



MOLECULAR CHARACTERIZATION AND BIOCONTROL POTENTIAL OF *Ampelomyces quisqualis* AGAINST POWDERY MILDEW (*Golovinomyces cichoracearum*) IN SUNFLOWER

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ABSTRACT

Powdery mildew is a common disease in sunflower ecosystems, particularly during the flowering stage, and is caused by the obligate pathogen *Golovinomyces cichoracearum* (*G. ambrosiae*). The disease produces powdery growth on leaves, petioles, and flowers, leading to significant seed and oil yield losses. In present study, a mixed population of *G. cichoracearum* and *Podosphaera xanthii* was observed, with *G. cichoracearum* being predominant. To manage powdery mildew, twenty-one naturally occurring mycoparasitic fungi were isolated from different host plants. Based on culturo-morphological characteristics, ten isolates resembling *Ampelomyces* spp. were selected for further study. Under laboratory conditions, these isolates produced pycnidia within 7 days. Molecular characterization using ITS (18S and 28S rDNA regions) revealed genetic diversity among the isolates. Pycnidiospore concentration of 10^8 spores mL^{-1} effectively parasitized the conidia and conidiophores of *G. cichoracearum* *in vitro*. Among the isolates, HP 003 (*A. quisqualis* AQ 003; NCBI accession ON502948) showed superior pycnidial production and faster mycelial growth, resulting in reduced disease severity under field conditions. The mycoparasitic activity was associated with the production of hydrolytic enzymes and secondary metabolites. GC-MS analysis identified 39 compounds, including 2-methylfuran and 3-pentanol, which are implicated in metabolic pathways and plant defense responses. These findings highlight the potential of *A. quisqualis* AQ 003 as an eco-friendly biocontrol agent against powdery mildew disease in sunflower crop.

Keywords: *Ampelomyces*, powdery mildew, mycoparasite, sunflower

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is an important oilseed crop, serving as a major source of edible oil. In India, it is cultivated over 1.83 million ha area, with an annual production of 1.25 million t and the average productivity of 683 kg ha^{-1} (www.indiastat.com). The crop is predominantly grown under rain-fed conditions, often without seed treatment, making it highly vulnerable to various biotic and abiotic stresses. Among these, diseases caused by fungi, bacteria, and viruses significantly affect crop performance, leading to an estimated seed yield loss of 25-40% (Parkash *et al.*, 2021). One of the most prominent foliar diseases affecting sunflower is powdery mildew, caused by *Golovinomyces cichoracearum* (DC) 1805. This disease has become increasingly common in major sunflower-growing regions of India, adversely affecting the commercially cultivated varieties and hybrids (Sujatha *et al.*, 2016). The pathogen thrives under conditions of moderate temperature (20-25°C) and high humidity. Infection typically begins on lower mature leaves and spreads upward, producing a characteristic white powdery appearance due to the formation of conidia. In

severe cases, the infection can extend to stems, flowers, and heads. Outbreaks during flowering stage are especially damaging, as they interfere with normal plant growth and result in significant seed yield reductions. To manage powdery mildew effectively, an integrated approach is recommended, including the adoption of cultural practices, application of appropriate fungicides, and the use of resistant hybrids (Sudisha *et al.*, 2010).

Excessive and repeated application of fungicides with identical modes of action has led to the development of resistance in powdery mildew pathogens (Rallos and Baudoin, 2016; Csikós *et al.*, 2020; Pintye *et al.*, 2020). Furthermore, these chemical treatments may exert deleterious effects on plant physiology and contribute to the decline of biodiversity (Petit *et al.*, 2012). In response to these limitations, alternative plant protection strategies have increasingly been investigated (Geiger *et al.*, 2010). Among these, the use of biological control agents represents a promising and sustainable approach. However, the efficacy of biocontrol agents is influenced by host characteristics, environmental conditions, and the intrinsic potential of microbial agents employed (Montesinos and Bonaterra, 2009).

The inconsistent performance of biocontrol agents under field conditions against powdery mildew has prompted the search for novel and more effective microbial candidates for use in commercial agriculture. In this context, the hyperparasite *Ampelomyces quisqualis* Ces. 1852, a pycnidial fungus widely distributed across the various members of Erysiphaceae (Angeli *et al.*, 2012), has attracted considerable attention. The pycnidiospores of *A. quisqualis* germinate on the host plant surface and penetrate nearby powdery mildew hyphae, subsequently growing intracellularly from cell to cell through septal pores. This colonization leads to the destruction of infected mildew mycelium and the formation of new intracellular pycnidia within 5-7 days after penetration (Hashioka and Nakai, 1980; Sundheim and Krekling, 1982). Furthermore, conidia produced within these intracellular pycnidia can persist in infected leaf tissues until the following season, thereby contribute to the overwintering of fungus (De Bary, 1870; Yarwood, 1939).

Plants possess inherent defense mechanisms against pathogenic infections, which may be further enhanced through the application of specific compounds or beneficial microorganisms (Walters *et al.*, 2009). In this context, the present study was undertaken to identify an efficient *Ampelomyces* isolate for the management of powdery mildew infection in sunflower. Accordingly, the objectives of this research were formulated to characterize powdery mildew infection in sunflower, and isolate and characterize naturally occurring mycoparasitic fungi as well as evaluate their efficacy under field conditions.

MATERIALS AND METHODS

Morphological characterization

The leaves of sunflower exhibiting characteristic powdery patches were collected individually in butter paper bags from experimental fields of the Department of Oilseeds, TNAU, Coimbatore (India) during the period July to October, 2021. Morphological characterization was carried out by examining the shape and structural features of conidia and conidiophores using a phase contrast microscope (GT12 Leica DM 2000 LED). In addition, transverse sections of infected leaves were prepared to facilitate the detailed observation of conidiophores and conidia.

Molecular characterization of powdery mildew

Genomic DNA was extracted from powdery mildew-infected sunflower leaves following the CTAB method (Knapp and Chandlee, 1996). Mycelial growth was carefully scraped from the infected leaf surfaces and transferred into sterile centrifuge tubes containing 1 mL 2% CTAB extraction buffer [10 mM tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB, 0.1% β -mercapto-ethanol, and 0.2% PVP]. The samples were incubated at 65°C for 1 h. An equal volume of chloroform: isoamyl alcohol (24:1) was then added to each tube, mixed thoroughly by vortexing, and centrifuged

at 12,000 rpm for 5 min to separate the phases. The aqueous supernatant was carefully transferred to fresh sterile tubes and mixed with an equal volume of ice-cold isopropanol to precipitate the DNA, followed by incubation at 25°C. The DNA precipitate was recovered by centrifugation, and the resulting pellet washed with 0.1 M ammonium acetate in 70% ethanol and incubated for 15 min. The pellets were then air-dried and resuspended in TE buffer (10 mM tris-HCl, 1 mM EDTA, pH 8.0). Finally, DNA concentration and purity were determined spectrophotometrically (Eppendorf AG).

PCR amplification was performed using the powdery mildew-specific primers, PMITS1 (5'-TCGGACTGGCCYAGGGAGA-3')/PMITS2 (5'-TCACTCGCCGTTACTGAGGT-3') (Cunnington *et al.*, 2003), *Erysiphe*-specific primer pair EryF (5'-TACAGAGTGCGAGGCTCAGTCG-3')/EryR (5'-GGTCAACCTGTGATCCATGTGACTGG-3') (Parthasarathy *et al.*, 2017), *G. cichoracearum*-specific primer G1 (5'-TCCGTAGGTGAACCTGCGGAAGGAT-3')/G2 (5'-CAACACCAAACCA CACACACGGCG-3') (Hirata *et al.*, 2000), *Podosphaera xanthii*-specific primers S1 (5'-GGATCA TTAGTGAGCGCGAGGCCCCG-3')/S2 (5'-CGCCGCCCTGGCGCGAGATACA-3') and *Leveillula taurica*-specific primers L1 (5'-CCCTCCACCCGTGTCGACTCGTCTC-3')/L2 (5'-CTGCGTTTA AGAGCCGCCGCGCCGAA-3') (Khodaparast *et al.*, 2001), with PCR conditions of 5 min of denaturation at 94°C; followed by 35 cycles consisting of 40 sec at 94°C, 60 sec at 62°C and 1.5 min at 72°C; and a final extension period of 6 min at 72°C. Furthermore, the extracted DNA samples were subjected to multiplex PCR amplification using a cocktail of primer pairs S1/S2, G1/G2, EryF/Ery R and L1/L2 with each sample analysed individually. This approach enabled the detection and differentiation of the powdery mildew pathogens *Golovinomyces cichoracearum* and *Podosphaera xanthii* infecting sunflower, following the protocol of Chen *et al.* (2008). The amplified PCR products obtained with the primer pair PMITS1/PMITS2 were purified using the QIAquick Gel Extraction kit (28704). The purified amplicons were subsequently sequenced (Bioserve Biotechnologies Pvt. Ltd., Hyderabad (India) and the resulting sequences were submitted to the NCBI database.

Mycoparasite isolation and characterization

Powdery mildew-infected leaves exhibiting altered colouration of powdery growth on host surface were collected for the isolation of mycoparasitic fungi. The mildew colonies showed a distinct change in colour from white to grey, depending on the extent of mycoparasitic infection. The presence of pycnidia and pycnidiospores was confirmed through observations under a stereozoom microscope (Labomed CZM4) and a phase contrast microscope (GT12 Lieca DM 2000 LED).

A total of twenty-one mycoparasitic isolates were obtained from the naturally infected powdery mildew samples collected from different host plants, including sunflower, sesame, *Clitoria*, *Euphorbia hirta*, *Phyllanthus niruri*, tamarind, and *Andrographis alata*. Morphological identification of pycnidia and pycnidiospores was carried out using a phase contrast microscope. Following confirmation, pycnidiospore suspensions were prepared in sterile water and plated onto Czapek Dox agar (CDA) medium supplemented with 0.5% chloramphenicol and carrot juice extract under aseptic conditions. The pure cultures were maintained on CDA medium at room temperature (25 ± 2°C) and sub-cultured at intervals of 6-8 weeks. Among the 21 isolates, ten strains exhibiting morphological similarity to *A. quisqualis* were selected for detailed characterization. These isolates were evaluated for colony topography, mycelial characteristics (septation and pigmentation), growth pattern, zonation, colony colour, and margin, with observations recorded at 15 days after inoculation. Each isolate was cultured individually by placing a disc of actively growing mycelium at the center of a Petri-plate containing CDA medium and incubated at room temperature.

Molecular characterization of mycoparasites

Ten isolates of *A. quisqualis* were cultured individually in 250 mL Erlenmeyer flasks containing 150 mL potato dextrose broth and incubated at room temperature for 14 days. The resulting mycelial biomass were harvested separately by filtration through sterile filter paper. Approximately 1 g mycelium from each isolate was ground into a fine powder using liquid nitrogen, and genomic DNA was extracted following the CTAB method (Knapp and Chandlee, 1996).

The internal transcribed spacer (ITS) region and large subunit (LSU) region of all 10 isolates were

amplified using primer combinations ITS1, ITS2, ITS4, and ITS5 (White *et al.*, 1990), along with LSU primers LR0R, LR5, LR07, and LR7 (Vilgalys and Hester, 1990). Additionally, species-specific primers AQF264/AQR462 (Tollenaere *et al.*, 2014) and AQ1/AQ2 were employed to amplify the mycoparasitic isolates. PCR amplification was performed in a 25 µL reaction mixture containing 50 ng template DNA, 5 mM of each dNTP, 20 pmol of each forward and reverse primer, and 0.5 U Taq DNA polymerase, under the conditions as under:

Primer name	Description (0.05 µmole)	PCR conditions	
ITS 1/ ITS 2	TCCGTAGGTGAACCTGCGG/ GCTGCGTTCTTCATCGATGC	Initial denaturation	95°C 2 min
		Denaturation	95°C 30 sec
		Annealing	55°C 30 sec
		Extension	72°C 60 sec.
		Final extension	72°C 10 min
ITS 5/ ITS 4	GGAAGTAAAAGTCGTAACAAGG/ TCCTCCGCTTATTGATATGC	Initial denaturation	94°C 2 min
		Denaturation	93°C 60 sec
		Annealing	53°C 2 min
		Extension	72°C 2 min
		Final extension	72°C 10 min
ITS 1/ ITS 4	TCCGTAGGTGGACCTGCGG/ TCCTCCGCTTATTGATATGC	Initial denaturation	94°C 5 min
		Denaturation	94°C 30 sec
		Annealing	52°C 40 sec
		Extension	72°C 90 sec
		Final extension	72°C 10 min
LR 0R/ LR 7	TACTACCACCAAGATCT/ AGATCTTGGTGGTAGTA	Initial denaturation	94°C 5 min.
		Denaturation	94°C 60 sec.
LR0R/ LR 5	ACCCGCTGAACTTAAGC/ TCCTGAGGGAAACTTCG	Annealing	50°C 60 sec.
		Extension	72°C 2 min.
		Finalexension	72°C 10 min
AQ1F/ AQ1R	ATGGTAGCTTCGCGGTAATG/ GCAGGTAAACAGCAGGAG TAA	Initial denaturation	95°C 5 min
		Denaturation	95°C 30 sec
		Annealing	55 °C 40 sec
		Extension	72°C 90 sec
		Finalexension	72°C 10 min
AQF 264/ AQR 462	GATGAAGAACGCAGCGAAAT/ GCTGCCAATTGCTTTGAGAT	Initial denaturation	95°C 10 min
		Denaturation	95°C 30 sec
		Annealing	60°C 30 sec
		Extension	72°C 30 sec
		Final extension	72°C 5 min

Among the ten isolates, the PCR product of fast-growing isolate AQ 003 was purified using QIAquick gel extraction kit (28704) and subsequently sequenced (Bioserve Biotechnologies (I) Pvt. Ltd., Hyderabad, India). The obtained sequences were subjected to BLAST analysis in NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Nucleotides>) for identification, followed by phylogenetic analysis using MEGA version 11.0 software (Tamura *et al.*, 2021). Phylogenetic trees were constructed using the maximum likelihood method (Tamura *et al.*, 2021), and the sequences were deposited in the NCBI database.

***In vitro* evaluation of mycoparasitic activity**

The mycoparasitic potential of *Ampelomyces quisqualis* isolate AQ 003 against the powdery mildew pathogen was assessed under *in vitro* using cavity slide and detached leaf assays.

Preparation of powdery mildew conidial suspension: Fresh conidia of *G. cichoracearum* were collected from young infected sunflower leaves. A conidial suspension (10^8 spores mL⁻¹) was prepared by immersing approximately 15 infected leaf segments in 25 mL sterile distilled water in a 50 mL centrifuge tube. The tube was shaken for 1 min to release the conidia, after which the leaf segments were

removed. The presence of conidia in the suspension was confirmed using a phase contrast microscope.

Pathogenicity test: The pathogenicity of *G. cichoracearum* was confirmed by spraying the conidial suspension (10^8 spores mL⁻¹) onto 28 day old sunflower plants (10 plants) grown under glasshouse conditions. Control plants were sprayed with sterile distilled water. Development of characteristic powdery growth on inoculated leaves confirmed pathogenicity. The experiment was conducted in a completely randomized design with three independent replications.

Preparation of pycnidiospore suspension: The *A. quisqualis* isolate AQ 003 was cultured on Czapek Dox agar plates and incubated at $25 \pm 2^\circ\text{C}$ for 15 days. Pycnidiospores were harvested by adding 1 mL sterile distilled water to the culture plates and gently dislodging the spores. A standardized suspension (10^8 spores mL⁻¹) was prepared for further assays.

a) Cavity slide method: Cavity slide assays were conducted by placing slides inside Petri-plates and applying three treatments: i) powdery mildew conidial suspension alone (10^8 spores mL⁻¹), ii) a combination of powdery mildew conidia and *A. quisqualis* pycnidiospores (10 μL each), and iii) *A. quisqualis* pycnidiospores alone. Each treatment was replicated four times. The plates were incubated at $20 \pm 1^\circ\text{C}$ under dark conditions for 24 h. Germination of powdery mildew conidia and evidence of mycoparasitism were observed at 6 h intervals using a phase contrast microscope (Deshmukh *et al.*, 2019).

b) Detached leaf assay: Healthy and powdery mildew-infected sunflower leaves were collected from the glasshouse and placed in 150 mm Petri plates containing mannitol sucrose medium. Three treatments were established: i) healthy leaves (control), ii) infected leaves without treatment (control), and iii) infected leaves treated with *A. quisqualis*. In treatment group, 100 μL pycnidiospore suspension (10^8 spores mL⁻¹) of isolate AQ 003 was applied to the infected leaves. Each treatment was replicated five times. All plates were incubated at $20 \pm 1^\circ\text{C}$ and monitored regularly. Mycoparasitic activity was assessed using a phase contrast microscope and scanning electron microscopy (Deshmukh *et al.*, 2019).

Glasshouse study

In addition to *in vitro* assays, the mycoparasitic efficacy of *A. quisqualis* isolate AQ 003 was evaluated under glasshouse conditions using 45 day old sunflower plants. The experiment consisted of three treatments: i) healthy plants without inoculation (control), ii) plants inoculated with *G. cichoracearum* conidia alone, and iii) plants inoculated with both powdery mildew conidia and *A. quisqualis* spore suspension. Each treatment included five plants and was replicated three times. Powdery mildew infection was established by spraying a conidial suspension (10^8 spores mL⁻¹) on plants in treatments ii) and iii). Following the appearance of disease symptoms, a spore suspension of *A. quisqualis* (10^8 spores mL⁻¹) was applied at a concentration of 1.0% to the plants in treatment iii. The effectiveness of *A. quisqualis* in suppressing powdery mildew was evaluated by calculating the percent disease index (PDI) according to the method described by Wheeler (1969).

Enzymatic activities in culture media

The enzymatic activities of *A. quisqualis* isolate AQ 003 were evaluated by culturing the fungus on specific indicator media, and activity was determined based on the formation of characteristic zones or colour changes surrounding the mycelial growth. All the assays were conducted independently in CRD and repeated five times.

Peroxidase and polyphenol oxidase activities: These activities were assessed by inoculating *A. quisqualis* (AQ 003) onto potato dextrose agar (PDA) medium and incubating for 96 h. Following incubation, 0.5% pyrogallol and 0.5% hydrogen peroxide were added to detect peroxidase activity, while 0.5% pyrogallol alone was used for polyphenol oxidase assay. The plates were further incubated at 28°C for 24 h, and the development of a brown colouration at the colony margin indicated positive enzyme activity (Gramss *et al.*, 1998).

Cellulase activity: Cellulase activity was determined by inoculating a mycelial disc onto minimal agar medium supplemented with 1% carboxymethyl cellulose (CMC) and incubating for 7 days. The plates were then flooded with 1% Congo red solution for 15 min, followed by washing with distilled water and treatment with 1 M NaCl for 15 min. The appearance of a yellowish opaque zone against a red background indicated cellulase activity (Hankin and Anagnostakis, 1975).

Amylase and xylanase activities: These activities were evaluated using a basal medium containing yeast extract, NH_4NO_3 , KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, supplemented with soluble starch (10 g L^{-1}) for amylase and birch xylan (10 g L^{-1}) along with wheat bran (5 g L^{-1}) for xylanase. The pH of media was adjusted to 6.0. Formation of a clear halo zone around the fungal colony was considered a positive reaction (Peraza-Jiménez *et al.*, 2022).

Laccase activity: Laccase activity was assessed using a modified medium containing fructose, malt extract, yeast extract, KH_2PO_4 , $(\text{NH}_4)_2\text{SO}_4$, MgSO_4 , CaCl_2 , MnSO_4 , ZnSO_4 , and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, adjusted to pH 4.8. The formation of a blue-green oxidation halo around the colonies indicated positive laccase activity (Sivakumar *et al.*, 2010).

Lipase activity: Lipase activity was evaluated using a medium composed of yeast extract, MgSO_4 , glucose, and KH_2PO_4 , supplemented with 2% (v/v) olive oil. The development of an orange fluorescent halo around the colonies was considered indicative of lipase production.

GCMS analysis of secondary metabolites

The isolate *A. quisqualis* AQ 003 was grown in potato dextrose broth (Product DM 1403, Central Drug House Pvt. Ltd.) at room temperature ($\pm 25^\circ\text{C}$) for 15 days and centrifuged at 120 rpm at 25°C . The culture broth was separated from the mycelium through Whatman No. 1 filter paper to obtain the cell-free supernatant. The filtrate was extracted with ethyl acetate in 1:1 ratio and allowed to shake for 2 h in rotary shaking incubator. The ethyl acetate fraction was then condensed with a rotary flask evaporator and dissolved in 10 mL HPLC grade methanol. The methanolic extract was used to analyse secondary metabolite compounds from the isolate AQ003. GCMS analysis was performed using the Pekin Elmer Clarus SQ8C instrument with DB-5 MS capillary standard nonpolar column and the compounds were identified through the NIST Standard Reference Database using CAS registry number (<https://webbook.nist.gov/chemistry/cas-ser/>).

Liquid bioformulation

Molasses yeast broth and Czapek Dox broth was added with the best suitable additive trehalose 10 mM (Parthasarathy, 2018) for the liquid bio-formulation of *A. quisqualis*. 15 days old culture with spore load of 2×10^6 spores mL^{-1} was used as inoculum @ 10 mL L^{-1} . Trehalose was added to enhance the shelf life of formulation and stored in controlled temperature (25°C).

Field study

A field trial was conducted to assess the bioefficacy of liquid formulation of *A. quisqualis* against *G. cichoracearum* along with wettable sulphur as chemical fungicide standard check. Five treatments with four replications were adopted using randomized block design. The treatments were T₁) foliar spray (FS) of *A. quisqualis* @ 1.0% immediately after the occurrence of disease, followed by 2nd and 3rd spray at 15 days interval, T₂) FS of *A. quisqualis* @ 2% immediately after the occurrence of disease, followed by 2nd and 3rd spray at 15 days interval, T₃) FS of *A. quisqualis* @ 3.0% immediately after the occurrence of disease, followed by 2nd and 3rd spray at 15 days interval, T₄) FS of wettable sulphur @ 0.1% immediately after the occurrence of disease, followed by 2nd and 3rd spray at 15 days interval and T₅) untreated control (water spray).

Statistical analysis

Statistical analysis was done using IRRISTAT version 92 developed by the International Rice Research Institute Biometrics Unit, the Philippines (Gomez and Gomez, 1984). Data were subjected to ANOVA at significant level ($P < 0.05$) and means were compared by Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Morpho-molecular characterization of powdery mildew pathogens

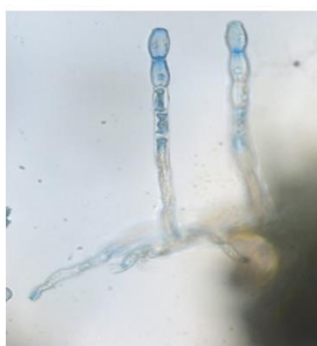
Powdery mildew symptoms in sunflower were initially noticed as white powdery colonies on the upper surface of lower leaves, which progressively spread to the leaf blade, petiole, and stem, causing chlorosis and eventual defoliation under severe infection (Fig. 1). At advanced stages, the superficial mycelium expanded and coalesced, covering the entire aerial plant parts, notably in flowering stage. This disease progression pattern corroborates earlier reports indicating that powdery mildew causes premature senescence and significant yield losses under tropical conditions (Vipin Parkash *et al.*, 2021).

Microscopic examination revealed the presence of two distinct conidial morphotypes (Fig. 2). Molecular characterization using species-specific primers differentiated the pathogens. Sample SPM1 produced a 454 bp amplicon corresponding to *Podosphaera xanthii*, while SPM2 yielded a 418 bp fragment specific to *Golovinomyces cichoracearum*. No amplification was observed for *Erysiphe* sp. or *Leveillula taurica*, indicating their absence in the sampled population. Multiplex PCR validated the coexistence and distinction of these two species. Sequencing results were deposited in NCBI (Accession No. PP029065 for *P. xanthii* and PP029066 for *G. cichoracearum*) [Fig. 3].

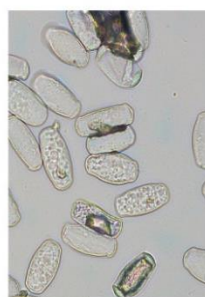
Interestingly, the early-stage infections were predominantly associated with *G. cichoracearum*,



a) Powdery mildew infection on sunflower leaves



b) Conidiophore and conidia of Sunflower powdery mildew



G. cichoracearum



P. xanthii

c) Powdery mildew conidia at 40X in phase contrast microscope,

Fig. 1: Natural occurrence of powdery mildew on sunflower; a) Powdery coatings observed on the upper surface of leaves; b) Conidia and conidiophore structures photographed in phase contrast microscope by lactophenol staining, c) Powdery mildew conidia at 40X in phase contrast microscope, less fibrosin bodies in *G. cichoracearum* and more and prominent fibrosin bodies in *P. xanthii* conidia

whereas severe infections involved mixed populations of both pathogens, suggesting a possible successional or synergistic interaction. Morphologically, *P. xanthii* conidia were ellipsoidal and contained fibrosin bodies in mature stages, whereas *G. cichoracearum* lacked these structures. These observations are in agreement with earlier descriptions (Sujatha *et al.*, 2016). Phylogenetic analysis supported these findings, with *P. xanthii* clustering closely with previously reported isolates, while *Golovinomyces cichoracearum* grouped with *G. ambrosiae* and *G. orontii*. Notably, as per Braun and Cook (2012), some isolates initially classified as *G. cichoracearum* may correspond to *G. ambrosiae*, highlighting the taxonomic complexity within the genus.

Isolation & characterization of mycoparasite (A. quisqualis)

The presence of mycoparasitism was indicated by a distinct colour change in

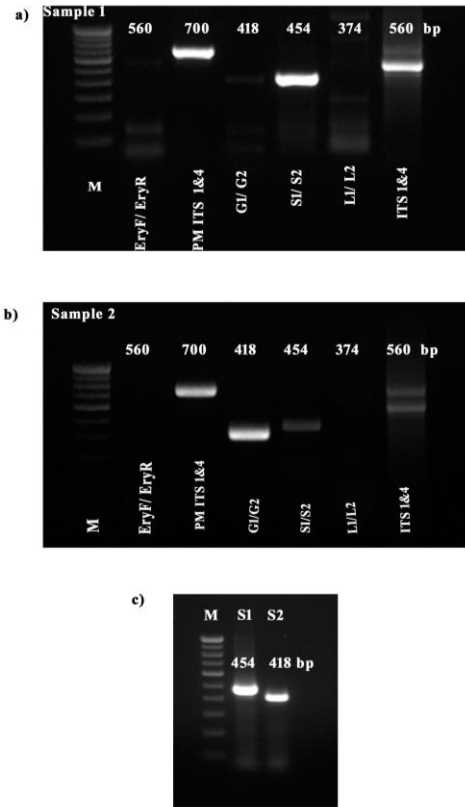


Fig. 2: PCR amplification of powdery mildew infection on sunflower; a & b: Two DNA samples from sunflower powdery mildew were amplified with powdery mildew specific primers (PM ITS - 700 bp; *E. pisi* - 560 bp; G1/G2 - 418 bp; S1/S2 - 454 bp; L1/L2 - 374 bp) and universal primers (ITS - 550 bp) separately; c) Powdery mildew specific primers of S1/S2, G1/G2, EryF/EryR, L1/L2 were used simultaneously to identify the genus from two samples. M - Marker

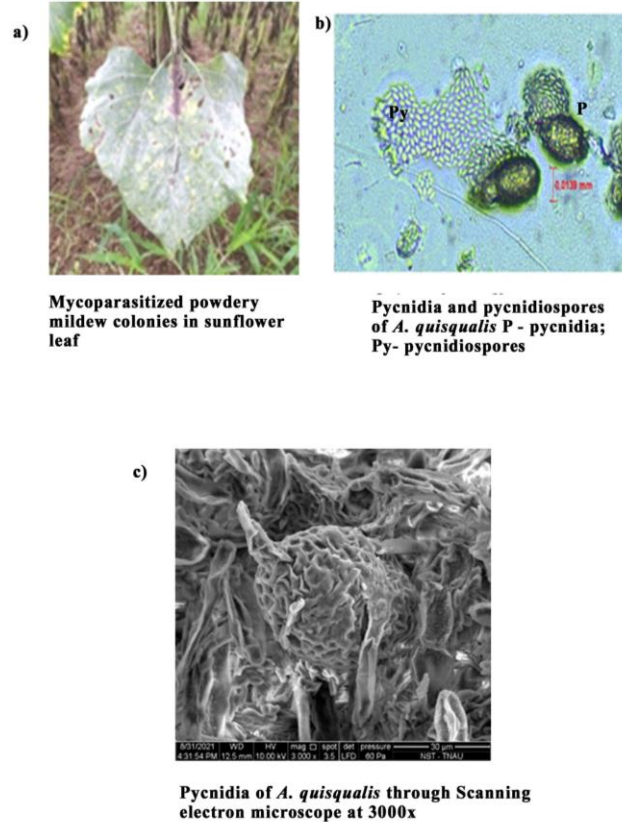


Fig. 3: Pycnidia and pycnidiospores of *A. quisqualis*; a) Mycoparasitized powdery mildew colonies in sunflower leaf; b) Pycnidia and pycnidiospores of *A. quisqualis* were observed under the phase contrast microscope at 40X magnification. c) Pycnidia of *A. quisqualis* emerged through the powdery mildew colony by disintegrating the conidia and conidiophore, Surface view of pycnidia visualized in Scanning electron microscope at 3000x magnification with 30 μ m length, 60 pa pressure.

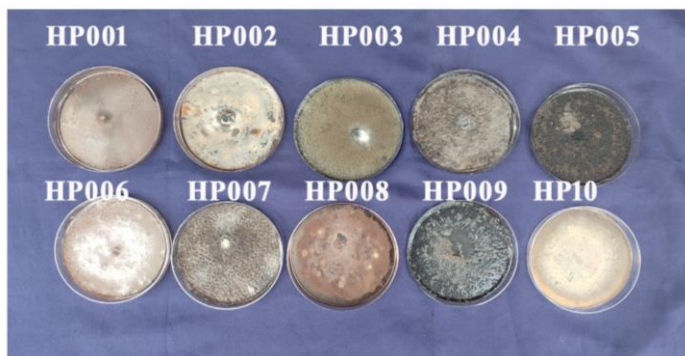


Fig. 4: Isolates of *A. quisqualis*; Morphological variations of ten mycoparasites of powdery mildew pathogen on Czapek Dox agar medium isolated from different hosts

powdery mildew colonies from white to grey on infected leaves (Fig. 4). Microscopic observations confirmed the formation of pycnidia and pycnidiospores typical of *Ampelomyces quisqualis*. The pycnidia were dark brown to black, spindle-shaped, and stipitate, while the pycnidiospores were hyaline, unicellular, and oval to cylindrical, consistent with previous descriptions of the species (Nemeth *et al.*, 2021). Twenty one isolates were obtained from various host plants, of which ten were morphologically and culturally similar to *A. quisqualis*.

Although minor variations were observed among the isolates, all isolates showed septate, hyaline hyphae and colonies with diverse morphologies, including radial, fluffy, and appressed growth patterns, with colour ranging from greyish white to brownish black (Table 1; Fig. 5). Such variability within *Ampelomyces* spp. has been previously reported (Kiss *et al.*, 2004), and may reflect the adaptation to different hosts or environmental conditions.

Table 1: Culturo-morphological characters of *Ampelomyces quisqualis* isolates on 15th day of inoculation on Czapek Dox agar medium

Isolates	Hosts	Powdery mildew pathogens	Colony colour	Zonation	Topography	Margin	Mycelium (septation/ colour)	Mycelial growth (mm)
HP 001	Sesame	<i>E. cichoracearum</i>	Brownish black	Absent	Radial	Smooth	Septate, hyaline	80.0
HP 002	Clitoria	<i>Oidium</i> sp.	Brownish black	Absent	Radial	Smooth	Septate, hyaline	79.0
HP 003	Sunflower	<i>Golovinomyces Cichoracearum</i>	Brownish black	Absent	Radial	Irregular	Septate, hyaline	86.0
HP 004	Amman-pacharisi	<i>Podosphaera xanthii</i>	Greyish white	Present	Fluffy	Irregular	Septate, hyaline	85.0
HP 005	Keelanelli	<i>Oidium</i> sp.	Brown	Absent	Radial	Smooth	Septate, hyaline	83.0
HP 006	Tamarind	<i>Oidium</i> sp.	Brown	Absent	Radial	Irregular	Septate, hyaline	82.0
HP 007	Sunflower	<i>Golovinomyces Cichoracearum</i>	Grey	Absent	Radial	Smooth	Septate, hyaline	80.0
HP 008	Sesame	<i>E. cichoracearum</i>	Grey	Present	Fluffy	Smooth	Septate, hyaline	80.0
HP 009	Siriyanangai	<i>Andrographis paniculata</i>	Grey	Absent	Radial	Smooth	Septate, hyaline	79.0
HP 010	Sesame	<i>E. cichoracearum</i>	Greyish white	Absent	Appressed fluffy	Smooth	Septate, hyaline	74.0

Molecular characterization using ITS and LSU regions yielded consistent amplicon sizes across all the isolates, confirming their fungal identity. Species-specific primers validated their identity as *A. quisqualis*. Among the isolates, strain AQ 003 exhibited highest mycelial growth and pycnidial production. Sequencing of actin gene (Accession No. ON502948) and subsequent phylogenetic analysis revealed that the isolate AQ 003 clustered with the reference strain DSM 222 (MTCC 975), confirming its identity (Fig. 6). Interestingly, the relatively rapid growth of isolate AQ 003 (79-85 mm within 15 days) contrasts with earlier reports describing *Ampelomyces* spp. as slow-growing fungi (Kiss *et al.*, 2004), suggesting strain-specific variability with potential implications for biocontrol efficiency.

Mycoparasitic activity under in vitro and glasshouse conditions

The mycoparasitic potential of *A. quisqualis* isolate AQ 003 was clearly demonstrated in *in vitro* study. In cavity slide assay, pycnidiospores germinated and actively parasitized powdery mildew

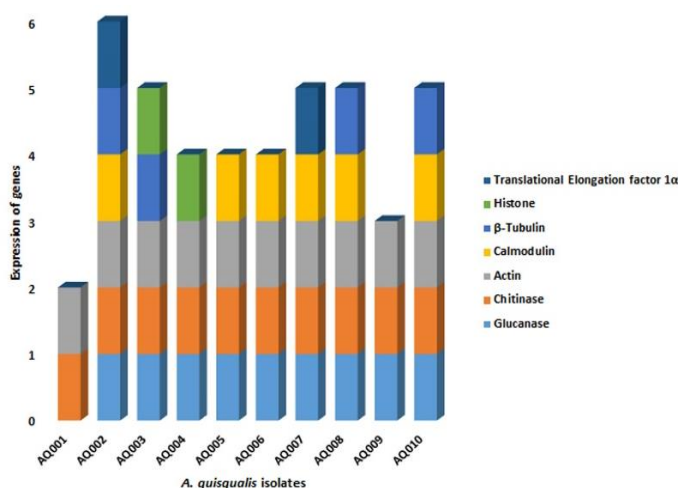


Fig. 5: Expression of defense and housekeeping genes in various isolates of *Ampelomyces quisqualis*

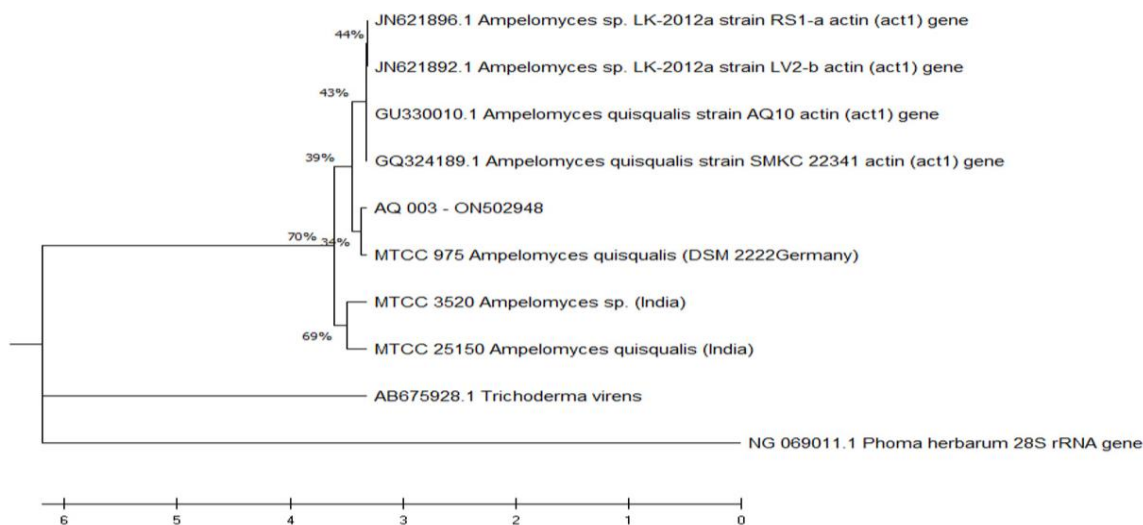


Fig. 6: Phylogenetic relationship based on actin gene sequences in *Ampelomyces quisqualis* isolate AQ 003 – ON502948; The phylogenetic tree was constructed based on maximum likelihood method and Tamura-Nei model. Evolutionary analyses were conducted in MEGA X

conidia, leading to the germination inhibition and structural disruption (Fig. 7). In detached leaf assays, rapid colonization and visible parasitism were observed within 7 days, indicating strong antagonistic capability at room temperature ($25 \pm 2^\circ\text{C}$) [Fig. 8]. Under glasshouse conditions, isolate AQ 003 significantly reduced powdery mildew severity. The plants inoculated with *G. cichoracearum* alone exhibited 55.5% disease incidence, whereas those treated with isolate AQ 003 showed a reduced incidence of 30.25%. The reduction in disease severity may be attributed to the intra-cellular parasitic nature of *A. quisqualis*, which penetrates powdery mildew hyphae, suppresses The mechanism of action involves direct penetration of host hyphae, formation of pycnidia within pathogen structures, and subsequent depletion of nutrients, ultimately limiting pathogenic growth

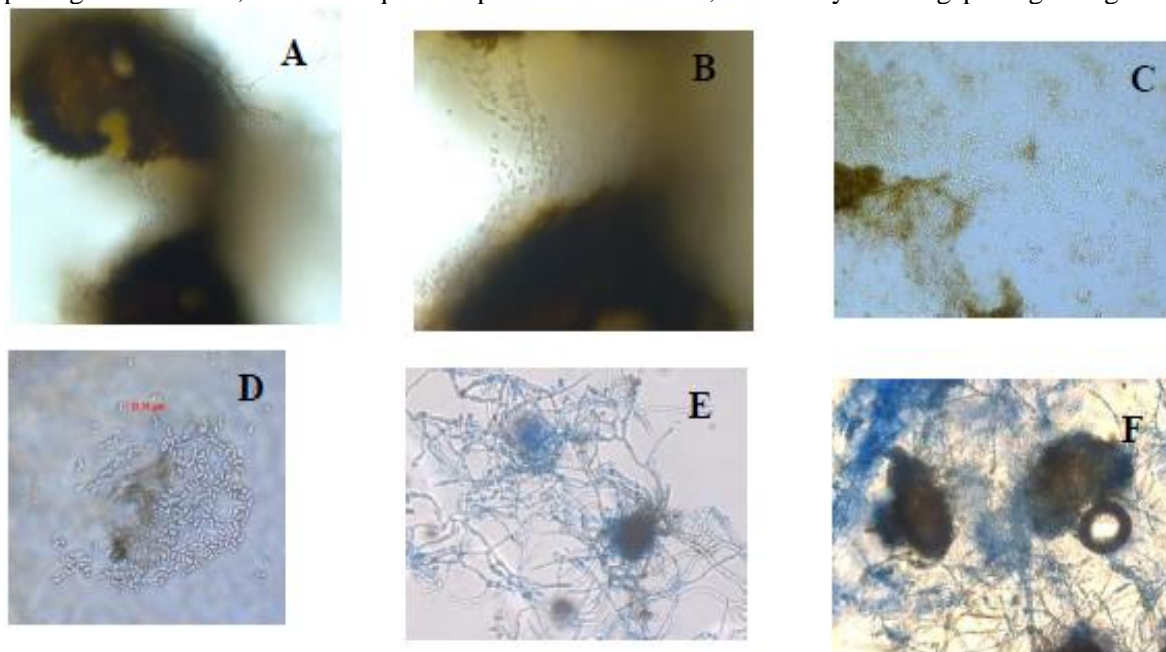


Fig. 7: Germination of *A. quisqualis* AQ 003 in cavity slide; A-C) Pycnidiospores released from the pycnidia viewed through phase contrast microscope A & B - 40X, C - 10X; D) Pycnidiospores at 40X; E) Gemination of pycnidiospores at 40X; F) Pycnidia of *A. quisqualis* AQ 003 at 40X

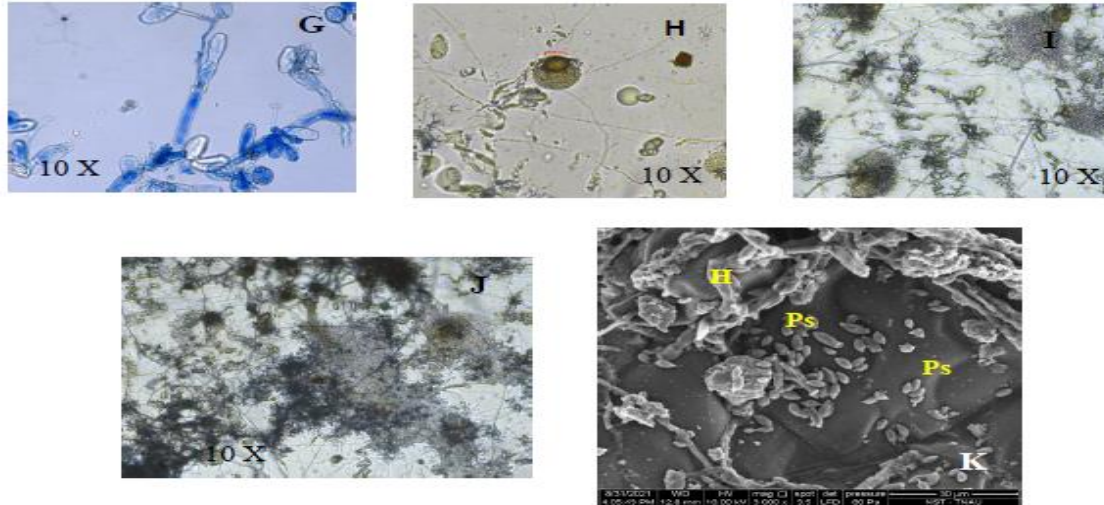


Fig. 8: Mycoparasitism of *Ampelomyces quisqualis* AQ 003 on *G. cichoracearum*; Upper left: Conidia of *G. cichoracearum* on uninoculated control leaves; Upper middle: Release of pycnidiospore from the pycnidia of *A. quisqualis* on treated leaves; Upper right: Hyphal germination of *A. quisqualis*; Lower left: Suppression of *G. cichoracearum* germination on the leaves; Lower right: SEM image of *in vitro* mycoparasitism of *A. quisqualis* pycnidiospores (Ps) on hyphae of powdery mildew (H).

and spread (Sztejnberg *et al.*, 1989; Romero *et al.*, 2003). The absence of conidial development in treated samples further supports the effectiveness of isolate AQ 003 as a mycoparasite.

Enzymatic activities and their role in mycoparasitism

A. quisqualis AQ 003 showed cellulase, amylase, xylanase, and peroxidase activities, while polyphenol oxidase, lipase, and laccase activities were absent (Fig. 9). The production of cellulolytic and hemicellulolytic enzymes is particularly significant, as these enzymes facilitate degradation of

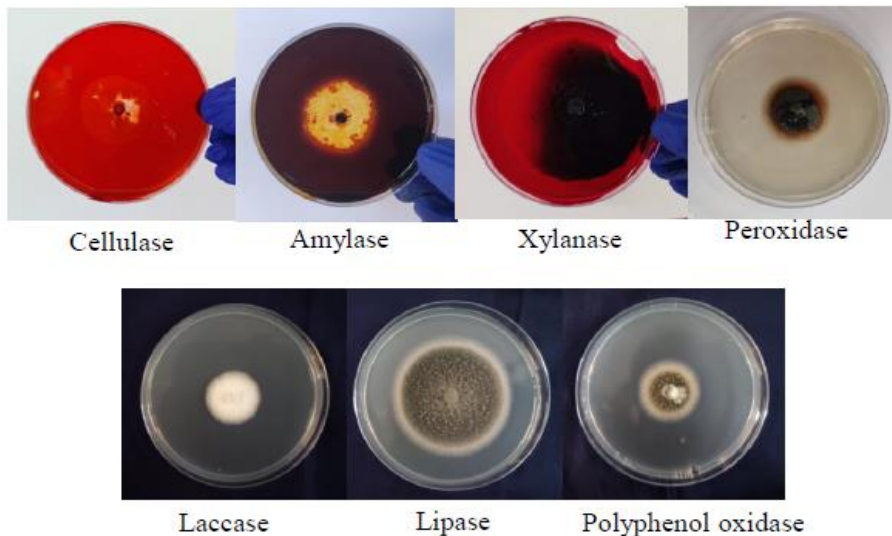


Fig. 9: Showing the cellulolytic & hydrolytic enzyme assay of *A. quisqualis* under *in vitro* conditions

fungus cell wall, thus facilitate the pathogenic penetration and colonization on host. Peroxidase activity suggests the involvement of oxidative processes that may contribute to host cell wall degradation or detoxification of reactive oxygen species. The absence of laccase enzyme indicated that the isolate relies more on hydrolytic rather than oxidative mechanisms for parasitism. Overall, these enzymatic capabilities enhance the ecological fitness and antagonistic potential of *A. quisqualis*.

Table 2: GCMS analysis of secondary metabolites present in ethyl acetate fraction of *A. quisqualis*

S. No.	Compound name	Retention time (min)	Peak area (%)	Role of secondary metabolites in KEGG pathway
1.	Cyclobutanone, 2-methyl-4-hydroxy-	2.614	1.358	-
2.	2,2-Difluoroethanol, TMS derivative	2.699	0.908	-
3.	Furan, 2-methyl	3.004	4.488	Involved in methane metabolism and act as a precursor for acetyl CoA pathway
4.	Trichloromethane	3.139	1.221	-
5.	Benzene	3.389	1.919	Involved in degradation of chlorobenzene, chlorocyclohexane, benzoate and aromatic compounds, also precursor for catechol in metabolic pathway
6.	5-Azacytidine	3.484	0.924	Involved in cysteine and methionine metabolism
7.	1-Pentanol	3.790	2.315	Lipid compound
8.	1-Butanamine	3.845	3.213	-
9.	Allomatrine	4.080	0.750	-
10.	Cyclobutene, 2-propenylidene-	4.320	1.025	-
11.	Butanoic acid, 2-methyl-, methyl ester	4.360	0.740	-
12.	Octane	0.740	0.797	Lipid compound
13.	Cyclotrisiloxane, hexamethyl-	4.760	5.061	-
14.	1-Butanol, 3-methyl-, acetate	5.790	2.901	Involved in butanoate metabolism
15.	2-Heptanone	6.010	0.439	Fatty acyls compounds
16.	à-Pinene	6.906	0.751	Cyclic monoterpenes
17.	Benzaldehyde	7.371	0.910	Involved in microbial metabolism in diverse environments and in xenobiotics biodegradation with toluene degradation
18.	Cyclotetrasiloxane, octamethyl	7.546	2.051	-
19.	1-Octen-3-ol	7.636	0.456	Fatty acyls
20.	1-Bromo-3,7-dimethyl -2,6-octadiene	7.841	2.293	-
21.	Butanoic acid, butyl ester	7.921	1.199	Involved in butanoate metabolism
22.	Hexanoic acid, ethyl ester	7.961	2.846	Fatty acyls
23.	à-Phellandrene	8.226	1.560	Menthane monoterpenoids
24.	p-Cymene	8.556	2.983	Involved in cymene degradation, dioxin and cumate degradation
25.	á-Phellandrene	8.701	14.035	Menthane monoterpenoids
26.	Cyclotrisiloxane, hexamethyl-	9.057	7.777	-
27.	ç-Terpinene	9.162	1.540	Prenol lipids
28.	Octane, 2-chloro-	9.292	0.657	Fatty acyls
29.	6-Hydroxy-4'-methoxyflavone	9.602	0.490	-
30.	Dodecane	9.837	0.529	Fatty acyls
31.	1,2-Epoxy-nonane	9.932	0.425	-
32.	Cyclopentasiloxane, decamethyl-	10.322	1.006	-
33.	1-(p-Tolyl)butan-1-one	10.582	1.236	-
34.	Butanedioic acid, 2,3-bis(benzoyloxy), (2R,3R)	10.872	0.802	-
35.	Rhodamine 6G cation	12.058	1.072	-
36.	Benzothiazole	12.153	1.230	-
37.	Cyclohexasiloxane, dodecamethyl-	13.158	0.471	-
38.	Caryophyllene	15.324	0.698	Involved in biosynthesis of sesquiterpenoid, triterpenoid, and secondary metabolites viz., tylosin, mycinamicin, oleandomycin, pikromycin/ methymycin
39.	Tetraethylene glycol	16.174	7.673	-

*The roles indicated were identified in KEGG pathway using the compound name obtained from the GCMS analysis as a preliminary level feature and further used for biological interpretation of compound.

Secondary metabolites and their antifungal role

GC-MS analysis of *A. quisqualis* isolate AQ 003 revealed the presence of 39 compounds, including major constituents such as α -phellandrene, cyclotrisiloxane hexamethyl, tetraethylene glycol, and 2-methylfuran (Table 2; Fig. 10). Many of these compounds are known to possess antimicrobial properties and are involved in key metabolic pathways. For instance, α -phellandrene has been reported to disrupt fungal cell membrane integrity, leading to leakage of cellular contents (Zhang *et al.*, 2017). Similarly, 2-methylfuran is associated with reactive oxygen species interactions, contributing to antifungal activity (Alizadeh *et al.*, 2020). Other compounds such as cyclotrisiloxane derivatives have been reported to inhibit pathogenic microorganisms (Keskin *et al.*, 2012). The presence of these metabolites suggests that, in addition to direct mycoparasitism, the isolate AQ 003 exerts indirect antagonistic effects through chemical inhibition.

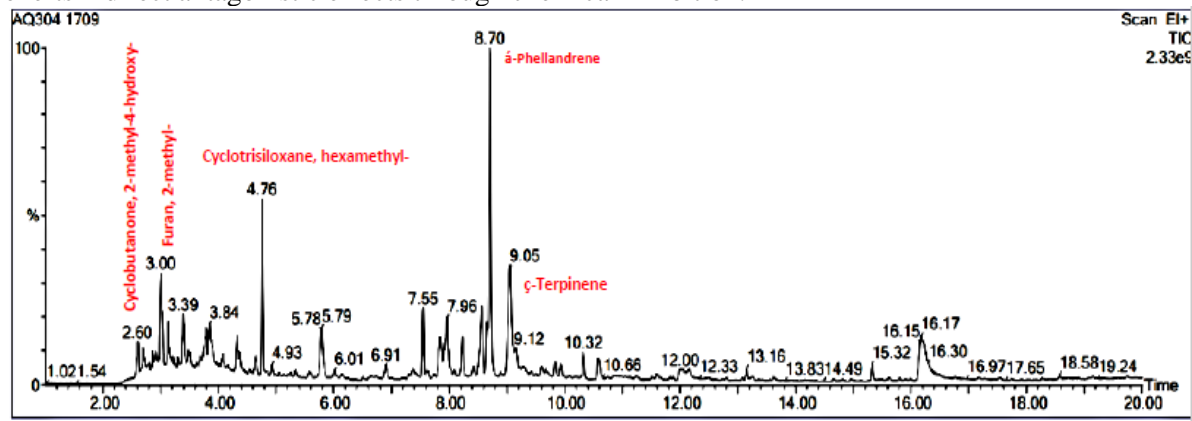


Fig. 10: Chromatogram with GCMS analysis of secondary metabolites *A. quisqualis* AQ 003

Formulation and field performance

Optimization of growth conditions revealed that mannitol (30 g) supplemented with trehalose (10 mM) supported maximum mycelial growth (89.12 mm) and favoured 100% pycnidial production, indicating its suitability for mass multiplication and formulation development. The field evaluation demonstrated that three foliar applications of *A. quisqualis* isolate AQ 003 at 3% significantly reduced disease severity, with a percent disease index (PDI) of 10.18 and 71.85% reduction over control (Table 3). Although wettable sulphur (0.1%) recorded a slightly lower PDI (8.17), the isolate AQ 003 provided substantial disease control while offering an environmentally safe alternative method. Lower concentrations of isolate AQ 003 were less effective but still demonstrated notable disease suppression.

Table 3: Efficacy of liquid formulation of *A. quisqualis* against sunflower powdery mildew

Treatments (foliar sprays)	Percent disease index (%)**				Percent reduction over control
	45 DAS	60 DAS	75 DAS	Mean*	
<i>A. quisqualis</i> AQ003 @ 1.0%	9.5 ^c (17.94)	15.0 ^d (22.79)	35.0 ^d (36.27)	19.83	45.17
<i>A. quisqualis</i> AQ003 @ 2.0%	9.0 ^a (17.45)	12.5 ^c (20.70)	13.0 ^c (21.13)	11.50	68.20
<i>A. quisqualis</i> AQ003 @ 3.0%	9.05 ^b (17.46)	10.0 ^b (18.35)	11.5 ^b (12.92)	10.18	71.85
Wettable sulphur @ 0.1%	12.0 ^d (20.26)	8.0 ^a (16.43)	4.5 ^a (12.25)	8.17	77.41
Control (untreated water spray)	13.5 ^e (21.55)	30.0 ^e (33.21)	65.0 ^e (53.73)	36.17	0.00

*Values are means of four replications. In a column, means followed by a common letter are not significantly different at 5% level by DMRT; **Values in parentheses are arcsine transformed values

Conclusion: The present study clearly demonstrates that powdery mildew in sunflower is caused by a complex of pathogens, primarily *G. cichoracearum* and *P. xanthii*. The mycoparasite *A. quisqualis* AQ 003 exhibited strong biocontrol potential through multiple mechanisms, including direct parasitism, production of cell wall-degrading enzymes, and secretion of antifungal metabolites. Its effectiveness under both laboratory and field conditions highlights its potential as a sustainable and

eco-friendly alternative to chemical fungicides for the management of powdery mildew in sunflower.

Ethics approval and consent to participate: This article does not contain any studies with human or animal participants performed by any of the authors.

Conflict of interests: The authors declare that they have no competing interests.

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Authors contribution: Lingan Rajendran - conceptualization, designing of experiments, Archana Sornakili - Morpho, microscopic, molecular characterization of powdery mildew pathogen and in vitro efficacy of *Ampelomyces*, Shanmugapackiam Subbiah - Isolation of pycnidial fungi, pure culturing, culture maintenance and morphological characterization, Durgadevi Dhakshinamoorthy - Culture maintenance, Thiruvengadam Raguchander - Designed the experiments on powdery mildew characterization and *Ampelomyces* isolation protocol and characterization.

Availability of data: Whole data relevant to the experiments are included in the paper.

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