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Genetic population structure and trichothecene genotypes of *Fusarium graminearum* isolated from wheat in southern Brazil

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A sample of 140 *Fusarium graminearum* isolates from Rio Grande do Sul, Brazil, representing three populations at least 150 km from one other, were examined for trichothecene genotype based on PCR amplification of portions of the *Tri3*, *Tri12* genes and a species-specific (Fg16F/R) primer. Genetic diversity was assessed in a sample of 103 *F. graminearum* lineage 7 (*F. graminearum sensu stricto*) isolates using amplified fragment length polymorphism (AFLP) markers. The 15-ADON genotype was dominant, followed by the NIV genotype (2–18% prevalence), across all three populations. All NIV-type isolates were in lineage 2 (*F. meridionale*) and all 15-ADON-type isolates were in lineage 7. Isolates with the same haplotype were rare and genotypic diversity was uniformly high (≥98% of the count), suggesting that recombination has played a significant role. The number of migrants (N_m) was estimated between 5 and 6 across all loci and all populations, but the high frequency of private alleles (up to 30%) suggests a historical, rather than contemporary, gene flow. Regarding linkage disequilibrium, 0·8, 1·5 and 2·2% of the locus pairs from the three populations were in disequilibrium, which is lower than values reported in other locations. Thus, Brazilian populations differ from those found in Europe, North America and most of Asia in the presence of a significant frequency (7·8%) of isolates of the NIV genotype in lineage 2.

Keywords: chemotype, deoxynivalenol, *Gibberella zeae*, small grains, *Triticum aestivum*, wheat scab

Introduction

Gibberella zeae (anamorph: *Fusarium graminearum*) causes fusarium head blight (FHB), a re-emergent disease for wheat, barley and other small grains worldwide (Goswami & Kistler, 2004). In Brazil, FHB of wheat is caused exclusively by the *F. graminearum* species complex (Angelotti *et al.*, 2006; Scoz *et al.*, 2009), which encompasses at least 13 phylogenetic species (O'Donnell *et al.*, 2008; Yli-Mattila *et al.*, 2009). FHB results in important yield loss of wheat in Brazil (Panisson *et al.*, 2003), principally in southern production regions, where humid subtropical climate and extensive no-till farming practices prevail (Del Ponte *et al.*, 2009a). Of particular concern are the the B-type trichothecene mycotoxins deoxynivalenol (DON) and nivalenol (NIV) produced by the fungus, which can render harvested grain and its by-products unsuitable for human or animal consumption (Desjardins, 2006). A recent mycotoxin survey in Brazilian commercial wheat grain found both DON and NIV toxins in the samples (Del Ponte *et al.*, 2009b). Such findings confirm the ability of the fungal populations to produce both trichothecenes, as demonstrated in a previous study that found both DON and NIV chemotypes in a regional population of the *F. graminearum* species complex (Scoz *et al.*, 2009).

Fusarium graminearum populations from around the world have been extensively characterized by various traits, including vegetative compatibility, mycotoxin production and DNA-based methods, amongst others (Gilbert *et al.*, 2002). Molecular markers such as amplified fragment length polymorphisms (AFLPs) have been used to characterize *Fusarium* populations isolated from wheat in the Americas (Zeller *et al.*, 2004; Schmale *et al.*, 2006; Ramirez *et al.*, 2007). Other markers also have been used to characterize *F. graminearum* populations from around the world (Suga *et al.*, 2004; Tóth *et al.*, 2005; Fernando *et al.*, 2006; Guo *et al.*, 2008; Gale *et al.*, 2011). Most of these studies have been done with *F. graminearum* from wheat, where there are high levels of diversity both between and within individual field populations in Europe (Miedaner *et al.*, 2001; Tóth *et al.*,

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2005), China (Gale *et al.*, 2002), Australia (Akinsanmi *et al.*, 2006), Canada (Guo *et al.*, 2008) and the USA (Zeller *et al.*, 2004; Schmale *et al.*, 2006). In Japan, high diversity levels were observed in wheat populations of *Fusarium asiaticum*, a member of the *F. graminearum* species complex (Karugia *et al.*, 2009).

The analysis of *F. graminearum* populations from multiple crops including cereal (wheat and barley) and non-cereal (potato and sugar beet) hosts grown in the upper midwest of the USA suggested low population differentiation because of the high gene flow levels and that a large single population was affecting those crops in the region (Burlakoti *et al.*, 2008). This concurs with previous conclusions of a panmitic population in the USA from studies of both epidemic and airborne *F. graminearum* populations on various spatial scales (Zeller *et al.*, 2003a, 2004; Schmale *et al.*, 2006). Population subdivision based on the trichothecene genotypes of *F. graminearum* prevalent in both the northern (Gale *et al.*, 2007) and southern (Gale *et al.*, 2011) USA has been inferred from population structure analysis. Similar subdivisions based on trichothecene genotypes were not found in Japanese populations of *F. asiaticum* (Karugia *et al.*, 2009).

Thus far, the population structure of *F. graminearum* in South America is poorly understood compared to other production regions and there is only one published study of genetic diversity in fungal populations from the region (Ramirez *et al.*, 2007). Although multiple phylogenetic species and chemotypes occur in South America (Scoz *et al.*, 2009; Sampietro *et al.*, 2010), little is known about the structure of the populations in these countries. It is hypothesized here, based on previous findings around the world, that individuals within a population are genetically diverse and randomly mating, and that the three populations are not genetically distinguishable from one another. Hence, the objectives of this study were to (i) identify *F. graminearum* isolates from three populations at the lineage level and determine their trichothecene genotypes and (ii) characterize the genetic structure of *F. graminearum* lineage 7 populations present in the major wheat-growing area in southern Brazil.

Materials and methods

Study area and sampling

Commercial wheat grain samples originated from fields grown in the main wheat-producing regions of the northern part of the state of Rio Grande do Sul, Brazil, during the 2007 cropping year, and were received as part of a systematic yearly survey for fungi and mycotoxin content in wheat (Del Ponte *et al.*, 2009b). The grain samples for this study came from three subregions separated by at least 150 km and all had >10% incidence of *Fusarium*-damaged kernels and >30% incidence of *F. graminearum* infection. The three sampled locations were Cruz Alta, Passo Fundo and Nonoai (Fig. 1). The northern production region of the state has a subtropical humid climate and is between 28°S and 30°S latitude.

Isolate collection, purification and DNA extraction

Two hundred kernels from each field sample were examined in a standard seed health blotter test (7 days'

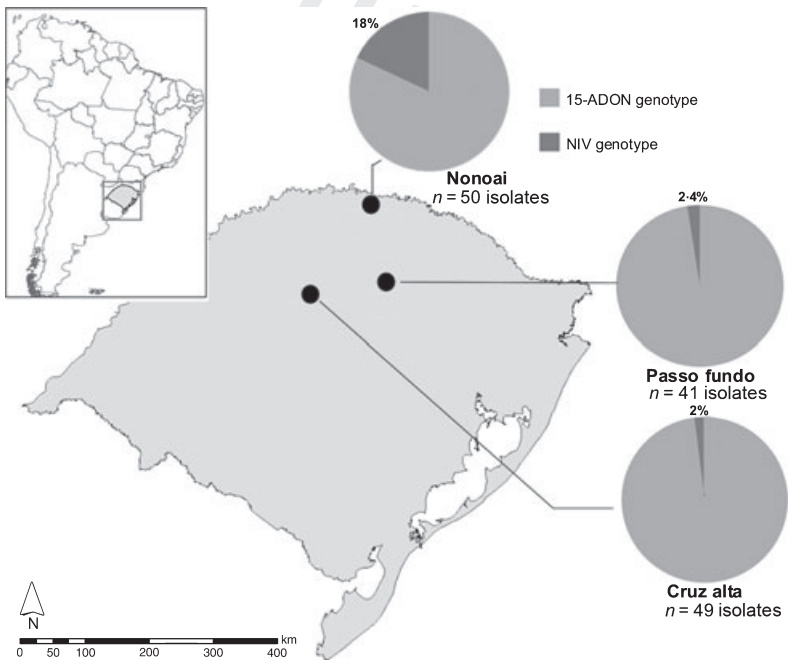

Figure 1 Areas in southern Brazil where three *Fusarium graminearum* species complex populations were sampled during the 2007 growing season, showing the proportions of 15-acetyl-deoxynivalenol (15-ADON) and nivalenol (NIV) trichothecene genotypes.

incubation of seeds in a 12-h light/dark cycle at 25 ± 1°C). Pure cultures were established following a single-spore subculture and long-term preservation carried out as described previously (Scoz *et al.*, 2009). Single macroconidia were identified with a stereomicroscope (×70 magnification), transferred to carnation leaf agar (CLA) and potato dextrose agar (PDA), and incubated as described above for 15 days for identification (Leslie & Summerell, 2006). Isolates initially identified as *F. graminearum* were kept as spore suspensions in 15% glycerol and frozen at −80°C.

Fungal isolates were grown in complete medium (Leslie & Summerell, 2006) and incubated on an orbital shaker (150 rpm) for at least 3 days at 25 ± 1°C. The resulting mycelia were harvested by filtration through non-gauze milk filters (Ken AG). Excess water was removed by blotting mycelia between clean paper towels, and dried mycelia were stored frozen at −20°C. DNA was extracted with a cetyltrimethylammonium bromide (2% CTAB) method (Leslie & Summerell, 2006).

PCR species and chemotype determination

Prior to trichothecene genotyping, DNA from all isolates were amplified by PCR with the Fg16F/R primer which produces polymorphic products (~400–500 bp) with DNA from members of the *F. graminearum* species complex (Nicholson *et al.*, 1998). Three trichothecene types (3-ADON, 15-ADON and NIV) were determined by a multiplex PCR assay that amplifies portions of the *Tri3* and *Tri12* genes (Ward *et al.*, 2002). PCR amplification reactions contained 20–30 ng fungal DNA in a total volume of 25 μL containing 1·5 mM·$MgCl_2$, 2 U *Taq* DNA polymerase, 20 μM dNTPs and each primer at 1 μM. PCR amplification consisted of an initial step at 94°C for 10 min, followed by two cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s. The annealing temperature was stepped down every two cycles to 58, 56, 54, 53, 52 and 51°C, then 50°C for 21 cycles, with a final amplification step at 72°C for 10 min. Resulting PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide at a final concentration of 0·5 μg mL^{-1} and visualized under UV light.

AFLP analysis

The AFLP procedures were performed as in Vos *et al.* (1995) modified by Leslie & Summerell (2006). All buffers and enzymes were used according to the manufacturer's instructions or standard protocols (Sambrook *et al.*, 1989). The presence or absence of polymorphic AFLP bands ranging from 200 to 500 bp in length was manually scored, and the data recorded in a binary, present (1)/absent (0) format. All bands in this size range were scored, including those that were monomorphic. Bands migrating at the same position were assumed to be homologous and to represent the same allele and locus. Bands of differing mobility were treated as independent loci with two alleles (present or absent). Irresolvable bands and missing data were treated as missing data. Multiple runs of DNA from the same isolate were at least 98% similar, so a cut-off of 98% similarity was used to identify clones. Eleven reference isolates identified as belonging to the *F. graminearum* complex were included: *F. austroamericanum* NRRL 2903 (lineage 1), *F. meridionale* NRRL 28436 (lineage 2), *F. boothii* NRRL 26916 (lineage 3), *F. mesoamericanum* NRRL 25797 (lineage 4), *F. acacia-mearnsii* NRRL 26754 (lineage 5), *F. asiaticum* NRRL 13818 (lineage 6), *F. graminearum sensu stricto* NRRL 31084 (lineage 7), *F. cortaderiae* 29297 (lineage 8), *F. brasilicum* NRRL 31281, *F. vorosii* NRRL 37605 and *F. gerlachii* NRRL 36905 (no lineage number) (O'Donnell *et al.*, 2000, 2004; Starkey *et al.*, 2007) as standards on each gel.

Genotypic diversity and population analysis

The degree of genetic similarity was estimated with the SINQUAL program (version 2·01 NTSYS.pc, Exeter Software; Rohlf, 1990), taking into account all pairs of isolates tested according to the Dice coefficient (Sd): $Sd = 2 \cdot N_{XY}/(N_X \cdot N_Y)$; where N_X and N_Y are the number of fragments amplified in isolates X and Y, respectively, and N_{XY} is the number of amplified fragments shared by both isolates. Genetic similarity dendrograms were constructed by using the UPGMA method with arithmetic averages protocol of NTSYSPC 2·0 (Numerical Taxonomy System) software package (Rohlf, 1990). Bootstrap values of dendrograms were obtained with PAUP version 4·0 (Swofford, 2003).

Populations were clone-censored, i.e. only one isolate of each clone was retained for analyses of genetic diversity and linkage disequilibrium. Genotypic diversity (G) was extimated for each population as described by Milgroom (1996) and the index for each population was normalized by dividing each estimated G by the number of genotypes identified in that population. Also estimated were: (i) allele frequencies of polymorphic loci and gene diversity within and between populations as described by Nei (1973), (ii) G_{ST} (fixation index) as described by Nei (1973), (iii) N_m (effective migration rate) as described by McDermott & McDonald (1993) and (iv) genetic identity among populations as described by Nei (1978) with POPGENE version 1·32 (Yeh *et al.*, 1997). All data analysed were treated as haploid with dominant markers. G_{ST} and N_m were estimated with both the complete dataset and with a subset of data that included only those loci for which both alleles were present at ≥5% frequency in at least one of the three populations. The results of these analyses were compared to determine if the inclusion of loci with rare alleles in these analyses altered the estimates of genetic differentiation of the populations. POPGENE was also used to estimate linkage disequilibrium for AFLP loci if both alleles at both loci were present in the population at a frequency of ≥5%. Two-locus gametic disequilibria were calculated between all pairs of these loci, and chi-squared tests for significance conducted as described by Weir (1979).

Results

Species and trichothecene type composition

A total of 140 isolates from the Cruz Alta (49 isolates), Passo Fundo (41 isolates) and Nonoai (50 isolates) populations were recovered and identified. In the PCR reaction with the Fg16F/R primers, all isolates produced either a 450- or 500-bp fragment, confirming that they belong to the *F. graminearum* species complex. Isolates representing 11 of the known phylogenetic species within *F. graminearum* were included as standards for comparison with the Brazilian isolates. In a UPGMA analysis of AFLP branching patterns for 111 isolates, 103 clustered with the standard isolates of lineage 7 (showing 81% similarity) and the remaining eight isolates (1/38 from Cruz Alta, 1/39 from Passo Fundo and 6/33 from Nonoai) clustered with isolates of lineage 2 (showing 80·6% similarity). Regarding the trichothecene genotype of the 140 isolates, the 15-ADON genotype was the most common trichothecene genotype in all three populations, followed by the NIV genotype (11/140), in proportions that varied from ~2% in two populations to 18% in the Passo Fundo population. All lineage 7 isolates had the 15-ADON genotype and all lineage 2 isolates had the NIV genotype (Fig. 1).

Genetic distance

A sample of 103 isolates representing only lineage 7 from the Cruz Alta (38 isolates), Passo Fundo (38) and Nonoai (27) populations was used in the AFLP analysis. The use of three primer pair combinations (*Eco*RI-AA+*Mse*I-AT, *Eco*RI-CC+*Mse*I-CT and *Eco*RI-TG+*Mse*I-TT) resulted in 18, 31 and 32 AFLP bands, respectively. Of the 89 AFLP bands, 56% were polymorphic in Cruz Alta, 69% in Passo Fundo and 56% in Nonoai (Table 1). Across the populations, the genetic similarity between any pair of isolates averaged 81% (64–100%) and within populations it ranged from 68% to 100% for Cruz Alta, to 64–100% for Passo Fundo and 70–98% for Nonoai.

Genotypic diversity and recombination

Seven of the 103 isolates had an AFLP haplotype that was the same as that of another isolate. Specifically, two pairs of identical isolates were detected in the Cruz Alta population and three identical isolates were detected in the Passo Fundo population. Normalized genotypic diversity ($\hat{G}$) was high (≥98% of the count) in all populations (Table 1).

Allele frequencies were generally very similar among these three populations (data not shown), as were the mean gene diversities. There were seven loci with private alleles (both allelic forms present in one population but not in the other) in the Cruz Alta population, but none had both alleles at a frequency of >5%. In the Passo Fundo population there were 17 private alleles, with both of the alleles at three of these loci present at a frequency of >5%. In the Nonoai population there were four private alleles, of which one had both alleles at a frequency of >5% (Table 1). The mean frequency of the 28 private alleles across all populations was ~5·3%.

For the full set of 79 polymorphic loci, the average gene diversity was 0·129 for the combined population (0·123–0·135 range across individual populations) (Table 1). When 45 loci with rare polymorphic alleles were removed from the analysis, the mean gene diversity estimates for the combined population increased from 0·129 to 0·246.

Values of G_{ST} (fixation index or differentiation among populations as a result of population subdivision) for individual loci ranged from zero, i.e. either no divergence or equal allele frequencies, to 0·495 (Table 2). The mean G_{ST} across all 79 loci was 0·073 (N_m (effective migration rate) > 6·4 across all 79 loci) (Table 2). Similar results were obtained when analysing a subset of 34 loci for which the frequency of the rarer allele was >5% (mean G_{ST} = 0·083 and N_m > 5·5) (Table 2).

For the calculation of two locus linkage disequilibria, there were 3081 possible pairwise comparisons for the 79 AFLP loci. The null hypothesis of two-locus linkage equilibrium ($P < 0·01$) was rejected in favour of the alternative hypothesis of two-locus linkage disequilibrium for

Table 1 Comparison of estimates of genotypic diversity for three *Fusarium graminearum* lineage 7 populations from the state of Rio Grande do Sul, Brazil, based on AFLP loci

Population	All populations	Cruz Alta	Passo Fundo	Nonoai
Sample size	103	38	38	27
Percentage polymorphic loci		63	77	63
No. of haplotypes		36	36	27
No. of private alleles		7	17	4
Mean frequency of private alleles		0·038	0·048	0·102
Range of private alleles		0·026–0·053	0·026–0·079	0·037–0·296
$\hat{G}$[a]		1·0	0·987	0·982
Mean gene diversity[b]				
79 loci		0·123	0·135	0·129
34 loci		0·242	0·254	0·241

[a]Calculated as described by Milgroom (1996) from comparisons of AFLP allelic data at 79 AFLP loci. $\hat{G} = 1/\Sigma pi^2$, where pi is the observed frequency of the multilocus genotype in a population.

[b]Calculated as in Nei (1973).

Table 2 Statistics on population genetic differentiation between Cruz Alta, Passo Fundo and Nonoai clone-censored populations of *Fusarium graminearum* (lineage 7) calculated from all polymorphic loci, and for the 34 loci for which the frequency of both alleles (band and no band) was >5%

Statistic	79 loci	34 loci
Mean frequency diversity[a] (range)	0·129 (0·017–0·497)	0·246 (0·017–0·497)
Fixation index (G_{ST})[a] (range)	0·073 (0·001–0·495)	0·083 (0·002–0·495)
Effective migration rate (N_m)[b] (range)	6·386 (0·510–417·5)	5·516 (0·510–207·3)
Genetic identity[c]	0·984	0·960

[a]Calculated as in Nei (1973).
[b]Calculated as in McDermott & McDonald (1993).
[c]Calculated as in Nei (1978).

68 (2·2%), 47 (1·5%) and 26 (0·8%) of the locus pairs for the Cruz Alta, Passo Fundo and Nonoai populations, respectively. The null hypothesis of two-locus linkage equilibrium was rejected ($P < 0·05$) for 139 (4·5%), 111 (3·6%) and 71 pairs of loci (2·3%) from the Cruz Alta, Passo Fundo and Nonoai populations, respectively, and for 233 pairs of loci (7·6%) from the combined population.

Discussion

This study is the first of the genetic diversity of *F. graminearum* epidemic populations from Brazil. Ninety-three percent of the isolates grouped with standards of *F. graminearum* lineage 7 and 7% (eight isolates) grouped with standards of lineage 2. These results are consistent with a previous analysis of a regional population of 82 isolates collected from 25 municipalities in 2006. In that study, isolates with the NIV genotype also belonged to lineage 2 and those with the 15-ADON genotype all belonged to lineage 7. The NIV genotype isolates in the present populations consistently produced ~500-bp fragments with the Fg16F/R primer pair, as did the lineage 2 isolates in the previous study (Scoz *et al.*, 2009) and NIV genotype lineage 2 isolates from Nepal (Carter *et al.*, 2000). The dominance of NIV genotypes in the lineage 2 isolates is consistent with previous analyses of isolates collected in other locations (Ward *et al.*, 2002).

These results reinforce the evidence for the dominance of *F. graminearum* lineage 7 causing wheat head blight in South America, as previously shown in Argentina and Uruguay (Zeller *et al.*, 2003b; Ramirez *et al.*, 2007). In these populations, lineage 7 was the dominant, or the only, *F. graminearum* lineage causing FHB in wheat, although some isolates of lineages 2 and 3 have been recovered from cereal hosts other than wheat (Sampietro *et al.*, 2010, 2011). Similar patterns of lineage 7 dominance have also been observed in North America and Europe (Zeller *et al.*, 2003a, 2004; Tóth *et al.*, 2005; Schmale *et al.*, 2006).

There is, as yet, no explanation for the widespread distribution of lineage 2 isolates in wheat and barley (P. Astolfi, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, unpublished data) in Brazil and not in the same crops in other South American countries. O'Donnell *et al.* (2000) suggested that lineage 2 originated in South America or Africa, but isolates of this lineage are known from Asia and Australia (O'Donnell *et al.*, 2000, 2004). Lineage 2 isolates from maize in Nepal also have a consistent NIV genotype pattern (Desjardins & Proctor, 2011), similar to those from maize from northwestern Argentina (Sampietro *et al.*, 2011).

In this study, isolates with the same AFLP genotype, i.e. clones, were rare; 7/103 isolates had a non-unique AFLP genotype. Correspondingly, the normalized genotypic diversity ($\hat{G}$) was high (≥98% of the count) in all three subpopulations analysed. The high genotypic diversity with relatively few clones strongly suggests that recombination played a significant role in the life history and population structure of *F. graminearum* in Brazil. Such findings are in agreement with previous studies on *F. graminearum* populations spanning three continents, in which haplotype diversity within individual populations ranged from approximately 30 to 100% with a median of 81%, and nearly all isolates sampled had unique haplotypes in 17 of 29 populations analysed (Miedaner *et al.*, 2008). In the USA, populations from two intensively and hierarchically sampled fields 500 km apart had an average of 67% unique haplotypes detected on spatial scales as small as (0·25 m^2), and rarely more than one isolate of the same haplotype was recovered from the same wheat head (Zeller *et al.*, 2003a, 2004).

Gene flow prevents genetic isolation because of drift. The genetic identities (Nei, 1978) of the three subpopulations evaluated here were all close to 1. The low G_{ST} values also indicated minimal geographic subdivision among the subpopulations and yielded N_m values between 5 and 6 across all loci and subpopulations, suggesting that the level of gene flow was approximately six times greater than that needed to prevent populations from diverging by genetic drift. These data support the hypothesis that the three subpopulations sampled are all of a larger population. This conclusion is tempered, however, by the relatively large number of loci (28/79) with private alleles in one of the three subpopulations. The relatively high frequency of some of these private alleles (up to 30%) may indicate that the observed migration levels reflect historical, rather than contemporary, gene flow. Other studies on gene flow in *F. graminearum* suggested up to 2000 migrants between populations in the USA (Zeller *et al.*, 2004), between seven and 30 in China (Gale *et al.*, 2002), between five and 20 in western Canada (Mishra *et al.*, 2004) and between two and 2000 in Argentina (Ramirez *et al.*, 2007).

Sexual recombination in *F. graminearum* has been observed from heterozygous perithecia under laboratory conditions with a moderate level of outcrossing (Bowden & Leslie, 1999). Under field conditions, recombination has been inferred only from high genotype diversity by

using VCGs and one or more molecular markers (Zeller *et al.*, 2003a). A population in which recombination occurs regularly should have relatively little linkage disequilibrium (LD), while one in which recombination is rare should have many more loci in LD. Almost 4·4% of the locus pairs scored in the present study were in LD. These values are lower than those found in other populations from USA and Argentina (Zeller *et al.*, 2003a, 2004; Schmale *et al.*, 2006; Ramirez *et al.*, 2007). The essential lack of observable LD in these Brazilian populations is a strong argument for them to be mating effectively randomly and exchanging genetic information relatively freely.

From an ecological perspective there may be particular epidemiological factors affecting FHB epidemics in southern Brazil that may impact the lineage diversity and evolutionary history of *F. graminearum* populations in the country. For example, inoculum sources are abundant because of the highly intensive and historical no-till cropping in southern Brazil for over 20 years. In this subtropical environment, inoculum is available all year round and sexual structures on crop residues may form and release airborne ascospores at any time of year (Reis, 1988; Panisson *et al.*, 2002), potentially infecting other hosts used in crop rotational systems such as maize and soyabean, from which multiple lineages and trichothecene genotypes have been isolated (E. M. Del Ponte, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, unpublished data). Further analysis of fungal populations from the country, either from wheat or other cereal hosts, and combined genetic diversity studies with populations from other countries in South America will help to shed light on the macro-scale population biology of *F. graminearum*.

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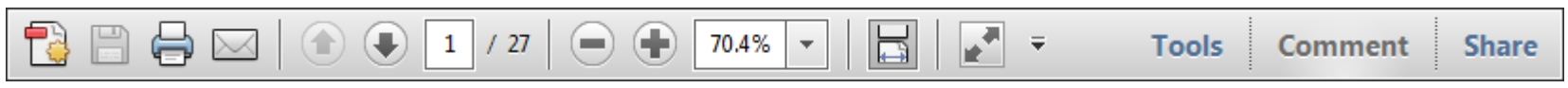
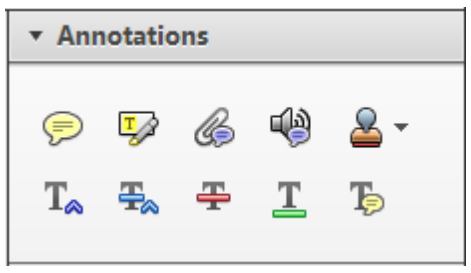
Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

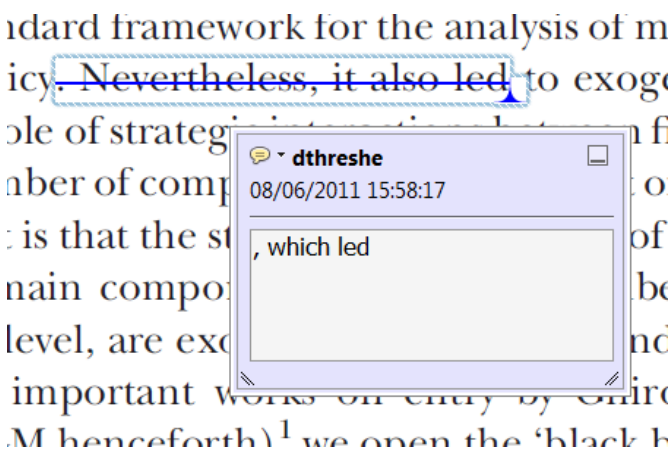


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

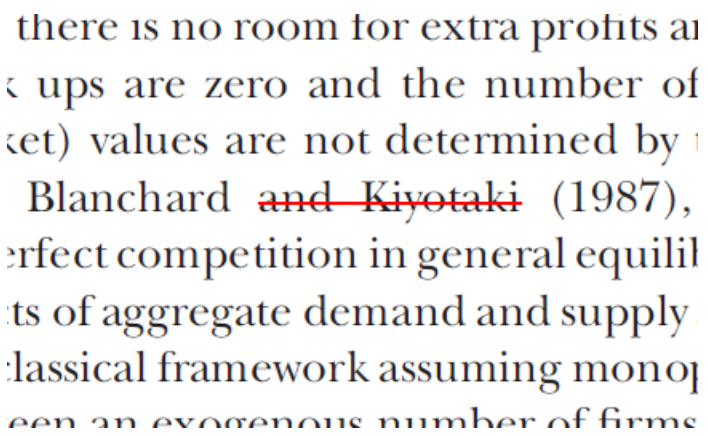


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

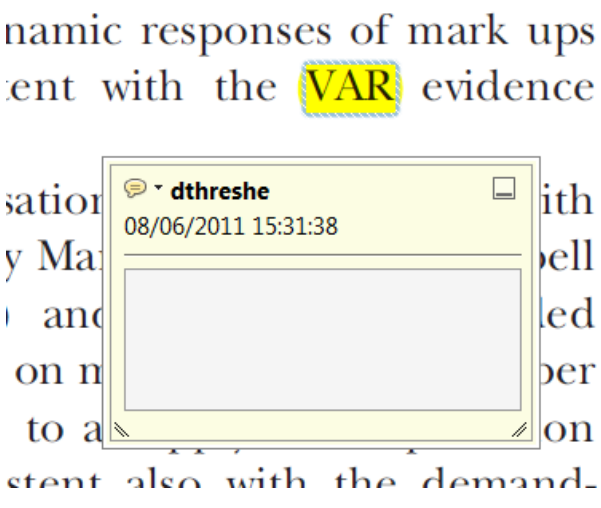


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

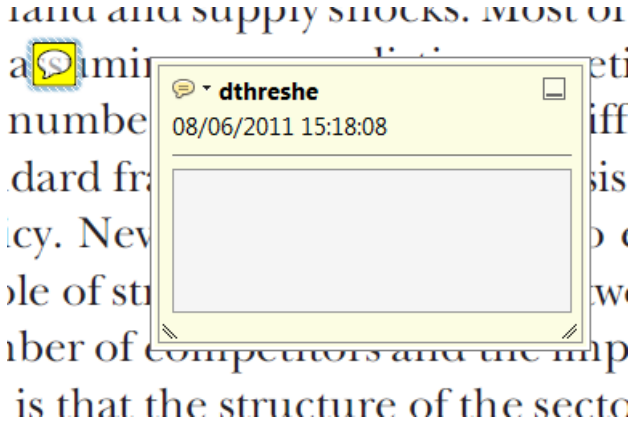


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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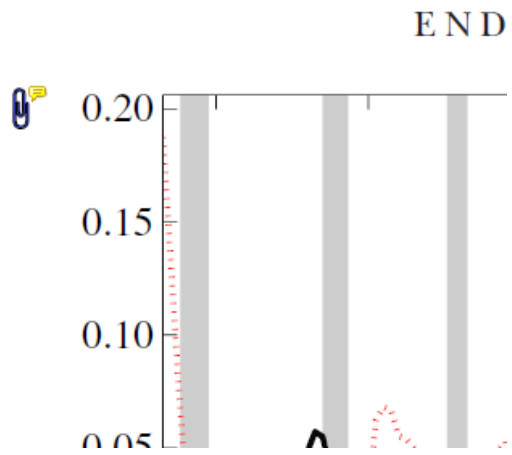
5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

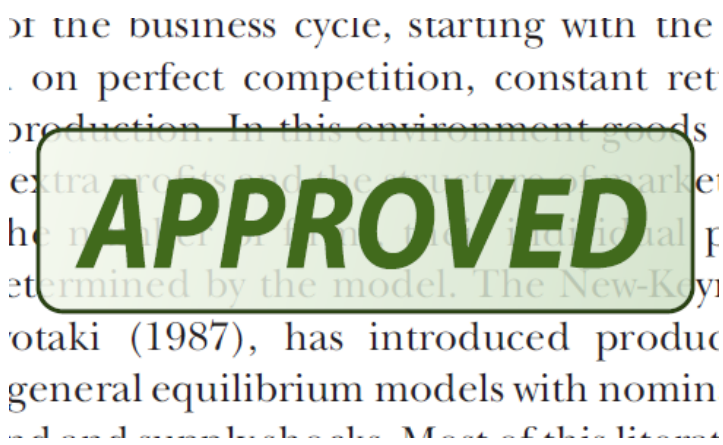


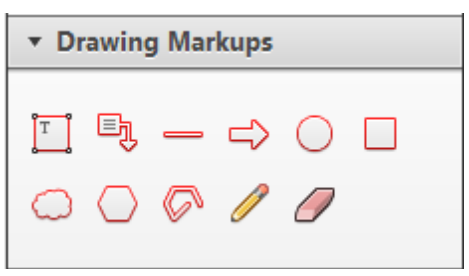
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

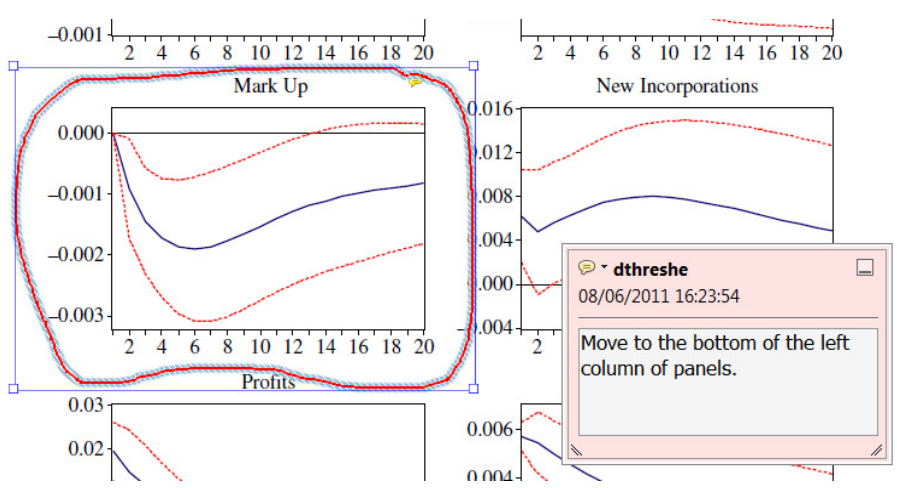
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).





7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: