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Entomopathogenic potential of indigenous *Simplicillium subtropicum* (Hypocreales: Cordycipitaceae) isolates from Tamil Nadu, India, against the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae)

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Abstract

Background *Aphis gossypii* Glover (Hemiptera: Aphididae) is an economically important polyphagous pest species infesting many agricultural and horticultural crops causing severe yield losses. In order to develop biopesticides for the management of aphids in an environmentally safe manner, 6 indigenous entomopathogenic fungi *Simplicillium subtropicum* were isolated from soils of Tamil Nadu and their pathogenicity was tested against *A. gossypii* under laboratory conditions after characterization.

Results Phenotypic and molecular characterization was performed for all the isolates for species identification. Results of the pathogenicity test showed that the isolate S6 (PP446637) performed well among other isolates causing 58.33% mortality on 7 days after treatment (DAT) @ 1×10^8 spores/ml. The LC_{50} and LT_{50} values were found to be 2.36×10^7 spores/ml at 7 DAT and 160.20 h @ 1×10^8 spores/ml, respectively. SEM images of *A. gossypii* treated with the isolate S6 (PP446637) revealed that initial phase of infection, *i.e.,* adhesion, formation of germ tube and appressoria starts within 24 h post-infection (hpi), hyphal development and penetration of cuticle occurred within 48 hpi and extrusion of mycelium and conidiogenesis occurred within 72 hpi.

Conclusion This study reveals the entomopathogenic potential of indigenous *S. subtropicum* isolates against *A. gossypii* and suggests their use for the eco-friendly management of aphids.

Keywords Entomopathogenic fungi, *Simplicillium subtropicum*, *Aphis gossypii*, Scanning electron microscope (SEM)

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Background

The cotton or melon aphid, *Aphis gossypii* Glover, 1877 (Hemiptera: Aphididae), is a critical polyphagous pest species infesting wide range of cultivated crops including Malvaceae (cotton, okra), Cucurbitaceae (melon, marrow, zucchini, watermelon), Solanaceae (potato, chili pepper, sweet pepper, eggplant) and ornamental plants including chrysanthemum and Hibiscus (Ebert and Cartwright 1997).

Cotton or melon aphid affects the plants by sucking the sap on the underside of leaves, leading to leaf chlorosis and curling. In addition, the honey dew secretion by the aphid causes physical contamination by forming sooty mold which severely hinders their photosynthesis process and weakens them. Also, it indirectly affects the plants by acting as a vector to many plant diseases (Andrews and Kitten 1989). Some of the viruses transmitted by cotton aphid include citrus tristeza virus (Yokomi and Garnsey 1987), cucumber mosaic virus, yellow vein mosaic virus in okra, pepper veinal mottle virus (PVMV) and potato Y virus (PVY) (Sertkaya and Sertkaya 2005).

Thus, *A. gossypii* causes damage throughout the crop period with significant decline in yield and farmers mainly rely on synthetic chemical pesticides for their management which disturbs the ecosystem and leaves harmful pesticide residues in the environment. The increasing awareness on ill effects of pesticide residues in agricultural produce, fruits and vegetables is of great concern. The development of resistance in *A. gossypii* to many commonly used insecticides is also notable (Patima et al. 2019).

Among the alternative eco-friendly management approaches, microbial biopesticides especially entomopathogenic fungi (EPF) attract considerable attention due to their broad host range and unique mode of action of directly penetrating through the insect integument resulting in mycosis. Globally, many EPF such as *Lecanicillium lecanii*, *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces* sp. and *Nomuraea rileyi* have been used to control aphids and various insect pests (Shah and Pell 2003). Also, the genus *Simplicillium* branched off from the genus *Verticillium* section Prostrata, for their unique nature of producing solitary phialides, have been reported as entomopathogens to various sucking pests including aphids (Sujithra et al. 2021).

Therefore, the present study was undertaken to isolate the indigenous EPF from different locations of Tamil Nadu to utilize them for the management of *A. gossypii*. The isolated fungi identified using morphological characters and molecular characterization were performed for species confirmation. Then, they were screened for their pathogenicity against *A. gossypii*. Ultramicroscopic studies were also conducted for the better understanding of trophic interactions between the isolate and the aphid.

Methods

Soil sampling

A survey was conducted during 2022–2023 years, and 94 soil samples were randomly collected from various districts of Tamil Nadu, India. About 200 g of the soil was collected at 5 different spots in the same sampling site at the depth of 10–15 cm using sterilized hand trowel which were then combined to get approximately 1 kg of the sample. Hand trowel was cleaned and sterilized using 70% ethanol after every sampling. Samples were then brought into Insect pathology laboratory of Department of Agricultural Entomology, Tamil Nadu Agricultural University, shade dried, processed and stored at 4 °C for further use (Tuininga et al. 2014).

Isolation of fungus

Serial dilution method was used for the isolation of fungi from soil samples. One gram of soil sample was weighed, serially diluted with sterilized distilled water and 1 ml of 10^{-2} and 10^{-3} dilutions were inoculated each into Petri plates along with Potato Dextrose Agar (PDA) media in triplicates. All the plates were incubated at 28 ± 1 °C and observed for growth after 3 days of incubation up to 7 days and distinct fungal colonies were inoculated into Petri plates using sterile inoculation needle. The sub culturing process was repeated until pure cultures were obtained. The pure cultures were maintained in PDA slants and stored at 4 °C for further use.

Characterization of entomopathogenic fungi

Phenotypic characterization

For morphological identification of the fungal strains, 15-day-old cultures were selected and macroscopic traits of the colonies like color on upper and lower side of the plates, shape, texture and elevation were observed. Also, microscopic observations were performed on the arrangement of the phialides and structure of the spores using phase contrast microscopy (Euromex iScope®) (Humber 2012).

Molecular characterization

DNA extraction The CTAB (cetyl-trimethyl ammonium bromide) method was used for the extraction of genomic DNA from the isolates as described by Zhang et al. (2010). The 10 mg of fungal mycelium was scrapped from the 10-day-old culture plates using sterile surgical blade, kept in sterilized mortar and pestle and crushed by adding 1 ml of 2% CTAB buffer. The contents were transferred into an Eppendorf® tube and incubated in water bath at 65 °C for 1 h. Afterward, it was centrifuged using

refrigerated centrifuge for 10 min at 12,000 rpm at 4 °C and the supernatant was collected. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and again centrifuged at 12,000 rpm for 10 min for phase separation. Again the supernatant was collected and an equal volume of ice cold iso-propanol was added and incubated overnight at − 20 °C. After that, it was centrifuged for 15 min at 13,000 rpm and the supernatant was discarded. The resultant pellet was washed with 70% ethanol, air-dried and dissolved in 30 µl of nuclease free water. The DNA concentration and purity were determined using Nano-drop Spectrophotometer (Thermo Scientific®, USA).

PCR amplification and sequencing of isolated DNA The universal primers ITS-1(ITS-1-F 5′-TCGGTAGGTAGG TGAACCTGCGG-3′) and ITS-4 (18s-ITS-4-R 5′-CAG GAGACTTGTACACGGTCCAG-3′) were used for the amplification of internal transcribed spacer (ITS) regions of rDNA of the fungal isolates (White et al. 1990). The PCR amplifications were performed in a total volume of 40 µl which included 20 µl of master mix (smart prime), 8 µl of nuclease free water, 4 µl of each forward and reverse primers and 4 µl of genomic DNA. A negative control without DNA sample was also maintained. The amplification temperature was set as 40 cycles of 95 °C for 10 min, 60 °C for 1 min, and 72 °C for 1 min.

Then, gel electrophoresis was used to determine the PCR amplification using 1% agarose gel with ethidium bromide and visualized under UV light using a Gel documentation unit for band confirmation. The amplified PCR products were Sanger sequenced by Syngenome (OPC) Private Limited, Coimbatore. The obtained sequences were edited using BIOEDIT software 7.2 and compared to sequences in NCBI database using BLAST software. All the sequences of this study were compared to already published sequences present in NCBI database. The phylogenetic tree was constructed using MEGA 11.0 software using neighbor joining tree statistical method and Kimura-2 parameter model.

Pathogenicity test against *A. gossypii*

Insect rearing

Aphids used in this study were reared in cotton plants at Insectary of Tamil Nadu Agricultural University, Coimbatore. Co 17 variety of cotton which is susceptible to aphids were grown in pots and *A. gossypii* infested leaves collected from fields were inoculated and allowed to multiply. Sowing was done at fortnightly intervals and aphids were allowed to spread on fresh plants.

Preparation of spore suspension

For the preparation of spore suspension, the fungal spores were scraped from well sporulated culture plates using a sterile surgical blade and transferred into 10 ml sterile distilled water containing 0.05% Tween 80®. The suspensions were homogenized and filtered using cheese cloth to remove the mycelium. The concentration of the spores was determined using improved Neubauer hemocytometer under phase contrast microscopy (Euromex iScope®) and adjusted according to the needs by dilution (Habtegebriel et al. 2016).

Bioassay

Preliminary pathogenicity test was conducted for the spore concentration of 1×10^8 conidia/ml, following the methodology described by Porter (2009). Fresh, healthy cotton leaves were collected and placed in the plastic containers with 1% agar. Thirty 3rd instar aphid nymphs were released and topically sprayed with the spore suspension using glass atomizer. The aphids sprayed with sterile distilled water containing 0.05% Tween 80® served as the control. The experiment was laid out in a completely randomized design and replicated four times. The mortality rate of insects was assessed on 3, 5 and 7 days after treatment (DAT). The dead insects were collected using sterile needle and placed in the moist filter paper to allow mycelial growth. The spores on the surface of the cadavers were observed under the microscope for confirmation. The median lethal concentration (LC_{50}) and median lethal time (LT_{50}) were calculated where the concentrations used ranged from 1×10^4 to 1×10^8 conidia/ml.

Ultramicroscopic observation of infected cadavers

Ultramicroscopic observations were conducted using a scanning electron microscope (SEM) to study the development of fungus in the insect body. Third instar nymphs were sprayed with 1×10^8 conidia/ml spore suspension and samples were collected at 24, 48 and 72 h post-infection (hpi) for processing. Firstly, they were fixed using 4% glutaraldehyde in 0.05 M phosphate bufer (pH 7.3), followed by rinsing with phosphate buffer for three times. Then, they were dehydrated using 30, 50, and 70% ethanol at 30 min interval. They were immersed in 100% ethanol for 45 min for critical point drying (Ganassi et al. 2010). The dried samples were then placed on pin stubs in different orientations using double-sided tape and sputter coated with gold. Then the observations were made under SEM FEI Quanta 250 (Netherlands) having an Everhart Tornley Detector with tungsten as an electron source.

Statistical analysis

Data obtained from various experiments were analyzed using SPSS (Statistical Package of Social Sciences) software version 21. The data in numbers were subjected to $\sqrt{x+0.5}$ transformation and the data in percentage were subjected to arcsine transformation (Gomez and Gomez 1984). Least significant difference (LSD) at $p<0.05$ was used to evaluate the difference between treatments. Abbott's formula was used for correcting the percent mortality data (Abbott 1925). The relationship between the concentration and mortality of the insects was calculated using the Probit analysis (Finney 1971). The LC_{50} and LT_{50} values of the promising isolate were calculated with 95% confidence limits.

Results

An extensive survey was carried out in 13 districts of Tamil Nadu from different agricultural and horticultural ecosystem from 2022 to 2023 and a total of 94 soil samples were collected. Among the locations, 6 entomopathogenic fungi were isolated from 4 regions of 3 districts. The details of the locations surveyed and EPF isolated are enlisted in Table 1.

Phenotypic characterization

Morphological and cultural characteristics of the isolates showed that they belonged to the genera *Simplicillium*. The isolates showed white (front) and brown (reverse) colony color, floccose texture and raised elevation. Growth rate of the colony ranged from 1.41 to 2.41 mm per day and the radial growth of the colony on 15th day after inoculation ranged from 50.88 to 59.50 mm. Observations on the microscopic parameters showed that all the isolates had solitary phialides and globose-shaped conidia with a size ranging from 1.829 to 2.090 µm in diameter and the sporulation rate of the isolates varied from 0.42×10^8 to 1.68×10^8 spores/ml (Table 2). Among the isolates, S6 (TNAU CBE 3) exhibited the highest growth rate, radial growth and sporulation rate, while S1 (TNAU TRY) had the lowest (Fig. 1).

Molecular characterization

The identity of the fungal isolates at molecular level was confirmed by partial sequencing of the 18S rDNA and ITS region. The amplified product length was approximately 550 bp in all the six isolates. The sequences obtained were subjected to nucleotide BLAST and submitted to NCBI database and the accession numbers were obtained, viz., PP446658, PP446656, PP446638, PP446639, PP446652 and PP446637. In blast analysis, all the six isolates had 100% similarity with Genbank sequences of the respective entomopathogenic fungi in NCBI database. The phylogenetic tree revealed that all the isolates S1 (PP446658), S2 (PP446656), S3 (PP446638), S4 (PP446639), S5

Table 1 Details of soil samples collected during the study period (2021–23)

S. no.	Districts	Collection zones	No. *n* samples	Habitat	Latitude	Longitude	EPF isolated
1	Coimbatore	TNAU farm	8	Cultivated lands	11.0122°	76.9354°	S2 (TNAU CBE1)
		Thondamuttur	7	Cultivated lands	10.9962°	76.8275°	S3 (TNAU CBE2) S6 (TNAU CBE3)
2	Erode	Sathiyamangalam	4	Cultivated lands	11.5034°	77.2444°	–
		Ariyappampalayam	10	Cultivated lands	11.3947°	77.0836°	–
3	Perambalur	Veppanthattai	6	Cultivated lands	11.3410°	78.7864°	–
4	Tirichirapalli	Melarasoor	5	Cultivated lands	11.0262°	78.9905°	–
		Kallagam	5	Cultivated lands	11.0222°	78.9965°	–
		Kumulur	3	Cultivated lands	10.9252°	78.8260°	S1 (TNAU TRY)
5	Dindigul	Natham	5	Cultivated lands	10.2400°	78.1685°	–
		Karanthamalai	5	Hill	10.2631°	78.1817°	–
6	Virudhunagar	Srivilliputhur	13	Cultivated lands	9.5025°	77.6448°	–
7	Tenkasi	Sankarankovil	4	Cultivated lands	9.1205°	77.6193°	–
		Alangulam	3	Cultivated lands	9.1510°	77.6802°	S4 (TNAU TKI1) S5 (TNAU TKI2)
8	Thoothukudi	Kovilpatti	5	Cultivated lands	9.1727°	77.8715°	–
9	Salem	Attur	2	Cultivated lands	11.6299°	78.1763°	–
10	Namakkal	Mohanur	3	Cultivated lands	11.0604°	78.1388°	–
11	Sivagangai	Manamadurai	2	Cultivated lands	9.6901°	78.4521°	–
12	Theni	Periyakulam	2	Cultivated lands	10.1283°	77.5998°	–
13	Cuddalore	Manjakuppam	2	Cultivated lands	11.7586°	79.7652°	–

Table 2 Morphological and cultural characteristics of *Simplicillium* isolates

Isolate code	Colony color		Colony shape	Colony texture	Elevation	Conidia shape	Conidia size (µm) diameter	Growth rate (mm Day^{-1})	Radial growth on 15th day (mm)	Sporulation ($\times 10^8$ spores/ml)
	Front side	Reverse side								
S1 (TNAU TRY)	White	Brown	Round	Floccose	Raised	Globose	1.893	1.41 (1.38)^{c}	50.88 (7.17)^{b}	0.42 (0.96)^{d}
S2 (TNAU CBE1)	White	Brown	Round	Floccose	Raised	Globose	1.912	1.59 (1.45)^{bc}	52.50 (7.28)^{b}	0.56 (1.03)^{cd}
S3 (TNAU CBE2)	White	Brown	Round	Floccose	Raised	Globose	1.829	1.84 (1.53)^{b}	53.00 (7.31)^{b}	1.16 (1.29)^{b}
S4 (TNAU TKI1)	White	Brown	Round	Floccose	Raised	Globose	1.876	2.18 (1.64)^{a}	53.88 (7.37)^{b}	1.23 (1.31)^{b}
S5 (TNAU TKI2)	White	Brown	Round	Floccose	Raised	Globose	2.090	1.63 (1.46)^{bc}	52.63 (7.29)^{b}	0.66 (1.08)^{c}
S6 (TNAU CBE 3)	White	Brown	Round	Floccose	Raised	Globose	1.929	2.41 (1.71)^{a}	59.50 (7.75)^{a}	1.68 (1.48)^{a}
SE(d)								0.050	0.125	0.043
CD (0.05)								0.106	0.264	0.092

All values are mean of four replications. Figures in the parentheses are square root ($\sqrt{(x+0.5)}$) transformed values. Values sharing same alphabets in superscript are statistically on par based on LSD ($P<0.05$)

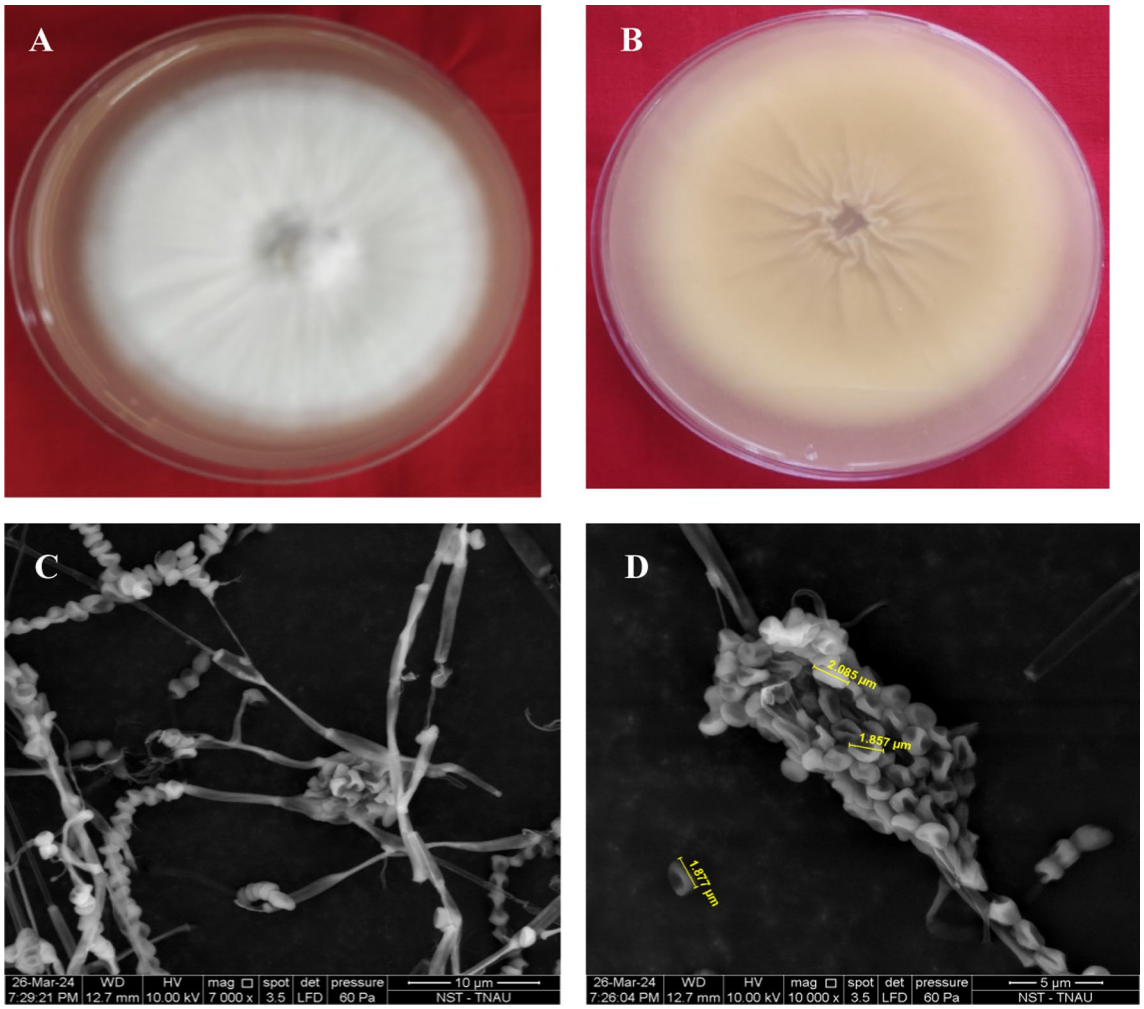

Fig. 1 **A** Front and **B** Reverse view of *Simplicillium subtropicum* (S6) colony on PDA media. **C** Scanning electron microscope image showing hyphae with chains of conidia. **D** Cluster of conidia observed under SEM

(PP446652) and S6 (PP446637) had a phylogenetic affinity to *S. subtropicum* (Fig. 2).

Bioassay

Preliminary screening for pathogenicity of isolates against *A. gossypii* was carried out, and the results are furnished in Table 3. The isolate S6 (PP446637) recorded the highest percent mortality of 6.67, 26.67 and 58.33% followed by S4 (PP446639) which recorded 2.50, 20.00 and 43.33% on 3, 5 and 7 DAT, respectively. The isolates S1 (PP446658) and S2 (PP446656) were on par with each other and recorded the lowest percent mortality of 9.17 and 11.67%, respectively, on 7 DAT, whereas *A. gossypii* in control remained symptomless showing no mortality. Mycelium recovered from dead aphids showed similar morphological characters as original isolates thus satisfying Koch's postulates.

Concentration and time mortality responses

The concentration and time mortality responses of *A. gossypii* to the virulent isolate S6 (PP446637) were carried out, and the results are presented in Table 4. The mortality percent of *A. gossypii* increased with the increase in conidial concentration and time. At three, five and seven days after treatment, mortality of *A. gossypii* ranged from 0.00 to 7.50%, 2.50 to 28.33% and 6.67 to 59.17%, respectively. The highest percent mortality of 59.17% was recorded at the spore concentration of 1×10^8 conidia/ml followed by 1×10^7conidia/ml (51.67%) and 1×10^4 conidia/ml being recorded the least at 7 DAT. The median lethal concentration (LC_{50}) was found to be 2.36×10^7 spores/ml with the fiducial limits of 6.24×10^6 to 8.95×10^7 spores/ml ($\chi^2=2.20$) at 7 DAT. The median lethal time (LT_{50}) was calculated as 160.20 h with the confidence limits of 138.26 to 185.63 h ($\chi^2=0.73$) at concentration of 1×10^8 conidia/ ml.

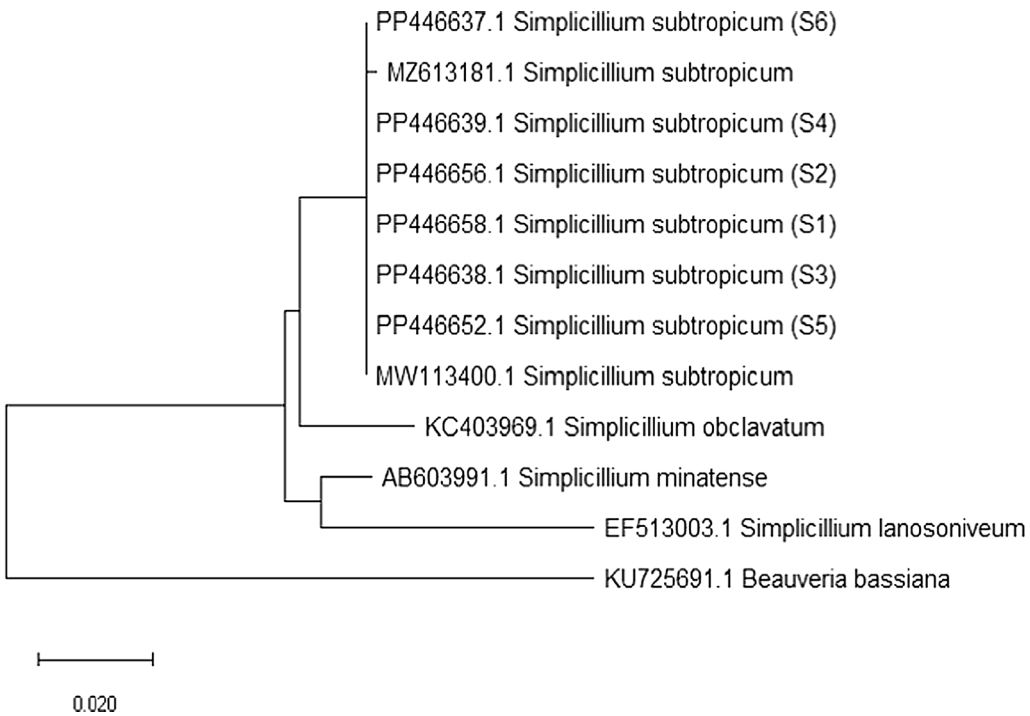

Fig. 2 Phylogenetic tree comparing the isolates S1, S2, S3, S4, S5 and S6 to other isolates in the NCBI database with *Beauveria bassiana* as an out-group

Table 3 Effect of different *Simplicillium* isolates on the third instar nymph of *Aphis gossypii*

Isolates	Percent mortality (days after treatment)*		
	3	5	7
S1 (PP446658)	0.00 (0.00)^{c}	5.00 (12.74)^{e}	9.17 (17.44)^{e}
S2 (PP446656)	0.00 (0.00)^{c}	7.50 (15.83)^{de}	11.67 (19.92)^{e}
S3 (PP446638)	0.83 (2.63)^{bc}	13.33 (21.01)^{c}	30.00 (33.19)^{c}
S4 (PP446639)	2.50 (6.37)^{b}	20.00 (26.53)^{b}	43.33 (41.16)^{b}
S5 (PP446652)	0.00 (0.00)^{c}	9.17 (17.57)^{cd}	19.17 (25.91)^{d}
S6 (PP446637)	6.67 (14.72)^{a}	26.67 (31.07)^{a}	58.33 (49.80)^{a}
Control	0.00 (0.00)^{c}	0.00 (0.00)^{f}	0.00 (0.00)^{f}
SE(d)	2.587	1.823	1.311
CD (0.05)	5.434	3.830	2.754

*@1×10^{8} spores ml^{-1}. All values are mean of four replications ($n = 30$). Figures in parentheses are arcsine transformed values. Values sharing same alphabets in superscript are statistically on par based on LSD ($P < 0.05$)

Table 4 Concentration-mortality responses of *Aphis gossypii* treated with different spore concentrations of S6 (PP446637)

Treatments (spores ml^{-1})	Percent mortality (days after treatment)		
	3	5	7
10^{8}	7.50 (15.83)^{a}	28.33 (32.15)^{a}	59.17 (50.29)^{a}
10^{7}	1.67 (5.26)^{b}	20.83 (27.11)^{b}	51.67 (45.96)^{b}
10^{6}	0.00 (0.00)^{c}	9.17 (17.57)^{c}	18.33 (25.24)^{c}
10^{5}	0.00 (0.00)^{c}	5.83 (13.85)^{c}	10.00 (18.31)^{d}
10^{4}	0.00 (0.00)^{c}	2.50 (7.89)^{d}	6.67 (14.72)^{e}
Control	0.00 (0.00)^{c}	0.00 (0.00)^{e}	0.00 (0.00)^{f}
SE(d)	1.824	1.879	1.646
CD (0.05)	3.831	3.946	3.457

All values are mean of four replications ($n = 30$). Figures in parentheses are arcsine transformed values. Values sharing same alphabets in superscript are statistically on par based on LSD ($P < 0.05$)

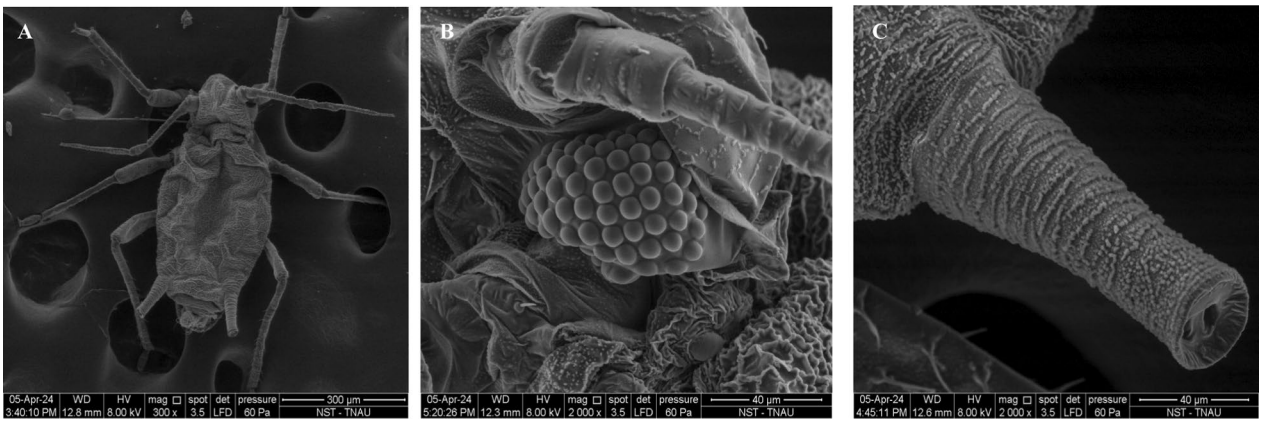

Fig. 3 Scanning electron microscope images of healthy third instar nymphs of *Aphis gossypii*. **A** Full view of healthy aphid. **B** Closeup of head region showing compound eye and antenna. **C** Closeup view of cornicle

Ultramicroscopic changes in *A. gossypii* during *S. subtropicum* infection

SEM images of healthy 3rd instar nymphs of *A. gossypii* were compared to treated nymphs at 24, 48 and 72 h post-infection (hpi). SEM images of aphid with no signs of infection are presented in Fig. 3. At 24 hpi, adhesion of conidia can be seen on the body region, legs, antennae and cornicles. Also, formation of germ tubes and appressoria by some adhered conidia were also observed as shown in Fig. 4. At 48 hpi, development of hyphae was observed in many parts of the body and also they started penetrating the cuticle (Fig. 5). At 72 hpi, extrusion of hyphae from different body regions can be seen. The mycelium coming out of the cuticle, overgrown and covered the entire body region and mycelial network bearing conidia were observed in different parts including legs and antennae (Fig. 6).

Discussion

Entomopathogenic fungi were widely used as biocontrol agents for the pest management throughout the world. Also, EPF of aphids were safer and a better alternatives to insecticides (Kim et al. 2001). But most of the commercial products of EPF face the problem of non-adaptability to different agro-ecological conditions. This can only be overcome by isolating EPF from local sites which are adapted and can perform well in that particular environmental condition. Accordingly, out of 94 soil samples collected from various regions of Tamil Nadu, 6 local isolates of *S. subtropicum* were isolated using serial dilution method. Nonaka et al. (2012) also isolated 18 species of *Simplicillium* including *S. subtropicum*, *S. aogashimaense, S. cylindrosporum, S. obclavatum* and *S. sympodiophorum* from the soil samples in Japan using serial dilution method.

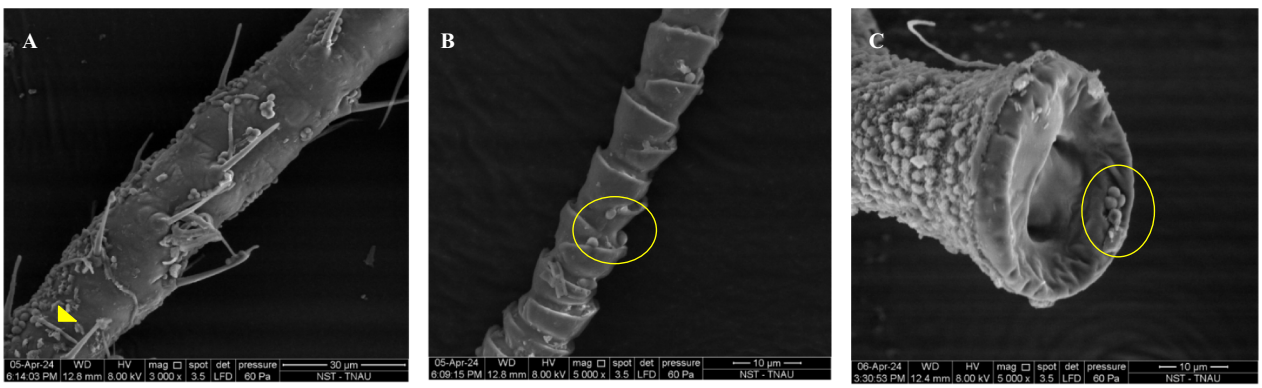

Fig. 4 Scanning electron microscope images of third instar nymphs of *Aphis gossypii* infected with *Simplicillium subtropicum* (24 hpi). **A** Cluster of conidia adhered to the leg region and arrowhead indicating the conidia with germ tube. **B** Conidia adhered to the antennal region (circle). **C** Adherence of conidia on the cornicle (circle).

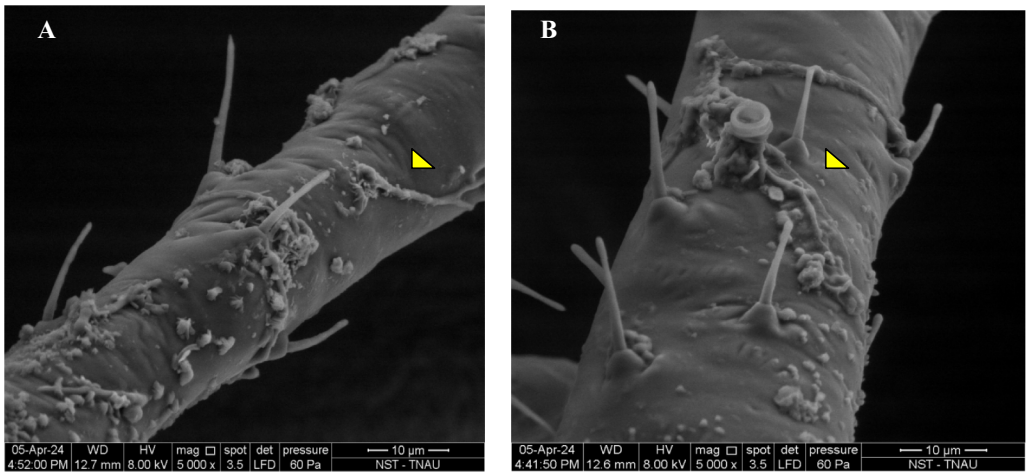

Fig. 5 Scanning electron microscope images of third instar nymphs of *Aphis gossypii* infected with *Simplicillium subtropicum* (48 hpi). **A**, **B** Developing hyphal clusters on the leg region and arrowheads showing the hyphae penetrating the cuticle

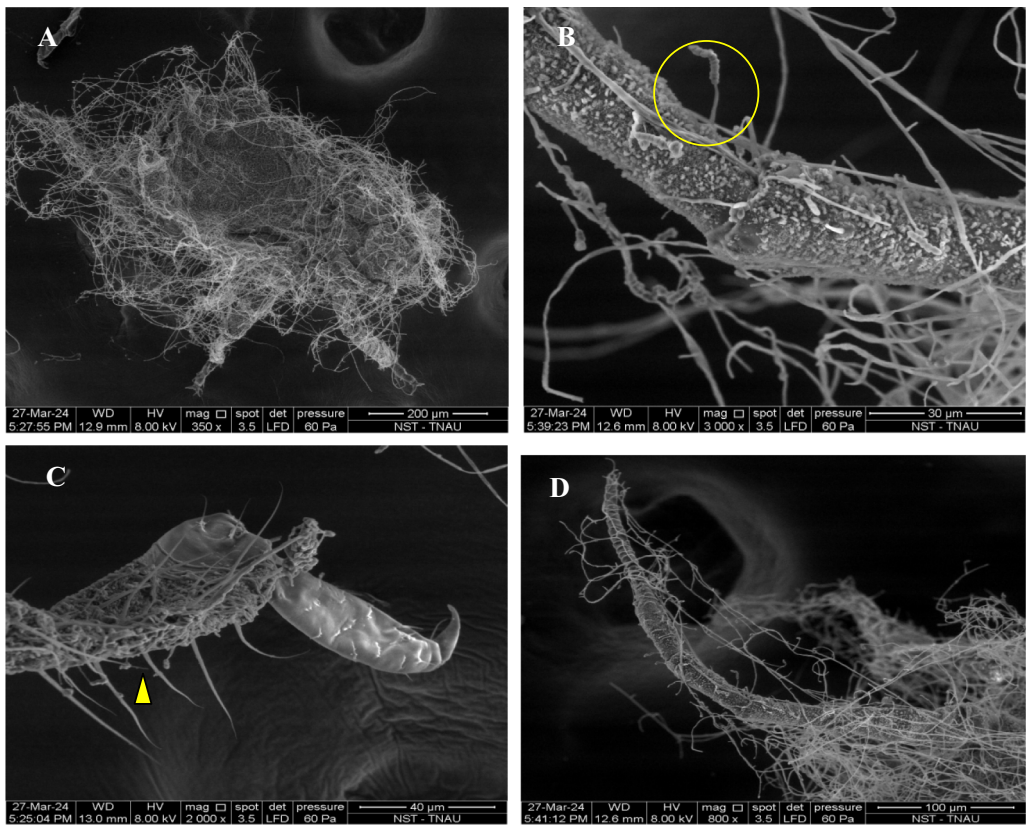

Fig. 6 Scanning electron microscope images of third instar nymphs of *Aphis gossypii* infected with *Simplicillium subtropicum* (72 hpi). **A** Lateral view of mummified cadaver. **B** Leg region covered with mycelium producing conidia (circle). **C** Extruding hyphae with conidia in leg region (arrowheads). **D** Antennal region covered with mycelial network

Morphological characterization of the isolates revealed that their colony color was white in the front side and brown in the reverse side of the plates. The texture of the colony was floccose with raised elevation. Growth rate per day and radial growth of the colony on 15th day after inoculation ranged from 1.41 to 2.41 mm and 50.88

to 59.50 mm, respectively. Bekele (2022) also mentioned that *Simplicillium* sp. colony was whitish with floccose aerial mycelium and grown up to 30–40 mm diameter in PDA after 10 days after inoculation. Coming to microscopic parameters, all isolates had solitary phialides and globose-shaped conidia with the size of 1.829–2.090 µm diameter and sporulation rates ranged from 0.42×10^8 to 1.68×10^8 spores/ml. Among the isolates, S6 (TNAU CBE 3) showed highest growth rate, radial growth and sporulation rates. This is supported by Nonaka et al. (2012) who stated that *S. subtropicum* produces solitary phialides and subglobose to ellipsoidal conidia.

For molecular identification, 18S rRNA genes of the isolates were amplified using primers, ITS-1 and ITS-4 in approximately 560 bp and the accession numbers were obtained. This is in line with Baró Robaina et al. (2024), who amplified 28S rDNA region of *S. lanosoniveum* isolate at 505 bp. The phylogenetic tree constructed at 1000 bootstraps replication verified the evolutionary relationship of the study isolates with other isolates in the NCBI database.

Pathogenicity studies on the *S. subtropicum* isolates against *A. gossypii* revealed that they caused percent mortality of 0.00 to 6.67% on 3 DAT; 5.00 to 26.67% on 5 DAT and 9.17 to 58.33% on 7 DAT. This indicates that *S. subtropicum* can infect *A. gossypii* on 3 DAT based on their virulence. The isolate S6 (PP446637) recorded the highest percent mortality of 6.67, 26.67 and 58.33% on all the days observed and selected for further studies. The median lethal concentration (LC_{50}) and median lethal time (LT_{50}) of S6 (PP446637) were found to be 2.36×10^7 at 7 DAT and 160.20 h at concentration of 1×10^8 conidia/ ml, respectively.

Entomopathogenic nature of the genera *Simplicillium* was reported by many in earlier studies including Chen et al. (2017) (*S. lanosoniveum* against aphids), Skaptsov et al. (2017) (*S. lanosoniveum* against *Coccus hesperidum*), Sujithra et al. (2021) (*S. lanosoniveum* against *Aleurodicus rugioperculatus*) etc. Also many insect associated species like *S. coccinellidae, S. hymenopterorum*, *S. neolepidopterorum*, *S. scarabaeoidea, S. coleopterorum, S. guizhouense*, and *S. larvatum* were studied by Chen et al. (2022). Especially, Chen et al. (2017) reported that *S. lanosoniveum* isolate Cs0701 had high virulence against *A. gossypii* and *Ceratovacuna lanigera.* But data on the pathogenicity of *S. subtropicum* to *A. gossypii* is lacking. So, for the better understanding of their interaction, ultramicroscopic changes in *A. gossypii* during *S. subtropicum* infection were studied using SEM.

SEM images of *A. gossypii* treated with *S. subtropicum* isolate S6 (PP446637) revealed that the adhesion and germination of conidia of the fungus on the insect cuticle and formation of appressoria, a structure responsible for penetration (Lopez-Llorca and Claugher 1990) occurs at 24 hpi. At 48 hpi, the hyphae started growing over the cuticle and penetrated them. Extrusion of hyphae from the insect body and formation of new conidia (conidiogenesis) occurred at 72 hpi, covering entire body, giving mummified appearance. The results were supported by Ganassi et al. (2010) who also observed the initial phase of infection by *L. lecanii* on the aphid *Schizaphis graminum* within 24 h of treatment. Also, within 72 h after treatment, hyphal development and conidiogenesis were recorded. Similar trends were also observed under SEM by Maketon et al. (2013) during the infection of *B. bassiana* on different aphid species and Haron et al. (2020) on *S. graminum* treated with *B. bassiana.*

Conclusion

Indigenous *S. subtropicum* were isolated from soils of Tamil Nadu, India, using serial dilution method. Further, morphological and molecular characterizations were performed for species confirmation. Laboratory bioassays revealed that the isolate S6 (PP446637) performed well against *A. gossypii* among the other isolates. Also, ultramicroscopic changes in the *A. gossypii* during infection by *S. subtropicum* were studied using SEM. This study evaluated the potential of indigenous *S. subtropicum* isolates to be used for the biocontrol of *A. gossypii* and further studies to test their potential against *A. gossypii* and other aphid species under laboratory as well as field conditions were recommended.

Abbreviations

PDA	Potato dextrose agar
CTAB	Cetyltrimethylammonium bromide
ITS	Internal transcribed spacer
DAT	Days after treatment
LC_{50}	Median lethal concentration
LT_{50}	Median lethal time
SEM	Scanning electron microscope
EPF	Entomopathogenic fungi
hpi	Hours post-infection

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Author contributions

KM carried out the research experiments and produced the manuscript. JNS, NS, SU, MS and PK provided the technical guidance and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data associated with this study has been deposited at NCBI GenBank Database under the Accession Numbers PP446658, PP446656, PP446638, PP446639, PP446652 and PP446637.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All the authors give their consent to publish the submitted manuscript as "Original paper" in EJBPC.

Competing interests
The authors declare that they have no competing interests.

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