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Diversity of Powdery Mildew Mycoparasite *Ampelomyces quisqualis* under Natural Ecosystem and Its Molecular Characterization

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The powdery mildew are the most common diseases which widely affect crops in many countries. The mildew infection appeared on leaves, petiole, buds, inflorescence and other tender tissues of crops causing 65% of the crop loss. Even though the fungicidal spray to control the disease, the residual and environmental effects are causing long term ecological imbalance in cropping system. Recently, an alternative and eco-friendly mycoparasite associated with powdery mildew pathogens was used against *Erysiphe* sp*, Leveilulla* sp*, Sphaerotheca* sp and *Oidium* sp. This study aimed to mitigated the use of the expensive and harmful fungicides to save human health and to reduce the financial costs of controlling the fungal infections of crops in the field by using a commercial biofungicide naturally associated with different species of powdery midew fungi. Our results exhibited that the isolation of the mycoparasite *Ampelomyces quisqualis* from the mildew pathogens was closely associated with most of the genera of powdery mildew pathogens. The natural mycoparasitization efficiency of *A. quisqualis* was observed and higher (81%) efficiency was

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recorded in *Erysiphe cichoracearum* followed by 76% in black gram powdery mildew caused by *Erysiphe polygoni*. A total of 20 mycoparasitic *Ampelomyces* isolates were enumerated from pycnidia of 6 different powdery mildew species that naturally infected their host plants. The pycnidial morphological variations of *A.quisqualis* and the largest size of pycnidia was found in the *Erysiphe chichoracearum* (73.54µm length × 42.15µm width) shows maximum efficiency in natural parasitization. The molecular characterization of *A.quisqualis* isolates based on using rDNA ITS region was carried out and sequenced. The phylogenetic analysis was performed using the Maximum likelihood technique was shown the distinct relatedness with five *Ampelomyces* isolates made in the present study were clustered. This is the first and detailed study on diversity of *Ampelomyces* and quantification of natural mycoparasitism of different genera of powdery mildew of *A. quisqualis.*

Keywords: Bhendi; mycoparasitism; ampelomyces quisqualis; biocontrol; powdery mildew.

1. INTRODUCTION

The obligate biotrophic powdery mildew fungi are causing infection to more than 10,000 host plant species, including important vegetables and horticultural crops [1]. To protect the crops from powdery mildew pathogens, fungicides are indiscriminately applied frequently with higher dosage which causes fungicide resistance. In addition to that the fungicides are causing harmful effect on biodiversity, natural ecosystem and causes the residual fungicides problem in food [2]. The physical and biological approaches have been proposed to support and replace chemical management of powdery mildews. The mycoparasites (fungi that parasitize other fungi) are naturally abundant in the majority of the powdery mildew infection in terrestrial environmental conditions especially in biotrophic interaction [3,4]. Numerous mycoparasites have been investigated extensively and economically used as bio-control agents [5]. *Ampelomyces quisqualis* is an unique mycoparasite of Erysiphales fungi [6] and it is classified as an endoparasitic nature because of its conidia penetrate into *Pyllactinia xanthii* and generate pycnidia inside powdery mildew structures [7]. The mycoparasite inhibits the growth of *P. xanthii* haustoria, hence limiting the pathogen's nutrition absorption.

The cross-inoculation assay of *A. quisqualis* studied by Liang et al. [8], for mycohost specificity. The studies have shown that a strain of *A. quisqualis* isolated from a species of powdery mildew fungi can infect different powdery mildew species, suggesting that there is no mycohost-specificity. Variations in mycohost phenotype and differentiation in that specific of *Ampelomyces* sp. from bhendi host caused in the infections in different powdery mildew pathogens *viz., Leveillula taurica* and *Erysiphe polygoni* in bell pepper and black gram respectively [9].

The usage of *Ampelomyces* spp as controller of the different strain of grapevine powdery mildew was successfully investigated by Kiss, [9]. Liyanage et al., [10] showed that the diversity of *Ampelomyces* strains belong to genetically-distinguished groups, based on sequence of internal transcribed (rDNA-ITS) regions with nuclear ribosomal DNA. Under this circumstance the present study focuses on isolation of *Ampelomyces* mycoparasitic strains in the powdery mildew pathogen, as well as investigating the morphological variation for identification and its mycoparasitic efficiency in natural conditions and to study the genetic diversity of *Ampelomyces* isolates using ITS rDNA.

2. MATERIALS AND METHODS

2.1 Collection of Samples and Processing

Powdery mildew-infected plant samples were collected from various regions of Tamil Nadu, India. A total of 526 samples were collected from horticultural and agricultural cropping ecosystem in field environmental conditions and samples were stored in refrigerator. The isolation of *Ampelomyces* pycnidia was carried out under stereo microscope with the magnification of 20X. The live samples were stored in the growth chamber for one week with the modified temperature of 23 ± 1^0C and 83% RH, 12:12 dark to light ratio according to Braun [11].

2.2 Enumeration of Mycoparasite from Powdery Mildew Infection

The presence of *Ampelomyces* pycnidia in the mycelium of the Erysiphales species was examined through microscopic studies. Pycnidia were observed by placing a spore suspension in a slide and covering it with a cover slip. The advanced microscopic equipments such as stereomicroscope, light microscope, phase contrast and Scanning electron microscope were used to document the host parasitic relationship. The qualitative and quantitative morphological structures of *Ampelomyces* pycnidia and pycnidiospores was measured according to Angeli, 2014.

2.3 Isolation of *Ampelomyces* sp. using Pycnidia Picking Method

The powdery mildew infected leaves parasitized by *Ampelomyces* spp. were used for isolation in potato dextrose agar medium. The pycnidia were examined using a stereo microscope were picked using a sterilized insulin needle and placed on potato dextrose agar (PDA; Himedia, Mumbai). Streptomycin sulphate 0.3% was added to the culturing medium to avoid any contamination. Plates were incubated at a temperature of 20±2°C and monitored the growth and development. A total of twenty isolates were obtained from different cropping system under natural ecosystem.

2.4 Morphological Examination of *Ampelomyces* sp.

The sub-cultured twenty ten days old culture isolates of *Ampelomyces* spp were used for morphological studies using phase contrast microscope. The radial growth five replicates each was assessed, as well as the height, texture, and colour of both Petri plate side The morphological parameters such as pycnidia, pycnidiospores, and the presence of petiolate in each isolate were measured at 100X magnifications. To study the characteristics of mycelium scanning electron microscope, a ten days old culture was chopped, scraped with a needle and mounted on aluminum stubs using double-sided adhesive tape, coated with gold palladium.

2.5 Extraction of Genomic DNA

The twenty isolates of *Ampelomyces* sp. were cultured in 100ml Erlenmeyer flasks containing 20ml PDA broth, after 10 days incubation, mycelium was collected. Total fungal DNA was extracted from 100 mg of mycelium by cetyl trimethyl ammonium bromide CTAB method (Möller et al., 1992). The purified DNA was dissolved in 50µl TE buffer (Tris 10mM + EDTA 1mM pH 8.0). Integrity of genomic DNA (gDNA) was checked in 1.5 per cent agarose gel (HiMedia, Mumbai). The quality and quantity of DNA was assessed by using NanoDrop1000 spectrophotometer (Thermo Fisher Scientific NanoDrop 2000c, USA). The concentration of DNA was adjusted to 50 ng/µl and stored at 4°C for further use [12].

2.6 PCR Amplification

Ampelomyces sp. cultures were identified molecularly using the conserved ribosomal internal transcribed spacer (ITS) region. Using the universal primer pairs ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), we amplified the ITS regions between the small nuclear 18S rDNA and the large nuclear 28S rDNA, including 5.8S rDNA [13,14]. All PCR reactions were carried out using a Mastercycler® Nexus X2 PCR cycler (MA, USA) using the following parameters: 1) initial denaturation at 95°C for 10 min, followed by 35 cycles at 94°C for 30 s, 60°C for 45 sec and 72°C for 1 min and a final extension at 72°C for 8 min, the electrophoresis was carried out on 1.0 percent agarose gels. The Bio-Rad Gel Doc EZ Imaging System was used to view the PCR products (Biotium, Hayward, CA).

2.7 Sequencing of ITS Region and Analysis

The PCR products were eluted and sequenced further at Barcode Scientific in Bangalore, India. Partial nucleotide sequences of rDNA ITS region of isolates of *Ampelomyces* sp were downloaded from NCBI database (www.ncbi.info). The program the Basic Local Alignment Search Tool-Nucleotide or BLASTn server, was used to edit and align the ITS sequences and the similarity between strains of 18S rRNA gene sequences was calculated using ClustalW [15,16]. The aligned sequences were deposited in the GenBank database.

2.8 Phylogenetic Analysis

The phylogenic tree was built with 1000 bootstrap replications in Mega X [17] using the Maximum Likelihood approach based on the Tamura 3-parameter model [18]. The *Pythium insodium* reference sequence for 18S rRNA were

Table 1. *A.quisqualis* isolated from the mycoparasitized samples collected from different 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.	AQ-TNAU-DST-01	*Abelmoschus esculentus* L.	*Erysiphe cichoracearum* DC.	Brown	73.54	42.15	Round	9.54	Cylindrical and curved at both the edges	81
2.	AQ-TNAU-DST-02	*Vigna unguiculata* L.	*Erysiphe polygoni* DC.	Brown	56.18	41.22	Pyriform	8.27	Cylindrical shaped	76
3.	AQ-TNAU-DST-03	*Sesamum indicum* L.	*Oidium erysiphoides*	Dark brown	48.77	40.21	Round	8.42	Oval shaped	52
4.	AQ-TNAU-DST-04	*Abelmoschus esculentus* L.	*Erysiphe cichoracearum* DC.	Dark brown	52.08	38.96	Oval	7.27	Cylindrical and curved at both the edges	78
5.	AQ-TNAU-DST-05	*Capsicum annuum* L.	*Leveillula taurica* (Lev.)	Dark brown	49.25	36.27	Oval and tapering at both the edges	9.22	Oval shaped	43
6.	AQ-TNAU-DST-06	*Abelmoschus esculentus* L.	*Erysiphe cichoracearum* DC.	Brownish black	41.25	36.23	Oval	8.14	Cylindrical and curved at both the edges	72
7.	AQ-TNAU-DST-07	*Vitis vinifera* L.	*E. necator* Schwein.	Dark brown	42.61	29.05	Round	9.01	Cylindrical and curved at both the edges	61
8.	AQ-TNAU-DST-08	*Tagetes erecta* L.	*Leveillula taurica* (Lev.)	Dark brown	32.62	28.25	Cylindrical	8.74	Oval shaped	31
9.	AQ-TNAU-DST-09	*Capsicum annuum* L.	*Leveillula taurica* (Lev.)	Brownish black	42.15	34.31	Round	8.52	Cylindrical and curved at both the edges	36
10.	AQ-TNAU-DST-10	*Abelmoschus esculentus* L.	*Erysiphe cichoracearum* DC.	Brown	40.78	33.13	Oval	7.61	Oval shaped	73
11.	AQ-TNAU-DST-11	*Vitis vinifera* L.	*E. necator* Schwein.	Brown	43.01	28.47	Oval and tapering at both the edges	9.11	Oval shaped	69
12.	AQ-TNAU-DST-12	*Rosa sp.*	*Sphaerotheca pannosa*	Dark brown	31.77	29.15	Oval	8.55	Cylindrical and curved at both	28

S. No	**Isolates**	**Plant host**	***A. quisqualis* parasitic on powdery mildew pathogens**	***A. quisqualis***						**Mycoparasitism (%)**
				Pycnidium				**Pycnidiospore (µm)**		
				Color	**Length**	**width**	**Shape**	**Length**	**Shape**	
									the edges	
13.	AQ-TNAU-DST-13	*Morus alba*	*Phyllactinia corylea*	Dark brown	51.13	37.21	Round	8.31	Cylindrical and curved at both the edges	64
14.	AQ-TNAU-DST-14	*Vigna unguiculata* L.	*Erysiphe polygoni* DC.	Dark brown	48.05	37.14	Cylindrical	9.34	Oval shaped	66
15.	AQ-TNAU-DST-15	*Sesamum indicum* L.	*Oidium erysiphoides*	Brownish black	42.12	35.12	Round	8.16	Oval shaped	53
16.	AQ-TNAU-DST-16	*Abelmoschus esculentus* L.	*Erysiphe cichoracearum*	Whitish	41.02	45.1	Round	9.01	Oval shaped	70
17.	AQ-TNAU-DST-17	*Vitis vinifera* L.	*E. necator* Schwein.	Black	32.62	41.22	Cylindrical	8.74	Oval shaped	63
18.	AQ-TNAU-DST-18	*Tagetes erecta* L.	*Leveillula taurica* (Lev.)	Dark brown	42.15	40.21	Round	8.52	Oval shaped	52
19.	AQ-TNAU-DST-19	*Vigna unguiculata* L.	*Erysiphe polygoni* DC	Black	41.78	39.96	Oval	7.61	Oval shaped	66
20	AQ-TNAU-DST-20	*Cucumis sativus*	*Erysiphe cichoracearum*	Grey	45.01	37.27	Round	9.11	Oval shaped	58

obtained from GenBank data and used as an out group in phylogenic tree analyses.

3. RESULTS

The powdery mildew diseased samples were collected from different locations of Tamilnadu, India during 2018–2020 and its severity along with presence of mycoparasitic infections were recorded. The mycoparasitic infections were noticed in different genera of powdery mildew pathogens *viz., Erysiphe, Uncinula, leveillula* and *Sphaerotheca*. The maximum mycoparasitization (81%) of *Ampelomyces* sp. pycnidia was recorded with genera of *Erysiphe* powdery mildews (Table 1).

3.1 Morphological Characterization and Identification of *Ampelomyces* sp.

The morphology of Ampelomyces sp. pycnidia were studied and shown with different shape such as ovoid, ellipsoid, or globose. The size of the pycnidia and pycnidiospores were ranged in length from 72.09 to 119.57 µm (major axis) and width from 28.45 to 47.12 µm (minor axis) (Fig. 1). The variations were in pycnidial shape depends on the genera of powdery mildew pathogens.

Pycnidiospores varied in length from 9.54 to 6.15 µm and in width from 5.71 to 3.21 µm. Pycnidial range in color from light brown to dark brown, while pycnidiospores are olive green in color. Out of 20 isolates two isolates were examined in depth for their morphological characters (Fig. 2). The morphological parameters of pycnidial shape and size were listed in the Table 1.

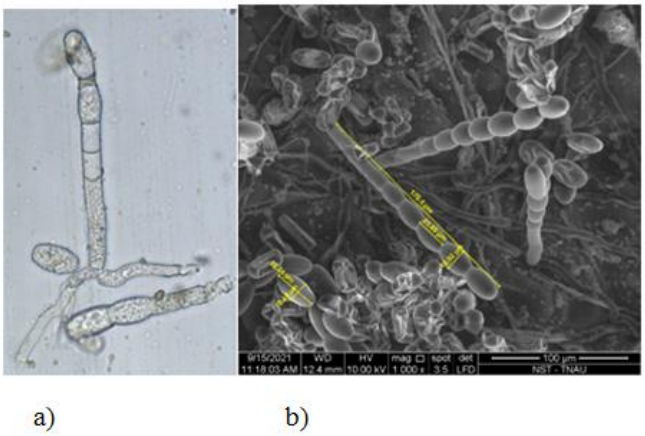

Fig. 1. Bhendi powdery mildew *Erysiphe chichoracearum* microstructures; (a) conidiophore of bhendi powdery mildew; (b)chain of conidia. (Scanning electron microscope) (Scale bar = 10 µm)

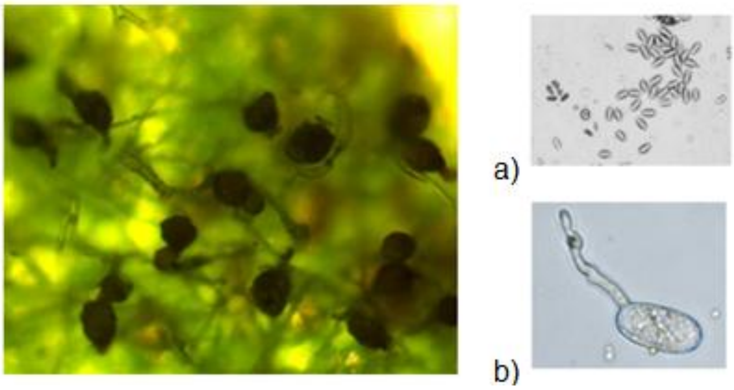

Fig. 2. Pycnidium on the surface of a bhendi powdery mildew; (a) pycnidiospores of *Ampelomyces* spp.; (b) conidia with germ tube

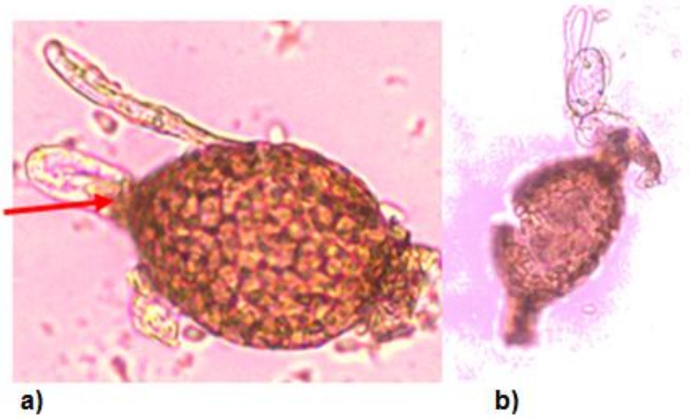

Fig. 3. ***Ampelomyces*** **spp. microstructures (a) pycnidia produced in the conidiophores of bhendi powdery mildew arrow indicates conidia of bhendi powdery mildew; (b) dehisced pycnidium by apical rupture**

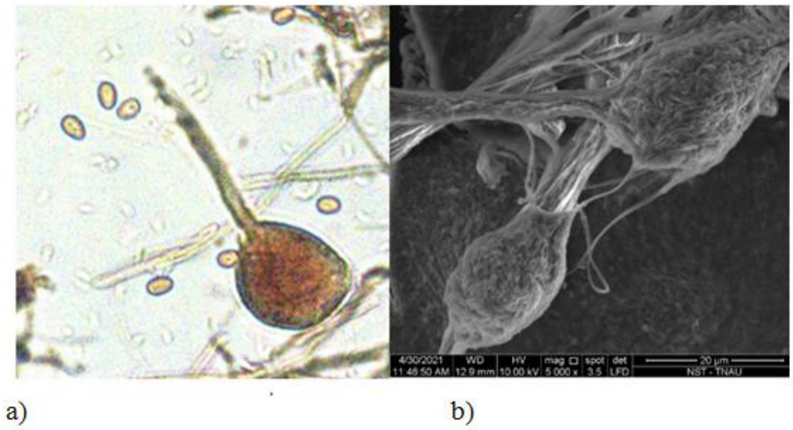

Fig. 4. Microscopic image of ***Ampelomyces*** **pycnidia; (a) pycnidia and pycnidiospores produced in** ***Ampelomyces*** **isolate; (b) Scanning electron microscopic image of pycnidia of** ***Ampelomyces*** **isolate AQTNAU-DST01**

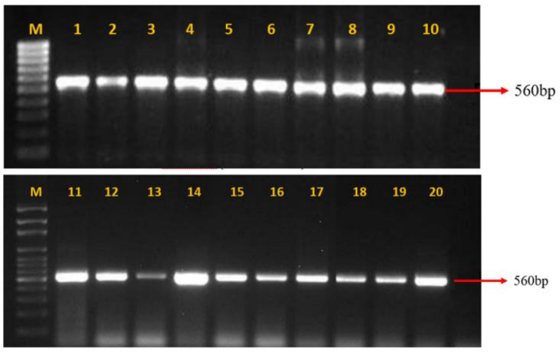

Fig. 5. Molecular identification of ***Ampelomyces*** **species of ITS region,1–16: M) DNA marker, 1) AQTNAU-DST1, 2) AQTNAU-DST2, 3) AQTNAU-DST3, 4) AQTNAU-DST4, 5) AQTNAU-DST5, 6) AQTNAU-DST6, 7) AQTNAU-DST7, 8) AQTNAU-DST8, 9) AQTNAU-DST9, 10) AQTNAU-DST10,11)AQTNAU-DST11, 12)AQTNAU-DST12, 13) AQTNAU-DST13,14) AQTNAU-DST14,15) AQTNAU-DST15, 16)AQTNAU-DST16, 17) AQTNAU-DST17, 18) AQTNAU-DST18, 19) AQTNAU-DST19,20) AQTNAU-DST20**

The pycnidia are unicellular, hyaline, and elongated to pyriform in shape, measuring around 46.36 × 10.82 µm of pale brown angular textured (Fig 4). The hyphal lengths were developed 48 hours after injection. Following inoculation of a single mature pycnidium in the middle of PDA medium, fungal colonies expand slowly and concentrically.

3.2 Molecular Identification and Phylogenic Analysis

The sequence of ITS regions were shown 97% sequence homology with GenBank sequences with BLASTn analysis. The sequences were submitted in NCBI GenBank, and OM424627, OM424628, OK236008, OM190525 and OM190526 were assigned as accession number for AQTNAU-DST01, AQTNAU-DST02, AQTNAU-DST03, AQTNAU-DST04 and AQTNAU-DST05 isolates, respectively. The phylogenic analysis shown that the isolated *Ampelomyces* were grouped with other reported *Ampelomyces* on Gen bank. The Maximum likelihood of 18s rDNA sequences of Species of *Ampelomyces* constructed with bootstrap values more than 500 (from 1000 replicates) are indicated at the nodes as percentage that varied with each cluster has been shown in Fig 6. Phylogenetic analysis based on 18s rDNA sequences of different species of *Ampelomyces* isolates were analyzed and the results revealed that 6 different clusters were formed in phylogenic tree. Cluster 1 was the biggest clade, with isolates, including three *Ampelomyces* isolates (AQTNAU-DST01, AQTNAU-DST03, and AQTNAU-DST04) isolated in this study. AQTNAU-DST01 shown 90% similarity with other *Ampelomyces* strains in the cluster I. Further two isolates *Ampelomyces* (AQTNAU-DST02 andAQTNAU-DST05) were clubbed together to form a cluster 2 with other different *Ampelomyces* isolates. There were multiple subgroups within this cluster. In the dendrogram, *Pythium insodium* has created a separate cluster (out group) (Fig. 6). Using the ITS region, we discovered that the 10 isolates obtained from various areas in Tamilnadu had sequence homology with isolates from other regions, including India, China, and Korea.

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 ITS 4 to validate the initial identification and identify the clear taxonomic position. All twenty isolates were amplified with 560 base pairs. The amplification were identical with prior identity and the amplified 18S-rDNA (ITS 1 and ITS 4) region was purified individually and sequenced by sangar dideoxy sequencing in NCBI. (Fig. 5).

4. DISCUSSION

The powdery mildew is an economically important disease that affects a wide range of crops and caused significant loss in yield. The high occurrence of the infection was noticed throughout the season especially under micro climatic condition. Cortesi et al. [19], reported that the source of inoculum was high in spring season in bhendi crop and inoculum was survived in the weed hosts.

In this research, we isolated 20 *Ampelomyces* isolates from five different pathogenic isolates of powdery mildew in Tamilnadu. All the isolates are showing the potential powdery mildews infection [20] the morphological characteristics of all the mycoparasitic activity against *A. quisqualis* isolates were identical and similar with earlier reports [8,21-28]. The investigations clearly indicated that the variations of pycnidia and its spores showed different species of powdery mildew pathogens in various plant species. It is supported by Puzanova [29] and Pintye et al. [30] who reported that there was no specific host specialization for morphological characters with *Ampelomyces* isolates and they mentioned that cross mycoparasitic activity with wide range powdery mildew pathogens

In all the examined strains, the colour and shape of the pycnidia varied from light to dark (brown or grey) and from ovoid to ellipsoid. Similar results were reported by Kim et al., [31] who reported that the color of pycnidium ranged from light brown to dark brown. At present, the morphometric studies of the isolates of *A. quisqualis* revealed that the size of pycnidia was variable in the range 72.09 to 119.57 µm; pycnidiospore ranged length from 9.54 to 6.15 µm and in width from 5.71 to 3.21 µm. This was in accordance with the findings of Liang et al. [8] who reported that the pycnidia were pyriform to globose measuring about 36-123 × 22-45 µm and it contained unicellular guttulated conidia which measured about 4.2-7.5 µm in length.

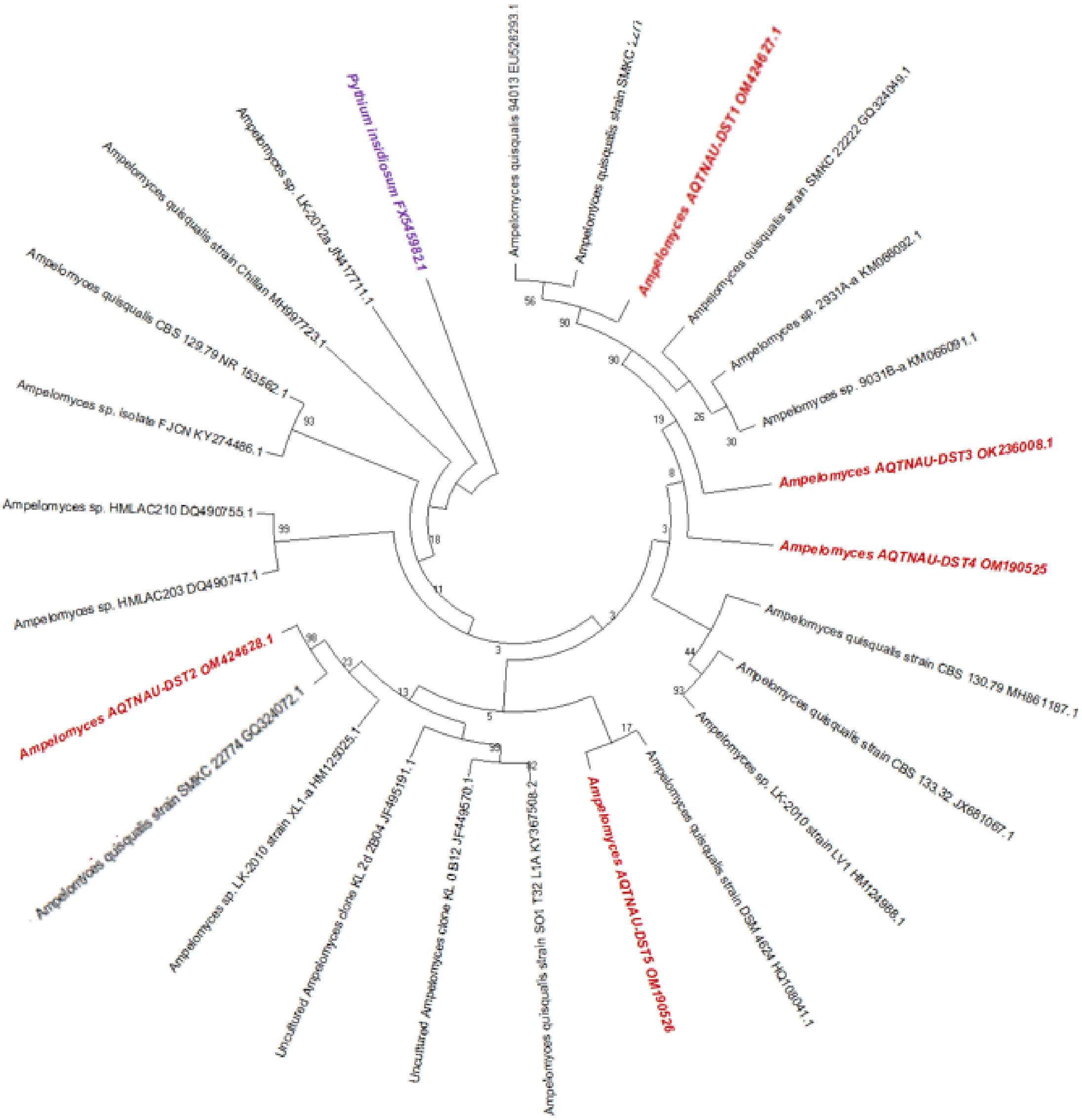

Fig. 6. Phylogenic tree generated from 18s rDNA sequence of *Ampelomyces* sp. using Maximum likelihood analysis in MEGA10

The mycelia of powdery mildew-infecting *Ampelomyces* spp. were hyaline and the pycnidia were light brown to dark brown in color, with olive green pycnidiospores. However, Lee et al. [32] and Angeli et al., [6] found color differences in the mycelium and pycnidia of several mycohosts belonging to the genus *Erysiphe,* ranging from olive green to light and dark brown. Pycnidia and pycnidiospores in *Ampelomyces* isolates from powdery mildews varied in shape depending on the fungal structure in which they were formed. They were pear shaped, spindle-shaped or nearly spherical when they were formed inside *E. necator* conidiophores, hyphae or chasmothecia [33].

In the present investigation, ITS-PCR analysis were attempted using ITS 1 and 4 universal primers pertaining to 18s rDNA Intergenic Transcribed Spacer region. The isolates of *Ampelomyces quisqualis* collected from different powdery mildew hosts were identified as *Ampelomyces quisqualis* based on the amplified as a fragment of 560 bp and it has been confirmed that all the isolates were *Ampelomyces* sp. The sequencing of the isolates showed 94 – 98% homology with the respective *Ampelomyces* sp.Our results were highly in agreement by those of Kiss (1997), Angeli *et al.* (2012 and Jamali (2015 who have reported that the size of the PCR product of *A. quisqualis* varied from 500-600 bp. The 18s rDNA gene sequencing analysis has an important role in the identification process of *Ampelomyces quisqualis* up to species level through 18s rDNA sequencing (Liyange *et al.,* 2018).

The Neighbour joining method of phylogenetic tree revealed that the similarity in clustering pattern with other species *Ampelomyces quisqualis* reported earlier. The phylogenic tree generated by our analysis contains six distinct Cluster. These results was confirmed further to conclude as *Ampelomyces quisqualis* and previous reports of Park et al. [34], Angeli et al., [6] and Nemeth et al., [35] were shown the molecular characterization and diversification of *Ampelomyces* isolates formed four distinct clades. Likewise, ITS rDNA region of *Ampelomyces* sp in our current investigation revealed the diversity of six different phylogenic groups comprising of anoutgroup of *Pythium insodium*. *Ampelomyces* isolate AQTNAU-DST03, AQTNAU-DST04 which formed cluster 2. The cluster 3 was comprising of AQTNAU-DST 02, AQTNAU-DST01 and AQTNAU-DST05.

Mycoparasitism has been shown to be an important biological control mechanism [36]. *Ampelomyces* sp infects the host powdery mildew, which reduces the growth of mycelium. It infects and produces spores in the mycelium and hyphae of powdery mildew. This has been confirmed by other studies on powdery mildew, including grapes and various crops [20]. *A. quisqualis* with a significant reduction in the expression of powdery mildew symptoms caused by *E. chichoracearum* in bhendi crop. Similarly, Shishkoff and Mcgrath [37] have shown that the parasite reduces growth and can eventually kill powdery mildew colonies.

These results suggest that *Ampelomyces* sp. mycoparasites and other disease progression and suppressed their sporulation from infected plants and improved plant vigor and growth after reducing powdery mildew infection. Several experiments have demonstrated that *Ampelomyces* sp. used as a biological control agent for the management of powdery mildew [26,38-41].

5. CONCLUSION

It follows that the ability of *A. quisqualis* in controlling powdery mildew infection is essential in integrated disease management. As an AQ10 bio fungicide, this mycoparasite is currently one of the most advanced in commercial development of a fungal biocontrol product against powdery mildew. The ecological interactions between plants, powdery mildew, and *Ampelomyces* can be further explored to better understand the role of fungal antagonists in the population dynamics of plant parasites. Continued use of fungicides creates a resurgence among pathogens and also affects beneficial microflora. In these cases, the use of effective biological control agents for disease management is necessary. Future studies aimed at improving the effectiveness of biological control agents and their delivery methods may further improve yield.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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