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Research Article

Exploring eco-sensitive strategies for effective powdery mildew management in grapevines

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ABSTRACT: Grapevine powdery mildew is one of the most important plant diseases widely affecting crops in many countries. The main aim of the present study was to use *Ampelomyces quisqualis* isolates to suppress the powdery mildew of grapes under field conditions. The findings revealed a strong correlation between the mycoparasite, *Ampelomyces quisqualis* and the grape powdery mildew pathogens, suggesting its potential for effective control. The results revealed that a single spray of *A. quisqualis* @ 2.0 per cent (MDU1) succeeded by two sprays of *Trichoderma asperellum* @ 2.0 per cent (Tasp,7) were found to be effective against *Erysiphe necator*. In addition, liquid formulation of *A. quisqualis* isolate MDU1 (2% w/v) as a foliar spray at 2% containing 2×10^6 spores ml^{-1} the during initial occurrence of the disease up to three sprayings at 15 days interval was found to be best. Thus, a liquid formulation of *A. quisqualis* isolate MDU1 proved effective in managing grapes powdery mildew disease under field conditions. The rDNA ITS region of *A. quisqualis* isolates was analyzed at the molecular level, and the resulting sequences were subjected to GC-MS analysis. The secondary metabolite identification using GC-MS revealed the presence of antimicrobial compounds, including squalene with the highest peak of 4.643 percent, octadecanoic acid with 3.862 percent, tetradecanoic acid with 3.600 percent, and 9,12-octadecadienoic acid (Z,Z) with 1.451 percent. These bioactive compounds revealed by GC-MS analysis in crude extracts of *A. quisqualis* had a stronger antifungal activity against *E. necator*.

KEYWORDS: *Ampelomyces quisqualis*, compatibility, GC-MS, hyperparasitism, powdery mildew, *Trichoderma asperellum*

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INTRODUCTION

Grapevine (*Vitis vinifera* L.) is an important fruit and cash crop in India and its production is mainly obstructed by several biotic and abiotic stresses. Among the biotic stresses, particularly the powdery mildew disease is considered as the major concern, caused by *Erysiphe necator* Schwein (previously *Uncinula necator* (Schwein.) which is an obligate parasite belonging to the phylum Ascomycota (Vimala and Suriachandraselvan, 2009). Powdery mildew is a major disease affecting both cultivated and wild grapevine species worldwide, resulting in significant yield and economic losses (Gadoury *et al.,* 2012; Dahivelkar *et al.*, 2017). Disease severity varies from year to year, depending mostly on environmental conditions, the presence of inoculum (disease history), and the vulnerability of the vines. As a result, the steps that need to be taken to prevent losses, may differ from season to season (Dahivelkar *et al.*, 2017). The disease affects the crop in all seasons, resulting in lower crop yields up to 40 per cent incurring significant economic losses (Siddappa *et al.*, 2013). Many infected buds perished over the extreme winter resulting in yield losses in grapevines planted under cold agro-ecological environments. The pathogen can infect all green tissues of the plant, including leaves, shoots, flowers, and bunches, but flower and berry infections cause the most economic damage (Calonnec *et al.*, 2004; Gadoury *et al.*, 2012). Furthermore, the powdery mildew spreads to green shoots and causes red and brown lesions, and around bloom, grape bunches get affected, resulting in a drop in fruit as well as grape production. When haustoria enter into the epidermal cells of berries, the fruit die, or their skin shows scarring and spitting. Because of off-flavor and lack of freshness, infected grapes become unmarketable (Sadek *et al.,* 2022).

Chemical fungicides have become widely used all over the world, which have a negative impact on biodiversity, natural ecosystems, and the residual fungicides problem in food (Fernandes *et al.*, 2020). Many mycoparasites have undergone thorough research and are now economically viable as effective bio-control agents (Keerthana *et al.,* 2022). *A. quisqualis* is a pycnidial hyperparasite on powdery mildew disease that is distributed widely across the world. A highly specific, environmentally friendly, and cost-effective fungus has been extensively studied as a bio-control agent against powdery mildew fungi (Keerthana *et al.,* 2022).

The main focus of *A. quisqualis* research has been on its potential as a biocontrol agent against powdery mildews in different crops (Keerthana *et al*., 2022; Sharma, 2006). This mycoparasite enters and destroys the host cytoplasm, leading to the death of the parasitized powdery mildew cells (Whipps, 2001; Kiss *et al*., 2014). In this specific study, *A. quisqualis* strains were selected from various regions of Tamil Nadu, exhibiting intracellular pycnidia formation and slow radial growth *in vitro* at room temperature (Keerthana *et al*., 2022). Biocontrol agents combat plant pathogens through direct antagonistic interactions, which involve actions like mycoparasitism, production of antibiotics, volatile metabolites, and secretion of lytic enzymes (Jayalakshmi *et al*., 2021; Ramamoorthy *et al*., 2015). Additionally, they exert indirect antagonistic activities such as competing for nutrients and space and inducing host resistance (Whipps, 2001; Kohl *et al*., 2019). Weindling (1932) reported that *Trichoderma* species primarily display antagonistic activity by secreting toxic metabolites. Both *Trichoderma* species and *A. quisqualis* are known for their strong mycoparasitic abilities, enabling them to effectively control various plant infections by suppressing fungi. However, there is limited research on the combined use of *Trichoderma* sp. and *A. quisqualis* for biological control against powdery mildew disease in grapes. Therefore, this study aimed to evaluate the effectiveness of *Trichoderma* sp. and *A. quisqualis*, both *in vitro* and under field conditions, to combat powdery mildew disease in grapes caused by *E. necator*. The research also focused on *A. quisqualis* hyperparasitism in conjunction with *Trichoderma* sp.

MATERIALS AND METHODS

Collection of samples and processing

Plant samples infected with powdery mildew were collected from various regions (Theni, Dindigul, Coimbatore and Krishnagiri) of Tamil Nadu, India. A total of 120 samples were gathered from various horticultural and agricultural cropping ecosystems under field environmental conditions and subsequently stored in a refrigerator. Besides that, 25 vineyards were sampled. The vineyards were chosen at random depending on the incidence of powdery mildew. The sampling in vineyards encompassed conventionally managed vines, organically managed vines, and abandoned (untreated) vineyards. For each vineyard and year, four sets of 25 leaves were randomly collected from the fifth leaf of the shoot. Visual assessments were conducted to determine the percentage of infected area on the upper leaf surface (characterized by whitish, powdery spots) and the number of infected leaves in each replicate. From this data, the severity of the disease (expressed as the percentage of infected leaf area) and disease incidence (represented as the percentage of infected leaves) were calculated. *Ampelomyces* pycnidia were isolated under a stereo microscope with a 20X magnification. The live samples were then kept in a growth chamber for one week, maintaining a modified temperature of 23±1°C and 83% relative humidity, with a 12:12 dark-to-light ratio (Braun *et al.,*1987).

Enumeration of mycoparasite from powdery mildew infection

Microscopic examinations were carried out to identify the presence of *Ampelomyces* **p**ycnidia within the mycelium of the *Erysiphales* species. To observe the pycnidia, a spore suspension was placed on a slide and covered with a cover slip. The interaction between the host and the parasite was documented using sophisticated microscopic equipment, including a stereomicroscope, light microscope, phase contrast microscope and Scanning Electron Microscope (SEM). The qualitative and quantitative characteristics of *Ampelomyces* pycnidia and pycnidiospores were measured during the study (Angeli, 2009).

Isolation of *Ampelomyces* sp. using pycnidia picking method

Pycnidia were extracted from leaves infected with powdery mildew and parasitized by *Ampelomyces* sp. They were then observed using a stereo microscope and carefully collected using a sterilized needle. Subsequently, the collected pycnidia were placed on potato dextrose agar (PDA; Himedia, Mumbai) for further analysis (Goh, 1999). To avoid contamination, 0.3% streptomycin sulphate or 2% chloramphenicol was added to the culturing medium. Plates were incubated at 20±2°C, and growth and development were monitored. Twenty isolates were collected from various cropping systems in a natural ecosystem.

Morphological examination of *Ampelomyces* sp.

Morphological studies of *Ampelomyces* sp. isolates were conducted using a phase contrast microscope after 20 days of growth. The radial growth was assessed in five replicates, and the height, texture, and color of each isolate were documented. At 100X magnification, morphological parameters such as pycnidia, pycnidiospores, and the presence

of petiolate structures were measured for each isolate. For further analysis, a ten-day-old culture was chopped and scraped with a needle as described by Sharma (2006), then mounted on aluminum stubs using double-sided adhesive tape coated with gold-palladium. The characteristics of the mycelium were studied under a SEM.

Extraction of genomic DNA

Twenty isolates of *Ampelomyces* sp. were cultured in 100ml conical flasks containing 20 ml of PD broth for a duration of ten days, after which the mycelium was collected. The total fungal DNA was then extracted from 100 mg of mycelium using the CTAB method (Moller *et al*., 1992). The extracted DNA was purified and dissolved in 50µl of TE buffer with a pH of 8.0 (Tris 10mM + EDTA 1mM). The integrity of the genomic DNA (gDNA) was assessed using a 1.5% agarose gel (HiMedia, Mumbai). The quality and quantity of the DNA were determined using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific NanoDrop 2000c, USA). The DNA concentration was adjusted to 50 ng/l and stored at 4°C for future use (Sambrook *et al*., 2006).

PCR amplification

The identification of *Ampelomyces* sp. cultures was carried out using molecular techniques targeting the conserved ribosomal Internal Transcribed Spacer (ITS) region. We amplified the ITS regions between the small nuclear 18S rDNA and the large nuclear 28S rDNA, including 5.8S rDNA, using the universal primer pairs ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990, Hirata *et al*., 1996). PCR reactions were conducted using a Mastercycler® Nexus X2 PCR cycler (MA, USA) under the following conditions: initial denaturation at 95℃ for 10 mins, followed by 35 cycles at 94℃ for 30 seconds, 60℃ for 45 seconds, and 72℃ for 1 min. A final extension step was performed at 72℃ for 8 mins. The PCR products were visualized using the Gel Doc™ apparatus (Bio-Rad) on 1.2 per cent agarose gel.

Isolation of *Trichoderma* sp. from the rhizosphere

Trichoderma isolates were primarily isolated from roots and the basal end of either rootstock or self-rooted plants from both young and mature vineyards, as previously described (Urbez *et al*., 2014) and plated on *Trichoderma* Selective Medium (TSM). The TSM plates were placed in an incubator at 22°C for a duration of two days. Subsequently, the growth of multiple fungal colonies was observed, and *Trichoderma* sp. were identified by examining the morphology of conidiophores and the arrangement of phialides (Figure 1). The fungal colonies of *Trichoderma* sp. were then transferred to sterile Petri plates containing PDA medium. To further confirm the identification of *Trichoderma* isolates, molecular methods were employed.

Selected grape genotype

The widely cultivated and popularly grown, Thompson Seedless genotype, which is susceptible to powdery mildew, served as the test material. This genotype was predominant and occupied the largest area in the regions of Theni and Dindigul. These grape genotype, Thompson Seedless is a seedless variety of grapes and produces thin clusters of large berries in large, long bunches with the yield potential of 20-25 t/ha.

Experimental site

The field trial took place in Theni during the cropping season of 2021-2022. Theni is located at the foothills of the Western Ghats, with the Grape Research Station (GRS) situated at coordinates 9.45ºN latitude and 77.20ºE longitude. The soil type in this region is red soil, with a pH ranging from 6.9 to 7.4. The climate is tropical, and the cropping season experiences mild winters. This location is known for being a hot spot for powdery mildew disease development, and a

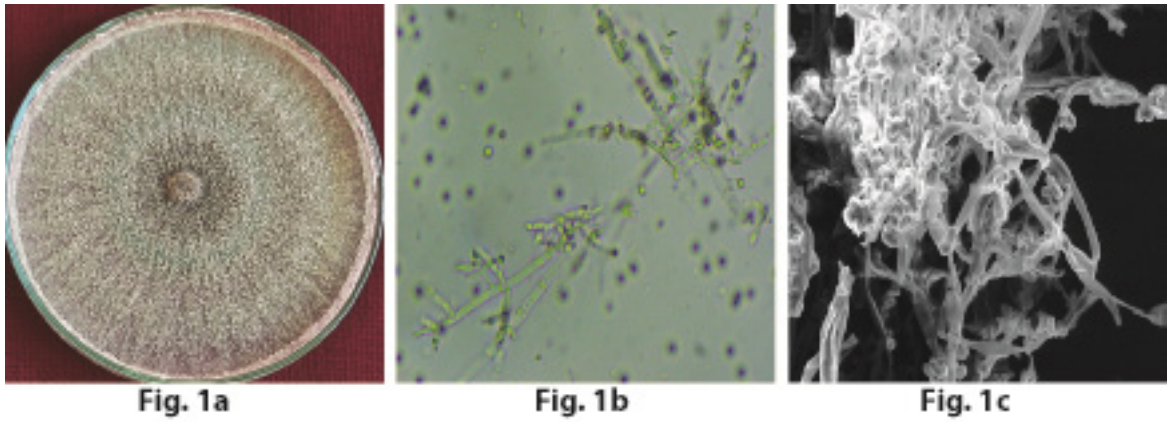


Figure 1 (a). *Trichoderma asperellum* isolate. (b). Phialide morphology. (c). SEM image.

Table 1. Average weather data of different cropping seasons during 2021-2022 at Theni and Dindigul

Particular of traits and weather parameters		Theni/Dindigul district							
Genotype used		Thompson Seedless	H -23/A	Muscat C	Flame Seedless	Charak-3	Jumbonath	Manjari Shyama	Arka Kanchan
Method of disease establishment		Natural	Natural	Natural	Natural	Natural	Natural	Natural	Natural
Mean maximum T°C		28.57	28.57	28.57	28.57	28.57	28.57	28.57	28.57
Mean minimum T°C		21.71	21.71	21.71	21.71	21.71	21.71	21.71	21.71
Mean maximum RH (%)		78.43	78.43	78.43	78.43	78.43	78.43	78.43	78.43
Mean minimum RH (%)		71.14	71.14	71.14	71.14	71.14	71.14	71.14	71.14
Total rainfall (mm)		883.00	883.00	883.00	883.00	883.00	883.00	883.00	883.00
Sunshine hrs (hours)		40.15	40.15	40.15	40.15	40.15	40.15	40.15	40.15
Wind velocity (km/hr)		13.10	13.10	13.10	13.10	13.10	13.10	13.10	13.10
PDI (%)	Leaf	19.23	17.89	10.27	7.45	13.25	15.48	11.23	6.22
	Bunch	15.67	18.26	7.15	6.89	11.39	18.25	13.67	7.36

*T – Temperature
RH – Relative Humidity
PDI – Per cent Disease Incidence

wide range of high-yielding grape varieties are cultivated here. The climatic conditions in these regions are favorable for powdery mildew disease due to the high perpetuation of powdery mildew inoculum in the surrounding grape-growing areas. Detailed weather parameters during the crop growth period in these two locations are provided in Table 1.

Observations and data analysis

Two fields were selected to monitor the percentage of disease incidence in both infected plants and grape bunches, and in each field, 20 plants were chosen at random and the per cent leaf area of the grapes affected by powdery mildew was visually assessed using a 0-9 scale. (Saari and Prescott, 1975; Azmat *et al*., 2012; Nongmaithem *et al*., 2017). The disease score was converted to Per cent Disease Index (PDI) by following the formula given by Wheeler (1969). The PDI was calculated by using the following formula,

$$PDI = \frac{\text{sum of all numerical ratings}}{\text{Total no. of leaves observed}} \times \frac{100}{\text{Maximum grade in the score chart}}$$

Testing the efficacy of *A. quisqualis* (MDU1) for the management of grape powdery mildew under field condition

Field trials were conducted in three cropping seasons during September – October 2021, January 2022 - February 2022 and May-June 2012 at GRS in the Theni district of Tamil Nadu, to test the comparative efficacy of liquid formulation of the *A. quisqualis* (MDU1) against powdery mildew of grapes. The experiment was laid out in a randomized block design with eight treatments and three replications mentioned in Table 2. The initial spraying of liquid formulations of *A. quisqualis* and *T. asperellum* were given as soon as the minimal powdery mildew symptom appeared followed by second and third spray was given fifteen days after the prior spray. Regular observations were made on per cent disease incidence periodically, and also the grape yield (t/ha) was recorded at the time of harvest for all the treatments. The survival and mycoparasitism of *A. quisqualis* (MDU1) was observed by microscopic image analyzer and scanning electron microscopy.

Statistical analysis

The data were statistically analyzed using R studio statistical package. Prior to statistical Analysis of Variance (ANOVA), the percentage values of the disease was converted to arcsine transformation. The data were subjected to ANOVA at significant level (P<0.05), and means were compared by Duncan's Multiple Range Test (DMRT).

Hyperparasitism of *A. quisqualis* with *T. asperellum*

The hyperparasitism assay was conducted using the dual-culture method on PDA plates. *A. quisqualis* and *T. asperellum* were positioned at opposite ends of the 90mm PDA plate, maintaining a distance of 1cm from the edge. The two fungi were allowed to grow under suitable environmental conditions during the experiment. Plates were incubated at 26°C in dark place & five replicates of the paired cultures were performed. The Interaction between the two fungi, i.e., *A. quisqualis* and *T. asperellum*. After 15-20 days, the hyperparasitism growth between *A. quisqualis* and *T. asperellum* were examined by visual observation, microscopic examination & SEM Analysis. Furthermore, five glass slides were prepared, each coated with a thin layer of PDA, and inoculated with *A. quisqualis* and *T. asperellum*, maintaining a distance of 1.5 cm between them. These slides were then placed in a Petri dish containing moist filter paper to create a

Table 2. Effect of liquid formulation of *A. quisqualis* (MDU1) on the incidence of powdery mildew and yield of grapes under field conditions (THENI – Field Trial)

T. No.	Treatments	Disease incidence (Per cent disease index)				Per cent disease reduction over control at 45 DAS	yield (t/ha)	Per cent yield increase over control
		BS	15 DAS	30 DAS	45 DAS			
1	Foliar spray (FS) of *A. quisqualis* MDU1@ 1.0%conc.immediately after the occurrence of disease followed by 2nd and 3rd spray at 15 days interval	10.68^{a} (19.04)	13.95^{c} (21.90)	14.87^{c} (22.64)	15.95^{c} (23.50)	50.53	15.44^{e} (23.14)	20.16
2	FS of *A. quisqualis* MDU1@ 2.0% conc. Immediately after the occurrence of disease followed by 2nd and 3rd spray at 15 days interval	10.78^{a} (19.15)	13.76^{cd} (21.74)	14.68^{c} (22.49)	15.71^{c} (23.31)	51.27	16.38^{cd} (23.88)	27.47
3	FS of *A. quisqualis* MDU1@ 3.0% conc. immediately after the occurrence of disease followed by 2nd and 3rd spray at 15 days interval	10.85^{a} (19.18)	13.59^{d} (21.60)	14.49^{c} (22.34)	15.59^{c} (23.22)	51.64	15.72^{de} (23.36)	22.34
4	FS of *Trichoderma asperellum* Tasp7@ 2.0% conc. immediately after the occurrence of disease followed by 2nd and 3rd spray at 15 days interval	10.81^{a} (19.19)	14.57^{b} (22.40)	16.86^{b} (24.20)	17.76^{b} (24.89)	44.91	15.23^{e} (22.97)	18.52
5	FS of *A. quisqualis* MDU1 with *Trichoderma asperellum* Tasp7@ 2.0% conc. immediately after the occurrence of disease followed by 2nd and 3rd spray at 15 days interval	10.74^{a} (19.10)	11.49^{e} (19.78)	11.68^{ef} (19.95)	11.84^{ef} (20.09)	63.28	18.72^{a} (25.64)	45.68
6	FS of *A. quisqualis BRBaq*@ 2.0% conc. immediately after the occurrence of disease followed by 2nd and 3rd spray with *Trichoderma asperellum* Tasp7 (1.0%) at 15 days interval	10.72^{a} (19.09)	13.61^{d} (21.62)	13.86^{de} (21.82)	13.92^{de} (21.87)	56.82	17.08^{bc} (24.42)	32.92
7	FS of *A. quisqualis* MDU1@ 2.0% as 1st spray followed by 2nd and 3rd spray with *Trichoderma asperellum* Tasp7 at 2% conc. at 15 days interval	10.63^{a} (18.98)	11.41^{e} (19.71)	12.53^{de} (20.70)	12.89^{de} (21.00)	60.02	17.28^{b} (24.57)	34.47
8	Untreated control	10.82^{a} (19.15)	16.45^{a} (23.86)	23.36^{a} (28.84)	32.24^{a} (34.54)	0.00	12.85^{f} (21.01)	0.00

BS: Before spraying. DAS: Days after spraying.
Values are mean of three replications.
Figures in parentheses represent arcsine transformation.
Means in a column followed by same superscript letters are not significantly different according to DMRT at $P \leq 0.05$.

humid chamber. The sealed Petri dishes were incubated in the dark at 25°C. Daily observations under a microscope were made to study the characteristic hyperparasitism interactions between the two fungi on the slides.

Preparation of crude extracts of *A. quisqualis*

To prepare the crude extracts of the effective *A. quisqualis*, a 9mm mycelial disc from an actively growing effective *Ampelomyces* isolate (MDU1) was transferred into 200ml of potato dextrose broth and incubated for seven days at 23±1°C. The culture filtrates were obtained by passing the extracts through Whatmann no.1 filter paper and then centrifuging them for 15 mins at 9000 rpm. Metabolites were extracted from the culture filtrates using ethyl acetate as the solvent. The solvent containing Volatile Organic Compounds (VOCs) was concentrated using a rotary evaporator until the solvent was completely evaporated. The final product was filtered through a 0.4µm bacterial filter after being diluted with 2ml of ethyl acetate.

Gas Chromatography-Mass Spectrum analysis (GCMS) of crude extracts of *Ampelomyces quisqualis*

Gas chromatography equipped with a Mass detector turbo mass gold, and using an Elite-1 (100% Dimethyl Poly

Siloxane) column measuring 30 m x 0.25 mm ID x one mM df, was utilized to identify various VOCs of the effective *A. quisqualis*. The analysis was conducted under the following conditions: helium was used as the carrier gas at a flow rate of 1 ml/min, the oven temperature was programmed from 110 ℃ (for 2 mins) to 280 ℃ (for 9 mins), the injector temperature was set at 250 ℃, and the total GC time was 45 mins. Ethyl acetate extracts were injected into the chromatography at a volume of 1.0 µl. A computer algorithm was employed to identify the major VOCs present in the samples, and the analysis results were compared to the library database of the National Institute of Standards and Technology (NIST) and the AMDIS software program. This GC-MS analysis was conducted at the Centre of Innovation for Excellence, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai.

RESULTS AND DISCUSSION

Powdery mildew of grapes caused by *E. necator* is one of the most common and widespread diseases in India, affecting both on and off-season crops. Because crops are grown throughout the year in various geographical regions of India, the disease occurs in epidemic proportions practically every year (Banyal *et al.*,1998; Kapoor and Kumar, 1996). In the present study, the powdery mildew disease severity and the presence of mycoparasitic infections were recorded from the samples collected from various locations in Tamil Nadu, India, during 2021 and 2023 (Figure 2). Mycoparasitic infections were found in 25 distinct locations. However, none was found in the samples collected from other locations. Twenty isolates showed the highest levels of pycnidial myco-parasitization of *Ampelomyces* sp. The light microscopic

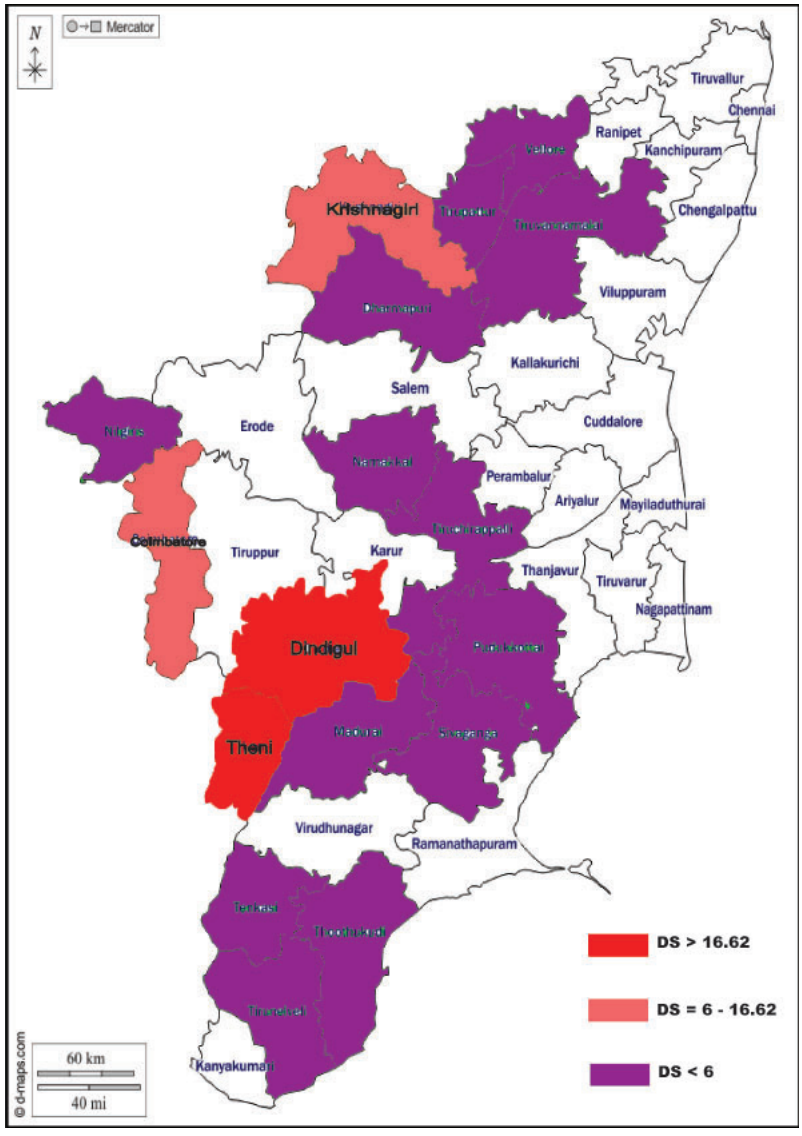


Figure 2. The map displays the surveyed districts for the years 2021 and 2022, with color-filled areas indicating the severity of disease (DS). Districts shaded in violet represent low disease severity (less than 6%), pink areas indicate a disease severity ranging from 6% to 16.62%, and red areas represent districts with disease severity greater than 16.62%.

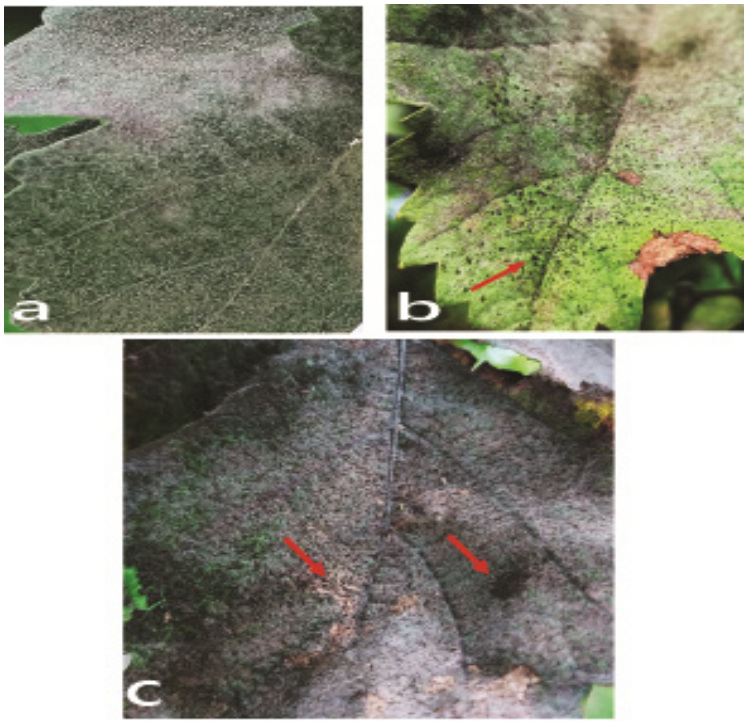


Figure 3. Different stages of *A. quisqualis* infection of powdery mildew fungal colonies: (a). healthy colonies of powdery mildew on the surface of grape leaf. (b). powdery mildew colonies infected with *A. quisqualis* (the brown/black color spots are the pycnidia produced by *Ampelomyces*). (c). powdery mildew colonies totally destroyed by *Ampelomyces*, 1–2 weeks after infection.

(LM) examination of grape powdery mildew colonies collected from Tamil Nadu, India, provided clear evidence of *Ampelomyces* sp. infection, even though *Ampelomyces* sp. has previously been reported to parasitize grape powdery mildew fungus (Figure 3 a-c).

Morphological characterization and identification of *Ampelomyces* sp.

Morphological analysis of *Ampelomyces* from naturally parasitized powdery mildew fungi revealed that the mycoparasite's hyphae were slender, slightly coloured, and located inside the powdery mildew fungi's hyphal cells, conidiophores, and conidia. During the initial stage, mycelia were septate and hyaline. In mature colonies, it changed from greyish-white to brownish-black. Some fully grown culture plates exhibited zonation, with its margins appearing smooth, wavy, or irregular. The mycoparasite's pycnidia varied in shape (round, ovoid, flask, pyriform, globose) and colour (olive green to brown with a reticulate pattern). The pycnidia's size also varied, ranging from 56.24 to 74.20 × 50.23 to 63.81 µm. Pycnidiospores were unicellular, hyaline, and oval in shape, ranging in size from 9.63 to 15.77 × 2.29 to 3.50 µm (Table 3, Figure 4). The hyphal bodies appeared 48 hours after the inoculation. Fungal colonies grew slowly and concentrically after a single mature pycnidium was inoculated in the middle of PDA medium.

Molecular identification

The ITS region of fungal DNA is extremely valuable for molecular systematics at the species level as well as within species (for example, identifying geographic races). Variation among individual rDNA repeats can sometimes be noticed within the ITS and IGS regions due to their higher degree of variation than other regions of rDNA. In the present study, the sequence was shown 97 per cent sequence homology with GenBank sequences with BLASTn analysis. Using the ITS region, we discovered that the ten isolates from different areas of Tamil Nadu shared sequence homology with isolates from other regions such as India, China, and Korea. To validate the initial identification and identify the clear taxonomic position, the Internal Transcribed Spacer (ITS) regions (ITS1 and ITS4) and 5.8S gene area of 18S rDNA were initially amplified with the primers ITS1 and ITS4. Five hundred sixty base pairs were amplified in all twenty isolates are presented in Figure 5 (Zhou *et al.*, 2001). The amplification was identical to the prior identity, and the amplified 18S-rDNA (ITS 1 and ITS 4) region was purified separately and sequenced at National Center for Biotechnology Information (NCBI) using sangar dideoxy sequencing.

Table 3. *Ampelomyces quisqualis* isolated from the mycoparasitized samples collected from various regions of Tamil Nadu

S. No	Isolates	Plant host	*A. quisqualis* parasitic on powdery mildew pathogens	*A. quisqualis*						Mycoparasitism (%)
				Pycnidium				Pycnidiospore (µm)		
				Color	Length	width	Shape	Length	Shape	
1.	AQMDU-1	*Vitis vinifera* L.	*E. necator* Schwein.	Olive green	32.62	41.22	Cylindrical	8.74	Oval shaped	63
2.	AQMDU-2	*Vitis vinifera* L.	*E. necator* Schwein.	Brownish black	42.12	35.12	Round	8.16	Oval shaped	53
3.	AQMDU-3	*Vitis vinifera* L.	*E. necator* Schwein.	Dark brown	49.25	36.27	Oval	9.22	Oval shaped	43
4.	AQMDU-4	*Vitis vinifera* L.	*E. necator* Schwein.	Brownish black	56.12	38.22	Cylindrica	8.16	Oval shaped	51
5.	AQMDU-5	*Vitis vinifera* L.	*E. necator* Schwein.	Dark brown	52.62	74.25	Cylindrical	8.74	Oval shaped	31
6.	AQMDU-6	*Vitis vinifera* L.	*E. necator* Schwein.	Brownish black	42.15	34.31	Round	8.52	Cylindrical shape	36
7.	AQBRB-1	*Vitis vinifera* L.	*E. necator* Schwein.	Dark brown	42.61	29.05	Round	9.01	Cylindrical and curved at both the edges	61
8.	AQBRB-2	*Vitis vinifera* L.	*E. necator* Schwein.	Brown	43.01	28.47	Oval and tapering at both the edges	9.11	Oval shaped	69
9.	AQBRB-3	*Vitis vinifera* L.	*E. necator* Schwein.	Dark brown	48.05	37.14	Cylindrical	15.84	Cylindrical and curved at both the edges	66
10.	AQBRB-4	*Vitis vinifera* L.	*E. necator* Schwein.	Brownish black	32.62	41.22	Cylindrical	13.73	Oval shaped	62

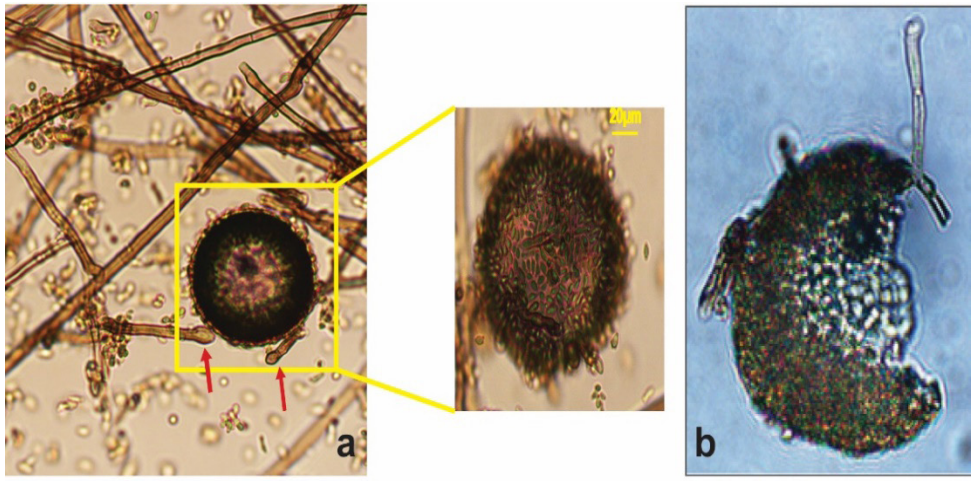


Figure 4. Microscopic image of *Ampelomyces* pycnidia shows: (a). pycnidia and pycnidiospores produced in *Ampelomyces* isolate (red arrow indicates conidia attached to conidiophore). (b). Mature chasmothecia releasing an ascus spores.

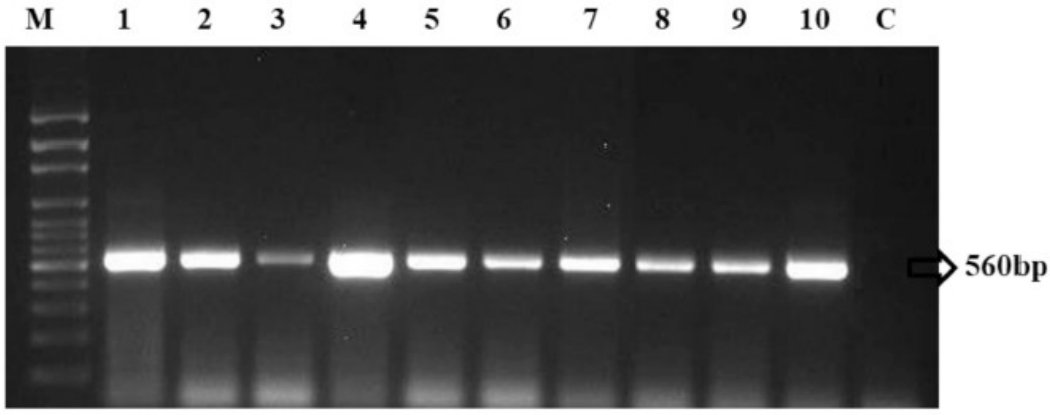


Figure 5. Molecular identification of *Ampelomyces quisqualis* species of ITS region.

Figure 6. Effect of liquid formulation of *A. quisqualis* (MDU1) on the incidence of powdery mildew and yield of grapes under field conditions (Field Trial).

Effect of *A. quisqualis* (MDU1) liquid formulation on grape powdery mildew disease incidence and yield under field conditions

In the field trial, which was conducted at Grape research station in Theni district, the exclusive bioagent *A. quisqualis* with *T. asperellum* at 2.0 per cent spray at 15 days interval was found to be individually significantly superior recording 11.84 PDI which accounted for 63.28 per cent disease reduction over control and this treatment recorded the highest significant yield of 18.72 t/ha as against the control which recorded 12.85 t/ha pod yield. This was followed by the application of *A. quisqualis* at 2.0 per cent, followed by two spraying of *T. asperellum* at 2.0 per cent and *A. quisqualis* (BRBaq) at 2.0 per cent, followed by two spraying of *T. asperellum* at 1.0 per cent which were on par with each other, respectively, recording 12.89 PDI (60.02 per cent disease reduction over control) and 13.92 PDI (56.82 per cent disease reduction over control). The above treatments, respectively, recorded 17.28 t/ha (34.47 per cent yield increase over control) and 17.08 t/ha (32.92 per cent yield increase over control) (Table 2, Figure 6). Among the different dosage levels, the 2.0 and 3.0 per cent concentrations of *A. quisqualis* were statistically on par, respectively, recording 15.71 PDI (51.27 per cent disease reduction over control) and 15.59 PDI (51.64 per cent disease reduction over control). *T. asperellum* (2.0%) was less effective compared to *A. quisqualis* in reducing the powdery mildew disease incidence.

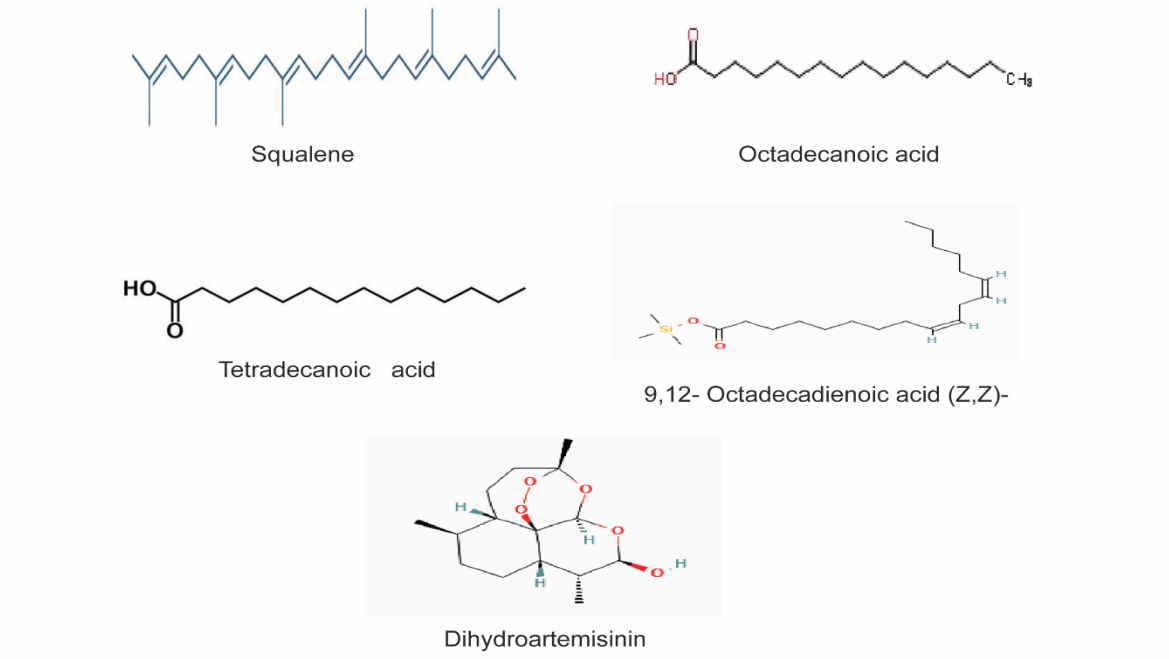
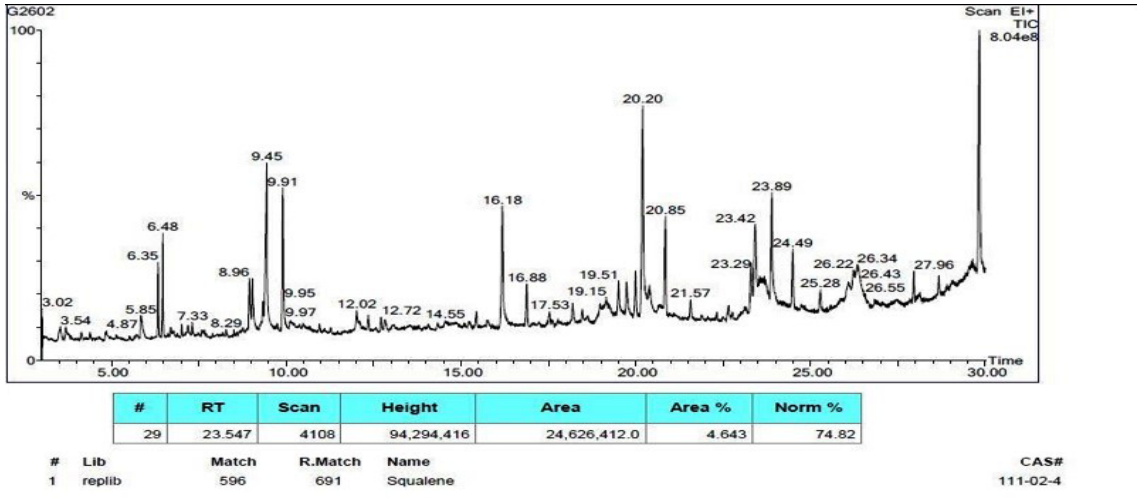


Figure 7. Chemical structure of important antifungal compounds produced by *A. quisqualis*.



#	RT	Scan	Height	Area	Area %	Norm %
29	23.547	4108	94,294,416	24,626,412.0	4.643	74.82

#	Lib	Match	R.Match	Name	CAS#
1	replib	596	691	Squalene	111-02-4

Figure 8. Total ion chromatogram of secondary metabolites identified from *A. quisqualis* by GC-MS analysis.

Detection of secondary metabolites by Gas Chromatography-Mass Spectrometry (GC-MS)

In the present study, the secondary metabolites produced from *Ampelomyces* crude extract using methanol solvent were subjected to GC-MS analysis. The compound's identity was confirmed using the NIST Library 2005 and the AMDIS software programme. The crude extracts of the AQS3 isolate contained approximately 40 secondary metabolic compounds. Among the various compounds, squalene has the highest peak of antimicrobial activity at 4.643 percent, followed by octadecanoic acid at 3.862 percent, tetradecanoic acid at 3.600 percent, and 9,12-octadecadienoic acid (Z, Z) at 1.451 per cent (Figure 7). Similarly, the lowest peak exhibiting compounds, namely 2-Hexadecanol, 1-Tricosanol, and 2-propenyl ester, were detected with 0.485, 0.519, and 0.560 percent, respectively (Table 4, Figure 8). In a manner similar to the research conducted by (Naznin *et al*., 2014), it was hypothesized that certain VOCs might possess inhibitory effects on *E. necator*. The study involved the isolation of several VOCs from *Ampelomyces* sp., which were found to be responsible for mitigating disease symptoms and reducing the population of the pathogen.

Hyperparasitism of *Ampelomyces quisqualis* with *Trichoderma asperellum*

In the dual-culture experiment, *A. quisqualis* was compatible with *T. asperellum*. The microscopic study of hyperparasitism reveals that the conidiophore of *T. asperellum* carry the conidia of *Ampelomyces,* and also,

Table 4. Secondary metabolites identified from crude extracts of *Ampelomyces quisqualis* through GCMS

Sl. No.	Retention time	Peak area per cent	Compound name	Molecular weight (g/mol)	Molecular formula	Biological properties	References
1.	3.699	0.560	2-propenyl ester	86.09	$C_4H_6O_2$	Antifungal	Wang *et a*l. (2012)
2.	5.855	0.885	4 H-Pyran-4- one, 2,3-dihydro-3,5- dihydroxy 6-methyl-	144.126	$C_6H_8O_4$	Antibacterial	El-Benawy *et al*. (2020)
3.	6.350	0.929	Cyclohexano,1-methyl-4 (1-methylethyl)	156.269	$C_{10}H_{20}O$	Antifungal	Wang *et al*. (2010)
4.	6.480	1.348	Dihydroartemi sinin	284.352	$C_{15}H_{24}O_5$	Antimicrobial	Dai *et al*. (2021)
5.	8.956	1.174	3-Decenoic acid	170.252	$C_{10}H_{18}O_2$	Antifungal	Ma *et al*. (1980)
6.	9.046	1.165	L-Glutamine	146.146	$C_5H_{10}N_2O_3$	Antitoxin	Wischmeyer *et al.* (2003)
7.	12.02	0.580	Dodecanoic acid	200.322	$C_{12}H_{24}O_2$	Antifungal	Walters *et al.* (2003)
8.	16.14	3.600	Tetradecanoic acid	228.376	$C_{14}H_{28}O_2$	Antifungal	Li *et al.* (2012)
9.	16.84	0.883	1-Nonadecene	266.513	$C_{19}H_{38}$	Antifungal	Jayasuriya *et al*. (2003)
10.	18.199	0.543	Pentadecanoic acid	242.403	$C_{15}H_{30}O_2$	Antifungal	Jenkins *et al*. (2015)
11.	19.735	1.051	9-Hexadecenoic acid	270.457	$C_{17}H_{34}O_2$	Antifungal	Wang *et al*. (2012)
12.	19.995	1.038	Dibutyl phthalate	278.348	$C_{16}H_{22}O_4$	Antifungal	Czubacka *et al*. (2021)
13.	21.566	0.485	2-Hexadecanol	242.447	$C_{16}H_{34}O$	Antifungal	Li *et al.* (2012)
14.	23.302	1.451	9,12-Octadecadieno ic acid (Z,Z)-	280.452	$C_{18}H_{32}O_2$	Antifungal	Wang *et al*. (2012)
15.	23.547	4.643	Squalene	410.73	$C_{30}H_{50}$	Antimicrobial	Awa *et al*. (2012)
16.	23.892	3.862	Octadecanoic acid	284.484	$C_{18}H_{36}O_2$	Antifungal	Awa *et al*. (2012)
17.	27.958	0.519	1-Tricosanol	340.636	$C_{23}H_{48}O$	Antiviral	Chatterjee *et al*. (2018)
18.	29.629	0.501	Digitoxin	764.95	$C_{41}H_{64}O_{13}$	Antifungal	Elbaz *et al*. (2012)

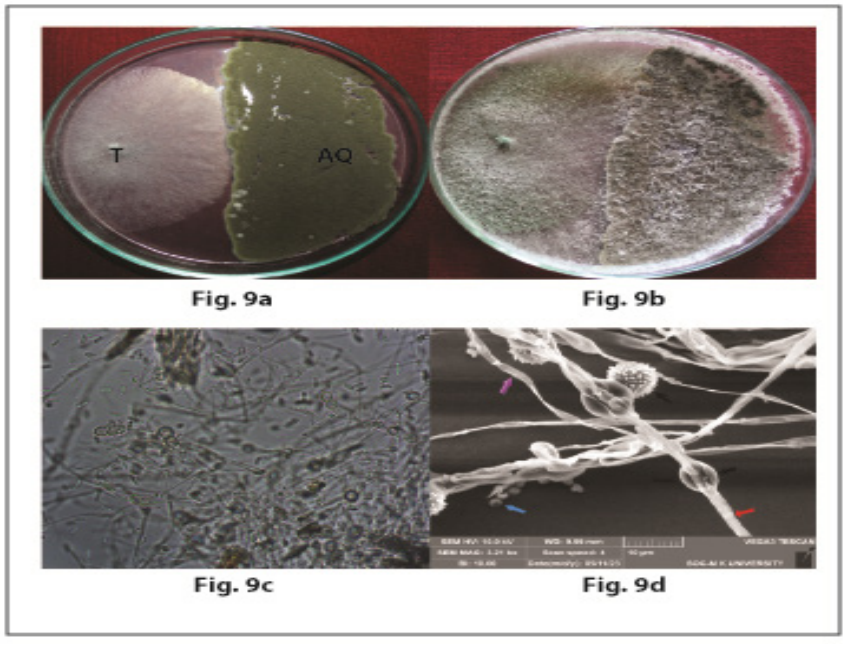


Figure 9(a). Dual-culture of *Ampelomyces.* **(b).** *Trichoderma* showing compartibilty. **(c).** Germination of *Ampelomyces and Trichoderma* spores under a microscope during hyperparasitism. **(d).** SEM image of hyperparasitism between *Ampelomyces* and *Trichoderma* (black arrow represent adherence of *Trichoderma* spores over *Ampelomyces* conidia and *Trichoderma* spore bounded over conidiophore of *Ampelomyces* and purple, red arrow shows conidiophore of *Trichoderma*, *Ampelomyces* respectively).

conidia of *Trichoderma* was attached with conidia of *Ampelomyces.* Another SEM analysis reveals that entire conidia of *Ampelomyces* was encircled with *Trichoderma* spores, and *Trichoderma* spore bounded over conidiophore of *Ampelomyces* (Figure 9).

CONCLUSION

In recent years, powdery mildew caused by *E. necator* has become a significant challenge in grape production in Theni district. The use of fungicides to manage powdery mildew has led to reduced diversity of beneficial microorganisms and the emergence of resistant pathogens. This study explores the eco-friendly management of grape powdery mildew using a liquid formulation of the mycoparasitic antagonist *A. quisqualis*. The results revealed that the exclusive spraying with the *A. quisqualis* (MDU1) with *T. asperellum* (Tasp7) at 2.0 per cent was the best in managing the powdery mildew incidence on grapes. It was observed that, the single spray of *A. quisqualis* (MDU1) at 2.0 per cent succeeded by two sprays of *T. asperellum* at 2.0 per cent (Tasp7) was effective compared to the single spray of *A. quisqualis* at 2.0 per cent (MDU1) followed by two sprays of *T. asperellum* 1.0 per cent (Tasp7). This is mainly because early reduction of powdery mildew disease incidence by *A. quisqualis* at 2.0 per cent supports the further rapid mycoparasitism of *T. asperellum* on the sprayed leaf surface. From these results, exclusive biocontrol spray, it is very clear that application of liquid formulation of *A. quisqualis* isolate MDU1 @ 20 ml litre-1 thrice at 15 days interval was effective in reducing the grapes powdery mildew incidence. *Ampelomyces quisqualis* stands out as an eco-friendly and highly specific mycoparasite, particularly effective against powdery mildews. Further investigation into the ecological interactions among plants, powdery mildew fungi, *Ampelomyces*, and *Trichoderma* can provide valuable insights into the role of fungal antagonists in shaping plant-parasite population dynamics.

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