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RESEARCH ARTICLE

Characterization of *Ampelomyces* and its Potentiality as an Effective Biocontrol Agent against *Erysiphe cichoracearum* DC Causing Powdery Mildew disease in Bhendi (*Abelmoschus esculentus* (L.) Moench)

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ABSTRACT

Ampelomyces is one of the most naturally occurring mycoparasitic fungi on powdery mildews. A detailed survey was conducted in major bhendi (*Abelmoschus esculentus* (L.) Moench) growing regions of Coimbatore district of Tamil Nadu to assess the incidence of powdery mildew disease. The results revealed that disease incidence ranged from 38.04 to 69.54 per cent. From the surveyed areas, ten isolates of *Ampelomyces* spp were isolated using water agar medium. All the isolates were identified by their morphological characters and microscopic studies. Initially, the mycelia were hyaline and septate in nature over a period of time the color of the matured colonies in various medium turned greyish white to brownish black. Most of the isolates showed radial, flat to the submerged growth pattern. The pycnidia of different isolates of *Ampelomyces* varied in their shape and were mostly ovoid, pyriform to globose in shape. The size of pycnidia varied from 56.24 – 74.20 × 50.23 - 63.81µm. The pycnidial production was found to be more in the isolates *viz.,* AQB1 and AQB7. Pycnidiospores are unicellular and guttulate in shape. An *in vitro* mycoparasitic activity on detached leaves of bhendi and a detailed microscopic analysis of the interactions between mycoparasite and *Eryisphe cichoracearum* was documented. Fungicide application is one of the most effective methods to manage powdery mildew disease. However, the frequent use of these fungicide has many disadvantages like the development of resistance to pathogens, residual toxicity and environmental pollution. Hence, we search for an alternative means for disease management. The genus *Ampelomyces* is considered to be the potential biocontrol agent against the powdery mildew pathogens.

Keywords: *Ampelomyces,mycoparasitic fungus, bhendi, biocontrol agent*

INTRODUCTION

Powdery mildew is a common fungal disease characterized by a white powdery covering on aerial plant parts. It grows well in favorable environmental conditions with high humidity and moderate temperatures. Taxonomically, powdery mildew belongs to order *Erysiphales*, which contains a number of genera and species distributed worldwide over diverse host range. This disease affects many types of plants including, grasses, vegetables, fruits, forest trees and agricultural crops, which results in huge annual yield loss every year. Hence, considerable management methods are suggested by many plant protection scientist from time to time. Although, a number of methods *viz.* physical, chemical and biological are now in practice to control the powdery mildew diseases on various hosts. Chemical fungicide is the most commonly used method in the control of powdery mildew (Zhao *et al.,* 2012). But the best method of control is prevention (Gautam and Avasthi, 2016). However, due to the negative effects of fungicide on the natural balance, there is an increasing interest in the use of biological control agents in recent years. There are a number of biocontrol agents available in nature which help the plant to control the fungal pathogen. An example of such type of biocontrol association is mycoparasitism, is defined as the association of two fungi where one acts as a parasite over the other. This term along with mycoparasite was introduced by Butler (1957) to elucidate the complex interrelationships between a fungal host and parasite.

Ampelomyces is one of the naturally occurring mycoparasites which affects mycelium, conidial spores and ascocarps of powdery mildew (Kiss *et al.,* 2004). This mycoparasite colonizes a large area of the target site, competes for the plant substrates and nutrients thereby causes the death of pathogens due to starvation. It acts like a hyperparasite

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which penetrates the pathogen and infects it by forming pycnidia within powdery mildew hyphae, conidiophores and chasmothecia. Pycnidial fungi belonging to the genus *Ampelomyces* have been documented to be intracellular mycoparasites of powdery mildew (Szentiványi *et al*, 2003). Genus *Ampelomyces* belong to the class of *Coelomycetes* that are widespread, thermophilic and adapted to various climatic conditions (Sucharzewska *et al.,* 2011). The concentration of *Ampelomyces* conidia is an important factor affecting their germination. Germination has been shown to decrease dramatically when conidia are at a concentration of more than 10^6 conidia ml^{-1}, due to the production of self-inhibitory substances. The presence of host fungi is recognized by *Ampelomyces* and a water-soluble substance from conidia of powdery mildew fungi has been shown to stimulate the germination of *Ampelomyces* conidia *in vitro* (Gu and Ko,1997). After penetration, the hyphae of the mycoparasite continue to grow and produce their intracellular pycnidia after 5 to 8 days in the mycelia of their fungal host (Hashioka and Nakai 1980; Sundheim and Krekling 1982). The natural occurrence of *Ampelomyces* is important for assessing its potential as biological control agent against powdery mildews with favorable conditions such as high humidity or moisture, temperature around 25 °C and the presence of host is essential but this mycoparasite have ability to survives and it active against the powdery mildew even at temperatures below 12 °C (Jarvis and Slingsby 1997; Philipp and Cruger 1979). The present study was aimed to understand the cultural and morphological characteristics of mycoparasite associate with *Erysiphe cichoracearum in vitro.*

MATERIAL AND METHODS

This work has been carried out at the Department of Plant Pathology, TNAU, Coimbatore.

Survey

An intensive and systematic survey was conducted to assess the severity of the powdery mildew disease on bhendi and other crops. For the survey we had selected fifteen leaves randomly from diseased plants and the per cent disease index (PDI) was worked out as per the standard grade chart is given by Jamadar and Desai (1997).

Powdery mildew disease score (0 - 9 scale)

Grade description (% leaf area infected)

0	No sign or symptoms
1	0-10% infection
3	11-15% infection
5	16-25% infection
7	26-50% infection
9	>50% infection

The Per cent disease index (PDI) was described by Mc Kinney's (1923).

PDI = Sum of numerical ratings / Total number of leaves observed × 100/ Maximum disease grade

The survey was also extended to collect the different isolates of *Ampelomyces* mycoparasitized samples in bhendi from the different locations of Coimbatore district. Infected samples were brought to the laboratory for further studies. The identification of mycoparasitic organism was made on the basis of morphological characteristics like pycnidial size, pycnidial shape and conidial morphology. The *Ampelomyces* infected leaves were incubated in the plant growth chamber for 2 weeks at 25± 2°C (>70% RH) under 8:16 dark to the light ratio (Liyanage *et al*., 2018). The growth of *Ampelomyces* and its mycoparasitism over the powdery mildew of bhendi were photographed every day fora time period of 2 weeks. Fresh samples collected from the field were wrapped in a sterilized moist paper towel, sealed in zip-lock plastic bags, and stored at 5 °C.

Isolation and identification

Ampelonlyces mycoparasites were detected with a stereomicroscope by the presence of their brown intracellular pycnidia in powdery mildew colonies. The area of mildew colonies was measured as well as the area of the parasitized mildew mycelia indicated by pycnidia of *Ampelornyces.* Light microscopy was used to study the structural aspects of mycoparasitism on samples (Kiss *et al*.,1997). Pycnidia of *Ampelomyces* species were isolated from powdery mildew infected bhendi leaves by transferring them with sterilized needles into Petri plates containing water agar supplemented with 200µg of streptomycin per ml (Hijwegen and Buchenauer, 1984), cultured at 25 °C using a 12-hour cycle of fluorescent illumination. After a colony had been formed the mycelium was sub cultured on modified Czapek-Dox medium supplemented with 2% malt extract and 0.5% chloramphenicol. (Liyanage *et al.,* 2018). The recipe of the modified Czapek-Dox medium is as follows: NaNO3 (1.5g), KH2PO4 (0.5g), KCl (0.25g), MgSO4 (0.25g) and malt (10g) in 500ml of distilled water. The isolates of *Ampelomyces* were grown separately in Czapek-Dox medium by placing a disc of the actively growing mycelium of each isolate at the center of the Petri plate and incubated at room temperature. The isolates were identified based on morphological characters by means of visual and microscopic observations. The morphological characters of different isolates of *Ampelomyces viz*., zonation, colony color, mycelial growth, pycnidial formation and topography were studied at 15th day after inoculation as described by Sharma (2006).

Cultural and morphological examination

The cultural behaviour and morphological characteristics of the pycnidia and conidia of the various *Ampelomyces* isolates were evaluated on 10 different solid media viz., Sucrose nutrient agar, Glucose nutrient agar, Czapeksdox's agar, V-8 agar, Oat meal agar, Potato dextrose agar, Potato sucrose agar, Carrot extract agar, Potato carrot agar and Host extract agar. The color of the mycelia and pycnidia was assessed by visual observation of colonies grown for 15 days at 25°C in the dark. We also observed the colony growth, growth pattern and color of the matured colony on different synthetic and non-synthetic media (Sharma, 2006).

Physiological and Nutritional influence

The radial growth rate of the *Ampelomyces isolates* was evaluated at different temperatures of 15°, 20°, 25°, 30° and 35° C on Petri dishes containing PDA. Cultures were incubated in the dark for 15 days and the radial growth of the colonies was evaluated by measuring the diameter of each colony twice a week. All of the experiments were conducted with three replicates (plates) per isolates (Angeli *et al.,* 2011). Likewise, the different pH and the nutrient requirement for growth of *Ampelomyces* isolates such as carbon and nitrogen sources were evaluated as like the same procedure followed above. The different pH evaluated on radial mycelial growth was 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0. The different carbon sources used were sucrose, mannitol, maltose, cellulose, starch and dextrose and different nitrogen sources are calcium nitrate, peptone, ammonium chloride, ammonium sulphate, ammonium nitrate, sodium nitrate and potassium nitrate (Sharma, 2006).

In vitro screening against *Erysiphe cichoracearum*

Cavity slide assay

Pycnidiospore suspensions of each isolate of *Ampelomyces* were collected separately by adding 5 ml of sterile distilled water to each Petri plate containing individual isolate of actively growing *A. quisqualis* and surface of colonies were rubbed smoothly with sterile brushes to dislodge the pycnidiospores. The pycnidiospore concentration of each isolate was adjusted equally to 10^8 spores ml^{-1} using a Neubauer hemocytometer. Mycoparasitism of *Ampelomyces* isolates (10^8 spores ml^{-1}) on *E. cichoracearum* (10^8 spores ml^{-1}) using cavity slides containing mannitol sucrose (MS) solution (mannitol, 20 g l-1, sucrose, 10 g l-1, tetracycline hydrochloride, 25 mg l-1) as described by Szentivanyi and Kiss (2003), was placed in a Petri plate (R.H. = 100%) and incubated under sterile dark condition for 7 days (Zhao *et al*., 2012). Then the mycoparasitic activity of *Ampelomyces* over mildew pathogen was confirmed by using light microscopy (Parthasarathy, 2018).

Detached leaf assay

Detached mildewed bhendi leaves had treated with different concentration of conidial suspension of *Ampelomyces*@ 10^2, 10^3, 10^4,10^5,10^6 spores/ ml at the rate of 3-4 drops of 15µl and water as control (Kiss *et al.* 1997 and Sharma 2006). Inoculated leaves and water controls were placed in Petri dishes filled with one layer of sterilized blotter paper anda thin layer of cotton wetted with distilled water, then incubated under the dark condition at 25°C for 7 days in a BOD incubator (Zhao *et al*., 2012). Three replications were maintained. The penetration, intra hyphal growth and pycnidial formation of the mycoparasites were also documented by microscopy.

Cross infection ability of Ampelomyces spp in vitro

The mycoparasitic efficacy of *Ampelomyces* virulent isolate on different powdery mildew fungi was studied using detached leaf assay. The germination and mycoparasitism of *Ampelomyyces* potential strain (10^8 spores ml^{-1}) against different powdery mildew fungi (10^8 spores ml-1) *viz*., *Erysiphe cichoracearum*, *E. necator, E. polygoni, Leveillula taurica*, *Phyllactinia corylea, Oidium parthenii* was tested as described by Szentivanyi and Kiss (2003). The detached leaves having both the powdery mildew pathogens and mycoparasite were placed in a Petriplate (R.H. = 100%) and incubated underthe dark condition at 25°C for 7 days in a BOD incubator (Zhao *et al*., 2012). Three replications were maintained and mycoparasitic activity was recorded after the incubation period using microscopic observation.

PCR amplification using ITS region

For the fungal molecular study, ITS regions of rRNA genes were sequenced from the mycelium of fungal isolates collected from Coimbatore district. Total DNA was extracted from the mycelia by CTAB (cetyltrimethylammonium bromide) (Zolan and Pukkila, 1996). The PCR was performed in a Master Cycler (Eppendorf, Germany) in a total volume of 20µl using 0.5 µl tubes. The PCR mixtures contained 10X buffer (with 2.5 mmol l^{-1} MgCl2) - 2 µl; 2 mmol l^{-1} dNTP mixture - 2 µl; 2 mol l^{-1} primer - 5 µl; Taq DNA polymerase 3U; water - 8 µl and 50 ng of template(Kiss, 1997; Kiss, 1998). The genomic rDNA ITS region was amplified using a combination of primers ITS-1 (5' -TCCGTAGGTGGACCTGCGG - 3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC- 3') (White *et al.,* 1990). The PCR conditions were 5 min preheating step at 95^{0}C followed by 35 cycles consisting of denaturing at 95^{0}C for 30 sec, 52^{0}C annealing for 30 sec, extension at 72^{0}C for 30 sec and with a final extension at 71^{0}C for 5 min (Szhentivanyi *et al*., 2005). The PCR products were analyzed by electrophoresis on a 1.5 per cent agarose gel,

visualized under UV light and photographed and documented with an Alpha Imager (Alpha Innotech, California, USA).

PCR amplification using specific primer pair

The reaction mix for specific primer for *Ampelomyces* amplification of the DNA consisted of 20 µl vol, (0.25 mM each of primer pair, 0.25 mM dNTP,1.5 mM MgCl2, 50-80 ng of template DNA, 2 U of Taq DNA polymerase an 1x PCR buffer mix) and the sequence of the ITS specific primers were as follows: AQ-F264, 5'-GATGAAGAACGCAGCGAAAT-3', AQ-R462, 5'-GCTGCCAATTGCTTTGAGAT-3'. PCR was undertaken using a Mastercycler gradient (Eppendorf, Germany) using 0.5 µl tubes. Conditions were 10 min preheating step at 95^{0}C followed by 40 cycles consisting of denaturing at 95^{0}C for 30 sec, 60^{0}C annealing for 30 sec, extension at 72^{0}C for 30 sec and with a final extension at 72^{0}C for 5 min (Tollenaere *et al.,* 2014). The PCR products were analyzed by electrophoresis on a 1.5 per cent agarose gel, visualized under UV light and photographed and documented with an AlphaImager (Alpha Innotech, California, USA). The amplified PCR product was sequenced and the nucleotide sequences were deposited in the GenBank database.

RESULTS AND DISCUSSION

Survey

The roving survey was conducted in bhendi growing areas of Coimbatore district. The observations on the incidence of Powdery mildew on bhendI leaves were recorded and per cent disease index (PDI) was calculated. The results are presented in Table 1. The result revealed that in general, the per cent disease index of powdery mildew ranged from 38.04 - 69.54 per cent. A maximum disease

Table 1. Survey and collection of isolates of Ampelomyces spp. in bhendi growing areas of Coimbatore districts in Tamil Nadu

Villages	Crop stage	GPS co – ordinates		Mean PDI of powdery mildew*
		Lattitude	Logitude	
Poochipatti	Flowering	11.3790ºN	77.6404ºE	50.56^g (45.32)
Vellimallaipatinam	Fruit formation	10.9862ºN	76.7769ºE	61.49^d (51.64)
Devarayapuram	Flowering	10.9976ºN	76.8159ºE	54.3^f (47.47)
Vedapatti	Maturity	11.0024ºN	76.8923ºE	67.10^b (55.00)
Thudialur	Fruiting	11.4910ºN	77.7412ºE	58.09^e (49.66)
Onampalayam	Vegetative	11.0102ºN	76.8671ºE	64.34^c (53.33)
Narasimpuram	Fruit formation	16.8080ºN	81.2230ºE	52.23^g (46.28)
Thondamuthur	Flowering	10.9899ºN	76.8409ºE	69.54^a (56.50)
Orchad, TNAU	Vegetative	11.0069ºN	76.8923ºE	47.49^h (43.56)
Poosaripalayam	Fruit formation	11.0062ºN	76.9333ºE	38.04^i (38.08)

PDI- Per cent Disease Index, * Values are means of three replications
Figures in parentheses represent arcsine transformation.

Means followed by a common letter are not significantly different at 5% level by DMRT at P ≤ 0.05 incidence of 69.54 per cent disease index was observed at Thondamuthur village in Coimbatore district followed by 67.10 PDI at Vedapatti village. A minimum incidence of 38.04 per cent disease index was recorded at Poosaripalayam village (Fig.1). From the present study, it is evidenced that the incidence of powdery mildew disease and its occurrence is wide spread in major bhendi growing areas of Coimbatore district. The disease occurs in almost all seasons leading to yield losses between 17 to 86.6 per cent (Sridhar and Poonam Sinha, 1989; Gogoi *et al*., 2013 and Younes and Abo-Elyousr, 2014).

This finding corroborates with the earlier study of Athira *et al*. (2017), they reported the Thondamuthur village of Coimbatore district showed the maximum incidence of powdery mildew incidence.

Table 2. Morphological characters of different isolates of Ampelomyces spp. on Czapex-Dox medium

Isolate	Topography	Colour of mature colonies	Zonation	Margin	Colony growth	Pycnidia shape	Pycnidia size(µm)		Pycnidio spores	Pycnidio spores size (µm)	
AQB1	Septate, Hyaline	Pale brown to black	Present	Wavy	Slow	Globose	70.76	63.81	Unicellular, hyaline, oval	13.68	5.21
AQB2	Septate, Hyaline	Pale brown to black	Absent	Smooth	Moderate	Ovoid	71.85	54.46	Unicellular, hyaline, oval	14.41	5.89
AQB6	Septate, Hyaline	Brown to white	Absent	Wavy	Rapid	Pyriform	56.24	50.23	Unicellular, hyaline, oval	9.63	5.51
AQB7	Septate, Hyaline	Dull white to ash	Present	Smooth	Slow	Globose	74.20	58.40	Unicellular, hyaline, oval	15.77	6.27
AQB8	Septate, Hyaline	Greyish black	Present	Irregular	Rapid	Round	59.04	50.54	Unicellular, hyaline, oval	10.45	5.50
AQCU1	Septate, Hyaline	white to black	Present	Wavy	Moderate	Globose	64.62	55.55	Unicellular, hyaline, oval	11.47	5.35
AQS1	Septate, Hyaline	Ash	Present	Wavy	Rapid	Ovoid	69.03	51.36	Unicellular, hyaline, oval	12.67	5.57
AQS2	Septate, Hyaline	Ash to black	Absent	Smooth	Rapid	Round	66.11	57.06	Unicellular, hyaline, oval	12.38	6.34
AQG1	Septate, Hyaline	Brownish ash	Absent	Wavy	Rapid	Oval	59.89	51.00	Unicellular, hyaline, oval	10.65	5.45
AQG2	Septate, Hyaline	Brownish grey	Absent	Smooth	Moderate	Oval	64.56	54.27	Unicellular, hyaline, oval	11.43	5.47

Isolation and identification

Totally 10 isolates of *Ampelomyces* were collected from bhendi and other crops. Morphological analysis of *Ampelomyces* from the naturally parasitized powdery mildew fungi indicated that hyphae of the mycoparasite are slender, slightly coloured and located inside the hyphae, conidiophores and conidia of the powdery mildew fungi. Pycnidia of the mycoparasite were variable in shape (round, ovoid, ellipsoid or cylindrical) and the color ranged from olive green to brown with a reticulate pattern. The size of pycnidia varied and ranged from 56.24 – 74.20 × 50.23-63.81 µm; pycnidiospore is 15.77-9.63 × 6.27 - 5.21 µm in length (Table 2). Mycelium is septate and hyaline during the initial stage. Later it turns greyish white to brownish black in matured

Table 3. Cultural characters of Ampelomyces spp (AQB7) in different synthetic and non synthetic media

Medium	Mean mycelial growth (mm)*	Colony growth	Growth pattern	Mature Colonies	Days to cover full plate	Days to Produce pycnidia	Pycnidial production in the medium
Synthetic media							
Sucrose nutrient agar	41.82^{b} (40.29)	Rapid	Radial	Ash	13	6	Higher
Glucose nutrient agar	42.67^{a} (40.79)	Rapid	Radial	Ash to white	13	7	Higher
Czapeks dox agar	44.33^{a} (41.74)	Slow	Flat	Brownish black	15	10	Moderate
Oat meal agar	39.27^{b} (38.80)	Moderate	Fluffy	Whitish grey	14	11	Higher
V-8 agar	38.33^{c} (38.25)	Slow	Flat	Yellowish brown	15	12	Lower
Non synthetic media							
Potato dextrose agar	44.67^{a} (41.94)	Moderate	Radial	Ash	12	8	Higher
Potato sucrose agar	41.37^{b} (40.03)	Slow	Radial	Greyish black	12	6	Higher
Carrot extract agar	39.26^{b} (38.80)	Rapid	Flat	Brownish black	15	4	Very high
Potato carrot extract agar	40.67^{b} (39.62)	Moderate	Radial	White to brown	13	8	Very high
Host extract agar	41.33^{b} (40.01)	Rapid	Submerged	Brown	12	5	Higher

*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 ≤ 0.05

colonies. The well-grown culture of *Ampelomyces* was viable in Czapex dox and PDA to the maximum period of 60 days. Among the 10 different isolates collected from various mycoparasitized hosts, the isolate AQB7 shows higher virulence. Therefore, which was used for further screening and validation work. The present study revealed that morphological characteristics of all the ten isolates slightly vary in their mycelium, topography, color,the margin of colonies, zonation, colony growth, pycnidial shape and size, pycnidiospore shape and size. The results was in accordance with the reports of Angeli *et al*. (2012), they reported the pycnidial color of *Ampelomyces* varies from light to dark (brown or green) with the age of the culture. The length and width of the pycnidia varies with the ranges of 30 - 90 µm and from 25 to 55 µm, respectively. These results are confirmed by the reports of Sztejnberg *et al*., 1989; Liang *et al*., 2007; Jamali, (2015); Gautam and Avasthi, (2016) on basis of morphological characters.

Table 4. Effect of different temperature and pH on the radial mycelial growth and sporulation of Ampelomyces spp (AQB7)

Different levels of Temperature			Different levels of pH		
Temperature (°C)	Mean mycelial growth (mm)*	Production of pycnidia**	pH	Mean mycelial growth*	Sporulation
15°C	33.00c	+	5.0	28.62^{e}	-
20°C	36.67^{b}	+++	5.5	30.29^{de}	++
25°C	44.00^{a}	++++	6.0	33.43^{bc}	++
30°C	26.00^{d}	++	6.5	34.67^{b}	++++
35°C	3.50^{e}	-	7.0	35.33^{a}	+++
15°C	33.00^{c}	+	7.5	34.00^{bc}	++
			8.0	32.67^{cd}	+

*Values are mean of three replications. ** - : Nil; +: 25%; ++: 50%; +++: 75%; ++++: 100%.

Means in a column followed by same superscript letters are not significantly different according to DMRT at $P \leq 0.05$.

Cultural and morphological examination

The growth of *Ampelomyces* was studied and compared in five synthetics and five nonsynthetic media. The radial growth, colony color and growth habit of the fungus were recorded. The results showed that maximum radial means mycelial growth in synthetic media (44.33 mm) was observed in Czapex dox agar and followed by Glucose Nutrient agar (42.67mm). Likewise in the case of nonsynthetic media, the maximum radial means mycelial growth was observed in potato dextrose agar (44.67mm) and followed by potato sucrose agar (41.37mm) at 15 days after inoculation. The colony color was found to vary from whitish grey to brownish black, mostly ash to brown in color and the growth varied from flat, radial, fluffy and submerged growth (Table 3). The media *viz*., Czapek-Dox agar, Sucrose nutrient agar, Potato dextrose agar, Carrot extract agar supported pycnidial production. The cultural character of *Ampelomyces* isolates was tested on different solid media. The results showed that, totally ten different media comprised of five synthetic and five non-synthetic media chosen for growing the cultures. The radial growth was assessed and the nature of the colony and sporulation were studied and recorded on solid media. Among the different solid media tested, there was a variation on colony color and growth rate of mycelium and pycnidiospore formation largely varied, due tothe nutritional status of individual medium. This result was in conformity with the earlier reports of Kiss *et al.* (2004) and Angeli *et al*. (2012). They reported the maximum mycelial growth was observed in PDA and sucrose nutrient agar. Then, more amount of conidial production was observed in host extract and carrot extract media in our experiment. This result was in line with Gu (1998), and he reported that carrot extract medium was best for the conidial formation of *Ampelomyces.*

Physiological and nutritional requirement

The optimum temperature required for growth and pycnidial production of the *Ampelomyces*, the culture was incubated at a different range of temperatures *viz*., 15°, 20°, 25°, 30° and 35° C on Petri dishes containing CDA. The maximum growth (44.00 mm) was recorded at 25°C followed by 20°C (36.67mm) and 15°C (33.00), respectively. Lowest growth (3.50 mm) was recorded at 35°C (Table 4).

Table 5. Effect of different carbon sources on the radial mycelial growth of Ampelomyces spp. isolate (AQB7)

Carbon sources			Nitrogen sources		
Sources	Mycelial growth (mm)*	Production of pycnidia**	Sources	Mycelial growth (mm)*	Production of pycnidia**
Sucrose	41.67[a]	+++	Peptone	42.33[a]	-
Dextrose	39.67[ab]	+++	Calcium nitrate	37.00[c]	+++
Maltose	37.43[ab]	++	Ammonium nitrate	34.33[d]	++
Mannitol	43.33[a]	++++	Potassium nitrate	40.33[b]	++
Cellulose	33.00[ab]	++	Ammonium sulphate	31.67[de]	-
Starch	25.57[b]	++	Sodium nitrate	39.00[b]	+++
Control	20.06[b]	-	Ammonium chloride	20.67[e]	-
			Control	24.82[e]	-

*Values are mean of three replications. ** - : Nil; +: 25%; ++: 50%; +++: 75%; ++++: 100%.
Means in a column followed by same superscript letters are not significantly different according to DMRT at P ≤ 0.05.

Similarly, pycnidial production was found at 15°C, 20°C, 25°C and 30°C except at 35°C at 15th day after inoculation. While, the optimum pH for growth and pycnidial production of the *Ampelomyces* was evaluated from the experiment. The results revealed that out of the seven pH ranges (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0), the pH 7.0 and 6.5 were found to be significantly superior, and recorded radial mycelial growth of 35.33 mm and 34.67 mm, respectively after 15 days incubation. In this experiment, the maximum pycnidial production of *Ampelomyces* was recorded in the Petri plates maintained at pH 7.0. Least mycelial growth (28.62 mm) was recorded at pH 5.0 as shown in (Table 4).The experimental results revealed that the essential nutrient required for the growth and sporulation of *Ampelomyces* from the different carbon and nitrogen sources were taken under the study.The results showed that mannitol and sucrose were the best carbon sources required by *Ampelomyces* by recording the mean radial growth of (43.33 mm) and (41.67mm) at 15 days after inoculation. The pycnidial formation was lower in the starch asa sole carbon source in the 15th day after inoculation. Mannitol, sucrose and dextrose supported the abundant production of pycnidia. Least radial growth was observed in medium containing starch (25.57 mm) even after 15 days of incubation (Table 5). As in the case of various nitrogen sources used to assess their effect in supporting the growth and development, peptone was found to be the best nitrogen source followed by sodium nitrate by recording 42.33 mm and 40.33 mm radial mycelial growth, respectively. Least growth was observed (20.67 mm) in ammonium chloride after 15 days of incubation. Peptone showed the maximum mycelial growth and abundant pycnidial production in sodium nitrate and potassium nitrate (Table 5). In the present study, *Ampelomyces* was grown at different temperatures *viz*., 15, 20, 25, 30 and 35°C. Sztejnberg *et al*. (1990) reported that on Czapek agar medium, the optimum temperature for spore germination and pycnidia formation by *A. quisqualis* was at 20 and 25°C, This report was in conformity with our experimental results. The results agree with Angeli *et al*.(2011), who reported the mycoparasitic fungus *Ampelomyces* could grow under a wide range of temperature from 15 to 30°C. The results are also in accordance with the previous study conducted by Liang *et al*. (2004), where the optimal temperature for spore germination was observed at 25°C. They also reported that *Ampelomyces* could grow at a temperature of 10 to 30°C. Yuan *et al*. (2006) also supported this study that *Ampelomyces* could grow at a temperature of 10 to 30°C and the optimal temperature for its growth and sporulation was at 20°C. Liang *et al*. (2004) indicated that the optimal pH value for the growth and sporulation of *Ampelomyces* was 5.0 to 7.0 and the spores could not germinate at pH lower than 2.0 or higher than 10.0. In this study, we observed that the fungus grew at all the pH levels tested and pH 7.0 was found most suitable for the growth of the fungus by recording maximum mycelial growth of 35.33 mm followed by pH 6.5 with 34.67 mm. The results are in accordance with the earlier work done by (Athira, 2015; Parthasarathy, 2018). They reported the maximum mycelial growth at the pH 7.0. Another supporting evidence carried out by Sharma

(2006), who reported that maximum mycelial growth and sporulation of *Ampelomyces* was obtained at pH 6.5. This was also supported by Yuan *et al*. (2006) that the optimal pH for the growth and sporulation of *A. quisqualis* was found to be at pH 7.0. The results are in conformity with Mhaskar (1976) who reported that *A. quisqualis* showed good growth and sporulation between pH 5.5 to 7.0. From our investigations, the maximum radial mycelial growth of *Ampelomyces* was observed in mannitol closely followed by sucrose and starch recorded the lowest mycelial growth. The results are in true to with Liang *et al*. (2004), who reported that among the different carbon sources, mannitol was found to be the best for mycelial growth and glucose was the best for sporulation. This was supported by (Sharma,2006: Athira, 2015). Angeli *et al*. (2011) reported that the glucose and sucrose were best carbon sources for the growth of *Ampelomyces.* From our experimental result, peptone showed maximum radial mycelial growth followed by potassium nitrate and sodium nitrate. This result was in conformition with the results of Athira *et al*. (2015) and Parthasarathy *et al.* (2018). Further was supported by Liang *et al*. (2004) by stating that peptone and yeast extract were better for mycelial growth, air-dried weight and sporulation of *Ampelomyces*. The results are in accordance with the previous study conducted by Yuan *et al.* (2006), they reported that peptone was the optimal nitrogen source for the growth of *Ampelomyces*. Rao and Mhaskar (1976) reported that peptone and aspartic acid was found to be good for the growth and sporulation of *Ampelomyces*

In vitro screening against *Erysiphe cichoracearum*

Efficacy of *Ampelomyces* against *Erysiphe cichoracearum in vitro*

Ten isolates of *Ampelomyces* spp were tested against *Erysiphe cichoracearum* under *in vitro* by cavity slide assay and detached leaf assay. All the ten isolates effectively mycoparasitized the propagules of the pathogen on cavity slide assay during incubation period. The mycoparasitism of virulent isolate of *Ampelomyces* spp on the powdery mildewed surface of detached leaf were found to show rapid colonization of *Ampelomyces* on conidiophores, conidia and mycelia of *E. cichoracearum* within 7 days of incubation at room temperature (30°C±2). The results obtained from the detached leaf assay experiment revealed that among the different concentration of conidial suspension of virulent isolate, the concentration at the rate of 10^8 conidia / mlwas found to show maximum parasitism against powdery mildew pathogen by recording 35.85 per cent intensity of mycoparasitism and followed by (35.28%) at the concentration of 10^7 conidia / ml (Table 6, Fig.2). The present study was aimed to explore the mycoparasitism and virulence potential of the isolates of *Ampelomyces* against *E. cichoracearum in vitro* both by cavity slide assay and detached leaf assay. The result obtained from this study was in accordance with the report of Parthasarathy (2018), he reported the conidial concentration of about 10^8 conidia/ml is found to be effective for mycoparasitism in both cavity and detached leaf assay.

Table 6. Effect of Ampelomyces spp AQB7 on the growth of Erysiphe cichoracearum on detached leaves

Spore concentration of antagonist/ml water	Mean growth inhibition (%)*
10^4	30.32^{d} (33.41)
10^5	32.36^{c} (34.67)
10^6	34.27^{b} (35.83)
10^7	35.28^{a} (36.44)
10^8	35.85^{a} (36.78)
Control (water)	-

*Values are mean of three replications. Figures in parentheses represent arcsine transformation. Means in a column followed by same superscript letters are not significantlydifferent according to DMRT at P ≤ 0.05.

Cross infection ability of Ampelomyces spp in vitro

The cross infectivity nature of a virulent isolate of *Ampelomyces* spp, naturally isolated from bhendipowdery mildew fungus, *E. cichoracearum* was further screened against several powdery mildew fungi.

Table 7. Effect of Ampelomyces AQB7 on different powdery mildew fungi *in vitro*

Plant host	Powdery mildew pathogen	Detached leaf assay (Per cent mycoparasitism)*
Bhendi	*Erysiphe cichoracearum*	34.08^{a} (35.72)
Sunflower	*Erysiphe cichoracearum*	24.68^{de} (29.79)
Cowpea	*Erysiphe polygoni*	29.51^{bc} (32.91)
Dolichos bean	*Erysiphe polygoni*	27.32^{cd} (31.51)
Grapes	*Erysiphe necator*	21.83^{ef} (27.86)
Cucumber	*Erysiphe cichoracearum*	30.85^{abc} (33.74)
Mulberry	*Phyllactinia corylea*	27.75^{cd} (31.79)
Sesamum	*Erysiphe cichoracearum*	29.37^{bc} (32.82)
Mexican fire plant	*Leveillula taurica*	19.81^{f} (26.43)
Parthenium	*Oidium parthenii*	32.91^{ab} (35.01)

*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 ≤ 0.05.

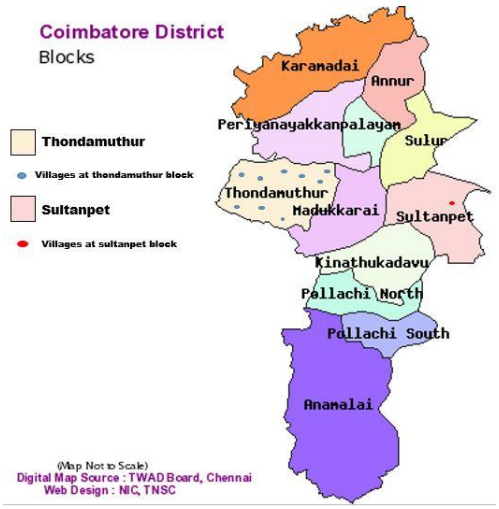

Figure 1. Surveyed areas for the incidence of powdery mildew disease and collection of isolates of Ampelomyces in Coimbatore district

The intensity of mycoparasitism on the *E. cichoracearum* mildew on the detached leaf surface of different hosts *viz*., in bhendi, sesamum, sunflower, cucumber, grapes, parthenium, cowpea, lab lab, mulberry, mexican fire (weed host) ranged from 19.81-34.08 per cent.

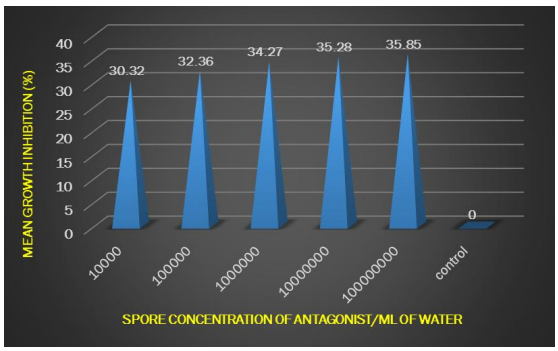

Figure 2. Effect of Ampelomyces spp (AQB7) on the growth of Erysiphe cichoracearum on detached leaves

The genus *Oidium* on *Parthenium* exhibited maximum mycoparasitism per cent (32.91%) compared to other genera tested. Meanwhile, other than *Erysiphe* genus tested, mycoparasitism of *Ampelomyces* sppon *L. Taurica* on Mexican fire plant (weed host) showed least mycoparasitic per cent of about 19.81. Similarly, the intensity of mycoparasitism was observed on the other genera *Phyllactinia* in mulberry (27.75%). In addition, the mycoparasitism was also observed on *Erysiphe necator* in grapevine and *Erysiphe polygoni* on *Dolichos* bean with intensities of 21.83% and 27.32%, respectively (Table 7). In this experiment, the mycoparasitism by *Ampelomyces* was observed on all the tested powdery mildew pathogens. The intensity of mycoparasitism was found to vary with

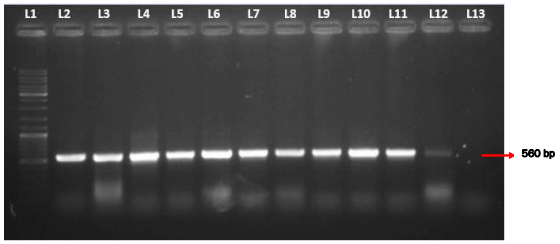

Figure 3. ITS amplification of Ampelomyces approximately at 560 bp

Lane1-1KB ladder ; Lane2-AQB1 ; Lane3-AQB2; Lane4-AQB6; Lane5-AQB7; Lane6-AQB8; Lane7-AQCUI; Lane8-AQS1; Lane9-AQS2; Lane10-AQG1; Lane11-AQG2; Lane12- Positive control (TNAU 123); Lane13- Negative control (water).

the genera and species due to microclimate, plant host nutrition, the persistence of other mycoparasites and life stage of the parasitic host, etc. In this experiment, the AQB7 isolate of *Ampelomyces* was found to be an effective mycoparasite, in terms of rapid and intensified mycoparasitism, cross-infectivity nature, highest per cent of inhibition against pathogen than all the other isolates tested and hence AQB7 isolate was taken up for further experiments. To ensure the virulence and cross-infectivity potential of the isolate AQB7, it was again subjected to detached leaf assay against six different genera of powdery mildew fungi *viz*., *Erysiphe* spp., *L. taurica, Oidium parthenii* and *Phyllactinia corylea*. The experimental results

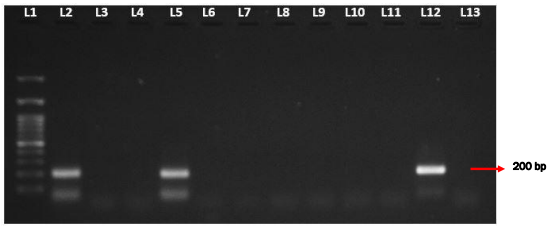

Figure 4. Specific primer amplification of Ampelomyces spp at 200 bp

Lane1-100bp ladder ; Lane2-AQB1 ; Lane3-AQB2; Lane4-AQB6; Lane5-AQB7; Lane6-AQB8; Lane7-AQCUI; Lane8-AQS1; Lane9-AQS2; Lane10-AQG1; Lane11-AQG2; Lane12- Positive control (TNAU 123); Lane13- Negative control (water).

showed that the virulence nature was due to rapid colonization and cross infectivity of *Ampelomyces* spp (AQB7) in major (*Oidium* and *Oidiopsis*) mycelial and conidiogenesis groups. Similarly, Hijwegen and Buchenauer (1984) proved the cross-infectivity nature of *A. quisqualis* isolated from *Erysiphe* spp. on *Medicago lupulina*to unrelated genus *S. fuliginea* on *Cucumis sativus*. This was in accordance with the findings of Liang *et al*. (2007), they reported that six authentic *A. quisqualis* isolates obtained froma culture collection, produced intercellular pycnidia in the conidiophores of *P. xanthii* and *Golovinomyces orontii*. The result was supported by some other evidence reported by Sztejnberg *et al*. (1989), Kiss *et al*. (2004), Szentivanyi *et al*. (2005), Liang *et al*. (2007) and Kiss (2008). The cross-inoculation

experimental results have repeatedly shown that *Ampelomyces* mycoparasites collected from a given powdery mildew fungal host can readily infect other fungal host species also. The experimental results indicated the absence of strict specialization of the *A. quisqualis* in relation to different powdery mildew fungi. Also, it seems to be the most common and exclusive internal parasite of *Erysiphales* though it has been reported early, to infect the species of *Mucorales* and *Plasmopara* (Hawksworth, 1981) and also, *Brasilomyces* (Hanlin and Tortolero, 1984).

Molecular characterization

PCR amplification of ITS region of ten isolates of *Ampelomyces* spp. was performed using the universal primers of forward ITS1 and reverse ITS4 to confirm the initial identification of genus, *Ampelomyces* which amplified a fragment of 560bp corresponding to the region of the 18s-28s rDNA intervening sequence for *Ampelomyces* spp (Fig.3). Further, using the specific primer pair of *Ampelomyces,* the isolates were subjected to PCR and it was amplified around 200 bp only in the isolate AQB1 and AQB7 (Fig.4) and then sequenced. The sequences were deposited in Gene Bank with Accession Nos. MK942572 and MK937681. The sequence was matched with *Ampelomyces* spp. In this course of work, the isolation of DNA from ten isolates of *Ampelomyces* spp was done separately. PCR based detection was done using the universal primers of forward ITS1 (5'CTTGGTCA TTTAGGAAGTAA-3') and reverse ITS 4 (5'TCCTCCGTTATTG ATATGC-3'). The *Ampelomyces* genus was amplified as a fragment of 560 bp. These results are in accordance with the results of Kiss (1997), Angeli *et al*. (2012), Pintye *et al*. (2012), Jamali (2015) and Liyanage *et al*. (2018). Bryan *et al*. (1995) reported that ITS regions have been used successfully to identify and differentiate closely related fungal species. The amplification of DNA sequences through the polymerase chain reaction (PCR) has found widespread application in the diagnosis and detection of fungi (Louis *et al*. 2000). Further, the isolates were subjected to PCR with the specific primer pair of *A. quisqualis* andit amplified an amplicon of 200 bp. The results are in accordance with the results of the previous study conducted by Tollenaere *et al*. (2014).

CONCLUSION

The mycoparasitic fungus *Ampelomyces* is naturally occurring and can able to survive at 20 to 30°C. The isolate of *Ampelomyces* was found to be effective against the powdery mildew disease caused by *Erysiphe cichoracearu*m on bhendi crop. Though cultural and molecular identification were done. Further, characterization using house keeping primers and formulation development are essential to use *Ampelomyces* as a successful biocontrol agent against powdery mildew diseases under field conditions.

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