International Journal of Plant & Soil Science

33(22): 12-21, 2021; Article no.IJPSS.76169
ISSN: 2320-7035

Rapid Detection of Airborne Inocula of Grapevine Mildews Using PCR and LAMP Assay

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Authors' contributions

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

Article Information

DOI: 10.9734/IJPSS/2021/v33i2230677
Editor(s):
(1) Dr. Ahmed Medhat Mohamed Al-Naggar, Cairo University, Egypt.
Reviewers:
(1) Fatma Abdel-Motaleb Mostafa, Mansoura University, Egypt.
(2) Dalia Gamil Aseel, City of Scientific Research and Technological Applications, Egypt.
Complete Peer review History: https://www.sdiarticle4.com/review-history/76169

Original Research Article

Received 12 August 2021
Accepted 24 October 2021
Published 27 October 2021

ABSTRACT

Grapes powdery mildew and downy mildew caused by Erysiphe necator and Plasmopara viticola respectively are the most devastating diseases worldwide resulting in significant loss of yield and quality. Epidemics of grapevine mildews are caused by airborne inocula such as conidia and sporangia. Rapid detection of airborne inocula will help to face up timely management strategies under field conditions. The aim of the current study was to design a suction spore trap to trap the airborne mildew inocula and their early detection by molecular methods of PCR and LAMP assay. A total of twelve airborne inocula samples were collected the weekly intervals from 3 to 14 standard weeks of 2021 during the cropping season. The presence of airborne inocula of *E. necator* was detected on standard weeks 3,6,10 and 13 through PCR assay which yielded an amplicon of 470 bp. Similarly, airborne inocula of *P.viticola* were detected on standard week 6 only through PCR which yielded an amplicon of 520 bp. A rapid, highly specific , sensitive Loop mediated isothermal amplification (LAMP) assay was performed to detect the *E. necator* and *P. viticola* using six sets of LAMP primers constructed by targeting rDNA region of ITS and the 5S rRNA and CesA4 a gene, respectively. LAMP assay efficiently detected the presence of airborne inocula of E.necator in most

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of the samples collected from standard week 3 – 14 except 7, 8, and 9. However, the presence of airborne inocula of P.viticola from standard week 3 – 14 was confirmed by LAMP assay. The LAMP assay is absolutely the best in identifying airborne inocula of grapevine mildews compared to PCR and phenotypic microscopic observation.

Keywords: Erysiphe necator; Plasmopara viticola; suction spore trap; airborne inocula; PCR; LAMP assay.

1. INTRODUCTION

Grapevine (*Vitis vinifera* L.) is an important fruit crop and it is cultivated worldwide predominantly for the production of fresh fruits and manufacturing of wine and raisins. The production was hindered by ascomycete fungus *Erysiphe necator* Schw. [syn. *Uncinula necator* (Schw.) Burr.] causing powdery mildew and Oomycete pathogen Plasmopara *viticola* [(Berk. & Curt.) Berlese & de Toni] causing downy mildew. Both the pathogens are obligate biotrophic that relay fully on a host cell to complete its life cycle. In powdery mildew, matured colonies are greyish, and produce airborne conidia the adaxial surface cause 65 percent yield loss [1], and the downy mildew colonies are whitish which produce airborne sporangia the abaxial surface cause 50-100 percent yield loss when conditions favor infection of flower and berries [2]. Spore trapping is developing technology for the detection of airborne inoculum density in the field and the need for timely application of fungicide spray. The air samples that measure directely amount of airborne pathogens can provide an accurate forecast the risk of severe epidemics for the diseases and also reduce the number of unnecessary chemical sprays [3]. To investigate biological particles in the air caught by a sampling device with a design format suitable for viewing particles under a microscope or detection using molecular techniques. The Burkhard seven days volumetric spore trap based on Hirst spore trap for *Mycospherella brassicicola* [4] and cost- effective impaction spore trap for monitoring the presence of airborne inocula of *Erysiphe necator* [5]. Sampling procedures may involve a passive collection of spores by gravitational deposition and/or sampling specific volumes of air with "active" spore-trapping devices [6]. For molecular detection, Loop-mediated isothermal amplification (LAMP) is an emerging and rapid on field detection technique in molecular biology by employing a set of six oligonucleotide primers that specifically recognize the distinct sequences the target DNA template [7]. The loop primers minimize the time required to amplify target DNA half and make the LAMP assay an efficient, time-saving and rapid detection technique in the modern world [8]. LAMP has also been shown to be less sensitive to PCR inhibitors, thereby requiring less DNA purification for high sensitivity [9]. LAMP assay may be conducted using relatively inexpensive heat sources, such as water baths or block heaters [10]. The amplified LAMP product can be detected by the deposition of magnesium pyrophosphate precipitate as a byproduct of LAMP assay which can be visualized by the addition of chemical reagents resulting in color change [11] and alternatively observed in gel electrophoresis.

In this study, we have developed a model of suction spore trap to trap the powdery mildew and downy mildew airborne inocula in the grapevine field. The airborne inocula were monitored at weekly intervals qualitatively. Visual identification of mildew inocula from air samples could be achieved through microscopy which is time-consuming and requires technical persons. Alternatively, various nucleic acid based technologies have been developed for detecting airborne pathogens and reduce the time required for assessing samples. One such molecular technique is Loop mediated isothermal amplification assay was used in this study for the molecular detection of airborne inocula samples. The objective of this study was rapid detection of airborne mildew inocula using suction spore trap coupled with LAMP assay.

2. MATERIALS AND METHODS

2.1 Designing of Suction Spore Trap

The Suction spore trap was designed a 110 mm diameter PVC pipe with three slit. The sampling plates were placed through the slits to collect airborne inocula in the grapevine field. The spore trap contains a 2.4 W - 12V DC motor attached the lower end of the trap coupled to a fan that runs at a maximum speed of 300 rpm and stands were adjustable the type to a definite height. Power supply to the DC motor was provided by

a solar panel with module dimensions (300 x 350 x 21mm) and peak power voltage 18 V, peak power current 0.56 A, and weight 1.3 kg. The following parameters the solar panel are measured under STC: 1000 w/m2 insolation at a particular distribution of AM 1.5 cell temperature of 25°C (Fig. 1). Suction spore trap that suck the air via inlet from the top and moves it the outlet of the trap the bottom. The airborne inocula were collected on a sampling plate coated with silicone vacuum grease. The spore trap was operated continuously from 3 to 14 standard weeks during the cropping season of 12 January to 7 May of 2021 and sample was collected at weekly intervals for further processing.

2.2 Sampling Plate Preparation

The suction plates were made up of stainless steel with a length of (65mm), width (50mm), and thickness (2mm) for trapping airborne inocula. Suction plates were immersed in hexane for 24 hours and then rinsed with water of 2-3 successive rinses. Suction plates were then autoclaved for 30 minutes and air- dried under an aseptic environment. Using gloved hands, the suction plate was transferred to the laminar airflow chamber and covered with a very thin layer of silicone vacuum grease (Dow Corning). The greased suction plates were placed in a sterile container for fixing a spore trap. Two sampling plate was fixed in the suction spore trap for effective trapping of airborne inocula.

2.3 Suction Spore Trap Deployment

A suction spore trap was deployed a commercial grapevine field situated in Appachipannai Cumbum of Theni district, Tamil Nadu. Within this grapevine field, the suction spore trap was placed in an area where the mildew disease intensity was highest in the previous season. The spore trap was placed at a height of 1.5m and installed one spore trap per hectare. The elevation of this field plot was 391 mean sea level. The mean precipitation was 4.06mm with maximum, minimum, and average temperatures of 35°C, 24°C, and 30°C respectively. The maximum, minimum and average relative humidity was 95%, 47%, 74% respectively. Grapevines were approximately fifteen years of age and the vines are spread over a pandhal mounted at a height 2m above the ground. The sampling plate in the spore tarp was replaced at weekly intervals and store at 4°C until they were processed for DNA extraction.

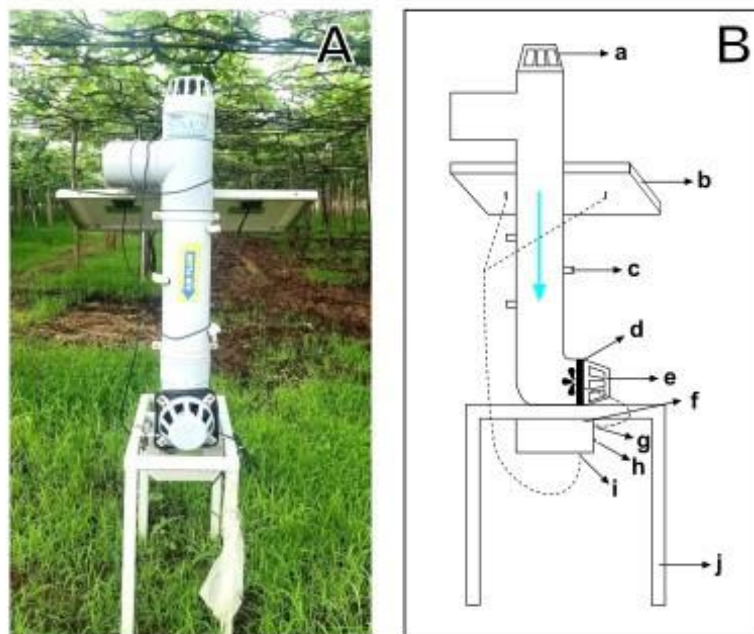

Fig. 1. (A) Suction spore trap design used to monitor the air-borne inocula in grapevine field. (B) Suction spore trap components : (a) Inlet (b)18 V Solar panel (c)stainless steel sampling plate (d)2.4W-12V DC motor (e)Outlet (f)Rechargeable battery (g)Motor connector (h)on/off switch (i)Solar panel connector (j) Spore trap stand . Dashed lines are 2.1mm thickness of 12V -5A power supply electric cable

2.4 Airborne Mildew Inocula Observation through Microscope

Sampling plates were collected a weekly interval from the spore trap installed in the grapevine field and transferred to the laboratory under aseptic conditions for further observation. The content of the sampling plate was gently scraped and transferred a clean sterile microscopic slide and observed a Phase contrast microscope (Leica DM 2000 LED). The images of mildew inocula photographed were photographed using software (LAS version 4.11.0) by Leica Microsystems (Switzerland) Ltd.

2.5 Genomic DNA Extraction from Air Borne Mildew Inocula

The genomic DNA was extracted from the sampling plates following the CTAB method with some modification [12]. The airborne inocula which are sticking in silicone vacuum grease over the sampling plate are scrapped off using a sterilized toothpick stick. Then, it is transferred to the sterile 14mL falcon snap- cap tube contained 300µl CTAB extraction buffer (50Mm Tris – HCl, PH 8.0; 0.7M NaCl and 1 percent CTAB (W/V) and high speed vortexed for a minute. The content in the falcon snap cap tube was transferred to a microcentrifuge tube incubated at 60°C for 20 minutes and then add an equal volume of phenol:chloroform: isoamyl alcohol (25: 24:1) centrifuged at 13,000 x g for 10 min. The aqueous phase was transferred to a 1.5 ml micro centrifuge tube and DNA was precipitated the addition an equal volume of ice-cold isopropanol and incubated at -20°C overnight. The DNA was collected by centrifugation at 13,000 x g at 4°C for 10 min. The pellet was washed twice with 70 percent ethanol, air-dried, and resuspended with 30µl nuclease- free water. The genomic DNA concentration was determined using a Nanodrop ND-3300 Fluor spectrometer (Nano Drop products, Thermo Scientific, Wilmington, DE,USA) was used to determine the DNA concentration and Purity of extracted DNA were determined.

2.6 Detection of Grapevine Mildew Airborne Inocula through PCR

A conventional PCR assay was performed by amplification of cytochrome b (cyt b) region of Powdery mildew pathogen *E.necator* using species specific primers cytbF (TGTTGTAATATTTATTTTAATG) and cyt b R (TGGGTTAGCCATAATATAA) primers [13]. The 20 µl reaction volume consisted of 10 µl master mix (2X concentration), 2 µl of forward and reverse primer and 2 µl of DNA template and the volume made up to 20 µl by using nuclease-free water. The reaction was carried out using the following PCR cycle: initial denaturation of 3 min at 94°C followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 42°C for 1 min, extension at 72°C the 90s and a final extension at 72°C for 7 min. Similarly, downy mildew pathogen *P.viticola* was detected through PCR assay performed by amplification of NADH dehydrogenase subunit 9 (NAD9) and apocytochrome b (COB) region using specific primer NAD9 & COBF (GTATAATTTATTTAAAATAAG), NAD9& COB R (CCAAACATATCCCAAATTTC) primer. PCR reaction volume set up in 20 µl mixture and the PCR cycle conditions are initial denaturations of 3 min at 94°C followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 1 min, extension at 72°C for the 90s and a final extension at 72°C for 7 min. Finally, amplified products were confirmed by gel electrophoresis (GeNei$^{TM)}$ in 1% agarose gel prepared in 1 X TAE (Tris-acetate EDTA) buffer (PH 8.8). Agarose gel electrophoresis was performed at 80V for 1 h and documented in a gel documentation unit (UVITECH, Cambridge).

2.7 Detection of Grapevine Mildew Airborne Inocula through LAMP Assay

The airborne inocula of Powdery mildew pathogen *E.necator* and Downy mildew pathogen *P.viticola* were detected by LAMP reaction. The six sets of LAMP primers targeting *E.necator* rDNA region encoding the ITS 1, ITS 2 and 5S ribosomal RNA sequences were used for LAMP assay [14]. Similarly, six sets of LAMP primers for *P.viticola* targeting cellulose synthase 4 (Ces A4) gene [13]. The LAMP primers were used to detect airborne inocula of mildew pathogens in the grapevine field is given in (Table 1). The 25 µl LAMP reaction mixture contained 1.4 µM each of the FIP and BIP primers, 0.2 µM each of the F3 and B3 primers, 0.4 µM each of the LF and LB primers, 8U of *Bst* DNA polymerase (New England Biolabs), 2.5 µl of the 10× Thermopol reaction buffer, 1.4 mM each of dNTPs, 0.8 M betaine, 4 mM MgSO4, 120 µM hydroxyl naphthol blue and 2.5 µl of template DNA. The reactions were carried out in a block heater incubated at an isothermal temperature of 65°C for 60 min followed by heat denaturation at 80°C for 2 min in order to terminate the reaction. The

result of the LAMP assay was observed to change the colour from violet to sky blue colour.

3. RESULTS

3.1 Phynotyphic Observation of Airborne Inocula Sample

An airborne inocula sample from the suction spore trap was collected weekly from the grapevine field. The collected samples were examined under a phase-contrast microscope. The identity of the *E.necator* was confirmed by its phenotypic characteristic of hyaline barrel shaped conidia (Fig. 2A), whereas the *P.viticola* as hyaline oval or lemon-shaped sporangia (Fig. 2B). The presence and absence of airborne conidia and sporangia during the cropping season were given in (Table 2).

3.2 Detection of Airborne Inocula of Grapevine Mildew through Conventional PCR

PCR detection of airborne inocula of grapevine powdery mildew pathogen *E.necator* were DNA was extracted from the sampling plates of suction spore trap for a period of 12 weeks from the standard week of 3 to 14 of 2021 and subjected to PCR amplification using a species specific primer targeting cytochrome b gene. The PCR reaction yielded an amplicon size of 470 bp for the DNA extracted on 3rd, 6th, 10th and 13th standard weeks. The samples from other standard weeks did not yield any amplicon of the cytochrome b gene (Fig. 3). This result indicated that presence of *E.necator* conidia in the air on each standard week.

Similarly, the presence of airborne inocula of grapevine downy mildew pathogen *P.viticola* was detected through PCR amplification with species-specific primer NAD9 and COB gene of pathogen. The PCR reaction yielded an amplicon size of 520 bp .Out of 12 samples collected from 3 to 14 standard weeks, samples from the 6th standard week only yielded an amplicon 520bp and the samples of all other standard weeks did not yield any amplicon (Fig. 4). This result indicated the presence of *P.viticola* sporangia on the 6th standard week alone.

3.3 Detection of Airborne Inocula of Grapevine Mildews Using LAMP Assay

The DNA extracted from the sampling plate of the suction spore trap was continuously subjected to the LAMP assay for rapid and specific detection of airborne inocula of grapevine mildew pathogens. In the present study, LAMP assay was made visualization by adding hydroxyl naphthol blue (HNB) as an indicator dye. In LAMP assay, the development of sky blue colour indicates a positive reaction whereas violet colour indicates a negative reaction. LAMP assay for the detection of airborne inocula of grapevine powdery mildew pathogen *E.necator* was performed with LAMP primers targeting ITS 1, ITS 2, and 5S ribosomal RNA gene. Out of 12 samples tested through LAMP assay, 9 samples showed positive reaction and 3 samples showed a negative reactions. The DNA samples obtained during standard weeks 3 to 6 showed positive reaction. DNA samples extracted during standard week 7th, 8th, 9th did not show a sky blue colour and they were remained violet. The DNA samples from 10 to 14 standard weeks again showed positive reaction as the violet colour changed to sky blue. The presence and absence of airborne inocula of grapevine mildew were further confirmed through agarose gel electrophoresis with ladder-like banding pattern for positive reaction and their absence for negative reaction (Fig. 5).

Similarly, a LAMP assay for the detection of airborne inocula of grapevine downy mildew pathogen *P.viticola* was performed with LAMP primers targeting cesA4 the gene. Out of 12 DNA samples collected from the standard week of 3 to 14 were tested through LAMP assay for the detection of downy mildew pathogen, all the DNA samples showed positive reaction by showing sky blue colour. This indicates the presence of airborne inoculum of *P.viticola* throughout the cropping season (Figure 6).The presence and absence of airborne inocula of grapevine mildew pathogens through molecular detection were given in (Table 3).

4. DISCUSSION

Grape downy mildew and powdery mildew are the most important disease of grapes worldwide and cause a yield reduction of 80% and 60-70 % respectively [15,16]. Grapevine mildews are managed by about 20 rounds of highly toxic and highly residual fungicides [17]. Due to this EU minimum residual level in grapefruits is exceeded for many fungicides that are used to manage grapevine mildews [18]. Since the mildew pathogens are airborne, early detection of pathogens in the air before symptom development and subsequently based fungicidal

spray will reduce the number of spraying and increase the quality of fruits. Mildew pathogens have a latent period of 7-18 days for the development of symptoms. Hence, early detection of airborne grapevine mildew in the field during cropping season will provide information on the possible occurrence of mildew diseases and subsequent spread.

Table 1. Sequence of LAMP primers for *Erysiphe necator* and *Plasmopara viticola*

Pathogen	Primer	Sequence (5' -3')
Erysiphe necator	FIP EN	ACCGCCACTGTCTTTAAGGGCCTTGTGGTGGCTTCGGTG
	BIP EN	GCGTGGGCTCTACGCGTAGTAGGTTCTGGCTGATCACGAG
	F3 EN	TCATAACACCCCCCTCAAGCTGCC
	B3 EN	AACCTGTCAATCCGGATGAC
	LF EN	AAACTGCGACGAGCCCC
	LB EN	ACTTGTTCCTCGCGACAGAG
Plasmopara viticola	FIP PV	TCCCCAGCTCTTCGTGGTTCTTCGATGGAGACGCAGCATAC
	BIP PV	AGGGAGTCGCAAATTCAAGGGCGTACATCTTGGCGCCTTCC
	F3 PV	CCAAGCTCTTGTCGCAGTC
	B3 PV	CGACGTCGCTGTGAAAGT
	LF PV	GGCCTTGGTCAGTTCATTGATG
	LB PV	TGGCCAAGAGTTGGCGCAAG

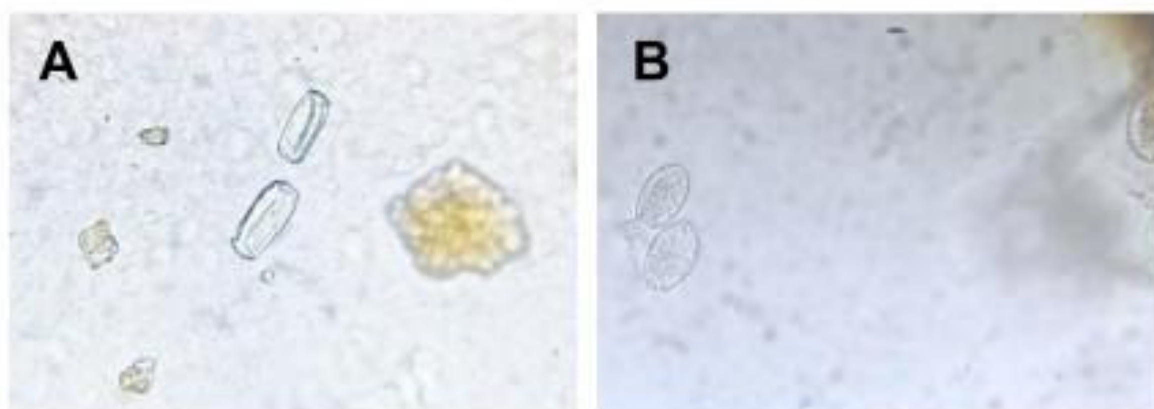

Fig. 2. Airborne inocula of mildew pathogen from suction spore trap observed under a phase-contrast microscope at 40X magnification. A) Barrel-shaped hyaline and single-celled conidia of *E. necator*. B) Oval or lemon-shaped hyaline sporangia of *P. viticola*

Table 2. Microscopic observation of airborne conidia of powdery mildew and sporangia of downy mildew

Sl.no	Standard weeks	*E.necator*	*P.viticola*
1.	3	-	+
2.	4	-	+
3.	5	-	-
4	6	-	-
5.	7	-	-
6.	8	-	+
7.	9	-	-
8.	10	+	-
9.	11	-	-
10.	12	-	-
11.	13	+	-
12.	14	-	-

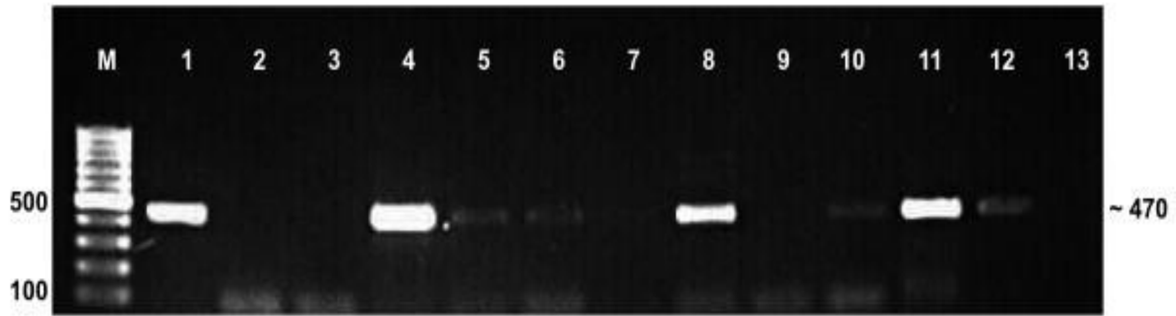

Fig. 3. Agarose gel electrophoresis of PCR amplicons of cyt b gene of *Erysiphe necator* samples from suction spore trap. Total DNA extracted from the sampling plate of spore trap was used as a template. M – 100 bp ladder, DNA samples from Lane 1 – Lane 12 are the spore trap DNA samples collected from 3-14 standard week, Lane 13 – Nuclease free water (Negative control)

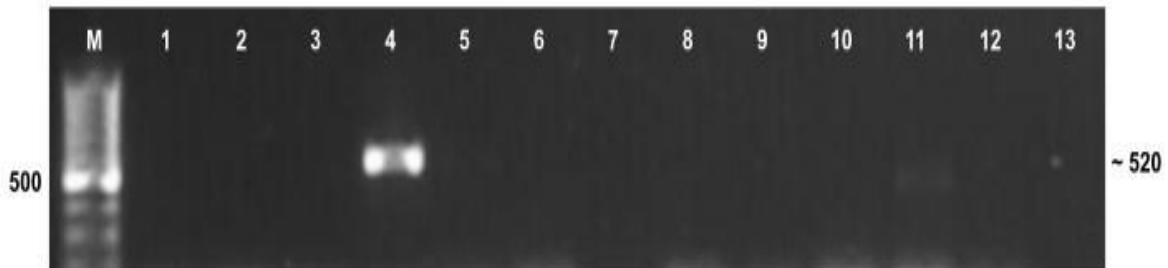

Fig. 4. Agarose gel electrophoresis of PCR amplicons of NAD9 and COB gene of *Plasmopara viticola* samples from suction spore trap. Total DNA extracted from the spore trap sample was used as a template. M – 100 bp ladder, DNA samples from Lane 1 – Lane 12 are spore trap DNA samples collected from 3-14 standard week. Lane 13 – Nuclease free water (Negative control)

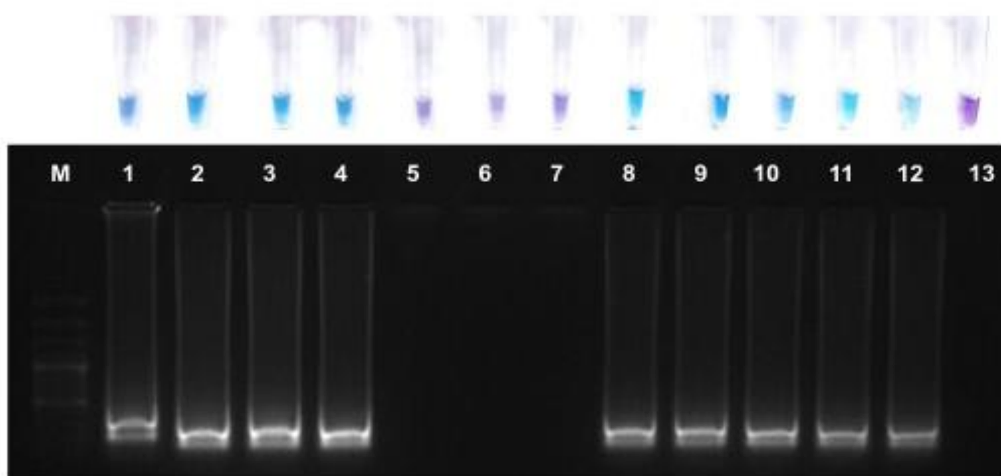

Fig. 5. Visualization of *Erysiphe necator* in LAMP assay.Total DNA extracted from the spore trap samples was used as a template. Agarose gel electrophoresis of LAMP products. M – 3000 bp ladder, Lane 1 – Lane 12 is spore trap DNA samples collected from standard week 3 - 14. Lane 13 – Nuclease free water (Negative control)

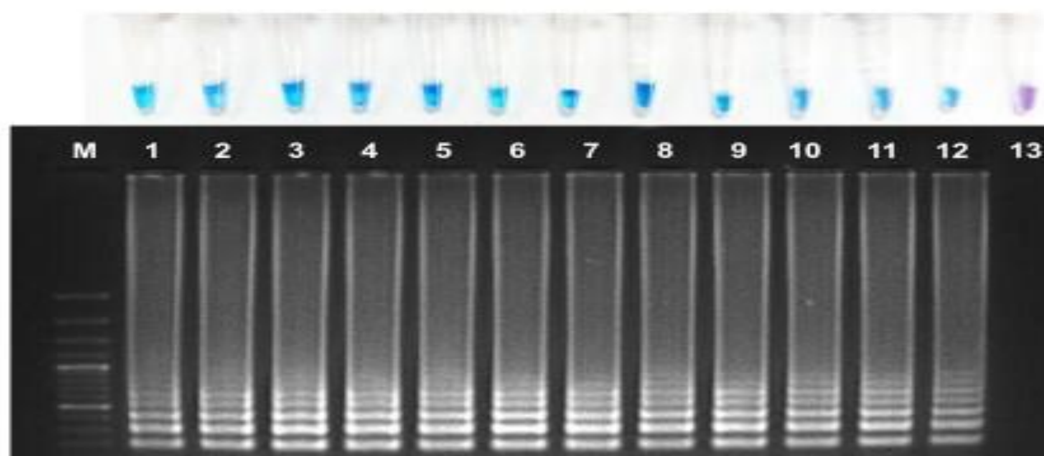

Fig. 6. Visualization of Plasmopara *viticola*in LAMP assay. Total DNA extracted from the spore trap sample was used as a template. Agarose gel electrophoresis of LAMP products. M – 3000 bp ladder, Lane 1 – Lane 12 is spore trap DNA samples collected from standard week 3 -14. Lane 13 – Nuclease-free water (Negative control)

Table 3. Molecular detection of airborne inocula of grapevine mildew pathogens by PCR and LAMP assay

Sl.no	Standard weeks	*Erysiphe necator*		*Plasmopara viticola*	
		PCR	LAMP	PCR	LAMP
1.	3	+	+	-	+
2.	4	-	+	-	+
3.	5	-	+	-	+
4.	6	+	+	+	+
5.	7	-	-	-	+
6.	8	-	-	-	+
7.	9	-	-	-	+
8.	10	+	+	-	+
9.	11	-	+	-	+
10.	12	-	+	-	+
11.	13	+	+	-	+
12	14	-	+	-	+

In the present study, we have developed and installed a low-cost solar operated suction spore trap for the detection of grapevine mildew airborne inocula. The microscopic observation of airborne samples revealed that the presence of *E.necator* conidia on standard weeks 10 and 13. Similarly, sporangia of *P.viticola* could be observed from standard weeks 3, 4, and 8. *E.necator* conidia and *P.viticola* sporangia were identified based on the phenotypic characters. Since the conidia and sporangia of grapevine, mildews were hyaline and resembled other powdery mildew and hyaline fungal spores; it was very difficult to confirm the presence of mildew pathogens in the air. The phenotypic observation through microscopy was tedious and laborious, it's required a highly skilled technical person. Hence, the samples of airborne inocula were subjected to molecular detection methods like PCR and LAMP assay.

PCR amplification of DNA extracted from airborne samples revealed the presence of *E.necator* from the 4 samples out of 12 collected. The absence of powdery mildew conidia during 4,5,7,8,9,12 standard week samples might be due to the prevalence of unfavorable weather conditions for the development of mildew on grapevine [19] or influence of PCR inhibitors [20] or insufficient quantity of pathogen DNA for PCR reaction [21]. Similarly, the presence of downy mildew sporangia in the air was studied through PCR in different standard weeks. Out of 12 samples tested, airborne samples from the 6th standard week alone yielded an amplicon of 520bp and confirmed the presence of *P.viticola*

sporangia. A DNA from all other samples did not yield any amplicon indicated the absence of airborne sporangia.

Loop- mediated isothermal amplification is an efficient time saving and rapid detection method in the modern world for the early detection of plant pathogens [22,17]. A by-product of the LAMP assay is a large amount of magnesium pyrophosphate precipitate, which allows for the visual assessment of target DNA amplification [23]. By using LAMP assay for the early detection of rice false smut pathogen *Ustilaginoidea virens* [24] and *colletotrichum gleosporoides in* soybean [25]. The grower conducted a LAMP assay protocol for the detection of field inocula of grapevine powdery mildew caused by *E.necator* in commercial vineyards [14]. The samples of airborne inocula from different standard weeks were subjected to LAMP assay with LAMP primers constructed by targeting the rDNA region encoding the ITS1, ITS2, and 5S ribosomal RNA sequences for *E.necator* and CesA4 gene for *P.viticola*. The result of the LAMP assay revealed that the presence of airborne inocula of *E.necator* from standard week 3 to 6 and standard week 10 to 14. Similarly, the presence of *P.viticola* was detected from all the samples collected from different standard weeks. This indicates the effectiveness of the LAMP assay in detecting airborne inocula of grapevine mildew.

5. CONCLUSION

Early detection of mildew pathogens through molecular methods will help to design the management strategies for the control of grapevine mildews. Since the phenotypic observation was tedious and skill- oriented, molecular methods become an alternative. Since the PCR-based detection was influenced by thermocycler, PCR inhibitors, and DNA concentration. Highly specific, sensitive, and rapid detection by LAMP assay would become an alternative method of early detection of grapevine mildews.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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