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www.publish.csiro.au/journals/app *Australasian Plant Pathology*, 2004, **33**, 109–114

# Pathotypes of *Cochliobolus sativus* on barley in Australia

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*Abstract*. Pathotypes of *Cochliobolus sativus* have been reported overseas but variation in the Australian population has not been investigated nor have the optimum conditions for the identification of variation been established. Preliminary experiments showed that maximum separation of infection responses of seedlings to infection by *C. sativus* conidia was obtained at 21°C using inocula containing $10^4$ conidia/mL and a dew period of 40 h. Under these conditions, the existence of pathotypes in the Australian population of *C. sativus* was demonstrated. Using 20 lines, six pathotypes were identified among 34 isolates of the fungus. A differential set consisting of 12 barley cultivars is proposed.

## Introduction

*Cochliobolus sativus* (anamorph *Bipolaris sorokiniana*) causes a common and sometimes severe foliar disease called spot blotch on barley in northern New South Wales and Queensland. It also causes common root rot, seedling blight and head blight of barley. In addition to barley, *C. sativus* attacks many grass species including the cereals, wheat, rye and triticale.

Diseases such as those caused by *C. sativus* are commonly managed by growing resistant cultivars. However, the development of resistant cultivars is complicated if the pathogen population contains members (pathotypes) differing in virulence gene composition. Pathotypes of the fungus have been identified in a number of countries (Valjavec-Gratian and Steffenson 1997; Zhong and Steffenson 2001) using three differential lines. However, there is no documented evidence of the occurrence of pathotypes in the Australian population of *C. sativus*.

The experiments reported in this paper were designed, firstly, to establish optimal conditions for separation of seedling host responses to facilitate pathotype detection. Once the optimal duration of dew period and spore concentration had been established, 34 Australian isolates were tested for evidence of the existence of pathotypes. Seventeen barley cultivars with varying reactions to *C. sativus* as well as resistant cultivars of wheat, rye and triticale were included in the initial set of differential cultivars.

## Methods

### *Establishing optimum conditions*

*Fungal isolate, culturing, inoculum preparation, inoculation and incubation.* Isolate WI 1902 of *C. sativus* from the Queensland Department of Primary Industries Hermitage Research Station Fungal Collection was used to determine the optimum conditions for separation of host responses. WI 1902, collected in the Mary Valley, Queensland, in 1984, grows well in culture and has given consistent and reproducible results in trials screening for spot blotch resistance.

A single-conidium culture was grown on starch-nitrate agar (SNA, Dodman and Reinke 1982) in the dark at 25°C for 14 days. After this time, mycelium from a single agar plug placed in the centre of a 90 mm Petri dish covered 90% of the plate and sporulated profusely. Conidia were harvested by pipetting 5 mL of distilled water containing 3 μL/L of polyoxyethylene sorbitan monolaureate (Tween 20) wetting agent into each plate and brushing the agar surface with a small artist's brush. The resulting suspension was poured from the plate, which was then washed with distilled water to remove remaining conidia. The suspension was passed through a 400 μm filter to remove the larger fragments of mycelium and agar and placed on a magnetic stirrer to ensure that conidia remained in suspension. The conidial concentration of the suspension was determined using a haemocytometer slide and averaging 18 counts. This suspension was diluted with distilled water to obtain the desired concentration. Five concentrations (5000, 10000, 20000, 40000 and 80000 conidia/mL) were used.

Plants were inoculated with the conidial suspension using a Paasche artist's airbrush connected to an air compressor at 185 kPa. The suspension was sprayed from ~40 cm and aimed directly at the leaves. Plants were inoculated from four sides giving an even and thorough coverage of inoculum onto the leaves.

After inoculation, plants were placed in dew chambers housed in a controlled environment where temperature was maintained at 21°C. A 12 h photoperiod (0.24 μmol/s/$m^2$) was provided by high pressure

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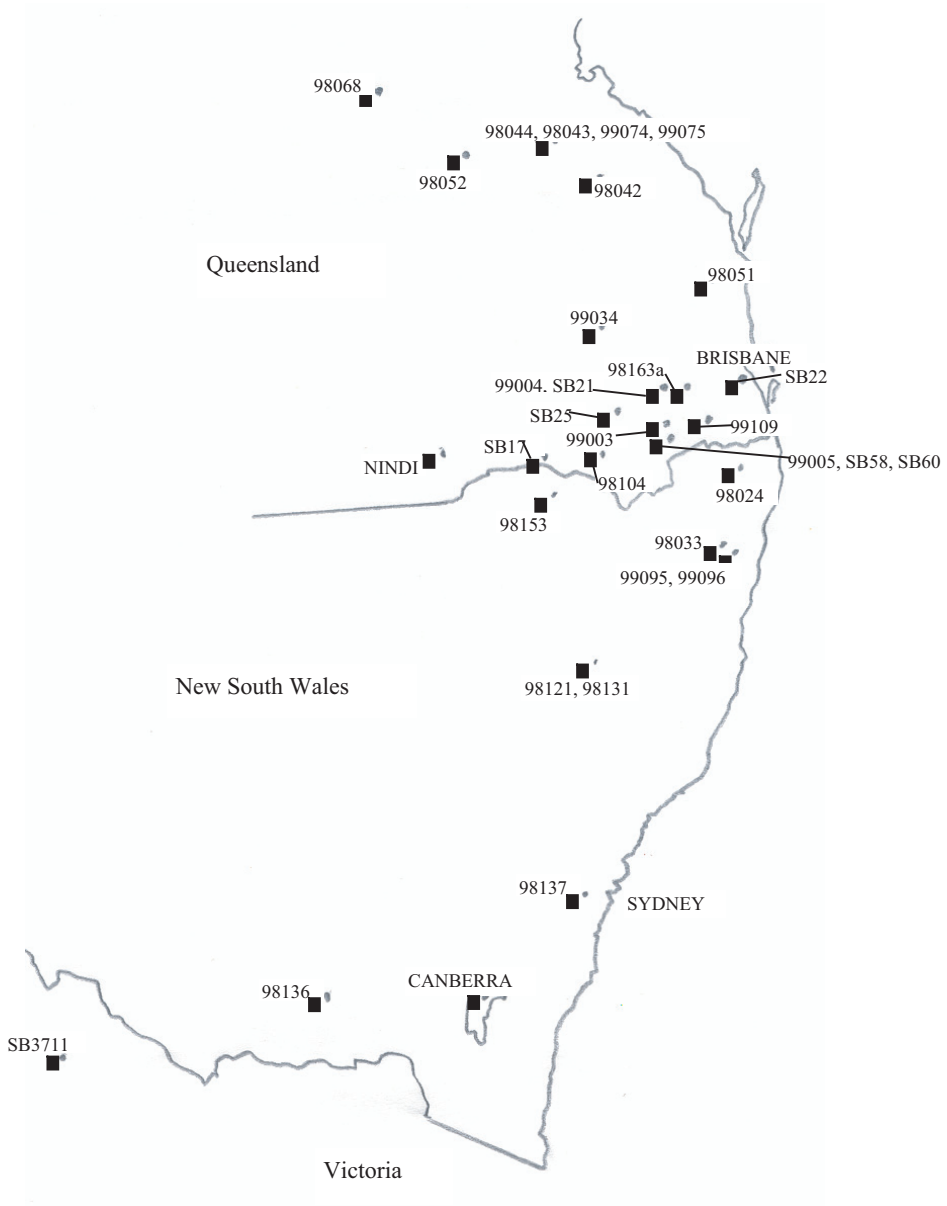


**Fig. 1.** Sources of isolates used in this study (except 99108 from Western Australia).

sodium vapour lamps. Plants were removed from the dew chambers 6, 12, 24, 36, 48 or 72 h after inoculation.

*Plant material and cultivation.* Five barley cultivars and one wheat cultivar were selected for this study because they exhibited a range of infection responses to *C. sativus* in previous studies. The barley lines were Larker (resistant), Klaxon/Tallon-34 (moderately resistant), Gilbert (moderately resistant to moderately susceptible), Klaxon/Tallon-45 (moderately susceptible) and Skiff (susceptible). The wheat cultivar was Hartog (resistant).

Plants were grown in 100-mm-diameter pots containing a soil mix consisting of loam, peat and vermiculite (2:1:1 by volume) and 2.5 g/L of GF306N basal fertiliser (GrowForce Australia). The pH was adjusted to 6.5 using 7.5 g of agricultural lime/L. Eight to ten seeds of two cultivars were sown at opposite sides of each pot. Each genotype was replicated three times with the pots randomised within each replicate. Pots were maintained in a glasshouse with a temperature range of 13–26°C, watered daily and fertilised with Aquasol at the recommended rate 4 and 10 days after seedling emergence. Plants were inoculated 12 days after sowing (growth stage 13, Zadoks *et al*. 1974). Following inoculation and incubation in the dew chambers, plants were returned to the glasshouse, watered daily and fertilised with Aquasol 4 and 10 days later.

*Disease assessment and data analysis.* The response to infection of the second leaf of each line was recorded 12 days after inoculation (growth stage 15) using a numerical infection scale of 1–9 with 1 being very resistant and 9 very susceptible (Fetch and Steffenson 1999).

Analysis of variance was used to determine the relationship between conidial concentration and dew period for each line and lines of best fit were plotted using an exponential relationship.

**Table 1. Isolates of *Cochliobolus sativus* used for pathotype testing**

| Accession no.[A] | Origin | Host | Source | Pathotype | Group[B] |
|---|---|---|---|---|---|
| 98033 | Copmanhurst, NSW | Barley | Leaf | 177 | 1 |
| 98068 | Gindie, Qld | Barley | Leaf | 177 | 1 |
| 98153 | North Star, NSW | Barley | Leaf | 177 | 1 |
| 98163a | Gatton, Qld | Barley | Leaf | 177 | 1 |
| SB60 | Hermitage, Qld | Barley | Seed | 377 | 1 |
| 98042 | Monto, Qld | Barley | Leaf | 377 | 1 |
| 98043 | Biloela, Qld | Barley | Leaf | 377 | 1 |
| 98051 | Logan Point, Qld | Barley | Leaf | 377 | 1 |
| 98052 | Bauhinia Downs, Qld | Barley | Leaf | 377 | 1 |
| 98121 | Tamworth, NSW | Barley | Leaf | 377 | 1 |
| 98136 | Lockhart, NSW | Barley grass[C] | Leaf | 377 | 1 |
| 99003 | Allora, Qld | Barley | Leaf | 377 | 1 |
| 99004 | Grainco seed lot, Qld | Wheat | Seed | 377 | 1 |
| 99034 | Jandowae, Qld | Barley | Leaf | 377 | 1 |
| 99075 | Biloela, Qld | Triticale | Leaf | 377 | 1 |
| 99095 | Grafton, NSW | Triticale | Leaf | 377 | 1 |
| 99096 | Grafton, NSW | Wheat | Leaf | 377 | 1 |
| 99108 | WA | Barley | Seed | 377 | 1 |
| 99109 | Aratula, Qld | Barley | Leaf | 377 | 1 |
| SB22 | Redlands, Qld | Barley | Leaf | 737 | 1 |
| 98044 | Biloela, Qld | Wheat | Leaf | 000 | 2 |
| 98104 | Inglewood, Qld | Barley grass | Leaf | 000 | 2 |
| 98131 | Tamworth, NSW | Barley grass | Leaf | 000 | 2 |
| 99005 | Hermitage, Qld | Barley | Stubble | 000 | 2 |
| 99074 | Biloela, Qld | Rye | Leaf | 000 | 2 |
| NINDI | Nindigully, Qld | Barley | Roots | 000 | 2 |
| RED | Not recorded | Barley | Roots | 000 | 2 |
| SB17 | Billa Billa, Qld | Wheat, barley | Stubble | 000 | 2 |
| SB21 | Toowoomba, Qld | Wheat | Seed | 000 | 2 |
| SB25 | Millmerran, Qld | Barley | Roots | 000 | 2 |
| SB3711 | Woomelong, Vic | Barley | Seed | 000 | 2 |
| SB58 | Hermitage, Qld | Barley | Leaf | 000 | 2 |
| 98137 | Cobbitty, NSW | Barley | Leaf | 404 | 3 |
| 98024 | Kyogle, NSW | Barley | Leaf | 003 | 4 |

[A]Department of Primary Industries Queensland Hermitage Research Station, Warwick, Accession Numbers.
[B]Groups were determined by cluster analysis of infection response data.
[C]*Critesion murinum.*

*Pathotype testing*

*Fungal isolates, culturing, storage, inoculum preparation, inoculation and incubation.* Thirty-four isolates of *C. sativus* from a wide geographical range were used in this study (Fig. 1, Table 1). Isolates were cultured from various hosts, plant parts and also from host stubble. All were collected between 1995 and 1999 except for isolate SB17, which was collected in 1985. The identity of a selection of isolates as *B. sorokinianum* was confirmed by Dr R. Shivas, Department of Primary Industries, Queensland.

To obtain the isolates, infected source material was placed on moist filter paper in a Petri dish and incubated at 21°C with a 12 h photoperiod provided by two black light tubes and one white light fluorescent tube from a distance of 20 cm. After 2 days, conidia had formed. Several single conidia were transferred to V8 agar (Waller *et al*. 2002) and one of the resulting colonies was then sub-cultured to SNA. Conidia were harvested and filtered as previously described. The suspension was vacuum filtered through a Millipore 8 μm filter until moisture was no longer observed. Conidia were then scraped from the filter membrane into vials using a sterilised scalpel. The vials were placed in a desiccator for up to 3 days and conidia dried to ~15% moisture before storage in a refrigerator at 4°C until needed.

Inoculum was prepared by mixing the dried conidia with water. Distilled water was added to the conidia drop by drop while stirring with a glass rod until a paste formed. Additional distilled water was then added slowly to ensure conidia were adequately dispersed. The concentration of the suspensions was determined using a haemocytometer slide and averaging six separate counts. The concentrated suspension was then diluted to $10^4$ conidia/mL.

Plants were inoculated with the conidial suspension as described above using a hand held Wattyl pressurised spray unit. Inoculated plants were incubated under the conditions previously described.

*Genotypes.* Twenty lines, including Australian and international cultivars and experimental lines, were selected as a preliminary differential set (Table 2). The Australian lines and cultivars were selected because they gave a range of infection responses in previous field and glasshouse screenings. The international lines and cultivars were chosen as they have known resistances to pathotypes originating in North America. Lines were sown and maintained as described above.

**Table 2. Lines used to distinguish pathotypes of *Cochliobolus sativus* and the reasons for their inclusion**

| Line | Country of origin | Head type | Reason for inclusion |
|---|---|---|---|
| Barley cv. Beecher | Australia | 6 row | Resistant in Australian trials |
| Barley cv. Bonanza | USA | 6 row | Resistant in Australian trials |
| Barley cv. Bowman | USA | 2 row | Once resistant in North Dakota |
| Barley cv. Larker | USA | 6 row | Susceptible in North Dakota |
| Barley cv. Heartland | Canada | 6 row | Resistant in Canada |
| Barley NDB112 | USA | 2 row | Resistant in North Dakota |
| Barley QB 1991–1 | Australia | 2 row | Moderately resistant to common root rot |
| Barley cv. Klaxon/Tallon-34 | Australia | 2 row | Moderately susceptible in Australia |
| Barley cv. Stirling | Australia | 2 row | Moderately susceptible in Australia |
| Barley cv. Gilbert | Australia | 2 row | Moderately susceptible in Australia |
| Barley CI 1227 | USA | 2 row | Resistant in North Dakota |
| Barley CI 6311 | USA | ? | Resistant in North Dakota |
| Barley ND5883 | USA | 2 row | Susceptible in North Dakota |
| Barley cv. Klaxon/Tallon-45 | Australia | 2 row | Susceptible in Australia |
| Barley cv. Lindwall | Australia | 2 row | Susceptible to common root rot |
| Barley cv. Lofa Abed | Middle East | ? | Resistant in Scottish trials |
| Barley cv. Skiff | Australia | 2 row | Susceptible in Australia |
| Rye cv. Ryesun | Australia | – | Alternative host, resistant control |
| Wheat cv. Hartog | Australia | – | Alternative host, resistant control |
| Triticale cv. Madonna | Australia | – | Alternative host, resistant control |

*Disease assessment and data analysis.* Disease was assessed as above and lines with infection responses 1–4 were classed as resistant while those with responses 5–9 were classed as susceptible. Each isolate was given a pathotype number using coded triplet nomenclature (Limpert and Müller 1994). The relationships among isolates and genotypes were determined by cluster analysis.

## Results

### *Establishing optimum conditions*

The duration of the dew period had a marked effect on infection (Fig. 2). After 6 h dew, even at the highest concentration of conidia on the most susceptible line, there was no evidence of infection. After 12 h dew, host reactions were apparent although not as well developed as at 24 h. In the more susceptible cultivars Skiff, Klaxon/Tallon-45 and Gilbert, the response to infection did not increase significantly with dew periods longer than 24 h. In the more resistant hosts, Klaxon/Tallon-34, Larker and the wheat cultivar Hartog, the response to infection continued to develop slightly with dew periods up to 72 h.

The lowest concentration of conidia (5000 conidia/mL) did not induce an adequate infection response even on Skiff, a cultivar known to be highly susceptible to spot blotch (Fig. 2). At the other extreme, the two highest concentrations (40 000 and 80 000 conidia/mL) induced Skiff, Gilbert, Klaxon/Tallon-34 and Klaxon/Tallon-45 to express susceptibility beyond their expected levels after only 24 h of dew. At concentrations of 10 000 and 20 000 conidia/mL and appropriate dew periods, all lines gave infection responses similar to those observed in previous trials.

### *Pathotype testing*

Six pathotypes were detected and denoted using coded triplet nomenclature based on the infection response of each genotype (Tables 1, 3). The two most common were 377 (15 isolates) and 000 (12 isolates). These two pathotypes comprised 79% of isolates. The remaining seven isolates could be separated into a further four pathotypes, 177 of which four isolates were tested, 003, 404 and 737 (one isolate each).

Cluster analysis of the infection response data of the 34 isolates separated the six pathotypes into 4 distinct groups (Table 1). Group 1 consisted of pathotypes 177, 377 and 737, group 2 of pathotype 000, group 3 of pathotype 404 and Group 4 of pathotype 003. Groups 1 and 2 contained 32 isolates (94%). There was only one isolate each in groups 3 and 4.

The hosts for group 1 (Table 1) were barley, wheat, triticale and barley grass (*Critesion murinum*). Group 2 pathotypes were isolated from barley seed, wheat, rye, barley grass and the common root rot phase of *C. sativus* on barley. Barley was the only host for groups 3 and 4 but only one isolate of each was tested. Further investigation may expand the host range for these groups.

Barley cvv. Beecher, Bonanza, Bowman, Heartland, Klaxon/Tallon-34, Larker, NDB112, QB1991-1, Rye cv. Ryesun, Triticale cv. Madonna and Wheat cv. Hartog were resistant to all 34 isolates tested and consequently were grouped together by cluster analysis. A Euclidean distance of 0.85 clearly separated resistant and susceptible genotype responses. No genotype was susceptible to all 34 isolates tested.

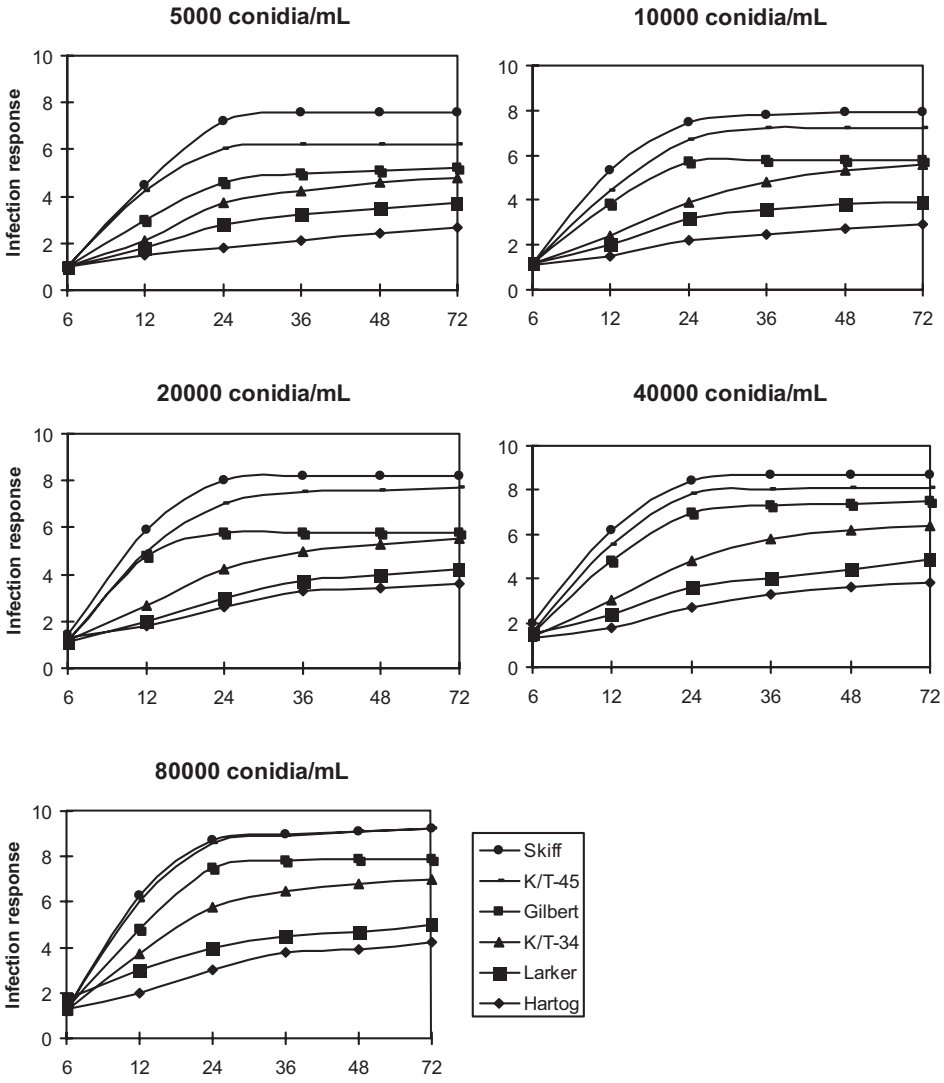


**Fig. 2.** Response of five barley cultivars and wheat cv. Hartog to infection by conidia of *Cochliobolus sativus* applied at varying concentrations and exposed to different dew periods. Infection response is based on a scale on which 1 represents very resistant and 9 very susceptible. (K/*t* = Klaxon/Tallon)

## Discussion

The optimal conditions for separation of host responses of barley seedlings and hence pathotypes of *C. sativus* in the glasshouse at 21°C were inocula containing 10000–20000 conidia/mL and a dew period of 24–48 h. The optimum separation of infection responses occurred after 36 h incubation. Since inoculation is traditionally carried out late in the afternoon, a 36 h dew period would be impracticable so a 40 h dew period is recommended to accommodate the working day. Since concentrations of 10000 and 20000 conidia/mL gave similar responses, 10000 conidia/mL is recommended because it requires less inoculum.

Six pathotypes were recognised using the conditions described above. Two pathotypes comprised 79% of the population tested. In turn, the six pathotypes could be arranged into four groups by cluster analysis. Groups 1 and 2 contained 94% of isolates. There were no apparent relationships between pathotypes and their geographical origin, host or source.

Similar studies in North Dakota using barley cultivars NDB112, Bowman and ND5883 as differentials (Table 3) and isolates from a number of countries identified three pathotypes designated 0, 1 and 2 (Valjavec-Gratian and Steffenson 1997; Zhong and Steffenson 2001). Based on the same differential host set, Australian pathotypes 000, 003 and 404 correspond to pathotype 0 and 177, 377 and 737 to pathotype 1. Pathotype 2 has been found only in North Dakota and is virulent on the line Bowman. No Australian

**Table 3. Response of seedlings of recommended differential lines to Australian pathotypes of *Cochliobolus sativus* (pathotypes are denoted using coded triplets)**

| Differential line | Virulence value | Resistance/susceptibility category | | | | | |
|---|---|---|---|---|---|---|---|
| Barley NDB112[A] | 4 | R[B] | R | R | R | R | R |
| Barley cv. Bowman [A] | 2 | R | R | R | R | R | R |
| Barley cv. Klaxon/Tallon-34 | 1 | R | R | R | R | R | R |
| Barley cv. Stirling | 4 | R | R | R | R | S | S |
| Barley cv. Gilbert | 2 | R | R | R | S | S | R |
| Barley CI 1227 | 1 | R | R | S | S | S | R |
| Barley CI 6311 | 4 | R | R | S | S | R | R |
| Barley ND5883 [A] | 2 | R | R | S | S | S | R |
| Barley cv. Klaxon/Tallon-45 | 1 | R | R | S | S | S | R |
| Barley cv. Lindwall | 4 | R | R | S | S | S | S |
| Barley cv. Lofa Abed | 2 | R | S | S | S | S | R |
| Barley cv. Skiff | 1 | R | S | S | S | S | R |
| Pathotype | | 000 | 003 | 177 | 377 | 737 | 404 |
| (Number of isolates tested) | | (12) | (1) | (4) | (15) | (1) | (1) |

[A]Differential lines used by Valjavec-Gratian and Steffenson (1997) and Zhong and Steffenson (2001).
[B]R = resistant, S = susceptible.

isolate was virulent on Bowman indicating a clear difference between the North Dakota and Australian populations.

Twenty cereal lines were used as a preliminary differential set. Clearly, the number of lines should be reduced to a more manageable differential set of approximately 12 genotypes. This many lines would be efficient for pathotype numbering as coded triplets are applicable to subsets of three genotypes. This number was achieved by eliminating wheat, rye and triticale and five of the eight lines that were resistant to all isolates tested. The remaining three resistant barley lines were retained as that group of lines included two of the North Dakotan differentials. An appropriate differential set for future pathotype determination in Australia is indicated in Table 3. It is recommended that rye, wheat and triticale also be included in pathotype screening as they are commercially important genera that *C. sativus* may also infect. All three are resistant to currently known pathotypes.

This differential set contains barley cultivars Bowman, ND5883 and NDB112, the lines used by Valjavec-Gratian and Steffenson (1997) and Zhong and Steffenson (2001) in their pathotype surveys, as well as lines that differentiate local pathotypes. Adoption of this set of genotypes can clearly define the pathotypes already identified as well as provide a basis for comparison with overseas virulences.

## Acknowledgements

We thank Dr R. G. Shivas, Department of Primary Industries, Queensland, for confirming the identity of *C. sativus* isolates, Ms Kerry Bell, Department of Primary Industries Queensland, Toowoomba for her advice and assistance with statistical analyses, and Mrs Andrea Adkins, School of Land and Food Sciences, University of Queensland, for her help with graphics and presentation.

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Received 5 February 2003, accepted 1 September 2003