Journal of Biological Control, 35(3): 181-186, 2021, DOI: 10.18311/jbc/2021/29506

Research Article

Natural occurrence of entomopathogenic fungus, *Aschersonia aleyrodis* on citrus whitefly, *Dialeurodes citri* (Ashmead) in Kinnow mandarin in Punjab, India

SANDEEP SINGH[1*], BONAM RAMANUJAM[2], RAJWINDER KAUR SANDHU[1], A. KANDAN[2], B. POORNESHA[2] and RUPA KUNDU[2]

[1]*Department of Fruit Science, Punjab Agricultural University, Ludhiana – 141004, Punjab, India*
[2]*ICAR-National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru – 560024, Karnataka, India*
**Corresponding author E-mail:* sandeep_pau.1974@pau.edu

ABSTRACT: Surveys were conducted during 2017 and 2018 in the citrus orchards of Punjab, India to record the incidence of different insect pests and their natural enemies. During October-December, Entomopathogenic Fungus (EPF), *Aschersonia aleyrodis* was found to infect nymphs and pupae of citrus whitefly, *Dialeurodes citri* on the lower leaf surface of Kinnow from the orchards of Hoshiarpur, Ludhiana, Mansa and Fazilka districts.The fungus was isolated from the infected nymphs and pupae and morphological studies were conducted to confirm the identity of the entomopathogenic fungus. *Aschersonia aleyrodis* was reported for the first time on *D. citri* under Punjab conditions and this EPF also confirmed by amplification and sequencing of beta tubulin gene showed 99.40 per cent identity in NCBI, GenBank. Hence further studies on the host range, interaction with other insect pests and parasitoids, survival and longevity should be conducted to explore the potential of this fungus as microbial biocontrol agent for citrus whitefly.

KEY WORDS: *Aschersonia aleyrodis*, biological control, citrus whitefly, entomopathogenic fungi, Punjab

(Article chronicle: Received: 04-03-2021; Revised: 23-08-2021; Accepted: 25-08-2021)

INTRODUCTION

The citrus fruits (Family Rutaceae) comprising of Mandarins (Kinnow), sweet oranges, lime, lemon and grapefruits are subtropical to tropical perennial fruit trees (Weisskopf and Fuller, 2014). Citrus is a major fruit crop of India occupying an area of 1003 thousand ha with a total production of 12546 thousand metric tons, out of which mandarins covered an area of 428 thousand ha with production of 5101 thousand metric tons (Anonymous, 2018). The citrus fruits are of major economic significance in Punjab with cultivation on an area of 57.29 thousand ha, production of 1281.63 thousand metric tons and productivity of 39.61 thousand kg/ha (Anonymous, 2019). Kinnow ranks first among citrus fruits by occupying an area of 53.05 thousand ha and production of 1246.82 thousand metric tons with productivity of 23.51 thousand kg/ha. A number of insect-pests attack this crop and about 45 insect-pests have been reported infesting different plants parts of citrus trees in Punjab (Singh *et al.,* 2016). The major insect-pests are citrus psylla (*Diaphorina citri*), citrus leaf miner (*Phyllocnistis citrella*), citrus whitefly (*Dialeurodes citri*), citrus blackfly (*Aleurocanthus woglumi*), aphids (*Toxoptera aurantii, Aphis gossypii and Myzus persicae*), mites (*Eutetranychus orientalis*), leaf folder (*Psorosticha zizyphi*), bark-eating caterpillar (*Indarbela quadrinotata*), citrus thrips (*Scirtothrips citri*), mealybugs (Four species, *Planococcus citri, Planococcus lilacinus, Nipaecoccus viridis* and *Maconellicoccus hirsutus)* and fruit flies (*Bactrocera dorsalis and Bactrocera zonata*) (Singh *et al.* 2016; Anonymous, 2019). Among these, citrus whitefly, *D. citri* is a major insect-pest causing serious damage to this crop by sucking sap from phloem tissues (Flint, 2015). Both nymphs and adults suck sap from plant tissues and reduce the vigour of plant. Severely infested plants turn yellowish green and sooty mould develops on the honeydew excreted by whitefly giving black appearance to the foliage. This black fungus may cover the foliage completely, thus interfering with the physiological properties of the plant (Fasulo and Weems, 2017).

The use of insecticides to control whitefly is both expensive as well as lead to resistance development in the

pest populations and results in decimation of natural enemies. Non-chemical approaches for pest management should be looked for to minimise the harmful effects of chemicals on environment. Biological control of insect pests using Entomopathogenic Fungi (EPF) has the potential to manage insect pests in a non-pesticidal way. EPFs have been tested as microbial control agents and their ability to infect sucking insect-pests by penetration of the cuticle, unlike virus and bacteria, make them a suitable pathogen of whitefly (Fransen, 1990).

Entomopathogenic fungi belonging to genus *Aschersonia* have been reported to be pathogenic to whiteflies and scale insects (Meekes *et al.,* 2002; Qiu *et al.,* 2013). *Aschersonia aleyrodis* is a promising fungal species against whiteflies and is considered as a prospective fungus for whitefly management (Meekes *et al.,* 2000). It has wide tolerance to relative humidity, longer persistence on the leaf surface, compatible with other natural enemies and has the ability to infect sap sucking insects by direct or secondary contact (Fransen and Lenteren, 1994; Qiu *et al.,* 2013). In this paper, we report the natural occurrence of *Aschersonia aleyrodis,* the biological control of citrus whitefly, *D. citri*, by an EPF, *A. aleyrodis* from Punjab, India.

MATERIALS AND METHODS

Roving and fixed plot surveys were carried out during 2017 and 2018 in the citrus groves of the Punjab, India to record the biodiversity of emerging insect pests and their biological control agents. Twenty trees from each of the orchards were surveyed randomly to record the presence or absence of insect-pests and their natural control. During surveys in October-December, some orange coloured fungal growth was observed on the leaves of Kinnow plants in the orchards from Districts Hoshiarpur, Ludhiana, Mansa and Fazilka of Punjab. Studies were taken up to identify the fungal growth on the citrus whitefly, *D. citri*, nymphs and pupae completely. Leaf specimens having fungal growth on nymphs and pupae were collected and sent to ICAR-National Bureau of Agricultural Insect Resources, Bengaluru, India for identification.

Isolation and morphological identification of *Aschersonia aleyrodis*

Infected nymphs and pupae collected from the leaves of Kinnow mandarin leaves were surface sterilized using 4% Sodium hypochlorite and later rinsed with sterile water three times. The insect bits were plated on Sabouraud's Dextrose Yeast extract broth (SDYA) medium (Dextrose 20 g, Mycological peptone 10 g, yeast extract 5 g, in 1 L distilled water) and the plates were incubated at 26 ± 1^0C for one week. The fungal growth observed on the insect bit was purified and the pure culture was observed for the colony morphology and microscopic characteristics.

Molecular based identification of entomopathogenic fungi

DNA was extracted from 50-100 g of lyophilized mycelium of the fungus from 5-7 day old cultures grown in Potato Dextrose Broth, following CTAB (Rogers and Bendich, 1985). Extracted DNA was suspended in EB buffer (10 mM Tris–HCl, pH 8.5) and stored at -20°C until used. Total DNA concentration was measured in spectrophotometer at 280 nm. PCR amplification of the beta tubulin gene FTUB1: 5'-AACAACTATGCCCGTGGYCACTAC-3' and RTUB1: 5'- CCACCGAAGGAGTGGAAGA-3' same as those used in Bischoff et al. (2009).

Each PCR reaction consisted of 25 µL reaction mixture containing 1X Taq buffer, 0.4-0.6 mM of each primer, 0.2 mM of dNTP mix, 1U of Taq DNA polymerase (GeNei) and 20-50 ng/µl template DNA. The PCR amplification had one initial cycle at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min. The PCR products were separated on 1% agarose gel containing 0.05% of EtBr in 0.5x TBE buffer and visualized in a gel documentation system. The PCR products were sequenced in an ABI 3130XL genetic analyser at Eurofins, Bangalore.

Sequence analysis

Beta tubulin sequences in FASTA format were imported into the sequence alignment application of MEGA X (Kumar *et al*. 2018) software package and multiple sequence alignments were performed with the ClustalX2 algorithm using default parameters. The Basic Local Alignment Search Tool (BLAST) (*Altschul et al*. 1990) was used to query the National Center for Biotechnology Information (NCBI) non-redundant nucleotide database with *A. aleyrodis* sequence data. The sequence details were analyzed carefully and base substitution mutations were identified manually. The sequences were submitted to NCBI for GenBank Accessions (Table 1). Sequence divergences between individual *A. aleyrodis* and *A. placenta* were calculated using the Kimura 2-Parameter distance model (Kimura 1980) and graphically displayed in a Neighbor-joining (NJ) tree (Saitou and Nei 1987) by the program MEGA X (Kumar *et al*. 2018). Tree robustness was evaluated by bootstrapping (Felsenstein 1985) with 2,000 replicates with the outliers *Beauveria bassiana* (EU604134.1) beta tubulin sequences.

RESULTS AND DISCUSSION

During surveys, orange coloured fungal growth was observed on the leaves of Kinnow plants in the orchards from

Table 1. Microbial cultures beta tubulin sequences used for the phylogenetic studies

Sl. No.	Microbial culture	Strain Name	Accession No.
1.	*Achersonia aleyrodis*	Aa1	MW894659.1
2.	*Achersonia aleyrodis*	PC578	DQ070052.1
3.	*Achersonia aleyrodis*	CR19	DQ070046.1
4.	*Achersonia aleyrodis*	ARSEF7394	DQ070049.1
5.	*Achersonia aleyrodis*	MCA2465	DQ070053.1
6.	*Achersonia aleyrodis*	PC553	DQ070051.1
7.	*Achersonia aleyrodis*	ARSEF7393	DQ070048.1
8.	*Achersonia aleyrodis*	PC434	DQ070040.1
9.	*Achersonia aleyrodis*	PC413_1	DQ070039.1
10.	*Achersonia aleyrodis*	ARSEF7339	DQ070037.1
11.	*Achersonia aleyrodis*	ARSEF7344	DQ070036.1
12.	*Achersonia aleyrodis*	ML175_4	DQ070050.1
13.	*Achersonia aleyrodis*	CR08	DQ070045.1
14.	*Achersonia placenta*	CBS34984	DQ070060.1
15.	*Achersonia placenta*	AFR28	DQ070067.1
16.	*Beauveria bassiana*	MRCIF40	EU604134.1

Fig. 1. Fungal growth of *Achersonia aleyrodis* on leaf surface

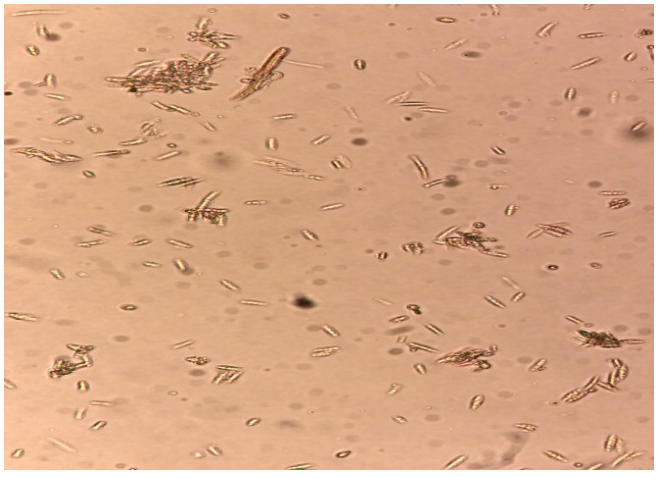

Fig. 2. Conidia of *Achersonia aleyrodis*

Districts Hoshiarpur, Ludhiana, Mansa and Fazilka of Punjab. This fungal growth on the citrus whitefly, *D. citri*, nymphs and pupae was identified as an EPF, *Aschersonia aleyrodis*. The growth of this fungus on the SDYA medium was fast with filamentous hyphae, white to yellowish white, with a peripheral circle. The colour of the conidial mass varied from light yellowish orange to reddish orange (Figure 1). Anamorphic stroma was observed as thin pulvinate structure along with hypothallus. Conidiomata 5-15 per stroma, conidiogenous cells arise singly or in whorls not branched, cylindrical, slightly tapering, truncate at apices. Conidial masses reddish orange, orange or light yellow, thickened in centre. Conidia fusiform unicellular, hyaline, guttulate, ends acute, 10–12x1.5–2.0 µm (Figure 2). Paraphyses abundant in the hymenium, hyaline and filiform. (Liu *et al.,* 2006; Wang *et al.,* 2013).

Achersonia aleyrodis infects the whitefly nymphs by attaching its conidia to the cuticle of the insect. Germ tube formation takes place and it penetrates inside the body of insect through cuticle. Appresorium formation also helps in the penetration of fungus. Then hyphal bodies are formed and infect all the tissues of the insect body. Haemocoel gets filled with the mycelial mass of the EPF and insect dies at this stage. Later under humid environment, fungus will protrude and yellowish-orange mycelium becomes visible on the insect's dead cadaver. Later, this fungal growth completely covers the insect body and conidial formation takes place. These conidia further get disseminated and infect other insects (Fransen and Lenteren, 1993).

Achersonia aleyrodis is also pathogenic to all the

larval stages of other whiteflies, e.g. greenhouse whitefly, *Trialeurodes vaporariorum* Westwood and *Bemisia tabaci* (Gennadius) (Osborne and Landa, 1992). Although eggs are not susceptible to this fungus but the first instar larvae hatched from them become infected by conidia surviving on the leaf surface (Meekes *et al.,* 1994). This EPF has been introduced into citrus plantations in Florida in the early 1900's to control *D. citri* and *D. citrifolii* in citrus (Lacey *et al.,* 2003). Also, in the Azerbadijan region, several strains of *A. aleyrodis* were introduced from China, India, Japan, Vietnam, USA and Cuba between 1958-1973, for the control of citrus whitefly (McCoy *et al.,* 1988). *Achersonia aleyrodis* is also compatible with parasitoid, *Encarsia formosa* Gahan and fungus does not infect whitefly larva if it is already parasitized by *E. formosa* (Fransen and Lenteren, 1993). *Achersonia aleyrodis* has been found very effective in controlling *D. citri* in the citrus orchards of Fujian, China (Gao *et al.,* 1985). High infection levels of *A. aleyrodis* were reported on silverleaf whitefly, *Bemisia argentifolii* and *T. vaporariorum* (Lacey *et al.,* 1996). This fungus was found to be naturally controlling the population of scale insect, *Saisettia* spp. on tea in North East India (Debnath, 2016). Zhang *et al* (2017) reported the high pathogenicity of *A. aleyrodis* against second and third instars and pupae of *B. tabaci* under laboratory and greenhouse conditions. Another species, *A. placenta* has been reported to be pathogenic to citrus whitefly, *D. citri* in China (Wang *et al.,* 2013). *Achersonia aleyrodis* was found to be highly pathogenic to mulberry aphid and citrus whitefly in Jorhat, India (Dutta *et al.,* 2013). *Achersonia placenta* was recently reported infecting citrus whitefly in Bali, Indonesia (Sudiarta *et al.,* 2019).

Sequence variation and genetic diversity of *A. aleyrodis*

Aschersonia aleyrodis strain Aa1 was collected from leaves of Kinnow plants in the orchards from Districts Hoshiarpur, Ludhiana, Mansa and Fazilka of Punjab. The beta tubulin gene was amplified and sequenced using the universal barcoding primer and used to generate the 663-bp product. Individual sequence was trimmed and deposited in NCBI, USA (Accession No. MW894659.1). The annotation of 13 *A. aleyrodis*, 2 *A. placenta* and one *B. bassiana* beta tubulin sequences showed 610 average identical pairs, out of which 14 transitionsal pairs, 6 transversional pairs and R value is 2.3 among the *A. aleyrodis* and *A. placenta* beta tubulin sequences analyzed (Table 1). Among the 13 *A. aleyrodis* and 2 *A. placenta* sequences, parsimony informative 43 sites and singleton 17 sites were noted.

Phylogenetic analysis

The Neighbour-joining tree (NJ tree) was constructed based on 16 sequences including one beta tubulin sequence of *A. aleyrodis* used in this study and 12 sequences of *A. aleyrodis*, 2 sequences of *A. placenta* and one *B. bassiana* from NCBI, Genbank using MEGA X. Based on a strict consensus NJ tree, two major clades for *Aschersonia* spp. in which one for *A. aleyrodis* and another clade for *A. placenta* (DQ070060.1 and DQ070067.1). *B. bassiana* (EU604134.1) acted as an outgroup. The optimal tree with the sum of branch length = 0.295199. Among the one major clade, the *A. aleyrodis* (MW894659.1) used in this study is closely related to *A. aleyrodis* (DQ070052.1).

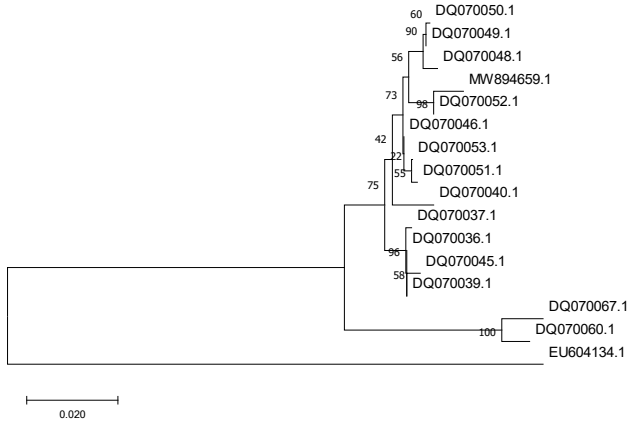

Fig. 3. Tree topology based on beta tubulin sequences

The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. This analysis involved 16 nucleotide sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 717 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

The entomopathogenic fungus, *A. aleyrodis* has the potential to control citrus whitefly, *D. citri* population. Although good results of control of whitefly has been observed, but still, detailed studies on relationship between host plants, insect-pest and fungal performance are needed. Different strains of this fungus from different locations should be screened on their pathogenicity towards *D. citri*. The exploitation of EPFs for insect pest control may result in long lasting, stable ecosystem as they will exert steady control of pest population. This EPF, *A. aleyrodis* has not been recorded on citrus whitefly, *D. citri* in Punjab, India and this is the first report of infection of *A. aleyrodis* on citrus whitefly, *D. citri* in Punjab.

ACKNOWLEDGEMENT

The authors are thankful to the Head, Department of Fruit Science, Punjab Agricultural University, Ludhiana, Project Coordinator, ICAR-AICRP on Fruits, Bengaluru and Director, ICAR-National Bureau for Agricultural Insect Resources, Bengaluru for providing funds and facilities to carry out this study.

REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol*. **215**: 403–41. https://doi.org/10.1016/S0022-2836(05)80360-2

Anonymous. 2018. Horticultural statistics at a glance 2018. Government of India. 490 pp.

Anonymous. 2019. Package of Practices for Cultivation of Fruits. Punjab Agricultural University, Ludhiana, 187 pp.

Butt TM. 1990. Fungal infection processes - A mini review. Proceedings and abstracts, 5th International Colloquium on Invertebrate Pathology and Microbial Control, Adelaide, Australia, pp. 121–4.

Bischoff JF, Rehner SA, Humber RA. 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia.* **101**: 512–30. PMid: 19623931. https://doi.org/10.3852/07-202

Clustal W and Clustal X version 2.0. *Bioinform.* **23**: 2947–8. PMid: 17846036. https://doi.org/10.1093/bioinformatics/btm404

Debnath S. 2016. Studies on entomopathogenic fungus *Aschersonia aleyrodis* infectious to scale insect pest, *Saisettia formicarii* of tea, *Camellia sinensis* (L.). O. Kuntze in Assam. *Indian J Fundamental Appl Life Sci.* **6**(3): 14–24.

Dutta P, Pegu J, Puzari KC. 2013. Current status and future prospects of entomopathogenic fungi in North East India. *KAVAKA.* **41**: 75–86.

Fasulo TR, Weems HV. 2017. Citrus whitefly, Dialeurodes citri (Ashmead) (Insects: Hemiptera: Aleyrodidae). http://entnemdept.ufl.edu/creatures/citrus/citrus_whitefly.htm

Flint ML. 2015. Whiteflies Integrated Pest Management for Homes, Gardens and Landscape. PEST NOTES Publication 7401.

Fransen JJ. 1990. Natural enemies of whiteflies: Fungi. In: Gerling D, ed. Whiteflies: Their Bionomics, Pest status and Management. Intercept Ltd, Andover, UK, pp. 187–210.

Fransen JJ, Lenteren JC. 1993. Host selection and survival of the parasitoid *Encarsia formosa* on greenhouse whitefly, *Trialeurodes vaporariorum*, in the presence of hosts infected with the fungus *Aschersonia aleyrodis. Entomol. Exp. Appl.* **69**: 239–49. https://doi.org/10.1111/j.1570-7458.1993.tb01747.x

Fransen JJ, Lenteren JC. 1994. Survival of the parasitoid *Encarsia formosa* after treatment of parasitized greenhouse whitefly larvae with fungal spores of *Aschersonia aleyrodis. Entomol. Exp. Appl.* **71**: 235–43. https://doi.org/10.1111/j.1570-7458.1994.tb01790.x

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution.* **39**: 783–91. PMid: 28561359. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Gao RX, Ouyang Z, Gao ZX, Zheng JX. 1985. A preliminary report on citrus whitefly control with *Aschersonia aleyrodis. J Fujian Agric College*. **14**(2): 127–33.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* **35**: 1547–9. PMid: 29722887 PMCid: PMC5967553. https://doi.org/10.1093/molbev/msy096

Lacey LA, Fransen JJ, Carruthers R. 1996. Global distribution of naturally occurring fungi ofBemisia, their biologies and use as biological control agents. Gerling D, Mayer R, eds. Bemisia: Taxonomy, Biology, Damage and Management. Intercept Ltd, Andover, UK. pp. 401–33.

Lacey LA, Wraight S, Kirls A. 2003. Entomopathogenic fungi for control of *Bemisia* spp.: Foreign exploration and implementation. USDA Miscellaneous publication. pp. 1343.

Liu M, Chaverri P, Hodge KT. 2006. A taxonomic revision of the insect biocontrol fungus *Aschersonia aleyrodis,* its allies with white stromata and their hypocrella sexual states. *Myco Res.* **110**: 537–54. PMid: 16769508. https://doi.org/10.1016/j.mycres.2006.01.013

McCoy CW, Samson RA, Boucias, DG. 1988. Entomogenous fungi. Ignoffo CM, Mandava NB, eds. Entomogenous protozoa and fungi. pp. 151–236. https://doi.org/10.1201/9780429487187-2

Meekes ETM, Fransen JJ, Lenteren JC. 1994. The use of entomopathogenic fungi for the control of whiteflies. Mededelingen van de Faculteit der Landbouwwetenschappen Rijksuniversiteit Gent. **59**(2a): 371–7.

Meekes ETM, Fransen JJ, Lenteren JC. 2002. Pathogenicity of Aschersonia spp. against whiteflies *Bemisia argentifolii* and *Trialeurodes vaporariorum. J Invertebrate Path*. **81**(1): 1–11. https://doi.org/10.1016/S0022-2011(02)00150-7

Meekes ETM, Voorst S, Joosten NN, Fransen JJ, Lenteren JC. 2000. Persistence of the fungal whitefly pathogen, *Aschersonia aleyrodis*, on three different plant species. *Myco Res.* **104**(10): 1234–40. https://doi.org/10.1017/S0953756200002513

Osborne LS, Landa Z. 1992. Biological control of whiteflies with entomopathogenic fungi. *The Flo Entomol*. **75**(4): 456–71. https://doi.org/10.2307/3496127

Qiu J, Song F, Mao L, Tu J, Guan X. 2013. Time-dose-mortality data and modeling for the entomopathogenic fungus *Aschersonia placenta* against the whitefly *Bemisia tabaci. Canadian J Micro*. **59**(2): 97–101. PMid: 23461516. https://doi.org/10.1139/cjm-2012-0569

Saitou N, Nei M. 1987. The Neighbor-joining method: A new method for reconstructing phylogenetic *trees. Mol. Biol. Evol.* **4**: 406–25.

Sudiarta IP, Suputra IPW, Wirya GNAS. 2019. New report of insect pathogenic fungi (*Aschersonia* sp.) of citrus whitefly (*Dialeurodes* sp.) in Bali Indonesia. *Agric Vet Sci*. **3**(1): 22–7.

Wang P, Song X, Zhang H. 2013. Isolation and characterization of *Aschersonia placenta* from citrus orchards and its pathogenicity towards *Dialeurodes citri* (Ashmead). *J Invertebrate Path*. **112**: 122–8. PMid: 23160084. https://doi.org/10.1016/j.jip.2012.10.005

Weisskopf A, Fuller DQ. 2014. Citrus Fruits: Origins and Development. Smith C, ed. Encyclopedia of Global Archaeology. New York: Springer; https://doi.org/10.1007/978-1-4419-0465-2_2173

Zhang C, Ali S, Musa PD, Wang XM, Qiu BL. 2017. Evaluation of the pathogenicity of *Aschersonia aleyrodis* on *Bemisia tabaci* in the laboratory and greenhouse. *Biocontrol Sci Tech.* **27**(2): 210–21. https://doi.org/10.1080/09583157.2016.1274878