

Identity and toxicity of *Fusarium* species isolated from wilted chickpea

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Summary. Four cultures of fungi, identified as *Fusarium oxysporum* f. sp. *ciceris*, were received from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), India. The cultures were race 1, a race designated as V2, race 2, and an isolate from Jabalpur which, on geographical evidence, was probably race 4, the races being defined on the basis of the reaction to them of a set of differential cultivars of the host. A further isolate, also identified as *Fusarium oxysporum* f. sp. *ciceris*, but of unknown race was received from the Thal region of Pakistan. Culture filtrates of all the isolates, grown on a defined medium, were toxic to cells of chickpea leaflets, separated enzymatically from the plant, but filtrates of the Thal isolate were the most toxic. Isolate toxicity was affected by incubation temperature and time, the maximum toxicity for the Thal isolate being 72 units activity ml⁻¹ cultural filtrate when it was grown at 20°C for 12 days but only 7 units activity ml⁻¹ when grown at 30°C for the same period. When ribosomal DNA sequences of the four ICRISAT isolates were compared, those of race 1 and V2 were identical for all 392 bps, suggesting that V2 is a variant of race 1. The Jabalpur isolate was a 99% (390/392 bps) match with race 1 and V2 and race 2 was a 97% (391/400 bps) match with race 1 and V2. Although the Thal isolate gave a 99% match with the race 1 and V2 for the first 262 bps (one gap) and a 89% match (59/66 bps) for bps 338–403, there was considerable divergence in the region from 263 to 337 bps. Similar results were obtained when the Thal isolate was matched with race 2 and 4. The four ICRISAT isolates were confirmed as *Fusarium oxysporum* but various *formae speciales* in the GenBank database such as *vasinfectum* and *vanillae* were equally well matched. No sequence for *F. oxysporum* f. sp. *ciceris* was present in the GenBank database. In contrast, the Thal isolate gave an almost exact match with *Fusarium acutatum* (407/408 bps) and the morphology of the Thal isolate, when viewed under the microscope, accorded well with the description of *F. acutatum* given in the literature.

Key words: *Cicer arietinum*, *Fusarium oxysporum* f. sp. *ciceris*, *Fusarium acutatum*, wilt, toxin.

Introduction

On a global basis, chickpea (*Cicer arietinum* L.) is the third most important pulse crop, only bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.) being more widely grown (FAOSTAT data, 2004

[www.fao.org/faostat/]). However, in South Asia chickpea is pre-eminent, with India currently accounting for about 65% of world production (FAOSTAT, 2004). With regard to diseases of chickpea, wilt, caused by *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo & K. Sato, is considered to be second in importance only to blight caused by *Ascochyta rabiei* (Pass.) Lab. on a global scale, but to be of first importance in southern India where temperatures are generally too high for *Ascochyta* (Trapero-Casas and Kaiser, 1992). Losses due to wilt vary from 10–15% (Jiménez-Díaz and Trapero-Casas, 1985; Jalali and Chand, 1991) but have been as high as 70% in some years in northern In-

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dia and Pakistan (Grewal and Pal, 1970). In Tunisia, the complete destruction of the chickpea crop has been reported on some occasions (Halila *et al.*, 1984; Halila and Harrabi, 1990). Because of the losses it can cause, Fusarium wilt is a major threat to chickpea production in India, Iran, Nepal, Burma, Spain and Tunisia (Jalali and Chand, 1991). The pathogen can survive in the soil and on crop residues as chlamydospores for up to 6 years (ICRISAT, 1985) and it is seed borne (Haware *et al.*, 1978), so that having clean seed is very important if the plant is to be grown in areas where the disease is not prevalent.

Eight races of the pathogen, designated 0, 1A, 1B/C, 2, 3, 4, 5 and 6 have been reported worldwide on the basis of the differential disease reaction of ten chickpea lines (Haware and Nene, 1982; Jimenez-Diaz *et al.*, 1993). Races 0 and 1B/C cause a chlorotic syndrome whereas the other races cause wilting. The different races are generally found in particular areas. Races 0, 1B/C, 5 and 6 are present in the Mediterranean countries and in California, while race 1A is found in California but also in India (Jiménez-Díaz *et al.*, 1993; Halila and Strange, 1996; Jiménez-Gasco *et al.*, 2001, 2004). Races 2, 3 and 4 are present only in India but each occurs in particular areas (Haware and Nene, 1982): race 1 is widespread in central and peninsular India, while race 2 is more common in northern India, and both these races are more virulent than races 3 and 4, which are present in Punjab and Haryana states (Haware *et al.*, 1992; Barve *et al.*, 2001).

Species of *Fusarium* are well known to produce mycotoxins and phytotoxins and some of the toxins produced, such as the trichothecenes, are toxic to both animals and plants (Desjardins *et al.*, 1992 and 1995). In plants, chlorosis and wilting are common symptoms of toxicosis and, as noted above, these symptoms are characteristic of the two phenotypes of chickpea plants infected with *F. oxysporum* f. sp. *ciceris*. Although toxins are thought to play a role in a plant disease syndrome, particularly if the symptoms are expressed at a distance from the site of infection, they are usually difficult to extract from the infected plant, and are more often isolated from axenic cultures of the pathogen. The five isolates of the fungus were therefore grown on a defined medium and the filtrates assayed by a live/dead assay with cells isolated from chickpea leaflets. The finding that filtrates from

the Thal isolate, when grown at 20°C, were at least ten times as active as filtrates from the other isolates raised questions about the identity of the isolates. These isolates were therefore scrutinised for differences in morphology and rDNA sequence.

Materials and methods

Fungal isolates, culture and storage

Four isolates of fungi causing wilt of chickpea were supplied by the International Crops Research Institute for the Semi Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, 502 324, India. They were identified as race 1 (whether 1A or 1B/C not specified) and race 2 of *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Snyder (Haware and Nene, 1982). The other two isolates were of unknown race and were given the names V2 and Jabalpur. A fifth isolate was obtained from wilted chickpea seedlings collected in the Thal area of the Punjab, Pakistan.

In order to obtain spores of the isolates, they were grown on Czapek-Dox Cation Liquid Medium (CDCLM; 30 ml in 250-ml flasks) consisting of Czapek Dox liquid medium (Oxoid, Basingstoke, UK) supplemented with ZnSO₄·7H₂O, 0.05 g l⁻¹; CuCl₂·6H₂O, 0.02 g l⁻¹; MnCl₂·4H₂O, 0.02 g l⁻¹; CaCl₂·2H₂O, 0.1 g l⁻¹; CoCl₂·6H₂O, 0.02 g l⁻¹ (Hamid and Strange, 2000). After incubation at room temperature for 5 d on an orbital shaker (throw 5 cm diam, 120 rpm) the cultures were filtered through four layers of sterile muslin cloth and the spores were pelleted by centrifugation at 3000 g for 15 min. They were washed twice by resuspension in sterile distilled water and centrifugation before suspension in 10% sterile glycerol at 10⁷ spores ml⁻¹. Aliquots (1 ml) were placed in sterile Nunc tubes (1 ml; Nalge Nunc International, Roskilde, Denmark) and stored in liquid nitrogen.

Toxin production

Roux bottles (1 litre) containing 100 ml CDCLM were inoculated with a spore suspension of the fungal isolates (100 µl; 10⁷ spores ml⁻¹) and incubated at 20°C. At 3-day intervals, cultures were harvested by filtration through four layers of muslin cloth and the filtrates were centrifuged at 10,000 g for 30 min before freeze-drying. Residues were dissolved in a minimal volume of methanol and the solutions diluted 1:9 in holding buffer before assay (Hamid and Strange, 2000).

Growth of plants

Seeds of chickpea (*Cicer arietinum* L.) cultivar ILC 482, kindly supplied by Ohamed Kharrat of the Institut de la Recherche Agronomique de Tunisie, Tunis, were soaked in water overnight and sown the following day in a 1:1 mixture of John Innes Compost No. 2 and silver sand in plastic pots (180 mm diam.). Seven holes of 3-cm depth were made in the growth medium of each pot and two seeds were placed in each hole and covered. Pots were watered heavily soon after sowing. Plants were raised in a greenhouse (70–80% relative humidity; 20±3°C), thinned to six plants per pot at the seedling stage and watered regularly. Daylight was supplemented with fluorescent light (200 $\mu\text{Em}^{-2}\text{s}^{-1}$) for 16 h per day during the winter months.

Bioassay

Toxin preparations were assayed on cells separated from the leaflets of chickpea plants (cv. ILC 482) essentially according to the method of Hamid and Strange (2000). In brief, cells were separated from chickpea leaflets by vacuum-infiltrating an enzyme solution in buffered osmoticum and gently stirring until the leaflets disintegrated. After washing in buffered osmoticum the cells were added to a dilution series of toxin preparations in a microtest plate (50 μl /well) and incubated for 3 h. Cell viability was ascertained using the vital dye fluorescein diacetate and the results were converted to probit percent cell death. These values were plotted against the \log_2 dilution factor and the dilution giving a probit value of 5 (= 50% cell death after correction for viability of controls) was arbitrarily defined as one unit of activity. The toxicity of any preparation was obtained by reading the dilution factor on the x axis corresponding to a probit value of 5 on the y axis and converting this to the arithmetic value. This was multiplied by 20 to give the number of units ml^{-1} as only 50 μl of toxin preparation was used in the wells of the assay plate.

Morphology of the fungal isolates

Spore suspensions (50 μl : 10^7 spores ml^{-1}) of the fungi were placed centrally on Petri dishes containing potato dextrose agar (PDA) and incubated at 20°C for 1–3 weeks in the dark. They were examined macroscopically for colony characteristics and microscopically for spores and associated structures.

DNA extraction

DNA was extracted from plugs of mycelium, cut from the edges of fungal colonies growing on PDA with a No. 3 cork borer by the Chelex mini-prep method of Challen *et al.* (2003).

PCR

Two primers, ITS 1 (5'-TCC GTA GGT GAA CCT GCG-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), which recognize conserved sequences at the 3' end of the 18S gene and the 5' end of the 28S gene of ribosomal DNA (White *et al.*, 1990) were obtained from MWG (Mill Court, Milton Keynes, UK). The reaction mixture consisted of template DNA (2.0–2.5 pg, 4 μl of each primer at 2.5 picomoles μl^{-1} and one "Ready to Go" PCR bead (Amersham Pharmacia Biotech Inc., Uppsala, Sweden). In some cases MgCl_2 was added to final concentrations of 1.5, 2.5 and 3.5 mM. Water was added to give a final volume of 25 μl . PCR was performed in a Progene Thermal Cycler (Techne Ltd., Cambridge, UK) using a programme consisting of 2 min at 94°C (denaturation) followed by 35 cycles of 30 sec at 94°C (denaturation), 30 sec at 55°C (annealing) and 2 min at 72°C (extension). At the end of the programme the tubes were held at 4°C until required for analysis. λ DNA (2 μl from a stock solution of 25 pg μl^{-1} ; New England Biolabs, Hitchin, UK) was used as a positive control and tubes lacking template DNA as a negative control. Amplicons were purified with a QIAquick PCR purification kit (Qiagen, Crawley, UK) according to manufacturer's instructions.

Sequencing and sequence analysis of the purified PCR products

The purified PCR products were sequenced with the WellRed dye-labelled dideoxy-terminator cycle sequencing kit using ITS 1 and ITS 4 as primers. This kit contained the dye-labelled terminators ddUTP, ddGTP, ddCTP, ddATP, dNTP mix solution, thermostable DNA polymerase and pyrophosphatase-sequencing buffer (Beckman Coulter Inc., CA, USA). Sequencing was performed on a Beckman CEQ2000XL instrument with an eight-capillary automated DNA sequencing and fragment analysis system. The read length of the capillaries (DNA separation array, 33–75B) was 700 bp with an accuracy of 98.5%. Sequences were aligned using Sequencher 4.1 (Gene Codes Corporation, MI,

USA). Homologies with sequences in the GenBank database were identified using BLAST (<http://www.ncbi.nlm.nih.gov/blast>). Alignment was carried out using the Sequencher program and Clustal W (<http://www.ebi.ac.uk/clustalw>).

Results

Toxicity of culture filtrates of isolates grown at 20°C and 30°C

There was considerable variation among isolates in toxin production, with the highest titres being obtained from the Thal isolate when grown at 20°C; however, at 30°C the titres for this isolate dropped markedly from a peak of 72 units ml⁻¹ culture filtrate at 20°C to 7 units ml⁻¹ (Fig. 1). Jabalpur was the second most toxic isolate, but the activity of this isolate was greater when it was grown at 30°C than at 20°C (Fig. 1).

During these experiments it was noticed that, although the Thal isolate gave rise to mycelium that was both red and white and filtrates that were red, the mycelium of some Thal cultures was white and their filtrates colourless. These latter cultures had only about one-tenth the activity of the red cultures. Repeated single sporing of red cultures did not eliminate the production of colourless cultures.

Morphology of the isolates

Isolates of race 1, race 2 and V2

Colonies of race 1 on PDA produced dense aerial mycelium that was even and white whereas mycelium of V2, while also dense, was not so high as with race 1 and was less even. In contrast, the mycelium of race 2, though still dense, was appressed. The closest colour matches to the obverse of race 1 and race 2 were Goldenrod 1 and Gold 1

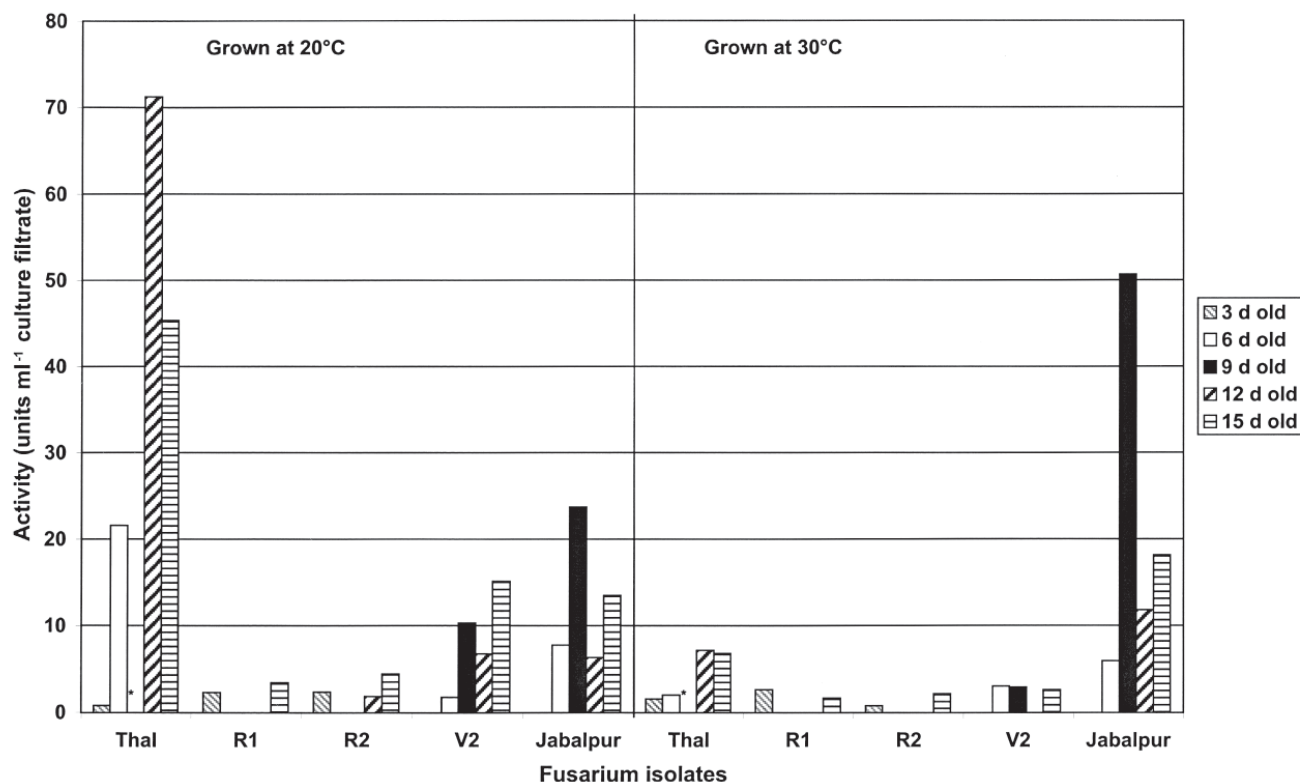


Fig. 1. Time course of toxin production by five isolates of *Fusarium* from chickpea at two temperatures. Chickpea cells, isolated from the plant, were employed in an assay of the toxicity of culture filtrates and the results expressed as units of activity where 1 unit of activity was the amount of toxin required to kill 50% of the cells (for details see text). * No data.

respectively, while that of V2 lay between these two [HYPE's Color Specifier for Netscape v. 3: (<http://users.rcn.com/giant.interport/COLOR/1ColorSpecifier.html>)]. All these isolates produced abundant microconidia (7.5–12.5×2.5 µm) on simple phialides, the larger microconidia being divided by a single septum. No macroconidia were seen but thick-walled chlamydospores (7.5 µm diam) were present in race 2.

Isolate of Jabalpur

The mycelium of this isolate was floccose and aggregated in many spikes that reached the lid of the Petri dish. The obverse had a deep purple circle (DarkGoldenrod 4 as determined by HYPE's Color Specifier for Netscape v. 3) of about 5 mm diameter at the point of inoculation of the plate. A circular but irregular band, about 5 mm in width, of the same colour about 23 mm from the point of inoculation was also present. Microconidia had the same dimensions as those of races 1, 2 and V2 except that none had septa. Chlamydospores were absent. Since this isolate was from Jabalpur, it was likely to be race 4, which is prevalent in this region of India.

Thal isolate

This isolate was similar to the description of *Fusarium acutatum* given by Nirenberg and O'Donnell (1998). Colonies had abundant and dense, white aerial mycelium that reached the lid of the Petri dish. The obverse had a deep purple circle, as did the Jabalpur isolate (DarkGoldenrod 4 as determined by HYPE's Color Specifier for Netscape v. 3) of about 5 mm diameter at the point of inoculation but the rest of the plate had a patchy purple to grey coloration. The microconidia of this isolate were 7.5–20×2.5 µm and sometimes had a single septum. Chlamydospores were present (5.0–7.5 µm diam.).

DNA sequencing and BLAST homologies

The ribosomal DNA (392 bps) of isolates of races 1 and V2 was identical and differed by only two base pairs from the Jabalpur isolate (Fig. 2). Race 2 differed from race 1/V2 by the interpolation of a total of 8 base pairs in the sequence (Fig. 2). In contrast, the Thal isolate had multiple interpolations of bases and substitutions which occurred mainly in the region from 263 to 337 bps (Fig. 2).

A BLAST search revealed total homology of the 392 bps of race 1/V2 and other *formae speciales* identified in GenBank as *vasinfectum* (7 entries), *melonis* (2 entries), *vanillae* (4 entries), *luffae* (1 entry), *lagenariae* (1 entry), *niveum* (2 entries), *dianthi* (2 entries), *radicis-lycopersici* (1 entry), *lilii* (1 entry) as well as many of which the *forma specialis* name was not given. No entry matched the sequence of the Jabalpur isolate completely, but one entry for *F. oxysporum* f. sp. *vanillae* and one for *F. oxysporum* f. sp. *radicis-lycopersici* differed by only 1 bp, as did 18 entries for which the *forma specialis* was not identified. With race 2 there was greater divergence, with no entry matching more than 391 out of the 400 bps. Here the list of the closest matching entries was very similar to that of race 1/V2. In contrast, the Thal isolate differed by the inclusion of only one extra base as compared with the database entry identified as *Fusarium acutatum*.

Discussion

Several toxins from various *formae speciales* of *F. oxysporum* have been described as causing wilt symptoms in their host plants. These toxins include fusaric acid from the banana pathogen *F. oxysporum* f. sp. *cubense*, beauvericin from the pathogen of muskmelon *F. oxysporum* f. sp. *melonis*, and several polyketide toxins from the cotton pathogen, *F. oxysporum* f. sp. *vasinfectum* (Thangavelu *et al.*, 2001; Moretti *et al.*, 2002; Bell *et al.*, 2003).

On chickpea, different races of *F. oxysporum* f. sp. *ciceris* cause two distinct disease syndromes, chlorosis and wilt. Other symptoms on chickpea are epinasty of the leaves, discoloration of the vascular tissue and ultimately collapse of the plant (Haware, 1990; Hamid *et al.*, 2001). These symptoms suggest that phytotoxins are involved in the disease although the evidence in the literature for this is scant. Kaur *et al.* (1987) found that partially purified toxin from this fungus inhibited callus growth in chickpea, and Rao and Padmaja (2000) reported that crude culture filtrates, when diluted to 30% with water, caused wilting of 1-week-old chickpea seedlings in 4–5 days. The present investigation was undertaken to ascertain whether any of the symptoms of *F. oxysporum* f. sp. *ciceris* could be explained at least in part with toxins produced by the pathogen.

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V2      AGCCCCGCTCCCGG-TAAAACG-GGACGGCCCGCC---AGAGGACCCCTAAACTCTGTTTC 55
R2      AGCCCCGCTCCCGGATAAAAACGAGGGCGGCCGCCAAAGAGGACCCCTAAACTCTGTTTC 60
R1      AGCCCCGCTCCCGG-TAAAACG-GGACGGCCCGCC---AGAGGACCCCTAAACTCTGTTTC 55
Jabalpur  AGCCCCGCTCCCGG-TAAAACG-GGACGGCCCGCC---AGAGGACCCCTAAACTCTGTTTC 55
Thal    AGCCCCGCTCCCGG-TAAAACG-GGACGGCCCGCC---AGAGGACCCCTAAACTCTGTTTC 55
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V2      -TATATGTAACCTTCTGAGTAAAACCATAAA--TAAATCAAAACTTTC-AACAACGGATCT 111
R2      ATATATGTAACCTTCTGAGTAAAACCATAAAATTAATCAAAACTTTC-AACAACGGATCT 119
R1      -TATATGTAACCTTCTGAGTAAAACCATAAA--TAAATCAAAACTTTC-AACAACGGATCT 111
Jabalpur -TATATGTAACCTTCTGAGTAAAACCATAAA--TAAATCAAAACTTTC-AACAACGGATCT 111
Thal    -TATATGTAACCTTCTGAGTAAAACCATAAA--TAAATCAAAACTTTC-CAACAACGGATCT 112
*****

V2      CTTGGTTCCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAA 171
R2      CTTGGTTCCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAA 179
R1      CTTGGTTCCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAA 171
Jabalpur CTTGGTTCCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAA 171
Thal    CTTGGTTCCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAA 172
*****

V2      TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATG 231
R2      TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATG 239
R1      TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATG 231
Jabalpur TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATG 231
Thal    TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATG 232
*****

V2      CCTGTTTCGAGCGTCATTTCAACCCCTCAAGCAC---AGCTTGGTGTGGGACTCG----- 282
R2      CCTGTTTCGAGCGTCATTTCAACCCCTCAAGCAC---AGCTTGGTGTGGGACTCG----- 290
R1      CCTGTTTCGAGCGTCATTTCAACCCCTCAAGCAC---AGCTTGGTGTGGGACTCG----- 282
Jabalpur CCTGTTTCGAGCGTCATTTCAACCCCTCAAGCAC---AGCTTGGTGTGGGACTCG----- 282
Thal    CCTGTTTCGAGCGTCATTTCAACCCCTCAAGCCCTCGGGTTTGGTGTGGGGATCGGCGAGC 292
*****

V2      -CGTTAATTCG---CGTTCCCTCAAATTGATTGGCGGTACACGTCG-AGCTTCCATAGCGTA 337
R2      -CGTTAATTCG---CGTTCCCTCAAATTGATTGGCGGTACACGTCG-AGCTTCCATAGCGTA 345
R1      -CGTTAATTCG---CGTTCCCTCAAATTGATTGGCGGTACACGTCG-AGCTTCCATAGCGTA 337
Jabalpur -CGTTAATTCG---CGTTCCCTCAAATTGATTGGCGGTACACGTCG-AGCTTCCATAGCGTA 337
Thal    CCTTTGCGGCAAGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTGCAGCTTCCATTGCGTA 352
* * * * *

V2      GTAGTAAAACCCCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCC-AACT 392
R2      GTAGTAAAACCCCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCC-AACT 400
R1      GTAGTAAAACCCCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCC-AACT 392
Jabalpur GTAGTAAAACCCCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCC-AACT 392
Thal    GTAGTAAAACCCCTCGCAACTGGTACGCGGCCGCGGCCAAGCCGTTAAACCCCCAACT 408
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Fig. 2. Alignment of ribosomal DNA sequences from five isolates of *Fusarium* obtained from chickpea. Note the differences of the Thal isolate from the other isolates, particularly between bases 263–337.

All the isolates in the study produced compounds in culture that were phytotoxic as determined by the live/dead cell assay. The highest titres were found in culture filtrates of the Thal isolate, followed by those from the Jabalpur isolate. However, toxicity also depended on the incubation temperature of the cultures, filtrates of the Thal isolate being far more toxic when grown at 20 than at 30°C, and, conversely, filtrates of the Jabalpur isolate grown at 30°C being more toxic than filtrates from isolates grown at 20°C. It may be significant that the Thal isolate comes from Thal, in the North Western Frontier Province of Pakistan, much of which is at high altitudes and consequently has a cool climate, whereas the Jabalpur isolate is from Jabalpur in central India, which has high summer temperatures of 30–45°C.

Preliminary chromatographic evidence (data not shown) suggested that the toxicity of the Thal isolate was associated with a red compound. The absence of this red compound both from colourless filtrates of the Thal isolate and from the filtrates of the other four isolates prompted a re-examination of their identity. Identification of species and subspecies of *Fusarium* on morphological grounds is notoriously difficult and liable to error. For example, Marasas (1985) found that about 50% of a collection of 200 *Fusarium* strains reported to be toxigenic had been incorrectly named on the basis of taxonomic concepts current at the time. In contrast, sequencing of ribosomal DNA provides unequivocal data that have been used to solve several problems of fungal identification and taxonomy (see for example Sugita *et al.*, 2004; Cunnington *et al.*, 2004).

In the present investigation, the four isolates provided by ICRISAT were confirmed as being *F. oxysporum* by comparison with ribosomal sequences in the GenBank database. However, the sequencing of this region was inadequate to differentiate the *formae speciales*. Moreover, it was unfortunate that *F. oxysporum* f. sp. *ciceris* was not represented in the GenBank database.

Jiménez-Gasco *et al.* (2002) showed that *F. oxysporum* f. sp. *ciceris* is monophyletic and later provided genetic evidence for a stepwise evolution of races of the pathogen correlating with the inferred DNA fingerprint lineages (Jiménez-Gasco *et al.*, 2004). Two models were presented: one that allowed only gains in virulence and another

that allowed both gains and losses in virulence when this was determined for a set of differential lines of chickpea. It would be interesting to know the mechanism(s) by which these gains and losses occur.

One possibility is that they are due to mutations controlling quantitative and/or qualitative differences in toxin production. Perhaps the fungus with a wider host range simply synthesises higher concentrations of toxin. Alternatively, the structure of the toxin end product may vary. Plant-pathogenic fungi often synthesise families of compounds having a similar molecular structure rather than synthesising only one single compound. For example, the strawberry pathotype of *Alternaria alternata* produces two related toxins, AF toxin I and AF toxin II. AF toxin I is toxic to both strawberry and pear but AF toxin II is toxic only to pear. Recently, Ito *et al.* (2004) showed that mutants of *A. alternata* produced by targeted disruption of the locus *AFTS1*, which encodes a protein similar to enzymes of the aldo-ketoreductase superfamily, did not produce AF toxin I, and were only pathogenic to pear, and not to strawberry. Thus quite subtle genetic modulations can alter the host range of a plant-pathogenic organism.

One mechanism by which fungi may acquire novel characteristics is by horizontal gene transfer (Rosewich and Kistler, 2000). In some instances such genes may be involved in toxin production. For example, two genes unique to *Cochliobolus heterostrophus* race T were found within 1.5 kb of each other on the Tox1B chromosome of the pathogen, one of which was essential for toxin production, suggesting that they were acquired by a horizontal transfer event (Rose *et al.*, 2002). In another study Hatta *et al.* (2002) showed that genes necessary for the synthesis of toxins by host-selective isolates of *A. alternata* were present on a 1.05-Mb chromosome that was dispensable for saprophytic growth. In the present case, it may well be that genes for production of toxins or their analogues, or even different toxins, increasing the host range of the pathogen, were acquired sequentially by such horizontal gene transfer events.

Fusarium acutatum is a pathogen of pigeonpea (O'Donnell and Cigelnik, 1997) and was described as a new species by Nirenberg and O'Donnell (1998). More recently, Kurmut *et al.* (2000) reported that it was the causal agent of root rot of *Vicia*

faba L. in the Sudan. However, it does not seem to have been recognised previously as a pathogen of chickpea. Consequently, its distribution and prevalence are unknown. As it is morphologically distinct from *F. oxysporum* f. sp. *ciceris*, screening for its presence should not be difficult and may yield interesting results.

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