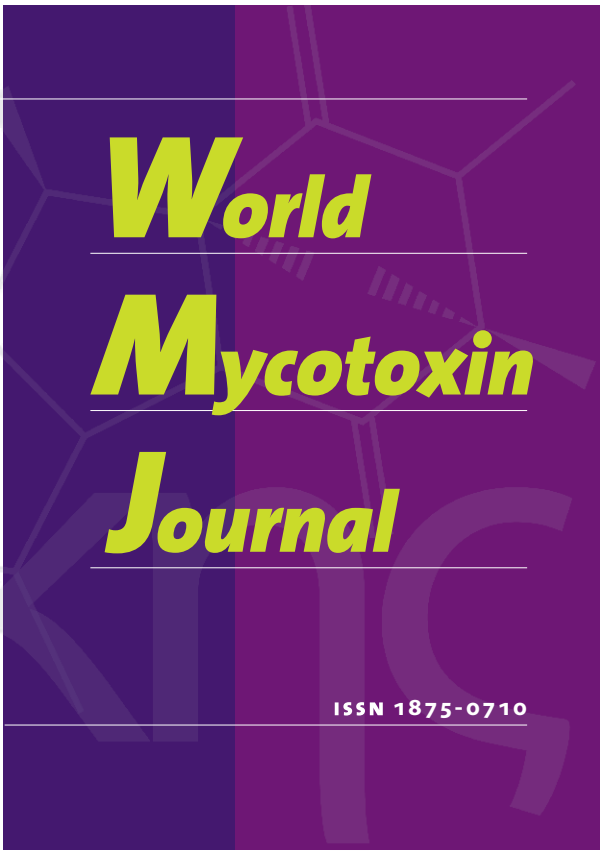

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Early detection of toxigenic *Fusarium graminearum* in wheat

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Abstract

Fusarium graminearum (Schwabe) contaminates agricultural crops and commodities with trichothecenes, mostly deoxynivalenol and its acetyl-derivatives. Current techniques available to detect final mycotoxin contamination products usually require an extended time lag between sampling and the corresponding report, and include different clean-up steps and eventually derivatisation. This study was aimed to develop a methodology to detect toxigenic *F. graminearum* prior to mycotoxin production. Headspace solid-phase microextraction coupled to capillary gas chromatography is shown to be useful to predict the potential of trichothecene mycotoxin formation by detecting the presence of *F. graminearum* at early stages of fungal growth in wheat cultivars, based on the detection of trichodiene (TRI), the volatile intermediate of trichothecenes. We showed that TRI is a useful marker to detect toxigenic *Fusarium* in wheat spikes from live plants, regardless of the actual development of *Fusarium* head blight (FHB). This is the first predictive methodology for FHB and trichothecene occurrence in field-collected samples. It might be a useful tool to help to prevent the risk of mycotoxin contamination.

Keywords: *Fusarium* head blight, trichodiene, volatile organic compounds, solid phase microextraction

1. Introduction

Success in cereal production is strongly related to quality control assessment both during pre-harvest and post-harvest handling and storage. The Food and Agriculture Organization of the United Nations (FAO) estimates that only at the stage of commercialisation there are close to 25% annual losses of the world production, rising to over 50% loss in less developed countries, both due to contaminant fungi and insect pests that affect grain overall quality (Mannon and Johnson, 1985). Within the sustainable agriculture paradigm new and ecologically safe alternatives against these pests are needed. With regard to fungal contamination at the pre-harvest stage, it is of utmost relevance to develop diagnostic tools for early detection of toxigenic fungi, aiming to help prevent/reduce contamination in the food chain. The ascomycete *Fusarium graminearum* is the major phytopathogenic agent of *Fusarium* head blight (FHB), a devastating disease that reduces crop yield and quality, contaminating wheat, barley and other crops with mycotoxins around the world (Miedaner *et al.*, 2008).

Mycotoxins are fungal metabolites that can cause acute and chronic toxicity either by ingestion, inhalation or dermal absorption. Trichothecene mycotoxins comprise two major groups of toxins, type A and B. Deoxynivalenol (DON), its acetyl-derivatives, and nivalenol (NIV) belong to the latter group. These mycotoxins affect animal and human health by inhibition of protein, DNA, and RNA synthesis, and deleterious effects on cell division and membranes (Rocha *et al.*, 2005). DON is also phytotoxic to a wide variety of plants, causing growth retardation, inhibition of seedling and green plant regeneration (Bruins *et al.*, 1993; McLean, 1996). Coleoptile growth of wheat cultivars was shown to be inhibited by DON; the inhibition degree was correlated to the levels of FHB resistance (Wang and Miller, 1999). Many *Fusarium* species are well known trichothecene-

producing fungi. Among the most aggressive species are *F. graminearum, Fusarium culmorum* and *Fusarium poae* (Liddell, 2003). Infection and further fungal colonisation of cereal heads affects crop yield, while major concern can arise by the potential trichothecene contamination of grain (Jennings *et al.*, 2004). Trichothecenes are difficult to remove and chemically stable, surviving storage and processing, even at high baking temperatures (Bullerman *et al.*, 1984).

FHB occurrence relies heavily on environmental conditions; warm temperatures and high humidity during a critical period around anthesis strongly favour pathogen infection (Osborne and Stein, 2007; Parry *et al.*, 1995). To date, FHB prediction in specific areas is based mostly on statistical data based on meteorological variables and history of previous infections in the area. FHB risk estimation can help to detect potential areas of disease development. Prediction models are developed for different wheat regions of Argentina, Canada, USA and Europe (Moschini and Fortugno, 1996; Prandini *et al.*, 2009). The use of such models may be helpful for decision-making purposes: to prevent/reduce yield losses and hazards for human and animal health based on the correct timing for chemical spray. They may also be helpful to predict the final level of contamination and better organise post-harvest management. However, no information on DON detection has been incorporated in available models to date.

Wheat grain is usually harvested 1-2 months after anthesis and grain filling, stages at which plants are more susceptible to *Fusarium* infection. Current methods to detect post-harvest *Fusarium* contamination are based on the direct detection of trichothecene mycotoxins in already contaminated food (Siegel and Babuscio, 2011; Turner *et al.*, 2009). In spite of the high sensitivity that can be achieved, they are useless at a predictive level. In the laboratory, trichothecene detection in grain and grain-derived products can be achieved with high sensitivity by a variety of techniques, among them, different chromatographic analyses (GC-MS, HPLC-MS, TLC), near-infrared spectroscopy, ELISA, and others (Eifler *et al.*, 2011). Most of these techniques require clean-up and/or derivatisation steps. The latest approach in post-harvest analyses is to detect contaminated grain samples by measuring DON levels using an electronic nose (Campagnoli *et al.*, 2011). This methodology seems to be very promising, circumventing purification/extraction steps.

Alternatively, a different approach is to detect fungal presence in stored grains. To this end, *Fusarium* detection by ELISA became popular because of easy use and relatively low cost, although with some restrictions for quantitative estimation or species differentiation (Iyer and Cousin, 2003). Quantitative PCR seems to be the ultimate method for *Fusarium* detection, although with relatively higher cost (Eifler *et al.*, 2011). So far, *Fusarium* detection can be done by both methods, either in the laboratory or in the field, although this approach has not been addressed yet. Fungal volatile organic components (VOC) are secondary metabolites proposed for many years as potential indicators of fungal activity that might be useful for early detection of grain spoilage, as reviewed by Magan and Evans (2000). In *Fusarium* spp., trichodiene (TRI) and other sesquiterpenes, together with minor amounts of short chain alcohols, esters, ketones, and hydrocarbons have been reported as the major VOC components (Demyttenaere *et al.*, 2004; Jelen *et al.*, 1997). TRI is the key metabolic intermediate to trichothecene formation (Beremand and Mc Cormick, 1992; Demyttenaere *et al.*, 2004; Tamm and Breitenstein, 1980). Based on VOC trapping from the headspace of fungal cultures and capillary gas chromatography (CGC) analysis, TRI was proposed as a volatile marker for trichothecene formation, and detection of toxigenic species. In these studies, the most popular method to measure volatile production was headspace solid-phase microextraction (HS-SPME) coupled to CGC (Demyttenaere *et al.*, 2004; Jelen, 2003; Jelen *et al.*, 1995, 1997, 2000; Perkowski *et al.*, 2007). *Fusarium* VOC profiling by SPME has also been used for screening fungal species isolated from infested wheat cultivar areas in Argentina (Girotti *et al.*, 2010). Among other techniques, an electronic nose has also been used to detect *Fusarium* volatiles in stored grains (Eifler *et al.*, 2011; Olsson *et al.*, 2002; Perkowski *et al.*, 2008; Presicce *et al.*, 2006).

The aim of this study was to develop a predictive methodology for trichothecene contamination at the pre-harvest level by detecting the presence of toxigenic *F. graminearum* in wheat cultivars, performing qualitative and quantitative evaluation of the volatile sesquiterpene production of different isolates, and measuring the dynamics of TRI production in wheat spikes by HS-SPME coupled to CGC.

2. Materials and methods

Fungal isolates

Toxigenic *F. graminearum* isolates were obtained from grain samples of common (*Triticum aestivum* L.) and durum wheat (*Triticum durum* Desf.) collected from different localities in the province of Buenos Aires (33°54' S 60°57' W to 34°52' S 57°58' W): Chacabuco (CH), General Alvarado (GA), Las Flores (LF), Rauch (R) and San Cayetano (SC). Grains were plated on potato dextrose agar medium (PDA) 2% (w/v) supplemented with 250 mg/l of chloramphenicol and 600 mg/l of pentachloronitrobenzene (75% wettable powder), and incubated at 25 °C for 5 days. A single spore culture was obtained from each isolate, subcultured on PDA, and maintained on vaseline at 4 °C.

For taxonomic identification, the isolates were placed on carnation leaf agar, incubated under fluorescent light at 22°C for 7 to 14 days, and identified based on culture features and conidial ontogeny, as suggested by various authors (Booth, 1971; Leslie and Summerell, 2006). The identity of the isolates was confirmed by running a species-specific PCR using the primer pair Fg16NF (ACAGATGACAAGATTCAGCGACA) and Fg16NR (TTCTTTGACATCTGTTCAACCCA) (Nicholson *et al.*, 1998). *F. graminearum* isolates used in this study were: UM.CIDEFI.Fg0039 (CH), UM.CIDEFI.Fg0042 (GA), UM.CIDEFI.Fg0048 (LF), UM.CIDEFI.Fg0135 (R), UM.CIDEFI.Fg0147 (SC) and as a control strain UM.CIDEFI.Fg0053 (CS) from the Mycology Collection of CIDEFI. These isolates were used as inoculum in the laboratory and field experiments.

Fungal cultivation

Fungal cultures on potato dextrose agar

Forty-ml vials containing 10 ml of sterile PDA were inoculated with a PDA plug (5 mm diameter) containing *F. graminearum* CS mycelium from the actively growing edge of a 7-day old culture (n=11). Vials only containing non-inoculated PDA medium were used as controls (n=3). All vials were sealed with a septum-containing lid and maintained at 26 °C in the dark until analysis.

Fungal cultures on rice

PDA plugs of 5 mm diameter were obtained from the actively growing edge of 7-day old culture isolates from different localities (CH, GA, LF, R, SC) and CS. They were cultured in 500 ml glass flasks with an aluminium perforated lid sealed with a rubber septum (Red septa 11 mm for 6890 GC; Agilent, Santa Clara, CA, USA) containing 50 g of sterile rice (RH 80%) as substrate (Greenhalgh *et al.*, 1983), there were ten replicates of each isolate.

Fungal cultures on liquid medium

For the production of inoculum to be used in field assays, CH isolate was cultured in Erlenmeyer flasks containing 50 ml of liquid Capellini Peterson medium (Booth, 1971) and incubated at 25 °C under constant agitation for 5 days. Macroconidia were harvested by centrifugation of the culture media at 3,500 rpm and 2 °C; then the spore pellet was suspended in sterile distilled water to a final concentration of ~10,000 spores/ml using a haemocytometer.

Field assays

Field trials were conducted at the Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Buenos Aires Province, Argentina (34° 54' 39" S and 57° 55' 39" W), on a typical Argiudol soil. Cultivars of wheat cv. Klein Chajá were seeded in 7 row plots (3 m length and 1.4 m width), at a density of 350 plants/m^2. At anthesis (Zadoks growth stage 65) (Zadoks *et al.*, 1974), 5 µl of the macroconidial suspension of the CH isolate were pipetted between the lemma and palea of a central floret of the spike (Figure 1). After inoculation, spikes were covered with a plastic bag to maintain ~100% RH for 48 h.

In another experiment, the same wheat cultivars were similarly seeded at the Experimental Julio Hirschhorn de la Facultad de Ciencias Agrarias y Forestales, UNLP (34° 59' 03" S and 57° 59' 48" W); in the area selected, occurrence of FHB was shown in previous years. FHB presence was confirmed by PCR as described above.

Headspace solid-phase microextraction sampling

Laboratory samples

Three SPME fibres carboxen/polydimethylsiloxane 75 mm (CAR/PDMS), polydimethylsiloxane 100 mm (PDMS) and polydimethylsiloxane/divinylbenzene 65 mm (PDMS/DVB) supplied by Supelco (Bellefonte, PA, USA) were tested. Fibres were pre-conditioned according to the manufacturer's instructions, and reconditioned before each analysis. Samples and controls were maintained at 25 °C for 20 min, and fungal VOC were extracted by HS-SPME after exposure for an additional 30-min period at 25 °C (Girotti *et al.*, 2010). VOC were analysed during a period between 3 to 15 days post-inoculation (dpi). In further analyses, each isolate was cultured in rice for 7 days and volatiles were extracted by HS-SPME using a PDMS/DVB fibre for 30 min at 25 °C, unless otherwise specified in the text (n=10). The relative abundance of sesquiterpenes versus culture time was compared in CS isolates at different time

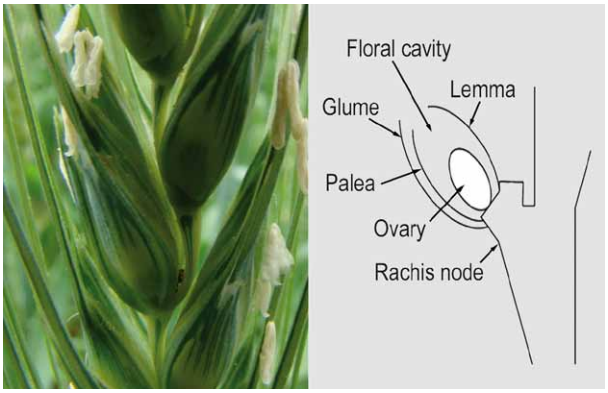

Figure 1. Schematic illustration of a wheat spikelet.

periods (2-22, n=16). The effect of the extraction time on the amount of TRI extracted was compared in CH isolates sequentially exposing the fibre in the same flask for 15, 30 and 60 min (n=12).

Field samples

Pre-inoculated wheat spikes were collected at different time periods (2, 3, 4, 6 and 7 dpi). There were eight replicates for each day, individually placed in 500 ml glass flasks with a lid sealed with a rubber septum, maintained at 26 °C in the dark for 1 day. Then, VOC were extracted by HS-SPME for 2 h at 25 °C.

Naturally infected wheat spikes were also collected during the first stages of grain fill, individually placed in 500-ml glass flasks under the same conditions, and treated as described for the inoculated spikes.

Headspace solid-phase microextraction sample analysis

CGC-flame ionisation detector (FID) analysis was performed using a Hewlett Packard 6890 gas chromatograph employing a non polar DB-5 capillary column (30 m length, 0.32 mm I.D., 0.25 µm film thickness) (J&W, Folsom, CA, USA). The injector was operated in the splitless mode at 250 °C, the oven temperature was programmed (40 °C for 2 min, 10 °C/min to 200 °C, 15 °C/min to 250 °C, with a holding time of 5 min at the final temperature). The FID temperature was set at 280 °C. CGC-MS analysis was performed on a HP 5975C VL Agilent mass selective detector (MSD) coupled to the CGC equipment. The MSD was set in the electron impact mode at 70 eV and operated in SCAN mode with a mass range of 35-650 amu, with the transfer line at 320 °C, the ionisation chamber at 230 °C, and the quadrupole set at 150 °C.

Trichodiene detection and quantitation

F. graminearum volatile sesquiterpenes, including TRI, were identified by HS-SPME coupled to CGC-MS by interpretation of their mass spectral fragmentation, spectral data comparison to MS libraries (NIST/EPA/NIH Mass Spectral Database 2005; Scientific Instrument Services, Ringoes, NJ, USA; Adams, 2007), and comparison of the Kovats retention indices (KI) (Kovats, 1965) with literature data (Demyttenaere *et al.*, 2004; Jelen *et al.*, 1997). Identification was performed as previously reported in this laboratory (Girotti *et al.*, 2010). KI values were calculated after analysis of C6-C20 *n*-alkane series (Supelco) under the same chromatographic conditions. TRI relative abundance was estimated from CGC-FID data as the ratio between the corresponding peak area and the sum of total peak areas between 13 and 16 min.

The amount of TRI and other sesquiterpenes was estimated by comparison of the corresponding peak area with that calculated from a calibration curve obtained after injection of known amounts of pentadecane.

Statistical analyses

The mean values of TRI extracted with different fibres, under different extraction conditions, and dpi in field assays were compared by analysis of variance (ANOVA) followed by Tukey's test ($P<0.05$).

3. Results

Fungal culture analyses

HS-SPME samples of *F. graminearum* volatiles obtained from the different isolates were analysed by CGC-FID and CGC-MS (Figure 2). The sesquiterpene fraction exhibited the characteristic mass spectra for $C_{15}H_{24}$ hydrocarbons, with molecular ion m/z 204. The fragmentation pattern and the KI of the major sesquiterpenes of *F. graminearum* isolates CH, GA, LF, R and SC (data not shown) were coincident with that reported for the CS isolate in previous studies performed in this laboratory, under the same chromatographic conditions as described here (Girotti *et al.*, 2010). The major peak was TRI eluting at 15.37 min with KI 1533, and fragmentation pattern m/z 109(100), 108(46.6), 67(33.9), 93(30.1), 81(17.7), 55(11.4), 41(13.4), 121(8.7) and 204(2.6), which is also in agreement with TRI data from the literature (Demyttenaere *et al.*, 2004; Jelen *et al.*, 1997). Figure 2A shows the VOC fingerprint of *F. graminearum* CS in the elution zone of sesquiterpenes, isolated after 7-days growth in rice and 30 min of HS-SPME sampling.

We also studied whether the relative amount of TRI varied with the culture time post-inoculation. VOCs were sampled by HS-SPME for 30 min from a 7-day old monosporic culture in rice, using a PDMS/DVB fibre (n=16). The relative abundance of TRI and other major volatile components of the CS isolate remained fairly unchanged over a period between 2 and 22 days (Figure 2B), although TRI values ranged from 7.6 to 112.5 ng, respectively. Quite similar profiles were obtained when fungi were grown in PDA, or after inoculation of wheat spikelets, although with lower relative abundance of TRI in PDA (24.61±1.19%, n=11) or wheat spikes (23.30±1.25%, n=16) as compared with the rice cultures (51.56±3.15%, n=6). Regardless the culture medium, TRI was the major component.

The performance of PDMS/DVB, PDMS, and CAR/PDMS fibres was compared in PDA-grown cultures over a period between 3 to 15 dpi (Figure 3). Under the experimental conditions, the total amount of TRI extracted was independent of the fibre used. TRI peaked a maximum at day 7, and then reduced by about 50% of that value from

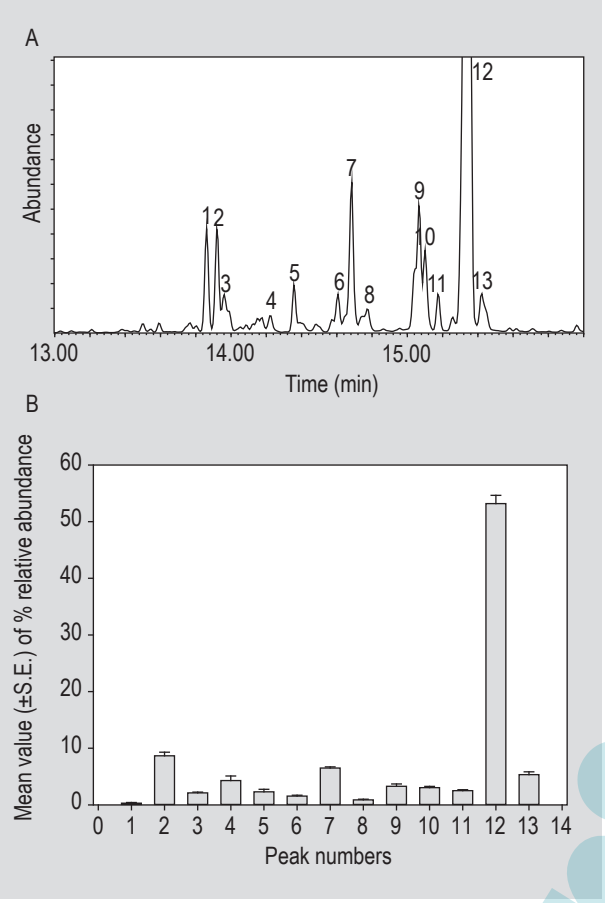

Figure 2. *Fusarium graminearum* volatile sesquiterpenes. (A) capillary gas chromatography-mass spectrometry profile after extraction by headspace solid-phase microextraction. Trichodiene and other sesquiterpenes were identified as previously reported (Girotti *et al.*, 2010); the corresponding Kovats retention indice values were: 1 – 1415 (longifolene), 2 – 1420 (barbatene), 3 – 1426 (unidentified sesquiterpene), 4 – 1444 (isobazzanene), 5 – 1453 ((E)-β-farnesene), 6 – 1473 (10-epi-β-acoradiene), 7 – 1480 (β-chamigrene), 8 – 1484 (aryl-curcumene), 9 – 1513 (β-bisabolene), 10 – 1516 (unidentified sesquiterpene), 11 – 1519 (unidentified sesquiterpene), 12 – 1533 (trichodiene), 13 – 1539 (α-farnesene). (B) Mean value (±SE) of the relative abundance of the major sesquiterpene components extracted with the polydimethylsiloxane/divinylbenzene fibre. Bars represent the mean values of 16 separate experiments.

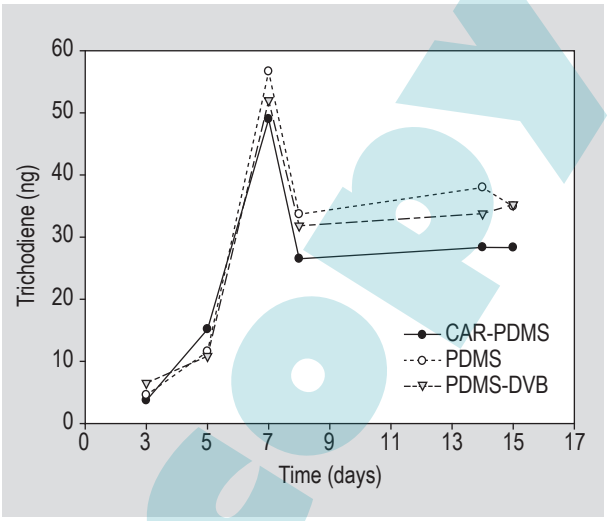

Figure 3. Effectiveness of solid-phase microextraction (SPME) fibres for trichodiene extraction. The amount of trichodiene extracted by headspace SPME with polydimethylsiloxane (PDMS), PDMS/divinylbenzene (DVB), and carboxen (CAR)/PDMS fibres is compared at variable intervals along a 15-day period. No significant differences were detected in the trichodiene amounts extracted with each fibre (P<0.0001).

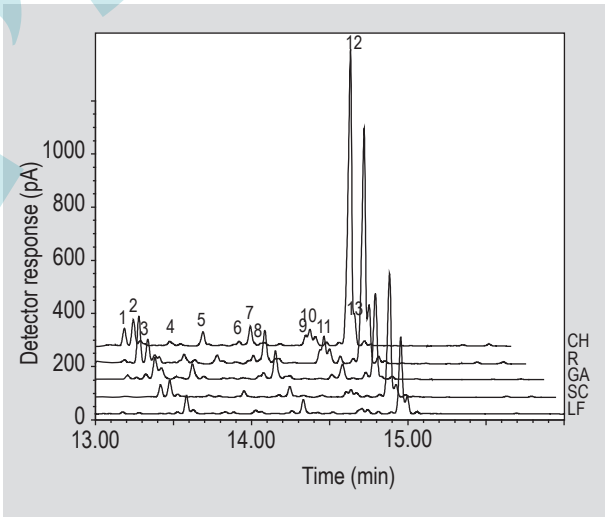

Figure 4. Capillary gas chromatography profiles of *Fusarium graminearum* isolated from different wheat cultivars in the Buenos Aires province. Sampling localities were Chacabuco (CH), General Alvarado (GA), Las Flores (LF), Rauch (R) and San Cayetano (SC). Peak numbers correspond to those in Figure 2A.

day 8 through day 15 post-inoculation. Thereafter, PDMS/DVB fibre was used in all further analysis.

Figure 4 shows the VOC profile of *F. graminearum* isolates obtained from different geographic locations in the wheat cultivated area of Buenos Aires province. The amounts of TRI and the major sesquiterpenes extracted by HS-SPME are compared for the five regions in Table 1.

No qualitative differences were observed by CGC-MS analyses, although the total amount of TRI varied from 19.56±4.20 to 57.41±5.20 ng, and sesquiterpenes ranged from 48.49±3.52 to 126.28±9.15 ng. The amount of TRI extracted differed significantly with the length of the extraction period (P<0.0001), varying from 13.22±1.62 to 23.11±0.20 and 33.02±1.42 ng after 15, 30 and 60 min of SPME extraction of the CH isolate, respectively.

Table 1. Amounts of major sesquiterpenes released by toxigenic *Fusarium graminearum* isolated from wheat cultivars collected in Buenos Aires province[1].

		Mean±SE (ng)				
Sesquiterpene	Peak[2]	Chacabuco	Rauch	General Alvarado	San Cayetano	Las Flores
Longifolene	1	16.85±3.08	11.48±3.06	0.75±0.33	4.08±0.58	0.63±0.20
Barbatene	2	8.75±0.92	7.79±1.50	3.86±1.17	4.78±0.80	4.99±0.87
Unidentified sesquiterpene	3	3.23±0.57	2.88±0.73	3.08±3.08	0.88±0.23	2.56±0.37
Isobazzanene	4	3.42±1.19	4.03±1.73	2.14±1.15	1.05±0.22	4.94±1.26
(E)-β-farnesene	5	3.15±0.38	3.41±1.33	0.53±0.34	2.22±0.48	0.67±0.32
10-epi-β-acoradiene	6	2.26±0.23	2.07±0.29	1.57±0.44	0.80±0.21	1.18±0.26
Aryl-curcumene	7	9.29±0.95	9.36±1.35	7.45±1.75	3.21±0.74	4.83±0.79
β-chamigrene	8	1.25±0.17	1.64±0.40	0.37±0.24	0.39±0.19	0.26±0.17
β-bisabolene	9	5.80±0.74	5.73±0.57	3.77±0.96	2.73±0.57	2.27±0.67
Unidentified sesquiterpene	10	4.08±0.57	3.29±0.45	2.78±0.84	1.64±0.28	1.29±0.31
Unidentified sesquiterpene	11	1.71±0.39	1.02±0.39	0.79±0.45	0.88±0.27	0.15±0.15
Trichodiene	12	57.41±5.20	51.25±13.88	19.85±4.50	43.05±5.20	19.56±4.20
α-farnesene	13	9.09±1.49	9.28±0.91	6.61±1.95	3.34±0.75	5.15±1.34
Total		126.28±9.15	113.24±10.87	53.54±4.90	69.06±3.19	48.49±3.52

[1] Data were obtained by headspace solid-phase microextraction and capillary gas chromatography analyses, as described in Materials and methods.
[2] Peak numbers correspond to those in Figures 2A and 4.

Toxigenic *Fusarium graminearum* detection in wheat spikes

After inducing the disease in wheat plants cultivated in field plots, the sesquiterpene profile obtained from one inoculated wheat spike showed a similar profile to that obtained from the fungal plate cultures, although with a smaller peak size (Figure 5, black line). The profile of a non-inoculated (control) wheat spike is also shown (Figure 5, in grey); no TRI was detected. Some minor unidentified components were also present in the wheat spikes.

We also explored the relationship between the time elapsed after inoculation and the detector response. Table 2 shows the amounts (ng) of TRI released from day 2 to day 7 post-inoculation. TRI was detectable from day 2, increasing approximately two-fold at day 4; after that period, the amounts released remained fairly constant up to day 7. Figure 6 shows fungal development on a wheat spike at different time periods after inoculation. No symptoms were detected after 2 days (Figure 6A); hyphae development from the inoculation site is evident after 7 dpi (Figure 6B).

Naturally infected spikes showed a quite similar CGC profile to that of artificially inoculated spikes, with 0.94±0.36 ng (n=4) of TRI measured at 1 dpi. Successive measurements of TRI content in the same flasks at 2 and 5 dpi were 1.80±0.59 ng and 4.57±0.53 ng, respectively.

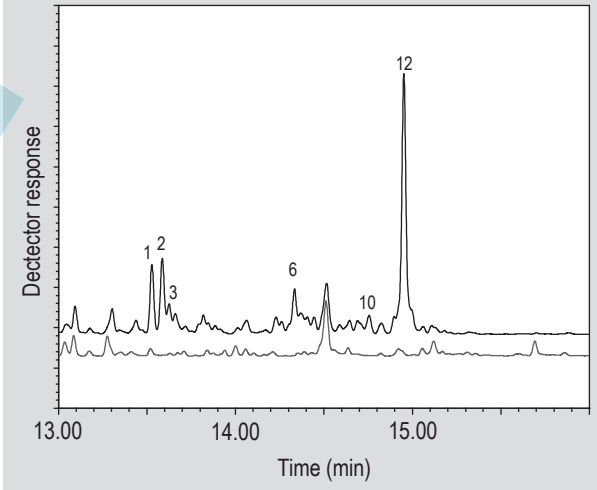

Figure 5. Capillary gas chromatography profiles of the volatile sesquiterpenes released from a non-inoculated (grey) and an inoculated wheat spike (black), after headspace solid-phase microextraction. Two days after inoculation, the inoculated and non-inoculated spikes were cut off and analysed as described in Table 2. Peaks numbered correspond to those detected in the fungal sesquiterpene profile shown in Figure 2A. Unidentified components released from wheat spikes eluted with Kovats retention indice values 1398, 1401, 1415, 1498 1551 and 1599, both in the inoculated and non-inoculated spikes.

Table 2. Trichodiene extracted from inoculated wheat spikes[1].

Days post-inoculation	Trichodiene mean±SE (ng)
2	1.36±0.33
3	1.41±0.41
4	2.84±0.38
6	2.66±0.43
7	2.16±0.44

[1] Spikes (n=8) were cut off after different days post-inoculation, each sample was analysed one day after maintaining at 26 °C in the dark, and headspace solid-phase microextraction sampling for 2 h.

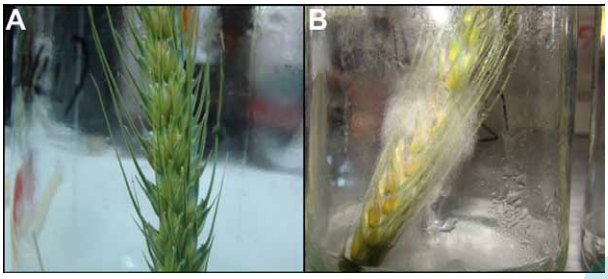

Figure 6. Wheat spikes. (A) Inoculated wheat spike after 2 days at 26 °C in the dark. (B) Same wheat spike (as in Figure 6A), showing *Fusarium* growth from the inoculation point, 5 days later.

4. Discussion

Early detection of TRI in the volatiles released by trichothecene-producing strains of *Fusarium sambucinum* and its absence in cultures of non-toxigenic strains led to propose TRI as a potential volatile marker to monitor fungal contamination (Jelen *et al.*, 1995). In the last decade, the use of fungal secondary volatile metabolites as indicators of food spoilage or contamination has been extensively addressed (Demyttenaere *et al.*, 2004; Jelen, 2003); Although there is general consensus on the role of TRI as a predictive marker, no information was available on its potential to detect the occurrence of trichothecene-producing *Fusarium* in the field before or at the initial stages of infection.

Analyses of *F. graminearum* VOCs by HS-SPME coupled to CGC showed that TRI was the major component in the sesquiterpene elution zone between 13 and 16 min (Figure 2A), regardless the time after inoculation (Figure 2B), the strain origin (Figure 4, Table 1) and the incubation media. Different fibre coatings tested showed no significant differences in the amount of TRI extracted at different time intervals between 2 and 15 dpi. The total amount extracted varied from ~5 ng at 2 dpi, peaked about 10 times that value (~50 ng) at 5 dpi, then decreased to 25-35 ng at longer periods post-inoculation (Figure 3). The reduction in VOC production from day 8 to longer periods was probably due to restricted fungal growth caused by O_2 depletion in the sealed vial (Greenhalgh *et al.*, 1983; Hutchinson, 1973).

Toxigenic *F. graminearum* isolates showed no structural differences in their volatile sesquiterpene components and their profiles were also qualitatively consequent with previous data (Girotti *et al.*, 2010). However, different isolates showed variable amounts of TRI. Although in a complex mode, variation in TRI amounts has been shown to be related to different aggressiveness of the isolates. The contribution of *Fusarium* mycotoxins to virulence was analysed by generating trichothecene-deficient mutants of the fungus after disruption of the gene *tri5* that encodes TRI synthase, the enzyme responsible for the first step in trichothecene biosynthesis. The resulting *tri5*$^-$ strains exhibited reduced virulence on wheat seedlings, although this response was variable depending on the host (Proctor *et al.*, 1995a) and the chemotype of the isolate (Maier *et al.*, 2006). The correspondence between TRI and trichothecene level (DON + NIV) was earlier shown by Perkowski *et al.* (2003) in naturally contaminated wheat grain samples. Thus, further assays for TRI quantitation from wheat field samples might help to determine its potential use as virulence marker.

Here we report the first evidence of TRI detection at early stages of infection in wheat plants, based on a simple protocol to collect volatiles by HS-SPME. *F. graminearum* spores germinate on the internal surface of the glumes and in the floral cavity within 12 h post-inoculation (Figure 1) (Pritsch *et al.*, 2000; Wanjiru *et al.*, 2002). The first microscopic indication of fungal colonisation in the palea was reported at 2 dpi (Brown *et al.*, 2010), although limited to subcuticular and intercellular hyphal growth. Using the green fluorescent protein (GFP) gene fused to the promoter of the TRI synthase gene *tri5*, the first fluorescence signals of the GFP were visible at 2 dpi and complete colonisation of the developing kernel was evident at 3 dpi (Ilgen *et al.*, 2009). At the same time, within the inoculated spikes, hyphae were still absent from the rachis node until 5 dpi (Pritsch *et al.*, 2000; Wanjiru *et al.*, 2002). Interestingly, the volatile sesquiterpene profiles of *F. graminearum* inoculated spikes at early stages of infection – when disease symptoms are not yet visible in the spike (Figure 5 and Figure 6A) – were quite similar to those obtained from laboratory cultures and those from naturally infected spikes (Girotti *et al.*, 2008) in the same elution zone.

We have shown that the presence of TRI in spikes is a strong indicator of toxigenic fungal occurrence. The amount of TRI released from infected wheat spikes after 2-3 dpi significantly increased two-fold after 4 to 7 dpi (Table 2). Ilgen *et al.* (2009) showed that there are two relevant stages during spike infection: colonisation of the developing kernel and invasion of the rachis node (between 4-7 dpi).

They also showed an increase in the expression of the TRI synthase (*tri5* gene) and correspondingly, in trichothecene biosynthesis. Thus, TRI detection in field collected samples can help detect the presence of toxigenic *Fusarium* in green wheat plants before grain formation (Girotti *et al.*, 2008). TRI can be detected in a single spike (>1 ng/spike) soon after colonisation (≥2 dpi); higher signals can be obtained by measuring TRI a few more days post-infection or by increasing the sample size (more than one spike per flask).

5. Conclusions

This is the first study addressing the early detection of trichothecene-producing *F. graminearum* in field samples based on the presence of TRI. The present methodology might help detect the toxigenic fungi in wheat cultivated areas at any stage during spike formation and flowering and also during grain storage and transport. Detection of the fungi in the spike does not depend on climatic conditions favourable to disease development. Under the conditions assayed, the minimum time required from collection to analysis was at least of 26 h. The extraction time depends on the period allowed for infection development; ranging from at least 120 min (~24 h post infection) to 15 min (≥48 h post infection, when fungal growth in the spikelets can be visually observed in the sealed container). Detection time can be shortened by increasing the extraction temperature, and/or the sample size (number of spikes per container) or the flask volume.

We have shown that the analysis of TRI in field samples is feasible and reliable, even without actual wheat infection or evidence of disease development. Thus, this is a predictive methodology that might allow to infer the potential development of FHB in wheat based on the presence or absence of TRI.

Here, we propose a change in the paradigm of the prevention of mycotoxins in food based on an early detection methodology to evaluate fungal presence in the field before the actual occurrence of detectable mycotoxin levels. Future comparative studies among available techniques might help show advantages and disadvantages of each one. Early toxigenic *Fusarium* detection can help improve existing FHB predictive models and might turn into a pre-harvest tool to reduce mycotoxin contamination in grain and derived products. The sesquiterpene chromatographic pattern was shown to help detecting trichothecene-producing *Fusarium* spp. as well (Girotti *et al.*, 2010). Thus this method might be useful to detect other FHB-producing *Fusarium* species.

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