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

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ABSTRACT

The current study isolated and identified *Alternaria alternata* isolate CVGCIPL 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 CVGCIPL as *A. alternata* isolate CVGCIPL. The newly registered sequence of *A. alternata* isolate CVGCIPL was submitted to the GenBank database MT415558.1 and deposited under accession number MT415558.

Keywords: Alternaria alternate, Green Scale, Insect, ITS-rDNA.

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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 *Clathrospora 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 appressoria 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. destruens* 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 *Zyginidia pullula* (Hemiptera: Cicadellidae) (Ozino, 1982). *A. alternata* exhibits the ability to control larvae of the blue cereal leaf beetle *Oulema melanopus* and *O. gallaeciana* (Coleoptera: Chrysomelidae) (Raizada, 1976). In Egypt, Shabana and Ragab (1997) isolated *A. infectoria*, which was considered as an entomopathogenic fungus, from eggs of fig wax scale insect *Ceroplastes rusci* (Homoptera: Coccidae). 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. alternat*a has the ability to infect many insects, including *Drosophila melanogaster* and *Ceratitis capitata*. The fungal mycelium of *A. alternat*a 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: Aphidoidea) (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 Gopkalo, 1980). *Alternaria* spp. stated as inhibiter 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 vigintioctopunctata* (Coleoptera: Coccinellidae) and beetles that attack other solanaceous plants (Sharma *et*

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 CVGCIPL from green scale insect.

MATERIALS AND METHODS

Isolation of *A. alternata* isolate CVGCIPL 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 CVGCIPL, 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 filter paper. Subsequently, dried pieces of green scale insect were placed onto potato dextrose agar (PDA) 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 CVGCIPL.

Isolation of fungal DNA from *A. alternata* isolate CVGCIPL

The fungal isolate CVGCIPL 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) in accordance with the method of Hamdia *et al*. (2016). The flasks were then incubated at 28 °C for 5 days in a rotary shaker at 150 rotations per min (rpm). Genomic DNA was isolated from *Alternaria* sp. isolate CVGCIPL via the Murray and Thompson (1980) method with some modifications was used for isolation of genomic DNA as reported in Hamdia *et al*. (2020). The dried pellet was suspended in 30 µL of deionised distilled water (ddH_2O) and left for 5 min at ambient temperature. Then, the dried pellet was centrifuged at 8000 rpm for 1 min and stored at −20 °C. The concentration of DNA was quantified spectrophotometrically using a NanoDrop spectrophotometer (Nanospec Cube, Germany) at $A_{260}/_{230}$ (absorbance at 260/230 nm) and analysed on 1% agarose gel. Moreover, 1 Kb DNA ladder (0.1 µg $µL^{-1}$, Promega Corporation, USA) was included into one of the wells for size determination and visual quantification.

Amplification of the rDNA of internal transcribed spacer regions

The presence of the ITS-rDNA region was detected in *Alternaria* sp. isolate CVGCIPL through PCR amplification with specific primer ITS4-Forward 5'TCCTCCGCTTATTGATATGC3' and ITS5-Reverse 5'GGAAGTAAAAGTCGTAACAAGG3' (White *et al*., 1990). PCR amplification conditions were based on the study of Hamdia *et al*. (2020). The quality and quantity of PCR products were determined by electrophoresis on 1.0% (w/v) agarose gels (Vivantis, USA) in 1× TAE buffer in accordance with Hamdia *et al*. (2020). The *Alternaria* sp. DNA band in the gel was visualised with a UV transilluminator and documented using a gel documentation system. PCR products were purified by using a PCR purification kit (Bioneer Company, Korea). Freshly purified DNA PCR product of *Alternaria* sp. isolate CVGCIPL was sent to Bioneer Corporation for sequencing.

Phylogenetic analysis of *A. alternata* isolate CVGCIPL

The ITS-rDNA consensus sequence obtained from cultures re-isolated from *Alternaria* sp. isolate CVGCIPL was subjected to 35 multiple alignment analysis downloaded from GenBank via the National Center for Biotechnology Information (NCBI) database. The DNA sequence was aligned through the ClustalW option available in BioEdit sequence alignment editor version 7. The evolutionary history of *Alternaria* sp. isolate CVGCIPL was inferred through maximum parsimony (MP), which indicates the minimal changes to produce data (MP) (Nei and Kumar 2000). Phylogenetic analyses were conducted on the ITS-rDNA sequences via MEGA version 6.6 (Tamura *et al*. 2013).

RESULTS

Isolation and identification of *A. alternata* isolate CVGCIPL

Fresh fungal culture of *Alternaria* sp. isolate CVGCIPL obtained from plate containing PDA medium was examined microscopically to identify the isolate CVGCIPL that belongs to the genus *Alternaria* (Figure 1a and b). The fungal mycelium of *Alternaria* sp. isolate CVGCIPL 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 CVGCIPL as *A. alternata* isolate CVGCIPL. Mycoparasitism by *Alternaria* sp. isolate CVGCIPL on green scale insect (Figure 1a) as displays the morphological characteristics of mycelium and conidia in (Figure 1b).

A. alternata isolate CVGCIPL 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 chitinous (polysaccharide molecules)) with some of wax (Bess 1958).

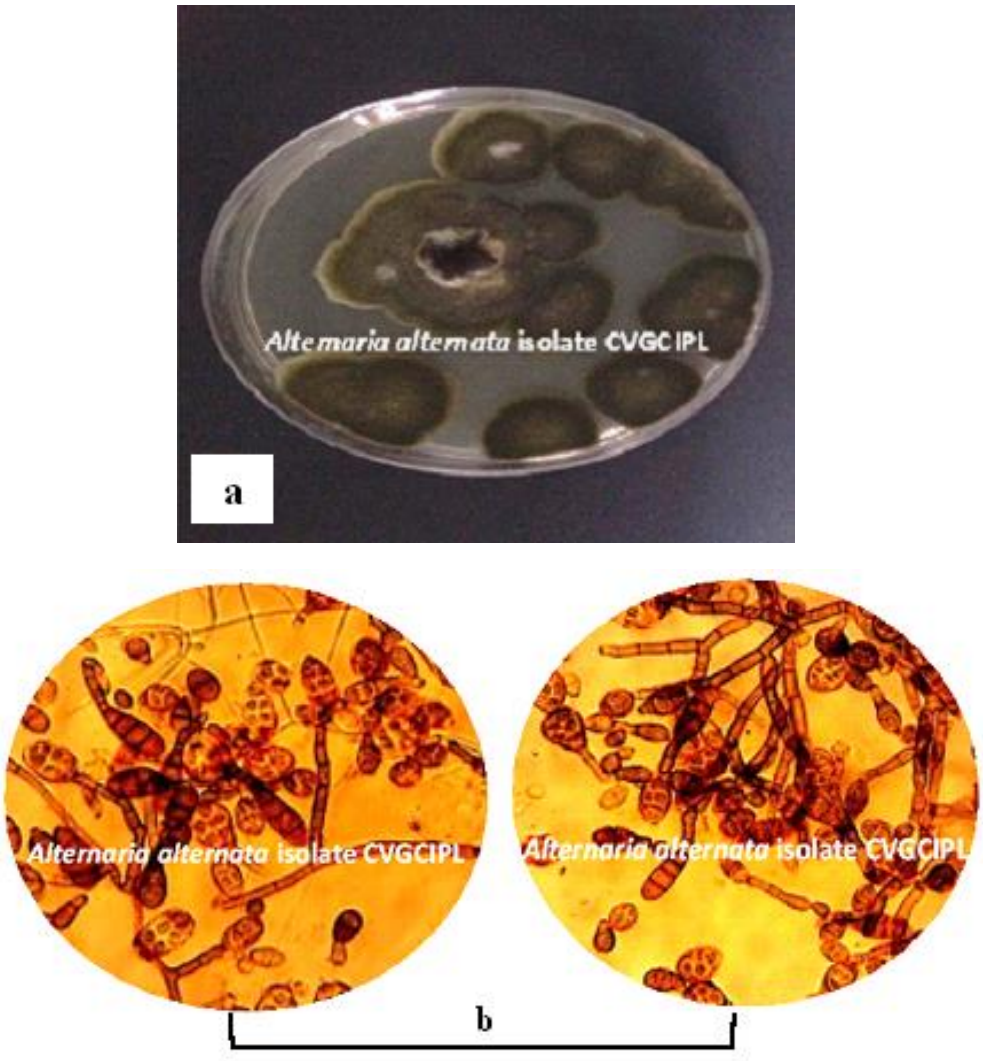
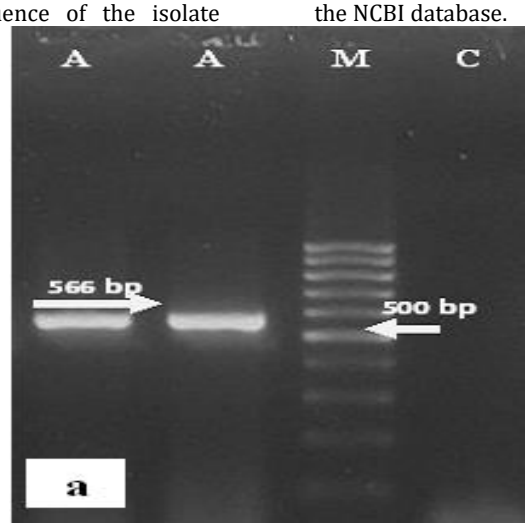


Figure 1. *A. alternata* isolate CVGCIPL under Microscope. (a) *A. alternata* isolate CVGCIPL; branches, hyphae and small conidia carry in more than one point (polyplastic). (b) *A. alternata* CVGCIPL spores; small conidia, ramoconidia.

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.



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  1 CAAATTAATA ATTACAACTT TCAACAACGG ATCTCTTGGT TCTGGCATCG ATGAAGAACG
 61 CAGCGAAATG CGATAAGTAG TGTGAATTGC AGAATTCAGT GAATCATCGA ATCTTTGAAC
121 GCACATTGCG CCCTTTGGTA TTCCAAAGGG CATGCCTGTT CGAGCGTCAT TTGTACCCTC
181 AAGCTTTGCT TGGTGTTGGG CGTCTTGTCT CTAGCTTTGC TGGAGACTCG CCTTAAAGTA
241 ATTGGCAGCC GGCCTACTGG TTTCGGAGCG CAGCACAAGT CGCACTCTCT ATCAGCAAAG
301 GTCTAG
```

b

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.

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 clade 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).

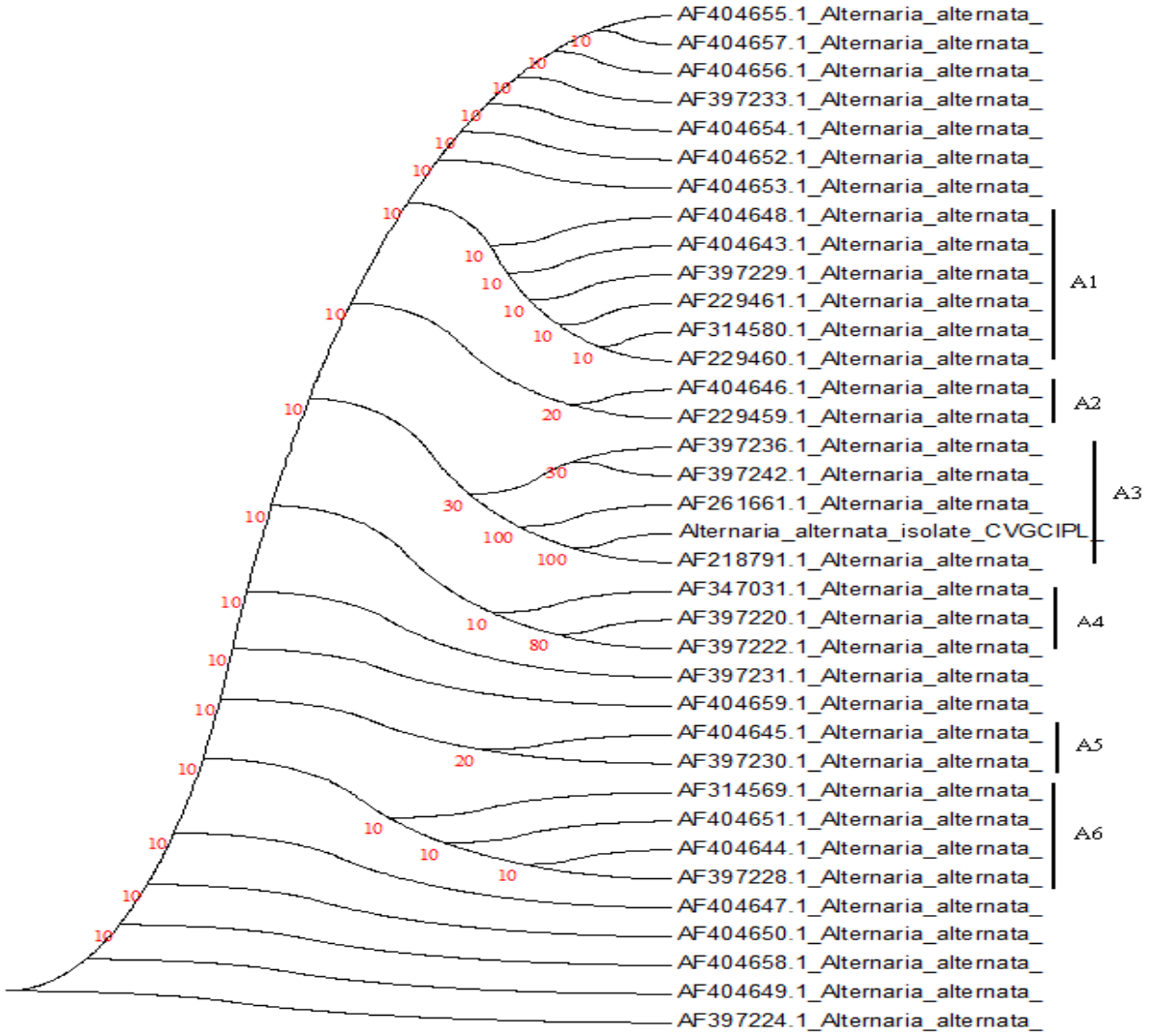

Figure 3. Maximum Parsimony analysis of taxa. The evolutionary history was inferred using the Maximum Parsimony method. The consensus tree inferred from 10 most parsimonious trees is shown. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The consistency index is 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.

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