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# Chickpea wilt: identification and toxicity of 8-*O*-methyl-fusarubin from *Fusarium acutatum*

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**Abstract**

*Fusarium acutatum* was isolated from wilting chickpea plants in Pakistan. Filtrates from cultures grown on a defined liquid medium caused permanent wilting of chickpea cuttings and killed cells, isolated enzymically from healthy plants, in a bioassay. Toxic activity was retained by a cyano solid phase extraction cartridge and the toxin was isolated by elution from the cartridge in acetonitrile and Si-gel thin layer chromatography of the eluate. Analytical HPLC of the compound on a cyano column with diode array detection gave a single peak with a homogeneous spectrum and $\lambda_{max}$ at 224 and 281 nm. NMR and mass spectral studies showed that the toxin was 8-*O*-methyl-fusarubin. The pure compound caused permanent wilting of chickpea cuttings and the LD50 value in the cell bioassay was 327 ng/ml.


*Keywords:* *Cicer arietinum*; Leguminosae; Chickpea; *Fusarium acutatum*; Wilt; Bioassay; NMR; Mass spectrum; Naphthoquinone; Phytotoxin

## 1. Introduction

Wilt is regarded as the most important disease of chickpea on the Indian subcontinent and the causal organism of the disease is usually ascribed to *Fusarium oxysporum* f. sp. *ciceri* but other species and formae speciales of *Fusarium* also cause wilting of a variety of plants (Jiminéz-Díaz et al., 1993; Srivastava et al., 1984; Di Pietro et al., 2003). The fungus that is the subject of the present investigation and which was isolated from wilted chickpea plants grown in the Thal area of Pakistan, did not accord morphologically with *F. oxysporum* f. sp. *ciceri* but was identified as *Fusarium acutatum*. This organism was originally reported as a pathogen of pigeon pea by O'Donnell and Cigelnik (1997) and described as a new species by Nirenberg and O'Donnell (1998). More recently, Kurmut et al. (2000) showed that it was the cause of root rot of *Vicia faba* L. in the Sudan.

Toxins of importance in plant disease syndromes are usually isolated from axenic cultures of pathogens. For example, the solanapyrone toxins which are produced by another pathogen of chickpea, *Ascochyta rabiei*, were isolated from cultures of the plant pathogen grown on a medium containing chickpea seed extract (Alam et al., 1989). Later a defined medium was formulated which allowed production of the toxins (Chen and Strange, 1991). In this investigation, essentially the same medium was used to grow cultures of *F. acutatum*. Filtrates were

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toxic to cuttings of chickpea and cells isolated from leaflets of the plant and the compound responsible was isolated and identified.

## 2. Results and discussion

When *F. acutatum* was grown on a defined medium consisting of Czapek-Dox nutrients and additional cations (Hamid and Strange, 2000), two types of cultures were obtained, those that were red and those that were colourless. Filtrates of the cultures were tested for their toxicity to cells isolated from chickpea leaflets using fluorescein diacetate to differentiate live and dead cells. This compound readily enters live cells with intact plasmamembranes and, once inside the cell, is metabolised by esterases to give free fluorescein. As plasmamembranes are impermeable to fluorescein, the compound accumulates and imparts a yellow-green fluorescence to such cells, which may be viewed under a fluorescence microscope. Using this assay to assess the toxicity of the two types of filtrates, those that were red were far more toxic than those that were colourless. Maximum activity of red cultures was attained after incubation of the fungus for 12 days at 20°C and exceeded 70 units of activity/ml culture filtrate where one unit of activity was defined as the amount of toxin required to kill 50% of the cells isolated from chickpea leaflets. In contrast, filtrates from colourless cultures did not exceed 10 units/ml.

The majority of the toxic activity of filtrates from red cultures of *F. acutatum* partitioned into ethyl acetate or could be extracted by a cyano-SPE cartridge. Thin-layer chromatography (TLC) of the ethyl acetate fraction, or of the acetonitrile eluate from the cyano cartridge, with $CHCl_3$–MeOH (8:1) gave a red spot at $R_f$ 0.57. When the silica from the TLC plate was eluted with acetonitrile only the area occupied by the red spot was active in the assay. RP-HPLC of the eluate on a cyano column with 10% acetonitrile as mobile phase revealed a single major peak with absorption maxima at 224 and 281 nm.

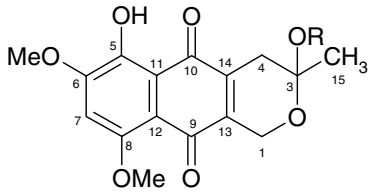
Cuttings of chickpea plants placed in culture filtrates of the pathogen or the pure toxin wilted permanently within 4 h. Such cuttings took up the equivalent of 1.5 and 25.5 μg of the toxin, respectively. In the cell bioassay, one unit of activity was the equivalent of 327 ng/ml of purified toxin.

$^1H$ and $^{13}C$ NMR data were collected at 400 MHz in a $CDCl_3$ solution. In the $^1H$-spectrum the compound displayed one aromatic singlet at $\delta$6.73 and a highly deshielded singlet at $\delta$13.16 which corresponded to an aromatic hydroxyl group. These signals and the presence of two methoxy singlets at $\delta$4.03 and 4.00 suggested a 1,2,4-trisubstituted naphthoquinone arrangement. Additional to the naphthoquinone signals the $^1H$-spectrum also contained a 2H double–doublet at $\delta$4.75 which is characteristic of a $CH_2$ adjacent to oxygen. Two further geminally coupled ($J$ = 18 Hz) 1H-doublets at $\delta$2.53 and 2.84 indicated the presence of a further isolated methylene moiety. The spectrum also contained a 3H singlet at $\delta$1.55 indicating a slightly deshielded aliphatic methyl group. Comparison of the observed signals and their coupling arrangements with the literature on known naphthoquinone natural products, showed that the isolated toxin corresponded to those of the previously reported 8-*O*-methyl-fusarubin (**1**) (Steyn et al., 1979; Tatum et al., 1985). Interestingly, two numbering systems have been utilised for this compound, which is also known as 9-*O*-methyl-fusarubin (Tatum et al., 1985). We have chosen to use that of Steyn et al. (1979). $^{13}C$ NMR data was collected to confirm structure assignment. All chemical shifts were in agreement with those previously reported (Steyn et al., 1979) as can be seen in Table 1.

Electrospray mass spectroscopy (ESI-MS) was carried out by direct infusion of a methanol solution of the isolated toxin. Atmospheric Pressure Chemical Ionisation (APCI) methods, deployed in the negative ionisation mode, indicated a molecular weight of 334. This was higher than the expected molecular weight of 320 for 8-*O*-methyl-fusarubin, although an ion corresponding to 320 was also observed in the spectrum. However, Tatum et al. (1985) showed that the addition of acidic methanol to 8-*O*-methyl-fusarubin causes the formation of 3-*O*-methyl-8-*O*-methyl-fusarubin (**2**) resulting from the methylation of the hydroxy group at C-3. The molecular weight of this compound is 334 and thus the observation of this ion in the mass spectrum, as an artefact of solubilization (**1**) in acidic methanol, served as further confirmation of the structural assignment.

Table 1
$^{13}C$ NMR chemical shifts in $CDCl_3$ solution using TMS as internal standard

| Carbon atom | Observed chemical shift | Literature chemical shift (Steyn et al., 1979) |
|---|---|---|
| 1 | 58.7 | 57.7 |
| 3 | 94.4 | 93.1 |
| 4 | 31.2 | 31.7 |
| 5 | 148.5 | 147.6 |
| 6 | 155.7 | 155.0 |
| 7 | 102.5 | 103.0 |
| 8 | 156.0 | 155.3 |
| 9 | 180.0 | 179.0 |
| 10 | 189.2 | 188.7 |
| 11 | 114.3 | 113.7 |
| 12 | 109.9 | 108.9 |
| 13 | 144.9 | 144.6 |
| 14 | 136.9 | 136.8 |
| 15 | 29.3 | 28.3 |
| 6-OMe | 56.8 | 56.1 |
| 8-OMe | 56.4 | 56.4 |




(**1**) R = H. 8-O-Methyl-fusarubin
(**2**) R = Me. 3-O-Methyl-8-O-methyl-fusarubin

Naphthazarin toxins, produced by species of *Fusarium*, of which 8-*O*-methyl-fusarubin is one, have been implicated in disease symptoms in citrus (van Rensburg et al., 2001), cotton (Bell et al., 2003) and pea (Holenstein and Défago, 1983). Their activity has been attributed to the production of the superoxide radical, ($O_2^-$) $H_2O_2$ and semiquinone radicals of the compounds (Medentsev and Akimenko, 1992). Such compounds attack membranes and could be responsible for the loss of the semi-permeability of the plasmamembrane as found in the cell assay reported in this paper. An attack on plasmamembranes could also explain wilting as chickpea is likely to depend in part on the turgor of parenchyma cells surrounding the stele for support. As a parallel, it is pertinent to point out that stem breakage, a prominent symptom of Ascochyta blight of chickpea, can be reproduced by solanapyrone A, one of the toxins produced by the causal agent of the disease, *A. rabiei*. Here, too, the loss of turgor of parenchyma cells surrounding the stele was invoked as an explanation of the phenomenon (Hamid and Strange, 2000).

## 3. Experimental

### 3.1. Isolation, spore production and storage of *F. acutatum*

Fungi were isolated from wilted chickpea seedlings collected in the Thal area of the Punjab, Pakistan. Collar regions from diseased seedlings were excised, surface sterilised with 2% sodium hypochlorite for 2 min, rinsed in distilled water and placed on petri plates containing Komada's medium (KM), ostensibly specific for *F. oxysporum* (Komada, 1975). Plates were incubated at 25 ± 2°C in an illuminated incubator with a photoperiod of 16 h for 5–7 d. Fungi developing from the chickpea material were sub-cultured and single spored on PDA. Spores were produced in shake culture on Czapek-Dox Cation Liquid Medium (CDCLM; 30 ml in 250 ml flasks) consisting of Czapek Dox liquid medium (Oxoid) supplemented with $ZnSO_4 \cdot 7H_2O$, 0.05 g $l^{-1}$; $CuCl_2 \cdot 6H_2O$, 0.02 g $l^{-1}$; $MnCl_2 \cdot 4H_2O$, 0.02 g $l^{-1}$; $CaCl_2 \cdot 2H_2O$, 0.1 g $l^{-1}$; $CoCl_2 \cdot 6H_2O$, 0.02 g $l^{-1}$ (Hamid and Strange, 2000). After 5 days, mycelium was removed by filtration through four layers of muslin cloth and the spores pelleted by centrifuging at 3000*g* for 15 min. They were resuspended in sterile distilled water and centrifuged twice more before finally resuspending them at $10^7$ spores/ml and storing them in liquid nitrogen.

### 3.2. Toxin production

Roux bottles (1 l) containing 100 ml CDCLM were inoculated with spore suspension of *F. acutatum* (100 µl; $10^7$ spores/ ml) and incubated at 20°C for 12 d. Cultures were filtered through four thicknesses of muslin and the filtrates were centrifuged at 10,000*g* for 30 min. MeOH was added to the supernatants to 20% (v/v) and after aging for 4–16 h the preparation was filtered again through four thicknesses of muslin. The MeOH was removed by film evaporation at <35°C and the aqueous remainder filtered through a 0.5 mm filter (Nalgene, Rochester, USA).

### 3.3. Solid phase extraction

Filtrates (100–150 ml) were introduced into a cyano SPE cartridge (5 g; Jones Chromatography, UK) after solvation and equilibration of the cartridge with 30 ml each of acetonitrile and ultra purified water. After washing with water (5 ml) and drying in a current of air the toxin was eluted in acetonitrile (10 ml).

### 3.4. Bioassay

Essentially the procedure of Hamid and Strange (2000) was followed.

#### 3.4.1. Thin layer chromatography

The acetonitrile eluates were spotted on Silica gel 60 $F_{254}$ TLC sheets with a concentrating zone and developed in $CHCl_3$–MeOH (8:1). Silica corresponding to zones containing a red compound at $R_f$ 0.57 was scraped from the plate and eluted in acetonitrile.

#### 3.4.2. HPLC

The acetonitrile eluates (20 µl) from TLC were injected into a cyano column (150 mm × 4.6 mm, protected with a guard column [20 × 4.6 mm] of the same material; Jones Chromatography, UK) and the column developed with 10% acetonitrile as mobile phase. Data were acquired on a Philips HPLC system consisting of a PU4100 quaternary pump, PU4021 diode array detector and a personal computer equipped with PU6003 diode array software for data handling.

#### 3.4.3. NMR

NMR was carried out on a Bruker Avance 400 MHz instrument. 8-*O*-Methyl-fusarubin (**1**) $^1$H NMR (400 MHz, $CDCl_3$, TMS as internal standard): δ1.55 (3H,

*s*, 15-$H_3$), 2.53 (1H, *d*, $J = 18$ Hz, 4-$H_{ax}$), 2.84 (1H, *d*, $J = 18$ Hz, 4-$H_{eq}$), 4.00 (3H, *s*, 8-OMe), 4.03 (3H, *s*, 6-OMe), 4.75 (2H, *dd*, $J = 18$, 2.8 Hz, 1-$H_2$), 6.73 (1H, *s*, 7-H), 13.16 (1H, *s*, 5-OH); $^{13}$C NMR (100 MHz, $CDCl_3$, TMS as internal standard): $\delta$29.33 (15-C), 31.20 (4-C), 56.40 (8-OMe), 56.82 (6-OMe), 58.66 (1-C), 94.36 (3-C), 102.49 (7-C), 109.89 (12-C), 114.28 (11-C), 136.91 (14-C), 144.94 (13-C), 148.47 (5-C), 155.70 (6-C), 155.97 (8-C), 180.01 (9-C), 189.23 (10-C).

#### *3.4.4. ESI-MS*

Direct infusion ESI-MS was carried out on a ThermoFinnigan LCQ by Atmospheric Pressure Chemical Ionisation (APCI) in negative ionisation mode.

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