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PHENOTYPIC AND MARKER ASSISTED EVALUATION OF AGGRESSIVENESS TOWARD WHEAT IN SOME ROMANIAN *FUSARIUM* POPULATIONS

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ABSTRACT

Aggressiveness toward wheat of 56 new *Fusarium* accessions, obtained from random naturally infected grain samples of bread wheat, durum wheat and triticale collected across eight year/location combinations from Romania, phenotypically and by molecular tools was investigated. Molecular techniques allowed identification of *Fusarium* species and the analysis of polymorphism within fungal isolates.

A large overall variation of aggressiveness, phenotypically expressed as reduction of coleoptiles length (ranging from 2.1 to 48.2 % of control), in two independent trials performed in seedling stage, on average over three varieties, was registered. Field point inoculations at anthesis of 90 *Fusarium* isolate x wheat varieties combinations also revealed variability of several components of aggressiveness in adult stage: *severity* (14.4-64.8%), *AUDPC* (104.9-527.1) and *FDK* (8.1-43.7%), respectively. The presence of *TRI5* gene involved in DON biosynthesis was detected in most isolates, allowing the assumption of their high toxigenic potential associated with the other aggressiveness traits. Both DON chemotypes, 3ADON and 15ADON, were identified in *Fusarium* populations from Albota, Brasov, Livada and Simnic. Similarity between records obtained in seedling and adult stage for the most aggressive of *Fusarium* isolates, suggests that phenotypic selection, in conjunction with molecular tools, could be a reliable method to select the appropriate pathogen strains for breeding of resistance.

Key words: Aggressiveness, *Fusarium graminearum, F. culmorum,* molecular polymorphism, *TRI 5* gene.

INTRODUCTION

Fusarium graminearum [(teleomorph *Gibberella zeae* (Schwein.) Petch.] and *F. culmorum* are known as main producers of Fusarium head blight (FHB), a disease that threatens wheat production across the world and poses a significant public health hazard, because of association with mycotoxin accumulation in grains (Pestska and Smolinski, 2005). Deoxynivalenol (DON, vomitoxin), with its analogs, 15ADON and 3ADON, is one of the most common trichothecene toxin produced by *F. graminearum*, representing an aggressiveness factor on wheat (Bai et al., 2002). Large genetic variability in *F. graminearum,* with close relation among different lineages (*phylogenetic species*), was reported (Bowden and Leslie, 1999) and a QTL for aggressiveness linked to the *TRI 5* locus was detected (Cumagun et al., 2004).

Deployment of resistant germplasm is the most accepted component of the strategy to control FHB, in spite to the complex nature of both, host resistance and pathogen aggressiveness, influenced by environment and laborious screening systems (Miedaner et al., 2001; Bai and Shaner, 2004; Buerstmayr et al., 2009). Wheat resistance to FHB, regardless the components already described (Schroeder and Christensen, 1963), is a multigenic, quantitative trait, where no immune cultivar and clear host by species interaction are known (Van Euwijk et al., 1995; Bai and Shaner, 1996). In the last years, a shift in *Fusarium* population from North America toward an increased frequency and aggressiveness (growth rate and DON production), as effect of 3ADON chemotype that become more common than 15ADON chemotype, has been reported (Ward et al., 2008). Recently, the higher aggressiveness of 3ADON in terms of FHB development

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and DON accumulation than other *Fusarium* specific chemotypes has been experimentally proved (Zhang et al., 2011).

Marker assisted selection (MAS) for host resistance and pathogen aggressiveness were employed in the past years for a better understanding and validation of these traits in *Fusarium*/wheat pathosystem. QTLs associated with FHB resistance, Type I (resistance to initial infection), Type II (resistance to spread into the plant from inoculation point) and more recently with DON detoxification (Type III *sensu* Miller and Arniston, 1986) were identified on almost all wheat chromosomes (Van Sanford et al., 2001; Lemmens et al., 2005; Ma et al., 2006; Häberle et al., 2007; Buerstmayr et al., 2009; Liu et al., 2009; Zhang et al., 2010). PCR markers for trichotecene genes *(TRI)* expression of *Fusarium graminearum,* in order to predict occurrence of new chemotypes of *F. graminearum,* and new techniques aimed to identify resistance to FHB in wheat were developed (Chandler et al., 2003; Gosman et al., 2010). That's why basic information about pathogenic ability is needed, in order to maximize the breeding efforts toward the management of FHB risks.

Our objectives were to (i) estimate the phenotypic variation of aggressiveness in *Fusarium* isolates obtained from several local pathogen populations, under artificial inoculation in seedling and adult stages, (ii) use molecular markers for identification of *Fusarium* species and toxigenic potential, expressed as DON production.

MATERIAL AND METHODS

Fusarium isolates. 56 *Fusarium* accessions obtained at NARDI Fundulea from naturally infected grains of bread wheat, durum wheat and triticale, randomly sampled in 2008 (30 isolates) and 2009 (26 isolates) from five (Albota, Brasov, Fundulea, Livada, Simnic and Tg. Mures) and three locations (Albota, Fundulea and Livada) respectively, were analyzed in seedling stage, while for field experiments by point inoculation only ten *Fusarium* isolates selected from them were used (Table 1). Inoculum necessary for artificial inoculations, consisted of homogenized suspensions of conidia in distilled water (about 500000/ml), produced from *Fusarium* isolates cultured on both, Czapek Dox medium and sterilized wheat grains under black light (Philips TLO, 40 W/80).

Table 1. Number of *Fusarium* isolates obtained from local populations analyzed for aggressiveness under artificial inoculation in seedling and adult stage (Test series)

Origin					Test of aggressiveness 2009-2010	
Sampling year	Location	Host plant			Seedling 2009 (1)-2010 (2)	Adult 2010 (3)
		bread wheat	durum wheat	triticale		
2008	Albota	5	-	1	6	1
	Brasov	-	2	-	2	-
	Livada	14	-	5	19	6
	Simnic	1	-	-	1	1
	Tg. Mures	2	-	-	2	2
Total		*22*	*2*	*6*	*30*	*10*
2009	Albota		-	-	11	-
	Fundulea		-	-	7	-
	Livada		-	-	8	-
Total		*26*	-	-	*26*	-

Aggressiveness test

In seedling stage, aggressiveness was evaluated according to the protocol established by Ittu (1986), where this trait was expressed as reduction of coleoptile length in % of control.

Each *Fusarium* isolate from both groups (2008 and 2009) was used to inoculate eight

days old seedlings of three wheat entries, in separate tests performed in 2009 (Test 1) and 2010 (Test 2), respectively.

In adult stage aggressiveness was analyzed in 90 host x pathogen combinations, in terms of *severity* (damaged florets at 20 days post inoculation, %), area under disease progress curve (AUDPC) and *Fusarium* damaged kernels (FDK %). Plant material consisting of nine adapted Romanian varieties (Mustatea et al., 2009), released between 1991-2004, by NARDI Fundulea (Dropia, Boema, Dor, Delabrad2) and the wheat breeding centers from Turda (Ariesan, Apullum, Dumbrava), Albota (Trivale), and Simnic (Briana), were inoculated at anthesis, by artificial point inoculations in the FHB experimental field at Fundulea with ten *Fusarium* isolates extracted from the 2008 pathogenic populations (Test 3).

Genotyping. DNA isolation from each fungal isolate was performed by the method described by Stepien et al., 2008. Three pair primers for the identification of *F. graminearum* (Fg16F/Fg16R), *F. culmorum* (FC01F/FC01R) and *F. sporotrichoides* (Fspo1F/Fspo1R) were used in a multiplex PCR reaction according to Demeke et al. (2005). Molecular polymorphism of the 94 fungal isolates was analyzed by RAPD technique, using seven arbitrary primers (OPA17, OPA19, OPC06, OPG06, OPR05, UBC147, UBC180 and UBC186) from Operon Technology and University of British Columbia, respectively. The presence of *TRI5* gene was checked in a PCR reaction using *tox5-1/tox5-2* primer pair (Niessen and Vogel, 1998). The PCR products were analyzed by electrophoresis on 1.5% agarose gel in 0.5 x TBE (Sigma-Aldrich) stained with 0.3 μg/ml ethidium bromide. Pictures from electrophoresis gels provided the information for phylogenetic tree. Each band was considered as a locus (presence of the band was scored as 1 and its absence as 0). All bands were studied except weak and incomplete ones. Genetic distance was computed by Nei and Li (1979) formula, using TREECON 1.3b software package.

Dendrogram was prepared by UPGMA (Unweighted Pair Group Method with Arithmetical Averages).

Statistical analysis. Analysis of variance (ANOVA) was used to estimate contributions attributable to genotypes of the pathogen and host, regarding aggressiveness.

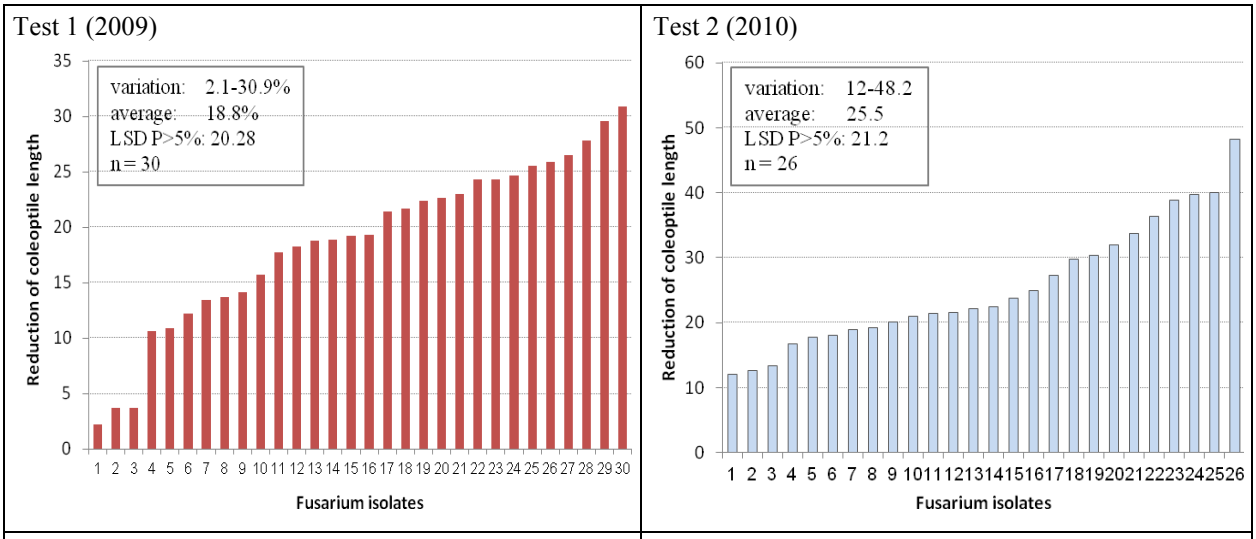
RESULTS AND DISCUSSION

Assessment of aggressiveness. A broad range of variation for aggressiveness toward the host was found among *Fusarium* accessions isolated from the pathogenic populations in 2008 and 2009, in wheat seedling trials performed in 2009 (Test 1) and 2010 (Test 2) (Figure 1).

Reduction of coleoptile length following the artificial inoculation with 30 *Fusarium* isolates in Test 1, ranged on average from 2.1 to 30.9% and from 12.0 to 48.2%, as compared with control in the group of 26 *F.* isolates analyzed in Test 2, respectively. An overall comparison between both groups suggests a relatively higher level of aggressiveness in *Fusarium* isolates extracted from the pathogen populations sampled in 2009 (Table 2).

Highly aggressive *Fusarium* isolates, inducing a reduction of coleoptiles length, exceeding 25.0% as compared with control, were obtained from naturally infected grain samples originated from different locations in 2008: Albota (FG1156, FG1182); Livada (FG1272, FG1226, FG1228) and Simnic (FC1056) and 2009, respectively: Albota (1494; 1475; 1485; 1552; FC1471); Fundulea (FS1625; 1626; FS1627) and Livada (FC1453; FG1445) (Table 3).

The experiment performed in adult stage (Test 3) showed a large variation of aggressiveness in terms of severity (%), AUDPC and FDK (%), among the ten analyzed *Fusarium* isolates toward nine wheat cultivars. The corresponding limits of variation for these phenotypic parameters were on average 14.4-64.8% (severity); 104.9-527.1 (AUDPC) and 8.1-43.7% (FDK) (Table 4).



Fusarium isolates 2008: 1)FG1266; 2)FG1216; 3)FG1143; 4)FG 1313; 5)FG983; 6)FG1220; 7)FG1169; 8)FG1318; 9)FG991; 10)FG1222; 11)FG1137; 12)FG1237; 13)FG1278; 14)FG1238; 15)FG1317; 16)FG1316; 17) FG1343; 18)FG1224; 19)FG1211; 20)FG1274; 21)FG1145; 22)FG1239; 23)FG1204; 24)FG1265; 25)FG1156; 26)FG1182; 27)FG1272; 28)FG1226, 29)FG1228; 30)FC1056.

Fusarium isolates 2009: 1)1443; 2)FG1445; 3)FC1447+; 4)FG1449; 5)FS1450; 6)FC1452; 7)FC1453; 8)FS1576; 9)FC1471; 10)1475; 11)1485; 12)1494; 13)1499; 14)FS1512; 15)1522; 16)1532; 17)1552; 18)FC+1553; 19)FC+1564; 20)1588; 21)1593; 22)1604; 23)FS1619; 24)FS1625; 25)1626; 26)FS1627.

Figure 1. Variation of aggressiveness toward three wheat genotypes, in *Fusarium* isolates selected in 2008 and 2009 (mean values for reduction of coleoptile length, % of control)

Table 2. Aggressiveness of 56 *Fusarium* isolates selected from eight year/location populations (means and ranges for reduction of coleoptile length, % of control under artificial seedling inoculation)

Isolate origin	Reduction of coleoptile length, % of control		
	Number of isolates	Location mean	Range
Year 2008			
Albota	6	18.2	3.7-26.0
Brasov	2	12.5	10.9-14.1
Fundulea	-	-	-
Livada	19	18.5	2.1-30.0
Simnic	1	31.0	-
Tg. Mures	2	23.4	22.4-24.3
Total	30	-	-
Year mean		*21.0*	*15.8-24.6*
LSD, P>5%	20.3		
Year 2009			
Albota	11	26.0	12.7-39.8
Brasov	-	-	-
Fundulea	7	28.5	18.9-48.2
Livada	8	22.0	12.0-36.4
Simnic	-	-	-
Tg. Mures	-	-	-
Total	26	-	-
Year mean		*25.5*	*14.5-41.5*
LSD, P>5%	21.2		

Table 3. The most aggressive *Fusarium* isolates extracted from local populations investigated in 2008 and 2009 (mean values for reduction of coleoptile length, % of control)

Isolate origin		*Fusarium* isolate code	Reduction of coleoptile length, % of control
Year	Location		
2008	Albota	1156	25.5
		1182	25.9
	Livada	1272	26.5
		1226	27.82
		1228	29.6
	Simnic	1056	30.9
2009	Albota	1494	29.7
		1475	30.3
		1485	31.9
		1552	38.8
		1471	39.8
	Fundulea	1625	27.3
		1626	40.0
		1627	48.2
	Livada	1453	33.8
		1445	36.4

Table 4. Components of aggressiveness under field point inoculation
(Fundulea 2009, ten isolates *vs*. nine wheat varieties, mean values)

Entry	Severity, %			AUDPC			FDK, %		
	Minimum	Maximum	*Mean*	Minimum	Maximum	*Mean*	Minimum	Maximum	*Mean*
FC1056	28.3	92.4	*61.8*	217.5	707	*527.1*	10.4	82.3	*43.7*
FG1265	11.4	75.0	*42.8*	89.5	536	*312.0*	1.9	59.1	*30.5*
FG1224	31.6	100	*64.8*	231	800	*489.0*	0.8	39.4	*29*
FG1272	15.1	65.7	*44.3*	145.5	462.5	*321.3*	0.7	46.5	*27.5*
FG1237	2.6	68.5	*48.9*	16	464.5	*329.6*	0	41.4	*26.6*
FG1145	20.8	62.7	*46.9*	193	458.5	*342.0*	6.3	49.0	*26*
FG1211	22.6	79.7	*54.1*	159	753	*439.9*	3.5	51.0	*26.0*
FG1204	16.5	57.3	*39.7*	113.5	437	*298.4*	3.2	54.6	*25.7*
FG1228	15.0	82.3	*40.7*	147	593.5	*326.6*	5.0	38.5	*22*
FG1239	8.3	23.9	*14.4*	72	145.5	*104.9*	0	18.3	*8.1*
Average	*17.2*	*70.8*	*45.8*	*138.4*	*535.8*	*349.1*	*3.2*	*48.0*	*26.5*
Minimum	*2.6*	*23.9*	*14.4*	*16*	*145.5*	*104.9*	*0*	*18.3*	*8.1*
Maximum	*31.6*	*100*	*64.8*	*231*	*800*	*527.1*	*10.4*	*82.3*	*43.7*
LSD for <5%			*11.9*			*79.9*			*10.1*

Similarly to the experiments carried on in seedling stage, *Fusarium culmorum (FC) 1056* isolated from Simnic in 2008 (Figure 1, entry number 30) expressed the highest level of relative aggressiveness in adult stage, in respect of AUDPC (527.1) and FDK, % (43.7). A good agreement between records obtained in both stages of wheat development, was also observed for other *Fusarium* isolates. Such findings suggest that phenotypic selection in conjunction with molecular tools could be a reliable method to select the appropriate pathogen strains for breeding of resistance.

Molecular analysis. Based on the expected amplicons produced by specific primers (Demeke et al., 2005) among all 94 fungal isolates analysed, DNA being obtained only from 78 of them, *Fusarium graminearum* was identified as being prevalent (87%) in populations sampled in 2008, while an increased incidence of *Fusarium culmorum* (24%) and other species from the *Fusarium* head blight complex, mainly *Fusarium sporotrichoides* was observed under natural conditions in the 2009 pathogenic *Fusarium* pools (Table 5).

Table 5. Structure of *Fusarium* populations isolated from wheat under natural conditions
(eight combinations year/location)

Origin of *Fusarium* populations													
Location	Year 2008							Year 2009					
	Isolates	**FG***		**FC****		Other***		Isolates	**FG**		**FC**		Other	
		No	*%*	No	*%*	No	*%*		No	*%*	No	*%*	No	*%*
Albota	18	**17**	*94*	**0**	*0*	**0**	*0*	11	**0**	*0*	**3**	*27*	**2**	*18*
Brasov	4	**3**	*75*	**1**	*25*	**0**	*0*	-	**-**	-	**-**	-	**-**	-
Fundulea	0	**0**	*0*	**0**	*0*	**0**	*0*	6	**0**	*0*	**0**	*0*	***3***	*50*
Livada	40	**35**	*88*	**0**	*0*	**5**	*11*	8	**2**	*25*	**3**	*38*	**2**	*25*
Simnic	2	**0**	*0*	**2**	*100*	**0**	*0*	-	**-**	-	**-**	-	**-**	-
Tg. Mures	6	**5**	*83*	**0**	*0*	**0**	*0*	-	**-**	-	**-**	-	**-**	-
Total	69	**60**	*87*	**3**	*4*	**0**	*0*	25	**2**	*8*	**6**	*24*	**7**	*28*

* *Fusarium graminearum (FG);*
** *F. culmorum (FC);*
*** *F. sporotrichoides* and other species.

RAPD analysis. Utilization of seven decameric primers suggested a relatively low molecular polymorphism in analyzed *Fusarium* isolates. Distinct differences were observed in some of the isolates, especially with OPA19 primer.

Based on the electrophoretic pattern of the amplicons, two clusters of strains with TREECON program were detected: one includes isolates *FG1228, FG1265, FG1182* and *FG1137*, while the others are grouped in the second cluster, which also contains several sub-clusters (Figure 2).

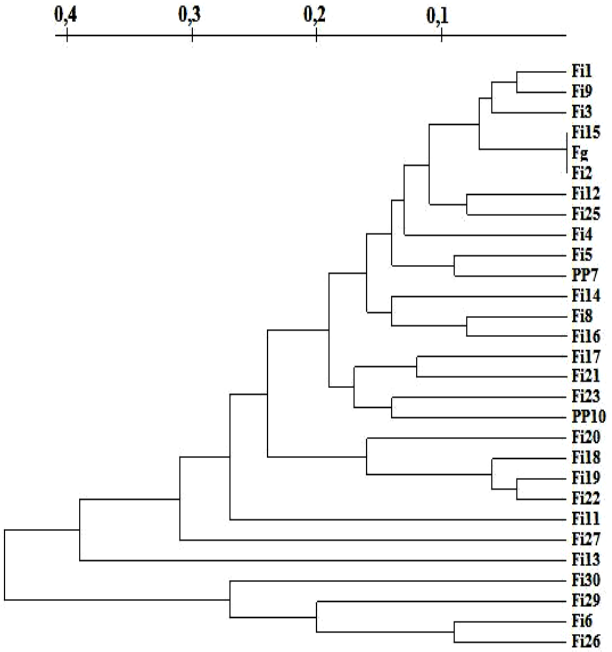


Figure 2. Dendrogram resulted after RAPD analysis of Romanian *Fusarium* isolates

(Legend: Fi1-FG 983; Fi9-FG 1216; Fi3-FG1143; Fi15-FG1278; Fi2-FG 991; Fi12-FG1226; Fi25-FG 1266; Fi4-FG1145; Fi5-FG1156; Fi14-FG1274; Fi8-FG1211; Fi16-FG1313; Fi17-FG1316; Fi21-FG 1222; Fi23-FG1272; Fi20-FG 1343; Fi18-FG1317; Fi19-FG 1318; Fi22-FG 1239; Fi11-FG1224; Fi27-FG1169; Fi13-FG1237; Fi30-FG1228; Fi29-FG1265; Fi6-FG1182; Fi26-FG1137)

The DON chemotype of some *Fusarium* isolates was determined using the primer set tox5-1/tox5-2, specific for presence of TRI5 gene (Niessen and Vogel, 1998).

The corresponding amplicon was found in all *Fusarium* isolates tested, except FG1266 and FG1216. This suggests that most isolates have the ability to produce DON (Figure 3).

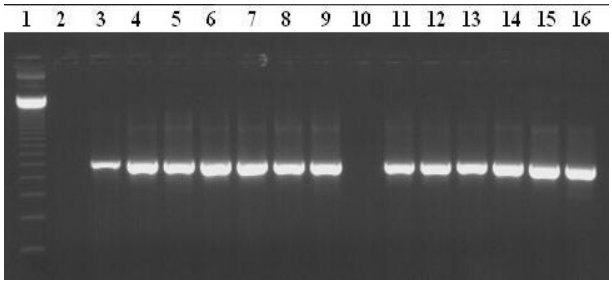


Figure 3. Amplification products obtained by PCR reaction carried out using DNA purified from *Fusarium* isolates and primer set *tox5-1/tox5-2*: 1 – ladder 123 bp (Roth); 2 – FG1266; 3 – FG983; 4 –FG991; 5 – FG1143; 6 – FG1145; 7 – FG1156; 8 – FG1182; 9 – FG1211; 10 – FG1216; 11 – FG1224; 12 – FG1226; 13 – FG1237; 14 – FG1274; 15 – FG1278; 16 – FG1313

The incidence of both DON chemotypes, 3ADON and 15ADON, was signaled in four *Fusarium* populations from Albota, Brasov, Livada and Simnic. The chemotype 3ADON was characteristic mainly to *F. culmorum* isolates (data not shown). Further investigations on aggressiveness of chemotype-specific *Fusarium* isolates and the corresponding resistance to FHB of different wheat varieties are demanded, as the information obtained could have an impact on development of resistant cultivars and on disease management.

CONCLUSIONS

A large variability of aggressiveness toward wheat in 56 new isolates originated from local Romanian *Fusarium* populations (eight year x location combinations) was revealed in assessment trials performed in seedling and adult stages.

The prevalence of *Fusarium graminearum* in the head blight complex of wheat and the ability to produce DON was rendered evident *via* molecular approach.

Phenotypic selection in conjunction with molecular tools could be a reliable method to select the appropriate pathogen strains for breeding of resistance to *Fusarium* disease in wheat.

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