First Record of *Alternaria Alternata* isolate CVGCIPPL Isolated from Green Scale Insect (*Coccus viridis*) on Citrus Plants

Hamdia Z. Ali*, Bassim H. Hassan and AbdulRahman A. AbdulRahman

Integrated Pest Management Center, Agriculture Research Directorate, Ministry of Science and Technology, Baghdad, Iraq

**Corresponding Author:** Hamdia Z. Ali  
**Email:** hamdiazali3@gmail.com

**ABSTRACT**

The current study isolated and identified *Alternaria alternata* isolate CVGCIPPL for the first time in Iraq from natural infection of green scale insect (*Coccus viridis*) on citrus plants during the 2020 season. Microscopic and phylogenetic analyses of the ITS-rDNA region sequence verified the identity of *Alternaria* sp. isolate CVGCIPPL as *A. alternata* isolate CVGCIPPL. The newly registered sequence of *A. alternata* isolate CVGCIPPL was submitted to the GenBank database MT415558.1 and deposited under accession number MT415558.

**Keywords:** *Alternaria alternata*, Green Scale, Insect, ITS-rDNA.

**INTRODUCTION**

*Alternaria* species are the causative agents of various crop diseases. They include a total of 299 species and infect more than 380 species of plants (Kirk et al., 2008; Nowicki et al., 2012). *A. alternata* fungus is considered to be in the Anamorph stage (asexual morphs), and the teleomorph stage (sexual reproductive stage, especially a fruiting body) of *A. alternata* is *Chalospora diplospora*. *A. alternata* survives for long time in the soil or in the leaves as conidia (Domsch et al., 1980; Timmer et al., 2003). According to Lawrence et al. (2016), *A. alternata* Nees (1816) is classified in Kingdom: Fungi, Phylum: Ascomycota, Class: Dothideomycetes, Order: Pleosporales, Family: Pleosporaceae and Genus: *Alternaria* (Fr.) Keissl. (1912). *A. alternata* is naturally found in the soil, water and indoors and is considered an opportunistic pathogen. In addition, *Alternaria* species cause at least 20% of agricultural spoilage and result in 80% of yield losses (Nowicki et al., 2012). Moreover, *A. alternata* (Fr.) Keissl. is commonly known as a saprophyte fungus through infested crop residue, and *Alternaria* spp. have been known to release secondary metabolites such as phytotoxic chemicals from different strains, which may be exploited in detecting the efficiency of biocontrol agents (Lawrence et al., 2016). *Alternaria* spp. have specific cells known as appressorium that play an important role in recognising the host through certain hydrophobic materials that are released from the host surface; these materials stimulate appressorium formation due to the accumulation of some dissoluble substances such as glycerol that use turgor pressure to penetrate the host and spread its pathogenicity and virulence through the melanin compound (Kimura and Tsuge, 1993). In addition, various species of *Alternaria* are known to produce more than 70 types of toxins (EFSA, 2011). Kaur et al. (2019) reported that α-glycosidase inhibitors produced from *A. deucrens* can be used to effectively protect against insects and pathogens. Magan et al. (2000) indicated that *Alternaria* spp. release volatile compounds such as hexan-1-ol. In the last four decades, *Alternaria* spp. have been used in biological pest management, and several studies concluded that *Alternaria* spp. play an important role in induced systemic resistance (ISR) in plants and produce active materials against pests/pathogens (Singh et al., 2012; Yang et al., 2012; Fatma et al., 2020). Hence, several studies used entomopathogenic fungi (EPF) related to *Alternaria* spp., especially *A. alternata* (Fr.) Keissler, in controlling insects effectively, such as onion thrips (Arthropoda: Thripidae) and *Thrips tabaci*. *A. alternata* was isolated from dead leafhopper insect *Zyginaida pullula* (Hemiptera: Cicadiellidae) (Ozino, 1982). *A. alternata* exhibits the ability to control larvae of the blue cereal leaf beetle *Oslema melanopus* and *O. gallaeciana* (Coleoptera: Chrysomelidae) (Raizada, 1976). In Egypt, Shabana and Ragab (1997) isolated *A. infectoria*, which was considered as a entomopathogenic fungus, from eggs of fig wax scale insect *Ceroplastes ruscii* (Homoptera: Cocciidae). They concluded that *A. infectoria* may be used as bio-inoculants against fig wax scale insect. In Greece, Kaur et al. (2013) extracted ethyl acetate from *A. alternata*, which was isolated from *Azadirachta indica* and used it as a biopesticide against tobacco caterpillar *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), which resulted in significantly increased larval mortality. Christias et al. (2001) found that *A. alternata* has the ability to infect many insects, including *Drosophila melanogaster* and *Ceratitis capitata*. The fungal mycelium of *A. alternata* can be isolated from different parts of dead aphids. *A. alternata* has been used as a toxin to induce the resistance of rose plants against rose aphids *Macrosiphum rosivorum* (Hemiptera: Aphidioidea) (Yang et al., 2012).

Based on the first evaluation of *Alternaria* sp., which was isolated from strawberry leaves by Amatuzzi et al. (2018), and later used successfully as biocontrol agents against European pepper moth *Duponchelia fovealis* (Lepidoptera: Crambidae) (Zeller, 1847). Saad et al. (2019) indicated that *A. alternata* plays an important role in suppressing cotton leaf worm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). Moreover, *A. alternata* can be used as biocontrol agents against other pests. *Alternaria* spp. were isolated from larvae of different species of *Aedes*, *Anopheles* and *Culex* (Rybalchenko and Podova et al., 1977). *Alternaria* spp. stated as inhibitor to the development of fruit fly *Drosophila melanogaster* (Diptera: Drosophilidae) has been through the active insecticidal material isolated from stable cultures of *Alternaria* spp. (Podova et al., 1977).

The application of EPF such as *A. alternata* as biocontrol agents has been the most recent approach in controlling spotted potato ladybird beetle *Henosepilachna vigintiactopunctata* (Coleoptera: Coccinellidae) and beetles that attack other solanaceous plants (Sharma et al., 2012).
al., 2012). Mantzoukas and Eliopoulos (2020) concluded that EPF play a critical role in integrated pest management programs and plant protection. Sharma and Sharma (2014) isolated Alternaria sp. from castor plant Ricinus communis and screened acetylcholinesterase compound by feeding the larvae. This material showed high inhibitory activity towered tobacco cutworm larvae or cotton leaf worm Spodoptera litura (Arthropoda: Lepidoptera) through re-isolation from infected larvae. The culture of Alternaria sp. has been utilised as medicine, biocontrol agent and insecticide. The present study isolated and identified A. alternata isolate CGCPI from green scale insect.

MATERIALS AND METHODS
Isolation of A. alternata isolate CGCPI isolated from green scale insect on citrus tree
The green scale insect was obtained from infected citrus fruit at Al-Mada’in orchard during the 2020 season. Dead green scale insect (Hemiptera: Coccidae), which was infected naturally by Alternaria sp. isolate CGCPI, was surface sterilised in bleach (1% chlorine) for 5 min. The specimens were washed in a laminar flow three times with sterilised water and then dried on sterilised paper. Subsequently, dried pieces of green scale insect were placed onto potato dextrose agar (PDA) medium and incubated at 28 ± 2 °C for 3–5 days. To classify the fungal pathogen of Alternaria sp., a single colony was spread onto water agar medium aseptically. Thereafter, it was transferred onto a plate containing PDA medium and incubated at 28 ± 2 °C for 3–5 days (Luo et al., 2018). Fresh fungal mycelium and spores of Alternaria sp. were morphologically examined under a compound microscope. Moreover, the green scale insect was used as a source for re-isolation of A. alternata isolate CGCPI.

Isolation of fungal DNA from A. alternata isolate CGCPI
The fungal isolate CGCPI of Alternaria sp. was used to extract genomic DNA. A 5 mm mycelial disc of fresh fungal mycelium cultured in a petri dish was transferred into 250 mL flasks containing 100 mL of potato dextrose broth medium supplemented with antibiotics (100 μg mL⁻¹ of streptomycin) and incubated at 28 ± 2 °C for 3–5 days. To classify the fungal pathogen of Alternaria sp., a single colony was spread onto water agar medium aseptically. Thereafter, it was transferred onto a plate containing PDA medium and incubated at 28 ± 2 °C for 3–5 days (Luo et al., 2018). Fresh fungal mycelium and spores of Alternaria sp. were morphologically examined under a compound microscope. Moreover, the green scale insect was used as a source for re-isolation of A. alternata isolate CGCPI.

Results
Isolation and identification of A. alternata isolate CGCPI
Fresh fungal culture of Alternaria sp. isolate CGCPI obtained from plate containing PDA medium was examined microscopically to identify the isolate CGCPI that belongs to the genus Alternaria (Figure 1a and b). The fungal mycelium of Alternaria sp. isolate CGCPI is slightly visible in Figure 1a. The examination results of fresh fungal mycelium and conidia, which were seen under the compound microscope, verified the identity of Alternaria sp. isolate CGCPI as A. alternata isolate CGCPI. Mycoparasitism by Alternaria sp. isolate CGCPI on green scale insect (Figure 1a) as displays the morphological characteristics of mycelium and conidia in (Figure 1b).

A. alternata isolate CGCPI attaches to the green scale insect via cell wall-degrading enzymes (CWDEs), and this mechanism involves morphological changes such as the formation of apical and intercalary, globose or lobate appressoria (Figure 1b). These parts provide high turgor pressure to support the penetration process into the host exoskeleton and facilitate the penetration of the mycelium into the insect body. The penetration hypha accumulates components of the exoskeleton at the tip of the appressoria and subsequently releases extracellular CWDEs towards the prey (insect) in a highly regulated fashion in order to penetrate the cuticle and the cell wall of the insect. Moreover, A. alternata inhibits the production and release of material by the insect via CWDEs before predation. Magan et al. (2000) confirmed that Alternaria spp. release volatile compounds such as hexan-1-ol that may degrade the exoskeleton (soft scales, which play a significant role in protecting the insect from biotic and abiotic stresses) of green scale insects and structured from main component (proteins (sclerotin) and chitin (polysaccharide molecules)) with some of wax.
Figure 1. *A. alternata* isolate CVGCIPL under Microscope. (a) *A. alternata* isolate CVGCIPL; branches, hyphae and small conidia carry in more than one point (polyplastic). (b) *A. alternata* CVGCIPL spores; small conidia, ramoconid.  

**PCR amplification of ITS-rDNA sequences of A. alternata isolate CVGCIPL**  
Based on the amplification of the ITS-rDNA region, which contains 5.8S rDNA, the selected primer pair (ITS 4 and ITS 5) provided a PCR product of approximately 566 bp (Figure 2a). The nucleotide sequence of the isolate CVGCIPL was verified as *A. alternata* through homologous analysis via BLAST analysis (Figure 2b). It was then submitted to GenBank and deposited under accession number MT415558. The sequence of isolate CVGCIPL was found to share 100% similarity with *A. alternata* found in the NCBI database.
Phylogenetic analysis of *A. alternata* isolate CVGCIPL

The evolutionary history was indicated via MP analysis, and the percentage of parsimonious trees, in which the associated taxa clustered together in the bootstrap test (1,000 replicates), is shown below the branches. The strict consensus tree was generated by summarising the most parsimonious tree that produced six clades. The phylogenetic analysis of the ITS-rDNA region showed that the location of *A. alternata* isolate CVGCIPL is nested within the second sub-clade of clad three, where it is clustered together with one isolate of *A. alternata* AF218791. The results of tree analysis of the ITS sequences conducted with homologous ITS-rDNA nucleotide sequences obtained from NCBI database, and high homology (bs = 100%) indicated to the highest degree of similarity and shared identity of our isolate *A. alternata* isolate CVGCIPL with *A. alternata* accession number AF218791 (Country; Antarctica, isolation source; ornithogenic soils in penguin colony areas, Windmill Islands) as presented in Figure 3. The analysis involved 36 nucleotide sequences; there were a total of 2405 positions in the final dataset. The evolutionary analysis was also conducted in MEGA version 6.6 (Tamura et al., 2013).

Figure 2. Amplification of ITS-rDNA region of *A. alternata* isolate CVGCIPL. (a) Lane M: 100 bp ladder (Promega, U.S.A); Lanes A, A.: PCR amplicon replicates of *A. alternata* isolate CVGCIPL. (b) The nucleotide sequence of the *A. alternata* isolate CVGCIPL.
Figure 3. Maximum Parsimony analysis of taxa. The evolutionary history was inferred using the Maximum Parsimony method. The consensus tree inferred from 10 most parsimonious trees is shown. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The consistency index is 0.996528 (0.900000), the retention index is 0.900000 (0.900000), and the composite index is 0.896875 (0.810000) for all sites and parsimony-informative sites (in parentheses). The percentage of parsimonious trees in which the associated taxa clustered together is shown below the branches. The MP tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm (pg. 126 in ref. [Nei and Kumar, 2000]) with search level 0 in which the initial trees were obtained by the random addition of sequences (100 replicates). The analysis involved 36 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2405 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.6 (Tamura et al, 2013).

CONCLUSION

On the basis of identification of endophytic A. alternata isolate CVGCIPL in current study which inclusive of ITS analysis through extracting the genomic DNA and shared a close relationship with A. alternata accession number AF218791. Therefore, A. alternata isolate CVGCIPL, which was isolated from dead green scale insect, can be used as a promising biocontrol agent against green scale insects in future work.

REFERENCES