Scientific
Research

Mango Malformation: I. Toxin Production Associated with *Fusarium* Pathogens

Wafaa Haggag M.[1], Hazza M.[2], Sehab A.[1], Abd El-Wahab M.[1]

[1]Department of Plant Pathology National Research Center, Dokki, Cairo, Egypt; [2] Science Faculty, Botany Department, Banha University, Banha, Egypt.
Email: wafaa_haggag@yahoo.com

ABSTRACT

Eight Fusarium species i.e. F. subglutinans, F. solani, F. oxyspoum, F. sterilihyphosum, F. proliferatum, F. moniliforme, F. avena and F. chlamydspore isolated from mango malformed disease were tested for their ability to cause mango malformation disease and their production of moniliformin and total fumonisins (FB1 + FB2) using HPLC. A evaluated for moniliformin production, seven isolates were toxin producers, the production levels ranging from 0.51 to 8.90 μg/ml. The higher levels were produced by Fusarium subglutinans (8.51 μg/ml). Moderate concentrations of moniliformin was produced by F.moniliforme (6.90 μg/ml), F. oxysporum (6.30 μg/ml), F. proliferatum (4.10 μg/ml) and F. sterilihyphosum (1.10 μg/ml). Separation and identification of Fumonisin that was isolated from the pathogen-causing disease are made by (HPLC). A evaluated for total fumonisin production (FB1 + FB2), seven isolates were toxin producers, the production levels ranging from 0.10 to 8.30 μg/ml. The higher levels were produced by F. moniliforme (8.30 μg/ml. Moderate concentrations of fumonisin was produced by F .proliferatum (0.64 μg/ml) and F. subglutinans (0.50 μg/ml). Strong positive correlations between moniliformin and total fumonisins (FB1 + FB2) activities and malformation disease incidence by F. subglutinans, F. solani, F. oxyspoum, F. sterilihyphosum, F. proliferatum was observed.

Keywords: *Fusarium, Mango Malformation, Moniliformin and Fumonisins*

1. Introduction

Mango (*Mangifera indica* L.) is the most important fruit grown in tropical and subtropical region of the world. Mango (*Mangifera indica* L.) is the most important fruit crop in Egypt. Mango Malformation is one of the most destructive mango diseases [1,2]. Losses due to malformation have not been accurately assessed because yield loss is not a linear function of disease severity [3]. During a survey of mango plantations in Sindh for investigating the association of fungi with mango malformation disease (MMD), six fungal species viz., *Fusarium nivale* (Fr.) Ces, *F. oxysporium, F. moniliforme, F. semitectum, Alternari alternata* and *Aspergillus niger* were isolated and identified on the basis of their colony characteristics and conidial morphology [4]. *F. sterilihyphosum* and *F. proliferatum* are first report association with mango malformation in [5]. As noted by [6] mango malformation probably involves two principles;1) the malformation inducing principle (MIP) which works through imbalance in growth substance and in conditioning of cells; 2)The toxic principle (TP) which causes growth retardation and toxicity symptoms. *Fuarium* species, particularly *F. moniliforme var subglutinans*, are likely source of MIP and TP, and the causal agent of disease. Singh and Dhillon [7] proposed that ethylene might play role in mango malformation by suppression of apical dominance, causing more isodiametric growth of rachides and shortening and thickening of secondary branches of malformed panicles. Ram [8] stated that the most probable role of malformin in the causation of mango malformation might be mediated through alteration of membrane permeability leading to efflux of IAA or its metabolite. He indicates the possibility that malformin antagonizes IAA action through efflux action of auxins of the malformed cells causing loss of apical dominance at a very early stage of panicle development. Malformed mango panicles have been shown to contain reduced levels of auxin [7,9,10]. Strains of *Fusarium proliferatum, F. subglutinans, F. anthophilum, F. annulatum, F. succisae, F. beomiforme, F. dlamini, F. napiforme,* and *F. nygamai* from a variety of substrates and geographic areas were

tested for the production of fumonisin B1 in culture. None of the cultures of *F. subglutinans*, *F. annulatum*, *F. succisae*, or *F. beomiforme* produced fumonisin B1 in culture. Strains of *F. proliferatum* produced fumonisin B1 in amounts ranging from 155 to 2936 ppm, of the species tested, *F. proliferatum i*s the most important producer of fumonisin B1 because of its association with corn and animal mycotoxicose such as porcine pulmonary edema. *F. napiforme* and *F. nygamai* also may be important because of their association with the food grains millet and sorghum [11].

Thus, objective of the present study is to study the production of toxins by pathogens involved in the causation of malformation.

2. Materials and Methods

Fusarium species isolated from mango malformed disease were tested for their ability to cause malformation. Mango seedlings cv. Sedekia (two years old) was inoculated with culture filtrate of *Fusarium spp.* by injection of the apical buds. Sterilized water was used as a control. Transplanted seedlings were monitored for development of malformation. At the end of the experiment (120 days), all surviving seedlings were examined for apical disease symptoms. Data were recorded on symptoms manifestation as diseases incidence and severity (from 1 - 4 scale).

2.1. Determination of Moniliformin and Fumonisin

Cultures were initially grown on agar slants for 7 - 10 days. A slant was macerated in 27 ml of sterile water. Aliquots of (2.5 ml) of the resulting suspension were added to 250 ml Erlenmeyer flasks containing 50 ml of inoculated medium made up of ultrapure water (1 L), NH_4Cl (3 g), $FeSO_4 \cdot 7H_2O$ (0.2 g). $MgSO_4 \cdot 7H_2O$ (2 g). KH_2PO_4 (2 g) peptone (2 g), yeast extract (2 g) malt extract (2 g) and glucose (20 g) after 48 h of incubation in the dark at 28°C on rotary shaker (220 rpm, 3.81 cm throw), the suspension was macerated and employed as moniliformin [12]. For production of moniliformin 2.5 ml aliquots were added to 250 ml Erlenmeyer flasks containing 50ml of production medium consisting of ultrapure water, $(NH_4)_2HPO_4$ (1 g). KH_2PO_4 (3 g), $MgSO_4$-$7H_2O$ (0.2 g) NaCL (5 g), Sucrose (40 g) and Glycerol (10 g), final pH (6.2). The flasks were covered in aluminum foil to protect the toxin from the light and incubated for 10 days [13].

2.1.1. Moniliformin Analysis

A 500 µl aliquots of culture filtrate was loaded onto a waters sep-pak®RP-18column pre-conditioned with water. The column was eluted with 2 ml of ion-pair solution (990:10 mix of 85:15 water/acetonitrile and 100:48:1.1 MKH_2PO_4/BU_4N^+OH [14]. A 100 µl aliquot was chromatographed on a Lichrosorb®RP-18 250*4.6 MM HPLC with the ion—pair solution described above. The moniliformin was detected with the same detector as above and concentration evaluated by integration at 230 nm. Concentrations were determined by reference to calibration curves established with standard provided by P. Scott of Health Canada [15].

2.1.2. Fumonisin Analysis

The cultured were filtered as above and analysis was performed on each replicate flask respectively as follows: A1 ml aliquot of filtrate was applied to abondElut Certify Π® (200 mg, varian) column preconditioned by aspiring methanol (6 ml) and water (6 ml) under vacuum. The minicolumns were then washed with water (6 ml) and methanol (6 ml). Fumonisins were eluted with 0.1% trifluoroacetic acid (TFA) in methanol (3 ml). Fumonisin was quantified by HPLC as follows [15]. The TFA/ methanol/methanol fraction from the clean-up column was concentrated to dryness and taken up in 1ml of methanol. A20 µL aliquot was transferred to 2 ml vial and dried under stream of nitrogen. The residue was re-dissolved in 100 µL of 0.05 M sodium borate buffer, pH 8.3 (adjusted with 1NHCl). A freshly prepared solution of 4-fluoro-7-nitrobenzenofurazan (NBD-F) [100µL of 22 Mm NBD-F (Molecular probes Inc.) in 95% ethanol] was added. After heating for 70 Secs at 70°C, the solution was quenched in an ice bath and made up to 500 µL with a 1:1 mixture of HPLC mobile phases A(0.05 M NaH_2PO_4/Methanol adjusted to pH 6.3 with 2N NaoH, 1:1) and B (acetonitrile/ H_2O, 8.2). A 20 µL aliquot was injected in duplicate onto aLichrosorb®5 µm RP-18250 MM × 4.6 MM column on a varian vista®5500 HPLC with a varian vista® CDS-401 data system. An 11 min linear geadient of o to 100% B followed by a 2 min plateau was run at a flow rate of 1 mL/min. the derivatized fumonisins were detected by their fluorescence at 490 nm after excitation at 450 nm (detector: water® model 420-E) are agent blank produced two main peaks at 6.3 min and 12.3 min. Concentrations were determined by refernce to calibration curves of fumonisin isolated in our laboratory (Miller *et al*., 1994).

2.2. HPLC Analysis and Method Development

The HPLC system consisted of a ternary solvent pump (Gynkotek model 480), auto sampler (Gynkotek Gina 50), decade electrochemical detector with a glassy carbon electrode (Antec) and a diode array detector (Gynkotek340S). Gynko soft software V5.60 was used to control the HPLC system and for data acquisition and analysis. The equipment was supplied by Dionex Softron (Idstein, Germany). Three columns, *i.e.* Multosphere C18 (3 µm;

125, 4 mm ID), Phenomenex Synergy MAX-RP C12 80A with TMS end-capping (4 µm; 150, 4.6 mm ID) and Phenomenex Synergi Polar RP (ether linkedphenyl phase with polar end-capping) were tested for the chromatographic separation of the above-mentioned substances. The Multosphere column was purchased from CS, Langer wehe, Germany and Phenomenex, Aschaffenburg, Germany supplied the Phenomenex columns. Peak identity was determined by means of retention time and UV spectra that were recorded for all samples in the range 200 - 400 nm.

3. Result and Discussion

Biochemical tests were carried out for studying the production of toxins by *Fusarium* pathogens involved in the causation of malformation. Eight fungi viz. *F. subglutinans*, *F. solani*, *F. oxyspoum*, *F. sterilihyphosum*, *F. proliferatum*, *F. moniliforme*, *F. avena* and *F. chlamydspore* were tested using susceptible Sadekia cultivar as inoculated as apical injection with culture filtrate (**Table 1**). Data pertaining to artificial inoculations revealed that effort to produce disease by apical injection with culture filtrate. For *Fusarium subglutinans* proved to be the dominant fungus with 100% sample's infection. Fungi *F. oxysporum*, *F. sterilihyphosum* and *F. proliferatum* showed moderate infection in induced typical malformation symptoms in inoculated mango seedlings.

Toxin production by *Fusarium* spp: determination of moniliformin and fumonisin

The ability of different *Fusarium* isolates to produce moniliformin was determined by grown the strains on liquid culture media (**Table 2** and **Figure 1**). Moniliformin was the secondary metabolite mostly produced by the *in vitro* cultures of the *Fusarium* isolates analyzed

Table 1. Comparative virulence of selected *Fusarium* isolates on inoculated mango cv. Sedekia seedlings.

Treatment	Injection buds with culture filtrate	
	Disease incidence %	Disease severity
F. subglutinans	100	4.0
F. solani	0.0	0.0
F. oxyspoum	25.0	0.3
F. sterilihyphosum	25.0	2.0
F. proliferatum	25.0	1.3
F.moniliforme	0.0	0.0
F.avena	0.0	0.0
F.chlamydspore	0.0	0.0
LSD	12.0	0.5

Table 2. Moniliform and fumonisin production by *Fusarium* isolates in liquid media.

Isolates	Concentration of moniliformin µg/ml	Concentration of fumonisin µg/ml
F. oxysporum	6.30	ND
F. proliferatum.	4.10	0.64
F. avenacum	3.88	0.16
F. chlamydospore	0.60	ND
F. subglutinans	8.51	0.50
F. sterilihyphosum	1.10	ND
F. solani	ND	0.10
F. moniliforme	6.90	8.30

ND = not detected.

using HPLC. A evaluated for moniliformin production, seven isolates were toxin producers, the production levels ranging from 0.51 to 8.90 µg/ml. There were found differences in moniliformin production among the *Fusarium* dependent on the species.

The higher levels were produced by *F. subglutinans* (8.51 µg/ml). Moderate concentrations of moniliformin was produced by *F.moniliforme* (6.90 µg/ml), *F. oxysporum* (6.30 µg/ml), *F. proliferatum* (4.10 µg/ml) and *F. sterilihyphosum (*1.10 µg/ml). The lowest concentration was obtained by *F. chlamydospore* (0.60 µg/ml).

Fumonisin was the secondary metabolite mostly produced by the *in vitro* cultures of the *Fusarium* isolates analyzed using HPLC (**Table 2** and **Figure 2**). A evaluated for total fumonisin production (FB1 + FB2), seven isolates were toxin producers, the production levels ranging from 0.10 to 8.30 µg/ml. There were found differences in fumonisin production among the *Fusarium* dependent on the species. The higher levels were produced by *F. moniliforme* (8.30 µg/ml). Moderate concentrations of moniliformin was produced by *F. proliferatum* (0.64 µg/ml) and *F. subglutinans* (0.50 µg/ml), The lowest concentration was obtained by *F. solani* (0.10 µg/ml).

F. subglutinans and *F. moniliforme* appear to have high level and different toxin profiles. This suggests that thèse fungi can cause malformation, necrosis via the production of phytotoxic metabolites. Some isolates of the species *i.e. F. subglutinans*, *F. sterilihyphosum*, *F. oxysporum* and *F. proliferatum* that were highly toxic to mango seedlings and produce moniliformin, suggesting that this toxins can be involved. *Fusarium* mycotoxins continue to occur in agricultural commodities as a result of fungal contamination hence presenting serious animal and human health problems. Various *Fusarium* species

have recently been found to produce several mycotoxins as moniliformin [9,11]. Moniliformin is formed in many cereals by a number of *Fusarium* species that include, besides *F. moniliforme*, *F. avenaceum*. *F. subglutinans*, *F. proliferatum* and others [4]. Malformin-like sub-

stances are somehow involved in the causation of malformation, with malformin-stimulated ethylene production [10] causing a hormonal imbalance and consequently disturbed metabolism inducing malformation.

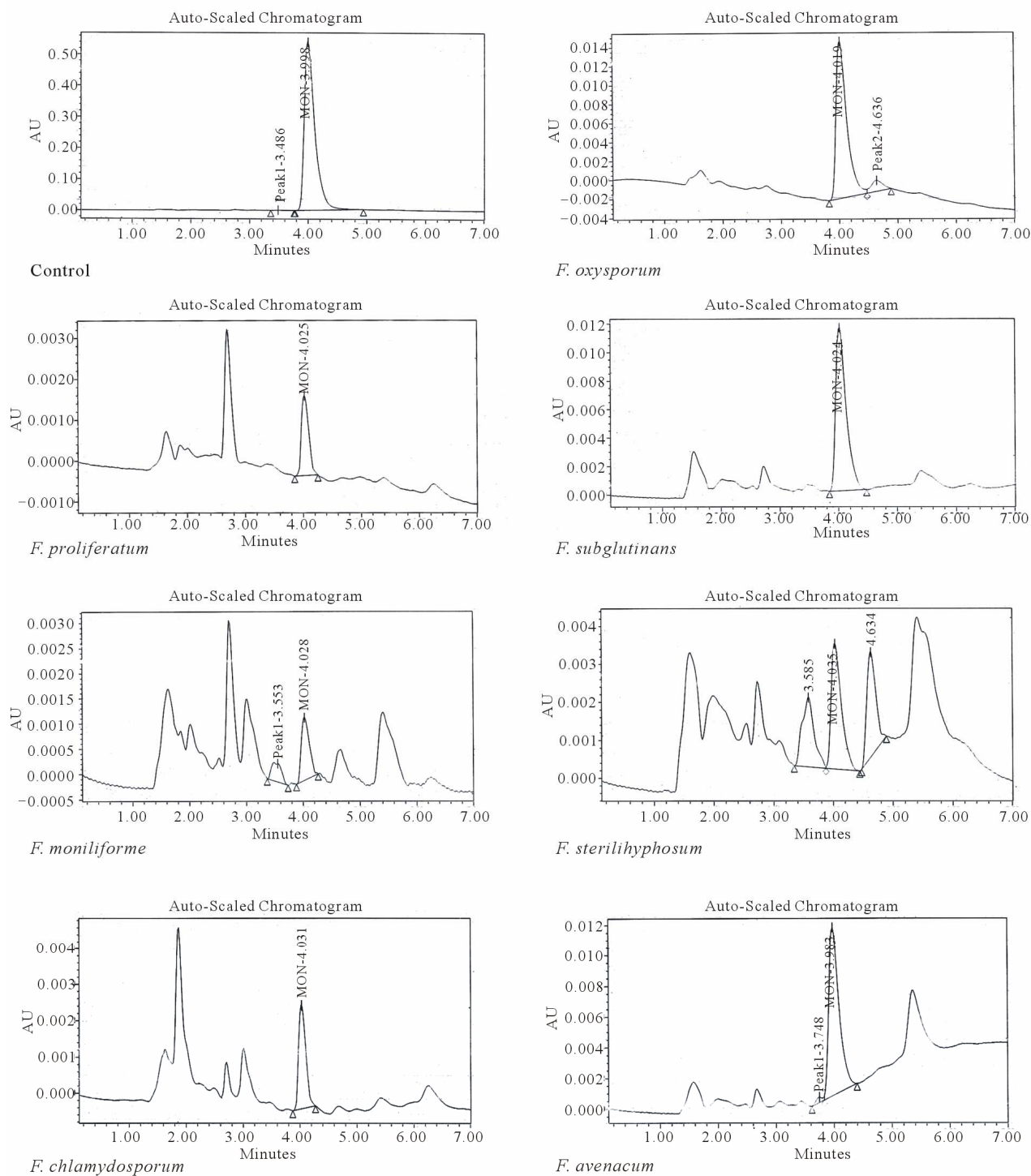Related, recently described species that have been

Figure 1. HPLC of moniliformin production by *Fusarium* isolates in culture media.
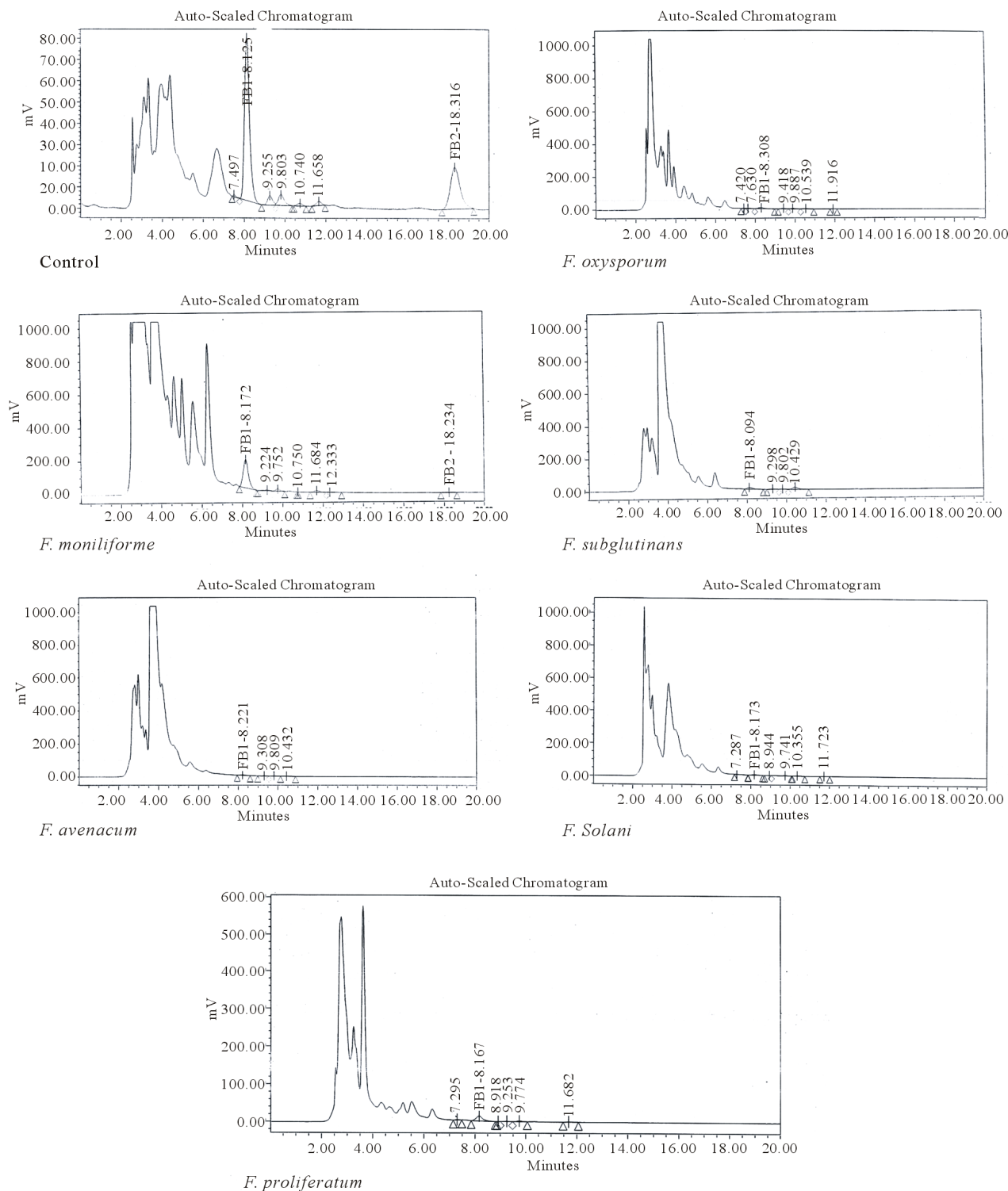
Figure 2. HPLC of fumonisin production by *Fusarium* isolates in culture media.

shown to produce fumonisins are *F. dlamini*, *F. napi-forme* and *F. nygamai* [11]. FB1 concentrations formed by *F. moniliforme* and *F. proliferatum* usually exceed those of FB2. The co-occurrence of these toxins may have synergistic harmful effects on the overall toxicity of the isolates, and may be a greater problem than initially lyte anticipated. Different *Fusarium* species dominated at different stages of development and a good correlation

was found between fumonisins and the presence of *F. moniliforme* and *F. proliferatum*. The occurrence of very high levels of fumonisin B# in some samples was correlated with the presence of strains producing abundant fumonisin B# in the laboratory [16].

4. Acknowledgements

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