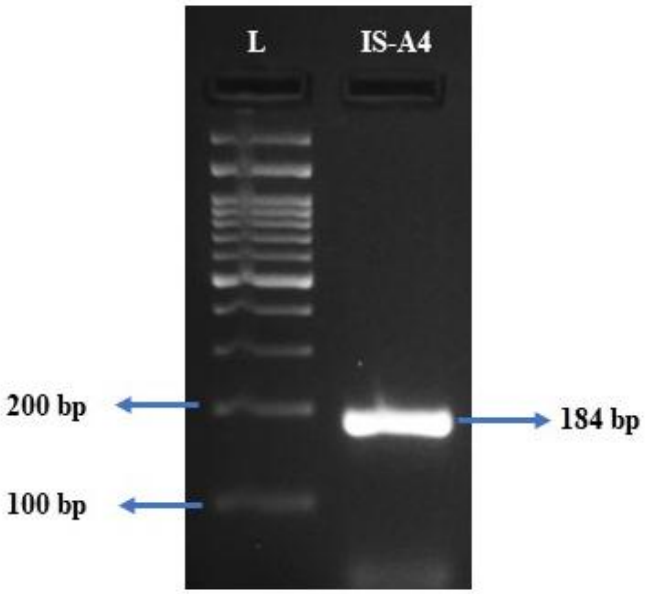


Fig. 5. confirmation of *Alternaria alternata* with specific primer AaF and AaR having product size of 184 bp.



CONCLUSION

Alternaria alternata causing Alternaria blight in marigold was characterized at molecular level using rDNA-ITS region. The rDNA-ITS analysis indicated that *Alternaria alternata* causing Alternaria blight in marigold is a distinct species from other reported *Alternaria* spp. infecting different host. In earlier findings, they have reported *Alternaria tagetica* (Tomioka *et al*, 2000) causing *Alternaria* blight in marigold.

REFERENCES

1. Basim, H., Basim, E. and Baki, D., Identification and characterization of Alternaria alternata (Fr.) Keissler causing Ceratonia Blight disease of carob (Ceratonia siliqua L.) in Turkey, *Eur. J. Plant Pathol*. **151:** 73-86 (2018).
2. Bhat, H. A., Ahmad, K., Ahanger, R. A., Qazi, N., Dar, N. A. and Ganie, S., Status and symptomatology of Alternaria leaf blight (*Alternaria alternata*) of Gerbera

(*Gerbera jamisonii*) in Kashmir valley, *Afr. J. Agric. Res*. **8(9):** 819-823 (2013).

3. Cotty, P. and Misaghi, I., Effect of light on the behavior of *Alternaria tagetica* in vitro and in vivo, *Phytopathology*, **75(3):** 366-370 (1985).
4. Dhiman, J. and Arora, J., Occurrence of leaf spot and flower blight of marigold (*Tagetes erecta* L.) in Punjab, *Journal of Research-Punjab Agricultural University.* **27(2):** 231-236. (1990).
5. Kumar, V., Haldar, S., Pandey, K., Singh, R. P., Singh, A. K. and Singh, P. C., Cultural, morphological, pathogenic and molecular variability amongst tomato isolates of Alternaria solani in India, *World J Microbiol Biotechnol*. **24(7):** 1003-1009 (2008).
6. Maiti, C. K., Sen, S., Charya, R. A. and Acharya, K., First report of *A. alternata* causing leaf spot on stevia. *Plant* Path. **56(4):** 723-725 (2006).
7. Sharma, M., Ghosh, R. and Pande, S., Occurrence of Alternaria alternata causing Alternaria blight in pigeonpea in India, *Adv Biosci Biotechnol*. **4(6):** 702-705 (2013).
8. Konstantinova, P. P., Bonants, J. M., Van Gent-Pelzer, M., Van Der Zouwen, P. and Van Der Bulk, R., Development of specific primers for detection and identification of Alternaria spp. In carrot material by PCR and comparison with blotter and plating assays, *Mycol Res*, **106(1):** 23–33 (2001).
9. Kordalewska, M., Brillowska-Dąbrowska, A., Jagielski, T. and Dworecka-Kaszak, B., PCR and real-time PCR assays to detect fungi of Alternaria alternata species. *Acta Biochimica Polonica*, **62(4):** 707-712 (2015).
10. Kusaba, M., & Tsuge, T., Phologeny of Alternaria fungi known to produce host-specific toxins on the basis of variation in internal transcribed spacers of ribosomal DNA, *Current Genetics.* **28(5):** 491-498 (1995).
11. Shome, S. and Mustafee, T., *Alternaria tagetica* sp. Nov. causing blight of Marigold (Tagetes sp.). *Current Science*, **35(14):** 370-371 (1966).
12. Simmons, E. G., *Alternaria*: An identification manual. CBS Biodiversity 6, CBS Fungal Biodiversity Centre, Utrecht, The Netherlands (2007).
13. Tomioka, K., Toyozo, S., & Koganezawa, H., Marigold leaf spot caused by *Alternaria tagetica* new to Japan, *J Plant Pathol*. **66(4):** 294-298 (2000).
14. Webster, J., Introduction to fungi, 3, Cambridge Univ. Press. Cambridge. New York, P. 669 (1986).
15. Xie, G., Tan, S. and Yu, L., Morphological and molecular identification of pathogenic fungal of post-harvest tomato fruit during storage, *AFR J MICROBIOL RES*. **6(22):** 4805-4809 (2012).