

Natural Pathogenic Fungi Infection Attributes of Malarial Vectors *Anopheles Maculipennis* S. L. and *Anopheles Superpictus* S. L. in Central Iran

Seyed Hassan Moosa Kazemi (✉ moosakazemi@tums.ac.ir)

Tehran University of Medical Sciences <https://orcid.org/0000-0001-8282-3630>

Tahereh Sadat Asgarian

Tehran University of Medical Sciences

Mohammad Mehdi Sedaghat

Tehran University of public Health

Saeedeh Javar

Tehran University: University of Tehran

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Abstract

Background: Due to the effect of synthetic and commercial insecticides on non-target organisms and the resistance of mosquitoes, in recent years non-chemical and environmentally friendly methods have become prevalent. In this research, we aimed to isolate entomopathogenic fungi with toxic effects on mosquitoes in natural larval habitats.

Methods: Larval mosquitoes were collected from Central, Qamsar, Niasar and Barzok Districts in Kashan County, Central Iran by standard dipping method, from April to late December 2019. Dead larvae and larvae with signs of infection to fungal mycelium detectable on the outer surface of its body were isolated from the rest of the larvae and were sterilized with 10% sodium hypochlorite for two minutes, then washed twice with distilled water and transferred to PDA (potato-dextrose-agar) and WA (water-agar) media and incubated at a temperature of $25 \pm 2^\circ \text{C}$ for 3 - 4 days. Larvae and fungi were identified morphologically based on identification keys.

Results: A total of 9789 larvae were collected from urban and rural areas in Kashan County. The genera i.e. *Anopheles* (7.89%), *Culiseta* (17.42%), and *Culex* (74.69%) including 13 species were identified. A total of 105 larvae including *Anopheles superpictus* s. l., *An. maculipennis* s. l., *Culex deserticola*, *Cx. perexiguus*, and *Culiseta longiareolata* were found to be infected by *Nattractia mangiferae*, *Aspergillus niger*, *A. fumigatus*, *Trichoderma* spp. and *Penicillium* spp. Fungi. *Penicillium* spp. was the most abundant fungus isolated and identified from the larval habitats while *An. superpictus* s.l. was the most infected mosquito species.

Conclusions: Based on the observations and results obtained from our study, isolated fungi had the potential for pathogenicity on mosquito larvae. Therefore, it is suggested that their effects on mosquito larvae be investigated in the laboratory. The most important point, however, is the proper way of exploiting these biocontrol agents to maximize their effect on reducing the population of vector mosquito larvae without any negative effect on non-target organisms.

Background

Transmission of malaria, filariasis, Japanese encephalitis, dengue fever, and other arbovirus diseases by mosquitoes has turned mosquitoes into the most important group of arthropods in medicine and health [1]. In Iran, mosquitoes are vectors of two protozoal, two bacterial, four filarial, and seven arboviral diseases [2, 3]. There are 70 species and 8 (or 12) genera of Iranian mosquitoes depending on the classification of the tribe Aedini [4]. *Anopheles* mosquito species are responsible for the transmission of malaria, but the majority of mosquito species from the genera of *Culex* and *Aedes* are responsible for the transmission of arboviruses to humans [5]. *Aedes aegypti*, a highly anthropophilic species, *Ae. albopictus* the highly invasive mosquito and *Culex* species are important targets for the prevention of arboviruses including dengue virus, chikungunya virus, yellow fever virus, Zika virus, Japanese encephalitis virus and West Nile virus [5, 6].

Globally, there were an estimated 229 million malaria cases in 2019 in 87 malaria endemic countries. The disease is a major endemic infectious disease in Iran, especially in the south and southeastern provinces including; the Sistan-Baluchestan, Hormozgan, and Kerman [7–12]. Most of the cases in the central counties of Iran were imported from other countries (90.4%), mainly from Afghanistan (56.5%) and Pakistan (16.3%). *Plasmodium vivax* was the causative agent of 93.75% of cases, followed by *P. falciparum* (6.25%). Above-15-year-old group contained the most malaria reported cases (66.7%) [13].

Anopheles species is responsible for the transmission of malaria. So far, seven malaria vectors have been recognized and reported in Iran, including: *Anopheles stephensi*, *An. culicifacies*, *An. dthali*, *An. fluviatilis*, *An. superpictus*, *An. maculipennis* and *An. sacharovi* [14]. The first five of these vectors can be found in the southeast of the country, together with the majority of malaria cases. Also, *An. pulcherrimus* has been considered as a potential malaria vector in this area based on immunological parasite detection (two-site immunoradiometric assay (IRMA)) [15].

Anopheles maculipennis s.l. distributed in Eurasia and North America and comprises nine Palearctic members [16, 17]. Some research indicated the occurrence of this malaria vector in central, the Caspian coast in the north, and North West of Iran [18–20].

Anopheles superpictus s.l. is distributed in Europe, Asia, and North Africa [21–25]. This species is one of the seven species of malaria vectors and reported in the Iranian Plateau, the slopes of the Alborz Mountains and southern Zagros, as well as the coastal plains of the Caspian Sea and the Persian Gulf in both malaria- endemic and non-endemic areas [8, 22]. Oshaghi et al in 2008 reported three genotypes X, Y, and Z in Iran. Interestingly, while the sympatric Y and Z genotypes appear to be exclusive to the populations from the southeastern part of the country, genotype X is geographically separated, and present in the North, the West, the South and the Central territories [23].

A malaria eradication programme was initiated in Iran in 1951 and changed to malaria control in 1985 due to the challenges and restrictions [14]. Iran has been in an elimination stage since 2010. In 2017, the total number of recorded cases was 89, and incidence cases decreased from 0.01/1000 cases in 2017 to zero in 2019 [14, 26].

The goal of all control methods is to reduce the size of vector populations. There is a risk of insecticide resistance and off-target effects on other arthropod species in chemical control [27]. Biological control is biodegradable and ecologically friendly [28]. Entomopathogenic fungi were first used on *An. gambiae* with a fungus from the genus of *Coelomomyces* [29]. Azari- Hamidian and Abaei reported *Coelomomyces* sp. from the larvae of *An. culicifacies* s.l. in Sistan and Baluchestan Province, Southeast Iran, where 5.8% of larvae were infected with the fungus [30]. The use of pathogenic insect fungi against mosquito larvae has been reported in many studies and fungi is proven to be an effective way of killing mosquito larvae. Unlike other biological control agents, pathogenic insect fungi can infect mosquitoes directly by penetrating the cuticle [31]. Some insect pathogenic fungi have been used effectively in recent years to control vector mosquitoes and have a wide range of species diversity. This group of pathogens is found among all phyla of fungi. The Ascomycota is the largest group of fungi. This group is extremely

ecologically diverse, just like the pathogenesis pathogen of plants, animals, and humans. Pathogenic insect ascomycetes include a large group of fungi that attack a wide range of insects and are the most common insect pathogens [32]. The entomopathogenic ascomycete fungi including *Metarhizium anisopliae*, and *Beauveria bassiana* have been reported as insecticides [33]. The use of *B. bassiana* for control of *Ae. aegypti* [34] and *Lagenidium giganteum* in California targeted to control *Cx. tarsalis* [35] reduced the survival rate, blood-feeding, fecundity, and disease transmission power of targeted mosquitoes. Two species, *Metarhizium anisopliae*, and *M. brunneum*, are pathogenic to a wide range of mosquitoes in the genera of *Aedes*, and *Culex* [36, 37]. In many studies, spores and secondary metabolites of insect pathogenic fungi have been reported as biocontrol agents against mosquitoes [38–41]. The fungal hyphae produces endotoxins and penetrates through the larval. These toxins cause larval damage and toxicity in the hemocoel and larval mosquito guts [42, 43]. Metabolites of *B. bassiana* caused changes in the body and tissues of treated *Cx. pipiens* larvae, especially in the cuticle and midgut [44].

This study aimed to isolate and identify entomopathogenic fungi associated with mosquito larvae in Kashan County, Central Iran, and their infection, effects on mosquito larvae, and the investigation of new ways of biological control for disease vector mosquitoes.

Methods

Study area

Kashan County is located in central Iran, the north of Isfahan Province. This county has four districts, including Central (51°24'43.2"E, 34°00'16.0"N), Qamsar (51°27'45.8"E, 33°45'30.5"N), Niasar (51°08'47.6"E, 33°58'39.3"N) and Barzak (51°13'44"E, 33°47'32"N) Districts. The climate of the county varies depending on ups and downs. The uplands are cold, foothills are temperate and plains, especially on the margins of the desert are tropical.

A total of 23 larval habitats were selected in Central, Qamsar, Niasar, and Barzok Districts. These larval habitats were natural or artificial, permanent or temporary, with or without vegetation, sunlight or shaded and clear or stagnant water (Fig. 1).

Larval sampling

Using a standard 350 ml capacity mosquito dipper, larvae and pupae of the mosquitoes were collected from April to late December 2019. Twenty dips were taken in each larval habitat in the morning (08:00–12:00 h) or afternoon (15:00–18:00 h). For sampling larvae from small water bodies, we used an eyedropper. Larvae were observed under a stereomicroscope. Dead larvae showing signs of infection and larvae and pupae with a white coating of fungal mycelium on the outer surface of their bodies were isolated from the rest of the larvae. Larvae of mosquitoes were identified based on a Culicidae identification key of Iran at the species level [45].

Isolation and diagnosis of fungi associated with mosquito larvae

A white coating of fungal mycelium is observed on the surface of some of the larvae and pupae (Fig. 2). These larvae and pupae were removed from the water of the larval habitat at the laboratory and were sterilized with 10% sodium hypochlorite for two minutes (to remove surface contaminants that conflict with the main pathogen), then washed twice with distilled water and the remaining water was removed and passed through the filter paper sterilizer [46]. They were then transferred to PDA (potato-dextrose-agar) and WA (water-agar) media. Parts of the body of some other larvae were degraded or broken, or the outer epithelial layer of the larva were cut and collapsed. Consequently, the outer surface of the larvae was wrinkled. Obviously, these larvae were sick (Fig. 3). Therefore, to determine the possibility of fungal infection, they were also transferred to PDA and WA media after surface disinfection, then incubated the Petri dishes in the incubator at a temperature of $25 \pm 2^\circ \text{C}$ for 3–4 days. Fungi were identified based on phenotypic characteristics and characteristics of the culture medium, such as shape and color of the fungus colony, filament growth pattern as well as microscopic properties such as shape, size, and color of spores, mycelium, and conidiophore structure [47].

Results

Larval sampling results

A total of 9789 larvae were collected from urban and rural areas of Central, Qamsar, Niasar, and Barzak Districts in Kashan County and were identified based on a valid diagnostic key and at the species level. Three genera, i.e. *Anopheles* (7.89%), *Culiseta* (17.42%), and *Culex* (74.69%), consist of 13 species were identified (Table 1). Some mosquito specimens were deposited in the museum of Medical Entomology, Tehran University of Medical Sciences (TUMS).

Table 1
Mosquito larvae identified in Kashan County, central Iran, 2019.

Districts	Mosquito larvae Identified
Central	<i>An. superpictus</i> s. l. (159), <i>Cs. longiareolata</i> (566), <i>Cx. theileri</i> (1028), <i>Cx. deserticola</i> (175), <i>Cx. hortensis</i> (150), <i>Cx. mimeticus</i> (15), <i>Cx. perexiguus</i> (205), <i>Cx. pipiens</i> (2651)
Qamsar	<i>An. maculipennis</i> s. l. (54), <i>An. superpictus</i> s. l. (453), <i>Cs. longiareolata</i> (355), <i>Cx. pipiens</i> (733), <i>Cx. theileri</i> (1233), <i>Cx. deserticola</i> (105), <i>Cx. hortensis</i> (31), <i>Cx. mimeticus</i> (39), <i>Cx. perexiguus</i> (29)
Niasar	<i>An. maculipennis</i> s. l. (15), <i>An. claviger</i> (1), <i>Cs. annulata</i> (43), <i>Cs. subochrea</i> (2), <i>Cs. longiareolata</i> (460), <i>Cx. deserticola</i> (68), <i>Cx. hortensis</i> (40), <i>Cx. pipiens</i> (37), <i>Cx. theileri</i> (176), <i>Cx. perexiguus</i> (71)
Barzok	<i>An. maculipennis</i> s. l. (22), <i>An. superpictus</i> s. l. (57), <i>An. turkhudi</i> (1), <i>Cs. longiareolata</i> (280), <i>Cx. deserticola</i> (92), <i>Cx. hortensis</i> (33), <i>Cx. pipiens</i> (237), <i>Cx. mimeticus</i> (14), <i>Cx. theileri</i> (118), <i>Cx. perexiguus</i> (31)

Fungi associated with mosquito larvae

Five species of fungi were isolated from mosquito larvae (Table 2). These fungi were isolated from larvae and pupae obtained from natural larval habitats in Qamsar and Barzak Districts. Five out of 13 mosquito species were found to be infected by fungi. A total of 105 larvae were infected with morphological or behavioral changes and fungal mycelium were observed in all infected larvae. *Natrassia mangiferae* was only isolated from *An. superpictus* s. l. larvae or pupae in Qamsar District in August. The white hyphae of *A. niger*, *A. fumigatus* and *Trichoderma* spp. had grown on the surface of the larvae, and penetrated the body. *Penicillium* spp. was identified from larvae whose parts of their bodies were wrinkled, degenerated, or broken (Figs. 4, 5). In this study, *Penicillium* spp. was isolated from 57 mosquito larvae (54.29% of infected larvae) and it was the most abundant fungus isolated, and identified from larval mosquito habitats in Kashan County (Fig. 6). This fungus was identified from larvae collected from a natural larval habitat with vegetation in Barzok. *Anopheles superpictus* s. l. had the highest number of larvae infected with the fungi in all larval habitats, and from 105 infected larvae collected, 59 larvae were related to this species (Table 2).

Table 2
Isolated fungi from mosquito larvae in Kashan County, central Iran, 2019.

Districts	Fungi Isolated	Mosquito larvae infected	Percentage
Qamsar	<i>Natrassia mangiferae</i>	<i>An. superpictus</i> s. l. (5)	<i>An. superpictus</i> s. l. (1.10)
	<i>Aspergillus niger</i>	<i>An. maculipennis</i> s. l. (2), <i>An. superpictus</i> s. l. (12)	<i>An. maculipennis</i> s. l. (3.70), <i>An. superpictus</i> s. l. (2.65)
	<i>A. fumigatus</i>	<i>An. maculipennis</i> s. l. (1), <i>An. superpictus</i> s. l. (7)	<i>An. maculipennis</i> s. l. (1.85), <i>An. superpictus</i> s. l. (1.55)
Barzok	<i>A. niger</i>	<i>An. maculipennis</i> s. l. (2), <i>An. superpictus</i> s. l. (4), <i>Cx. deserticola</i> (3), <i>Cx. perexiguus</i> (3)	<i>An. maculipennis</i> s. l. (9.1), <i>An. superpictus</i> s. l. (7.02), <i>deserticola</i> (3.26), <i>Cx. perexiguus</i> (9.68)
	<i>A. fumigatus</i>	<i>An. maculipennis</i> s. l. (1), <i>An. superpictus</i> s. l. (4)	<i>An. maculipennis</i> s. l. (4.55), <i>An. superpictus</i> s. l. (7.02)
	<i>Penicillium</i> spp	<i>An. maculipennis</i> s. l. (3), <i>An. superpictus</i> s. l. (25), <i>Cs. longiareolata</i> (14), <i>Cx. deserticola</i> (9), <i>Cx. perexiguus</i> (6)	<i>An. maculipennis</i> s. l. (13.64), <i>An. superpictus</i> s. l. (43.86), <i>Cs. longiareolata</i> (5), <i>Cx. deserticola</i> (9.78), <i>Cx. perexiguus</i> (19.35)
	<i>Trichoderma</i> spp	<i>An. superpictus</i> s. l. (2), <i>Cs. longiareolata</i> (2)	<i>An. superpictus</i> s. l. (3.51), <i>Cs. longiareolata</i> (0.71)

Discussion

Insect pathogenic fungi can grow in liquid and solid environments, and their spores can attack and kill mosquito larvae [48]. In the present study, five fungi species were identified from mosquito pupae or larvae. All of these fungi were isolated from larvae and pupae collected from natural larval habitats and this is the first report of natural infection of mosquito species with these fungi from Iran.

Effects of fungi mycelia and secondary metabolites on mosquito larvae

Nattrassia mangiferae is common in the tropics and is best known as a plant pathogen (the cause of die-back and trunk cankers in trees). It can also cause fungal infections in human nails [49]. This fungus had not previously been isolated from insects and we are the first to report this from mosquito larvae and pupae. *Aspergillus* has more than 180 species, some of them are pathogenic or allergenic to humans and animals. In different studies, several species of this fungus have been reported in mosquito larvae. Turkey et al. [46] and Khalaf et al. [50] isolated *A. candidis*, *A. niger*, *A. terreus*, and *A. flavus* from *Cx. quinquefasciatus* larvae. Balumahendhiran et al. [51] examined secondary metabolites of *A. flavus*, and *A. fumigatus* in the control of *Ae. aegypti*, *An. stephensi*, and *Cx. quinquefasciatus* mosquito larvae. Species of *Aspergillus* produce a variety of secondary metabolites, including aflatoxins. Secondary metabolites of *A. fumigatus* had the highest toxicity to *Cx. quinquefasciatus*, and *An. stephensi* larvae. Secondary metabolites of *A. niger* were also effective against the larvae of these three mosquito species [31]. In our study, also, it was found that two species of *Aspergillus*, including *A. niger*, and *A. fumigatus*, can grow in an aquatic environment on mosquito larvae and infect them.

In the present study, *Trichoderma* spp. were also isolated and identified from mosquito Larvae. This fungus is capable of attacking other organisms and microorganisms by producing antibiotics and other extracellular enzymes. Because of this ability, *Trichoderma* spp. have been known as biocontrol agents of plant pathogens for about 70 years [52]. This fungus is widely used in agriculture to control plant diseases as well as to increase crop yield. Podder and Ghosh investigated the effect of *T. asperellum* against anophelinae larvae and their study was the first report on the use of *T. asperellum* as mosquito larvicides. They observed that the internal tissues of the larvae were destroyed after larval death [53]. It has been confirmed that some fungal toxins can cause tissue damage and dehydration of the host tissues [54]. In a study in 2016, researchers reported *M. brunneum* blastospores kill *Aedes* larvae much faster than conidia of this fungus in natural habitat, in freshwater. Blastopores easily penetrated the larval cuticle and results in rapid larval death. Conidia cause stress-induced mortality, which takes a slightly longer time [48].

Effects of fungi on morphological or behavioral changes of mosquito larvae

In the present study, *Penicillium* sp. was isolated from wrinkled and degenerated larvae and the larvae whose parts of their cuticle were destroyed. Ragavendran et al. [55] studied the effect of larvicidal of seven fungal isolates and their metabolites on *Ae. aegypti* and *Cx. quinquefasciatus* in vitro and reported

that *Penicillium* sp. had the best larvicidal effect compared to other fungi. The mycelia extract of this fungus had toxic effects on many parts of the larval body, including thorax, abdomen, anal gills, such as the loss of external hair, crumbled epithelial layer of the outer cuticle and shrinkage of the larvae. After 30 minutes of exposure of the larvae to the fungal metabolites, the behavioral symptoms of the treated larvae were observed including upward, downward, horizontal and vertical movements of the larvae and damage at the bottom of the larval body. Damage to the cuticle layers was also one of the morphological changes in the treated larvae. In our study also *Penicillium* sp. was isolated from larvae that had morphological changes in cuticle layers. Lethargy and inactivity were among behavioral changes observed in infected larvae.

Fungi infection in this study was present in *An. superpictus* s. l. larvae. Omrani et al. reported the first case of a microsporidium infection (a microsporidium species from the genus *Parathelohania*) in *An. superpictus* s. l. from Iran [56]. *Parathelohania legeri* was reported in *An. maculipennis* s. l. about 110 years ago [57].

In addition to parasitic effects and their potential for mosquito control, mosquito associated fungi also have nonpathogenic interactions with mosquitoes such as impact on breeding site selection and impact on larval and adult feeding behavior. It has been demonstrated that secondary metabolites produced by *Trichoderma viride* have effects on attract gravid *Cx. quinquefasciatus* females and find oviposition sites. Studies on nonpathogenic fungi of mosquitoes are very scarce and have not been done in Iran. Therefore study of impact of pathogenic and nonpathogenic fungi on behavior of mosquitoes can help to develop new vector control strategies [58, 59].

Conclusion

Based on the observations and results obtained from our study and investigations of other researchers, entomopathogenic fungi have the potential for mosquito control and can efficiently kill mosquito larvae in laboratory and field conditions. We did not study the lethal effects of these fungi on larvae in the laboratory, and only reported natural fungi infection in mosquito larvae in their natural habitats. Therefore, it is suggested that their effects on mosquito larvae be investigated in the laboratory. The most important point, however, is the proper way of exploiting these biocontrol agents to maximize their effect on reducing the population of vector mosquito larvae without any negative effect on non-target organisms.

Declarations

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Competing interests

The authors declare that they have no competing interests

Authors' information

^{1,2,3} Department of Medical Entomology & Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. ⁴ Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran.

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Figures

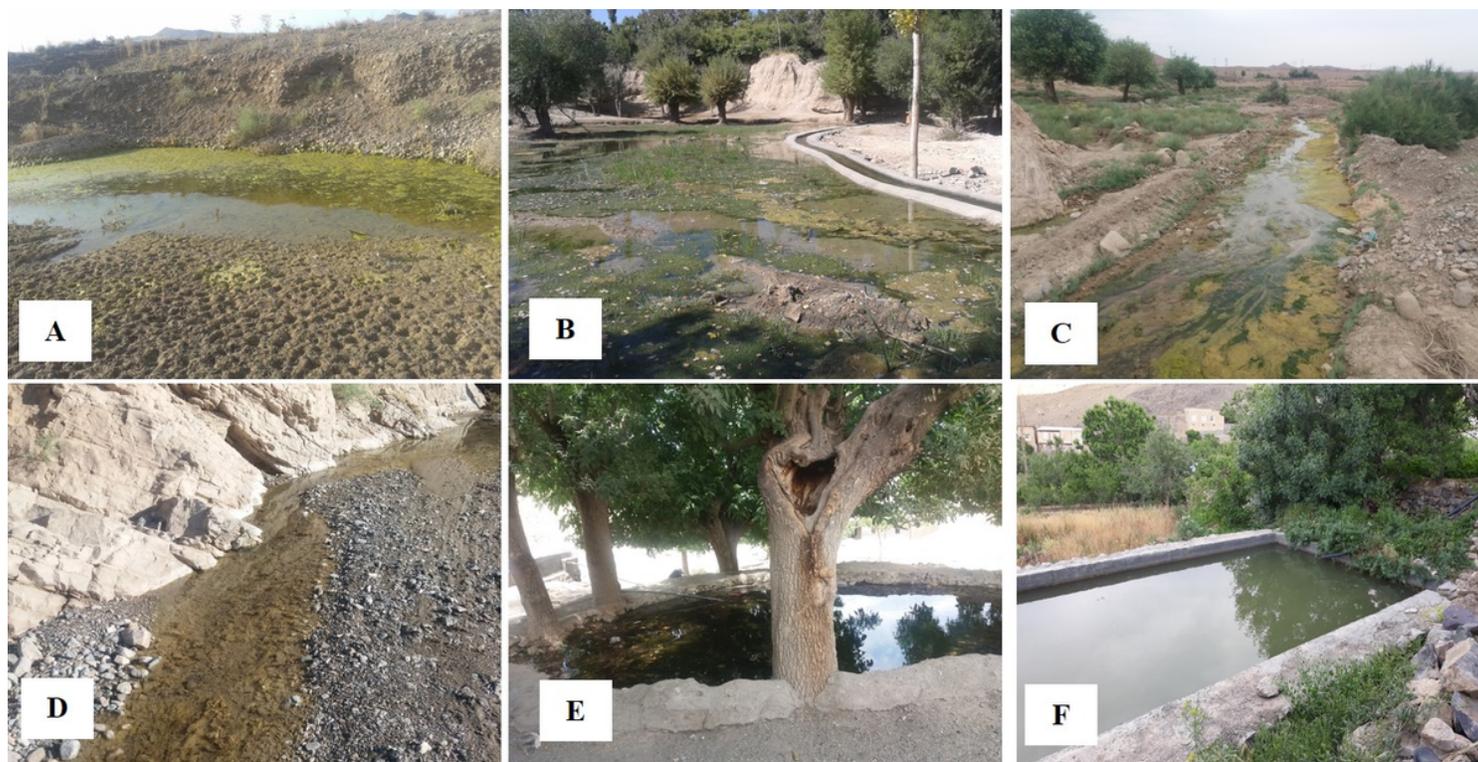


Figure 1

A, B, C, D, E and F General views of mosquito larval habitats in Kashan County, central Iran, 2019.

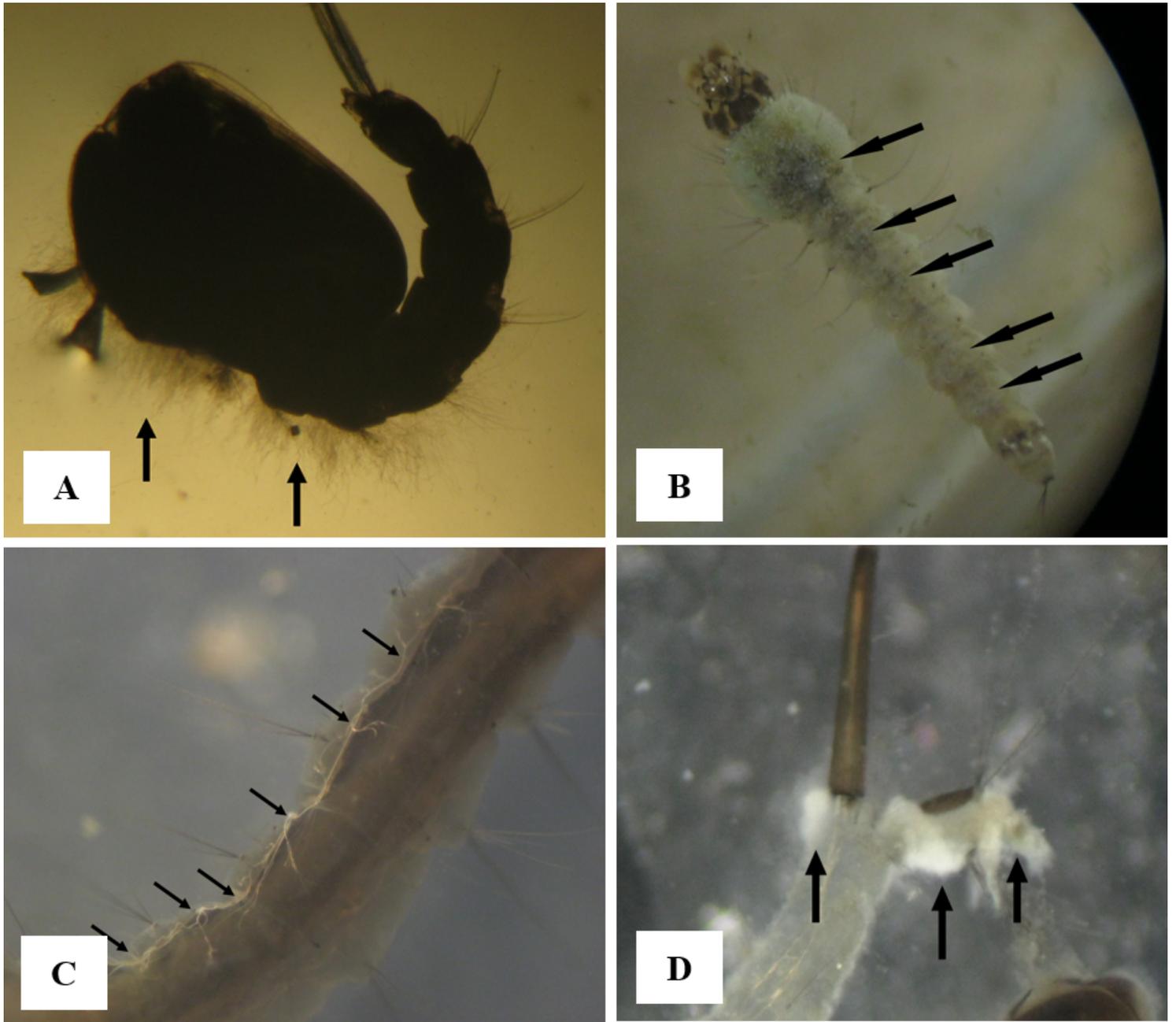


Figure 2

Stereo microscopic view of pathogenic fungi on the 4th instar larvae and pupae of mosquitoes in Kashan County, Central Iran, 2019. Fungal mycelium on A: *An. superpictus* s. l. pupa, B: *An. superpictus* s. l. larva, C: *Culiseta longiareolata* larva D: *Culex* spp. Larva (Arrows show fungal hyphae).

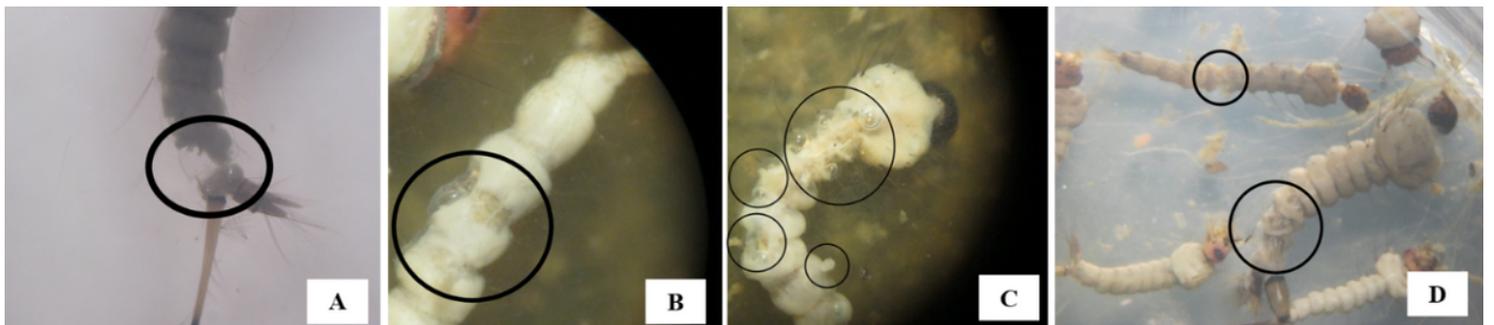


Figure 3

A, B, C, D: Stereo microscopic view of 4th instar mosquito larvae, Kashan County, Central Iran, 2019. The circles indicate crumbled epithelial layer of the outer cuticle, shrinkage of the larvae, and bumps on the surface of the larval bodies.

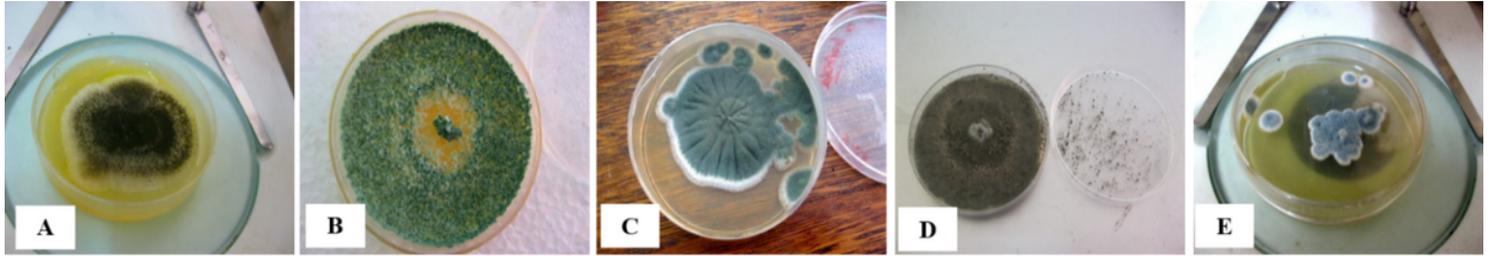


Figure 4

Fungi isolated from mosquito larvae and grown in PDA, Kashan County, central Iran, 2019. A: *Aspergillus niger*, B: *Trichoderma* sp., C: *A. fumigatus*, D: *Nattractasia magniferae*, E: *penicillium* spp.

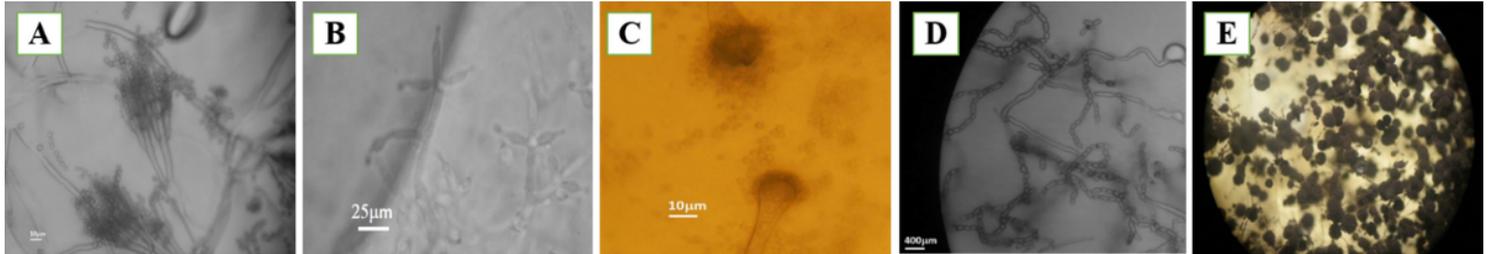


Figure 5

Microscopic image of fungi medium isolated from mosquito larvae in Kashan County, central Iran, 2019. A: *penicillium* spp., B: *Trichoderma* sp., C: *Aspergillus fumigatus*, D: *Nattractasia magniferae* and stereo microscopic view of E: *Aspergillus niger*.

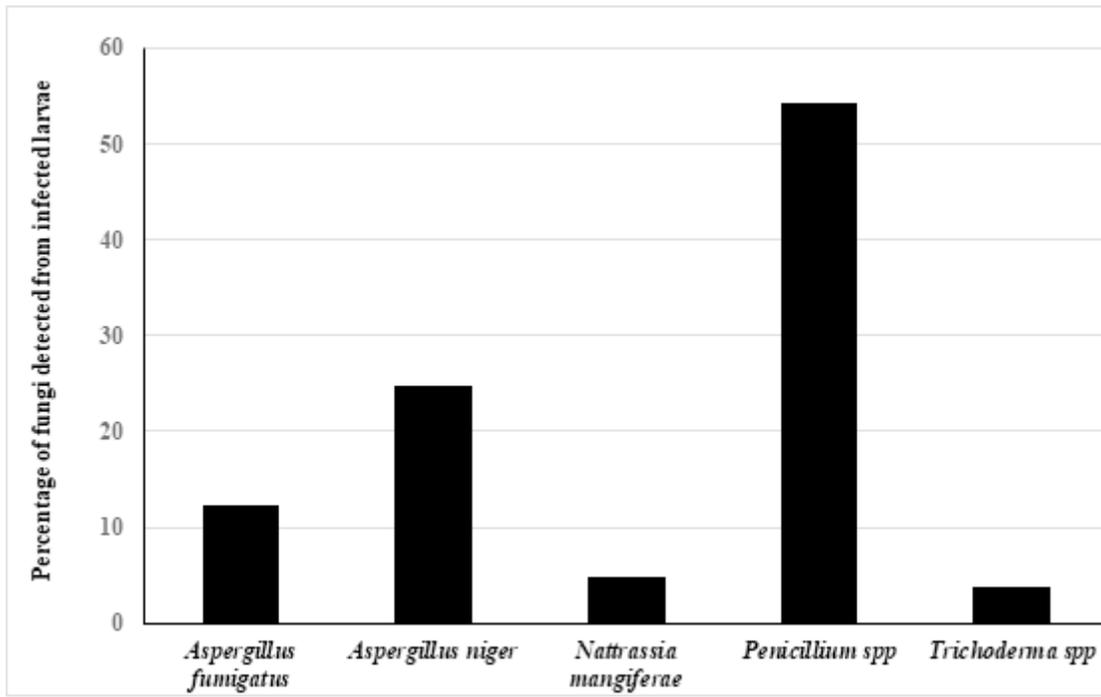


Figure 6

Percentage of fungi detected from infected larvae in Kashan County, central Iran, 2019.