



Verification of conventional pathotype screening for Australian isolates of *Cochliobolus sativus* using molecular fingerprinting.

Merrill Fordyce¹ and Stuart Meldrum^{1,2}

¹ Agency for Food and Fibre Sciences, QDPI, Hermitage Research Station, M/S 508, Warwick QLD 4370

² QFRI, Meiers Road, Indooroopilly QLD 4370

Abstract

The fungus *Cochliobolus sativus* (Ito & Kurib.) Drechs. ex Dastur (anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoemaker) is a common foliar disease of barley in northern New South Wales and Queensland. Physiological specialisation of *C. sativus* has been documented in Australia with six pathotypes recorded using coded triplet nomenclature (Meldrum et al 2000).

The aim of the work was to confirm the genetic variation identified from the Australian pathotype survey (Meldrum et al 2000) using DNA fingerprinting. Isolates representative of each of the six pathotypes of *C. sativus* identified in the survey were selected for the molecular study.

A DNA extraction protocol for *C. sativus* was obtained from the USA (Shaobin Zhong unpublished) and optimised prior to the commencement of molecular fingerprinting. RAPDs were used due to their low cost and their high levels of polymorphism. This paper reports both the refinements made to the DNA extraction protocol and the results from the RAPDs.

The molecular fingerprint patterns revealed similar differences to those identified in the pathotype survey for each of the six isolates. The good correlation between the DNA fingerprint patterns and the traditional method of pathotype classification indicates that molecular tools could be used to characterise Australian isolates of various pathogens other than *C. sativus*.

Introduction

In order to document the pathotype diversity in the Australian spot blotch population, a pathotype survey was conducted using thirty-four isolates of *C. sativus* and twenty differential lines (Meldrum et al 2000). Infection responses were evaluated and using coded triplet nomenclature (Limpert and Müller 1994), six distinct pathotypes were

identified. A cluster analysis of the data based on the resistant/ susceptible classification separated these pathotypes into four groupings.

For this study six isolates of *C.sativus* that were representative of the six identified pathotypes within the Australian population (Meldrum et al 2000) were selected from the Hermitage Research Station collection (Table 1). Three of the isolates selected came from the same grouping.

Table 1 Details of the *C sativus* isolates selected for molecular fingerprinting

| Isolate No. | Access. No. | Origin | State | Host | Source | Pt. No. | Group No. | DNA code |
|-------------|-------------|----------|-------|--------|--------|---------|-----------|----------|
| 11 | 98153 | Nth Star | NSW | Barley | Leaf | 177 | 1 | B |
| 17 | SB22 | Redlands | Qld | Barley | Leaf | 737 | 1 | C |
| 1 | 98042 | Monto | Qld | Barley | Leaf | 377 | 1 | E |
| 3 | 98024 | Kyogle | NSW | Barley | Leaf | 3 | 4 | D |
| 8 | 98137 | Cobbitty | NSW | Barley | Leaf | 404 | 3 | F |
| 29 | 99074 | Biloela | Qld | Rye | Leaf | 0 | 2 | A |

Materials and Methods

A DNA extraction protocol for *C. sativus* using fungal mycelium was obtained from Shaobin Zhong, a PhD student under the supervision of Professor Brain Steffenson when at the University of North Dakota, USA.

Conidia harvested from 10 to 12-day-old cultures were inoculated into a 500 mL flask containing 100 mL of liquid complete medium (Yoder 1988) with a final concentration of approximately 10⁶ conidia/mL.

After incubating at 28°C on a rotary shaker at 150 rpm for 24 hours, the resulting mycelium was harvested by pouring the solution through four layers of cheesecloth and then thoroughly washed with sterile distilled water. The mycelium was blotted dry on paper towels.

The mycelium was ground in liquid nitrogen with a mortar and pestle to a fine powder.

7 mL of DNA isolation buffer (50mM Tris-HCl at pH 8.0, 150 mM EDTA, 1% sarkosyl, and 300 µg proteinase K/ml) was added to the ground mycelium and stirred with a glass bar to homogenise the mixture.

After incubating at 65°C for 30 minutes, an equal amount of chloroform was added and the samples were shaken on a rotary shaker for 30 minutes.

Samples were centrifuged at 10,000 X g for 10 minutes at room temperature.

The aqueous phase containing DNA was transferred to a new 50ml centrifuge tube.

DNA was precipitated by adding an equal volume of isopropanol.

The precipitate was collected by centrifugation at 5,000 X g for 5 minutes then rinsed with 70% ethanol, and air-dried. The DNA pellet was resuspended in 500-1000 μ L of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA) or sterile distilled water.

Optimisation of the DNA extraction method for *C. sativus* formed a substantial part of this study and once achieved the DNA from each isolate was used to run RAPDs. This was to determine whether the differences between isolates identified from the pathotype survey could be distinguished using molecular fingerprinting techniques.

Results and Discussion

The DNA protocol used was modified in a number of ways to improve the yield and quality of the DNA extracted. It was decided that conidia would be easier to produce than mycelium and that an RNase treatment and two phenol chloroform steps should be reinstated as in Yoder (1988). A range of weights of conidia were tested from 0.1g to 2g to assess DNA yields. It was decided that 0.5g of conidia would give sufficient DNA yields for fingerprinting. Such a weight required 5 petri-dishes of *C.sativus* to be cultured per isolate.

The quality of the DNA was variable and a DNA purification step was deemed necessary to reduce the amount of degraded DNA in each sample. A gel purification kit (the Gibco BRL[®] Concert Gel Extraction System) was used once the DNA sample was cut from the agarose gel. Once all DNA samples were of equal volume and uniform quality RAPDs were conducted.

The six DNA samples were screened with a number of RAPD Operon primers. Molecular results confirmed those of the survey in revealing that pathotypes A, D and F (each representing a different grouping) were all different from one another. Pathotypes B and C, both from grouping 1, showed similar banding patterns as expected. However the third isolate chosen from grouping 1, designated E was different to B and C in the case of primer P1 (Figure 1). Further testing will be carried out to confirm these discrepancies.

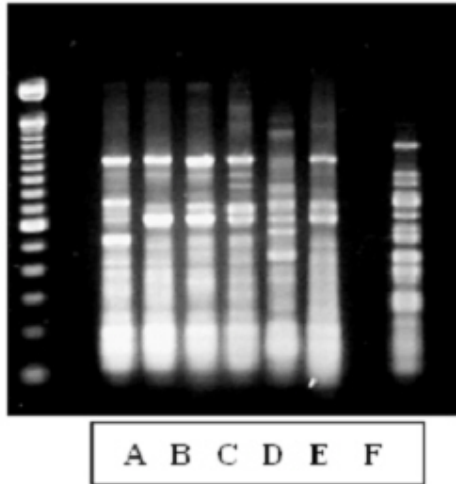


Figure 1 RAPD fingerprint for the six *C.sativus* isolates using Operon primer OP-P1

Conclusions

Determination of the accuracy of differential pathotyping versus molecular pathotyping has only been carried out in this study with a small subset of lines. It would be beneficial to further fingerprint the remaining 28 isolates used in the original pathotype survey to confirm correlations between the 2 methods. Currently in the USA many disease nurseries are screened in molecular marker laboratories as opposed to field and glasshouse screening (pers. comm Steffenson 2000). If the accuracy and cost is reasonable there is no reason why similar screening of spot blotch of barley in Australia cannot be carried out in this way.

Acknowledgments

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