Article

Application Marker-Assisted Selection (MAS) and Multiplex PCR Reactions in Resistance Breeding of Maize (*Zea mays* L.)

Aleksandra Sobiech [1,*], Agnieszka Tomkowiak [1], Jan Bocianowski [2], Bartosz Nowak [3], Dorota Weigt [1], Danuta Kurasiak-Popowska [1], Michał Kwiatek [1], Sylwia Mikołajczyk [1], Janetta Niemann [1] and Katarzyna Szewczyk [1]

[1] Department of Genetics and Plant Breeding, Poznan University of Life Sciences, Dojazd 11, 60-632 Poznan, Poland
[2] Department of Mathematical and Statistical Methods, Poznan University of Life Sciences, Wojska Polskiego 28, 60-637 Poznan, Poland
[3] Smolice Plant Breeding Sp. Z O. O. IHAR Group, Smolice 146, 63-740 Kobylin, Poland
* Correspondence: aleksandra.sobiech@up.poznan.pl

Abstract: Cultivated maize (*Zea mays* L.) is the oldest and one of the most important crop species in the world. Changing climatic conditions in recent years, warm weather, expansion of acreage and intensification of maize cultivation have resulted in an increase in the threat posed by diseases caused by, among others, *Fusarium* fungi. Breeding success in all plant species is determined by access to starting materials with possible high genetic diversity also in terms of disease resistance. Identification of parental combinations that produce offspring that are high-yielding and resistant to *Fusarium*, among other diseases, is one of the costliest steps in breeding programs. We used maize lines which, as a result of five-year field observations, were divided into resistant and susceptible to *F. verticillioides.* It is known that resistance to fusarium is a trait strongly dependent on environmental conditions. Due to the fact that the years of observation of the degree of infestation were hot and dry, the resistance of some lines could result from favorable environmental conditions. In view of the above, the aim of this study was to analyze the genetic basis of the resistance of these lines and to correlate molecular analyses with field observations. Comprehensive field and molecular analyses will allow the selection of reference lines that will be resistant to fusarium in the field and, at the same time, will have pyramidized resistance genes. Such lines can be used for crossbreeding to obtain fusarium-resistant varieties. In addition, an attempt was made to develop Multiplex PCR conditions for faster identification of the analyzed markers. As a result of the analyses, it was found that the resistance of the studied maize lines was correlated with the number of molecular markers identified in them. Both field and laboratory analyses have shown that the best line that can be used for crossbreeding as a source of fusarium resistance genes is the line number 25. It has a resistance level of 8–9 on the nine-point COBORU scale. In this line, as a result of molecular analyses, 10 out of 12 markers were identified (SSR 85, Bngl 1063, Bngl 1740, Umc 2082, Bngl 1621, Umc 2059, Umc 2013, SSR 93, SSR 105, STS 03) related to fusarium resistance genes, which may be the reason for such a high resistance to this pathogen. Similarly, 9 markers were identified for line number 35 (SSR 85, Bngl 1063, Bngl 1740, Umc 2082, Bngl 1621, Umc 2059, Umc 2013, SSR 93, STS 03). This line, however, was characterized by a slightly lower resistance at the level of 7–8. Line 254 turned out to be the least resistant, as the resistance was at the level of 4–5, and the number of identified molecular markers was 5. Lines numbered 25 and 35 can be successfully used as a source of fusarium resistance genes.

Keywords: maize; molecular markers; multiplex PCR; *Fusarium*

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1. Introduction

The maize (*Zea mays* L.), along with rice, is the most commonly cultivated crop for human and animal consumption. It is also a species grown for grain and feed. In 2022, the

largest producers of maize for grain were the United States of America (367.3 million tons), Mexico (337.8 million tons) and China (266.2 million tons). Maize ranks first place in terms of production volume among grain crops in the world (about 1 billion tons). In comparison, in 2000, the leading positions in the production of this species were occupied by the same countries; however, the amount of grain they produced was 251 million tons, 106 million tons and 32 million tons, respectively. These data confirm that over the past 20 years, the global maize production for grain has increased significantly [1].

The prevailing warm weather in the last decade of the 20th century, the expansion of acreage and intensification of corn cultivation, as well as the introduced agrotechnical simplifications, resulted in an increase in the threat caused by diseases to the amount and quality of yields. In addition, epidemics are facilitated by the emergence of new agrophage species, and the invasive movement of those already present, into neighboring areas. Based on studies conducted over the past few years, it is estimated that corn diseases cause yield losses of up to 30% each year [2]. The quality of the grain yield is also significantly affected because early plant infection by fungi and bacteria causes the grain to become diminished, deteriorating the feed value and quality of the forage obtained [3].

Diseases caused by fungi of the genus *Fusarium*, which are the main culprits of seedling rot, root rot and stem base rot, but also the most dangerous cob fusariosis, are now considered the most dangerous. Maize cob fusariosis, except in cases of severe occurrence, causes little yield loss, but greatly impairs the quality of grain and feed as a product for further processing [2,4]. Fungal species responsible for the infestation, in addition to secreting substances necessary for life, have the ability to produce secondary metabolites, so-called mycotoxins, accumulated in grains and other parts of the plant (trichothecenes, among others: T-2 toxin and diacetoxyscirpenol-DAS, ochratoxin A, zearalenone, deoxynivalenone-DON, HT-2 toxin, alphatoxins and others). These substances can cause many diseases in humans, including various types of allergies, hormonal disorders, cancer (they activate oncogenic cells) [5]. Their presence in feed also poses a major threat to animal health and life, especially for pigs and poultry, as they cause increased susceptibility to infectious agents that under standard conditions, without the additional action of toxigenic fungal metabolites, would not be able to cause disease. In addition, they negatively affect production and reproductive performance and, most importantly, the health and quality of the final product going to the consumer [6].

Fusarium resistance is a polygenic and is strongly influenced by environmental factors. This type of resistance is very complex which makes breeding difficult and results in most commercial maize hybrids having a lower level of resistance than is desirable [7].

Integrated pest management involves the development of non-chemical methods as an alternative to the most commonly used fungicides. Breeding for plant resistance to pathogens is a sustainable way to produce more crops without using inputs that are harmful to the environment and humans [8]. Stagnati et al. [9] mapped quantitative trait loci (QTLs) for *F. verticillioides* resistance in two maize populations. As a result of the study, they found that they were not consistent between populations. Analyzing the transcriptome and identifying areas associated with *F. verticillioides* resistance may help to better understand the processes that occur when plants are infected by these fungi.

Salah et al. [10] using marker-assisted selection (MAS) identified molecular markers linked to *F. verticillioides* resistance (QTL) genes: RAPD (OPA02), ISSR (AD8), SSR (SSR93, SSR105, SSR225 and SSR337) and STS (STS03). It was shown that the SSR and STS markers were located on chromosome 10. The use of SNP markers coupled to yield structure traits in maize and barley, showed greater precision than methods based on the study of metabolic pathways [11]. Maschietto et al. [12] demonstrated, the utility of SSR, GBS, and transcriptomics markers and QLT mapping to improve the selection of *F. verticillioides* resistant lines. Abdel-Rahman et al. [13] showed that regression analysis for the relationship between SSR markers and phenotypes of maize F_2 populations evaluated for *F. verticillioides* head blight severity was highly significant. This indicates that SSR markers were associated with resistance to the disease. SRR markers have been the most widely used markers

for many years due to their ease of use and relatively low price. Nowadays, more and more research is being conducted using SNP markers, as they occur at a much higher frequency in the genome than SRRs. Authors Jones et al. [14], Hamblin et al. [15] and Van Inghelandt et al. [16] point to the advantage of SNP markers over SRRs in studying the genetic diversity of inbred lines in maize.

Today, in public databases, more than one million SNPs can be found for maize. With the development of association mapping using SNPs, it will be possible to accelerate the identification and use of new agronomically useful alleles [17].

Marker-assisted selection (MAS) reduces financial expenses and increases productivity. By increasing the efficiency of selecting varieties for crossbreeding, breeders can improve breeding programs in less time [18].

The process, referred to as multiplex polymerase chain reaction (PCR), saves considerable time and money by simultaneously amplifying multiple sequences in a single reaction. Optimization of the method consisted of selecting the appropriate primer volume for all markers in each variant and adjusting the appropriate primer annealing temperature in order to obtain uniformly intense bands on the gel. Developing an effective multiplex PCR usually requires strategic planning and multiple attempts to optimize reaction conditions. The testing of a number of molecular markers related to maize plant resistance to *F. verticillioides* and the development of multiplex PCR conditions will provide breeders with tools ready to guide selection.

In view of the above, the aim of this study was to analyze the genetic basis of resistance to the fusarium of the studied lines and to correlate molecular analyses with field observations. Comprehensive field and molecular analyses will allow the selection of reference lines that will be resistant to fusarium in the field, and at the same time, they will be characterized by pyramidized resistance genes. Such lines can be used for crossing to obtain fusarium-resistant varieties. Additionally, an attempt was made to develop Multiplex PCR conditions for faster identification of the analyzed markers

2. Materials and Methods

2.1. Plant Material

Plant material consisted of 30 genotypes (15 resistant and 15 susceptible) to *F. verticillioides* donated to the Department of Plant Genetics and Breeding by Plant Breeding Smolice Ltd. IHAR Group. Lines used for the research were derived from hybrid varieties available on the Polish market. They are mainly characterized by grain types of Dent. Hybrids from which the inbred lines were derived belong to the BSSS and non-BSSS origin groups, mainly Iodent and Lancaster. The plant material is shown in Table 1. These lines were monitored for 5 years for resistance to fusarium. Throughout the years of observation, the resistance of each line remained constant (on a COBORU scale of 1–9; 1, susceptible; 9, resistant). In order to establish the genetic basis of resistance to the fusarium of these lines, the above experiment was set up.

Table 1. Plant material used in the experiment.

Resistant Genotypes		Susceptible Genotypes	
No.	**Genotype Number**	**No.**	**Genotype Number**
1	9	16	16
2	25	17	23
3	28	18	24
4	35	19	41
5	45	20	57
6	47	21	58
7	52	22	67
8	66	23	68
9	71	24	78
10	74	25	103
11	80	26	253
12	114	27	254
13	255	28	256
14	257	29	258
15	260	30	259

2.2. Methodology

2.2.1. Field Experiment

The experiment with 30 *F. verticillioides*-resistant and -susceptible maize genotypes was established in 10 m^2 fields belonging to Plant Breeding Smolice, Smolice, Ltd., Co., Poland Plant Breeding and Acclimatization Institute—National Research Institute Group (51°41′23.16″ N, 17°4′18.241″ E)—in a randomized complete block design in three replicates, in 2021. During the conduct of the experiments, observations were made on the degree of maize cob infection by *F. verticillioides*. The observations were carried out on eight dates: term 1—development of the first blister stage kernels, which contain about 16% of dry matter (BBCH 71); date 2—the beginning of early milk (BBCH 73); term 3—milk stage, middle kernels are milky, containing about 40% of dry matter (BBCH 75); term 4—nearly all kernels have reaches final volume (BBCH 79); date 5—the beginning of the kernel's denting maturity, kernels are soft, containing 45% of dry matter (BBCH 83); date 6—full denting maturity of the kernels, kernels with a typical color, containing about 55% of dry matter (BBCH 85); term 7—physiological maturity, visible black layering at the base of the kernel, with kernels containing about 60% of dry matter (BBCH 87); date 8—full maturity, hard and shiny kernels containing about 65% dry weight (BBCH 89).

Meteorological conditions during the 2021 growing season were favorable for the growth and development of maize, although frosts in April delayed sowing. The month of May, which is very important for the growth and development of maize, should be counted as cool (12 °C) and wet as the amount of precipitation was 76 mm. In contrast to May, June and July 2021 turned out to be dry (June 52.7 mm; July 65 mm) and warm (June 19.3 °C; July 20.9 °C) months. The dry and warm weather did not favor the spread of fungal diseases during this period. Intensive infestation of European corn borer (*Ostrinia nubilalis*) was also not observed. European corn borer feeds on maize and increases its susceptibility to fusarium by laying eggs from mid-June to the end of August. In August, an increased infestation of maize by *Fusarium* spp. was observed, which was due to very high rainfall (140.1 mm) and fairly high temperature (17 °C). The very dry months of September (42.3 mm) and October (19.2 mm) affected the inhibition of the development of fungal diseases including cob fusariosis.

2.2.2. DNA Isolation

Isolation of genomic DNA from 30 susceptible and resistant genotypes to *F. verticillioides* was performed using the reagent kit (Genomic Mini AX Plant) from A&A Biotechnology (Gdańsk, Poland).

The concentration and purity of the isolated DNA samples were measured by a DS-11 spectrophotometer from DeNovix. The isolated DNA matrix was brought to a uniform concentration of 50 ng μL^{-1} by dilution with distilled water.

DNA concentration ranged from 155 ng/µL for line 28 to 950 ng/µL for line 24. Purity of individual samples was also very good and ranged from 1.7 to 2.1 for absorbance 260/280 and 260/230.

2.2.3. Molecular Marker Analysis and PCR Amplification

Identification of markers (Table 2) coupled to *F. verticillioides* resistance genes was carried out using polymerase chain reaction (PCR) and primers proposed by Abdel-Rahman et al. [13] and Salah et al. [10] (Table 2).

The polymerase chain reaction (PCR) was conducted in a BIO-RAD T1000 thermocycler. Reagents from Promega were used to prepare the mixture. The composition of the reaction mixture volume (µL) of reaction components per 20 µL sample: Buffer (5× Green Go Taq, Flexi Buffer), 4 µL; 25 mM $MgCl_2$, 1.6 µL; 10 mM Ultrapure dNTPs Mix, 0.32 µL; DNA polymerase (Go Taq G2 Flexi), 0.17 µL; Nuclease-free water, 11.91 µL (reaction mixture for a single primer pair), 10.91 µL (reaction mixture for multiplex PCR); Starter F, 0.5 µL; Starter F, marker 2–0.5 µL (reaction mixture for multiplex PCR); Starter R, 0.5 µL; Starter 1,

marker 2–0.5 µL (reaction mixture for multiplex PCR); DNA, 1 µL. Reaction profile for PCR is shown below:

Table 2. Molecular markers associated with *F. verticillioides* resistance genes for maize.

Polymorphism	Marker	Primer Sequences		Product Size (bp)	Melting Temperature (°C)	Reference
		Rewers	Forward			
SSR	bnlg1621	GGATCTTCGTTGCAGTTCTT	CATCAGTGATCCTCCACCAT	135–160	54	[13]
	bnlg1063	GGAGACAACCCCGACGAC	GGTACCAGAGCCACAGATCC	105–120	55	[13]
	bnlg1740	TTTTCTCCTTGAGTTCGTTCG	ACAGGCAGAGCTCTCACACA	125–160	56	[13]
	umc2082	TAGCTGCCCCTCTTCCGTCT	GTCGTGGCGTAGAGACTAGGGT	100–130	54	[13]
	umc2059	CTCTTCGATCTTTAAGAGAGAGAGAG	ACACGAGGCACTGGTACTAACG	170–200	54	[13]
	umc2013	GGAAAAGGAGGAACAGTGTAAGCA	AGCGTGATCAGACGTACAATGCTA	110–130	54	[13]
	SSR85	GGGACGAGAGTCTGTTGTTGTTG	GTTGATGCATGTGACTCTGGAAAC	110–125	55	[13]
	SSR93	CGCCGTACAGACTGCTATGA	CACATGCTACGACTGCGATG	210	57	[10]
	SSR105	GTTCATCCTGATTCCCATCC	CAGCCTTGCTTCTACACCAC	200	58	[10]
	SSR255	TCGACGAGATACGCGACTAC	CAGTACAAAGCCGATCCAAG	200	55	[10]
ISSR	AD8	$(AGC)_6GC$		410	55	[10]
STS	STS03	CTTGTATCATCAGCTAGGGCATGT	GTGATCTGAACGCCAACCTC	300	54	[10]

PCR conditions:

94 °C/2 min.
95 °C/1 min. } 39×
54 °C */0.50 min. } 39×
72 °C/1 min. } 39×
72 °C/5 min.
4 °C/∞.

* The primer binding temperature was different for each marker and depended on the melting temperature of the primer. The temperatures for each marker are given in Table 2. Melting point for multiplex PCR is 54 °C.

2.2.4. Electrophoretic Separation

Electrophoresis was conducted in a 2% agarose gel in 1× TBE buffer out at 120 V for 1.5 h.

Composition of the 2% agarose gel: 1× TBE buffer, 150 µL; Agarose, 3 g; Midori Green DNA Stain, 7.5 µL.

2.3. Statistical Analysis

The normality of the distribution of the degree of *F. verticillioides* infection of the maize lines was tested using Shapiro–Wilk's normality test. The homogeneity of variance was tested using Bartlett's test. A one-way analysis of variance (ANOVA) was carried out to determine the main line effect on the variability of the degree of *F. verticillioides* infection. The genetic similarity for each pair of the investigated lines was estimated based on the coefficient proposed by Nei and Li [19]. The lines were grouped hierarchically using the unweighted pair group method of arithmetic means (UPGMA) based on the calculated coefficients [20]. The relationships between the lines were presented in the form of a dendrogram. Results were also analyzed using multivariate methods. The principal component analysis (PCA) was applied to present a multi-markers assessment of similarity for the tested lines. The association between molecular markers and the degree of *F. verticillioides* infection was estimated using regression analysis [21]. All analyses were conducted in GenStat 18.2 (VSN International Ltd., Hemel Hempstead, England, UK).

3. Results

3.1. Field Experiment

As a result of field observations, it was confirmed that genotypes identified as resistant were characterized by higher resistance on a 9-level COBORU scale (1, susceptible; 9, resistant) than susceptible genotypes. The empirical distribution of the degree of *F. verticillioides* infection was normal. The results of the analysis of variance for *Fusarium* resistance

($F_{29;60}$ = 8.55) showed variability of the tested lines at the significant level α = 0.001. The lines with numbers 255, 260, 9, 25, 28, 52, 71 were the most resistant at levels 8 and 9 (Table 3). It is worth noting that during molecular analyses, the highest number of molecular markers linked to *F. verticillioides* resistance genes were identified in these lines (Table 3). The least resistant at level 4–6 were lines with numbers: 254, 257, 24, and 67.

Table 3. Degree of *Fusarium verticillioides* infection of individual genotypes and number of identified markers.

Line No.	Observation of the Degree of Resistance			Number of Molecular Markers Identified
	I Repetition	II Repetition	III Repetition	
253	7	7	8	6
254	4	5	4	5
255	9	9	9	9
256	5	7	6	7
257	5	5	6	6
258	7	8	7	7
259	7	7	7	8
260	9	9	8	9
9	9	9	8	9
16	7	6	7	7
23	6	6	7	8
24	6	5	4	6
25	9	8	9	10
28	9	8	8	8
35	8	8	7	9
41	8	7	6	7
45	7	7	8	8
47	8	7	8	8
52	9	8	8	8
57	5	6	5	7
58	6	6	5	7
66	8	7	7	8
67	4	5	6	6
68	5	7	4	7
71	8	8	9	8
74	8	6	5	8
78	6	8	6	7
80	6	6	7	8
103	8	6	6	7
114	9	8	9	8

3.2. Identification of Molecular Markers Linked to Fusarium verticillioides Resistance Genes

The analyzed markers were taken from literature reports. Table 4 shows the distribution of molecular markers associated with *F. verticillioides* resistance genes in the tested lines. ("+" -visible stripe, "-"-no stripe). As can be seen from the analyses, ten of the 12 markers tested were identified for one line number 25 (Table 4). This line has a fusarium resistance of 8–9 under field conditions Table 3). For lines numbered 255, 260, and 9, nine of twelve markers were identified. These lines also exhibited resistance at the 8–9 level under field conditions. Similarly, for the line with number 35, nine markers were identified; this line, however, exhibited slightly lower resistance at the 7–8 level. The least resistant was line 254, in which resistance was at the level of 4–5, and the number of identified markers was five. The same was true for lines 257, 24 and 67, which were characterized by resistance at the level of 4–6 and the number of identified markers was six (Tables 3 and 4).

Table 4. Molecular markers linked to plant resistance to fusarium present in the analyzed lines.

Line No.	Molecular Marker											
	SSR85	**bngl1063**	**bngl1740**	**umc2082**	**bngl1621**	**umc2059**	**umc2013**	**SSR93**	**SSR105**	**SSR255**	**AD8**	**STS03**
253	-	+	+	+	+	+	+	-	-	-	-	-
254	-	+	+	+	+	+	-	-	-	-	-	-
255	+	+	+	+	+	+	+	-	+	-	-	+
256	-	+	+	+	+	+	+	+	-	-	-	-
257	-	+	+	+	+	+	+	-	-	-	-	-
258	-	+	+	+	+	+	+	-	-	+	-	-
259	-	-	+	+	+	+	+	-	+	-	+	+
260	+	-	+	+	+	+	+	-	+	+	-	+
9	+	+	+	+	+	+	+	-	+	-	-	+
16	+	-	+	+	+	+	-	-	+	-	-	+
23	+	-	+	+	+	+	+	-	+	-	-	+
24	+	-	+	+	+	+	+	-	-	-	-	-
25	+	+	+	+	+	+	+	+	+	-	-	+
28	+	-	+	+	+	+	+	+	-	-	-	+
35	+	+	+	+	+	+	+	+	-	-	-	+
41	+	-	+	+	+	+	+	-	-	+	-	-
45	+	-	+	+	+	+	+	-	+	-	-	+
47	+	-	+	+	+	+	+	-	+	-	-	+
52	+	+	+	+	+	+	+	-	-	+	-	-
57	+	-	+	+	+	+	+	-	-	+	-	-
58	+	+	+	+	+	+	+	-	-	-	-	-
66	+	+	+	+	+	+	+	-	-	+	-	-
67	+	-	+	+	+	+	+	-	-	-	-	-
68	+	-	+	+	+	+	+	-	-	+	-	-
71	+	+	+	+	+	+	+	-	-	+	-	-
74	+	+	+	+	+	+	+	-	-	+	-	-
78	+	-	+	+	+	+	+	+	-	-	-	-
80	-	+	+	+	+	+	+	-	+	-	-	+
103	+	-	+	+	+	+	+	+	-	-	-	-
114	+	+	+	+	+	+	+	-	-	+	-	-

Below, photos 1 and 2, and 3 and 4 show sample electrophoretic images showing the identification of bngl1621 and umc2059 markers for the 30 maize genotypes tested (Figures 1–4). For the bngl1521 marker, the following products were obtained by polymerase chain reaction: for lines 253, 254, 257, 258, 260, 16, 23, 66, 67, 68, 71, 74, 80 and 114, a specific product of 170 bp was obtained. For lines: 255, 256, 259, 9, 24, 25, 28, 35 41, 45, 47, 52, 57, 58, 78 and 103 a specific product of 175 bp was obtained (Figures 1 and 2). For the umc 2059 marker, specific products of four different sizes were obtained: 110 bp (for lines: 260, 9, 16, 23, 24, 25, 28), 120 bp (for line: 254), 125 bp (for lines: 253 and 258) and 130 bp (for lines: 257 and 35) (Figure 3). In Figure 4, a product of 110 bp was observed in all analyzed lines.

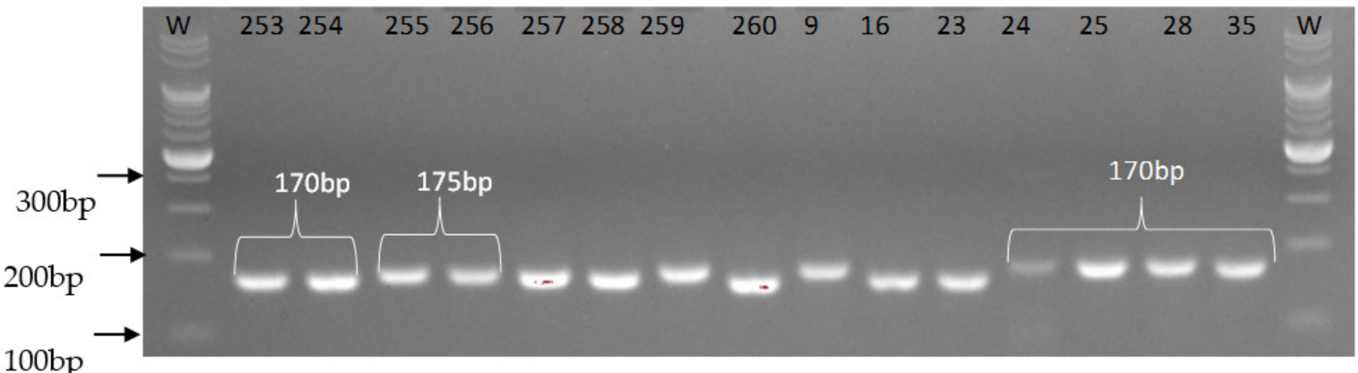

Figure 1. Electrophoretic image of PCR products in 2% agarose gel using bnlg1621 primer pair.

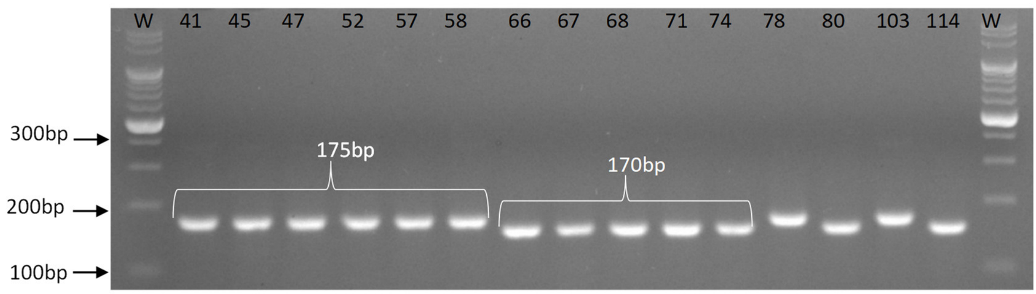

Figure 2. Electrophoretic image of PCR products in 2% agarose gel using bnlg1621 primer pair.

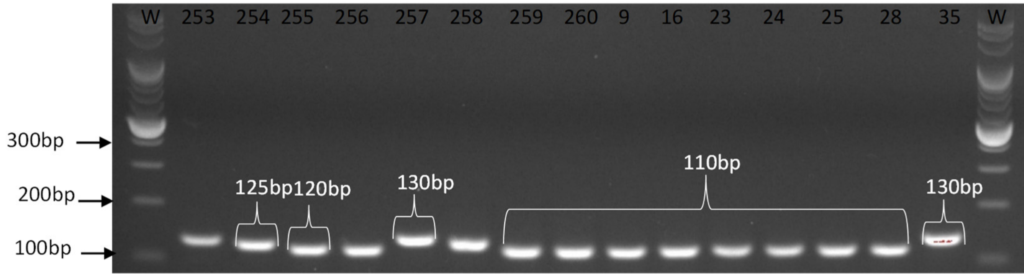

Figure 3. Electrophoretic image of PCR products in 2% agarose gel using umc2059 primer pair.

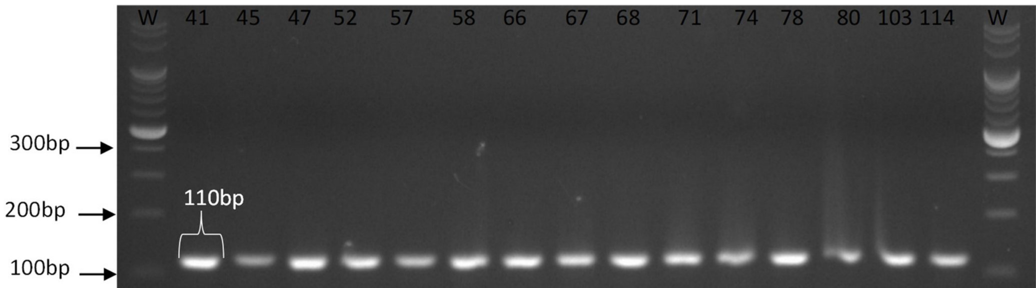

Figure 4. Electrophoretic image of PCR products in 2% agarose gel using umc2059 primer pair.

All markers, except AD8, were associated with *F. verticillioides* resistance of the maize lines (Table 5). Percentage variance accounted for particular markers ranged from 0.7% (for bngl1621 170 bp and bngl1621 175 bp) to 19.9% (for STS03 (Table 5).

The greatest genetic similarity (equal to 1) was found for lines 78 and 103, while the most diversity (equal to 0) for lines 28 and 258 (Figures 5 and 6).

Table 5. Characteristics of molecular markers associated with *Fusarium verticillioides* resistance.

Marker	Effect	Percentage Variance Accounted
SSR85	0.921 ***	9.9
bngl1063	0.565 ***	7.4
bngl1740 125 bp	−0.093 *	1.5
bngl1740 155 bp	−0.432 ***	6.7
bngl1740 160 bp	0.518 ***	2.8
umc2082 100 bp	0.503 ***	3.4
umc2082 115 bp	0.067 *	1.2
umc2082 120 bp	0.164 ***	4.7
bngl1621 170 bp	−0.521 ***	0.7
bngl1621 175 bp	0.521 ***	0.7
umc2059 110 bp	0.694 **	1.8
umc2059 120 bp	−2.7 **	11.2
umc2059 125 bp	0.417 *	7.5
umc2059 130 bp	−0.476 **	5.5
umc2013 120 bp	−0.224 *	2.8
umc2013 125 bp	−0.093 *	1.5
SSR93	0.486 **	3.6
SSR105	1.033 ***	11.3
SSR255	0.483 ***	5.7
AD8	0.06	
STS03	1.25 ***	19.9

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

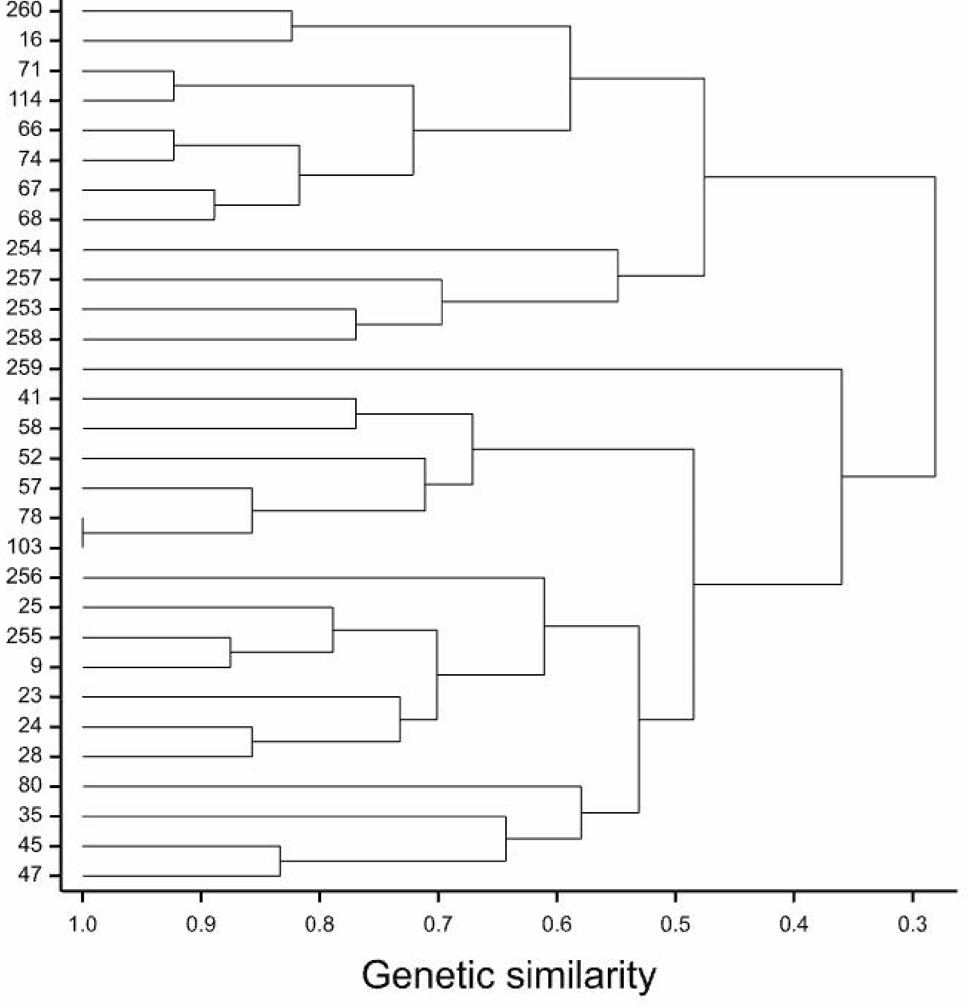

Figure 5. Dendrogram of cluster groupings of 30 maize lines based on all molecular markers.

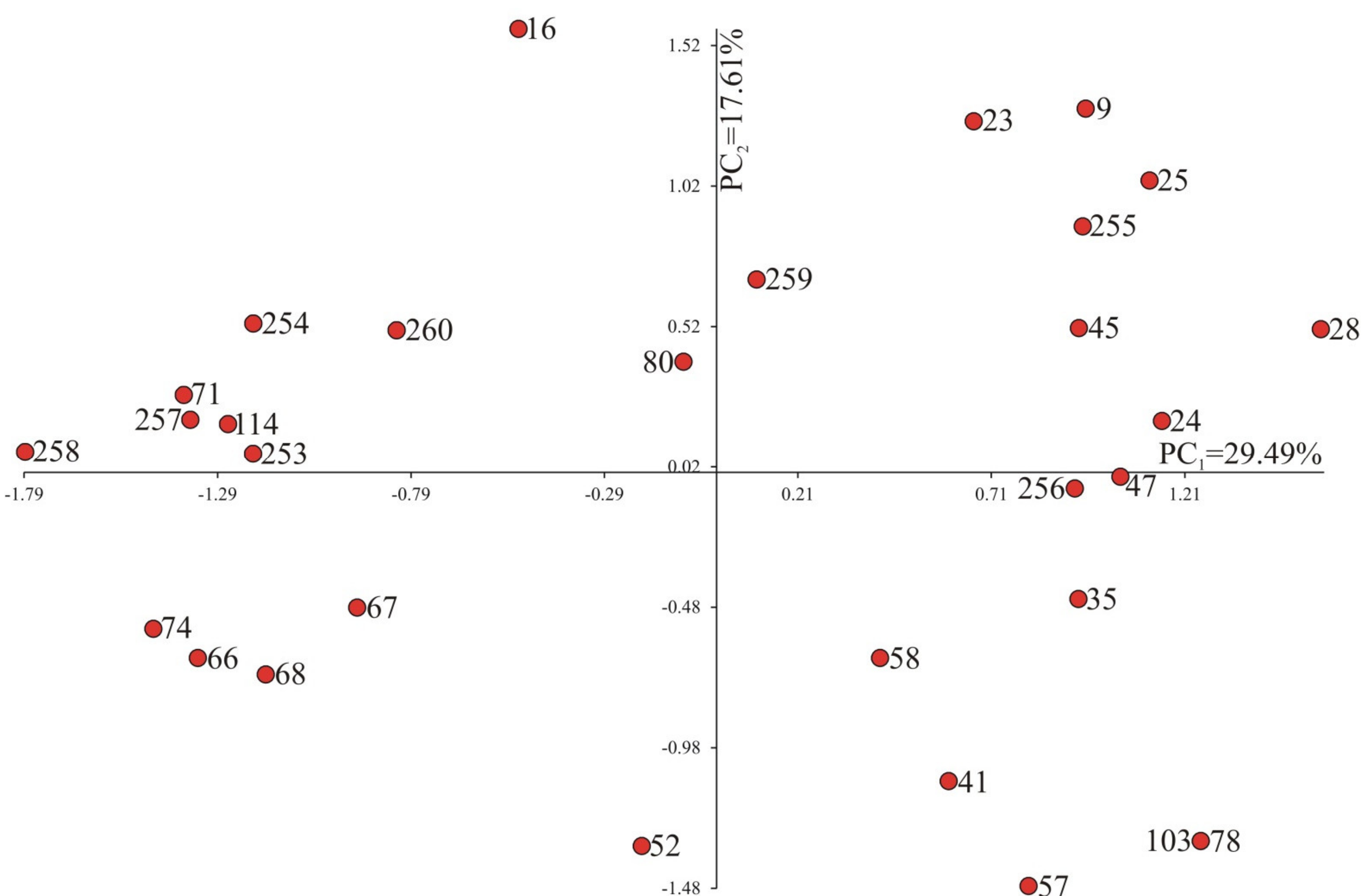

Figure 6. Distribution of 30 maize lines in the space of the first two principal components.

Analysis of the first two principal components for 30 maize lines regarding the all molecular markers is shown in Figure 6. In the graph, the coordinates of the point for a particular line were the values for the first and second principal component, respectively. The first two principal components accounted for 47.10% of the total variability between the individual lines.

3.3. Multiplex PCR Analyses

In order to simultaneously identify two markers associated with cob fusarium resistance genes, conditions were developed for multiplex PCR reactions. Figures 5 and 6 show the multiplex PCR results developed for the markers bnlg1621 and umc2059 (Figures 7 and 8). The multiplex PCR results corresponded to those from single marker analyses. Multiplex PCR was also developed for markers bnlg1621 and SSR85, bnlg1621 and bnlg1740, bnlg1740 and SRR85, STS03 and umc2013, and STS03 and SRR255 according to the methodology presented in Materials and Methods.

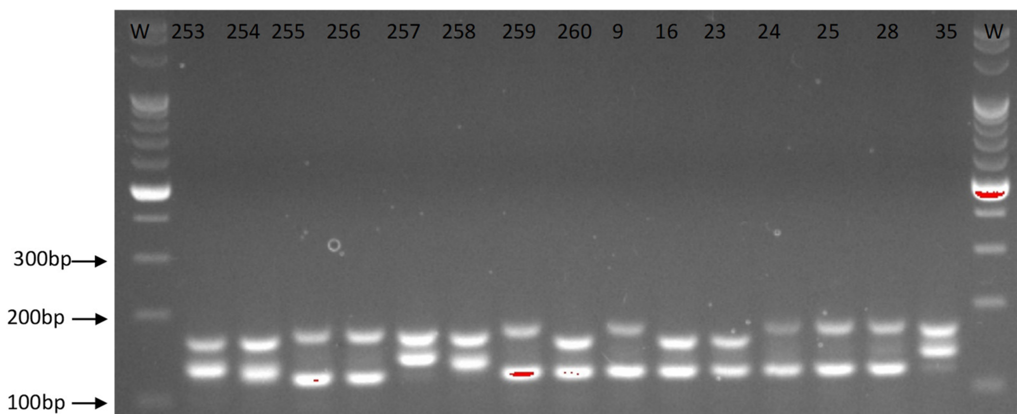

Figure 7. Electrophoretic image of PCR products after including bnlg1621 and umc2059 primer pairs in the reaction.

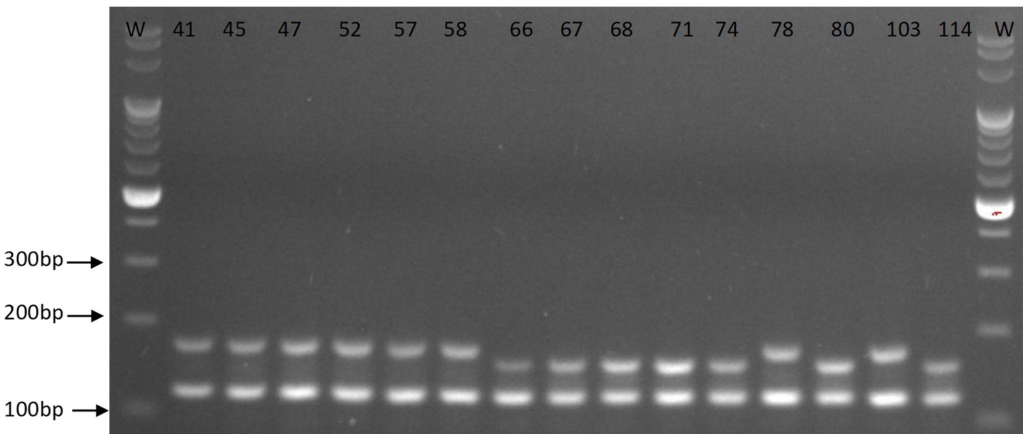

Figure 8. Electrophoretic image of PCR products after including bnlg1621 and umc2059 primer pairs in the reaction.

4. Discussion

Fungi of the genus *Fusarium* spp. are common pathogens among grain crops. They cause diseases associated with rot of ears, stems and roots, among others. Their negative effects cause large losses in yield. In addition, some races of these fungi have the ability to produce mycotoxins that are harmful to humans and animals [22]. Mycotoxins can be divided into three classes: fumonisins, compounds with a simple structure, cause complex and diverse harmful reactions; trichothecenes, a group of compounds with the most harmful effects on humans and animals, can cause fatal and chronic poisoning; zearalenones, the least harmful group of compounds, are toxic but cause death [23].

Mycotoxin toxicity is divided into four types: chronic, acute, teratogenic and mutagenic. The effect of acute poisoning is most often a decrease in kidney and liver function. In severe cases, it leads to the death of the individual [24].

As part of prevention, great emphasis is placed on the development of resistance breeding. Classical selection of resistant varieties based on phenotype analysis is time-consuming and costly. In view of the above, molecular biology tools are increasingly being used for selection. Sets of molecular markers are available that significantly augment traditional resistance breeding methods [25].

Maize resistance to *Fusarium* spp. is a quantitative trait determined by low-influence polygenes [12,26–28]. Over the past decade, many studies have been conducted on the genetic basis associated with resistance to the disease [29]. Genome-wide association and transcriptomic studies (transcriptomic and genome-wide association studies GWAS)

combined with QTL mapping have helped identify markers associated with increased resistance to *Fusarium* spp.

We know from literature reports that phytohormones, e.g., salicylic acid, jasmonic acid and abscisic acid, play a key role in maize's defense response to *Fusarium* spp. The induction of genes related to the benzoxazinoid biosynthetic pathway (i.e., an antimicrobial and insecticidal secondary compound in maize) has also been shown to be linked to plant defense mechanisms against pathogens. The involvement of genes encoding pathogenesis-related (PR) proteins, particularly β-1,3-glucanase, chitinase and pathogenesis-related protein 10 (PR10 protein), has also been observed in maize plant defense responses against *F. verticillioides* [30,31]. It is important to identify markers associated with genes affecting fusarium resistance to facilitate the selection of resistant genotypes. As a result of many studies to date, several SSR-type markers (SSR85, bnlg1063, bnlg1740, umc2082, bnlg1621, umc2059 or umc2013) have been selected for molecular-level selection of resistant varieties and lines.

As can be concluded from the analyses, the resistance of the maize plants tested was correlated with the number of molecular markers identified. An example is line number 25, in which ten (SSR 85, Bngl 1063, Bngl 1740, Umc 2082, Bngl 1621, Umc 2059, Umc 2013, SSR 93, SSR 105, STS 03) of the twelve markers tested were identified. This line is characterized by a *F. verticillioides* resistance of 8–9 under field conditions. For other lines with numbers 255, 260, and 9, nine (SSR 85, Bngl 1063, Bngl 1740, Umc 2082, Bngl 1621, Umc 2059, Umc 2013, SSR 105, STS 03 of twelve markers were identified. As in the case of the line with number 25, these lines also exhibited od resistance at the 8–9 level under field conditions. The same pattern was observed for the line with number 35, for which nine markers were identified. However, this line was characterized by a slightly lower resistance at the level of 7–8. The least resistant was line 254, in which resistance was at the level of 4–5 and the number of identified markers was five. The same was true for lines 257, 24 and 67, which were characterized by resistance at the level of 4–6, and the number of identified markers was six.

The molecular markers used for analysis in this work were taken from the literature of Abdel-Rahman et al. [13]. In their work, the authors studied *Fusarium* spp.—resistant and susceptible genotypes and two parental genotypes. This allowed them to select PCR reaction products responsible for increased resistance. In their study, the problematic markers were umc2082 and bngl1063. As many as four different products were obtained for the umc2082 marker. The resulting bands had similar lengths, making further analysis difficult. Park et al. [32] also studied this molecular marker to analyze the genetic diversity of maize in Korea. The course of the PCR reaction looked similar to the course in this work. However, Park et al. [32] in their work used a polyacrylamide gel for electrophoretic separation thus obtaining better separation of PCR reaction products. Hence, it can be inferred that by using this type of gel, a clearer electrophoretic image could be obtained. Abdel-Rahman et al. [13] obtained products of about 100 and 120 bp for this markers.

Sa et al. [33] evaluating the genetic diversity and structure of selected maize inbred lines studied the bnlg1621 marker, which is also analyzed in this work. According to the study, a total of as many as 15 different alleles were shown for this particular molecular marker, which occurred in the inbred lines studied. Their research work also analyzed other SSR-type markers, 50 in number, and a total of 381 alleles were distinguished in just 32 maize inbred lines.

As a result of the methods used, clear and readable electrophoretic images were obtained for all the molecular markers analyzed. The exceptions were the aforementioned markers umc2082 and bngl1063. Ignjatović-Micić et al. [34] studying the diversity as well as genetic structure of local maize populations also took into account the bngl1063 marker due to its high polymorphism. In their research work, they obtained a very clear electrophoretic image. However, they used different primer attachment temperatures (63.5 °C or 56 °C). The authors did not specify which exact temperature was used for this marker. However, the difference between those used in this paper is at least one degree. Therefore, it can

be concluded that using a slightly higher temperature for primer attachment would have yielded a better, clearer electrophoretic image.

Ashkani et al. [35] investigating multiplex PCR conditions using SSR markers concluded that such reactions often require extensive optimization to obtain relatively good results, and the reaction conditions themselves can differ significantly from single marker analyses. Very often, primers of both markers can hybridize with each other.

In order to improve the breeding cycle, multiplex PCR conditions were developed to identify two markers simultaneously. The multiplex PCR results corresponded to those from single marker analyses. Multiplex PCR was developed for markers bnlg1621 and umc2059 bnlg1621 and SSR85, bnlg1621 and bnlg1740, bnlg1740 and SRR85, STS03 and umc2013, STS03 and SRR255 according to the methodology presented in Materials and Methods.

Butrón et al. [2] investigated quantitative trait loci (QTLs) associated with resistance to corn cob fusariosis using a maize population called Multiparent Advanced Generation Intercross (MAGIC). The authors confirmed that maize cob fusariosis resistance is determined by quantitative trait genes, meaning that many loci manifest small additive effects [36–38].

In conclusion, it was found that the tested molecular markers may be useful for the selection of fusarium-resistant maize lines. In addition, multiplex PCR conditions were developed for the simultaneous identification of two markers. On the basis of the analysis of field experiments and molecular studies, maize lines with numbers 9, 25, 35, 255 and 260 were selected, which are characterized by very high resistance to fusarium and have accumulated markers linked with the genes of resistance to fusarium. These lines can be taken as reference lines and used in the breeding process as a source of fusarium resistance genes.

5. Conclusions

It is well known that advances in plant breeding depend on technologies to identify markers of quantitative traits. Statistical methods used in association mapping and genomic selection are playing an increasingly important role. Attempts are being made to identify and map new markers significantly associated with many traits, including *F. verticillioides* resistance. In recent years, there has been a change in the approach to selection. Instead of using single feature markers, multiple markers for these features are searched for, or the entire available pool of markers describing the population is used for selection. The research described in this paper shows that the presented markers can be successfully used for the selection of varieties resistant to *F. verticillioides*. In the course of molecular analyses and field observations, five lines were selected: 9, 25, 35, 255 and 260, which can be used to crossbreed in order to obtain varieties resistant to fusarium. In addition, conditions for multiplex PCR reactions were developed to shorten the breeding cycle. This tool can be used for the selection of resistant lines and varieties.

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