

National Diagnostic Protocol

Pyricularia oryzae
the cause of rice blast



NDP 14 V2

Pyricularia oryzae is now present in Australia. This protocol is being kept as a resource but will not be reviewed or updated.

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National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs although NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

<https://www.ippc.int/core-activities/standards-setting/ispms>

Process

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NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for *Pyricularia oryzae* is current as at the date contained in the version control box below.

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Further information

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1 INTRODUCTION

Rice blast is a disease of cultivated rice (*Oryza sativa*) caused by *Pyricularia oryzae* (*Pyriculariaceae*, *Magnaporthales*). This is generally considered the most important disease of rice worldwide because of its extensive distribution and destructiveness under favourable conditions. Infection of rice plants occurs from airborne conidia and symptoms appear as lesions or spots.

Pyricularia oryzae infects a range of grasses in several genera, including *Eragrostis*, *Panicum*, *Oryza*, *Setaria*, *Stenotaphrum* and *Triticum* (Klaubauf et al. 2014). *Pyricularia oryzae* is now recognised as a distinct species that can be differentiated from *P. grisea* and morphologically similar species by multi-gene DNA sequence analysis (Klaubauf et al. 2014).

1.1 Host range

Although rice (*Oryza sativa*) is the predominant host, *P. oryzae* can survive on and infect many other host plants within *Poaceae* (Appendix 9.1)

2 TAXONOMIC INFORMATION

Phylum: Ascomycota

Class: Sordariomycetes

Order: Magnaporthales

Family: Pyriculariaceae

Species: *Pyricularia oryzae* Cavara 1892

Synonyms: *Magnaporthe oryzae* B.C. Couch 2002

Common names: rice blast disease, rice rotten neck, rice seedling blight, blast of rice

3 DETECTION

3.1 Symptoms

Rice blast can affect most of the rice plant with the exception of the roots. Symptoms can be either lesions or spots: the shape, colour and size vary depending on varietal resistance, environmental conditions and the age of the lesions (Ou 1985).

Pyricularia oryzae infects and produce lesions on the following part of the rice plant: leaf (leaf blast), leaf collar (collar blast), culm, culm nodes, panicle neck node (neck rot) and panicle (panicle blast). The fungus can infect rice plants at any growth stage.

3.1.1 Leaf blast

Initial lesions/spots are white to gray-green with darker borders. Older lesions are white-grey, surrounded with a red-brown margin and are diamond shaped (wide centre and pointed toward either end). Lesion size is commonly 1-1.5 cm long and 0.3-0.5 cm wide (Figure 1). Under favourable conditions, lesions can coalesce and kill the entire leaf.



Figure 1. Leaf blast symptoms (photos: left, D.E Groth, right, R.D. Cartwright, USA).

3.1.2 Collar rot

Lesion is located at the junction of the leaf blade and leaf sheath and can kill the entire leaf (Figure 2)



Figure 2. Collar rot symptoms (photos: D.E. Groth, USA)

3.1.3 Neck rot

Symptoms of neck rot start at the base of the panicle (Figure 3), often leading to necrosis of the entire panicle. Infected panicles are white and unfilled (Figure 4). These symptoms can be confused with stem-borer attack, which also results in white heads.



Figure 3. Neck rot symptoms (photos: left, D.E Groth, right, R.D. Cartwright, USA)



Figure 4. White heads caused by neck rot (photo: R.D Cartwright, USA)

3.1.4 Panicle blast

Panicle appears brown or black (Figure 5).



Figure 5. Panicle blast symptoms (photo: D. E. Groth, USA)

3.1.5 Node infection

Infected nodes appear black-brown and dry and often occur in a banded pattern. This kind of infection often causes the culm to break, resulting with the death of the rice plant (Figure 6).



Figure 6. Node infection symptoms (Photos: left, D.E Groth, right, R.D. Cartwright, USA)

3.2 Sampling

The most common symptoms are likely to be found on the neck nodes and on flag leaf collars. It is recommended to collect samples displaying young, middle stage and mature lesions/symptoms to maximise the chances of a positive identification.

3.3 Other disorders and diseases with symptoms similar to rice blast

3.3.1 Brown spot on leaves caused by the fungus *Cochliobolus miyabeanus*

Young leaf blast symptoms can easily be confused with brown spot symptoms (Figure 10). Brown spot is caused by *Cochliobolus miyabeanus* (Ito & Kuribayashi) Drechs. ex Dastur, anamorph *Bipolaris oryzae* (Breda de Haan) Shoemaker [syns. *Dreschera oryzae* (Breda de Haan) Subramanian & P. C. Jain and *Helminthosporium oryzae*. Breda de Haan].

The conidia produced by *C. miyabeanus* are easy to differentiate from the conidia produced by *P. oryzae*. Conidia usually present a curved shape and possess numerous septa (6 to 14). Their size ranges from 63-153 to 14-22 μm (Figure 11).



Figure 10. Brown spot symptoms (photo: D. E. Groth, USA)

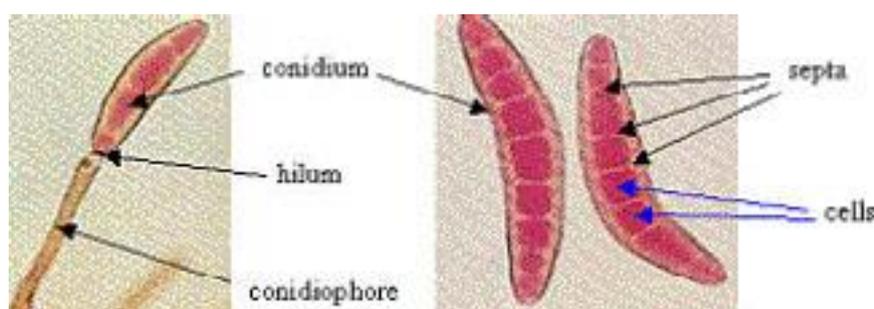


Figure 11. *Cochliobolus miyabeanus* conidia (photo: IRRI)

3.3.2 Sugarcane and maize stem borer

Bathytricha truncata is a native moth that feeds on a wide range of crops, including rice. After hatching, larvae burrow inside the rice stem. White head symptoms occur if the larvae penetrate the stem after the heading stage (Figure 12L). This symptom can be confused with the neck rot symptoms as the infected panicles appear white and are partly or completely unfilled.

The larvae pupate within the tiller and should be, therefore, easily detected (Figure 12R).



Figure 12. (L) White head caused by a stem borer attack of the tiller and (R) Larva (approx. 22 mm long) of the sugarcane and maize stem borer removed from inside a rice tiller (photos: M. Stevens).

3.3.3 *Stem necrosis on panicles caused by the bacteria *Pantoea ananas**

The necrosis/discoloration of the neck region in and below the panicle (Fig.13a) extended from within the rachis down the stem. The lesion is usually darker at the panicle base where the first primary branch of the panicle arose and extended no more than half way up the panicle axis. Stem discoloration can stop near the flag leaf collar or extend well into the sheath to the second node (Fig. 13b). In some stems, the tissue 1-2 mm either side of the top node was lighter in colour with the node itself being almost black. In some severely affected stems, the top node was black with uniformly brown tissue on either side (Fig. 13c).

Another symptom observed was a fine 'mottling' of brown and green tissue above and below the top node, though generally not extending into the stem area covered by the flag leaf sheath. Some flag leaves had a light brown to yellowish lesion on the collar extending 1-2 cm along one side of the leaf blade (Fig. 13c). These lesions rarely extended down the leaf sheath.' Similar symptoms were again observed at Leeton, in 2004.

Pantoea ananas does not seem to affect the number of grains on the panicle and the infected panicles are usually well filled. (Cother *et al.* 2004).



Figure 13. Symptoms on rice in NSW caused by *Pantoea ananas*: (a) necrotic stems (arrow); (b) lightbrown, moist lesions were not observed to extend below the second node; (c) necrotic rice stems showing (i) blackened top nodes, (ii) lesion on flag leaf sheath; (d) area of affected rice, cv. Amaroo at Whitton, NSW prior to harvest; (e) stem lesions on cv. Amaroo. (© NSW DPI)

4 IDENTIFICATION

Initial identification is based upon symptoms on rice plants, followed by the isolation of a fungus with the characteristic conidia of *Pyricularia*. Confirmation of *P. oryzae* identification can only be achieved through the DNA sequence data of three gene loci, or by restriction fragment length polymorphism (RFLP). Figure 7 is a flowchart for responding to a report of rice blast-like symptoms. In addition, an International Seed Testing Association (ISTA) method to detect *P. oryzae* on rice seeds is available (<https://www.seedtest.org/upload/cms/user/7-011.pdf>; accessed 1st October 2013).

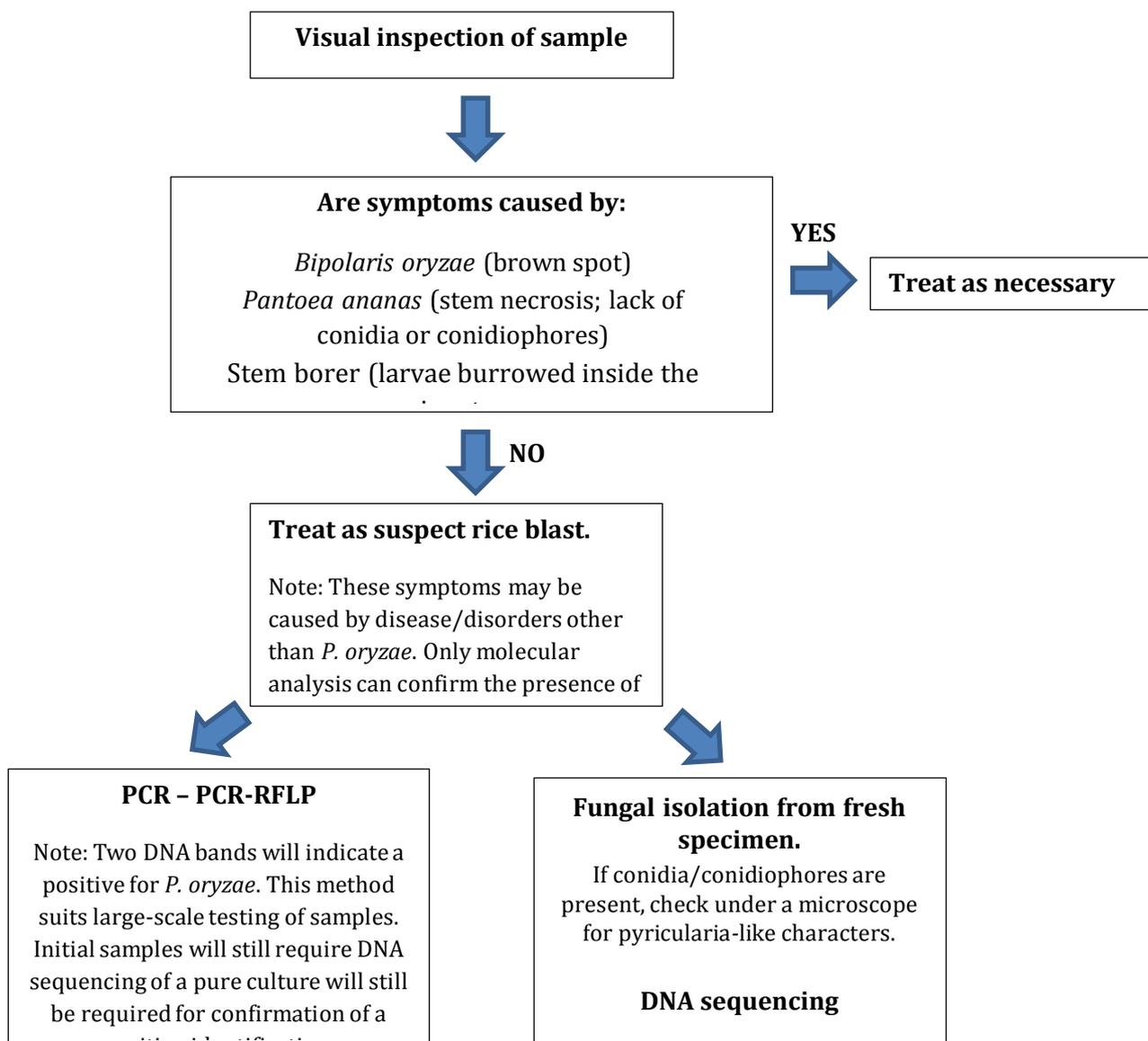


Figure 7. Flowchart for responding to a report of rice blast-like symptoms.

4.1 Morphological methods

4.1.1 *Inducing sporulation on rice tissue*

Samples showing typical symptoms usually need to be placed into a humid chamber to induce sporulation. Greer and Webster (2001) successfully induced sporulation on both panicle neck nodes and leaves by placing the samples on "three pieces of moist filter paper in glass Petri dish and incubated for 48 hours under continuous fluorescent lighting at room temperature".

4.1.2 *Inducing sporulation on media*

Pyricularia oryzae is easily isolated on (full or half strength) Potato Dextrose Agar (PDA). Initial cultures can then be sub-cultured onto PDA. Guochang and Shuyuan (2001) reported that cornmeal, rice straw agar, oatmeal agar and cornmeal agar are all suitable at inducing sporulation (27.8×10^4 , 10.6×10^4 and 9.4×10^4 conidia per mL, respectively). The cultures were grown for 12 days at 28°C under a continuous black light (20 W black light blue fluorescent bulb placed 30 cm above the cultures, wave length 310-420 nm). Bvindi (2010) reported good sporulation using the following protocol. *Pyricularia oryzae* isolates were grown for 15 days on oatmeal agar at 25°C under a 16: 8 hour light:dark cycle, then scratched to stimulated spore (conidia) formation exactly three days before inoculation.

4.1.3 *Identification of the causal agent: Pyricularia oryzae*

The following morphological descriptions are adapted from Subramanian (1968).

Septate conidiophores with up to 20 conidia emerge from the rice stomata. Conidia are pyriform (pear-shaped), and have 2 septa (sometimes 1 or 3 septa). The spores are usually 19-27 x 8-10 µm in size, almost hyaline but sometimes pale olive. Conidia have a characteristic basal appendage (hilum), 1.5-2.5 wide (Figures 8 and 9). Conidia mostly germinate from the apical or basal cells but can also germinate from the median cell. The teleomorphic stage of the fungus has never been found in nature.

Cultures greyish. Conidiophores single or in fascicles, simple, rarely branched, showing sympodial growth. Conidia formed singly at the tip of the conidiophore at points arising sympodially and in succession, pyriform to obclavate, narrowed toward tip, rounded at the base, (1-) 2 (-3)-septate, hyaline to pale olive, (14-) 19-23 (-40) x (6-) 7-9 (-13) µm, with a distinct protruding basal hilum. Chlamydo spores often produced in culture, thick-walled, 5-12 µm diam. (Subramanian 1968).



Figure 8. *Pyricularia oryzae* conidia (photo: C. Wang)

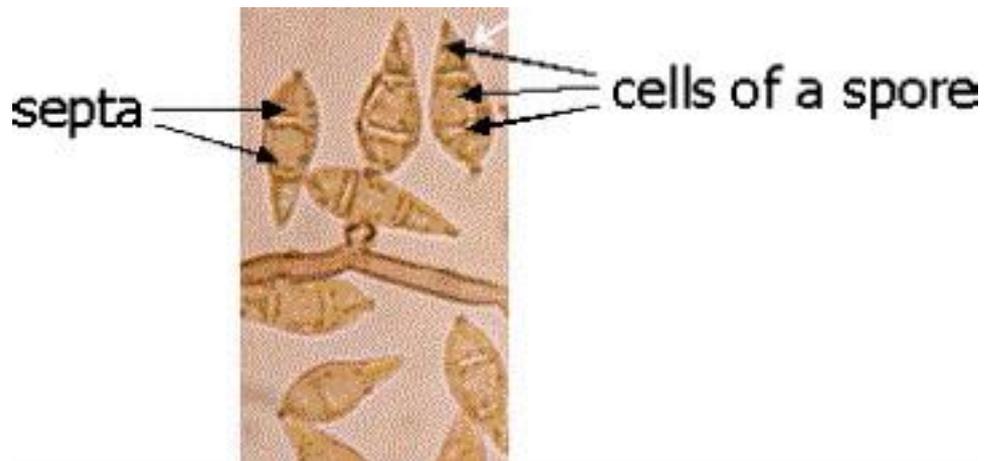


Figure 9. *Pyricularia oryzae* conidia and conidiophore (photo: International Rice Research Institute, Philippines)

4.2 Molecular methods

Laboratory requirements

- Personal protective equipment, i.e. gloves, laboratory coats,
- 2.0, 200, and 1000 μ L sterile barrier pipette tips
- 2.0, 20, 200, and 1000 μ L pipette tips
- Microcentrifuge
- Microcentrifuge tubes 1.5 and 2.0 mL.
- Benchtop vortexer.
- 0.2 mL PCR tubes
- Thermocycler
- Gel tray with suitable comb/s, electrophoresis tank and powerpack
- UV transilluminator
- Camera/gel documentation system

Components

- Puregene Bacteria/Yeast DNA Extraction kit (Qiagen)

Note: Any DNA extraction kit suitable for bacterial/yeast may also be used instead.

- PCR reagents (Table 4)
- Running buffer, 0.5x TBE (Table 9)
- Agarose
- Ethidium bromide (10 mg/mL)
- DNA Ladder
- 6X gel loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 30% v/v glycerol; store at 4°C)

4.2.1 Extraction of DNA from pure cultures using Puregene kit

- 1) Scrape mycelium from agar plate and transfer into 2.0ml Safe-Lock tube (Eppendorf).
- 2) Add approximately 500 μ l of 0.5mm beads (glass or zirconia/silicone; Daintree Scientific) into a 2.0ml Safe-Lock tube.
- 3) Add 300 μ l Cell Suspension Solution and 1.5 μ l Lytic Enzyme.
- 4) Homogenise tissue in a TissueLyser (Qiagen) for 2 mins at 30hz/s.

Note: An equivalent method of homogenisation may be used instead, such as a mortar and pestle with Liquid N₂,

- 5) Incubate at 37°C for 1 hr.
- 6) Spin the samples in a microcentrifuge at maximum speed (~17,000 rpm) for 1 min. discard the supernatant.
- 7) Add 300 μ l Cell Lysis Solution. Vortex for 5 secs.
- 8) Add 100 μ l Protein Precipitation Solution. Vortex for 20 secds.
- 9) Spin the samples in a microcentrifuge at maximum speed (~17,000 rpm) for 1 min.
- 10) Transfer the supernatant to a clean 1.5ml tube.

- 11) Add 1.5µl Rnase A. Vortex to mix thoroughly. Incubate on the bench at room temperature (~22°C) for 15 mins.
 - 12) Spin the samples in a microcentrifuge at maximum speed (~17,000 rpm) for 1 min.
 - 13) Transfer supernatant to a 1.5ml microcentrifuge containing 300µl isopropanol.
 - 14) Invert tube 50 times.
 - 15) Spin the samples in a microcentrifuge at maximum speed (~17,000 rpm) for 3 mins. Discard the supernatant.
- Note: A pale DNA pellet should be visible,**
- 16) Wash pellet with 300µl 70% ethanol. Invert tube 6 times.
 - 17) Spin the samples in a microcentrifuge at maximum speed (~17,000 rpm) for 3 mins. Discard the supernatant.
 - 18) Dry the pellet (either in a vacuum microcentrifuge, or on the benchtop at room temperature).
 - 19) Resuspend DNA pellet in 30µl DNA Hydration Solution. Vortex to mix thoroughly.
- Note: To facilitate DNA resuspension, either incubate at 65µC for 1 hr, or at 37°C overnight.**
- 20) Store at 4°C (or -20°C for long term storage).

4.2.2 Polymerase Chain Reaction (PCR)

DNA Quality Evaluation PCR

- 1) Prepare a reaction mix as described in Table 2 for the number of test samples, one positive control, a blank, and one extra. The positive control is DNA from *M. oryzae* which is available from the Plant Pathology Herbarium (BRIP), Dutton Park, Queensland.

Note: PCRs should be set up at a dedicated workbench away from the area where the DNA was extracted and electrophoresis is done.

- 2) Evaluation of the quality of the DNA, a PCR is conducted using primers for the internal transcribed spacer (ITS) region of the ribosomal RNA (Table 3).

Table 2. Volumes of reagents used in PCR reaction.

Component	Volume (µL)
Nuclease-free H ₂ O	10.5
Phusion Master Mix (HF Buffer)	12.5
Primer 1 (10 µM)	0.5
Primer 2 (10 µM)	0.5
Total	24

Note: PCR can also be conducted using Taq DNA polymerase.

Table 3. Primers sequences used in PCR to evaluate the quality of DNA extracted (White et al. 1990).

Primer	Sequence
ITS1	5'-TCCGTAGGTGAACCTGCGG-3'
ITS4	5'-TCCTCCGCTTATTGATATGC-3'

3) Add 1.0µl DNA template and place in a thermocycler using the following program:

Cycle 1	step 1	98°C	30 secs
Cycles 2 to 29	step 1	98°C	10 secs
	step 2	55°C	30 secs
	step 3	72°C	30 secs
Cycle 30	step 1	72°C	5 min
	step 2	10°C	10 min

Note: If using Taq DNA polymerase, follow the manufacturer's guide for thermocycling conditions.

4) Load 5µl PCR product with 1µl 6x loading dye on a 1.5% TBE gel at 80V for 60 mins (see Electrophoresis Procedure).

Samples should produce a PCR product of approximately 600bp in size, corresponding to the length of the ITS region.

4.2.3 Sequencing the actin, beta-tubulin and calmodulin gene regions for identification

Identification of the isolate is based upon the DNA sequences of the partial region of actin, beta- tubulin and calmodulin loci.

1) Prepare a PCR as described in Table 2 using the primer pairs in Table 4.

Table 4. Primers used to amplify each of the loci for DNA sequence-based identification (Couch and Kohn 2002).

Locus	Primer	Sequence
Actin	ACT-512F	5'-ATGTGCAAGGCCGGTTTCGC-3'
	ACT-783R	5'-TACGAGTGCCTTCTGGCCCAT-3'
Beta-tubulin	Bt1a	5'-TTCCCCCGTCTCCACTTCTTCATG-3'
	Bt1b	5'-GACGAGATCGTTCATGTTGAACTC-3'
Calmodulin	CAL-228F	5'-GAGTTCAAGGAGGCCTTCTCCC-3'
	CAL-737R	5'-CATCTTTCTGGCCATCATGG-3'

2) Add 1.0µl DNA template and place in a thermocycler using the following program:

Cycle 1	step 1	98°C	30 secs
Cycles 2 to 29	step 1	98°C	10 secs
	step 2	60°C	30 secs
	step 3	72°C	30 secs
Cycle 30	step 1	72°C	5 min
	step 2	10°C	10 min

3) Check that the PCR has worked by running 3µl of the PCR product in a TBE gel. Expected sizes of PCR: actin, 320bp; beta-tubulin, 550bp; and calmodulin, 500bp.

4) Purify the PCR products using the QIAquick PCR Purification Kit (Qiagen) (or another equivalent kit) according to the manufacturer's instructions.

5) The purified PCR products can either be prepared for sequencing with ABI Big Dye (Life Technologies) according to the manufacturer's instructions, or it can be directly submitted to a DNA sequencing facility (e.g. Australian Genome Research Facility or Macrogen Inc).

6) The resultant DNA sequences are compared against the reference sequences listed in Table 5. Similarities of 99-100% are considered to be a match.

Table 5. Actin, beta-tubulin, and calmodulin loci reference sequences for comparison (Couch and Kohn 2002).

	GenBank accession numbers		
	Actin	Beta-tubulin	Calmodulin
<i>Pyricularia grisea</i> (<i>syn. Magnaporthe grisea</i>)	AF395951	AF395997	AF396008
<i>Pyricularia oryzae</i> (<i>syn. Magnaporthe oryzae</i>)	AF395965	AF395984	AF396024

4.2.4 Electrophoresis Procedure

- 1) Determine the size of the gel and gel comb required (see Table 6 for example).
- 2) Place gel tray with appropriate comb(s) in the tray holding.
- 3) Weigh agarose (either 1.0 g or 1.5 g/100 mL of buffer) and place into suitable heat proof container.
- 4) Add the required amount of gel running buffer (0.5X TBE).
- 5) Heat until the agarose is completely dissolved, then allow to cool to approximately 50-55°C.
- 6) Add the required amount of ethidium bromide (final concentration 0.5 µg/mL) to agarose solution and mix gently.

Safety Note: Ethidium bromide is a carcinogen; always wear protective nitrile gloves and wash hands thoroughly afterwards. An alternative, such as GelRed ® can also be used instead of ethidium bromide. Ethidium bromide can either be added directly into the gel, or the gel can be stained with an ethidium bromide solution after it has been run.

- 1) Pour agarose into gel tray and allow to set.
- 2) Place the set gel in an electrophoresis tank and submerge with 0.5X TBE buffer to a depth of at least 1 mm above the gel surface.
- 3) Load 5 µL of DNA Ladder into the first well of the gel.
- 4) Add 1 µL of 6X gel loading dye per 5 µL of PCR product and load into the individual wells of the gel.
- 5) Connect the electrodes to a powerpack and apply a constant voltage of 130 Volts. Run the gel for a period of time suitable for the size of selected gel.
- 6) View the gel on a UV transilluminator, and record the image using a camera or a gel documentation system.

Table 6. Volume of gel solution required for specific gel tray sizes.

Gel tray size (cm)	Volume (mL) of gel solution (for 0.7 cm thickness)
7.7 x 9.5	50
14.5 x 15.0	150
18.3 x 25.0	300

Table 7. Components of TBE (Tris/borate/EDTA) electrophoresis buffer.

Component	10X stock solution (1 L)	5X stock solution (1 L)
Tris base	108 g	54 g
Boric acid	55 g	27.5 g
0.5M EDTA pH 8.0	40 mL	20 mL

For TBE, a working solution of 0.5X provides sufficient buffering power, and almost all agarose gel electrophoresis is carried out using this buffer. Alternative buffers, such as TAE (Tris, acetate, EDTA) and Lithium Borate (LB), may also be used. Stock and working solutions are stored at room temperature.

4.2.5 PCR Restriction Fragment Length Polymorphisms (RFLP)

- 1) Undertake DNA extractions (as per 4.2.1) on fresh or dried infected leaves.

Note: *Minimise the amount of uninfected leaf tissues by excising the lesions for DNA extractions*

- 2) Set up a PCR as per Table 2, using the primers for beta-tubulin gene.

Note: DNA template will need to be diluted 1 in 10 or 1 in 100.

- 3) Run the PCR using the thermocycling conditions in section 4.2.2.
- 4) Check that the PCR has worked by running 5µl onto a 1% TBE gel.
- 5) Purify the remaining PCR products, and elute in 20µl ddH₂O.
- 6) Prepare restriction enzyme digestion as per Table 8.

Table 8. *Hpa* II digestion of beta-tubulin PCR products.

Reagents	Volume (µL)
Purified PCR product	5
Buffer (10X)	3
<i>Hpa</i> II enzyme	0.5
ddH ₂ O	21.5
Total	30

- 7) Incubate at 37°C for 3 hours.
- 8) Run 15µl of the digested, and 5µl undigested PCR product on 1.5% TBE gel, at 80V for 60 mins.

Hpa II digestion of beta-tubulin PCR products from *P. oryzae* yields two DNA fragments, one at 188bp and the other at 362bp. Beta-tubulin PCR product from *P. grisea* and all other potential fungal pathogens do not have an *Hpa* II restriction site, and therefore remain undigested.

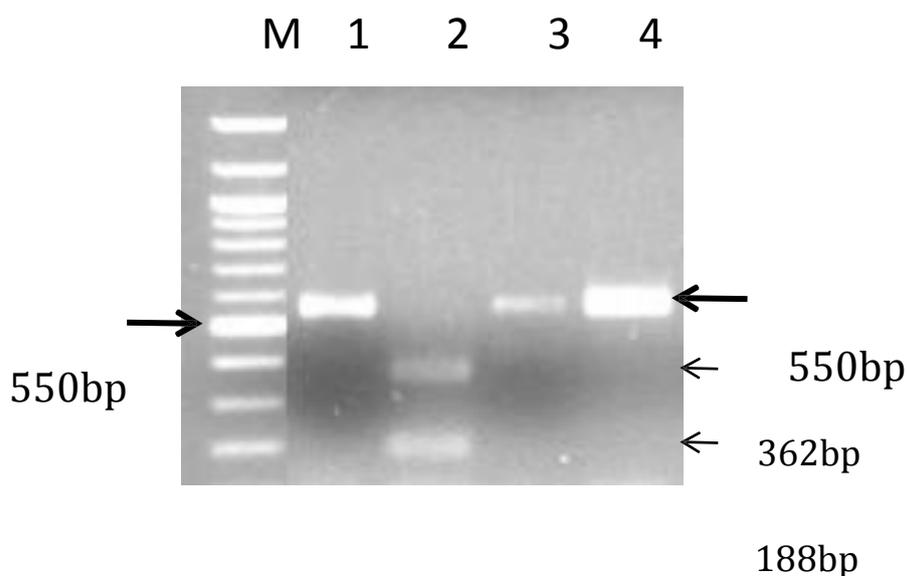


Figure 14. PCR-RFLP of the beta-tubulin gene. Marker (lane M); *P. oryzae* undigested beta-tubulin PCR (lane 1), and digested with *Hpa* II (lane 2); *P. grisea* undigested beta-tubulin PCR (lane 3), and digested with *Hpa* II (lane 4).

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8 APPENDICES (OPTIONAL)

8.1 Host table

Table 1. A non-exhaustive list of *P. oryzae* hosts.

<i>Anthoxanthium</i> sp.	<i>Eleusines indica</i>	<i>Panicum miliaceum</i>
<i>Avena sativa</i>	<i>Eleusines lehmanniana</i>	<i>Panicum repens</i>
<i>Brachiaria distachya</i>	<i>Eragrostis curvula</i>	<i>Panicum</i> sp.
<i>Brachiaria mutica</i>	<i>Festuca arundinacea</i>	<i>Phalaris</i> sp.
<i>Brachiaria plantaginea</i>	<i>Festuca</i> sp.	<i>Phyllostachys</i> sp.
<i>Brachiaria</i> sp.	<i>Hordeum vulgare</i>	<i>Rottboellia compressa</i>
<i>Ctenanthe appenheimiana</i>	<i>Lolium multiflorum</i>	<i>Rottboellia</i> sp.
<i>Ctenanthe setosa</i>	<i>Lolium perenne</i>	<i>Sasaella</i> sp.
<i>Digitaria adscendens</i>	<i>Lolium</i> sp.	<i>Setaria italica</i>
<i>Digitaria sanguinalis</i>	<i>Melinis minutiflora</i>	<i>Setaria viridis</i>
<i>Echinochloa colonum</i>	<i>Molinia japonica</i>	<i>Setaria</i> sp.
<i>Echinochloa crus-galli</i>	<i>Oryza australiensis</i>	<i>Stenotaphrum secundatum</i>
<i>Echinochloa</i> sp.	<i>Oryza rufipogon</i>	<i>Triticum aestivum</i>
<i>Eleusines africana</i>	<i>Oryza sativa</i>	<i>Zea mays</i>
<i>Eleusines boranensis</i>	<i>Oryza</i> sp.	<i>Zea</i> sp.
<i>Eleusines coracana</i>	<i>Panicum bisulcatum</i>	

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