SHORT COMMUNICATION

IDENTIFICATION OF DEOXYNIVALENOL AND NIVALENOL PRODUCING CHEMOTYPES OF Fusarium graminearum ISOLATES FROM DURUM WHEAT IN A RESTRICTED AREA OF NORTHERN ITALY

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SUMMARY

Fusarium graminearum is one of the main causal agents of fusarium head blight (FHB) in Italy. A population of 74 F. graminearum strains was collected from symptomatic durum wheat spikes from different fields around Bologna (Emilia-Romagna, northern Italy) between 2006 and 2008. The data obtained showed that the F. graminearum population is variable in an homogeneous restricted geographical area. F. graminearum sensu stricto strains were examined using specific primers for chemotypes based on the production of the trichothecenes deoxynivalenol (DON) and nivalenol (NIV). The strains were analyzed by multiplex PCR in the Tr12 sequence of the trichothecene gene cluster and assigned to one of three profiles of trichothecene chemotypes. All three fungal chemotypes were found. The 15-acetyldeoxynivalenol (15ADON) chemotype was the most frequent (87.2%), followed by the 3-acetyldeoxynivalenol (3ADON) (8.1%) and NIV (2.7%) chemotypes. The variability of the strains in such a restricted area leads us to hypothesize that there is similar variability of F. graminearum throughout Italy.

Key words: Fusarium head blight, durum wheat, Tr13, Tr12, DON and NIV chemotypes.

FHB can have a severe impact on production with yield decrease between 30 and 70% (Parry et al., 1995). It is responsible for reduced seed quality characteristics, such as lower protein levels, semolina colour and mycotoxin accumulation. Deoxynivalenol (DON) is a trichothecene mycotoxin mainly produced by F. graminearum that inhibits DNA, RNA and protein synthesis. It is responsible for hemorrhagic and anorexic syndromes, neurotoxic and immunotoxic effects in mammals (Visconti et al., 2004). For this reason, mycotoxin concentrations in food and feed are strictly regulated at the international level. For instance, the maximum DON level in durum wheat is defined by the legal limits set by the EU in the community regulations No. 856/2005 and its updated version No. 1126/2007. In recent years, DON has been found in FHB-infected durum and bread wheat kernels in several wheat-growing areas of Emilia-Romagna and other Italian regions (Lops et al., 1998; Pascale et al., 2002; Rossi et al., 2006). DON level in many durum wheat samples from Emilia-Romagna exceeded the legal limits in the 2007-2008 growing season (Rossi, 2008).

Between 1995 and 2007, F. graminearum was the most frequently isolated species from FHB heads (mean value 32.1%) in the Emilia-Romagna region (Pisi et al., 2008). Its population comprises two chemotypes that synthesize DON and nivalenol (NIV), respectively. DON-producing isolates can be further divided on the basis of the predominant acetyl DON derivative that produces either 3-acetyl DON (3ADON) or 15-acetyl DON (15ADON) (Miller et al., 1991).

In Italy the whole durum wheat production is used for food, therefore it is very important to know the prevalent chemotype in any given F. graminearum population, to evaluate possible mycotoxin contamination risks (Quarta et al., 2005). The knowledge of chemotype prevalence in different geographical areas is also very important for breeders when developing new wheat cultivars resistant to FHB, so as to have a low mycotoxin content. In the present study, the results obtained during a field investigation conducted between 2006 and 2008 are reported.
Chemotypes of *Fusarium graminearum* in Italy

FHB durum wheat heads from different cultivars were collected from 21 localities and 43 fields in the plains around the city of Bologna (Emilia-Romagna) (Fig. 1). Glumes, rachis and sub-glumes of five heads from each field were washed in sterile water, disinfected in 2% sodium hypochlorite solution for 2 min, rinsed twice in sterile water to eliminate hypochlorite residues, dried on sterile filter paper, placed in Petri dishes containing potato dextrose agar (PDA, Difco, USA) supplemented with neomycin and streptomycin sulphate (0.3 g l⁻¹) and incubated at 22°C in the dark for 5 days. The mycelium was then transferred onto fresh PDA plates and placed under near-ultraviolet (NUV) alternating light and dark (12 h photoperiod) for 10 days to favour sporulation. Colonies obtained from single spore cultures (Balmas et al., 2000b) were identified as *F. graminearum* according to morphological criteria (Nelson et al., 1983; Leslie and Summerell, 2006). DNA from 74 strains extracted using a CTAB (cetyl-trimethyl-ammonium bromide) method adapted from Lodhi et al. (1994), was analyzed with specific primers Fg16F/Fg16R under the conditions described by Nicholson et al. (1998), confirming that all strains belonged to *F. graminearum* sensu stricto (Fig. 2a).

The *F. graminearum* chemotypes were identified using a multiplex version (Starkey et al., 2007) of a chemotype-specific test previously validated by Ward et al. (2002). The technique described by Starkey et al. (2007) is simple and rapid, and allows a large number of strains to be screened. Primers designed in the region of the Tri12 gene located in the terminal gene cluster for trichothecene biosynthesis, can distinguish three subgroups depending on the type of β-trichothecene produced. One primer is common to all chemotypes (12CON) and the others are chemotype-specific for 15ADON (12-15F), 3ADON (12-3F) and NIV (12NF) (Starkey et al., 2007). PCR was performed using 10 ng of fungal DNA in a final volume of 10 µl containing 0.5 units of GoTaq Flexi DNA Polymerase (Promega, USA), 5X Green GoTaq Flexi Buffer, 0.2 mM dNTP, 2 mM MgCl₂ and 2.5 pmol of each primer (Invitrogen, UK). Amplification products were resolved on 1.5% agarose gels stained with ethidium bromide (0.4 µg ml⁻¹) and visualized under UV light, alongside a 100 bp DNA ladder (Promega, USA) (Fig. 2b).

The evolution of genes encoding β-trichothecene biosynthesis does not correlate with *F. graminearum* complex phylogeny, and chemotype polymorphism is believed to be trans-specific and has been maintained through multiple speciation events (O’Donnell et al., 2000; Ward et al., 2002). Environmental factors could influence the prevalence of a chemotype. For instance, trichothecenes have an ecological value with a significant impact on pathogen fitness (Ward et al., 2002). Chemotypes are also known as virulence factors (Proctor et al., 1995; Maier et al., 2006), even if their aggressiveness is determined by numerous different elements that play a role in relative competitive ability in particular host species (Qu et al., 2008b).

In the results presented by Ward et al. (2008), it was shown that a population of *F. graminearum* with the 3ADON chemotype replaced the dominant 15ADON population in western Canada, providing evidence for a selective advantage. This was confirmed by Guo et al. (2008) who showed that in Manitoba (Canada) the 15ADON chemotype remained predominant, but the 3ADON chemotype had increased in the south of the region. In a preliminary field trial conducted by us on a susceptible Italian cultivar of durum wheat, Simeto, 15ADON strains were found to be more pathogenetic than 3ADON strains (data not shown).

Fig. 1. Map of the area around the city of Bologna (Italy), indicating the sites for sampling.

Fig. 2. PCR products amplified from genomic DNA of *F. graminearum* strains using specific primers Fg16F/Fg16R (expected product 410 bp) (a), and chemotype-specific primers (b), with the 12CON primer common to all chemotypes, associated with other chemotype-specific primers 2-15F for 15ADON (expected product 670 bp), 12-3F for 3ADON (expected product 410 bp) and 2NF for NIV (expected product 840 bp). Strains from: Angelato (lane 1), Baricella (lanes 2 and 4), San Giorgio di Piano (lanes 3, 5 and 8), Colungu (lanes 6 and 7), Idice (lanes 9 and 10), San Pietro Capoluino (lanes 11 and 12). Lane M, 100 bp molecular marker and lane W, water.
All the three chemotypes were present in the *F. graminearum* population studied in this work. The most frequent strains were 15ADON (87.2%), followed by 3ADON (8.1%) and NIV producers (2.7%) (Table 1). These results were confirmed using primers designed for the Tri3 gene by Starkey et al. (2007) (data not shown). The 15ADON strains were found in all of the fields examined, except for three. Strains belonging to different chemotypes were simultaneously present in the same locality, Baricella, S. Giorgio di Piano and S. Pietro Capofiume (Table 1), but only in two cases in the same field, Baricella and S. Pietro Capofiume.

Our data on chemotype frequency are comparable with those reported by others. Gale et al. (2007) examined 712 strains of *F. graminearum sensu stricto* from different areas of the US, identifying 15ADON as the dominant chemotype, followed by 3ADON (5.1% of total), with only one isolate of the NIV chemotype. In South America the situation is different, for in southern Brazil and Argentina no strains of the genotypes 3ADON and NIV have been found in *F. graminearum sensu stricto* (Scoz et al., 2009; Reynoso et al., 2007). Scoz et al. (2009) reported that in southern Brazil the isolates representing the NIV genotype belonged to *F. meridionale*, which is another member of the *F. graminearum* complex (O'Donnell et al., 2008).

In Europe there is more variability. In western Russia and Finland, all *F. graminearum* isolates were found to be 3ADON, whilst in southern Russia 90% of the isolates was 15ADON (Yli-Mattila et al., 2008). Jennings et al. (2004) examined 101 isolates from England and Wales and found that the predominant chemotype was DON, with 95% of these being 15ADON producers, and 25% of the total were NIV strains. Waalwijk et al. (2003) identified the NIV chemotype in the Netherlands in similar proportions to those reported by Jennings et al. (2004), using 207 isolates examined in two consecutive years (2000-2001). Our investigation shows that isolates of the NIV chemotype were not so frequently identified in the surveyed areas (only two strains from two different localities) (Table 1). In addition, the 3ADON chemotype seems to be less competitive in Italy than in Northern Europe. To our knowledge, there has only been one previous study on Italian strains of *F. graminearum*, which investigated isolates from eight different regions, including Emilia-Romagna

### Table 1. Chemotypes of *Fusarium graminearum* strains isolated from the surveyed localities around Bologna.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Number of Fields</th>
<th><em>F. graminearum</em> strains</th>
<th>15ADON</th>
<th>3ADON</th>
<th>NIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argelato</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baricella</td>
<td>10</td>
<td>21</td>
<td>20</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Bologna</td>
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<td>3</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>Cadrano</td>
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<td>1</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>Castelfranco Emilia</td>
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<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Castel Maggiore</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>Colunga</td>
<td>1</td>
<td>2</td>
<td></td>
<td>2</td>
<td></td>
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<tr>
<td>Crespellano</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
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<tr>
<td>Crevalcore</td>
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<tr>
<td>Galliera</td>
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<td>1</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
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<td>2</td>
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<td>Malalbergo</td>
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<td>Mezzolara</td>
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<tr>
<td>Minerbio</td>
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<td>6</td>
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<tr>
<td>Molinella</td>
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<td>1</td>
<td>1</td>
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<td></td>
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<tr>
<td>San Giorgio di Piano</td>
<td>6</td>
<td>11</td>
<td>9</td>
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<tr>
<td>San Giovanni in Persiceto</td>
<td>1</td>
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<td></td>
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<tr>
<td>San Pietro Capofiume</td>
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<td>4</td>
<td>3</td>
<td>1</td>
<td></td>
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<tr>
<td>Sant’Agostino</td>
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<td>Sant’Antonio</td>
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<td>1</td>
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<tr>
<td>Total</td>
<td>43</td>
<td>74</td>
<td>66</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>%</td>
<td></td>
<td>100</td>
<td>87.2</td>
<td>8.1</td>
<td>2.7</td>
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(Gale et al., 2007). In this case, 3ADON appeared to be more competitive (21%), but only 19 strains were analyzed (15ADON, 58%; and NIV, 21%).

In China the situation is more complicated due to the simultaneous presence of F. graminearum lineage 7 (sensu stricto) and F. graminearum lineage 6 (F. asiaticum). This last lineage is not present in Europe or the USA. Ji et al. (2007) studied a population of 157 strains isolated from wheat, and found that most of the strains belonged to the 15ADON chemotype, three to 3ADON, two to NIV and five strains to F. asiaticum. A similar situation was found in maize in the same Chinese sampling area, whilst in barley in another area, the situation was exactly the opposite with F. asiaticum being predominant.

These data and those of Li et al. (2005), who reported that DON producer strains are present at an even higher level than NIV and with different proportions, can be explained by considering the differences related to the sampling area and changes observed over time in the regional composition of F. graminearum populations. Ji et al. (2007) stress the necessity of examining a large number of isolates from one sampling area to determine the real situation. A recent and important study by Qu et al. (2008a) on the genetic diversity in a population of 437 isolates from areas where FHB is endemic, supports this. They found that F. asiaticum was more frequent in warmer regions, whilst F. graminearum sensu stricto was more prevalent in cooler regions. Within this population, nine isolates of F. graminearum sensu stricto were analyzed for chemotype, all being 15ADON with no NIV (Qu et al., 2008b).

F. asiaticum is also present in Japan on wheat and barley, especially in the northern regions, while F. graminearum is prevalent in the southern regions. For F. asiaticum, 70% of the strains were NIV, 29% 3ADON and only 0.4% 15ADON, whereas for F. graminearum, 70% were 3ADON, 30% 15ADON with no NIV chemotype (Suga et al., 2008). The data from Asia differ from those of the Middle East. For example, in north Iran the majority (46/57) of F. graminearum strains belong to the NIV chemotype (Haratian et al., 2008).

In conclusion, this is the first time that a population of Italian F. graminearum exclusively isolated from durum wheat and in a specific locality has been examined for chemotypes. The durum wheat cultivars, especially in northern Italy, are at high risk of seed infection due to FHB-associated pathogens (Shah et al., 2005). This is an important aspect to consider, especially for the Emilia-Romagna region which, in a period of only three years, has increased the area given over to durum wheat from 22,256 to 46,467 ha, as part of a large quality grain project. Furthermore the variability of the strains in such a restricted area, with the presence of all three chemotypes, leads us to hypothesise that there may be a similar variability within F. graminearum populations in other geographical areas of Italy, albeit influenced by the different environmental characteristics. Future studies are therefore required to monitor wheat fields from all over Italy, especially where durum wheat prevails, to better understand the distribution of F. graminearum chemotypes.

ACKNOWLEDGMENTS

We thank the Ente Nazionale Sementi Elette teams of Bologna and Verona sections for technical field help.

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