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Grapevine Leaf Rust – Incursion Risk Analysis and Improvement of PCR Diagnostics (Project 1D)



FINAL REPORT to
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

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Research Organisation: **Department of Resources**

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Abstract

Studies were undertaken to illustrate the likelihood of a windborne re-introduction to Australia of *Phakopsora euvitis*, the cause of grapevine leaf rust (GLR), and to develop a better detection method using polymerase chain reaction (PCR).

Experiments showed that, in general, *P. euvitis* urediniospores were very sensitive to environmental conditions once they were detached from diseased leaves. Exposure to total (global) solar radiation caused near 100 % mortality after 4 h. Some spores remained viable over a two day period when they were shaded to simulate cloud cover, although they showed abnormal morphology upon germination.

The studies suggested that a windborne re-introduction of GLR to northern Australia is possible, but the risk low. Under normal wind conditions expected during the monsoon season, successful travel of spores would probably rely upon darkness and/or significant cloud cover during the day. Even if this were the case however, the small number and sparse distribution of the preferred host, *Vitis* spp., in northern Australia provides very little opportunity for deposition and successful infection of spores.

To enable development of a quantitative real time PCR (qRT-PCR) test, the large subunit of the ribosomal DNA sequences from many *Phakopsora* species were aligned to determine potential areas where a specific probe to detect *P. euvitis* DNA could be designed. However, the sequences available indicated a very high similarity between species within the *Phakopsora* genus making it very difficult to identify a probe specific for *P. euvitis*. As a result, the diagnostic test for GLR will continue to rely upon conventional PCR to amplify the D1/D2 region of the large subunit ribosomal DNA. This determines the presence of any species within the *Phakopsora* genus. Further differentiation to species level can then be conducted using restriction fragment length polymorphism (RFLP) which provides a DNA fingerprint unique to each species.

An effort to improve diagnostic capability by developing a method for early detection showed that amplification of the target *P. euvitis* gene from non-symptomatic *Vitis* spp. leaves was achievable. However the commonly used universal primers which target the 5' end of the fungal nuclear large subunit ribosomal gene also amplified segments of the 26S rRNA from *Vitis* spp. Differentiation of *Phakopsora* species by using RFLP to establish a DNA fingerprint was possible since the restriction enzymes (*Alu* I, *Rsa* I and *Sau* 96I) did not digest the plant gene.

Executive Summary

Following the successful eradication of the grapevine leaf rust (GLR) pathogen, *Phakopsora euvitis,* from Darwin (Northern Territory, Australia) in 2007, the possibility of windborne re-introduction to northern Australia and development of an improved diagnostic test using polymerase chain reaction (PCR) were investigated.

To investigate the possibility of windborne re-introduction, studies undertaken focused on two aspects of the process:

1. The characteristics of airflow between SE Asia and northern Australia and,
2. The mortality of detached urediniospores.

The airflow between SE Asia and northern Australia during the summer months (December to March) is influenced by the NW monsoon, defined by active and non-active periods. During the active period, airflow over northern Australia is from the NW direction across SE Asia and the Timor Sea. This could potentially disseminate viable spores of *P. euvitis* from other continents in the region. Active monsoons can last several days to weeks but generally about seven days. During non active periods, the airflow does not pose a risk of introducing disease.

In general, airflow occurs within the troposphere (between the earth surface and the stratosphere). Conditions in the troposphere can be below freezing at high altitudes, the temperature decreasing by 6.5 ºC per km increase in altitude (Danielson et al, *Meteorology,* 2003). Sub-zero temperatures would be lethal for the fragile, short-lived urediniospores. However, modelling by the Bureau of Meteorology, Darwin indicated that it is possible for "parcels" of air passing over or leaving the southern East Timor during active monsoon weather to maintain a low altitude (1 km and lower) trajectory and flow to Darwin within a short time frame. If this air contained urediniospores, the moderate temperature conditions experienced at these low altitudes would not be lethal. Urediniospores of *P. euvitis* have been shown to survive temperatures as low as 5 ºC (Ozoe and Kadowaki, 1971, Daly and Hennessy, 2007).

A study of urediniospore germination after exposure to three levels of solar irradiance; global solar radiation (GSR), solar radiation beneath an ultraviolet light filter (GSR-UV), and diffuse solar radiation (DSR) were undertaken. The viability of spores after 4 h of GSR was reduced to near zero. The GSR-UV treatment appeared to delay the loss of spore viability with some germination still occurring after 8 h of exposure. Under conditions where the spores were shaded to simulate cloud cover (DSR), the spores maintained a comparatively high viability. Solar radiation also seemed to cause dormancy, since following GSR and GSR-UV treatments the germination percentage increased after 48 h compared to that recorded after 24 h.

The effect of elevated temperatures on urediniospore viability was assessed. A significant reduction in germination resulted after treatment at 40 ºC and 45 ºC compared with that after 25 ºC and 35 ºC. Temperatures recorded during the solar irradiance study generally reached the mid 40's. Therefore, it is possible that the lethal effects of the treatments in the previous study were a combined effect of solar radiation and high temperature.

Aside from the implications of experimental outcomes described in this report, other factors that may influence the re-introduction of *P. euvitis* to northern Australia by windborne dissemination need to be considered. Factors such as grapevine distribution, spore release and escape dynamics, spore volume entering the airflow and the ability of spores to infect leaves after transport are either poorly understood or unknown. This is a problem not unique to GLR and applies to invasive species generally.

The conclusion from these studies is that a windborne re-introduction to northern Australia is possible but unlikely. In consideration of other factors outside the scope of this research, there is not enough information to quantify the risk. Under normal wind conditions expected during the monsoon season, successful travel of spores would probably rely upon darkness and periods of significant cloud cover during the day. Even if this occurred, the small number and sparse distribution of the preferred host, *Vitis* spp., during the monsoon season in northern Australia allows very little opportunity for deposition and successful infection of spores.

In addition to the above studies, efforts were made to design a novel diagnostic test for *P. euvitis* using qRT-PCR where the user is able to see results as they appear. Many studies which investigate the presence of fungal plant pathogens rely either on the pathogen being isolated and identified from pure cultures or DNA extracted directly from the spores. qRT-PCR would therefore improve the diagnostic process by providing faster results and significantly increasing the number of samples that can be analysed at one time. However, using current nucleotide sequence data available for all *Phakopsora* species, it was difficult to design specific probes and genetic markers which could pinpoint *P. euvitis* in a sample. When the test was implemented for plant tissue, the genetic markers not only amplified the target fungal gene

but also a plant gene. This significantly limits the ability to develop this genetic marker or this particular region of the fungal gene to specifically target *P. euvitis*. As a consequence, the research continued using the current conventional PCR test.

Isolates of the pathogen from GLR infected material in Yogyakarta, Indonesia were sequenced. Comparisons showed that they were the same as the isolates previously recorded in Australia and East Timor. Once the fungal gene was identified, we were able to use enzymes to cut the gene into different sized fragments. Upon separation on a high resolution gel these DNA fragments produced a unique fingerprint pattern. Using these two techniques, we concluded that even if the plant gene is present in the sample, we would still able to determine the presence and absence of *P. euvitis* in *Vitis* spp. leaves. The main reason is due to the use of selective enzymes which do not cut the plant gene, only that of *P. euvitis*.

The GLR diagnostic test could be significantly enhanced with more knowledge of the *P. euvitis* genetic makeup. Further research to ascertain the sequence of less conserved genes may allow specific *P. euvitis* probes to be designed that do not co-amplify plant DNA. *In planta* sampling includes the isolation of not only the target pathogen but also plant DNA or even other microbes which may also inhabit samples. In many cases, the plant DNA is in abundance and the amount of target DNA is minute. In this study, the application of the current test for GLR was expanded to detect the rust pathogen in non-symptomatic *Vitis* spp. leaves. We found that the test preferentially amplified the abundant plant DNA over the fungal DNA.

We would like to acknowledge the GWRDC for funding, Professor Yoshitaka Ono from Ibaraki University for his input throughout this project; Dr Siti Subandiyah, Ir Arif Wibowo and staff from the University of Gadjah Mada for assistance with field and laboratory experiments; Sam Cleland and Robin Hicks from the Bureau of Meteorology for their assistance with the modelling experiments; Dr Mark Hearnden from the Department of Resources for statistical analysis of experimental data.

Background

Grapevine leaf rust (GLR) was detected in Darwin (Northern Territory Australia) in 2001. Although it is unknown how it arrived, it has a high potential for spread via windborne spores called urediniospores. These spores can persist on vines year-round in tropical regions. The disease is widespread in East Timor and Indonesia and these countries may be a source of windborne inoculum capable of entering northern Australia.

Long distance aerial dispersal between continents is known to have occurred for a number of fungal plant pathogens. It is an important survival mechanism for many of these organisms. Rust pathogens, in particular, are renowned intercontinental travellers (Gregory, 1963; Zadocks, 1967; Isard *et. al.,* 2005; Viljanen Rollinson & Cromey, 2002). Unlike many fungi that can survive on dead and decaying organic matter, rusts are dependent on living host tissue for reproduction and survival (obligate biotrophs) and produce huge numbers of spores for wind dispersal from one host to another (Brown & Hovmoller, 2002).

To understand the potential for windborne re-introduction of GLR, it was necessary to identify wind patterns that may transport urediniospores to northern Australia and to assess their ability to survive conditions encountered during transport. For these spores to successfully traverse the Timor Sea from infested areas in Indonesia and East Timor, they must have the ability to survive for many hours or days at variable temperature, relative humidity and solar irradiance. Previous studies have shown that once spores of plant pathogens are detached from the host and exposed to the elements, solar irradiance is the major factor limiting viability.

Following the successful eradication of GLR from Darwin in 2007, research has focused on enhancing the ability to manage future incursions. This report details studies conducted with the aims of illustrating the likelihood of disease re-entry via windborne inoculum and developing a qRT-PCR assay. Evaluating the potential pathways of introduction is important for identifying opportunities to limit the risk and to help design appropriate surveillance. An appropriate and efficient response to exotic pest and disease incursions requires consideration of aerial as well as human assisted dispersal (Aylor, 2003). Surveillance could be vastly improved with the availability of a qRT-PCR test to detect the pathogen in infected leaves not yet showing symptoms and would enhance the prospects of eradication.

Project Aims and Performance Targets

Aims	Performance Targets
1. Investigate the possibility of windborne re-introduction of grapevine leaf rust to northern Australia.	12/2008
2. Develop a Real-Time Polymerase Chain Reaction (RT-PCR) test to identify the causal agent, *Phakopsora euvitis*.	12/2008
3. Develop the capacity to identify *P. euvitis* in leaf tissue, during the incubation period, when no symptoms are visible.	12/2008

Method

Study 1: Investigate the possibility of windborne re-introduction of grapevine leaf rust to northern Australia

- ***Airflow between SE Asia and northern Australia***

The Bureau of Meteorology (BOM) provided information about airflow between the SE Asian and Australian continents. Long-range movement (hundreds of kilometres) of spores of plant pathogens is generally modelled by trajectory analysis (Davis, 1987). Recent analysis to explain the introduction of serious exotic pathogens such as soybean rust into South America and the United States has been conducted using HYSPLIT (Hybrid Single-Particle Lagrangian Integrated Technology) (Isard *et. al.,* 2005), a particle dispersion/transport model. Examples of the direction, elevation and duration of travel of hypothetical parcels of air during the period of the NW monsoon (December to March) were generated using HYSPLIT V. 4.7. This model is used by meteorology bureaus to forecast the long-range aerial dispersal of biologically hazardous air pollutants. A HYSPLIT trajectory illustrates the likely path that a hypothetical parcel of air at a given location, elevation and time will follow and is able to compute the trajectories for a large number of particles. It can be used to simulate the dispersal of agents such as fungal spores emerging as a "plume" from a large source.

- ***Urediniospore mortality***

Experiments were conducted in Yogyakarta, Indonesia in 2008 where *P. euvitis* is endemic and widespread. Research was based at the University of Gadjah Mada (UGM).

a) Exposure to solar radiation

An experiment was designed to determine the effect of solar radiation on the germination of urediniospores. An experimental site was installed in the courtyard of the university and consisted of fencing an area of about 3 m in diameter with plastic (Fig 1) to protect the spores from gusts of wind.

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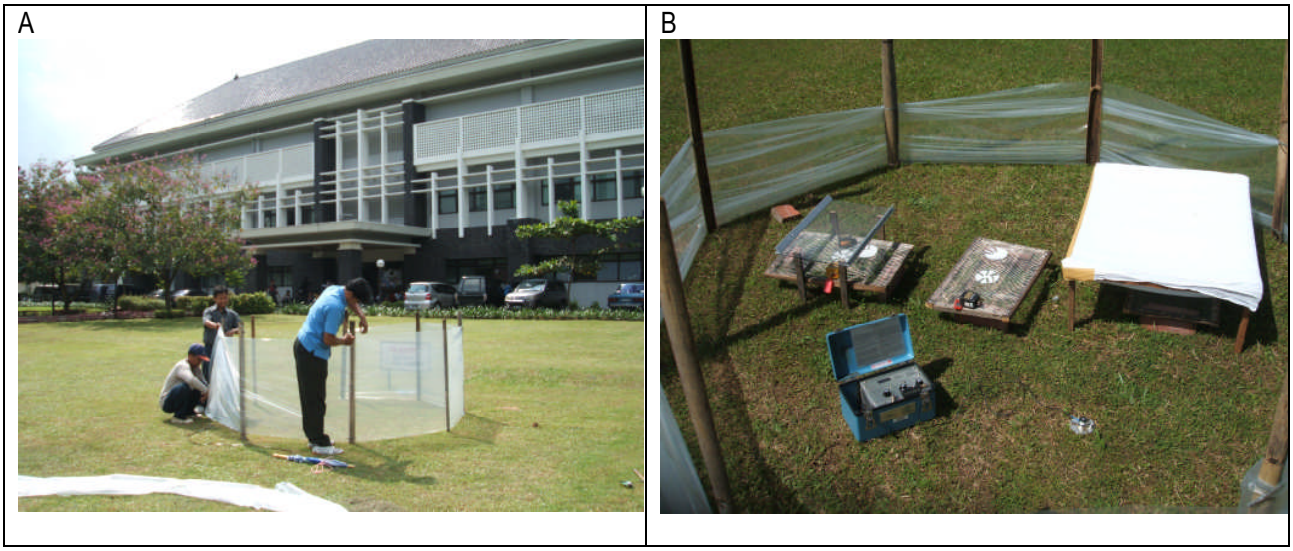


Fig 1. A. Installation of the experimental site. B. Treatments and equipment. University of Gadjah Mada, Indonesia (May 2008).

Urediniospores were exposed to solar radiation in the experimental site for two consecutives days. On the day prior to exposure, infected leaves were harvested from local *Vitis* spp. at approximately 10:00 h, when the overnight dew had dried. The maturity of the spores collected was considered as this may affect sensitivity to solar radiation but literature about similar rust pathogens suggests this is not an issue (Hwang, 1941). The leaves were taken to the university laboratory and kept refrigerated at 5 ºC overnight inside a sealed plastic bag. The following morning at 07:30 h, the spores were gently tapped from the leaves onto sterile, 9 cm filter paper disks, which were placed into uncovered plastic Petri dishes. At 08:00 h the Petri dishes were placed into the experimental site, above the ground, on wire grid platforms for allowing airflow. The spores on the disks were subjected to the following three treatments:

1. GSR; exposure to global solar radiation (Fig. 2).

2. GSR-UV; exposure to solar radiation beneath an ultraviolet (UV) filter. The UV filter consisted of a trans-illuminator cover, (PCR laboratory equipment) which blocks 99 % of total UV radiation (Fig. 3).

3. DSR; exposure to diffuse solar radiation beneath four layers of cheesecloth (also known as Cotton Crepe or Indian Cotton) (shaded control). The light intensity beneath the cloth at midday with a clear sky was equivalent to that recorded previously at midday during full cumulonimbus cloud cover (Fig. 4).

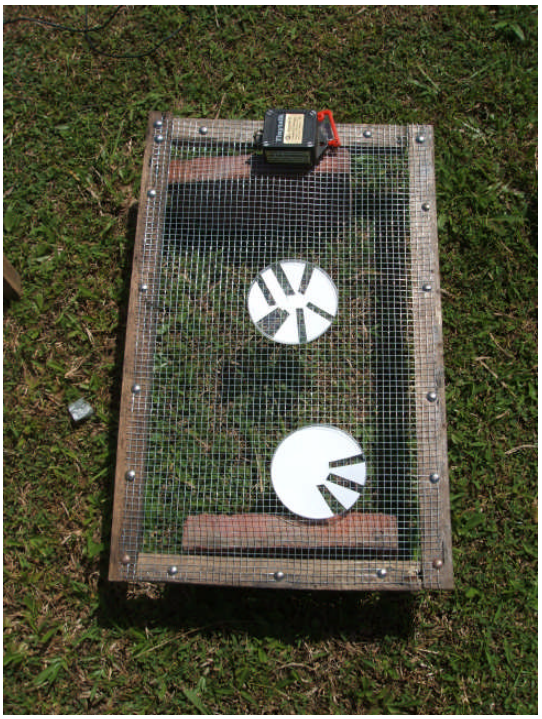


Fig. 2. "GSR" treatment.
Filter paper disks with urediniospores placed onto a wire grid platform and exposed to global solar radiation.

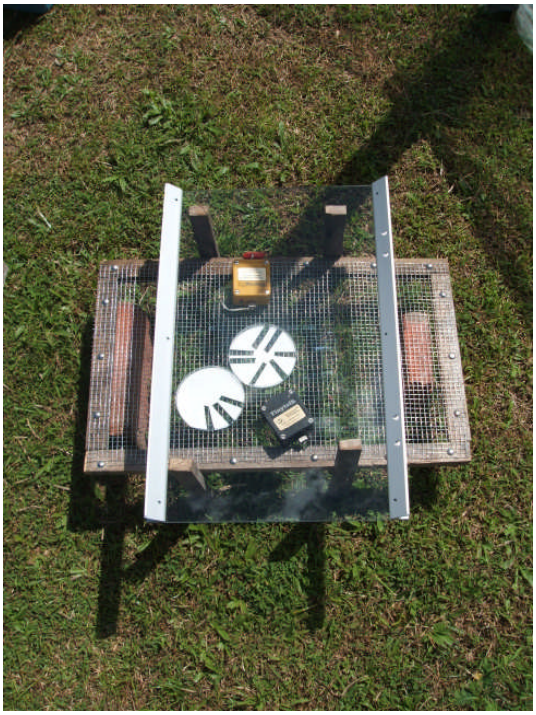


Fig. 3. "GSR-UV"" treatment.
Filter paper disks with urediniospores placed onto a wire grid platform and exposed to solar radiation beneath a UV filter.



Fig. 4. "DSR"" treatment.
Filter paper disks with urediniospores placed onto a wire grid platform and exposed to diffuse solar radiation beneath cheesecloth (shaded control).

The GSR-UV treatment was included in an attempt to separate the effects of UV radiation from that of total (global) solar radiation. The DSR treatment was included to simulate heavy cloud cover, similar to what would occur during monsoonal conditions. When simulating cloud conditions, it is important to use a white cover to create shade. Transmission of short-wave radiation, which is most photo-biologically active (causes the greatest damage to the DNA of organisms), is highest under white shade cloth and lowest under black (Healey and Rickert, 1998). One filter paper disk with urediniospores was used for each treatment. At the beginning of the experiment, each disk was sampled to determine the initial spore viability. They were then exposed to solar radiation from 08:00-16:00 h, refrigerated at 5 ºC overnight and returned to the experimental site for a repeat exposure on the second day. Each treatment was sampled at 2 h intervals during the exposure by cutting a piece measuring approximately 1 x 5 cm from the disks and touching it onto the surface of 20 g/L water agar (WA) in a Petri dish, leaving a spore-print. The plates were incubated in darkness at 25 ºC. Urediniospore germination was assessed after 24 and 48 h. Each time, the germination of 200 spores was assessed. Where no germination was evident, all urediniospores on the plates were viewed (ca. 2,000-3,000). Initial germination was variable between treatments and experiments and sometimes quite low (<50 %). For the results to be comparable, initial germination for all treatments was considered as 100, with subsequent germinations after treatment calculated as a proportion of this.

The light intensity (400-700 nm wavelength range measured in µmol S^{-1} m^{-2}) under the three treatments was recorded every hour using a LI-COR quantum sensor. Air temperature and relative humidity was recorded for each treatment every 15 min using Tiny Tag data loggers. Type and general duration of daily cloud cover was also noted.

The experiment was repeated three times over a seven day period during May 2008.

Data for all treatments (for all experiments combined) were fitted with exponential decay relationships for proportion germinating with time (16 h cumulative exposure to solar radiation) where maximum germination (intercept) =1:
Proportion germinating = $e^{-B.Time}$ where B = the slope of the relationship to an asymptote of 0.
The regression model is fitted via least squares. Errors are assumed Gaussian and independent with constant variance.

b) Exposure to elevated temperatures

This study involved treating detached urediniospores at different temperatures. The same procedure as in a) was used for preparing the treatments. The spores on the filter paper disks were subjected to the following four treatments inside controlled temperature/humidity chambers:

1. 25 °C (control)
2. 35 °C
3. 40 °C
4. 45 °C

The experiment was conducted over a one day period from 08:00-16:00 h. The same procedure as in a) was used for sampling the treatments and assessing germination, except that 100 spores were assessed each time.

The humidity for each treatment was maintained at around 50 %.

The experiment was conducted in September and repeated in December 2008.

Results were analysed as binomial proportions using the successes (i.e. germinations) and failures in a generalised linear model with binomial errors. The mean for each treatment here is the total proportion of germinations for all replicates combined.

Study 2: Develop a Real-Time Polymerase Chain Reaction (RT-PCR) test to identify the causal agent,* Phakopsora euvitis*.

Grapevine (*Vitis* spp.) leaf samples were collected from locally infected vines in Yogyakarta, Indonesia. Molecular experiments were initially conducted at the UGM to determine if the PCR tests could amplify *Phakopsora* DNA successfully. The remainder of the testing was conducted in Darwin at Department of Resources (DoR) molecular facilities.

DNA from *P. euvitis* was obtained by scraping spores from infected *Vitis* spp. leaves onto a microscope slide. The spores were mixed with 20 µL Microlysis-Plus (Microzone Ltd, United Kingdom) solution. The suspension was then placed in a PCR thermal cycler (Bio-Rad, Australia) and subjected to the following treatment.

Step 1. 65 ºC for 15 min

Step 2. 96 ºC for 2 min

Step 3. 65 ºC for 4 min

Step 4. 96 ºC for 1 min

Step 5. 65 ºC for 1 min

Step 6. 96 ºC for 30 s

Step 7. 20 ºC hold

The heat treatment lysed the spore cells and released the DNA into the solution.

To develop a qRT-PCR assay, a probe which specifically detects *P. euvitis* is required. DNA sequences of *P. euvitis* and other *Phakopsora* species were downloaded from GenBank (a DNA database freely available on the internet, http://www.ncbi.nlm.nih.gov/). These sequences were aligned to determine regions that were both similar and different among the *Phakopsora* species.

Study 3: Develop the capacity to identify* P. euvitis *in leaf tissue, during the incubation period, when no symptoms are visible.

DNA extractions were performed at the UGM. Leaves were sampled from 24 diseased and four non-diseased *Vitis* spp. vines growing nearby. Each leaf was assessed under a stereo-microscope to determine the presence and/or absence of spores prior to their selection for testing. 100 mg of tissue per sample was excised from 24 symptomatic, 22 non-symptomatic (from the diseased vines) and four healthy leaves and ground in 2 mL tubes using a sterile micro-pestle. Extraction of *P. euvitis* DNA from plant tissue was trialled using a commercially available test kit (Qiagen DNeasy Plant mini kit) following the manufacturer's instructions. The resulting total DNA (*Vitis* spp. and *P. euvitis*) solution was stored at -20 ºC until PCR testing.

The samples were subjected to a PCR test using NL1/NL4 primers which detects the D1/D2 region of the large subunit ribosomal DNA, a common fungal gene (O'Donnell, 1993). If *Phakopsora* DNA is present, a PCR product 560 bp in size is observed. Two types of Hot Start *Taq* polymerase were used to determine efficacy and efficiency. The Megamix- Royal (Micromon Ltd, United Kingdom) was trialled at the UGM and DoR and the Immomix Red (Bioline, Australia) was trialled only at the DoR. Another PCR test using ITS1F/LR15 primers (Gardes and Bruns, 1993, Chatasiri and Ono, 2008) that amplifies the complete ITS region, ITS 1 and 2, and the 5.8S ribosomal RNA region was also tested. PCR parameters used were; initial denaturation at 94-95 ºC for 10 min, 40 cycles of denaturation at 94 ºC for 1 min, annealing at 50 ºC for 1.5 min, extension at 72 ºC for 2 min then a final extension at 72 ºC for 10 min. The PCR products were visualised by gel electrophoresis on a 1 % agarose gel and using 1 x TAE buffer (40 mM Tris acetate, 1 mM ethylenediaminetetraacetic acid) for 40 min at 80V. PCR product size was determined by using the reference 1 Kb Plus DNA ladder (Invitrogen, Australia). At the DoR, tissue from healthy vines leaves growing in a glasshouse was included as a negative control.

Due to technical problems at the UGM, it was not feasible to sequence PCR products there. The products were purified and sequenced to confirm if *Phakopsora* DNA was amplified at the DoR. PCR products were prepared for sequencing using the Qiaquick PCR purification kit (Qiagen, Australia) and the DNA concentration was determined by running a known volume of DNA with a reference low mass ladder (Invitrogen, Australia) on a 1 % agarose gel. Sequencing reactions and capillary separation was conducted at the Bioscience North Australia facility located at the Charles Darwin University. Bioinformatic analyses were performed using the NCBI GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) "BlastN" search engine. This allows the user to search for matches against all nucleotide sequences deposited in GenBank. Phylogeny analysis was conducted using a freely available web based program (http://www.phylogeny.fr).

To further characterise the gene, it was necessary to determine the DNA fingerprint and differentiate the *Phakopsora* DNA at species level. This was achieved by cutting the PCR product into pieces using three different restriction enzymes (*Alu* I, *Rsa* I and *Sau* 96I). 5 µL of PCR product was digested with a restriction master mix containing 0.5 µL enzyme, 1 µL enzyme buffer and 3.5 µL of sterile distilled water. The digestion mixture was incubated for 2 h to overnight at 37 ºC. The fragments were then separated on an 8% polyacrylamide gel for 5 h at 150 V. Upon completion, the polyacrylamide gel was stained in an ethidium bromide (10 mg/mL) bath for 5-10 min followed by destaining in distilled water for 10 min. The unique DNA fingerprint was determined upon exposure to ultraviolet illumination.

Results & Discussion

Study 1: Investigate the possibility of windborne re-introduction of grapevine leaf rust to northern Australia

Wind is an important dispersal mechanism for many micro-organisms. They can be carried long distances between continents, remaining viable for long periods and then able to colonise new environments. Urediniospores of *Phakopsora pachyrhizi,* a cause of soybean rust, were able to traverse the Atlantic Ocean from Africa and establish disease in South America, a distance in the order of 5-8000 km. This means the spores were able to survive for days, possibly up two weeks in the air (Isard *et. al.,* 2006). To give an extreme example, a stored collection of nineteenth century Saharan dust samples made by Charles Darwin over the Atlantic Ocean contained fungi and bacteria that have remained viable for almost two hundred years (Gorbushina *et. al.,* 2007).

There are two distinct processes that result in spores of a plant pathogen becoming airborne. They are; the removal spores from the host and, the escape of spores from the canopy (Aylor, 1990). Once airborne, they are subject to many adverse conditions that limit the distance travelled and the ability to establish a population following deposition. In general, the risk of infection from aerially dispersed fungal spores is a function of: 1), spore production (population size); 2), spore release; 3), spore escape (from the infected plant canopy); 4), transport and dilution (by turbulence and washout by rain); 5), loss of viability during transport (exposure to solar radiation, desiccation etc); 6), deposition on host tissue; 7), area of host tissue per unit ground area; and 8), infection efficiency of deposited spores (susceptibility of host tissue and ability of spores to infect) (Aylor, 1986).

- ***Airflow between SE Asia and northern Australia***

Airflow between SE Asia and northern Australia during the summer is influenced by the NW monsoon, occurring from December to March. The season is defined by a cycle of active and non-active monsoon periods. During the period of the “active monsoon” over northern Australia air flows consistently in a NW direction across SE Asia, the Timor Sea and northern Australia (Fig 5). There are generally two to three of these major monsoon events, when sustained flows of moist air into the monsoon trough occur (BOM, n.d). These events may last several days to weeks but most commonly for seven days, occurring at intervals of about 40 days (ABC, 2000). During the periods when the monsoon is not active (“monsoon break”) and during the dry season, the airflow is not a risk (Figs. 6 and 7).

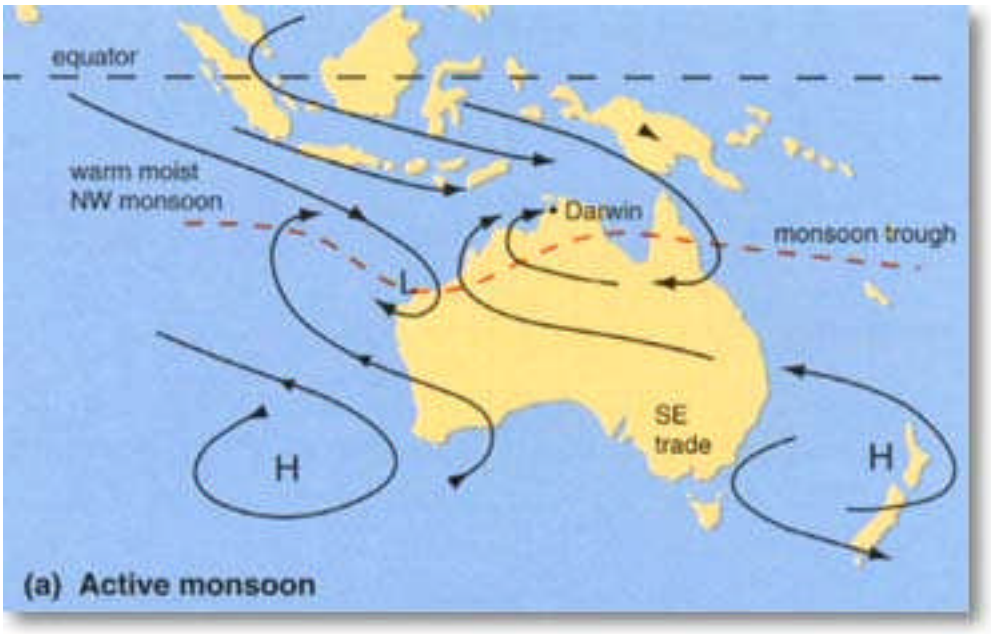


Fig. 5. Sustained NW airflow between SE Asia and northern Australia during the "active monsoon" period.
Courtesy of Bureau of Meteorology, 2008.

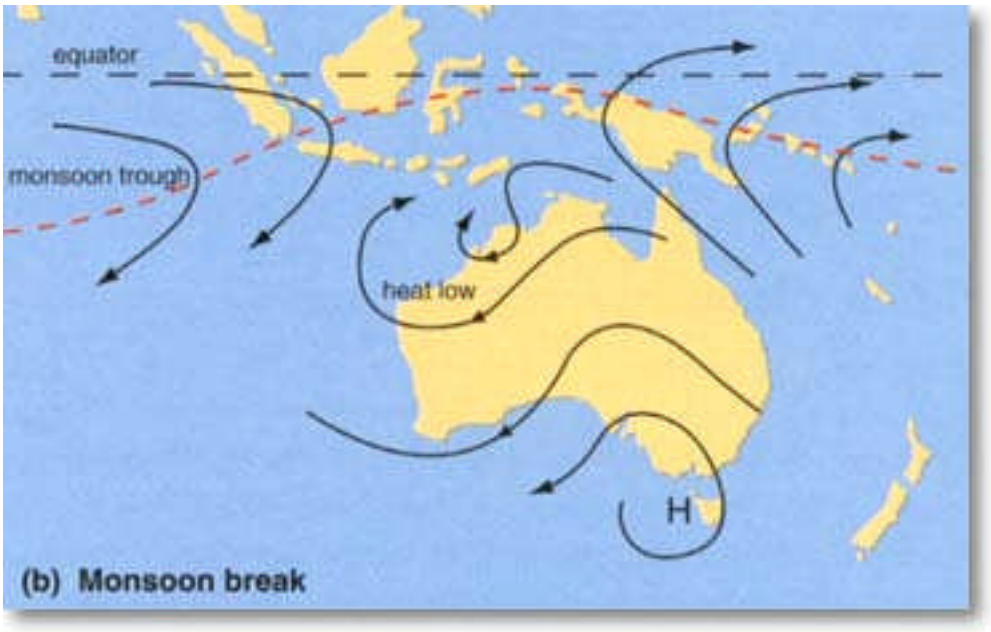


Fig. 6. Interruption to the prevailing NW airflow during the "monsoon break".
Courtesy of Bureau of Meteorology, 2008.

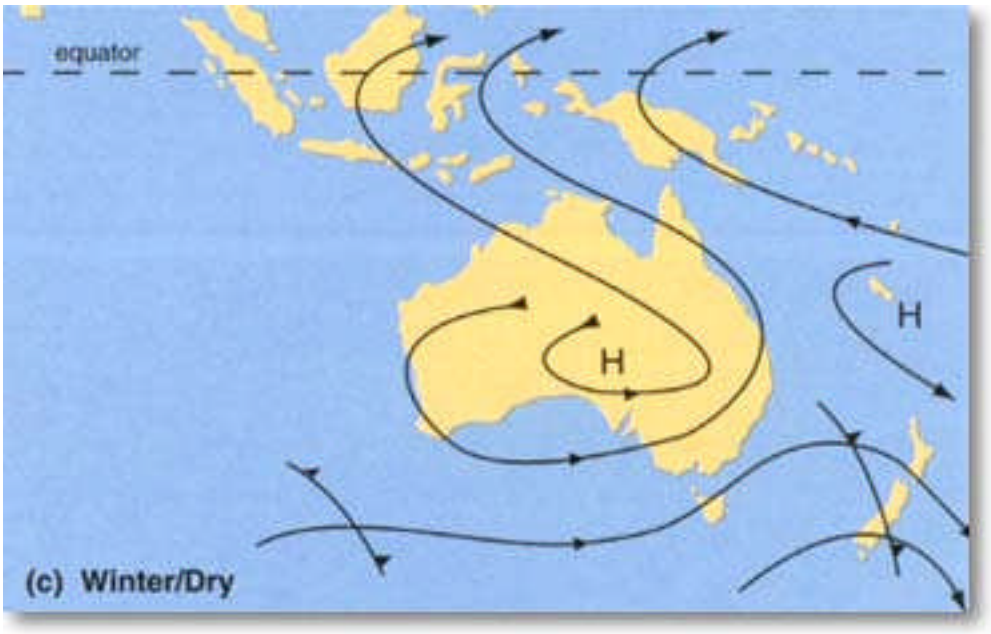


Fig. 7. SE airflow across northern Australia during the dry season.
Courtesy of Bureau of Meteorology, 2008.

Airflow occurs in what is known as the troposphere, the lowest layer of the atmosphere, between the earth surface and the stratosphere. The depth of the troposphere varies with latitude but is about 17 km at the equator (ESPERE-ENC, 2006). Conditions in the troposphere can get quite cold, below freezing at altitudes above about 4 or 5 km over the Tropics. While at the surface the temperature is approximately 30 °C, there is a decrease of about 6.5 °C per km increase in altitude in the lower troposphere (Danielson et al, *Meteorology,* 2003). Sub-zero temperatures would be lethal to the relatively fragile, short-lived urediniospores of *P. euvitis.* However, modelling by the Bureau of Meteorology, Darwin indicated it is possible for "parcels" of air passing/leaving the southern side of East Timor at low altitude (10-1000m) to maintain their trajectory, flowing all the way to Darwin within a short period of time (in a little over a day) during the active monsoon period.

Examples of predicted airflow using the Environmental Emergency Response version of HYSPLIT are provided below. Fig. 8 shows the predicted trajectory of airflow over northern Australia from its recorded origin in East Timor (forecast). Fig. 9 shows the trajectory of airflow traced back to its predicted place of origin from the recorded settling location in the Northern Territory (hind-cast). East Timor was chosen as a possible source of the disease in Darwin due to its close proximity (only 700 km from Darwin) and the fact that GLR has been confirmed there and is likely to be widespread.

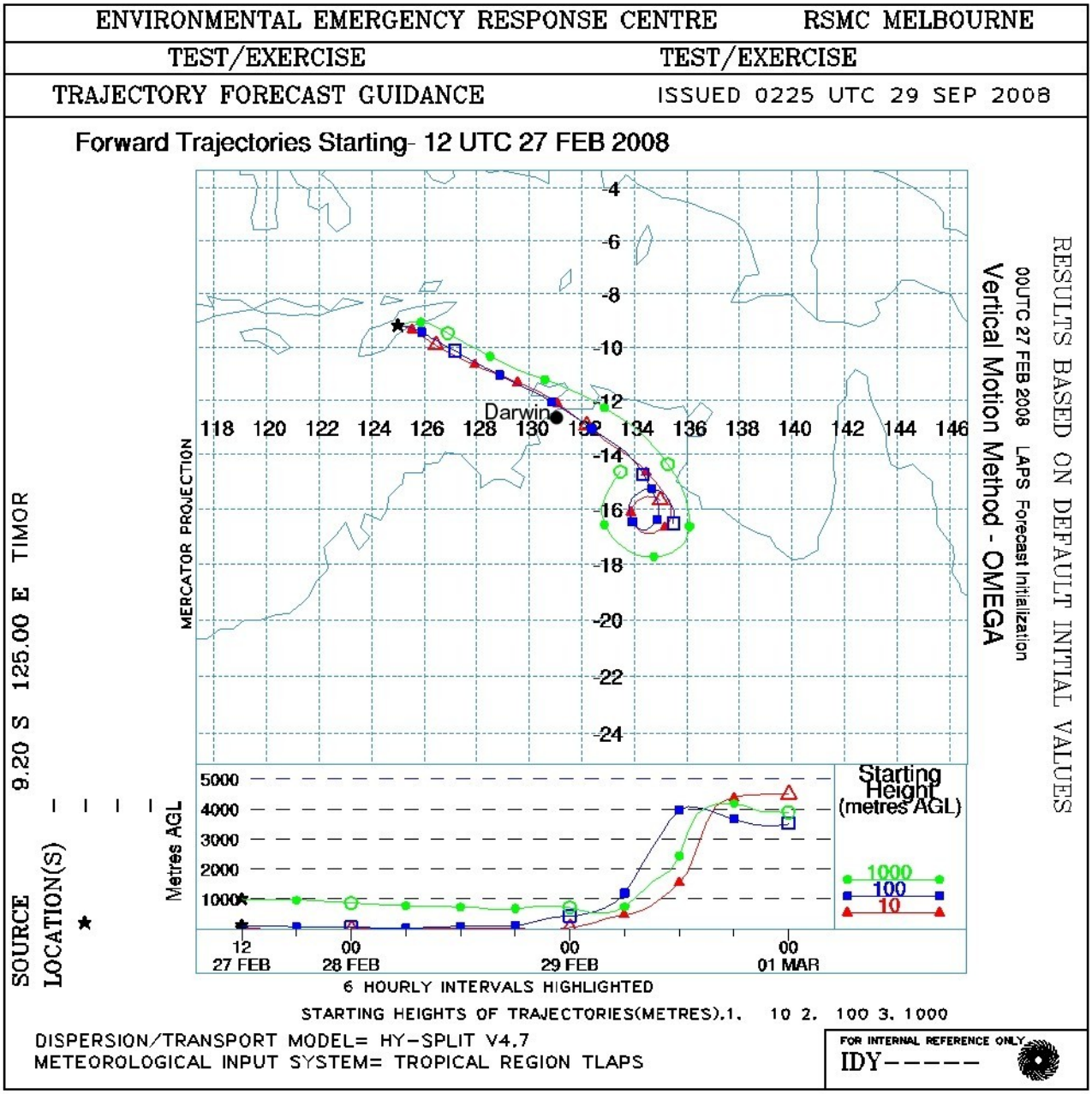


Fig 8. Computed forward trajectory of parcels of air flowing from East Timor over Darwin on 27 February 2008 at starting elevations of 10m (red triangles), 100m (blue squares) and 1000m (green circles).
Courtesy of Bureau of Meteorology, 2008.

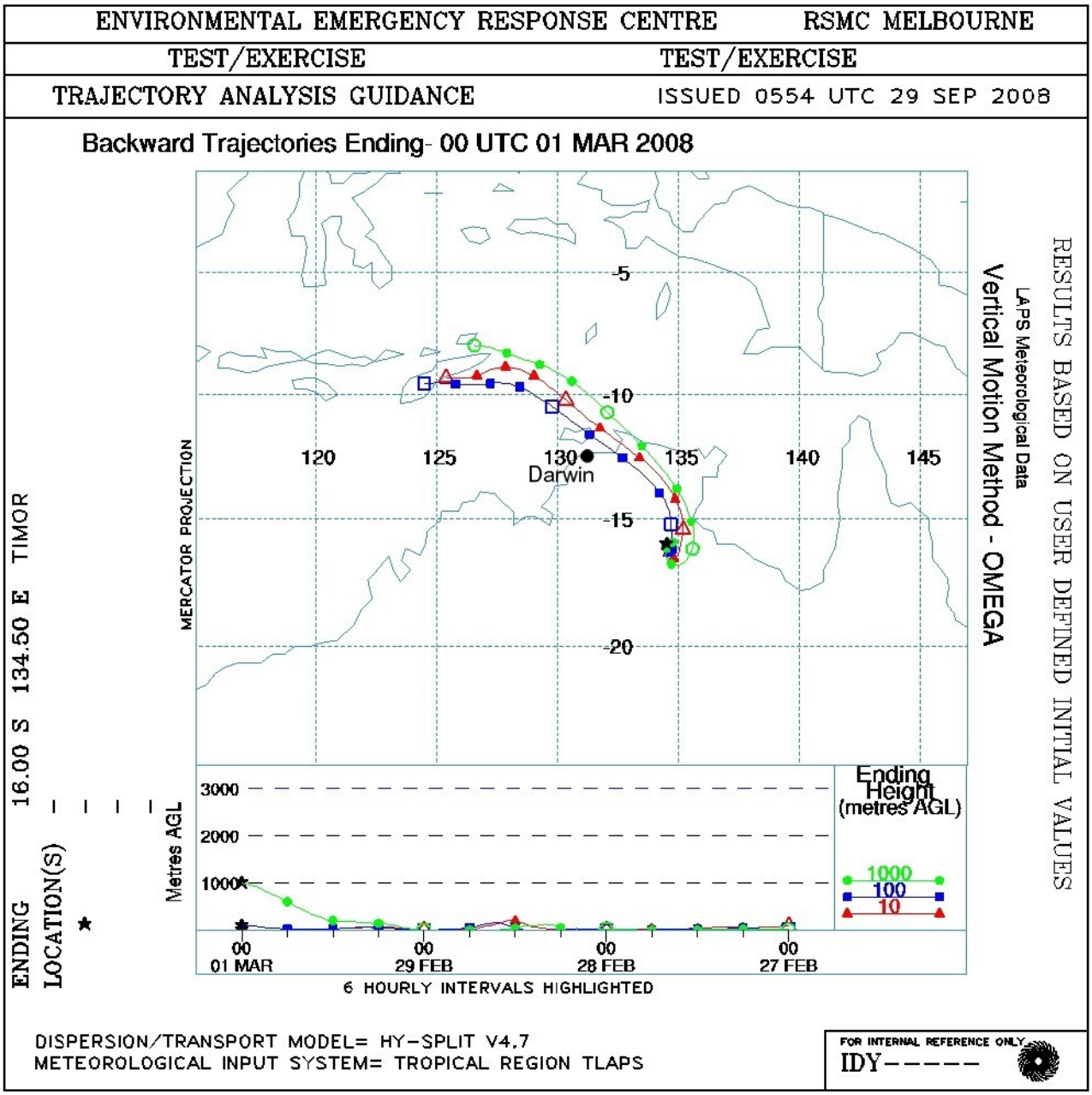


Fig 9. Computed backward trajectory of parcels of air recorded at their settling location in northern Australia on 27 February 2008 at starting elevations of 10m (red triangles), 100m (blue squares) and 1000m (green circles) (Courtesy of Bureau of Meteorology, 2008).

This modelling provides a useful visual representation of the possibility for windborne re-introduction of GLR from East Timor. Air temperature at 1000 m (the highest altitude modelled) will be a warm 20-25 °C. The forecast airflow maintained the low altitudes specified at the point of origin throughout the trajectory and passed over Darwin in around 24 h. If these parcels of air were laden with spores, a portion of them could be carried under ideal temperature conditions (low altitude) all the way to Darwin in only one day. Urediniospores of *P. euvitis* are capable of surviving temperatures as low as 5 °C (Ozoe and Kadowaki, 1971, Daly and Hennessy, 2007). Spores could potentially be transported in airflow another 3-4 km higher in the atmosphere before low temperature is likely to be a cause of mortality.

To more thoroughly understand the risk of windborne re-introduction of GLR from SE Asian locations such as East Timor, the HYSPLIT model could be used to generate trajectories for everyday during a monsoon season. The frequency with which "risk events" (i.e., those trajectories passing directly over Darwin within a reasonable time period) occur from chosen locations, altitudes, dates and times could be computed to give some idea of the total risk throughout the monsoon season.

Fungal spores can be "actively" or "passively" liberated from the host plant (Aylor, 1990). Passive liberation is by the action of wind and occurs for rust pathogens. Passively liberated spores are typically found in highest concentration in the atmosphere during the daytime, often reaching a peak around midday (Hirst, 1953) when the wind speed and turbulence near the ground are highest (Arya, 1988). The dynamics of urediniospore release and aerial concentration for *P. euvitis* in relation to wind conditions is

unknown but, this general information about rust pathogens could be used to target the morning to midday hours for modelling when the wind is most turbulent and atmospheric spore concentration perhaps highest.

Despite the possibilities of modelling, it would still not be possible to highlight all of the specific airflow trajectories that constitute a "risk event" for northern Australia. Also the value of doing this is questionable as there is a general lack of vital information, such as the distribution and concentration of *Vitis* spp. on East Timor and spore production and release characteristics specific to *P. euvitis*. In the case of soybean rust in the United States, to determine the likely source of the disease, an extensive collaborative effort between researchers from the United States, Africa and South America was required to construct maps and calendars of spore production in Africa and South America (Isard *et. al.,* 2005). This required detailed information including the number of hectares planted with soybean in these areas and peak spore release times. As this is an important commercial crop in these regions, good information could be sourced from literature, overseas scientists and from visits to determine accurate distributions (Isard *et. al*., 2004).

Conducting retrospective runs of the model for some of the years just prior to the detection of GLR could also be considered. "Risk events" could be aligned with information about spore mortality and rainfall over Darwin (which could have caused wet deposition of spores). However, this would be a veritable "stab in the dark", due to the lack of information about *Vitis* spp. distribution and *P. euvitis* biology, and unlikely to uncover how the disease was introduced. As well, it is unknown how long the disease has been present in Darwin. It may have been so for many years prior to detection. Reasonably accurate knowledge about when a disease is introduced to a new area is usually necessary for a productive investigation into the source and the method of introduction. By awareness of and monitoring for soybean rust in the United States, authorities were able to detect the disease in crops soon after it arrived in 2004. This enabled identification of a "risk event". Hurricane Ivan was subsequently blamed for transporting the disease from Columbia to the United States (Isard *et. al.,* 2005) (this type of extreme event is worth considering as a possibility for aiding windborne re-introduction of *P. euvitis* to northern Australia as opposed to normal seasonal airflows). The initial detection of *P. euvitis* in 2001 cannot be considered as a starting point in time for such an investigation.

The HYSPLIT model is also able to represent dispersion plumes of atmospheric contaminants. It can be adapted to forecast the dispersion of biological organisms, such as the Foot and Mouth virus and Anthrax, with input of specific biological information about the organism and the effect of environmental conditions. An example of the forecast dispersion plume from a simulated release of the Foot and Mouth virus is provided below (Fig. 10). This corresponds with the forecast trajectory in Fig. 8. The HYSPLIT model could also be adapted to forecast dispersion plumes for GLR. However, this would require significant model development with input of detailed information of the nature stated by Aylor (1986) and outlined above.

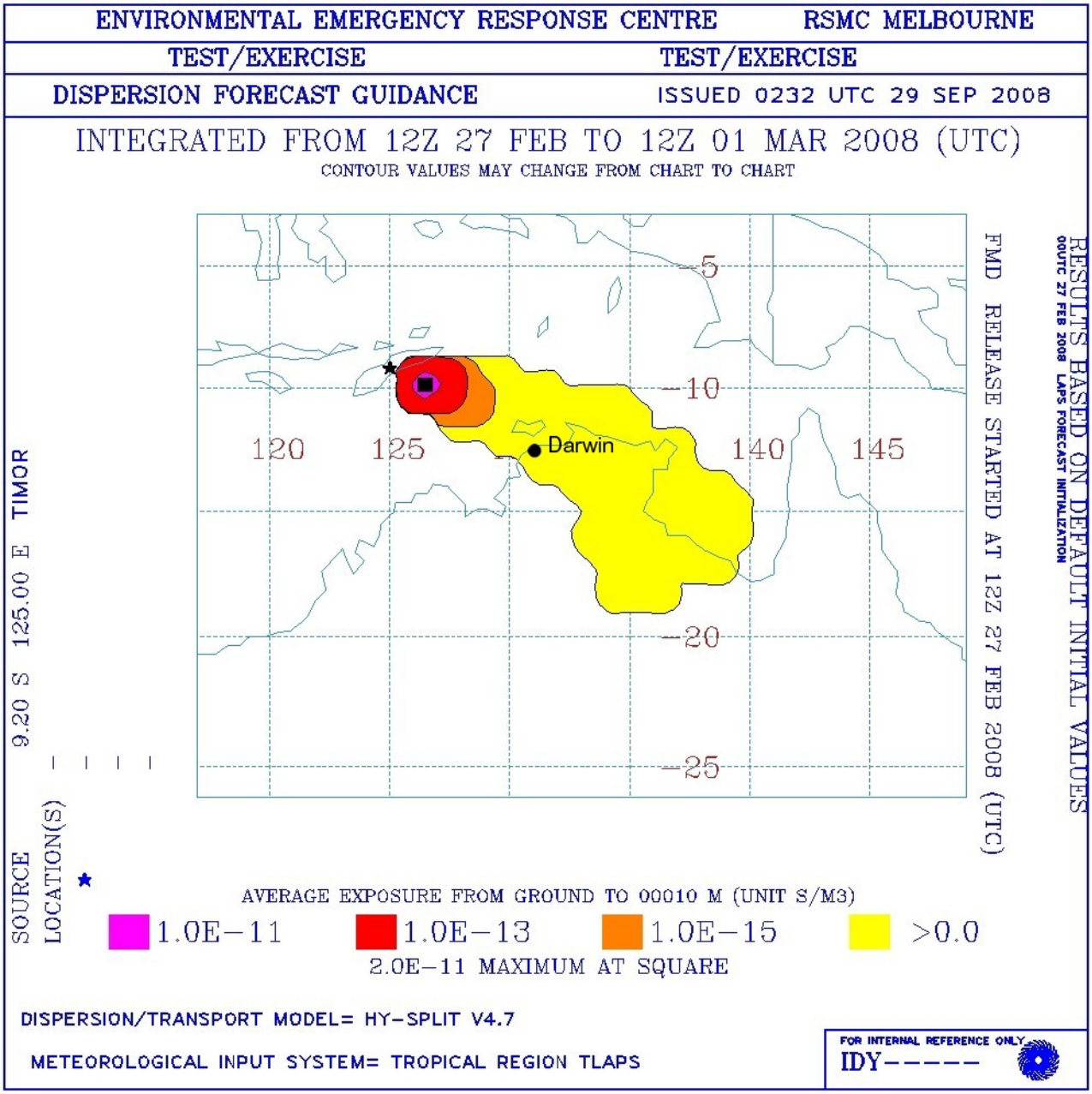


Fig 10. Computed dispersion plume of the Foot and Mouth virus originating from a selected location in East Timor. The colours indicate the concentration of the virus within the plume, ranging from most (pink) to least (yellow) concentrated.
Courtesy of Bureau of Meteorology, 2008.

- ***Urediniospore mortality***

Once spores of plant pathogens are exposed to the elements, solar radiation has been shown to have the greatest effect on spore viability. There have been numerous studies on many different fungal pathogens. Urediniospores of *P. pachyrhizi* are sensitive to solar radiation and for them to survive in the air during intercontinental travel this must occur under cloud cover (Isard *et. al.,* 2006). The authors concluded that temperature and humidity levels to which urediniospores would be exposed in the air during summer are within the range that *P. pachyrhizi* can tolerate. Hwang (1941) demonstrated a direct relationship between light intensity and loss of viability of urediniospores of a number of cereal rust species. Arabi and Jawhar (2003) found that the germinability of *Cochliobolus sativus*, the causal agent of barley spot blotch, was not greatly affected by temperature but rather by solar radiation. Bashi and Aylor (1983) found that sunlight rather than temperature and relative humidity was the most important factor determining survival of downy mildew pathogens *Peronospora destructor* and *P. tabacina.*

However, as would be expected, the resistance to damage from solar radiation varies between species. Urediniospores of the grass stem rust pathogen are apparently relatively resistant to damage from solar radiation (Maddison and Manners, 1972). Resistance can also potentially vary within species from different locations. Spores of pathogens from tropical latitudes for example might be expected to have a higher resistance to solar radiation than those of pathogens from temperate latitudes. Spore

pigmentation is important in relation to their survival when exposed to UV radiation. Generally, the darker the colour the more UV radiation required to de-vitalise the spore (Nagy, 1947). Survival of white and orange urediniospores of *Puccinia graminis* subsp. *tritici* was shown to be lower when exposed to UV radiation than grey and red spores (Dillon-Weston, 1931a, 1931b and 1932). Urediniospores of *P. euvitis* are orange. It is for the above reasons that specific information is required for individual pathogens about their resistance to solar radiation and meaningful studies on mortality need to be conducted on pathogens in their places of origin.

a) Exposure to solar radiation

Urediniospore germination results for *P. euvitis* after exposure to the three levels of solar irradiance (DSR, GSR-UV and GSR) are presented below (Fig. 10). The raw data is presented in Appendix 5, Table 2. Whilst there were some brief periods of cloud cover during the experiments (some low level cumulus cloud but mainly thin, high-level cirrus type cloud), the days during which the assessments took place were predominantly clear and sunny.

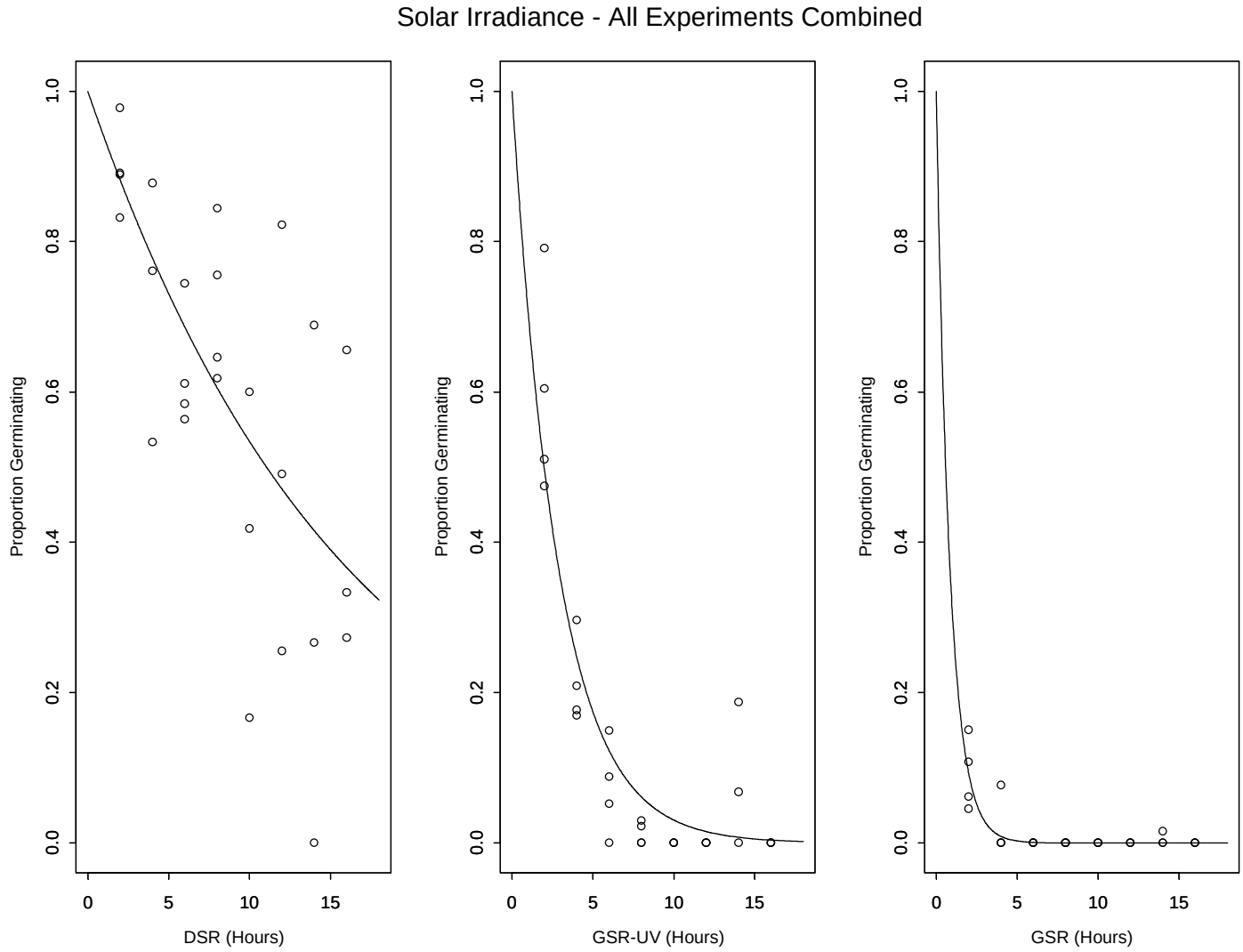


Fig. 10. Proportion of urediniospores germinating after incubation at 25 ºC for 24 h on water agar following exposure over two days to diffuse solar radiation (DSR), solar radiation minus UV (GSR-UV) and global solar radiation (GSR), sampled at 2 h intervals. Actual initial germinations were 44 %, 48 % and 46 % for DSR, GSR-UV and GSR treatments respectively.

Viability of spores exposed to just 4 h of global solar radiation (GSR treatment) was reduced to near zero. Similar results to these have been published for other fungi. Bashi and Aylor (1983) found that germination of downy mildew pathogens, *Peronospora destructor* and *P. tabacina,* after just 4 h exposure to direct solar radiation was zero. Exposure to total solar radiation for 4 h at midday completely inactivated the conidia of strains of the fungus *Metarhizium anispoliae* (Braga *et. al.,* 2001). The viability of sporangia of *Phytophthora infestans* was decreased by 95 % after 1 h of exposure on sunny days (Mizubuti *et. al.* (2000). The blocking of UV from the radiation reaching the spores (GSR-UV treatment) appeared to delay the loss of viability of spores, with a

proportion of around 0.4 germinating after 4 h exposure. Some germination still occurred after 8 h exposure. When the spores were shaded (DSR treatment) they maintained comparatively high viability, with proportions of around 0.7 and 0.4 still germinating after 8 h and 16 h exposure respectively.

In both the GSR and GSR-UV treatments there was an increase in the germination percentage after 48 h of incubation compared with that following 24 h incubation, indicating that exposure to solar radiation can also cause dormancy. Delay in germination due to exposure to UVB radiation has been reported previously. Ghajar *et. al.* (2006) found that high doses of UVB killed conidia and caused delays in germination of surviving conidia. Olga *et. al.* (1980) showed low doses of radiation in the UVA range delayed germ tube emergence of spores of the fungus *Cladosporium cucumerinum.* Braga *et. al.* (2001) found the germination of surviving conidia of three strains of the fungus *Metarhizium anispoliae* was delayed following exposure to both full-spectrum and UVA sunlight.

Although the viability results were based on the germinating proportion of the first 200 spores in each treatment, all spores of the GSR and GSR-UV treatments, following 4 h and 8 h of exposure respectively, were observed for germination. A very small number of them (1-10 spores) had germinated, but with highly abnormal germ tubes; being constricted, coiled and shortened. Although germination occurred, their abnormal appearance indicates probable DNA damage and the ability of these spores to infect a vine is questionable. The germ tube morphology of many of the spores under the DSR treatment when assessed following exposure for more than a day also appeared abnormal.

Most of the damaging effect of sunlight on biological organisms is caused by the UV portion of the spectrum, in particular, the UVB wavelengths (320-290 nm) (Diffey, 1991). UVA (400-320 nm) is not as photo-biologically active and UVC (290-200 nm) is highly absorbed by the ozone layer and does not reach the earth surface in an appreciable quantity (Diffey, 1991). As the ozone layer occurs in the stratosphere (above the troposphere), UVC is negligible in the troposphere also. Considering this, it is surprising that the viability of spores was reduced so rapidly under the GSR-UV treatment. It might be that the filter used did not block as much of the UV radiation as expected. It did not cover an area much wider than that containing the spores. UV light is readily scattered by particles in the air and reflection from surfaces. The spores therefore may have still been exposed to a significant amount of UV light even though they were under the filter. This effect was reported by Mizubuti *et. al.* (2000) in a similar study of the mortality of *Phytophthora infestans* sporangia.

Despite the rapidly lethal effects of solar radiation in this experiment, it is important to consider how this might apply under normal conditions of aerial transport. Urediniospores of rust fungi are often removed as clusters of two or more spores (Ferrandino and Aylor, 1984 and 1987). Although this means a reduction in infective units once the spores land on plant tissue, clusters may be especially important for dispersal under conditions of low spore viability as those within the cluster may be more likely to survive and germinate (Aylor and Ferrandino, 1985). The inner spores would be given protection from the lethal effects of solar radiation by the outer spores. No effort was made in this experiment to evenly disperse the spores over the surface of the filter paper for exposure to solar radiation and clusters were evident when checking germination under the microscope. Hence, the effects of solar radiation on clusters as opposed to single spores was somewhat accounted for.

Dehydration of spores was observed during the experiment as indicated by their crinkled shape. This could have contributed to some of the loss in viability. However, Eversmeyer and Kramer (1989) found no effect of desiccation on *Puccinia recondite* and *P. graminis,* the causes of wheat leaf and stem rust respectively, when held for 24 h at 20 °C and 30 % relative humidity. Ellison *et. al.* (1988) also found no correlation between humidity (15 % and lower) and viability including when coupled with high temperatures. Dehydration may not influence viability but it may affect the aerodynamic properties of the spores and therefore affect the distance they travel in the air. The relative water content of corn pollen plays an important role in the effective long distance dispersal (Aylor, 1999). Dehydration causes the shape to change, becoming crinkled, which decreases the settling speed of individual grains by about 34 % (Aylor, 2002) and presumably allowing them to travel further.

Average temperatures recorded during exposure under the GSR and GSR-UV treatments generally ranged within the mid 30's and 40's (°C) for the duration of the exposure, reaching these levels within the first 2 h (Figs. 11 and 12). Peaks in excess of 50 °C did occur on occasions. Although the daily temperatures recorded were similar for both treatments, the light intensity of the UV treatment was consistently 15-20 % lower. Relative humidity ranged between 35 % and 65 %. Temperatures recorded during exposure under the DSR treatment were generally in the low 30's, significantly milder than those recorded for the other two treatments. Relative humidity ranged between 45 % and 65 %, therefore being a little more stable than for the GSR and GSR-UV treatments.

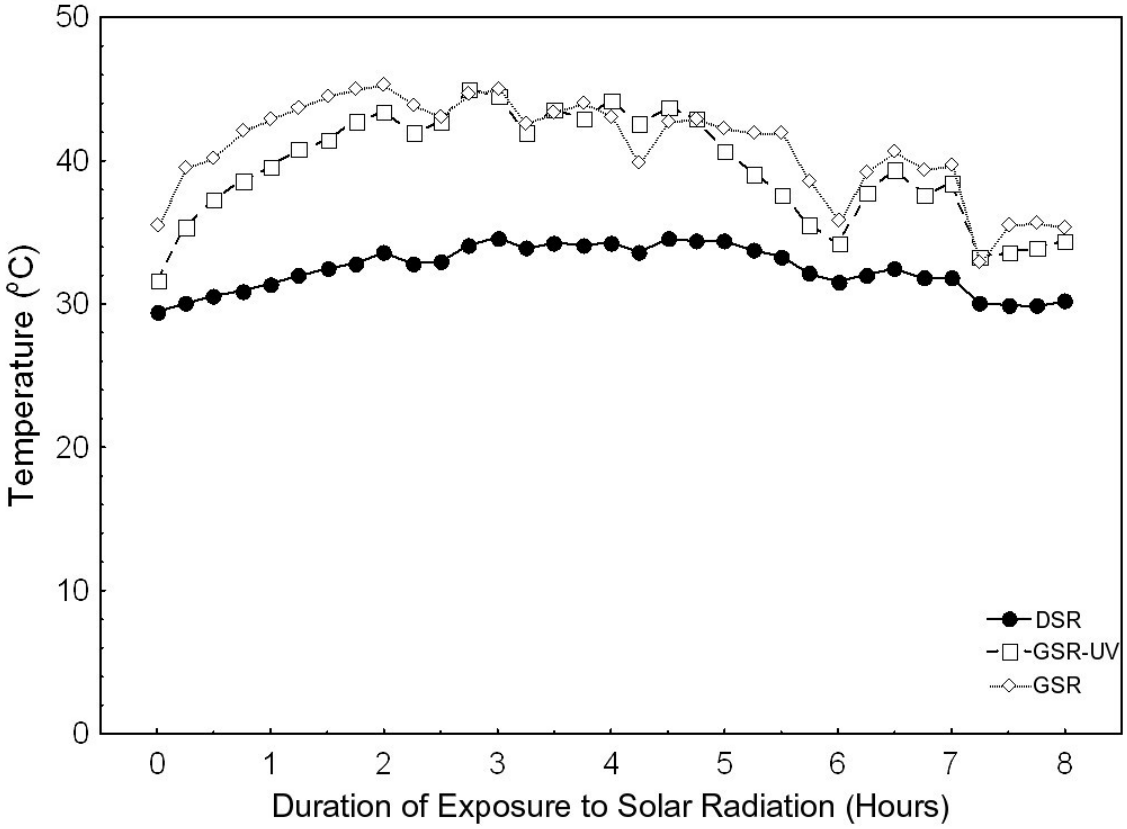


Fig. 11. Average daily temperatures recorded between 08:00 and 16:00 h during the first day of exposure of urediniospores to global irradiance (GSR), solar radiation minus UV (GSR-UV) and diffuse solar radiation (DSR).

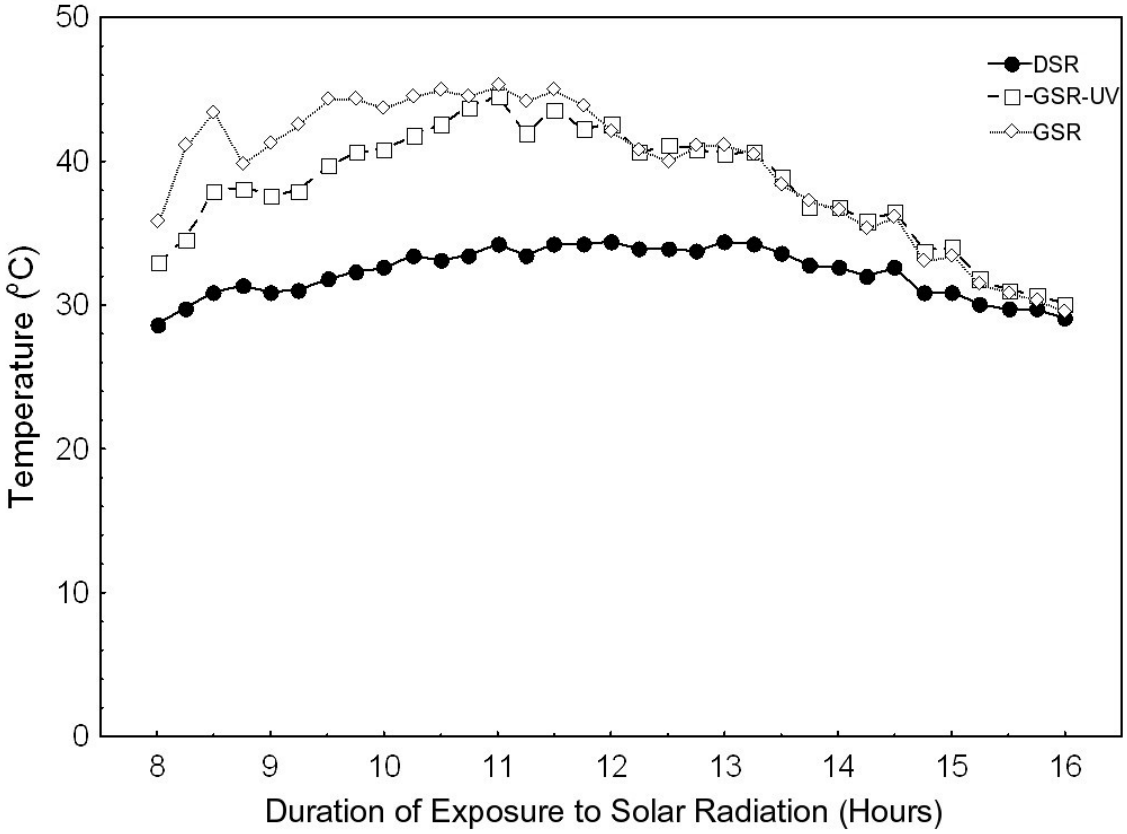


Fig. 12. Average daily temperatures recorded between 08:00 and 16:00 h during the second day of exposure of urediniospores to global irradiance (GSR), solar radiation minus UV (GSR-UV) and diffuse solar radiation (DSR).

The high temperatures recorded during the experiments under the GSR and GSR-UV treatments (direct light) are probably a result of the design of the data loggers (large surface area and heat absorption by the casing). If this is the case, then the method of exposing the spores on filter paper in Petri dishes may have also led to the spores experiencing temperatures higher than they normally would during aerial transport (although only the temperature adjacent to the spores could be measured, not the actual temperature of the spores). Aylor (2003a), in a study of dehydration of corn pollen in the air, suggests that because of their small size, individual pollen grains drifting in the air are expected to be at a similar temperature to the surrounding air. Pollen grains of corn are two to three times the size of the largest spores of *P. euvitis.* Hence, as the spores of *P. euvitis* may not have been at ambient temperature during this experiment, the effect of elevated temperatures needed to be investigated in a separate study.

b) Exposure to elevated temperatures

Although literature on other fungal pathogens indicates that solar radiation is the predominant cause of spore mortality, the effect of high temperatures on *P. euvitis* urediniospores required investigation considering those experienced during exposure to solar radiation in the previous experiments.

The raw data is presented in Appendix 5, Tables 3 and 4. Temperatures at 40 °C and 45 °C significantly reduced germination when compared to both 25 °C and 35 °C during this experiment. Therefore, there may have been a significant decline in viability due to artificially high temperatures under the GSR and GSR-UV treatments in the previous experiment, exaggerating the effects of solar radiation. This would probably not occur during aerial transport. Urediniospores may be more resistant than they appeared to be.

- ***Overall Evaluation of Windborne Re-introduction***

As well as airflow and urediniospore mortality, there are several more factors that will significantly affect the potential of the disease to re-establish in Australia. These include:

- Abundance and distribution of diseased *Vitis* spp. at the source location during the NW monsoon season.
- Intensity and turbulence of wind at the source location.
- Dispersal units (single spores versus clusters).
- Spore washout from the *Vitis* spp. canopies at the source location.
- Depletion during aerial transport through wet and dry deposition.
- Abundance of susceptible foliage in northern Australia during the NW monsoon season.
- Suitability of conditions for germination once spores land on foliage.
- Ability of the spores to infect following exposure and the proportion of these.
- Leaf penetration method of the spore.

To determine whether this is a realistic means by which the disease could re-enter Australia, much more detailed information on some of these factors is required.

Modelling airflow trajectories from SE Asia to northern Australia provides a useful visual representation of possible aerial dispersal of *P. euvitis* but is not sufficient to provide a measure of risk that such events pose. Pfender *et. al.* (2006), using CALPUFF (another atmospheric dispersal model) to estimate the dispersal and dry deposition of grass stem rust (*Puccinia graminis* subsp. *graminicola*) urediniospores, showed that under several weather scenarios few or no spores remain airborne above the canopy for longer than 1 h. However, under moderately turbulent wind conditions, a small proportion are elevated higher and remain suspended for longer. When large numbers of spores are released, a small proportion represents potentially millions of spores that could be transported hundreds of kilometres. Hence, wind turbulence can be important for spore release, escape and elevation high enough from the crop canopy to undergo long distance dispersal. It may be important for long distance dispersal of *P. euvitis* but the abundance of spores and the conditions necessary for their escape and suspension above the canopy are not known. Solar radiation can also be associated with spore release and influence the ability of large numbers to be aerially transmitted. Pfender *et. al.* (2006) found that reduced solar radiation and resultant lower temperature gradient was associated with a failure of spores to remain airborne an hour after release. Hwang (1941) commented that heavy spore showers of cereal rust species occur in Minnesota (USA) when days are long and bright. Hence, although solar radiation is highly lethal to urediniospores of *P. euvitis,* it may also play an important role in their ability to become airborne. Quantifying the escape of spores from a canopy is essential for calculating the potential for long-distance spread of a plant disease (Aylor and Taylor, 1983; Aylor *et. al.,* 1982). Quantification and modelling of this escape is difficult and complex (Pfender *et. al.,* 2006). To develop an understanding of wind conditions resulting in *P. euvitis* spores escaping a vine canopy requires a study of canopy structure and the distribution of disease within it. This information will be unique to the location as it depends on how the vines are grown.

Another matter worth considering is the ability of spores to infect foliage after deposition. Germination does not always correspond to infectivity (Zadocks, 1979; Rowell, 1984) and although many of the spores under the DSR treatment germinated, their ability to infect leaves after this exposure remains unknown. The ability to infect can be thought of in terms of infection efficiency (percentage of spores). The reported infection efficiency for *Puccinia graminis* subsp. *tritici* under optimal conditions is 15-25 % (Rowell, 1984). Due to the presence of clusters during aerial dispersal, this efficiency might drop to 10 % (100 spores deposited represents 70 dispersal units) (Pfender *et. al.*, 2006). This information isn't available for *P. euvitis* but is important in relation to the abundance of spores entering the airflow, deposition, and the abundance of *Vitis* spp. in the deposition area (i.e. Darwin suburbia).

Despite the lack of knowledge in the above mentioned areas, other key aspects of *P. euvitis'* lifecycle and its hosts are well understood. Some of these aspects are favourable as they significantly reduce the chances of windborne re-introduction to northern Australia. *P. euvitis* is very host specific and only able to readily infect *Vitis* spp. It can occasionally infect the native grapevines *Ampelocissus acetosa* and *A. frutescens* which grow in northern Australia. Whilst some fungi, such as *P. pachyrhizi* and *P. apoda* (a rust fungus of kikuyu grass) penetrate the leaves directly through the cuticle (Adendorff and Rijkenberg, 2000), *P. euvitis* only penetrates via stomata on the underside (Ozoe and Kadowaki, 1971; Leu and Wu, 1983; Y. Ono, pers. comm., January 23, 2009). Therefore, spores must either come in contact with the underside of the leaf by deposition directly or by being washed there. This greatly limits the opportunity for spores to infect if they happen to be deposited onto susceptible foliage. Pfender *et. al.* (2006) suggests that spores of fungi in the upper atmosphere undergo wet deposition. Wet deposition is important in disease epidemics as a large number of spores can accumulate in a small area during conditions conducive for infection (Aylor, 1986). For the same reasons it could also be important for the successful introduction of a plant pathogen into a new environment. This seems the most likely way spores caught in NW monsoonal flows would be deposited on vines in northern Australia. Dry deposition (settling spores) would generally occur inland in areas probably devoid of vines where the monsoonal flows terminate. It is possible the native hosts *Ampelocissus acetose* and *A. frutescens* could become infected within and beyond the range of domesticated *Vitis* spp. A small number of infections could build up disease on these hosts and provide inoculum that blows back onto cultivated vines in Darwin during the inconsistent wind movement of the monsoon breaks. However, during the recent eradication program that ended in 2007, 64 sites (over 1000 plants) of *Ampelocissus* spp. received ongoing surveillance and were monitored every 6-8 weeks (West *et. al.,* 2007). The disease was only detected once. This was on a plant growing on a suburban property and close to a heavily infected *Vitis* spp. vine. Only one leaf of more than 400 on the plant carried the disease. During the eradication program, 744 *Vitis* spp. vines were located on 589 properties. This small number of vines, further reduced due to removal during the program, and sparse distribution provides a very limited opportunity for spores to be deposited onto host foliage.

***Study 2: Develop a Real-Time Polymerase Chain Reaction (qRT-PCR) test to identify the causal agent,* Phakopsora euvitis.**

qRT-PCR has a distinct advantage over conventional PCR since it allows the user to observe the kinetics of the reaction in real time (i.e. see the products as they are amplified) (Mackay, 2007). This is only achievable for conventional PCR with an extra step, using agarose gels, where the end point of the test is determined. Real time PCR is able to detect very small concentrations of target DNA, hence making it a useful diagnostic tool.

The large subunit of the ribosomal DNA sequences from *Phakopsora* spp. obtained from the NCBI GenBank (Chatasiri and Ono, 2008) were aligned to determine potential areas where a specific probe to detect *P. euvitis* DNA could be designed (Appendix 5). The multiple sequence alignment of the ribosomal DNA sequences available indicated high similarities between the *Phakopsora* spp. (data presented in Appendix 5; differences highlighted in yellow). Efforts were made to design primers fPeD1D2 and rPeD1D2 targeting areas where the nucleic acid bases differed (data presented in Appendix 5; highlighted in blue). Conventional PCR results produced the expected 200 bp *P. euvitis* band for all tested samples with a faint positive in non-symptomatic samples (Fig 13). However, the primers were not specific and a larger band at ~800 bp was co-amplified in most samples. This band was confirmed to be the 26S rRNA *Vitis* spp. gene by sequencing (data not shown), thus eliminating the possibility of using this primer combination for the qRT-PCR test.

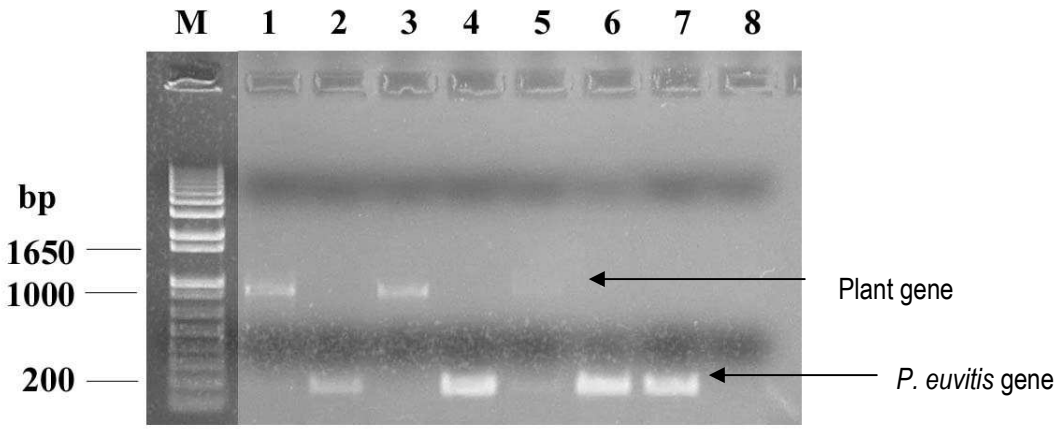


Fig 13. PCR test using f/r PeD1D2 primers. M = 1 Kb Plus ladder, lane 1, S1A; lane 2, S1D; lane 3, S8A; lane 4, S8D; lane 5, S15A; lane 6, S15D; lane 7, *P. euvitis* urediniospores and lane 8, sterile distilled water.

Study 3: Develop the capacity to identify* P. euvitis *in leaf tissue, during the incubation period, when no symptoms are visible.

- ***Optimising PCR test conditions***

DNA was successfully extracted from symptomatic and non-symptomatic *Vitis* spp. leaf samples as well as urediniospores. The target gene was amplified from diseased samples, *P. euvitis* urediniospores and some non-symptomatic leaves (Table 1) using the MegaRoyal Mix (Micromon, UK). An extra product, 1 Kb in size, was also detected and confirmed to be the 26S rRNA gene of *Vitis* spp. (Fig. 14).

Table 1. Amplification of fungal DNA from symptomatic and non-symptomatic leaf samples and *P. euvitis* urediniospores collected from grapevine leaf rust infected *Vitis* spp. growing in Yogyakarta, Indonesia (May 2008)

Sample No.	Source	Sample Location	Symptom	NL1/NL4 PCR
S1A	*Vitis* spp.	Rajawali St Catur Tunggal Depok Sleman Yogyakarta	Non-symptomatic	560 bp, 1 Kb
S1D	*Vitis* spp.	Rajawali St Catur Tunggal Depok Sleman Yogyakarta	Grapevine leaf rust	560 bp
S2A	*Vitis* spp.	4 Ringgodani St Catur Tunggal Depok Sleman Yogyakarta	Non-symptomatic	560 bp, 1 Kb
S2D	*Vitis* spp.	4 Ringgodani St Catur Tunggal Depok Sleman Yogyakarta	Grapevine leaf rust	560 bp
S3A	*Vitis* spp.	1 Mawar St Catur Tunggal Depok Sleman Yogyakarta	Non-symptomatic	560 bp, 1 Kb
S3D	*Vitis* spp.	1 Mawar St Catur Tunggal Depok Sleman Yogyakarta	Grapevine leaf rust	560 bp, 1 Kb
S4A	*Vitis* spp.	Anggajaya Condong Catur Depok Sleman Yogyakarta	Non-symptomatic	560 bp, 1 Kb
S4D	*Vitis* spp.	Anggajaya Condong Catur Depok Sleman Yogyakarta	Grapevine leaf rust	560 bp, 1 Kb
S5A	*Vitis* spp.	Mundgur St Condong Catur Depok Sleman Yogyakarta	Non-symptomatic	560 bp
S5D	*Vitis* spp.	Mundgur St Condong Catur Depok Sleman Yogyakarta	Grapevine leaf rust	560 bp, 1 Kb
S6A	*Vitis* spp.	Mundgur St Condong Catur Depok Sleman Yogyakarta	Non-symptomatic	560 bp, 1 Kb
S6D	*Vitis* spp.	Mundgur St Condong Catur Depok Sleman Yogyakarta	Grapevine leaf rust	560 bp
S7A	*Vitis* spp.	Wates St Km 10 Sleman Yogyakarta	Non-symptomatic	560 bp, 1 Kb
S7D	*Vitis* spp.	Wates St Km 10 Sleman Yogyakarta	Grapevine leaf rust	560 bp
S8A	*Vitis* spp.	Wates St Km 10 Sleman Yogyakarta	Non-symptomatic	1 Kb
S8D	*Vitis* spp.	Wates St Km 10 Sleman Yogyakarta	Grapevine leaf rust	560 bp
S9A	*Vitis* spp.	Wates St Km 10 Sleman Yogyakarta	Non-symptomatic	560 bp, 1 Kb
S9D	*Vitis* spp.	Wates St Km 10 Sleman Yogyakarta	Grapevine leaf rust	Negative
S10A	*Vitis* spp.	Wates St Km 4.5 Sleman Yogyakarta	Non-symptomatic	1 Kb
S10D	*Vitis* spp.	Wates St Km 4.5 Sleman Yogyakarta	Grapevine leaf rust	560 bp
S11A	*Vitis* spp.	NASA House Ringroad St Sleman Yogyakarta	Non-symptomatic	560 bp, 1 Kb
S11D	*Vitis* spp.	NASA House Ringroad St Sleman Yogyakarta	Grapevine leaf rust	560 bp

S12A	*Vitis* spp.	West Ringroad St Sleman Yogyakarta	Non-symptomatic	Negative
S12D	*Vitis* spp.	West Ringroad St Sleman Yogyakarta	Grapevine leaf rust	560 bp
S13A	*Vitis* spp.	Manwkan Plasakuning Ngaglik Sleman Yogyakarta	Non-symptomatic	Negative
S13D	*Vitis* spp.	Manwkan Plasakuning Ngaglik Sleman Yogyakarta	Grapevine leaf rust	560 bp
S14H	*Vitis* spp.	Blotan Wedomartani Ngemplah Sleman Yogyakarta	'Healthy'	Negative
S15A	*Vitis* spp.	Garuda Gejayan Depok Sleman Yogyakarta	Non-symptomatic	560 bp
S15D	*Vitis* spp.	Garuda Gejayan Depok Sleman Yogyakarta	Grapevine leaf rust	560 bp
S16 Atypical	*Vitis* spp.	Christanti Juwangen Kalasan, Yogyakarta	Leaf spots	560 bp
S16D	*Vitis* spp.	Christanti Juwangen Kalasan, Yogyakarta	No rust	560 bp
S17H	*Vitis* spp.	Wahyudi Juwangen Kalasan, Yogyakarta	'Healthy'	560 bp
S18H	*Vitis* spp.	Umbulharjo Yogyakarta	'Healthy'	560 bp
S19H	*Vitis* spp.	Umbulharjo Yogyakarta	'Healthy'	Negative
S20A	*Vitis* spp.	Umbulharjo Yogyakarta	Non-symptomatic	1 Kb
S20D	*Vitis* spp.	Umbulharjo Yogyakarta	Grapevine leaf rust	1 Kb
S21A	*Vitis* spp.	Umbulharjo Yogyakarta	Non-symptomatic	1 Kb
S21D	*Vitis* spp.	Umbulharjo Yogyakarta	Grapevine leaf rust	Negative
S22A	*Vitis* spp.	Umbulharjo Yogyakarta	Non-symptomatic	1 Kb
S22D	*Vitis* spp.	Umbulharjo Yogyakarta	Grapevine leaf rust	560 bp
S23A	*Vitis* spp.	Banteng Estate, Yogyakarta	Non-symptomatic	1 Kb
S23D	*Vitis* spp.	Banteng Estate, Yogyakarta	Grapevine leaf rust	560 bp
S24A	*Vitis* spp.	Banteng Utama, Yogyakarta	Non-symptomatic	560 bp, 1 Kb
S24D	*Vitis* spp.	Banteng Utama, Yogyakarta	Grapevine leaf rust	1 Kb
S25A	*Vitis* spp.	Banteng 3, Yogyakarta	Non-symptomatic	Negative
S25D	*Vitis* spp.	Banteng 3, Yogyakarta	Grapevine leaf rust	1 Kb
S26A	*Vitis* spp.	Jambon Estate, Yogyakarta	Non-symptomatic	560 bp, 1 Kb
S26D	*Vitis* spp.	Jambon Estate, Yogyakarta	Grapevine leaf rust	560 bp
S27A	*Vitis* spp.	Kriecak, Yogyakarta	Non-symptomatic	Negative
S27D	*Vitis* spp.	Kriecak, Yogyakarta	Grapevine leaf rust	Negative
P. euvitis	Spores	Rajawali St Catur Tunggal Depok Sleman Yogyakarta	Grapevine leaf rust	560 bp

.

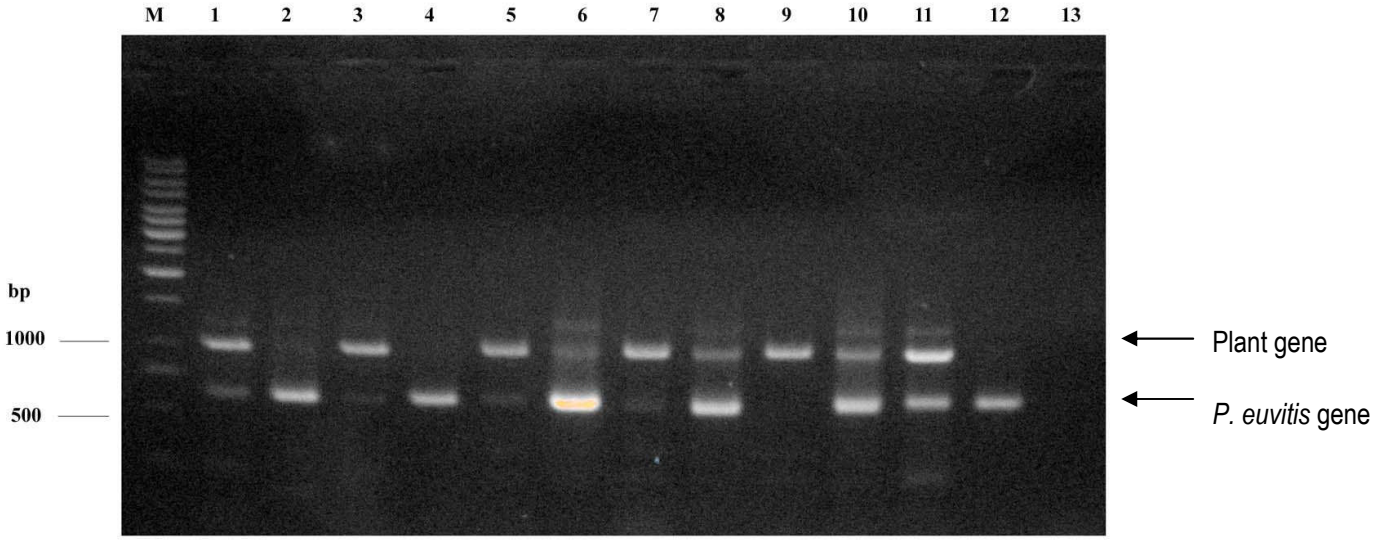


Fig. 14. NL1/NL4 PCR results for *Vitis* spp. leaf samples using MegaRoyal Mix (Micromon). M = 1 Kb Plus DNA ladder; lane 1 S1A; lane 2 S1D; lane 3 S2A; lane 4 S2D; lane 5 S3A; lane 5 S3D; lane 6 S3D; lane 7 S4A; lane 8 S4D; lane 9 S5A; lane 10 S5D; lane 11 S6A; lane 12 *P. euvitis* urediniospores and lane 13 sterile distilled water.

Two bands were observed in most samples (16 symptomatic and 15 non-symptomatic) including samples that were taken from vines with no visible symptoms. Both bands were gel extracted and purified for sequencing upon return to Australia. Bioinformatics confirmed the 560 bp to be *P. euvitis* and the 1 Kb band was the 26S rRNA gene from *Vitis* spp.

In an effort to optimise the PCR test to amplify only *P. euvitis* DNA several PCR parameters were altered. The annealing temperature was increased and the number of cycles was reduced. However, this only resulted in further amplification of non-specific bands. Another Hot Start *Taq* polymerase, Immomix Red (Bioline, Australia), was trialled and this seemed to improve the PCR result. Only one band was observed at 560 bp using NL1/NL4 primers (Fig. 15). However, the same size PCR product was also amplified from a healthy *Vitis* spp. sample collected from the DoR glasshouse. The PCR products from six samples (symptomatic and non-symptomatic), healthy and *P. euvitis* were purified and sequenced in both directions. Bioinformatic analyses of the consensus sequence showed that samples with obvious rust spores and *P. euvitis* were 99.89 % similar to the D1/D2 region (28S rRNA gene) of *P. euvitis* from Australia (GenBank Accession number AB354755) and East Timor (GenBank Accession number AB354757). The sequence from PCR products of non-symptomatic samples indicated a mixed template. BlastN searches showed similarity to the 26S rRNA gene from *Vitis* spp. The PCR product from the healthy sample was 100 % similar to the 26S rRNA gene from *Vitis* spp., thus confirming that *Vitis* spp. DNA was also amplified in the PCR test.

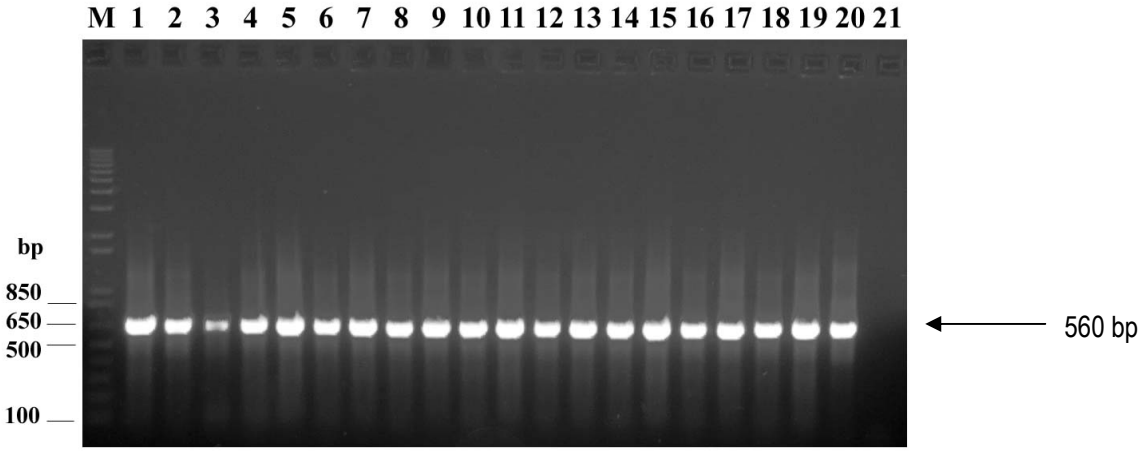


Fig 15. NL1/NL4 PCR using Immomix Red. Lane M 1 Kb Plus DNA ladder; lane 1 S18H; lane 2 S19H; lane 3 S20A; lane 4 S20D; lane 5 S21A; lane 6 S21D; lane 7 S22A; lane 8 S22D; lane 9 S23A; lane 10 S23D; lane 11 S24A; lane 12 S24D; lane 13 S25A; lane 14 S25D; lane 15 S26A; lane 16 S26D; lane 17 S27A; lane 18 S27D; lane 19 healthy *Vitis* spp. DNA (NT); lane 20 *P. euvitis* DNA and lane 21 sterile distilled water.

PCR results using ITS1F/LR15 were not clear cut. In most cases double bands or non-specific bands were present, the exception being DNA from the spores (data not shown). Several attempts were made to increase the stringency of the test, but the same results occurred. This was not unexpected since the use of the ITS region to identify fungal symbionts using universal ITS primers amplifies not only fungal DNA but also that of plants, protists and animals (White et al., 1990; Gardes and Bruns, 1993). In many situations, fungal DNA may be minimal relative to the plant host DNA and preferential amplification of plant DNA usually occurs (Gardes and Bruns 1993). In their study to design taxon-specific ITS primers which preferentially amplify mycorrhizae and rust DNA, Gardes and Bruns (1993) found that the combination of a fungal specific primer (ITS1-F) and a basidiomycete specific primer (ITS4-B) could target basidiomycetes DNA effectively and discriminated against ascomycete DNA. The primer combination produced also amplified plant DNA. In conditions where plant DNA and fungal DNA was present, the test showed that the fungal DNA was preferentially amplified to the exclusion of the plant host DNA. This was not observed in the study described here. In all instances, plant DNA was preferentially amplified and not fungal DNA.

- ***Restriction fragment length polymorphism (RFLP)***

The *Alu* I DNA fingerprint of the D1/D2 region of the 28S ribosomal DNA gene of *P. euvitis* amplified using Immomix Red was initially separated on a 1.5 % agarose gel electrophoresis for 1 hr at 80 V to ascertain the time frame required for complete digestion (Fig 16). The two expected *P. euvitis* bands at ~250 bp and ~ 360 bp (Ono and Imazu, 2001) were observed for symptomatic samples and *P. euvitis* urediniospores. *Alu* I did not cut the product for the healthy sample or the non-symptomatic samples as evident from the 560 bp band. Very faint bands at similar locations to the *P. euvitis* bands were observed for non-symptomatic samples but clearly this was overpowered by the intensity of the uncut 560 bp band.

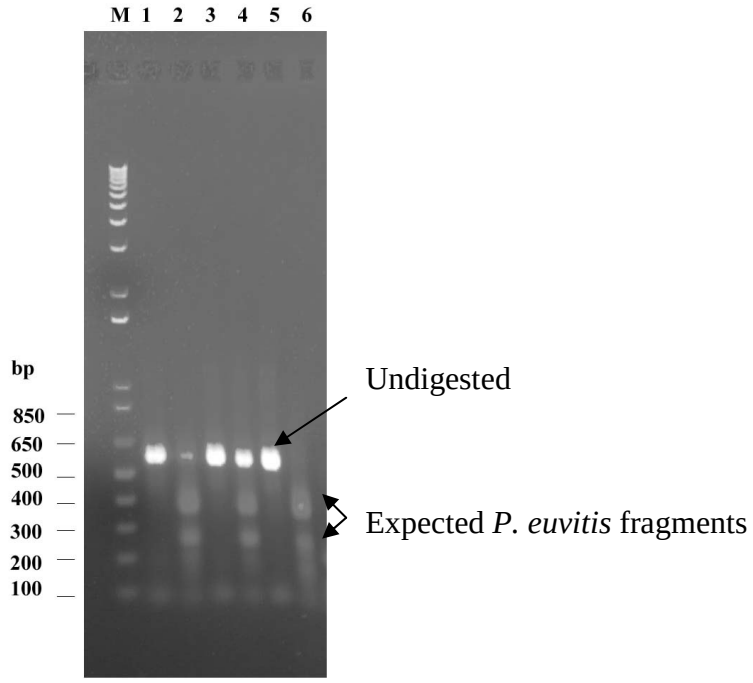


Fig 16. DNA fingerprint of the D1/D2 region of the 28S ribosomal DNA gene of *P. euvitis* extracted from symptomatic and non-symptomatic *Vitis* spp. leaf samples, generated using the restriction enzyme, *Alu* I. Lane M 1 Kb Plus DNA ladder; lane 1 S1A; lane 2 S1D; lane 3 S8A; lane 4 S8D; lane 5 healthy *Vitis* spp. (NT) and lane 6 *P. euvitis*.

When the RFLP was expanded to include more samples and DNA fragments were separated on an 8 % polyacrylamide gel, extra bands were observed even though the same digestion parameters used previously were repeated (Fig 17). This indicated partial digestions. The expected *P. euvitis* bands (indicated by arrows) were clearly evident amongst the many non-specific bands. Once again, these bands were also observed in non-symptomatic samples, albeit extremely faintly. Similar results were observed with samples amplified in Indonesia.

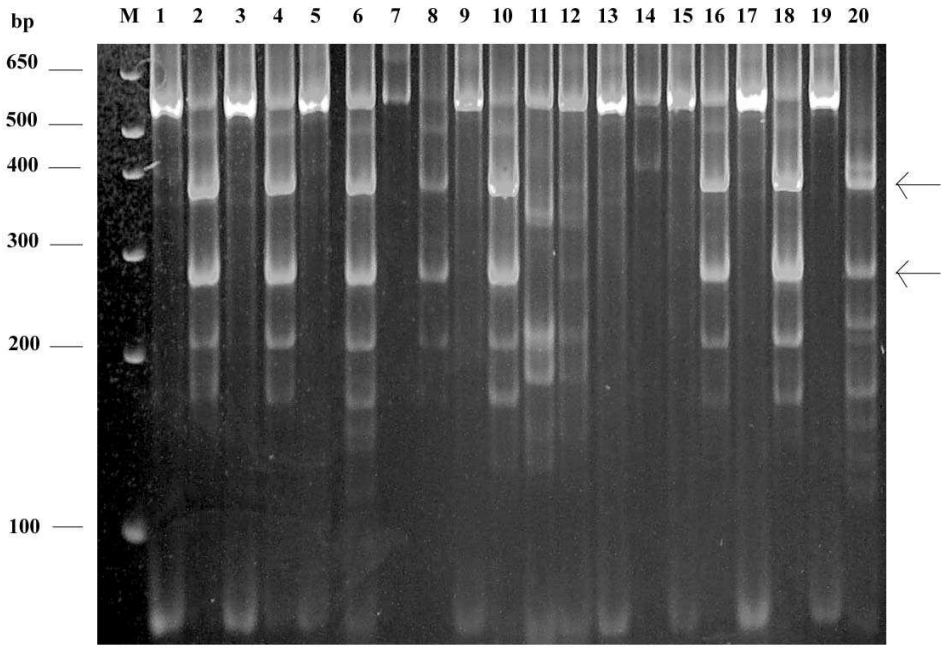


Fig 17. DNA fingerprint of the D1/D2 region of the 28S ribosomal DNA gene of *P. euvitis* extracted from symptomatic and non-symptomatic *Vitis* spp. leaf samples generated using the restriction enzyme, *Alu* I. Lane M 1 Kb Plus DNA ladder; lane 1 S1A; lane 2 S1D; lane 3 S8A; lane 4 S8D; lane 5 S9A; lane 6 S9D; lane 7 S12A; lane 8 S12D; lane 9 S15A; lane 10 S15D; lane 11 S16atyp; lane 12 S16D; lane 13 S17H; lane 14 S19H; lane 15 S22A; lane 16 S22D; lane 17 S23A; lane 18 S23D; lane 19 healthy *Vitis* spp. (NT) and lane 20 *P. euvitis*.

- ***Bioinformatics***

The sequences of the D1/D2 region of the 28S rRNA gene from *P. euvitis* urediniospores from Yogyakarta were aligned with those provided by Chatasiri and Ono (2008). Based upon the multiple sequence alignment, a phylogenetic tree was generated to ascertain if the Yogyakarta strain was in fact similar to that previously identified in Australia (Fig 18). Results indicated that the *P. euvitis* sample from Yogyakarta clusters with reference specimens from Australia and East Timor.

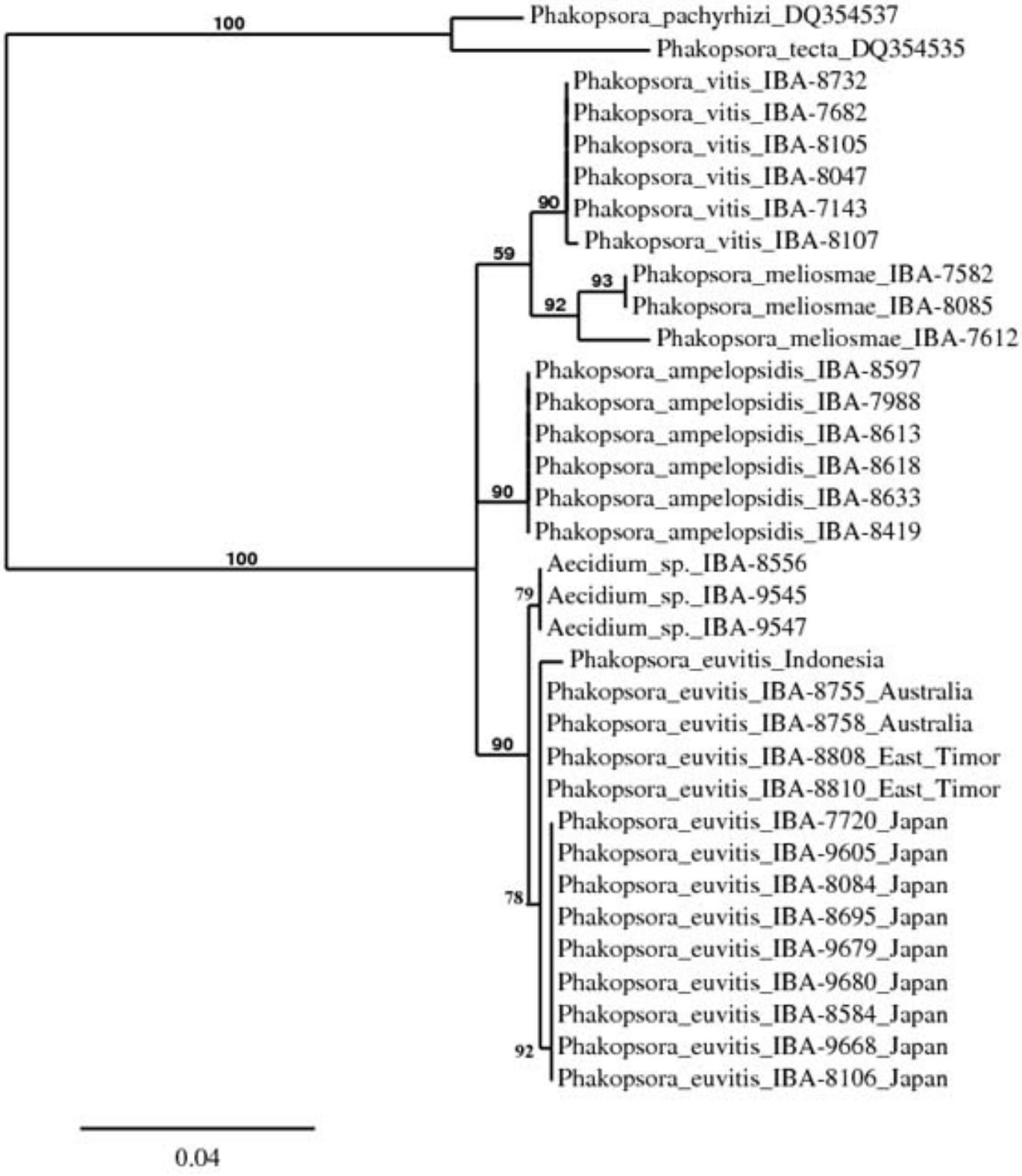


Fig 18. Bootstrapped phylogenetic tree inferred from the 28S rRNA gene of *P. euvitis* (http://www.phylogeny.fr), bootstrap values are indicative of 100 replicated trees.

Outcome and Conclusion

Study 1

In general, urediniospores of *P. euvitis* appear to be very sensitive to environmental conditions once they are detached from diseased leaves. In the studies conducted, solar radiation rapidly and significantly reduced spore viability (GSR and GSR-UV treatments), although some of the effect may be attributed to exposure to unnaturally high temperatures. There were a relatively high proportion of spores able to survive and germinate following exposure under the DSR treatment. However, their ability to infect a grapevine following this exposure was questionable. The observed morphology suggested damage to the DNA, likely to seriously hinder or eliminate their ability to infect. Further studies are necessary to test the pathogenicity of spores following exposure.

There is not enough information to quantify the risk of windborne introduction when other factors outside the scope of this study are taken into consideration. Factors such as grapevine distribution, spore release and escape characteristics, spore volume entering the airflow and the ability of spores to infect leaves after transport are poorly understood or unknown. This is a problem not unique to GLR. Even with a much studied pathogen like *P. pachyrhizi,* Isard *et. al.* (2005) reported that the relationship between weather conditions (temperature, humidity, wind speed and turbulence), crop growth stage, canopy density, the timing of spore release and how these influence escape of the spores from the canopy are critical yet poorly understood components of soybean rust dispersal. The authors state that "development of a comprehensive approach to understand and forecast aerial movements of invasive species is in its infancy. Further progress will require teams of professionals integrating biological knowledge and monitoring techniques with information technologies".

The results of this study suggest a windborne re-introduction of GLR is certainly possible but the risk of this occurring is low. Under normal wind conditions expected during the monsoon season, successful travel of spores would probably rely upon darkness and periods of significant cloud cover during the day. Even if this were the case however, the small number and sparse distribution of the preferred host (*Vitis* spp.) during the monsoon season in northern Australia gives very little opportunity for deposition and successful infection of spores.

Study 2

The current nucleotide sequences available for *Phakopsora* spp. are limited and more genomic information is required to improve current molecular diagnostics. At present, most work on fungi involves DNA extractions from pure cultures or spores. Limited research detailing extractions directly from plant tissue describes preferential amplification of plant DNA while using universal primers. The development of a specific *Phakopsora* probe is required and is an important step to quantify the pathogen DNA in infected *Vitis* spp. samples by using qRT-PCR (Li et al 2008). There are several requirements before a specific probe for *P. euvitis* can be designed, most important of which is identifying the less conserved target areas where the probe and associated primers can be designed. This is difficult using the information currently available. The genomic information of *P. euvitis* needs to be expanded to include other genes aside from conserved high copy rRNA genes. This would allow better choices for specific probe targets to improve current diagnostic tests. Obtaining other *Phakopsora* spp. DNA is vital to ensure the probe is highly specific and does not amplify these other rust species.

Study 3

The current diagnostic test using primers to target the D1/D2 region of the large subunit ribosomal DNA can be utilised to determine the presence of *Phakopsora* DNA in samples. DNA fingerprint and/or sequencing can be conducted to determine the identification of the amplified product. The *P. euvitis* isolate from Indonesia was confirmed by sequencing to be similar to the strains from Australia and East Timor. Our studies using direct tissue extractions indicated that plant DNA is preferentially amplified using either the universal primers for the large subunit ribosomal DNA or the ITS regions. However, it was still possible to determine the presence of *Phakopsora* based upon the DNA fingerprint where distinct bands can be distinguished from non-specific potentially plant DNA. This could be expanded further if other genes could be targeted so host plant DNA does not amplify.

Recommendations

Study 1

- If further studies on the mortality of *P. euvitis* urediniospores are conducted, they need to include bioassays to determine the infectivity of spores following exposure treatments.
- If the HYSPLIT model is to be adapted to forecast dispersion plumes for GLR, this will require significant model development. Detailed phenological studies are required to determine characteristics of *P. euvitis* and *Vitis* spp. that will influence aerial transport. These include spore production (population size), release, escape, transport and atmospheric dilution, infection efficiency and area of host tissue per unit ground area.
- Monitoring of sentinel vines in Darwin for GLR should be most intensive during the December to March period and two weeks after the beginning of monsoon events.

Study 2

- Further research needs to be conducted to expand the genomic knowledge of *P. euvitis* to enable probes to be designed to target more diverse genes.
- Alternatively, a qRT-PCR could be established where relative quantification can be achieved. This study may involve the use of relative qRT-PCR to detect the change in expression of the target (rust DNA) against the exogenous control (host plant DNA). A similar study was conducted for *Fusarium solani* f. sp. *glycines* in soybean roots (Gao et al 2004), where the fungus was detected using a mitochondrial subunit rRNA gene and the host plant DNA was detected using the *cyclophilin* gene.

Study 3

- Further research is required to expand the current knowledge of the genetic makeup of *P. euvitis* as stated in the recommendations for study 2. Once this has been established, a specific gene(s) can be used as the basis to improve the current diagnostic test.
- Further experiments are also recommended to determine the minimum number of *Vitis* spp. leaves and sampling area tested before a result is given. A previous study by Jurick II et al (2007) who compared the diagnostic protocols for Asian soybean Rust (ASR) showed differences in results (false positives) and disagreements between the protocols. This study can be expanded to include time course studies to determine the period from infection to when the fungus could be reliably detected. Methodology used by Lamour *et. al.* (2006) in the early detection of Asian soybean Rust caused by *P. pachyrhizi* could be modified to suit *P. euvitis*. The author inoculated soybean plants with varying concentrations of rust spores to establish different levels of infection. A time course study was conducted over 12 days where plant tissue was sampled at seven points in time. In this instance, both conventional and qRT-PCR assays were used, mainly because qRT-PCR could detect latent infection in plant tissue. Most importantly, they were able to use Asian soybean rust specific primers which did not amplify host plant DNA.

Appendix 1: Communication

1. Preliminary results detailing the detection of *P. euvitis* DNA using PCR was presented at the Australasian Plant Pathology Society Conference in Adelaide 2007. Attendees included international plant pathologists, scientists and researchers.

2. A national diagnostic protocol is currently being compiled detailing identification of *Phakopsora euvitis* using microscopy and molecular tools.

3. The results from this project will be discussed at the 2009 Australasian Plant Pathology Society Conference in Newcastle.

Appendix 2: Intellectual Property

N/A

Appendix 3: References

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Appendix 4: Staff

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Appendix 5: Raw Data

Table 2. Urediniospore germination following exposure to solar radiation and 24 hours incubation. For each treatment/exposure time, 200 spores were counted.
(May 2008)

Exposure	Treatment		
Time (h)	GSR	GSR-UV	DSR
0	93	95	87
2	9	58	78
4	1	21	70
6	0	8	55
8	0	2	63
10	0	0	31
12	0	0	41
14	0	7	29
16	0	0	35

Table 3. Urediniospore germination following exposure to elevated temperatures and 24 hours incubation. For each treatment/exposure time, 100 spores were counted.
(September 2008).

	Temp (°C)			
Time (h)	25	35	40	45
0	50	44	51	46
2	46	44	44	25
4	15	13	6	5
6	31	28	12	6
8	22	18	6	7

Table 4. Urediniospore germination following exposure to elevated temperatures and 24 hours incubation. For each treatment/exposure time, 100 spores were counted.
(December 2008).

	Temp (°C)			
Time (h)	25	35	40	45
0	28	32	36	28
2	40	27	5	1
4	41	16	5	9
6	29	21	5	4
8	18	8	3	4

Alignment of the large subunit ribosomal DNA(derived from sequences from Chatasiri and Ono, 2008).

```
                          1        10        20        30        40        50        60
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  Aecidium sp. IBA-9687   ----actttcagcaatgg----------------------atctcta----ggctctca
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P.ampelopsidis IBA-8419   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
P.ampelopsidis IBA-8597   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
P.ampelopsidis IBA-8613   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
P.ampelopsidis IBA-8618   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
P.ampelopsidis IBA-8633   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-8210   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
  Aecidium sp. IBA-8556   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
  Aecidium sp. IBA-9545   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
  Aecidium sp. IBA-9547   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-8755   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-8758   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-8808   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-8810   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-9605   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-7720   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-8084   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-8106   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-8584   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-8695   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-9668   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-9679   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
     P.euvitis IBA-9680   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
       P.vitis IBA-7143   ggattcccctagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
       P.vitis IBA-7682   ggattcccctagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
       P.vitis IBA-8047   ggattcccctagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
       P.vitis IBA-8105   ggattcccctagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
       P.vitis IBA-8732   ggattcccctagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
       P.vitis IBA-8107   ggattcccctagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
   P.meliosmae IBA-7582   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
   P.meliosmae IBA-8085   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
   P.meliosmae IBA-7612   ggattccccagtaacggcgagtgaagagggaaaagcccaaatttgtaatctggctcttt
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P.ampelopsidis IBA-7988   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
P.ampelopsidis IBA-8419   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
P.ampelopsidis IBA-8597   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
P.ampelopsidis IBA-8613   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
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P.ampelopsidis IBA-8633   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
     P.euvitis IBA-8210   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
  Aecidium sp. IBA-8556   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
  Aecidium sp. IBA-9545   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
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     P.euvitis IBA-8755   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
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     P.euvitis IBA-8810   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
     P.euvitis IBA-9605   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
     P.euvitis IBA-7720   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
     P.euvitis IBA-8084   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
     P.euvitis IBA-8106   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
     P.euvitis IBA-8584   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
     P.euvitis IBA-8695   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
     P.euvitis IBA-9668   tagagtccgagttgtaattttgagaactgttttctgtgctggaccatgtttaagtctgtt
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       P.vitis IBA-7682   tagagtccgagttgtaattttgagaactgttttcagtgctggaccatgtttaagtctgtt
       P.vitis IBA-8047   tagagtccgagttgtaattttgagaactgttttcagtgctggaccatgtttaagtctgtt
       P.vitis IBA-8105   tagagtccgagttgtaattttgagaactgttttcagtgctggaccatgtttaagtctgtt
       P.vitis IBA-8732   tagagtccgagttgtaattttgagaactgttttcagtgctggaccatgtttaagtctgtt
       P.vitis IBA-8107   tagagtccgagttgtaattttgagaactgttttcagtgctggaccatgtttaagtctgtt
   P.meliosmae IBA-7582   tagagtccgagttgtaattttgagaactgttttcagtgctggaccatgtttaagtctgtt
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   P.meliosmae IBA-7612   tagagtccgagttgtaattttgagaactgttttcagtgctggaccatgtttaagtctgtt
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P.ampelopsidis IBA-8419   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
P.ampelopsidis IBA-8597   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
P.ampelopsidis IBA-8613   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
P.ampelopsidis IBA-8618   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
P.ampelopsidis IBA-8633   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
     P.euvitis IBA-8210   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
  Aecidium sp. IBA-8556   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
  Aecidium sp. IBA-9545   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
  Aecidium sp. IBA-9547   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
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     P.euvitis IBA-8758   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
     P.euvitis IBA-8808   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
     P.euvitis IBA-8810   gaaaaacagcatcattgagggtgataatcccgttcatgatatggactaccagtgctctat
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   P.meliosmae IBA-7612   gaaaaacagcatcattgagggtgatagtcccgttcatgatatggactaccagtgcttttat
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   P.meliosmae IBA-7612   atctaaggctaaatataggtgagagaccgatagcaaacaagtaccgtgagggaaagatga
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P.ampelopsidis IBA-8419   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
P.ampelopsidis IBA-8597   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
P.ampelopsidis IBA-8613   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
P.ampelopsidis IBA-8618   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
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  Aecidium sp. IBA-8556   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
  Aecidium sp. IBA-9545   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
  Aecidium sp. IBA-9547   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
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     P.euvitis IBA-8758   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
     P.euvitis IBA-8808   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
     P.euvitis IBA-8810   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
     P.euvitis IBA-9605   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
     P.euvitis IBA-7720   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
     P.euvitis IBA-8084   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
     P.euvitis IBA-8106   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
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     P.euvitis IBA-9680   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
       P.vitis IBA-7143   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
       P.vitis IBA-7682   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
       P.vitis IBA-8047   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
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       P.vitis IBA-8732   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
       P.vitis IBA-8107   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
   P.meliosmae IBA-7582   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
   P.meliosmae IBA-8085   aaagaactttggaaagagagttaacagtacgtgaaattg--ctgaaagggaaacaattga
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P.ampelopsidis IBA-8597   agtt----------agccttgttattgccagttcagcccttctagggtgtattctggtg
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     P.euvitis IBA-8210   agtt----------agccttgttatttccagctcaaccttctagggtgtattctggtg
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P.ampelopsidis IBA-8633   attaacgaccaacatcagtctttgagtgctggataa--gggttttgggaatgtagcagtc
     P.euvitis IBA-8210   attaacgaccaacatcagtctttggtgctggataa--gggttttggaaatgtagcagtc
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