Genetic approaches to chemotype determination in type B-trichothecene producing Fusaria

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Abstract
This review summarises the genetic methods used for chemotype determination of the main Fusarium type B-trichothecene producing species. Literature on Fusarium chemotype epidemiology over the last 15 years is reviewed in order to describe temporal and spatial chemotype distribution of these fungi worldwide. Genetic approaches used for chemotype determination are also reviewed and discussed, highlighting successes and potential pitfalls of the technique. Results from both genetic and chemical approaches are summarised to compare reliability, advantages and limitations of the two methods. Potential applications of genetic chemotyping to toxigenic Fusarium species are evaluated in the light of improving food safety of agricultural products. The use of chemotype determination in population studies, toxin prediction as well as for breeding purpose is described.

Keywords: TRI genes, deoxynivalenol, nivalenol, acetylated deoxynivalenol, Fusarium culmorum, Fusarium graminearum species complex.

Introduction
Among the most studied and harmful toxins produced by Fusarium spp. are the sesquiterpene epoxides trichothecenes, secondary metabolites that inhibit eukaryotic protein synthesis and cause severe toxicosis in humans and other animals upon
ingestion of contaminated grain or their derivatives, affecting intestinal, immune
endocrine and neurologic functions (Maresca et al., 2013). Trichothecenes are also
highly phytotoxic and play a role in virulence on the host plants (Arunachalam and
Doohan 2013; Desmond et al., 2008; Ilgen et al., 2009; Proctor et al 2009; Scherm et al.,
2011).
Fusaria may produce different types of toxins depending on differences in the core
trichothecene cluster (TRI cluster), which includes two regulatory genes (TRI6 and
TRI10) and most of the biosynthetic enzymes required for the production of
trichothecenes (Alexander et al., 2009, 2011; Kimura et al., 2003; Lee et al., 2001).
Depending on the species and chemotype the number of functional core genes in the
cluster varies. In F. graminearum, for example, the trichothecene gene cluster consists of
10–12 contiguous genes as well as two other genes, Tri1 and Tri101, which are at
separate loci outside the main cluster. Fusarium trichothecenes can be grouped in two
classes based on the presence (B-trichothecenes) versus absence (A-trichothecenes) of
a keto group at the C-8 position (Ueno et al., 1973). The difference is due to catalytic
divergence of the cytochrome P-450 enzymes encoded by Tri1. While in F. graminearum
Tri1p oxygenates both C-7 and C-8 (which results in a hydroxyl at C-7 and a carbonyl at
C-8), in F. sporotrichioides, only C-8 is hydroxylated by Tri1p (Rep and Kistler, 2010).
Among type B-trichothecenes, those having a significant impact on safety issues are:
deoxynivalenol (DON), nivalenol (NIV), and their acetylated derivatives 3-
acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and 4-
acetyldeoxynivalenol (4-ANIV, syn. fusarenone-X).
Based on the type of trichothecene produced, different chemotypes have been described
so far for Fusarium species: chemotype I, producing DON and/or its acetylated
derivatives, and chemotype II, producing NIV and/or 4-ANIV (Sydenham et al., 1991).
The DON chemotype can be further split into chemotype IA (producing 3-ADON) and IB
(producing 15-ADON; Miller et al., 1991). The intact gene cluster in F. graminearum
results in strain producing NIV where TRI13 cytochrome P450 monoxygenase and TRI7,
the associated acetyltransferase, catalyze the C-4 hydroxylation and acetylation. In
DON/ADON producers, TRI13 and TRI7 show insertions and deletions which determine
the lack of functional enzymes able to hydroxylate in C-4 and transacetylate (Lee et al.,
2002). The acetylation position determining the 3-ADON or 15-ADON seems to be
caused by differential activity of TRI8 which encodes for a C-3 esterase (Alexander et
al., 2011).
Structural differences among toxin chemotypes may have relevant consequences on strain fitness, since the specific pattern of oxygenation and acetylation can modify the bioactivity and hence the (phyto)toxicity of these compounds (Alexander et al., 2009, 2011; Brown et al., 2002, 2004; Ward et al., 2002; Lee et al., 2002).

The discovery of a vast array of secondary metabolites produced by *Fusarium* species has fostered surveys of mycotoxin diversity in many different epidemiological and agricultural conditions. Surveys are routinely conducted in different geographic areas (Barros et al., 2012; Clear et al., 2000a, 2000b; Del Ponte et al., 2012; Desjardin et al., 2000; Edwards, 2009; Giraud et al., 2010; Goertz et al., 2010; Kim et al., 1993; Ok et al., 2011, 2014; Park et al., 2005; Seo et al., 1996; Tanaka et al., 1986; Vanheule et al., 2014; Wagacha et al., 2010; Yoshizawa and Jin, 1995) to identify major toxigenic risks in affected grains. Indeed, trichothecenes are continuously found in cereals and derived food products around the world (Adejumo et al., 2007; Bosch et al., 1992; Gonzales et al., 2008; Nielsen et al., 2014; Poapolathep et al., 2008; Roscoe et al., 2008; Scudamore and Patel, 2009). DON and NIV now represent the two major concerns for safety of wheat and barley products, being the two most abundant toxins detected, as recently reported in a large survey on Canadian grains (Tittlemier et al., 2013).

The purpose of this review is to summarise genetic methods used for chemotype determination of type B trichothecene producing *Fusarium* spp. Papers published during the last 15 years and reporting on the chemotype identified for sets of isolates were selected, and information on the investigated area as well as on the species and crop have been retained to generate a virtual description of known chemotype distribution worldwide. Moreover, potential applications and limits of genetic chemotyping of *Fusarium* are discussed.

We focus on the *Fusarium graminearum* species complex (FGSC, O'Donnell et al., 2000) which presently includes at least 16 species (Aoki et al., 2012), *F. culmorum* and *F. cerealis* ( Cooke) Sacc., since these species are considered among the most relevant pathogens on wheat and other cereals (Moss and Thrane, 2004, Osborne and Stein 2007). Production of a type B trichothecene (nivalenol) has been reported also from *F. poae* (Peck) Wollenw. (Jestoi et al., 2008; Thrane et al., 2004; Vogelsgang et al., 2008b) and *F. equiseti* (Corda) Sacc. (Kosiack et al., 2005). However, since they rarely produce significant amounts of other trichothecene B toxins (Kristensen et al., 2005), genetic chemotype determination does not offer additional valuable information and it is therefore not treated in detail here.
Part I.

Why determine the chemotype of an isolate?

Determining the chemotype of an isolate is carried out for two main reasons: 1) to obtain epidemiological information on the population colonising a crop in a given area, using chemotype as a proxy in the field;

2) to inform on the toxigenic risk that the presence of a certain chemotype may determine on the food or feed that is produced, with the long term perspective of developing preventive models to decrease the toxigenic risk.

Ward et al. (2002) demonstrated that polymorphism within TRI genes is trans-specific and appears to have been maintained by balancing selection acting on chemotype differences. Different trichothecene-type isolates do not just have different trichothecene profiles but can in some instances be regarded as different genetic populations (Mishra et al., 2009), even if they co-occur within the same area and some gene flow may take place between them as shown using VNTR and RFLP markers (Gale et al., 2007; Karugia et al., 2009b; Ward et al., 2008). Gene flow between different populations, yet within a species, seems comparatively limited even if they co-exist, although the factors that inhibit gene flow between populations in the same area are unknown so far (Karugia et al., 2009b). This original observation leads to the idea that monitoring chemotype diversity can be informative for characterising a field population. Indeed, the evolutionary dynamics of the core trichothecene cluster were demonstrated to be essentially uncoupled from the rest of the genome (Ward et al., 2008, Proctor et al., 2009).

However, as long as recombination frequency is low, chemotype could be considered as a marker for a genomic background specific to populations or individuals that are distinguished by a variety of phenotypic traits beyond chemotype. Because trichothecene production is associated with the spread of the disease after initial infection in wheat (Mesterházy, 2002), and trichothecene production is a factor affecting not only FHB but also seed diseases (Wang et al., 2006), finding a population with higher toxin production may suggest a stronger impact of the disease. For example, F. graminearum populations with 3-ADON chemotype seem to have a higher average toxigenic capacity in wheat and barley (as well as growth rate) in North America compared to NIV and 15-ADON populations (Foroud et al., 2012; Gilbert et al., 2010; von der Ohe, 2010; Ward et al., 2008). Conversely, 3-ADON populations do not differ for pathogenicity and sexual reproduction in different Fusarium species from different...
locations (Alvarez et al., 2010; Gilbert et al., 2010; Purahong et al., 2014; Schmale et al., 2011; Spolti et al., 2014b; von der Ohe, 2010). On another set of isolates, differences in the aggressiveness among chemotypes were reported by Malihipour et al. (2012), who suggested a gradient of aggressiveness from NIV to 15-ADON to 3-ADON chemotypes.

Analysing populations carrying the NIV chemotype compared to local DON populations, lower virulence for the NIV populations were observed in *F. asiaticum* from China (Puri et al., 2012; Shen et al., 2012; Zhang et al., 2012), in *F. graminearum* (Foroud et al., 2012; Miedaner et al., 2008), as well as in *F. culmorum* in rye (Miedaner and Reinbrecht, 2001). On the contrary, NIV population did not differ in pathogenicity to its DON population counterpart when two different pathogenicity scorings were used (Purahong et al., 2014). Discrepancies between results on aggressiveness among chemotypes can be attributed to the use of chemotype as a proxy of a population. Depending on the gene flow and variability of a certain population in a sampled area, pathogenicity characters may or may not be associated with chemotype data. Aggressiveness is a factor being influenced not only by the characters of the pathogen, but also by its interaction with the host and the environment. Indeed, when large set of resistant cultivars were assayed, the underlying genetic resistance seemed cross-applicable between chemotypes (Clear et al., 2013; Foroud et al., 2012; Horevaij et al., 2011; Perkowski et al., 1997).

In order to understand which factors do favour persistence and spread of a chemotype, the hypothesis was formulated that some fitness characters associated with a chemotype can favour its establishment in a given area, as observed in Canada and USA (Puri and Zhong, 2010; Ward et al., 2008), as well as in China for barley (Yang et al., 2008; Zhang et al., 2010a, 2010b) and wheat (Zhang et al., 2012). Indeed, regional difference in chemotype distribution may be influenced by environmental and cultural practices since chemotype variation may confer an adaptive potential to these pathogens and it is likely driven by natural selection: recent migration and introgression were suggested to be the way for new chemotypes with adequate fitness to become established into the resident populations (Desjardins et al., 2008; Gale et al., 2011; Zhang et al., 2012).

Of course chemotype diversity cannot *per se* explain all differences that are observed between groups of isolates with different phenotypic characters.. Comparison between population studies and chemotype description is needed (Wang et al., 2011) to clarify the level of variability of a chemotype within a group.
Working on a *F. culmorum* population from an international collection, Miedaner et al. (2013) showed that isolates with 3-ADON and NIV chemotype have a similar genetic background confirming that gene flow occurs also in *F. culmorum*. One hypothesis on the stability of *F. graminearum* 15-ADON chemotype status in New York state is that the original character favouring the spread of the 3-ADON chemotype in North America (Ward et al., 2008) has been transferred to the 15-ADON population, therefore enabling a balance between the two populations (Spolti et al., 2014b). Mechanistic studies are now starting to explore changes by taking into account genetic variability represented by chemotypes at the proteome and transcriptome level (Krishna et al., 2012; Pasquali et al., 2013b) as well as in gene knock-outs (Abou Ammar et al., 2013; Pasquali et al., 2013a). Similarly, the activity of toxin inhibiting compounds has been tested taking into account the different chemotypes (Boutigny et al., 2009, 2010; Kulik et al., 2014). These comparative studies may facilitate the understanding of evolutionary forces acting on the selection of the characters and at the same time may shed light on the mechanisms favouring a certain chemotype in a field, consequently affecting the amount and quality of toxins found in grains.

Among the factors that may have an effect on chemotype selection, fungicides have been suggested by Gale et al. (2007) and their effect was investigated in different papers. With respect to azoles, strobilurins and isopyrazam there seems not to be an effect of chemotype on resistance (Amarasinghe et al., 2013; Beyer et al., 2014; Dubos et al., 2011, 2013; Kulik et al., 2012; Spolti et al., 2014a). On the contrary, carbendazim (MBC) resistance, coupled to higher toxin production (Zhang et al., 2009), seems to be associated with the 3-ADON chemotype in Asia in *F. graminearum* and *F. asiaticum* where MBC sensitivity differed between NIV and DON chemotypes (Zhang et al., 2013a), but this difference was not evident in other studies (Qiu et al., 2014; Wang et al., 2010). Moreover, the 3-ADON chemotype revealed significant advantages over *F. asiaticum* producing NIV, including higher resistance to benzimidazoles (Zhang et al., 2012).

On a small set of isolates, chemotypes differed for fitness characters such as a higher resistance to thermal shock by the 3-ADON chemotype in *F. graminearum* (Vujanovic et al., 2012), or a higher adaptation to oxidative stress by the NIV chemotype (Ponts et al., 2007, 2009). Whether these characters are truly associated with chemotype has to be further investigated on a larger set of isolates.
Environmental factors may influence chemotype success. It has been proposed that chemotype specialisation may be driven by a certain host (Yli-Mattila et al., 2013). NIV-producing isolates were found to be more aggressive towards maize compared to DON-producers (Carter et al., 2002) and were associated, in *F. asiaticum*, preferentially to maize in China (Ndoye et al., 2012). Being NIV a pathogenicity factor in maize (Maier et al., 2006), findings that associate an increase in NIV population in areas where preceding crops was maize (Audenaert et al., 2009; Pasquali et al., 2010, Sampietro et al., 2011) are not surprising, despite this association is not consistent in all sampling worldwide. NIV chemotype has been also associated with rice cultivation (Davari et al., 2013; Gale et al., 2011, Lee et al., 2009; Umpierrez et al., 2013). Similarly Nielsen et al. (2012) reported a higher detection of 3-ADON chemotype in oats compared to barley and wheat in northern European conditions. Other reasons for chemotype spread has been postulated by Lee et al. (2012), suggesting that the persistence of the NIV chemotype in *F. asiaticum* (lineage 6) of FGSC is due to its role as a fitness factor towards other microbial communities. By comparing different species within the FGSC in wheat, Goswami and Kistler (2005) found that NIV was accumulated less abundantly by similarly aggressive isolates, hence speculating on its major toxicity accounting for similar pathogenicity results. It is nonetheless evident that aggressiveness is not due to toxin type and production as showed on *F. culmorum* in barley and rye by Miedaner et al. (2004). Gilbert et al. (2011) reported that under controlled conditions, 3-ADON isolates of *F. graminearum* colonise more abundantly the plants when co-inoculated with 15-ADON at 28°C but not at 20°C. This finding was not confirmed in nursery conditions (Clear et al., 2013) and on a different set of isolates (Spoliti et al., 2014b). By examining weather variables over the 5-year period on a set of field studies, Gilbert et al. (2014) found no correlation between recovery of a chemotype and temperature or precipitation. It seems therefore difficult to find a clear effect of climatic conditions on chemotype selection.

Within a food safety perspective, understanding the toxigenic potential of the isolates collected from a given area (field, region, county, state, and beyond) may help in guiding risk assessment on toxin contamination at the field/regional scale. Two examples that have been proposed to demonstrate the utility of monitoring chemotype are:

1. the appearance of a chemotype able to produce a toxin with higher toxigenicity in a certain environment [e.g., the toxicity of nivalenol compared to deoxynivalenol (Minervini et al., 2004) as well as reports of a NIV-producing population in Luxembourg (Pasquali et al., 2009), US (Gale et al., 2011), Uruguay (Umpierrez-
Failache et al., 2013; *F. asiaticum*, Brazil (Del Ponte et al., 2012) and China (Lee et al., 2001; Zhang et al., 2012; *F. asiaticum*) suggesting the need to monitor for the increased risk of NIV contamination in the grains.

2. The presence of a population of the pathogen that is able to produce on average a higher amount of toxin on a certain crop [e.g., the case of the 3-ADON population in US and Canada (Foroud et al., 2012; Gilbert et al., 2010; von der Ohe, 2010; Ward et al., 2008)].

It is therefore evident that a continuous monitoring of the chemotype situation may well inform on the risk and the type of population that are present over a certain environment. For this reason, a large set of methods were developed and surveys around the world were carried out. This set of data is considered in the second part of the review.

**Part II (methods and surveys)**

*Molecular genetics methods*

Detecting the presence of a certain chemotype requires a chemical method, based on the identification of the product in the substrate or directly in the grain. In some instances chemical analysis can be substituted with antibody-based detection methods, despite reliability of different kits is not fully confirmed and acetylated forms are often cause of cross reactivity phenomena (Tangni et al., 2010). Another complementary method is based on the determination of the genetic structure of the isolate(s) using PCR-derived methods applied to the pure culture or, more recently, also to the whole grain. Chemical analytical methods as well as rapid alternative methods have been revised extensively elsewhere (Cigić and Prosen, 2009; Josephs et al., 2004; Koch, 2004; Köppen et al., 2010; Kraska et al., 2001; Maragos and Busman, 2010; Ran et al., 2013) hence they will not be discussed here.

The history of chemotype diversity studies shows that while earlier works were focused on the strain production biochemistry, the appearance of seminal papers on the genetic determinants of the toxin [the genes involved in trichothecene production, mainly studied in *F. sporotrichioides* (Desjardins, 2009)] allowed researcher to postulate and finally to develop tools to differentiate chemotypes based on gene diversity. This information coupled with PCR flexibility and accessibility (Nicholson et al., 2003) led to a significant shift towards the use of genetic chemotyping methods.
The most widely used approach so far aims at detecting strains isolated from plant tissue. It consists in the isolation of single spore colonies from infected spikes and the determination of the chemotype on the isolated strains. Historically this approach led to the identification of trichothecene type B isolates from different crops by measuring the toxin produced by each isolate in vitro (Faifer et al., 1990; Gang et al., 1998; Mirocha et al., 1989). More recently, by genetic chemotyping, often combined to chemical confirmation, it has been feasible to obtain information on the distribution of a certain chemotype within a country (Zhang et al., 2012), a field (Suga et al., 2008) or on seed samples (Wang et al., 2012).

The first PCR method developed for discriminating between the DON and the NIV chemotypes (with no distinction between 3- and 15-ADON) was developed by analysing the polymorphism of TRI genes, particularly the TRI7 gene, which has insertions in the non-coding region in DON producers but not in NIV producers. Therefore, a simple sequence length assay allows to distinguish between NIV and DON chemotypes (Lee et al., 2001).

In 2002, the respective function of TRI7 and TRI13 was demonstrated to be linked to chemotype diversity (Lee et al., 2002), suggesting that both genes could be used for genetic chemotype distinction. Based on this finding and on additional sequencing work, Chandler et al. (2003) developed a set of primers to amplify TRI7 and TRI13 genes and successfully identified DON and NIV chemotypes in F. graminearum, F. culmorum, and F. cerealis by a double assay. Similarly, Waalwijk et al. (2002) developed a TRI13 primer pair to differentiate between DON and NIV chemotype according to difference in length of the amplified product.

A further optimization of the method developed by Chandler et al. (2003) was proposed by Quarta et al. (2005, 2006). A multiplex PCR assay, based on primer pairs derived from the TRI3, TRI5, and TRI7 genes allowed to identify 3-ADON, 15-ADON and NIV among F. graminearum, F. culmorum, and F. cerealis. The assay was also validated on plant material (Quarta et al., 2006).

After confirming that the TRI set of genes coevolved independently in the three chemotypes, Ward et al. (2002), based on the finding that reciprocally monophyletic groups (corresponding to each of the B trichothecene chemotypes) were strongly supported (bootstrap scores _93%) in TRI3, TRI11, and TRI12 gene trees, developed a method based on the polymorphism of two of these genes. TRI3 and TRI12 are at the
edges of the cluster and are well conserved within the chemotypes. The method proved
to be effective on *F. culmorum*, *F. cerealis* as well as on members of the FGSC (Starkey
et al., 2007). The analysis of polymorphisms in two genes of the cluster improved the
robustness of the assay.

The primers were further optimised for use in a Luminex® assay (Ward et al., 2008). The
multiple gene analysis successfully detected discrepancies among *TRI3* and *TRI12*
polymorphism in strains obtained from South Africa suggesting the presence of hybrid
species (Boutigny et al., 2011). Similarly, new species were detected in Asia and Africa
by combining the observation of TRI cluster recombination (O’Donnell et al., 2008; Yli-
Mattila et al., 2009) with aspecific signals from species specific identification probes from
the Luminex assay.The primer couple developed by Li et al. (2005) can discriminate
DON and NIV (3-ADON and 15-ADON cannot be distinguished by this assay). The
assay is based on the polymorphism found in the intergenic region between *TRI5* and
*TRI6*, generating products of two sizes according to the chemotype. It can be coupled
with other primers able to amplify a single chemotype as those used in Jennings et al.
(2004a, b), that were derived from Chandler et al. (2003) allowing discrimination of the
three chemotypes.

Another method based on different sizes of insertions in the *TRI13* gene was developed
by Wang et al. (2008). The advantage of this method consists in the use of a single
primer pair, being the detection of the three chemotypes linked to the presence of an
insertion that is variable according to the chemotype. The method was shown to
distinguish Chinese isolates of *F. graminearum* and *F. culmorum* strains.

An alternative method based on *TRI3/TRI6* polymorphism was developed by Suzuki et
al. (2010) and aimed to differentiate simultaneously *F. asiaticum* and *F. graminearum* as
well as their chemotypes. This approach proved successful for Japanese (Suzuki et al.,
2010) and Chinese (Puri et al., 2012) isolates in a multiplexing approach.

More recently, also polymorphism in the *TRI11* gene were used for chemotyping, as
reported by Zhang et al. (2010a) on *F. graminearum* isolates obtained from barley, and
subsequently used by Talas et al. (2011, 2012a). Similarly, Wang et al. (2012) presented
another multiplex assay based on the same gene.

A very promising approach is to develop qPCR multiplex detection and quantification of
the chemotypes. This would in principle allow to directly screen grains for the abundance
of each chemotype without further isolation of contaminating strains. A qPCR primer and
TaqMan® probe set, based on *TRI12* polymorphism, was developed by Kulik (2011) to
allow chemotype quantification in planta. A SYBR® green method based on the same
gene was used by Nielsen et al. (2012) to quantify chemotypes within a set of grains
from Denmark, suggesting also potentialities for studying the evolution of isolate
distribution in grain samples collected from historical seed collections. Both methods
have been used on isolates sharing similar geographic origin, but potentially they can be
used to quantify chemotypes in the field anywhere.

Table S1 summarises the methods for chemotype determination and lists primer set
used.

Critical points in genetic chemotyping assays

It is important to underline that without a proper chemical identification of the different
trichothecene B types, the genetic methods cannot be considered per se sufficient to
determine precisely the ability to produce the toxin by any isolate. Indeed, a number of
reports have integrated both chemical and genetic determination of the chemotype to
overcome this issue.

As many researchers pointed out, the production of a toxin does not always correspond
to a certain genetic chemotype (Tan et al., 2012). Sometimes various amounts of
different toxins (such as NIV and DON) can be produced by the same isolate (Gilbert et
al., 2001; Mugrabi de Kuppler et al., 2011). Moreover, several studies reported the co-
production of acetylated forms by single strains, although in different relative amount
(Alvarez et al., 2009; Christ et al., 2011; Kawakami et al., 2014; Korn et al., 2011;
Mugrabi de Kuppler et al., 2011; Sugiura et al., 1990; Spolti et al., 2014b; Szécsi et al.
2005; Talas et al., 2012b; Ward et al. 2002; Yli-Mattila et al., 2009).

Llorens et al. (2006) reported also co-production by modifying temperature and growth
conditions, confirming the role of temperature in influencing toxin production (Walker et
al., 2001). Indeed, cultural and laboratory parameters may play a role in the quality of
toxin produced (Llorens et al., 2004). At the same time also the crop influences the toxin
found as in the case of potato, which transforms DON into NIV, probably by enzymatic
activity (Delgado et al., 2011).

Nonetheless, chemical methods may have limitations related to the number of
processable samples and the identification of optimal conditions for toxin production. If
the methods are applied on single strains and not directly on the grains, similar problems
as those described for genetic chemotype may occur, since toxin production in vitro is
extremely variable and may not represent the real toxigenic potential of a given strain.
Only field inoculation in planta can show the real toxigenic capacity of each strain (Gang et al., 1998). In fact, it has been reported that toxin production is strain-dependent as well as substrate-dependent in many Fusarium species (Vogelgsang et al., 2008a). Toxin production is often variable among isolates (Spolti et al., 2014b) and some strains do not produce any toxin under laboratory conditions (Tan et al., 2012). Therefore, research on the effects of substrates on the induction/repression of toxin (Gardiner et al., 2009a; Jiao et al., 2008; Kawakami et al., 2014; Pinson-Gadais et al., 2008; Ponts et al., 2006; Sakamoto et al., 2013; Suzuki et al., 2013; Tsuyuki et al., 2011) as well as on chemical and physical parameters (Gardiner et al., 2009b; Hope et al., 2005; Marin et al., 2010; Ryu and Bullerman, 1999; Schmidt-Heydt et al., 2011) are active areas of research.

By considering the history of application of the different genetic chemotyping methods, it is evident that while on average all methods did work quite efficiently, inevitably some failures or contradicting results were obtained by using different methods. For example, the methods developed by Lee et al. (2001) and Chandler et al. (2003), when used by Desjardins et al. (2008) showed a different level of reliability in assigning the chemotype to maize isolates from Nepal. Despite both primer sets were designed on the sequence of the TRI13 gene (a pseudogene in DON producers) Lee’s primers that are located closer to the insertion/deletion sites of degeneration failed to amplify due to the lower degree of stability of the region, thus generating potential false negatives.

Similarly, the TRI13 length polymorphism method developed by Wang et al. (2008) provided inconsistent results in identifying the three chemotypes. The method was effective when applied to Chinese isolates (Wang et al., 2008) but failed to identify 15-ADON isolates of F. graminearum sensu stricto (s.s.) outside Asia (Pasquali et al., 2011) and were probably effective only on F. asiaticum (Amarasinghe et al., 2011), due to a difference in the size of insertion in the TRI13 gene.

The method by Quarta et al. (2006) showed contrasting results with some Argentinian isolates giving DON/NIV chemotypes while producing only DON by chemical analysis (Reynoso et al., 2011) as well as with two isolates from barley and wheat from Italy (Quarta et al., 2006), and with some Polish strains (Stephien et al., 2008).

So far, the method developed by Ward et al. (2002) and further improved and implemented in a Luminex® system (Ward et al., 2008) proved very reliable in discriminating polymorphisms linked to chemotype and it is probably the most used worldwide on international collections of B-clade isolates. Alexander et al. (2011)
reported a misamplification for three *F. meridionale* isolates, suggesting that methods targeting the functional domain generating the toxin may further improve the precision of a PCR test linking effectively a certain mutation / polymorphism in a gene with its function within the process of toxin biosynthesis. The recent identification of the TRI8 catalytic region as determinant of the 3-ADON generation (Alexander et al., 2011) may be promising, since a genetic method based on functional domains would further increase the reliability of the analysis. For a PCR assay to have broad utility, it must be able to detect not only an allele that results in an inactivated protein, but all the alleles resulting in an inactivated protein - an incredibly difficult task indeed (Reynosso et al., 2011). Despite that, knowledge on the linkage relationships and evolutionary dynamics involving selection on sets of genes at either end of the core trichothecene cluster allows to confidently use the most used method so far which includes TRI3-TRI12 polymorphism and well as those based on TRI3 and TRI7 polymorphisms. It is evident that a continuous monitoring using complementary methods is still needed (Nicholson et al., 2004; Desjardin et al., 2008), therefore PCR validation of the method should rely on chemical measures of toxin produced by each tested isolate.

**Surveys**

Chemotype studies worldwide have increased 20 times over the last 10 years. At present, data on chemotype distribution of FGSC are available from all continents (see Table 1 for details), being *F. graminearum* s.s. the most studied species. Given the lower general importance of *F. culmorum* as primary cause of FHB, less work has been devoted to chemotype determination in this species. However, since it was shown that toxin translocation may occur from roots (Covarelli et al., 2012; Winter et al., 2013) further attention on this species should be expected where environmental conditions favour foot and root (crown) rot disease (Scherm et al., 2013). Chemotype determination and population studies can therefore help in managing also crown rot diseases and their associated toxins (Rebib et al., 2014).

Historically, since the pioneering studies by Mirocha et al. (1989) and by Miller et al. (1991), it became apparent that a regional relationship could be sometimes postulated between the geographic origin and the production of of NIV or 15-ADON or 3-ADON as the major isomer (Bottalico and Perrone, 2002). This phenomenon can be due to different species colonising the region as not all species are able to produce all chemotypes (see Aoki et al., 2012 for a review in FGSG). For this reason a shift in
species could also be associated to a shift in toxin type (Astolfi et al., 2011; Audenaert et al., 2009; Beyer et al., 2014; Bottalico and Perrone, 2002; Malihipour et al., 2012; Pasquali et al., 2010; Yang et al., 2008; Yli-Mattila, 2010).

From an analysis of the chemotyping studies carried out since 2000, it is evident that the majority of reports were carried out on wheat while investigation on other cereals are more limited, although scattered information is available also for crops that are not notoriously target of trichothecene B contamination, such as asparagus, banana, etc..

Information from all continents are available but not all reports include complete information on the isolates analysed nor precise characterisation of the species that sometimes is based only on morphological observations or on the use of putatively species-specific primers, hence making it impossible to further using the dataset for comparison. It is desirable that a more coordinated effort, leading to common protocols for sampling, chemotype determining and data reporting in a more accessible way could facilitate the effort of understanding which factor do favour establishment and persistence of a certain chemotype.

The usefulness of genetic chemotyping studies was proven by the identification of novel groups and species. For instance, the NIV population reported in Louisiana by Gale et al. (2011) was then identified as a new species using multiple genotyping techniques (Sarver et al., 2011). Similarly, studies pinpointing inconsistencies among results of chemotyping on two TRI genes coupled with species-specific detection probes led to the identification of a new species in Ethiopia (O’Donnell et al., 2008).

Shift in species population has been reported in many surveys (Fredlund et al., 2013; Nielsen et al., 2011; Xu et al., 2005), but chemotype shift in certain areas is somewhat a novel report that would probably become more popular in the future (Beyer et al., 2014; Guo et al., 2008; Nielsen et al., 2012; Waalwijk et al., 2002; Ward et al., 2008). Analysing historical samples, Nielsen et al. (2012) showed that the presence of a certain chemotype is associated to historical periods. It is advisable that the availability of faster and more effective tools for chemotype determination would facilitate the identification of factors driving such shift.

For practical purposes, studying chemotype diversity for breeding against FHB susceptibility has been acknowledged (Gilbert et al., 2010; Gosman et al., 2010; He et al., 2013; Spolti et al., 2012; van der Ohe et al., 2010). In particular, testing local genetic diversity including toxin abilities in breeding programmes is considered essential to develop locally adapted varieties (Horevaj et al., 2011). Indeed, differences in the
characteristics of the pathogen (species/isolate) used in breeding programs (Warzecha et al., 2010) may affect reaction of host genotypes, leading to erroneous results, and therefore explaining different reactions to FHB in different geographical zones (Malihipour et al., 2012).

Whether chemotype diversity needs to be assessed constantly is a matter of debate. Studying the prevalence of 15-ADON and 3-ADON chemotype on barley, Clear et al. (2013) found no strong effect on the chemotype prevalence as being determined by resistance factors from the plant, suggesting on the contrary a potential role of micro-environmental factors. While it is evident that highly resistant cultivars do not recognise chemotype diversity as the pathogenic process is hindered (Foround et al., 2012), breeding activities that will likely explore the susceptibility to toxin accumulation need to consider also chemotype diversity as well as species interaction (Xu et al., 2007). Given the challenge to toxin contamination posed by new evolving populations of F. graminearum (Foroud et al., 2012), breeding programs should include a panel of isolates and chemotypes to better cover natural variability of populations causing FHB.

Future challenges

By acknowledging the potential informativeness of chemotype diversity, the table presented here should be considered with caution as it includes experiments carried out with different rigour, different methods and using different techniques. It would be therefore extremely valuable to generate a technically homogeneous and accessible map of chemotypes where homogeneous data and methods could allow a true comparison of the situation during years and among countries.

A small step towards improving research on chemotype diversity would be to include these data in any fungal collection. Instruction on how the sampling was performed as well as detailed information on cultural practices and location is indeed essential in order to address questions such as which conditions are more favourable to a given chemotype. Indeed, understanding which factor do play a role in chemotype prevalence may help limiting the toxigenic risk associated to the spread of a certain chemotype.

Further research on the tools for chemotype determination is needed. The different methods developed so far provide information that are at least partially discordant and may fail to predict real toxin production by the strains. At the same time chemical conditions simulating the behaviour of a strain in the field are not defined yet and do not allow any preventive approach. Considering the need for preventive tools to limit
mycotoxin contamination in food and feed, it appears essential developing molecular tools that are able to predict toxin contamination at an early stage of infection in the field based on the chemotype spread over a certain area. At the same time, it would be important to identify conditions regulating toxin production in the field, by taking into account the environment/plant/pathogen interactions.

Paramount in a food safety perspective is the role that the host plant can play in transforming a toxin, hence determining a different level of toxin contamination. The ability of potato to transform DON into NIV (Delgado et al., 2010) due to enzymatic activity requires further attention. Similarly, the general issue of masked mycotoxins (Berthiller et al., 2013), which can be partially prevented by monitoring the producing microorganism and not the product itself or all its closely related metabolites in any sample, requires further understanding at both the toxicological and epidemiological viewpoints.

A larger coverage of crops and environments where Fusarium species may produce type B-trichothecenes is also needed in order to better monitor all potential toxigenic risks in food and feed. It has been emphasised that understanding host-specific differences in pathogen composition is crucial in the development of pathogen and mycotoxin control strategies, and could lead to novel approaches to achieve improved resistance in commercial cultivars (Boutigny et al., 2011).

Exploitation of molecular approaches to chemotype quantification such as those based on qPCR in grains may help guiding epidemiological studies and may lead to a better understanding of correlations between fungal populations and toxin production (Yli Mattila et al., 2008, 2009b). There is a need for rapid and cheap tools able to predict effectively toxin productivity in the field. Bakan et al. (2002) attempted to build a marker for isolates based on strain toxin productivity, linking polymorphism of a regulatory region to toxin production but no further validation of the method was carried out. As toxin production is subject to a series of regulation layers (Audenaert et al., 2013; Gardiner et al., 2009a, 2009b; Hope et al., 2005; Mereji et al., 2010; Reverberi et al., 2010; Seong et al., 2009) actual diagnostic methods based exclusively on DNA polymorphism can probably be used as a simple warning method for toxin risk. Despite mapping a population by genetic chemotyping would evidently be only an approximation of the field situation, it can result anyway in an effective monitoring of potential food threats.

Finally, the discovery of novel metabolites belonging to type B-trichothecene (Fruhmann et al., 2014) is also suggesting that genetic chemotyping determination requires
continuous monitoring of the markers used that need to be coupled with genetic research on diversity in order to develop novel and more precise markers for toxin prevention.

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