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First report of entomopathogenic fungi, *Aspergillus tamarii* (Eurotiales: Trichocomaceae) on *Musca domestica* (Diptera: Muscidae) larvae

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Abstract

A fungus isolated from mango wasp was found to be pathogenic to houseflies. The isolated fungus was identified as Aspergillus tamarii using morphological characteristics and by comparative nucleotide sequence analysis of ITS regions. Fragments covering the region encoding ITS-1, 5.8S rDNA, and ITS-2 were amplified using the universal primers, ITS1 (TCCGTAGGTGAACCTGCGG) and ITS2 (TCCTCCGCTTATTGATATGC). The results of bioassays against early second instar larva showed dose-dependent mortality. Larvicidal activity was carried out at eight different concentrations viz, $1x10^6$, $1x10^7$, $1x10^8$, $2x10^6$, $2x10^7$, $2x10^8$, $3x10^6$, $3x10^7$, and $3x10^8$ spores/ml. Among the two different methods of bioassays, bait method showed highest mortality. Beauveria bassiana was used as a positive control. Tween 80 (0.05%) was used as negative control. Larval mortality commences from day 3 until pupation in both bait and immersion methods. The least LC_{50} value observed for bait method was $5.28x10^7$ spores/ml followed by immersion method, $5.12x10^9$ spores/ml. On the other hand, the larva treated with commercial fungal strain, Beauveria bassiana showed an LC_{50} value of $1.26x10^8$ spores/ml and $1.32x10^8$ spores/ml for bait and immersion methods, respectively. Visual observation of mycosis was noticed on treated larva. Treated larvae had extensive growth of fungal hyphae on the larval surface. Scanning Electron Microscopy studies of treated larvae clearly showed the process of fungal infection. Longitudinal section of the treated larva showed fungal hyphae on the haemocoel.

Keywords: *Musca domestica, Aspergillus tamarii, larvicidal, biocontrol agent*

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The housefly, *Musca domestica* L. (Diptera: Muscidae) is a global pest of humans and animals that carries many pathogens. Adult houseflies acquire microbes such as bacteria, protists, viruses, and helminth eggs during their continuous association with a microbe-rich environment. Over 200 different species of microbes have been isolated from wild-caught houseflies (Nayduch and Burrus, 2017). Pathogens from flies are transferred to animals and human beings by physical contact or through contamination of various food sources (Jacques *et al.,* 2017). Housefly transmits salmonellosis in cattle which affects colostrum thereby reducing milk production and deficient calves suffer from fever, diarrhoea, rapid dehydration, and death within 24-48 hours (Wray and Wray, 2000). Necrotic enteritis (NE) is an economically significant house fly-transmitted disease caused by the bacterium *Clostridium perfringens*, common in poultry farms worldwide (McDevitt *et al.,* 2006). Many strains of *Salmonella, Shigella dysenteriae,* and *Staphylococcus aureus* are viable on housefly excreta, leading to the active dissemination of these pathogenic bacteria. Houseflies can disperse pathogens several kilometers from the larval habitat such as farms, which could help the spreading of bacteria including anti-microbial resistant (AMR) strains to the surrounding region (Neupane *et al.,* 2020).

Housefly management is practiced using cultural, chemical, mechanical, physical, and biological control strategies. The cultural method includes manure management by composting, incorporation into the soil, and moisture mitigation (Tahir and Ahmad, 2013). Use of screens, air curtains, and high-speed fans can control fly population (Carlson *et al.,* 2006). Ultraviolet lights, sticky tapes, and electrocuting light traps can disrupt the dissemination of fly-borne pathogens (Urban and Broce, 2000). Attractant-based traps using food and/or pheromone baits are significant in integrated house fly management. Natural-based baits (molasses, milk, yeast, fermenting wheat bran) and pheromones such as Z-9 Tricosene are attractive to houseflies (Tang *et al.,* 2016).

Though insecticides are effective agents for the suppression of house fly population, resistance has been developed to several drug classes (Acevedo *et al.,* 2009). Biological measures such as *Bacillus thuringiensis* isolated from environmental sources, parasitic nematodes such as *Steinernema feltiae* and *Heterorhabditis heliothidis* have been evaluated as biopesticides against housefly larvae and adults (Geden *et al.,* 1986). Entomopathogenic fungi are naturally occurring fungi that kill insects and closely related arthropod hosts. They enter the host by invading the cuticle or are being ingested, where they quickly proliferate, disseminating hyphal bodies and releasing various toxins. After the death of the host, the fungus might sporulate on the exterior of the host cadaver, if humidity is high. Conidia are then dispersed to other uninfected individuals and the cycle continues. However, the infection/virulence in field conditions depends upon the host and environmental conditions (Mishra *et al.*, 2015).

The primary entomopathogenic fungi that have been evaluated for housefly control were *Metarhizium anisopliae, Beauveria bassiana,* and *Entomophthora muscae* (Kurtti and Keyhani, 2008). Following the death of the fly, large and sticky conidia are discharged into the environment where they can be encountered by healthy flies (Kalsbeek *et al.*, 1995). These characteristics of entomopathogenic fungi are significant for the development of fungal-based biopesticides for the management of houseflies. This study was designed to find new sources for entomopathogenic fungi that are necessary for effective fly management in poultry farms.

Materials and methods

Fungi

Single spore colonies were originally isolated from a mango wasp and incubated for 7 days at room temperature. Spores obtained from the first subculture were used for bioassays. Preliminary identification indicated that the fungus belongs to the genus *Aspergillus*. Spores harvested by scraping from the agar surface using a loop were stored at 4°C until used. Clumps of spores were then dispersed in 0.1% (vol/vol) Tween 80 and vortexed. The concentration of spores was estimated using a Neubauer chamber (Lacey, 2012).

House fly rearing

Adult houseflies (*M. domestica*) were collected from the chicken stall using sweep nets and reared in a metal cage (Fig. 1). Vitamin syrup was provided as adult feed. Coconut cake and rice husk (3:1) were provided as a larval medium. Milk was provided as an egg-laying substrate. Eggs were collected and placed in a larval medium. After pupation, the pupae were collected and kept in cages for adult emergence (Keiding, 1986).

Fig. 1 Housefly rearing

Pathogenicity test- Bait method

Plastic containers (15 cm× 15 cm) containing 10 grams of larval diet are mixed with one ml of different concentrations of selected entomopathogenic fungal spore suspensions. One ml of 0.05% Tween 80 was mixed with a larval diet as negative control and 10 larvae of 2nd instar larvae were added to the medium. Commercial preparation of *Beauveria bassiana* was used as a positive control. Each concentration was replicated 5 times. The assay was monitored till the larvae become pupae. The dead larvae were surface sterilised and placed in a moist condition for mycosis (Farooq and Freed, 2016).

Pathogenicity test - Immersion method

Ten larvae were immersed in the desired concentration of entomopathogenic fungal spores for a period of 60 seconds. The immersed larvae were then placed in the plastic container containing 10 grams of larval diet. The assay was monitored till the larvae become pupae. Commercially purchased *Beauveria bassiana* was used as a positive control. The dead larvae were surface sterilised and placed in a moist condition for mycosis (Farooq and Freed, 2016).

Polymerase chain reaction

A culture of *Aspergillus* sp. was grown in Potato Dextrose Broth for 1 week. The mycelial mat was harvested and then DNA was extracted according to a cetyltrimethylammonium bromide (CTAB) based method. Fragments containing the region encoding the ITS-1,5.8S rDNA and ITS-2 were amplified using the universal primers ITS1(TCCGTAGGTGAACCTGCGG) and ITS2 (TCCTCCGCTTATTGATATGC) as described previously (White *et al*., 1990). Polymerase chain reaction was performed with an initial denaturation at 94°C for 5 min, followed by denaturation of 94°C for 30 s, 37°C for 1 min and 72°C for 2 min, and a final extension of 72°C for 10 min in a thermocycler (Eppendorf® Mastercycler®). The amplicons were sequenced and multiple sequence alignment was performed using the BIOEDIT program. The phylogenetic tree was constructed using the NCBI tree viewer and deposited in the NCBI GenBank database.

Statistical analysis

The obtained data on larvicidal activity was subjected to One Way ANOVA. Significant differences between treatments were calculated by using Tukey's multiple-range tests. The LC_{50} values were calculated by using Probit analysis for larvicidal activity.

Scanning electron microscopy study of treated larva

SEM analysis was carried out following the method of Mishra *et al.* (2017). The impact of entomopathogenic fungal spores on housefly larvae was observed under TESCAN VEGA 3SBU Scanning Electron Microscope (SEM). The dead larvae were collected after treatment

with the highest spore concentration ($3x10^8$ spores/ml). The untreated larvae were used as control. Larvae were washed with ethanol to remove impurities and placed in moisture for the development of mycosis. All samples were stored in ethanol. Longitudinal section of the treated larvae was prepared to observe the hyphae within the body hemocoel. The dorsal side of the sample was fixed mounting on a stub just prior to coating with conductive material and examination under SEM.

Result and discussion

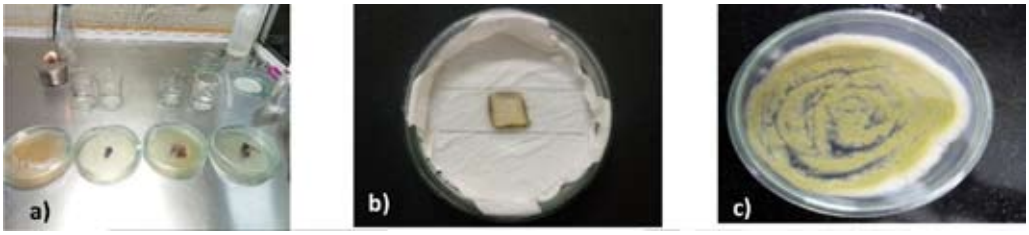
Isolation and identification of entomopathogenic fungi

The conidium of *A. tamarii* was globose, orange yellow and later turned brown. Each spore had a diameter of approximately 4.31µm. Morphological observations in the present study coincide with previous studies on the morphology of *Aspergillus* strain (Diba *et al*., 2007). Therefore, the fungal morphology revealed that the isolated fungi belong to the genus *Aspergillus* (Fig. 1a-e). Entomopathogenic fungi can be harvested from insect cadavers embedding external fungal sporulation (St Leger and Wang, 2007). Host switching among fungal biocontrol agents is important for the investigation of fungal virulence and survival according to target or non-target (Haarmann *et al*., 2009). This idea suggests the possibility of isolating novel fungal strains from insect cadavers. Further, the novel strain of entomopathogenic fungus of *M. domestica* was identified using comparative analysis of the nucleotide sequence of the ITS region. In present study, ITS-1 and ITS-2 were chosen for identification of *Aspergillus sps.* Balajee *et al*. (2007) recommended ITS1-5.8S rDNA-ITS2 as an appropriate locus for identifying *Aspergillus* strains. The ITS rDNA region of *Aspergillus tamarii* was sequenced and the sequence was deposited in the GenBank database (accession number OM978233). Comparison between the ITS sequence of *Aspergillus sp.* and those of available strains in GenBank showed the evolutionary relationship of the obtained strains with its ancestral origin.

Pathogenicity of Aspergillus tamarii against Musca domestica

The larvicidal activity of *A. tamarii* against housefly larvae was concentration dependent. In comparison with the immersion method, the bait method has shown the least $LC_{50.}$ Larval mortality commenced from day 3 until pupation in both bait and immersion methods (Table 1 and 2). Overall, the bioassay study demonstrated a significant impact on house fly larvae both in bait and immersion methods. Negative control (0.05% Tween 80) has shown no activity against housefly larvae using both bait and immersion methods. Positive control has shown 94% mortality by bait method and 74% mortality on day 4 by immersion method. This observation suggests that the spore adherence began after 24 hours followed by penetration of spores through the intersegmental region of the larva. The penetration of spores causes the release of toxins leading to the death of the host. These toxins act synergistically to produce full toxicity, making it difficult for insect populations to resist this entomopathogen (Bravo *et al*., 2006). Mwamburi *et al.* (2010) have also reported that the susceptibility of houseflies was concentration-dependent.

At the highest concentration ($3x10^8$ spores/ml) 62% mortality was observed on day 4 by bait method. About 94% mortality was observed for those larvae treated with *Beauveria bassiana* (Positive control). Compared to the immersion method, the bait method showed significant activity. The Least LC_{50} value was observed for the bait method ($5.28x10^7$ spores/ml) compared to the immersion method



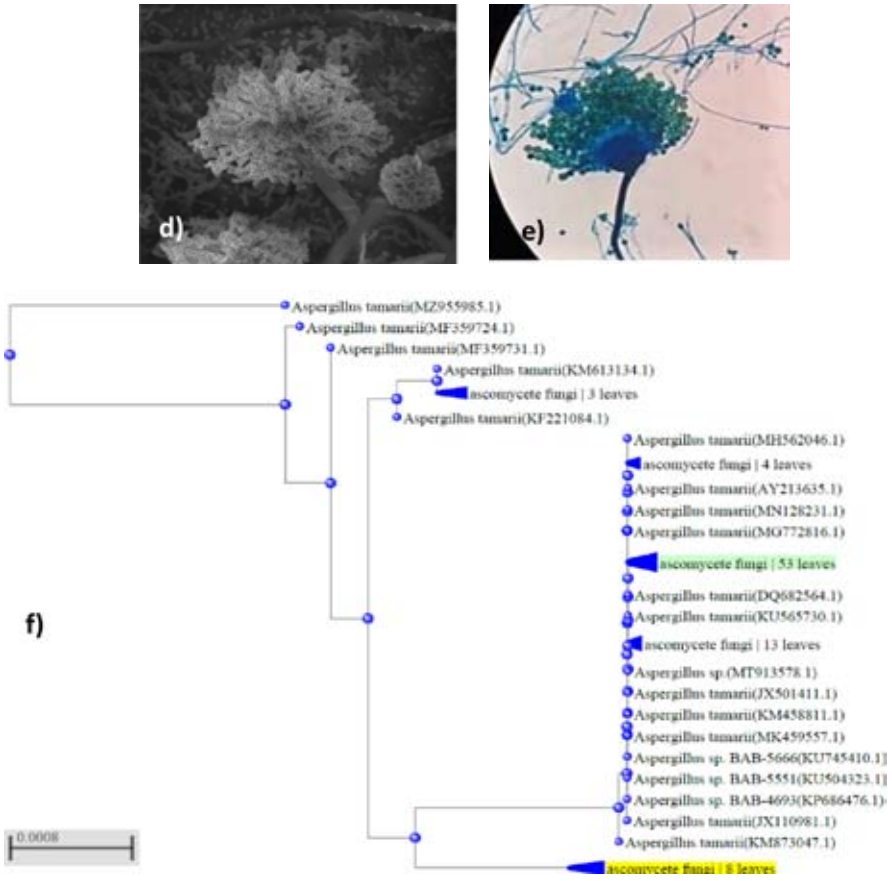


Fig. 1. Isolation and identification of *Aspergillus tamarii* (OM978233)
a) Isolation of fungi; b) Slide culture technique; c) *A. tamarii* grown on PDA; d) SEM image of colony morphology (350x); e) LCB stained Colony morphology (40x); f) Phylogenetic tree of classification.

(5.12x10^9 spores/ml) (Table 3).

Larval mycosis

In current study, after treatment, the conidia began to germinate at 8 hours and continued to grow up to 4 days. The infected larva shrinks and becomes yellow to dark brown in colour. The conidia were orange-yellow, globular, and thick with a very rough surface, becoming brown with age (Fig. 3 A, B, C)

After the successful adhesion of spores on the host cuticle, it penetrates the

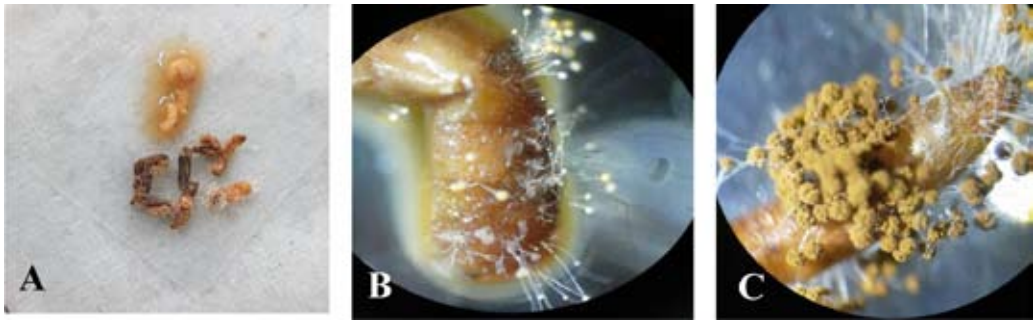


Fig. 3. Pathogenicity of *Aspergillus tamarii* (OM978233). A) The dead larva placed in a moisture chamber; B) 3 days after death showing hyphal growth and fungal sporulation (40x); C) 4 days after death showing hyphal growth, sporulation, and change in fungal colours (40x).

Table 1. Larvicidal activity of entomopathogenic fungi *Aspergillus tamarii* (OM978233) by bait method

Concentration (Spores/ml)	Corrected mortality (% ± SD)			
	***Aspergillus tamarii* (OM978233)**		***Beauveria bassiana* (Control)**	
	Day 3	**Day 4**	**Day 3**	**Day 4**
1 x 10^6	14±3.5^{ab}	18±2.1^{a}	12 ± 2.00^{a}	20 ± 0.0^{a}
1 x 10^7	20±3.9^{ab}	28±3.6^{ab}	20 ± 3.1^{ab}	30 ± 1.9^{b}
1 x 10^8	32±1.8^{bc}	40±1.7^{bc}	30 ± 3.2^{b}	42 ± 3.8^{c}
2 x 10^6	22±3.3^{b}	38±1.8^{bc}	46 ± 2.5^{c}	52 ± 1.9^{d}
2 x 10^7	34±3.5^{cd}	46±1.7^{cd}	52 ± 3.7^{cd}	60 ± 3.2^{d}
2 x 10^8	42±1.7^{d}	54±2.8^{de}	62 ± 3.8^{cd}	72 ± 2.0^{e}
3 x 10^6	38±4.5^{cd}	48±1.8^{c}	64 ± 2.4^{d}	76 ± 2.5^{e}
3 x 10^7	46±2.1^{de}	56±3.5^{de}	74 ± 2.5^{e}	86 ± 3.9^{f}
3 x 10^8	54±2.2^{e}	62±1.8^{de}	88 ± 1.9^{f}	94 ± 2.3^{f}

Means followed by the same letter superscript are not significantly different (Tukey's test, p <0.05)

Table 2. Larvicidal activity of entomopathogenic fungi *Aspergillus tamarii* (OM978233) by immersion method

Concentration (Spores/ml)	Corrected mortality (%±SD)			
	***Aspergillus tamarii* (OM978233)**		***Beauveria bassiana* (Control)**	
	Day 3	**Day 4**	**Day 3**	**Day 4**
1 x 10^6	0±0^{a}	0±0^{a}	12 ± 1.9^{a}	22 ± 2.7^{a}
1 x 10^7	0±0^{a}	0±0^{a}	20 ± 3.1^{a}	34 ± 2.4^{a}
1 x 10^8	0±0^{a}	2±1.7^{a}	32 ± 3.7^{a}	40 ± 3.1^{a}
2 x 10^6	8±1.7^{ab}	10±2.8^{ab}	44 ± 3.0a	52 ± 3.7^{a}
2 x 10^7	12±1.8^{bc}	18±1.7^{bc}	52 ± 1.9^{ab}	60 ± 0.0^{ab}
2 x 10^8	14±2.1^{bc}	22±3.3^{bcd}	62 ± 2.0^{bc}	70 ± 3.1^{bc}
3 x 10^6	20±2.8^{cd}	24±3.5^{cd}	68 ± 3.7^{cd}	74 ± 2.4^{cd}
3 x 10^7	28±1.7^{de}	34±2.2^{d}	78 ± 1.9^{d}	82 ± 2.2^{d}
3 x 10^8	36±2.2^{e}	48±3.3^{e}	90 ± 3.4^{e}	94 ± 2.3^{e}

Means followed by the same letter superscript are not significantly different (Tukey's test, p<0.05)

Table 3. Probit analysis of second instar larvae due to Entomopathogenic fungi *Aspergillus tamarii* (OM978233)

Fungal isolates	Method	Spores/ml	
		LC_{50}	**LC_{90}**
Aspergillus tamarii	Bait	5.28×10^7	6.47×10^7
	Immersion	5.12×10^9	6.79×10^9
Beauveria bassiana	Bait	1.26×10^8	2.74×10^8
	Immersion	1.32×10^8	2.77×10^8

surface and reaches the inner structure for vegetative and reproductive growth. This requires both enzymatic breakdown and mechanical pressures (Frisvad *et al*., 2007). Conidia germinates at the inter-segmental regions of the whole-body surface. The fungi penetrate the insect cuticle by germ tube formation or by establishing an appressorium that affixes the cuticle and produces a thin penetrating peg (Urquiza and Keyhani, 2013; Soliman, 2020). In present study, spores penetrated into the larval body and utilized the nutrients, and started germinating within the body. Longitudinal section of the treated larva has shown the hyphae and spores in the haemocoel. Extensive fungal growth was evident in haemocoel within four days after exposure to fungal conidia. The treated larva after 72 hours has shown the extension of hyphae over the

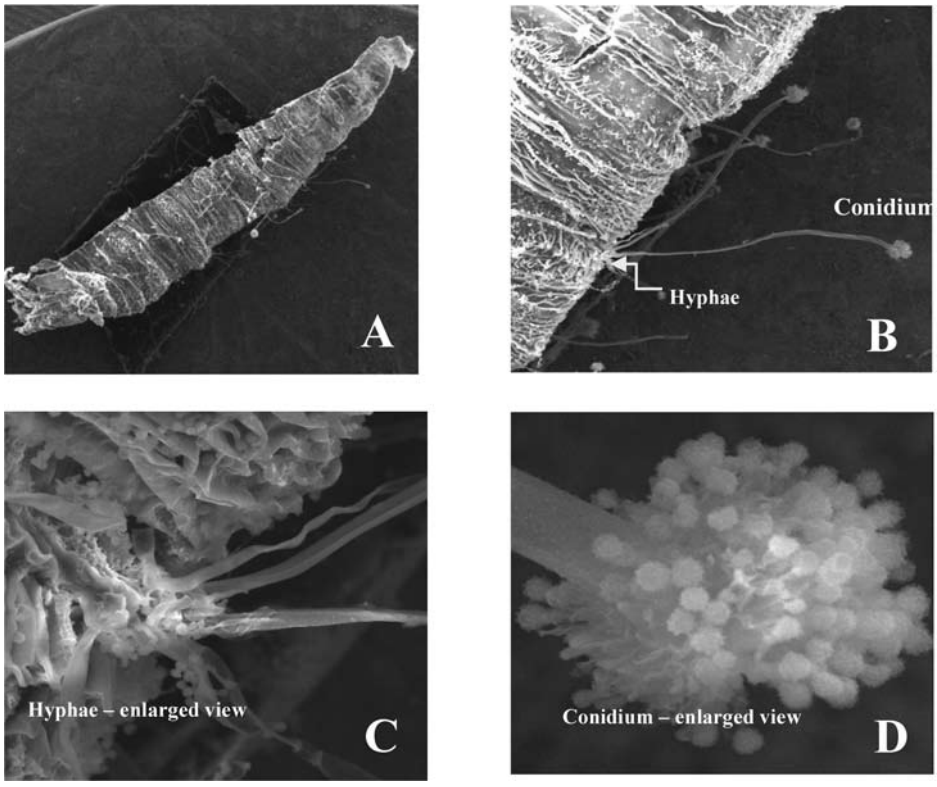


Fig. 4. A) L.S of *Aspergillus tamarii* (OM978233) treated housefly larva B) Intersegment of the treated larva (100x) C) Hyphae enlarged view (1800x) D) Conidium enlarged view (1500x)

integument from the inter-segmental region (Fig. 4 A, B). Insect death occurs due to various factors such as mycotoxins, tissue invasion, and inadequate nutrients (Islam *et al*., 2021). Further, it emerges from the intersegmental region utilizing the nutrient from the larva (Fig. 4 C). Hyphae were septate and the conidiophore was enlarged at the tip, forming a swollen vesicle (Fig. 4 D). Similarly, Kumar *et al*. (2004) have also observed the extensive fungal growth of *A. flavus* in the infected *B. mori* larvae 3–4 days post-inoculation. The cadaver-bearing spores are able to spread and infect other individuals (Kumar, 2007).

Conclusion

The current study provides the first systematic report of an *Aspergillus tamarii* (OM978233) strain as an entomopathogenic fungus against *M. domestica*. The bioassay results suggest that the *A. tamarii* (OM978233) strain has the potential to kill the house fly larva. Further, studies including growth optimisation, spore stability, and bio efficacy against houseflies under field conditions are needed before commercialisation.

Conflict of interest

The authors declare that they do not have any conflict of interest.

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