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# Molecular Detection of the Grapevine Pathogens *Plasmopara viticola* and *Erysiphe necator* from Airborne Inoculum Collector Cyclones

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**Abstract:** Grapevine (*Vitis vinifera* L.) varieties are particularly susceptible to the pathogens downy mildew *Plasmopara viticola* and powdery mildew *Erysiphe necator*. Conventional methods for identifying and classifying spores rely on time-consuming microscopic examinations susceptible to human error and requiring qualified personnel. The aim of the present work has focused on the establishment of a protocol for the rapid molecular detection of the fungal species *P. viticola* and *E. necator* from adhesive tapes used to trap spores in airborne inoculum collector cyclones. Four DNA extraction methods were tested. Subsequently, molecular detection of both pathogens was performed by validating some of the specific molecular markers available in the literature. PCR with the primers Nad9 cob-F/Nad9 cob-R and Uncin144/Uncin511 showed specific results for *P. viticola* and *E. necator*, respectively, and the best results were obtained with the T-CTAB method. The methodology developed in this work could be of great help for relating direct measurement of *P. viticola* and *E. necator* airborne inoculum to disease risk and detection of pathogens, which could be integrated into the early diagnosis of these grapevine pathogens, improving existing warning systems such as Decision Support Systems.

**Keywords:** Integrated Pest Management; early detection; DNA extraction; molecular markers; spores



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## 1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops globally due to its cultivated area and economic value [1–4]. *V. vinifera* varieties are particularly susceptible to the pathogens causing two of the most common grapevine diseases worldwide, grapevine downy mildew and grapevine powdery mildew [5]. Both diseases have an adverse effect on the crop, which can result in moderate to extreme yield loss [6]. Downy mildew is caused by the oomycete *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, while powdery mildew is caused by *Erysiphe necator* (syn. *Uncinula necator* (Schwein.) Burril) Schwein. Both pathogens are obligate biotrophic fungi parasites, which require living plants for survival, nutrition, and infection [7].

As regards morphology and symptoms, *P. viticola* shows dimorphism in its reproductive forms, with both sexual and asexual spores, and is therefore characterized by primary (sexual) and secondary (asexual) infection cycles that overlap during part of the season [8]. Oospores represent the sexual stage [9], and they germinate to produce sporangia, which are transported by wind or water and infect the wet leaves through stomata on the lower surface. The first visible symptoms are the so-called oil spots, identified as yellowish-green

lesions on the leaf surface. Sporulation can be observed on the abaxial side of the leaf and the surface of tendrils, inflorescences, and young berries [10]. On the other hand, *E. necator* is an ascomycete that produces colonies of superficial hyphae and asexual spores (conidia) [11]. The disease cycle is usually initiated in spring by ascospores that are released from the chasmothecia when exposed to favorable environmental conditions. The initiation of these chasmothecia requires hyphal fusion of opposite mating types [12]. Young ascospore colonies appear whitish and those that have not yet sporulated show a metallic sheen, while senescent colonies are greyish and may show cleistothecia at different stages of development [13].

Conventional methods for identifying and classifying fungi based on their spores rely on time-consuming microscopic examination of morphological characters [14–16]. Moreover, these techniques are susceptible to human error and require qualified personnel with a high level of experience in morphological recognition [16–19]. Different molecular techniques have gained prominence in recent years in the cultivation of grapevines, such as marker-assisted selection or genome sequencing to identify regions linked to pathogen resistance genes [20]. Today, these detection techniques are being performed faster, more efficiently, and at lower cost [17]. DNA-based methods could serve to get around some of the limitations and drawbacks of microscopy-based detections [21], especially if they make use of the polymerase chain reaction (PCR) [22]. In recent years, different molecular markers, based on the PCR technique, have been described for molecular detection from airborne inoculum samples of *P. viticola* [23] and *E. necator* [14,23].

Most disease forecasting systems rely on optimal weather conditions that favor the production and release of pathogen inoculum or host infection, assuming the inoculum is present. Nonetheless, air sampling for spores can be used to monitor plant pathogen populations [24,25]. It should be noted, therefore, that combining molecular diagnostics with airborne inoculum sampling can be of great value in more accurately predicting disease risk [26,27]. Aerobiological data can thus be used to model disease development and within disease forecasting systems [28]. A more accurate and cost-effective disease control program could result from initiating fungicide programs when airborne inoculum is detected prior to disease onset, rather than following an arbitrary schedule of preventive sprays [14]. Rapid and accurate diagnostics of diseases and inoculum detection could be essential for the quick implementation of effective crop protection strategies [29]. Furthermore, early diagnosis of pathogens could improve existing warning systems, such as that based on Decision Support Systems (DSSs), which are considered to be one of the main cornerstones of the implementation of Integrated Pest Management programs [30].

Therefore, the main objective of the present work was the molecular detection of *P. viticola* and *E. necator* from adhesive tapes used to trap spores in airborne inoculum collector cyclones. To achieve this, the optimization of a DNA extraction method and the validation of the specific markers in our samples were necessary.

## 2. Materials and Methods

### *2.1. Inoculation of Adhesive Tapes and Processing*

For the optimization of the DNA extraction methodology from adhesive tapes, 2 adhesive tapes (silicone fluid 1.000.000 cSt, 20 g polydimetylsiloxane and 980 mL dimethylether) (Lanzoni, Bologna, Italy) of the same type as those used to collect the spores with airborne inoculum collector cyclones were inoculated, one with *P. viticola* and the other with *E. necator*. From these 2 tapes, 24 samples were prepared, 12 with each of the two pathogens. For the inoculation, the adhesive tapes were impregnated with an inoculum of the corresponding fungus by dragging naturally infected plant material along the tape surface. This plant material, infected with *P. viticola* and *E. necator*, was taken from plants of the variety Mazuela, located in a commercially cultivated organic vineyard (41.360043, −1.179996) within the Cariñena PDO in the province of Zaragoza (Aragón, Northeastern Spain). In addition, 12 plant samples, including leaf (3) and grapes (3) without fungus infection, leaf infected with *P. viticola* (3), and grapes infected with *E. necator*, (3) were used as controls.

Infected plant material was confirmed to be infected by identifying symptoms visually and also by microscopy.

Once the DNA extraction methodologies with the best results were selected, new inoculated adhesive tapes (A and B) were used to confirm the results using additional sampling and inoculum tapes. Thus, two sampling tapes, A and B, were used, of the same type as those used to collect the spores with airborne inoculum cyclone samplers. They were manually inoculated using different methodologies, inoculum, and plant material. On the one hand, tape A was prepared by shaking a brush previously inoculated with powdery mildew and downy mildew, dropping the inoculum onto the tape. The infected grapevine leaves were incubated in a humid chamber for 48 h to increase the infection of both pathogens. These leaves were collected from a vineyard of the Tempranillo variety, located at the Higher Polytechnic School of Huesca, located in Huesca (Aragón, Spain), infected with *P. viticola* and *E. necator*. On the other hand, tape B was inoculated by rubbing the adhesive side of the tape directly against the surface of vine leaves infected with *E. necator*. These leaves were taken from plants of the variety Mazuela, located in a greenhouse at the facilities of the Agrifood and Technology Centre of Aragon, located in Zaragoza (Aragón, Spain). For both tape A and tape B, the infected plant material was confirmed to be infected by identifying the symptoms visually and also by microscopy.

In all cases, for the suitable processing of the samples, inoculate adhesive tapes were cut into 20 mm × 12 mm sections and placed on a glass slide (Twin Twin Frost, Labolan, Navarra, Spain) using sterile scissors and forceps. In order to confirm the presence of *P. viticola* and *E. necator* spores, an Olympus microscope, BH2 (Olympus, Tokyo, Japan), provided with a Flexacam C3 12MP (Leica, Wetzlar, Germany), was used to identify spores of both pathogens in the fragments. Pathogen spores were identified without any staining and using 10×, 20×, and 40× magnifications. Images were processed with Leica Microsystems Enersight software 1.0.2.97 (Leica, Wetzlar, Germany). Finally, 12 inoculated fragments of *P. viticola* and 12 of *E. necator* were used for the optimization of the DNA extraction methodology and 24 inoculated fragments of the tape A and 8 fragments of the tape B for the subsequent analysis. Adhesive tapes were stored in microtubes of 1.5 mL until their analysis.

### *2.2. DNA Extraction Methods*

For the extraction of fungal DNA from adhesive tapes, four methods were tested. Two of them were based on the CTAB DNA, TissueLyser-CTAB (hereinafter T-CTAB) and Ultrasounds-CTAB (hereinafter U-CTAB). The other two methods were direct extraction without prior sample preparation with commercial kits PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) (hereinafter PrepMan) and Extract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, St. Louis, MO, USA) (hereinafter Xnar). The DNA extraction by both CTAB methods was performed according to Doyle and Doyle [31], with minor modifications [32]. Briefly, the samples of the T-CTAB method were homogenized with the CTAB solution using a TissueLyser II (Qiagen, Venlo, The Netherlands) for 2 min at 30 Hz, while the samples of the U-CTAB method were sonicated with the CTAB solution in an ultrasounds-H bath (JP-Selecta, Barcelona, Spain) at 25 °C for 15 min and then homogenized with a lancet. For DNA extraction by PrepMan method, 200 µL of PrepMan Ultra Sample Preparation Reagent was added into the microtube with the sample, then vigorously vortexed, and finally incubated at 100 °C for 10 min. Lastly, DNA was extracted by Xnar method by adding 200 µL of the Extraction Solution to the microtube with the sample and vortexing briefly. Then, the microtubes were incubated at 95 °C for 10 min, and finally, 200 µL of the Dilution Solution was added. On the other hand, genomic DNA was extracted from young leaves and grapes of individual plants using a modified CTAB method by Garcés-Claver et al. [32].

The quality and quantity of DNA were determined spectrophotometrically using a NanoDrop ND-10000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and fluorometrically using a Qubit dsDNA BR Assay Kit (Invitrogen, Waltham,

MA, USA) on a Qubit 3 fluorometer (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. In addition, the quality of extracted DNA was determined by gel electrophoresis on 1.5% agarose gel (Nzytech, Lisbon, Portugal) stained with 5% GelRed (Biotium, Hayward, CA, USA) in TAE 1X at 100 V for 40 min and visualized on a G-BOX UV transilluminator (Syngene, Cambridge, UK).

### *2.3. Primer Selection for Molecular Detection of P. viticola and E. necator*

Specific PCR primer pairs, described by Basha et al. [23], Valsesia et al. [33], Falacy et al. [14], and Péros et al. [34] for the detection of *P. viticola* and *E. necator*, were used (Table 1). On the one hand, the cytb-F and cytb-R primers were designed on the basis of cytochrome b (cyt b) region of *E. necator*; the Uncin144 and Uncin511 primers were designed based on highly conserved regions specific to *E. necator* and mo3E11-F and mo3E11-R primers were designed to amplify a sequence of 150 bp length that contained the SSR motif $(CA)_8(CT)_{17}$, allowing an automatized detection of the two genetic groups in *E. necator* (sexual or asexual reproduction) in one PCR reaction. On the other hand, the Nad9 cob-F and Nad9 cob-R primers were designed on the basis of NADH dehydrogenase subunit 9, an apocytochrome b (COB) region of *P. viticola*, and the Giop-F/Giop-R primers were designed on the basis of the *Phytophthora* sp. KACC40449 sequence (Accession No. AF087474) with the forward primer within the ITS1 rDNA sequence and the reverse primer within the 5.8S rDNA sequence.

**Table 1.** Name and primer sequence, species for which they have been designed, size of the expected fragment, and bibliographic reference.

| Primer Name | Primer Sequence (5′-3′) | Specie | Expected Fragment Size | Reference |
|---|---|---|---|---|
| Nad9 cob-F | GTATAATTTATTTAAAATAAG | *P. viticola* | 520 bp | Basha et al. [23] |
| Nad9 cob-R | CAAACATATCCCAAATTTC | | | |
| Giop-F | TCCTGCAATTCGCATTACGT | *P. viticola* | 208 bp | Valsesia et al. [33] |
| Giop-R | GGTTGCAGCTAATGGATTCCTA | | | |
| Uncin144 | CCGCCAGAGACCTCATCCAA | *E. necator* | 367 bp | Falacy et al. [14] |
| Uncin511 | TGGCTGATCACGAGCGTCAC | | | |
| cytb-F | TGTTGTAATATTTATTTTAATG | *E. necator* | 470 bp | Basha et al. [23] |
| cytb-R | TGGGTTAGCCATAATATAA | | | |
| mo3E11-F | TTGGCTGGCTGTTGTGGT | *E. necator* | 221/150 or 131bp | Péros et al. [34] |
| mo3E11-R | CCGCGTGAAGTTGAAGATTT | | | |

For the amplification of the fragments corresponding to *P. viticola* and *E. necator* fungi, each PCR was performed in a total volume of 25 µL containing 2 µL of DNA; 0.5 µL of each primer pair (10 µM); 0.5 µL of dNTPs (2.5 mM); 0.8 µL of $MgCl_2$ (50 mM); 2.5 µL of 10x reaction buffer (20 mM Tris-HCl, pH 8.4 + 50 mM KCl); and 0.2 µL of Taq DNA polymerase (Nzytech). In addition, for samples extracted with the Xnar method, another PCR was performed according to the manufacturer's instructions in a total volume of 20 µL containing 5.2 µL of $H_2O$ MilliQ (Ultramatic Type 1, Wasserlab, Navarra, Spain), 10 µL of extract-N-Amp PCR ReadyMix, 0.4 µL of each primer (10 µM), and 4 µL of DNA.

Amplification reactions were performed in an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, USA) with the following programs: for Nad9 cob-F and Nad9 cob-R: 3 min denaturation at 94 °C, followed by 40 cycles of 30 s at 94 °C, 1 min at 51 °C, and 90 s at 72 °C, and a final extension cycle for 7 min at 72 °C; for Giop-F and Giop-R: 2 min and 30 s at 94 °C, 45 cycles of 30 s at 94 °C, followed by 1 min at 53 °C and 30 s at 72 °C, and a final extension cycle of 10 min at 72 °C; for Uncin144 and Uncin511: 2 min denaturation at 96 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 70 °C, and 30 s at 70 °C, and final extension cycle of 7 min at 70 °C; for cytb-F and cytb-R: 3 min denaturation at 94 °C, followed by 40 cycles of 30 s at 94 °C, 1 min at 42 °C, and 90 s at 72 °C, and a final extension cycle for 7 min at 72 °C; and for mo3E11-F and mo3E11-R: 4 min at 94 °C,

35 cycles of 1 min at 94 °C, followed by 1 min at 50 °C and 1 min at 72 °C, and a final extension cycle of 6 min at 72 °C.

PCR products were separated by gel electrophoresis in 1.5% agarose (Nzytech) in 1X TAE buffer, stained with GelRed at 5% (Biotium) and visualized under UV light in a G-Box UV transilluminator system (Syngene). In all cases, gels were performed in 1x TAE buffer (0.04 M Tris-Acetate, 1 mM EDTA) during 50 min at 90 V. To determine the size of the amplified bands, a 100 bp Kb Plus DNA marker (Invitrogen) was used for all electrophoreses. To check the specificity of the primers, in 7 of the total samples (2 tapes with *P. viticola*, 2 tapes with *E. necator*, 1 from a leaf sample with *P. viticola*, and 2 from grapes with *E. necator*), the amplified fragments were sequenced by the Sanger method (STAB VIDA, Caparica, Portugal) and compared in the BLASTn database.

### *2.4. Optimization of PCR Conditions*

To optimize the process time, we tried to establish the conditions for a multiplex PCR with the primers Nad9 cob-F/Nad9 cob-R and Uncin144/Uncin511. For the multiplex PCR, one sample of leaf infected with downy mildew (30) and two samples of grapes infected with powdery mildew (35 and 36), which had previously tested positive in PCR with these primer pairs, were used. The PCR conditions were as follows: 1 initial denaturation step of 3 min at 94 °C, 40 cycles of 30 s at 94 °C, 1 min of annealing at different temperatures (Table 2), and 90 s at 72 °C, and a final extension cycle of 7 min at 72 °C. To determine the optimal annealing temperature for the multiplex-PCR, the range between 51 °C and 65 °C was chosen because it covered the optimal annealing temperatures for both primers (Table 2).

**Table 2.** Annealing temperatures used in multiplex PCR for the elongation step. The letters A to H are the thermal cycler rows. Each row was assigned a temperature, the range chosen in this case being 51 to 65 °C, because these are the annealing temperatures of the two primer pairs Nad9 cob-F/Nad9 cob-R and Uncin144/Uncin511.

| **Thermal Cycler Rows** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** |
|---|---|---|---|---|---|---|---|---|
| Temperatures (°C) | 65 | 63.9 | 62.2 | 59.5 | 56.3 | 53.7 | 52 | 51 |

## 3. Results and Discussion

Integrated Pest Management (IPM) [35] is a global approach to pest control in agriculture that offers an alternative to conventional techniques, thus reducing excessive use of phytosanitary products. At the European Union level, and considering the Directive 2009/128/EC on the sustainable use of pesticides (SUD) and the Farm to Fork Strategy of the European Green Deal, IPM is increasingly recognized as a key strategy to achieve sustainable agriculture and protect the environment [36]. IPM consists of applying long-term control strategies that combine biological, cultural, and chemical methods to bring pathogen populations down to tolerable levels and keep pests below the economic threshold [37]. In this vein, IPM involves the coordinated use of all available control tactics [38], including the ones based on DSSs, which can be of great use for informing users about the risk of plant diseases and thus help to accurately target crop protection measures [39–41]. The importance of the use of DSSs lies in their potential to enhance the sustainability of crop production, leading to a reduction in the harmful effects of agrochemicals on the environment and lower economic costs for farmers [42].

### *3.1. Detection by Microscopy*

The control adhesive tapes inoculated with *P. viticola* and *E. necator* were visualized by light microscopy and the presence of the pathogens was confirmed (Figure 1).

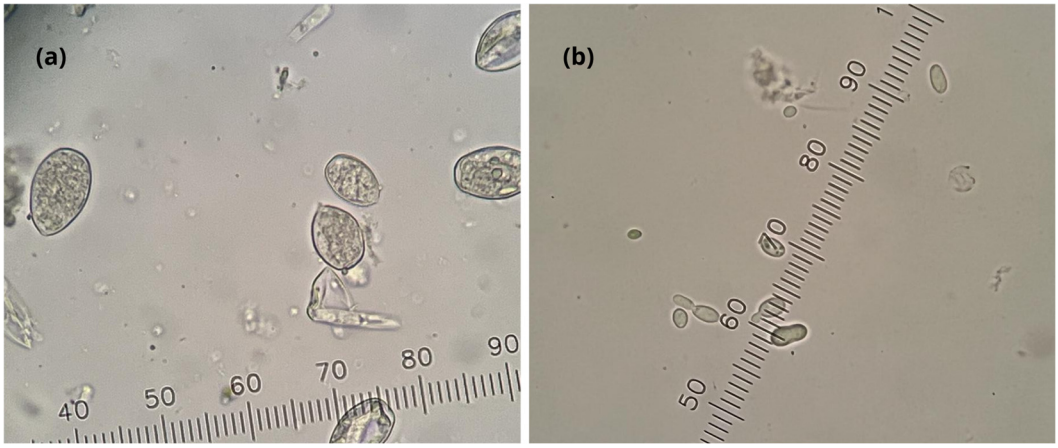


**Figure 1.** Inoculated adhesive control tapes visualized by light microscopy at 40× magnification: (**a**) *P. viticola* sporangia/zoosporangia; (**b**) *E. necator* spores and yeasts.

As can be seen in Figure 1, sporangia/zoosporangia of *P. viticola* can be distinguished on the left (a). Similarly, on the right (b), sporangia of *E. necator* can be found.

From the tape fragments observed under the microscope, in order to claim the presence of spores, 24 fragments were selected, and 12 fragments for *P. viticola* and 12 for *E. necator* were identified. The fact that no staining was used made it possible to use the same samples that were analyzed by microscopy for the optimization of the DNA extraction protocol. The tape fragments were distributed in the different extraction protocols so that each method had samples with different inoculum loads.

### *3.2. DNA Extraction and Quantification*

Firstly, agarose gel electrophoresis was performed to assess the quality of the extracted DNA. The amount of extracted DNA in the tape samples (samples 1 to 24) was so low that no band was observed. These results were as expected considering the low number of *P. viticola* sporangia/zoosporangia and *E. necator* spores visualized by microscopy. However, in the leaf tissue samples (samples 25, 26, and 27), in the leaf samples infected with downy mildew (samples 29 and 30), and in the grape samples infected with powdery mildew (samples 34, 35, and 36), banding was observed because the amount of DNA was higher, being mainly sourced from the plant.

DNA was then quantified spectrophotometrically in a Nanodrop device and the purity of the extracted DNA was assessed using 260/280 and 260/230 ratios. Finally, DNA was quantified by fluorometry in a Qubit device. This technique quantifies DNA concentrations more specifically and accurately than spectrophotometry as it selectively measures the DNA concentration of the sample. Concentration could not be measured by any of the techniques, electrophoresis, spectrophotometry, or fluorometry. This was due, on the one hand, to the fact that the concentration of the samples was below the detection limits of the equipment used. On the other hand, the kits used did not allow DNA purification compatible with the quantification techniques used. Only when DNA was extracted from an infected or uninfected plant sample could it be quantified, either by electrophoresis, spectrophotometry, fluorometry, or all. This was because the DNA came from the plant (Table S1).

### *3.3. Molecular Detection of Downy Mildew (P. viticola) and Powdery Mildew (E. necator)*

Molecular detection of downy mildew and powdery mildew was performed on the 24 inoculated samples and the 12 plant samples using two primer pairs specific for *P. viticola*, Nad9 cob-F/Nad9 cob-R [23] and Giop-F/Giop-R [33], and three primer pairs specific for *E. necator*, cytb-F/cytb-R [23], Uncin144/Uncin511 [14], and mO3E11-F/mO3E11-R [34].

3.3.1. Results Obtained with Primer Pairs Nad9 cob-F/Nad9 cob-R and Giop-F/Giop-R

Molecular detection of downy mildew with Nad9 cob-F/Nad9 cob-R primers [23] amplified the expected 520 bp fragment in all downy mildew-inoculated tape samples extracted with the U-CTAB (samples 4, 5, and 6), T-CTAB (samples 10, 11, and 12) and Xnar (samples 16, 17, and 18) methods (Figure 2). However, for none of the downy mildew tape samples extracted with the PrepMan method (samples 22, 23, and 24) was the expected fragment amplified. On the other hand, none of the tape samples inoculated with powdery mildew (samples 1, 2, 3, 7, 8, 9, 13, 14, 15, 19, 20, and 21) produced amplification.

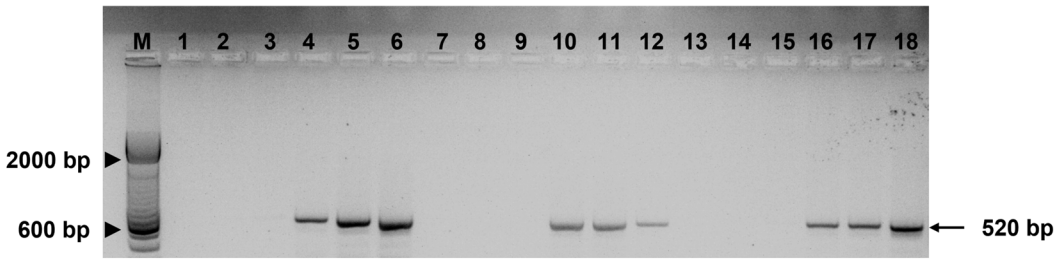


**Figure 2.** Amplification products corresponding to the expected 520 bp fragment obtained with the *P. viticola*-specific primers Nad9 cob-F/Nad9 cob-R on the *P. viticola*-inoculated tape samples (samples 4, 5, 6, 10, 11, 12, 16, 17, and 18). The samples inoculated with *E. necator* (samples 1, 2, 3, 7, 8, 9, 13, 14, and 15) did not show amplification. Lane (M) Marker: 100 bp DNA ladder.

Regarding the plant material samples, no amplification occurred in any of the samples of healthy grape tissue (samples 31, 32, and 33) and leaf (samples 25, 26, and 27) nor in the samples of grapes infected with powdery mildew (samples 34, 35, and 36) (Figure 3). On the other hand, of the plant samples infected with downy mildew, only the expected fragment was amplified in sample 30.

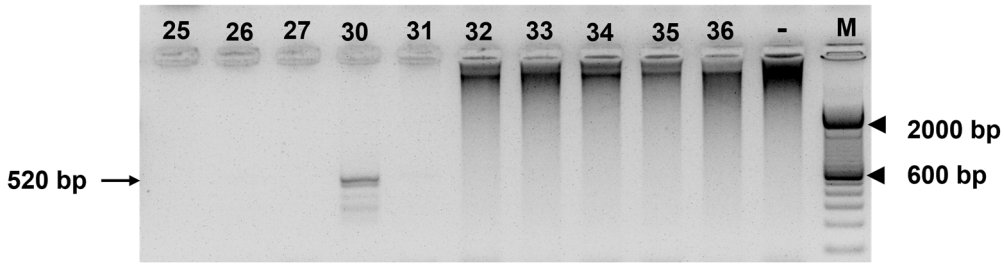


**Figure 3.** Amplification product corresponding to the expected 520 bp fragment obtained with the downy mildew-specific primers Nad9 cob-F/Nad9 cob-R in the downy mildew-infected leaf sample (sample 30). Lane (M) Marker: 100 bp DNA ladder.

The results obtained with the primer pair Nad9 cob-F and Nad9 cob-R in this work suggest that these are specific primers for *P. viticola*. Similarly, Basha et al. [23] obtained positive results for the molecular detection of downy mildew in airborne inoculum samples using this same primer pair.

In terms of the results obtained with the primers Giop-F/Giop-R [33], the expected fragment of 208 bp was amplified in the tape samples inoculated with *P. viticola* (samples 4, 5, 6, 10, 11, 12, 12, 16, 17, 18, 22, 23, and 24) and *E. necator* (samples 1, 3, 7, 15, and 21), as well as in the plant samples infected with *P. viticola* (samples 28, 29, and 30) and *E. necator* (samples 34, 35, and 36) and those of healthy grape plant tissue (32 and 33) (Figure 4).

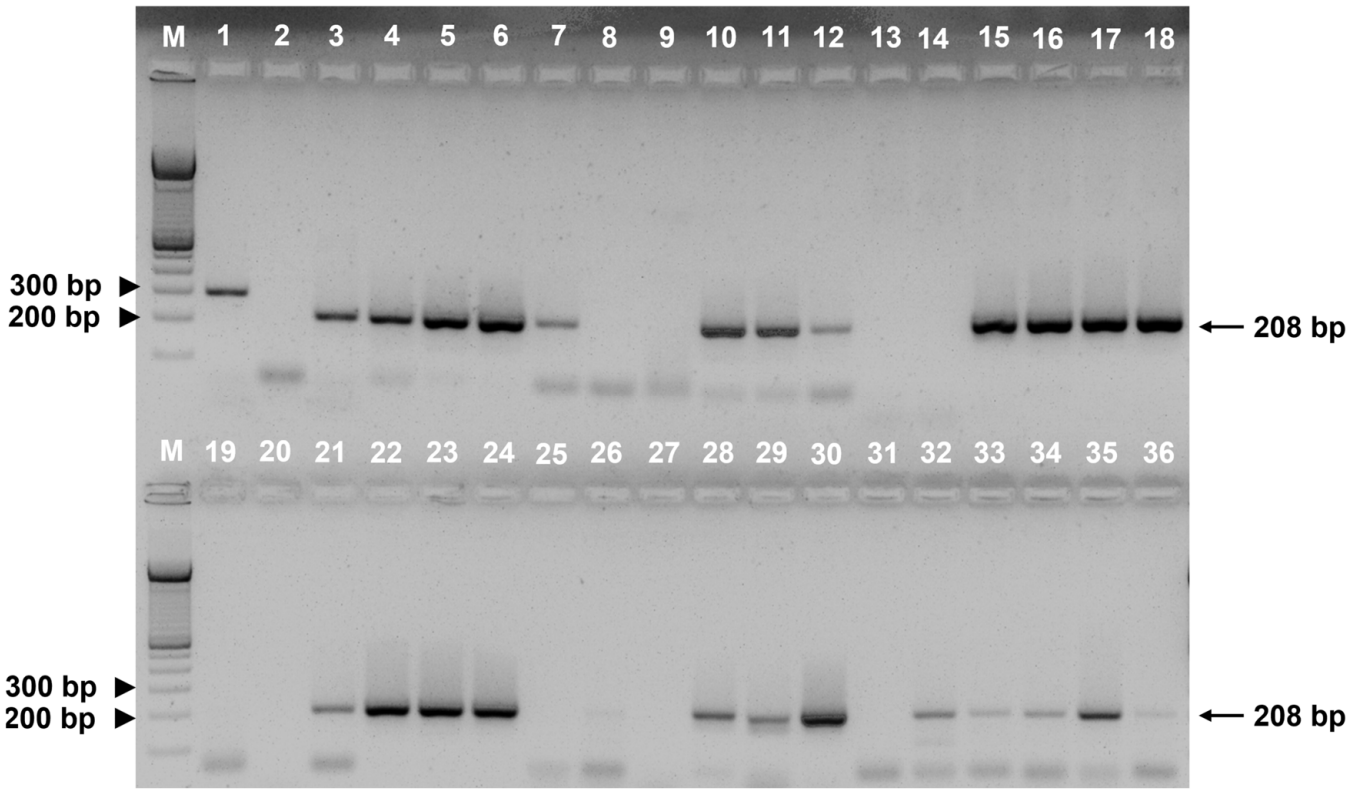


**Figure 4.** Amplification product corresponding to the expected 208 bp fragment obtained with the *P. viticola* primers Giop-F and Giop-R in the *P. viticola*-inoculated tape samples (samples 4, 5, 6, 10, 11, 12, 16, 17, 17, 18, 22, 23, and 24), on tapes inoculated with *E. necator* (samples 1, 3, 7, 15, and 21), on healthy grape plant tissue samples (samples 32 and 33), on leaf samples infected with *P. viticola* (samples 28, 29, and 30), and on grape samples infected with *E. necator* (samples 34, 35, and 36). Samples inoculated with *E. necator* (samples 2, 8, 9, 13, 14, and 19) and healthy leaf (samples 25, 26, 27) and grape (sample 31) plant tissue samples showed no amplification. Lane (M) Marker: 100 bp DNA ladder.

Some authors were able to use this primer pair successfully [43,44], although in their work they only worked with downy mildew and not also with powdery mildew as in our case. On the other hand, others obtained results similar to those of the present work, with the primer pair Giop-F/Giop-R amplifying the 208 bp fragment in both downy mildew and powdery mildew samples [45]. Therefore, it can be considered that the Giop-F/Giop-R primer pair is not specific for the detection of downy mildew, at least under the conditions studied in this work.

#### 3.3.2. Results Obtained with Primer Pairs cytb-F/cytb-R, mO3E11-F/mO3E11-R, and Uncin144/Uncin511

In the molecular detection of *E. necator* with the primer pair cytb-F/cytb-R [23], the expected 470 bp fragment was amplified in tape samples inoculated with both *E. necator* (samples 1, 2, 3, 7, 8, 13, 14, 15, 19, 20, and 21) and *P. viticola* (samples 5, 6, 10, 11, 12, 16, 17, 18, 22, 23, and 24), as well as in uninoculated plant samples of leaf (sample 26) and grape (samples 31 and 32) and those inoculated with *E. necator* (samples 34, 35 and 36) and *P. viticola* (samples 28 and 30) (Figure 5).

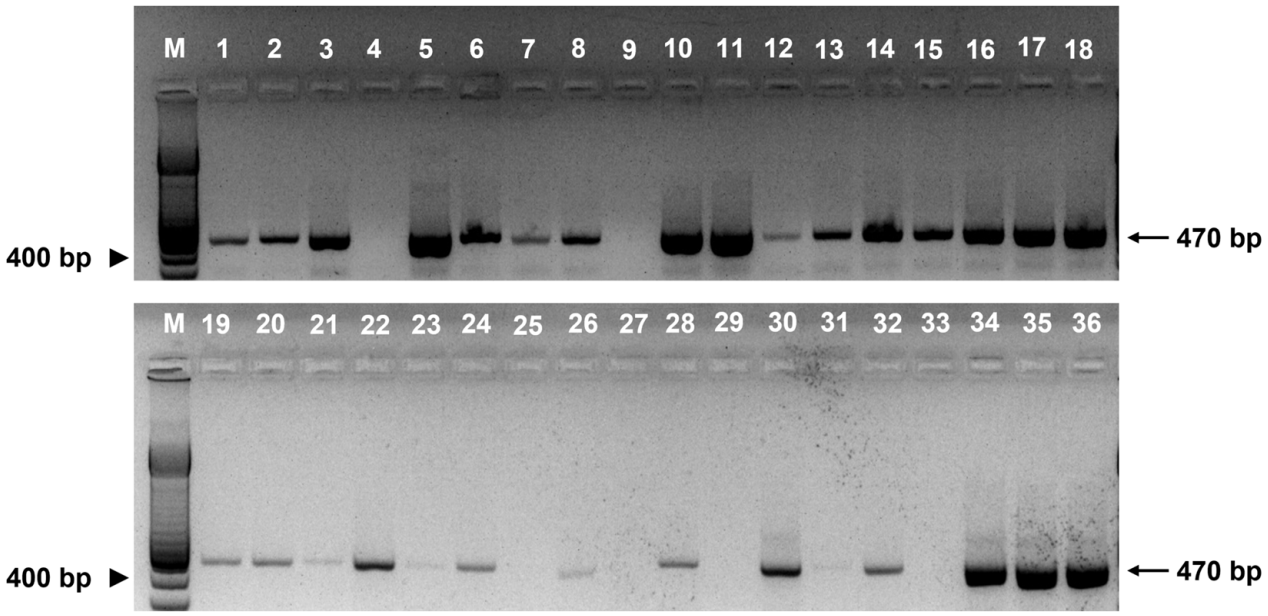


**Figure 5.** Amplification product corresponding to the expected fragment of 470 bp obtained with the *E. necator* primers cytb-F and cytb-R in the tape samples inoculated with *E. necator* (samples 1, 2, 3, 7, 8, 13, 14, 15, 19, 20, and 21), in the tape samples inoculated with *P. viticola* (samples 5, 6, 10, 11, 12, 16, 17, 18, 22, 23, and 24), in the tape samples inoculated with *P. viticola* (samples 5, 6, 10, 11, 12, 16, 17, 18, 22, 23, and 24), in samples of healthy plant tissue of leaf (sample 26) and grape (samples 31 and 32), in samples of leaf infected with *P. viticola* (samples 28 and 30), and in samples of grape infected with *E. necator* (samples 34, 35, and 36). The tape samples inoculated with *E. necator* (sample 9), the tape sample inoculated with *P. viticola* (sample 4), as well as the uninoculated plant samples of leaf (samples 25 and 27) and grape (sample 33) and those inoculated with *P. viticola* (sample 29) did not show amplification. Lane (M) Marker: 100 bp DNA ladder.

In contrast to the results presented in previous work, this primer pair did not show specificity for *E. necator* detection, at least under the conditions performed in this work. However, it should be noted that most of these works studied plant tissue, grape, and leaf infected only with *E. necator*, without considering any other pathogen [46,47].

Regarding the mO3E11-F/mO3E11-R primer pair, Péros et al. [34] identified polymorphisms among the different *E. necator* isolates tested, classifying two groups. Group A, which only reproduces asexually, showed a 131 bp product. Group B, which can also produce recombinant genotypes by sexual reproduction, showed bands of 150 and 221 bp. In this work, the expected fragment of 131 bp for group B isolates was amplified in a healthy grape plant tissue sample (sample 32). The expected fragments of 150 and 221 bp for group A isolates were amplified in a grape sample infected with *E. necator* (sample 36). For the rest of the samples, a banding pattern different to the expected one was observed as a result of the non-specific amplification. The multiple banding observed showed that it anchored to other areas of the genome, both *E. necator* and *P. viticola*, amplifying several fragments.

In contrast to Péros et al. [34], but as in previous work [45], the primer pair mO3E11-F and mO3E11-R did not perform specifically under the conditions of this work.

Finally, with the primer pair Uncin144/Uncin511 [14], the expected 367 bp fragment was amplified in the tape samples inoculated with *E. necator* (samples 7, 13, 14, 15). In tape samples inoculated with *P. viticola* (samples 4, 5, 6, 10, 11, 12, 18, 22, 23, and 24), no amplification occurred, except in samples 16 and 17 (Figure 6).

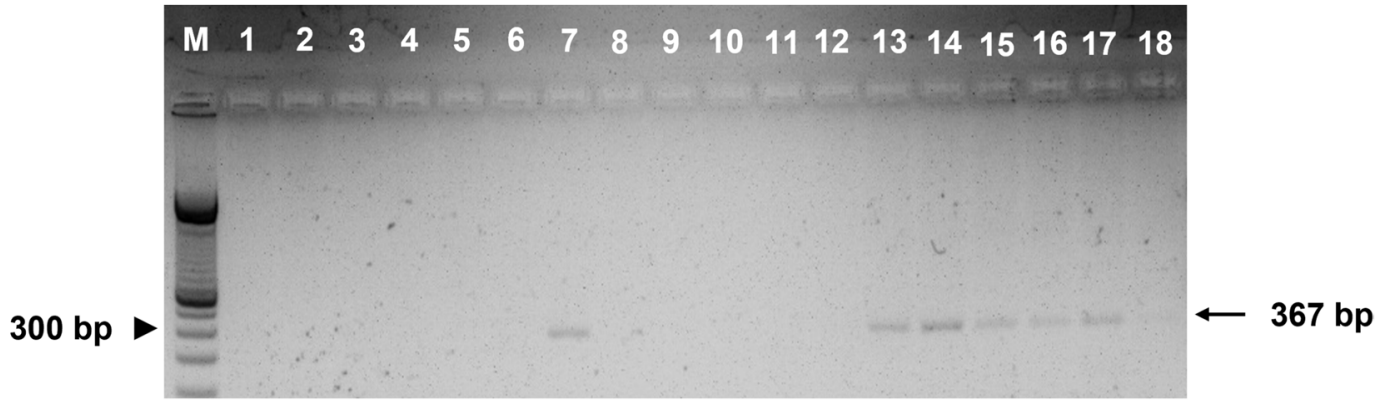


**Figure 6.** Amplification product corresponding to the expected 367 bp fragment obtained with primers Uncin144 and Uncin511 in the samples of tape inoculated with *E. necator* (samples 7, 13, 14, and 15) and in the samples of tape inoculated with *P. viticola* (samples 16 and 17). *E. necator* (samples 1, 2, 3, 8, and 9) and *P. viticola* (samples 4, 5, 6, 10, 11, 12 and 18) samples did not show amplification. Lane (M) Marker: 100 bp DNA ladder.

As for the plant material samples, amplification occurred in the *E. necator*-infected grape plant samples (samples 34, 35, and 36). However, no amplification was shown in samples of healthy leaf (samples 25, 26, and 27) and grape plant tissue (samples 31, 32, and 33) and samples of plant tissue infected with *P. viticola* (samples 28, 29, and 30).

Since the expected 367 bp fragment for *E. necator* was amplified in the *P. viticola* inoculated tape samples 16 and 17, both amplified fragments were sequenced in order to confirm the organisms present in them. In addition, two samples of tape inoculated with *E. necator* (13 and 14), one leaf sample infected with *P. viticola* (30), and two grape samples infected with *E. necator* (35 and 36) were sequenced. The sequences analyzed from samples 16 and 17 aligned with sequences belonging to both *E. necator* and *P. viticola*. This result confirms the presence of these pathogens and validates the specificity of the primers to specifically amplify the pathogens. Since the growth of both organisms is very similar to the naked eye, the presence of *E. necator* was not identified. Furthermore, in samples 13, 14, 35, and 36, only *E. necator* sequences were identified and in sample 30 only *P. viticola* (Appendix A). These results indicate that there is no matrix effect and that the primers Uncin144/Uncin511 are specific for *E. necator*. Similar results were obtained by Falacy et al. [14], who identified the presence of powdery mildew against other ectoparasitic fungi of the erysiphaceae family in samples collected from 48 hosts belonging to 26 different plant families. Similarly, Alimad et al. [48] detected *E. necator* from 29 isolates collected from different geographical regions of Syria and from different grapevine cultivars.

#### 3.3.3. Multiplex PCR

As shown in Figure 7, for all temperatures tested, the expected 367 bp fragment was amplified in the powdery mildew samples (35 and 36), while for the downy mildew sample (30), only the 520 bp fragment was amplified at 51 °C for both replicates. This temperature corresponds to the annealing temperature described by Basha et al. [23] for the Nad9 cob-F/Nad9 cob-R primer pair. These results confirm the possibility of using both primer pairs, Nad9 cob-F/Nad9 cob-R and Uncin144/Uncin511, in a single PCR, which would save time.

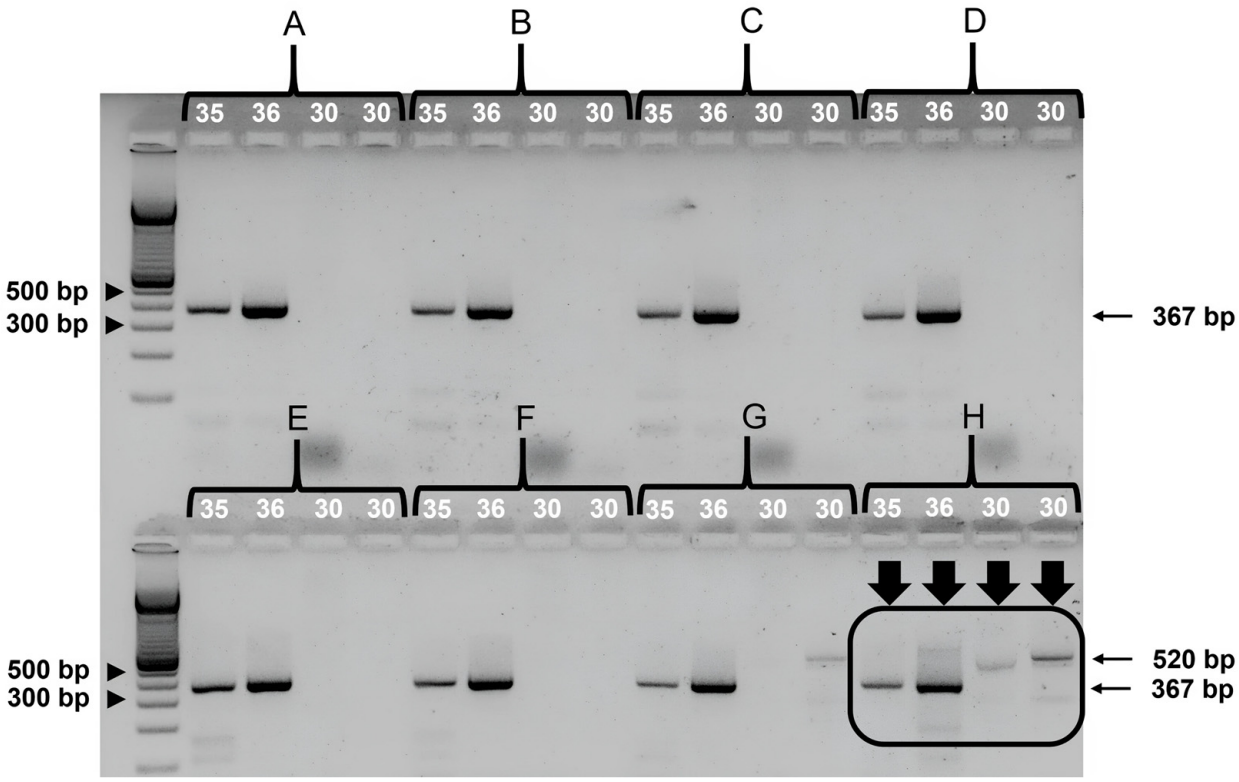


**Figure 7.** Multiplex PCR with primers Nad9 cob-F/Nad9 cob-R and Uncin144/Uncin511 on samples 30, 35, and 36. Amplification product corresponding to the expected fragment of 520 bp and 367 bp for *P. viticola* and *E. necator*, respectively, in all samples at 51 °C. The letters A/B/C/D/E/F/G/H correspond to the annealing temperatures of the thermal cycler rows indicated in Table 2. Lane (M) Marker: 100 bp DNA ladder.

### *3.4. Validation of the DNA Extraction Method and Selected Primers*

The T-CTAB and Xnar methods were chosen as they showed the best results in the optimization of the extraction method using additional inoculated tapes (A and B). On the one hand, tape A was divided into 28 fragments and randomly distributed between the two methods; 14 were extracted by the T-CTAB method (samples A1 to A14) and 14 by the Xnar method (samples A15 to A28). On the other hand, tape B was divided into eight pieces; four were extracted by the T-CTAB method (samples B1 to B4) and four by the Xnar method (samples B5 to B8).

First, the presence of powdery mildew and downy mildew was confirmed by light microscopy. On tape A, sporangia (zoosporangia) and mycelium (non-septate) of downy mildew were identified (Figure 8), while on tape B, inoculated only with powdery mildew, powdery mildew conidia were observed (Figure 9). It can also be seen in these images how on tape B there was a higher amount of powdery mildew inoculum than on tape A. This could be due to the fact that on tape A the pathogen was inoculated by shaking a brush, so the distribution of the inoculum may not be homogeneous over the whole surface. This was intended to simulate the conditions of airborne inoculum samples collected with a cyclone collector, in which the particles pass through the inlet hole and are deposited on the surface of the tape. Therefore, it is to be expected that the tapes are not completely covered with spores, resulting in areas with and without inoculum. However, pathogen distribution by rubbing was also performed on tape B in order to have a more homogeneous inoculum load and sufficient to ensure that the methods worked.

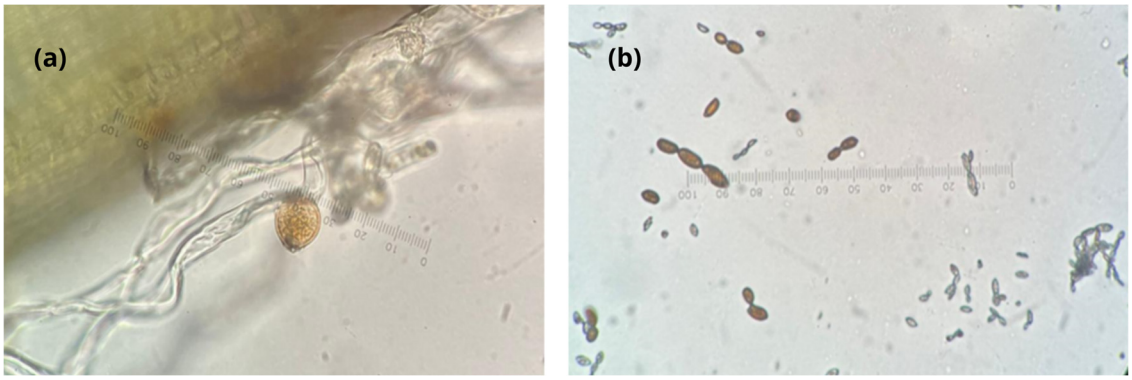


**Figure 8.** Pathogens seen under light microscope on tape A: (**a**) Sporangia/zoosporangia and mycelium (non-septate) of *P. viticola* at 40× magnification; (**b**) conidial chains of *E. necator* at 20× magnification.

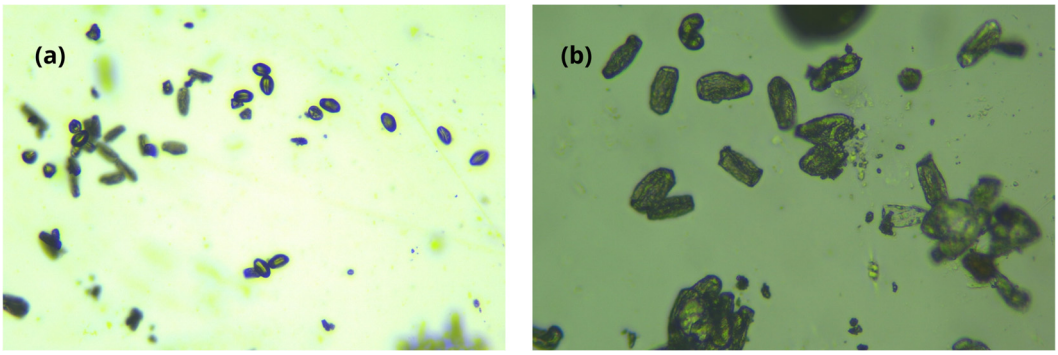


**Figure 9.** *E. necator* on tape B: (**a**) *E. necator* conidia viewed under a light microscope at 20× magnification; (**b**) *E. necator* conidia viewed under a light microscope at 40× magnification.

Given the characteristics of the two types of tape, the distribution of inoculum on tape A being more heterogeneous and with less inoculum, a longer length of tape A was taken than for tape B in order to have a higher probability of finding the presence of pathogens.

Based on the results obtained, the molecular detection of downy mildew and powdery mildew was performed on the 24 samples of tape A and 8 samples of tape B using two pairs of specific primers, Nad9 cob-F/Nad9 cob-R for *P. viticola*, previously described by Basha et al. [23], and Uncin144/Uncin511 for *E. necator*, previously described by Falacy et al. [14]. In addition, to rule out PCR errors, samples 10 and 11 for downy mildew and sample 36 for powdery mildew, from the optimization of the DNA extraction method, were included as positive controls.

In the molecular detection of *P. viticola* with the primers Nad9 cob-F/Nad9 cob-R, the expected 520 bp fragment was not amplified in any of the samples of tapes A and B by any of the DNA extractions (T-CTAB and Xnar). This may be due to the fact that the *P. viticola* content on tape A, which was the tape where the pathogen was inoculated, was insufficient to result in its amplification. In the positive controls (samples 10 and 11) of tape infected with *P. viticola* and extracted with the T-CTAB method, amplification of the expected 520 bp fragment occurred. On the other hand, with the primer pair Uncin144/Uncin511, the expected fragment of 367 bp was amplified in the tape A samples extracted with the T-CTAB method (samples A2, A3, A4, and A5), in the samples of tape B extracted with T-CTAB (samples B1, B2, B3, and B4) (Figure 10), and of the Xnar method from tape B (samples B5, B6, B7, and B8), as well as in the positive control (sample 36) of *E. necator*-infected grapes (Figure 11).

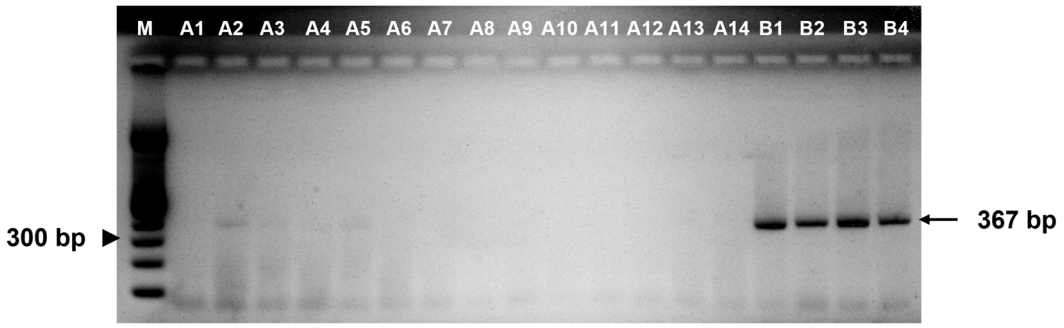


**Figure 10.** Amplification product corresponding to the expected fragment of 367 bp obtained with the primers Uncin144 and Uncin511 in the samples of tapes A and B extracted by the T-CTAB method (samples A2, A3, A4, A5, B1, B2, B3, and B4). Lane (M) Marker: 100 bp DNA ladder.

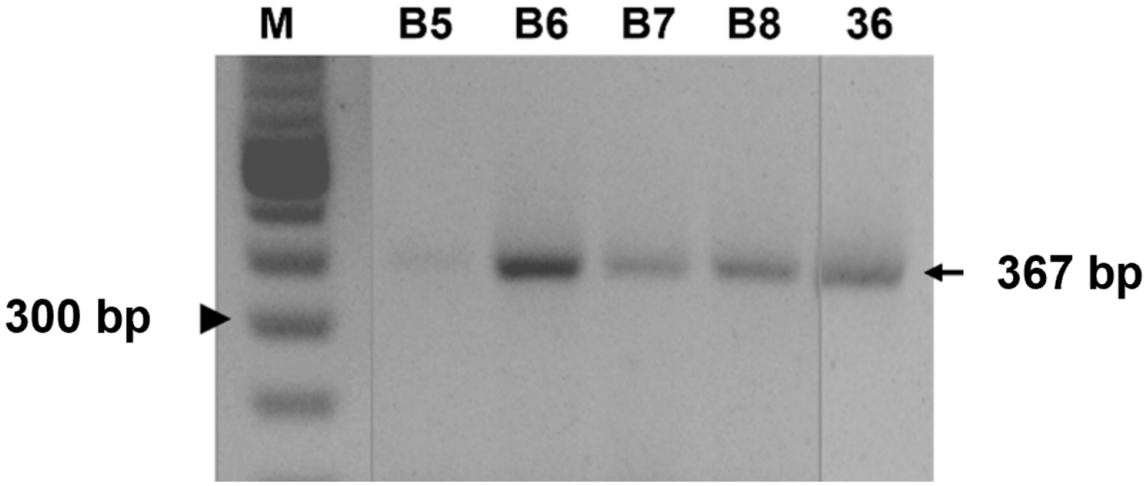


**Figure 11.** Amplification product corresponding to the expected fragment of 367 bp obtained with the primers Uncin144 and Uncin511 in the samples of tape B extracted with the Xnar method (B1, B2, B3, and B4) and in the positive control of grapes infected with *E. necator*, sample 36, used in the optimization of the method. Lane (M) Marker: 100 bp DNA ladder.

As can be seen from the Xnar method, no amplification occurred in any of the samples from tape A. These results could be due to the fact that tape A was inoculated by shaking a previously inoculated brush and therefore the inoculum distribution may not have been homogeneous. As the spores were distributed in this way, there may have been an absence of spores on these fragments or a concentration too low to be detected, which does not necessarily imply that the method is ineffective. However, the B tape was split into eight fragments and amplifications could be observed by both methods. In addition, the bands resulting from the B-tape samples had a higher intensity than in the A-tape samples, both using the T-CTAB method. This could be due to the fact that the amount of inoculum on tape B was higher than that on tape A. It should be noted that the inoculation was carried out by rubbing, which may have resulted in a more homogeneous method with a higher amount of pathogen.

Finally, validation confirmed the specificity of the primers. On the one hand, Uncin144 and Uncin511 correctly amplified both positive controls and samples containing *E. necator*. On the other hand, the primer pair Nad9 cob-F and Nad9 cob-R, although it did not amplify any samples, did amplify positive controls, indicating that the amount of pathogen may have been too small to be amplified. This validation showed that both extraction methods worked correctly on both A and B strips, even on A, which contained less inoculum and was less homogeneously distributed, thus simulating the collector cyclone tapes.

The DNA extraction methods tested in this work have some practical limitations that need to be taken into account. Although airborne inoculum collector cyclones can be a good option for collecting fungal spores for DNA analysis [49], it should be noted that detecting pathogens under field conditions can be difficult. It is expected that only a few spores can be captured in the field, and thus the spore concentrations in the samples collected by the cyclones are too low [50]. This could result in an amount of extracted DNA that is too low to be detected by standard PCR. These challenges mean that these DNA extraction methods and detection methodologies may need to be addressed in future work using advanced PCR variants such as nested PCR [51] or quantitative PCR (qPCR) [52].

## 4. Conclusions

By microscopy, *P. viticola* and *E. necator* propagules were identified in the tape samples inoculated without any staining, which allowed use of the same samples observed under the microscope in the DNA extraction process. Four methods were tested for DNA extraction from artificially inoculated tape with *P. viticola* and *E. necator*.

The Xnar method has some advantages over the methods based on the CTAB method (T-CTAB and U-CTAB), such as speed and simplicity, which make it an easy method to implement for the routine control of *P. viticola* and/or *E. necator* in any laboratory. Although the DNA concentrations extracted from the inoculated samples were so low that reliable results were not obtained with any of the three quantification methods tested (gel electrophoresis, spectrophotometry, and fluorometry), two primer pairs gave specific results, one for *P. viticola* and one for *E. necator*. On the one hand, with the primer pair Nad9 cob-F/Nad9 cob-R, positive results were obtained in the amplification of samples inoculated with downy mildew (*P. viticola*) and negative for those inoculated with powdery mildew, demonstrating specificity in the detection of downy mildew. On the other hand, the primer pair Uncin144/Uncin511 gave positive results for the amplification of samples inoculated with powdery mildew (*E. necator*) and negative results for those inoculated with downy mildew, demonstrating specificity in the detection of powdery mildew. The detection of downy mildew and powdery mildew using molecular markers offers numerous advantages over conventional microscopy, including obtaining results in less time, eliminating the human factor, and thus allowing objective interpretation of results. The obtained results are a starting point for further studies using samples with airborne inoculum that will allow direct measurement of *P. viticola* and *E. necator* airborne inoculum and subsequent integration into disease risk prediction models. Considering that preventive measures are one of the main principles of Integrated Pest Management, this early detection of the presence of airborne inoculum could be integrated into the early diagnosis of these grapevine pathogens, improving existing warning systems such as Decision Support Systems.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14112619/s1, Table S1: Sample code, sample types (tape, plant material), inoculated organism (*P. viticola* and *E. necator*), and method of DNA extraction.

**Author Contributions:** Conceptualization, J.B.-G., A.G.-C., J.J.B.-V. and O.F.; methodology, J.B.-G., A.G.-C., J.J.B.-V. and O.F.; validation, J.B.-G., A.G.-C., J.J.B.-V. and O.F.; formal analysis, J.B.-G., A.G.-C., J.J.B.-V. and O.F.; investigation, J.B.-G., A.G.-C., I.P.-L., J.J.B.-V. and O.F.; resources, A.G.-C. and J.J.B.-V.; writing—original draft preparation, J.B.-G., A.G.-C., I.P.-L., J.J.B.-V. and O.F.; writing—review and editing, J.B.-G., A.G.-C., J.J.B.-V. and O.F.; visualization, J.B.-G. and O.F.; supervision, A.G.-C. and J.J.B.-V.; project administration, A.G.-C. and J.J.B.-V.; funding acquisition, A.G.-C. and J.J.B.-V. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data presented in this study are available upon request from the corresponding author. The data are not publicly available due to their relevance to an ongoing Ph.D. thesis.

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## Appendix A

Sequences obtained from sequencing of samples 13, 14, 16, 17, 30, 35, and 36 during molecular detection of pathogens with primers NAD9-F and UNCIN144-F.

Sample 16 with primer NAD9-F—99.6% similarity to *Plasmopara viticola* (downy mildew):

ACAGTTACAGAATAATAATAAAATATTTTATTATATCTATGTTATATAGGTACT-
TATAAAAAATTTATAAAGTATTTAAAAAAAGAATTTATTTATTTTTATAATATAAA-
AAGATATTTTTTATATAGTATAAAGTAATTTTATATTTTTATTATGAAAATATTACA-
AAAATTTAGTCAGTATTTATTAAACGTTTTACCTATAGTAAACTATACTCTATATA-
AAAATGAATTGTGTATTAATATTTCTACAAATAAATTAGTTCCTATTTTATTGTTTT-
TTAAAAATCACACAAATAGTCAATTTAAAATTTTATCTGAAATTTGTGCTGTAGA-
TTATATTAATAAAAAAAAACGTTTTGAGATTATCTATAATTTATTAAGTATTCGTTT-
TAATAGTCGTTTAAAAGTTAAAATTACACTTAACGAATTACAACCTGTAAATTCA-
ATTATTAAAGTATATAAAAGTGCTAATTGGTGTGAAAGAGAAATTTGGGATATGT-
TTGGA

Sample 17 with primer NAD9-F—99.17% similarity with *Plasmopara viticola* (downy mildew):

ATATAATAAAATATTTTATTATATCTATGTTATATAGGTACTTATAAAACATTT-
ATAAAGCATTTAAAAAAAGAATTTATTTATTTTTATAATATCAAAAGATATTTTTT-
ATATAGTATAAAGTCATTTTATATTTTTATTATGAAAATATTACAAAAATTTAGTCA-
GTATTTATTAAACGTTTTACCTATAGTAAACTATACTCTATATAAAAATGAATTGT-
GTATTAATATTTCTACAAATAAATTAGTTCCTATTTTATTGTTTTTTAAAAATCACA-
CAAATAGTCAATTTAAAATTTTATCTGAAATTTGTGCTGTAGATTATATTAATAAA-
AAAAAACGTTTTGAGATTATCTATAATTTATTAAGTATTCGTTTTAATAGTCGTTT-
AAAAGTTAAAATTACACTTAACGAATTACAACCTGTAAATTCAATTATTAAAGT-
ATATAAAAGTGCTAATTGGTGTGAAAGAGAAATT

Sample 30 with primer NAD9-F—98.19% similarity with *Plasmopara viticola* (downy mildew):

ATAATAAAATATTTTATTATATCTATGTTATATAGGTACTTATAAAAAATTTAT-
AAAGTATTTAAAAAAAGAATTTATTTATTTTTATAATATAAAAAGATATTTTTTATA-
TAGTATAAAGTAATTTTATATTTTTATTATGAAAATATTACAAAAATTTAGTCAGTA-
TTTATTAAACGTTTTACCTATAGTAAACTATACTCTATATAAAAATTAATTGCGCA-
TTACTATTTCTACAAAAAAATTAGTTCCTATTTTATTGTTTTTTAAAAATCACTCA

Sample 35 with primer NAD9-F—100% similarity to *Plasmopara viticola* (downy mildew):

ATATAATAAAATATTTTATTATATCTATGTTATATAGGTACTTATAAAAAATTT-
ATAAAGTATTTAAAAAAAGAATTTATTTATTTTTATAATATAAAAAGATATTTTTTA-
TATAGTATAAAGTAATTTTATATTTTTATTATGAAAATATTACAAAAATTTAGTCAG-
TATTTATTAAACGTTTTACCTATAGTAAACTATACTCTATATAAAAATGAATTGTGT-
ATTAATATTTCTACAAATAAATTAGTTCCTATTTTATTGTTTTTTAAAAATCACACA-
AATAGTCAATTTAAAATTTTATCTGAAATTTGTGCTGTAGATTATATT

Sample 16 with primer UNCIN144-F—88.61% similarity with *Erysiphe necator* (powdery mildew):

CCTGGTCTGGCACTTTGAAAAAAGCCGGAAATGCGATAGGAATGTGAATT-
GCAAAATTTAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGCATTC-
CGAGGGGCATGCCTGTTCGAGCGGTGTAACACCCCCCTCGATGTGCCCTTGTGT-
TGGCTTCCGTGTTGGGGCTCTCCCCATTTTTGCGGTGCTTCTTAAATACAGTAGCG-
GTCCCCGCGTGGGCTCTACGTTTTATAAATTTCTTCCTCAAGATAAAACAACACTC-
GTAATCATGTTTTGATATATCTCTCTCTGCGG

Sample 17 with primer UNCIN144-F—97.24% similarity to *Erysiphe necator* (powdery mildew):

AACTGGGATTCTGAATGTGAATTGCAGAATTTAGTGAATCATCGAATCTTTG-
AACGCACATTGCGCCCCTTGGCATTCCGAGGGGCATGCCTGTTCGAGCGGCATAAC-

ACCCCCCTCAATCTGCCCTTGTGGTGGCTTCGGCGTTGGGGCTCGCCGCATT

Sample 13 with primer UNCIN144-F—100% similarity to *Erysiphe necator* (powdery mildew):

CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTTAG-
TGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGCATTCCGAGGGGCAT-
GCCTGTTCGAGCGTCATAACACCCCCCTCAAGCTGCCCTTGTGGTGGCTTCGGT-
GTTGGGGCTCGTCGCAGTTTT

Sample 14 with primer UNCIN144-F—99.38% similarity to *Erysiphe necator* (powdery mildew):

CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAG-
TAATGTGAATTGCAGAATTTAGTGAATCATCGAATCTTTGAACGCACATTGCGC-
CCCTTGGCATTCCGAGGGGCATGCCTGTTCGAGCGACATAACACCCCCCTCAA-
GCT

Sample 30 with primer UNCIN144-F—0% similarity to *Erysiphe necator* (powdery mildew):

CTCCGACCCGGACGAATCCAAGCGGCGATTCTGTTCGCGGACGAAGTGGA-
AGTGGGGCATCGGAGGCGTGTGCATGGAGGAGGGTCTGACAAGCCCCCTTCCA-
CAGGCCTTTTGGCGCACCCACCATATGGGGTTCCCCGAAGTGTTGAGGTGAAAT-
TAGTTGAATGAGTTGTCTGTCGGGCGGGTTTTACACGACCAAGCATGAAAATCGA-
GACTGGGTGCCGCGCGCGAGCCTCCTA

Sample 35 with primer UNCIN144-F—99.20% similarity to *Erysiphe necator* (powdery mildew):

CGATAAGTAATGTGAATTGCAGAATTTAGAGAATCATCGAATCTTTGAACG-
CACATTGCGCCCCTTGGCATTCCGAGGGGCATGCCTGTTCGAGCGTCATAACAC-
CCCCCTCAAGCTGCCCTTGTGGTGGCTTCGGGGTTGGGGCTCGTCGCAGTTTTG-
CGGTGGCCCTTAAAGACAGTGGCGGTCCCCGCGTGGGCTCTACGCGTAGTAAC-
TTGTTCCTCGCGACAGAGTGACGCTCGTGATCAGCCAA

Sample 36 with primer UNCIN144-F—100% similarity with *Erysiphe necator* (powdery mildew):

AACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATA-
AGTAATGTGAATTGCAGAATTTAGTGAATCATCGAATCTTTGAACGCACATTGC-
GCCCCTTGGCATTCCGAGGGGCATGCCTGTTCGAGCGTCATAACACCCCCCTCA-
AGCTGCCCTTGTGGTGGCTTCGGTGTTGGGGCTCGTCGCAGTTTTGCGGTGGCC-
CTTAAAGACAGTGGCGGTCCCCGCGTGGGCTCTACGCGTAGTAACTTGTTCCTC-
GCGACAGAGTGACGCTCGTGATCAGCCAAA

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