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RAPD MARKER ANALYSIS OF POLYMORPHISM AMONG *BOTRYTIS TULIPAE* ISOLATES OBTAINED FROM PLANTATIONS WITH DIFFERENT PLANT PROTECTION VARIANTS

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Abstract: A total of 15 isolates of *B. tulipae* collected from home grown tulips without chemical protection and two commercial tulip plantations were examined by RAPD fingerprint analysis. The first tulip plantation was protected by bulb treatment and foliage spraying with fungicides in the growing period and the second plantation – only by the application of fungicides in the growing period. In the previous study, a set of isolates obtained from a plantation with an extensive use of fungicides demonstrated a higher pathogenicity level measured by the inhibition of plant growth, the percentage of bulb and root necrosis in flower pot tests on forced tulips, and by the necrosis size in tests on leaf disks. The relationships between the groups and among isolates were determined by cluster analysis of mean character differences using UPGMA and NJ methods. Similarity index values ranged from 0.872 to 1; on average, the index value was 0.933. A mean similarity of genotypes indicated the highest genotype uniformity of isolates obtained from a plantation with the extensive use of fungicides.
3 groups of clusters, could be observed in the obtained dendrograms. The first cluster contains exclusively genotypes of isolates obtained from a plantation with an extensive use of fungicides, the second one only genotypes of isolates obtained from a plantation protected only by the application of fungicides in the growing period and the third – one genotype of previous group of isolates and four genotypes of isolates obtained from home grown tulips without chemical protection. The most distinct differentiation between the groups of isolates was observed by the amplification using primers G4, H20 and J13.
The results of this study revealed genetic similarity between isolates which were obtained from chemically protected plantations and demonstrated a higher degree of pathogenicity in comparison to the isolates which were obtained from unprotected plants and showed a lower degree of pathogenicity.

Key words: *Botrytis tulipae*, pathogenicity, fungicide, genetic relationship

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INTRODUCTION

Species of *Botrytis* are pathogens of many plant species. They are necrotrophs causing the decay of infected plant tissue. Their ability to infect host plants may result from a combination of four factors: 1) the possession of pathogenicity factors (e.g. toxins and cell-wall degrading enzymes) that may kill and invade plant tissue, 2) the ability to avoid or counteract plant resistance mechanisms, 3) the ability to survive outside host-plant tissue under less favourable conditions (e.g. low humidity, UV irradiation), 4) the ability to reproduce and disperse (Staats et al. 2005). Among many *Botrytis* species there are polyphageous species like *Botrytis cinerea* Pers. and species with a narrow host range, a representative of which is *B. tulipae* (Lib.) Lind – causal agent of tulip fire. The pathogen causes severe damage of tulips in all the regions of a tulip's plantation (Fox 1992)

B. tulipae belongs to few species of *Botrytis* which do not undergo sexual reproduction (Staats et al. 2005). It is parasexualism that is responsible for genetic diversity of the microorganisms reproducing without meiosis and fertilizing. Considering a huge amount of cell divisions, random parasexual processes may essentially determine the cause of genetic diversity of pathogenic fungi. This type of diversity may be a decisive factor in the occurrence of virulence alleles (Kozłowska and Konieczny 2003).

Reproduction strategies of fungi, mutations, gene flow, population size and age as well as selection are the factors affecting fungal genetic diversity. RAPD (Random Amplified Polymorphic DNA) is a powerful tool that is useful especially among fungi with asexual reproduction mode having a population structure composed of clonal lineages, since a large number of amplicons can be screened in a relatively short period of time. Furthermore, RAPD data are easy to interpret because they are based on amplification or nonamplification of specific DNA sequences, producing a binary data set that is easy to analyse (McDonald 1997). According to Moyano et al. (2003), both marker analysis – RAPD as well as AFLP (Amplified – Fragment Length Polymorphism) are useful in the study of genetic structure of the *Botrytis* population, although RAPD generated more polymorphism per *loci* than AFLP, and explained genetic relationships between the isolates in a better way.

Despite the importance of *B. tulipae* as a pathogen of tulips, there are lack of studies on the genetic structure of *B. tulipae* populations concerning pathogenicity and fungicide resistance. The aim of this work was to investigate genetic relationships among *B. tulipae* isolates obtained from a plantation with two different chemical control strategies and from home garden without chemical control using RAPD markers. In the previous test, the collected isolates showed a high diversity between groups concerning their pathogenicity to tulip cv. Apeldoorn in flower pots and leaf disks tests (Piwoni 2005).

MATERIALS AND METHODS

Isolates of *B. tulipae*

Three groups of *B. tulipae* isolates were obtained from necrotic spots on sprouts and leaves of tulips, in the spring before the first chemical treatment. The isolation was conducted in laboratory conditions on PDA in Petri dishes. Next, the hyphal tips were subcultured and stored on PDA slants. The isolates originated from two

commercial tulip plantations located near Puławy, and from home-grown tulips near Biała Podlaska, in Lublin province. On the first plantation, before planting, the tulip bulbs were dipped in fungicide suspensions: Kaptan 50 WP (50% of captan) at conc. 1%, Topsin 70 WP (70% of methyl tiophanate) at conc. 0.7%, Sumilex 500 SC (50% of procymidone) at conc. 0.2%, and sprayed at the growth period with: Dithane 80 WP (80% of mancozeb) – at conc. 0.2%, Sumilex 500 SC (50% of procymidone)at conc. 0.1% and Topsin 70 WP at conc. 0.1% (group B, isolate numbers: B50, B58, B67, B74, B76). Tulips grown on the second plantation were not treated before planting but only sprayed at the growth period with: Benlate 50 WP (50% of benomyl) at conc. 0.1%, Dithane M 45 WP at conc. 0.2%, Sumilex 500 SC at conc. 0.1% and Topsin 70 WP at conc. 0.1% (group W, isolate numbers: W1, W3, W4, W5, W12). Tulips grown in home garden were not protected (group K, isolate numbers: K2, K6, K13, K14, K15).

Pathogenicity of isolates

In the previous research, pathogenicity tests of three groups of isolates were carried out on forced tulips cv. Apeldoorn in flower pots (Piwoni 2005). A slice of *B. tulipae* mycelium was placed under a bulb according to the method described by Mańka (1989). A mean height of plants was measured and a mean percentage of necrosis on bulbs and roots was estimated.

In pathogenicity tests conducted by the method described by Hsiang and Chastagner (1991) in Petri dishes on leaf discs inoculated with *B. tulipae* mycelium disks, the mean size of leaf disk necrosis was measured (Table 1).

Table 1. Mean heigh of plants, percentage of necrosis on bulbs and roots and size of necrosis on the leaf disks in pathogenicity test and leaf disks test of *B. tulipae* isolates on tulip cv. Apeldoorn (Piwoni 2005)

Isolate No.	Mean height of plants [cm]	Mean % of bulbs necrosis	Mean % of root necrosis	Mean size of leaf disk necrosis [cm^2]
Control	42.1 ab	0.0 a	0.0 a	0.00 a
K2	44.2 a	30.0 bc	52.0 bc	0.80 bc
K6	37.6 abcd	52.0 bcde	62.0 bcd	0.54 ab
K13	44.1 a	48.0 bcde	58.0 bc	1.53 de
K14	30.4 bcde	44.0 bcd	76.0 cde	0.86 bc
K15	42.7 ab	32.0 bc	54.0 bc	0.84 bc
W1	28.3 bcde	52.0 bcde	94.0 e	0.87 bc
W3	38.2 abc	58.0 cde	74.0 cde	0.46 ab
W4	28.0 cde	72.0 def	95.0 de	0.56 ab
W5	39.9 abc	26.0 b	44.0 b	0.16 a
W12	24.3 def	78.0 ef	93.0 e	2.06 ef
B50	29.8 bcde	60.0 def	99.0 e	1.27 cd
B58	19.6 ef	58.0 cde	100.0 e	0.90 bc
B67	11.3 f	95.0 f	100.0 e	2.21 f
B74	12.8 f	68.0 def	99.0 e	1.98 ef
B76	23.7 ef	48.0 bcde	97.0 e	1.37 cd

Means in columns followed by the same letters are not significantly different at the level 0.05 in Duncan's test

Culture of *B. tulipae* isolates

The isolates were subcultured by transferring hyphal tips fragments on PDA in Petri dishes and incubated in darkness at 18°C for 14 days. For the analysis, the aerial mycelium was collected with wide ends of sterile pipette tips and replaced in sterile Eppendorf tubes. The collection of mycelium was repeated twice, each isolate from two separate Petri dishes.

DNA isolation

DNA was isolated according to the method by Milligan et al. (1992), with slight modifications. Mycelium was hand-ground under liquid nitrogen. The mixture of 400 µl of extracting buffer BE (0.1 M Tris pH 8.0; 0.5 mM EDTA pH 8.0; 0.5 M NaCl; 25 mM β-mercaptoethanol; 30 mM PVP) and 100 µl of 10% SDS pH 6.8 was added to the homogeneous tissue. The mixture was incubated for 20 minutes at 65°C, and after the addition of 850 mM of potassium acetate (pH 7.5) it was incubated for 5 minutes on ice. Next, the mixture was centrifugated for 30 minutes at 4°C by 12 000 rpm. The supernatant was removed to sterile Eppendorf tubes and after the addition 250 mM of potassium acetate (pH 7.5) incubated 5 minutes on ice. Next, the tubes were centrifugated for 10 minutes at 4°C by 12 000 rpm. The supernatant was transferred to new tubes. DNA was precipitated using isopropanol with 120 mM of sodium acetate (pH 5.2) at –20°C for 2 hours. Subsequently, the tubes were centrifugated for 15 minutes by 8 000 rpm at 0°C. The obtained pellets were rinsed twice with 70% ethanol and dried at room temperature. DNA was resuspended in 50 µl TE buffer (1M Tris pH 8.0; 0.5 M EDTA) with 0.01 U RNA-se and incubated for 30 minutes at 37°C. The obtained DNA solutions were stored at –20°C. The final concentration of DNA was measured on agarose gel and compared with mass standard MassRuler DNA Ladder (Fermentas, Lithuania)

RAPD analysis

RAPD reactions were performed according to the modified method of Williams et al. (1990). The reaction mixtures of 15 µl volume contained: 1 x PCR reaction buffer (10 mM Tris pH 8.8; 50 mM KCl; 0.08% Nonidet P40) (Fermentas Lithuania), 200 µM of each dNTP, 900 pM of primer, 1.5 mM $MgCl_2$, 70 ng of DNA template, 0.5 U *Taq* DNA polymerase (Fermentas Lithuania). Among 76 tested primers (Table 2), 14 of them were selected for the analysis.

Amplification reactions were performed with two DNA samples of each genotype. Simultaneously, the control reaction without DNA was conducted. PCR reactions were performed in Thermal Cycler T1 Biometra using the following conditions: 94°C for 3 minutes initially to denaturate DNA, followed by 44 cycles of 45 seconds, each at 94°C, 45 seconds at 37°C and 45 seconds at 72°C. These cycles were followed by a final extension of 7 minutes at 72°C.

PCR amplification products were size – separated by electrophoresis on 1.5% agarose gels stained with 0.01% ethidium bromide in TBE buffer (89 mM Tris- borate, 2.5 mM EDTA) for 2.5 hours at 120 V. The gels were photographed under UV light using gel documentation system PolyDoc.

The molecular size of analysed bands ranged from 150 bp to 2000 bp. The obtained banding patterns were compared with molecular size standard GeneRuler™ 100 bp DNA Ladder Plus (Fermentas Lithuania) using 'dnafrag' program version 3.03.

Table 2. Sequences of RAPD primers sequences

Primer name	5′–3′ sequence	Primer name	5′–3′ sequence
A-05	AGG GGT CTT G	L-02	TGG GCG TCA A
A-07	GAA AAG GGT G	L-03	CCA GCA GCT T
A-08	GTG ACG TAG G	M-02	ACA ACG CCT C
A-09	GGG TAA CGC C	M-07	CCG TGA CTC A
A-10	GTG ATC GCA G	M-09	GTC TTG CGG A
A-11	CAA TCG CCG T	M-11	GTC CAC TGT G
A-12	TCG GCG ATA G	P-06	GTG GGC TGA C
A-14	TCT GTG CTG G	T-01	CGC AGT ACT C
A-15	TTC CGA ACC C	T-02	GTC CTA CTC G
A-16	AGC CAG CGA A	T-02B	CTA CAC AGG C
A-17	GAC CGC TTG T	T-03	GTC CTT AGC G
A-18	AGG TGA CCG T	T-04	GTC CTC AAC G
A-20	GTT GCG ATC C	U-02	CTG AGG TCT C
B-08	GTC CAC ACG G	U-18	GGG CCG TTT A
D-06	ACC TGA ACG G	U-136	TAC GTC TTG C
D-07	TTG GCA CGG G	U-197	TCC CCG TTC C
D-10	GGT CTA CAC C	U-221	CCC GTC AAT A
D-11	AGC GCC ATT G	U-225	CGA CTC ACA G
D-16	AGG GCG TAA G	U-250	CGA CAG TCC C
F-05	CCG AAT TCC C	U-254	CGC CCC CAT T
F-17	AAC CCG GGA A	U-280	CTG GGA GTG G
G-01	GGG AAT TCG G	U-287	CGA ACG GCG G
G-02	TGC TGC AGG T	U-295	CGC GTT CCT G
G-03	CCA GTA CTT C	U-300	GGC TAG GGC G
G-04	GGA GTA CTG G	U-386	TGT AAG CTC G
G-05	AAC CCG GGA A	U-532	TTG AGA CAG G
G-06	TTC CCG GGT T	U-534	CAC CCC CTG C
G-07	CCT CTA GAC C	U-552	CTA AAT GGC G
G-08	CCG CAT CGC G	U-572	TTC GAC CAT C
G-09	TCG CGA CCG C	U-600	GAA GAA CCG C
G-10	CCG ATA TCC C	V-15	CAG TGC CGG T
H-17	CAC TCT CCT C	W-02	ACC CCG CCA A
H-20	GGG AGA CAT C	X-01	TGG CCA GTG A
J-05	CTC CAT GGG G	X-02	AGG GTA CCA G
J-09	TGA GCC TCA C	X-03	GTA GCT GAC G
J-10	AAG CCC GAG G	X-04	TCA GTC CGA C
J-13	CCA CAC TAC C	X-05	GAC AGG TAC C
J-19	GGA CAC CAC T	X-06	TCC GAG TCT G

Analysis of polymorphism

The presence or absence of each amplified band was scored as 1 and 0, respectively. Each band was assumed to represent a single genetic locus. Genetic similarity (SI – similarity indices) between pairs of all investigated genotypes was estimated according to Dice formula as recommended by Nei and Li (1979):

$$SI = 2N_{xy} / (N_x + N_y)$$

Where N_{xy} is the number of banding patterns that occurred in both compared genotypes X and Y. N_x and N_y are the numbers of banding patterns that occurred in genotype X and genotype Y respectively.

Construction of dendrograms

Obtained SI matrixes were analysed using Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) (Rohlf, 2001) and used to construct the dendrograms by UPGMA (unweighted pair-group method with arithmetic average) and NJ (neighbour joining) methods.

RESULTS

The obtained data of previous pathogenicity tests carried out on forced tulips cv. Apeldoorn in flower pots and leaf disks in Petri dishes indicated a significantly higher pathogenicity in trials with isolates obtained from a plantation with intensive fungicide protection (such as bulb treatment and foliage spraying) in comparison with the isolates obtained from a home garden without protection (Table 1) (Piwoni 2005).

Table 3. Characteristics of the applied RAPD primers

No.	Primer	No. of bands			
		total	polymorphic	monomorphic	specific for particular isolate
1	A-08	5	3	2	1
2	A-10	9	2	7	0
3	D-16	2	1	1	0
4	F-05	6	1	5	0
5	G-02	8	5	3	0
6	G-04	5	2	3	0
7	H-20	7	3	4	0
8	J-10	12	3	9	1
9	J-13	6	2	4	0
10	L-02	16	1	15	0
11	U-287	4	1	3	0
12	U-300	9	1	8	1
13	W-2	13	5	8	0
14	X-05	6	1	5	1
Total		108	31	77	4
Average/primer		7.7	2.2	5.5	0.3

Table 4. Similarity coefficient matrixes (using SI of Dice) between all pairs of isolates calculated on polymorphisms of RAPD markers

	B_50	B_58	B_67	B_74	B_76	K_2	K_6	K_13	K_14	K_15	W_1	W_3	W_4	W_5	W_12
B_50															
B_58	0.978														
B_67	0.984	0.973													
B_74	0.978	0.978	0.995												
B_76	0.978	0.978	0.995	1.000											
K_2	0.895	0.873	0.900	0.895	0.895										
K_6	0.895	0.873	0.900	0.895	0.895	1.000									
K_13	0.919	0.919	0.924	0.919	0.919	0.890	0.890								
K_14	0.894	0.883	0.899	0.894	0.894	0.989	0.989	0.900							
K_15	0.894	0.883	0.899	0.894	0.894	0.989	0.989	0.900	1.000						
W_1	0.973	0.962	0.989	0.984	0.984	0.889	0.889	0.935	0.888	0.888					
W_3	0.900	0.889	0.916	0.911	0.911	0.983	0.983	0.895	0.983	0.983	0.905				
W_4	0.962	0.951	0.978	0.973	0.973	0.889	0.889	0.924	0.888	0.888	0.989	0.905			
W_5	0.950	0.939	0.966	0.961	0.961	0.932	0.932	0.933	0.931	0.931	0.955	0.937	0.944		
W_12	0.967	0.956	0.972	0.967	0.967	0.872	0.872	0.940	0.881	0.881	0.983	0.888	0.972	0.938	

Similarity within groups: B – 0.983, K – 0.953, W – 0.941

Mean similarity between groups

	B	K	W
B			
K	0.898		
W	0.954	0.916	

Out of the preliminarily analysed 76 primers, 14 primers generating stable and polymorphic banding patterns were chosen. These primers were used in the reactions that were conducted for all investigated genotypes. The results are presented in Table 3. The total number of a obtained bands was 108, 31 of which were polymorphic. The band number amplified in one reaction scored from 2 for the primer D-16 to 16 for L-02, whereas a mean number of the amplified fragments was 7.7 for the primer. The number of polymorphic bands varied from 1 to 5 and on the average, primers generated 2.2 polymorphic amplicons. Among the analysed 14 primers only 4 have initiated amplification of specified products for particular isolate. In the presence of the primer A-08, U-300 and X-05, bands specific only for genotype K13 occurred, in the presence of J-10 primer – bands specific for genotype W4.

Estimation of genetic similarity and construction of dendrograms based on RAPD markers polymorphism

On the basis of the results of research work on polymorphisms of RAPD markers the matrix of genetic similarity indices after Dice was constructed (Table 4). The similarity index values ranged from 0.872 between K2 and W12 as well as K6 and W12 to 1 between B74 and B76, K2 and K6 as well as K14 and K15; the average index value was 0.933. A mean similarity of genotypes within group B was 0.983, K – 0.953, W – 0.941, which indicated the highest uniformity of B genotypes and the highest differentiation of W genotypes. The group of B genotypes differed most from genotypes K and was most similar to W genotypes.

The analysis of clustering with UPGMA (Fig. 1) and NJ (Fig. 2) methods was conducted with the support of SI matrix. The obtained dendrograms, independently of clustering method used, showed 3 groups of clusters.

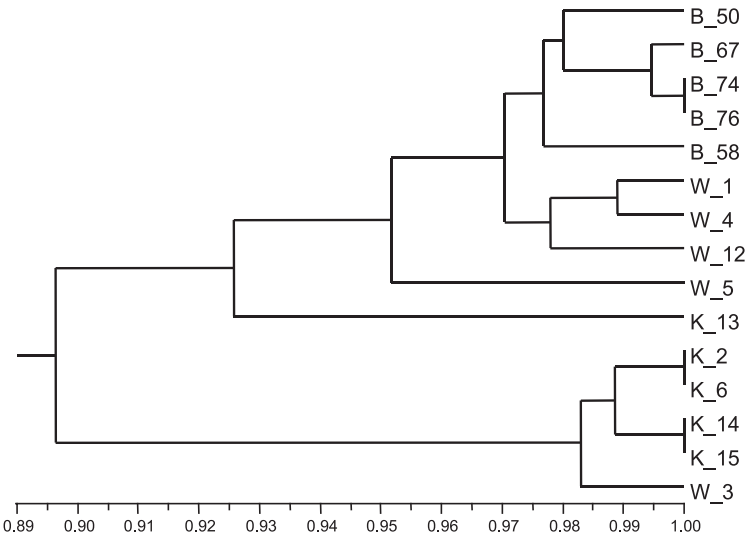

Fig. 1. Dendrograms of the analysed *B. tulipae* isolates obtained by UPGMA method using RAPD markers

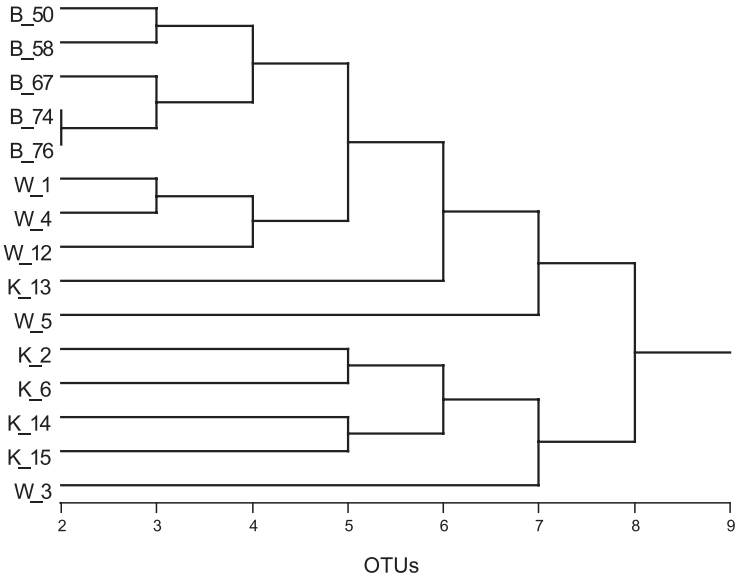

Fig. 2. Dendrograms of the analysed *B. tulipae* isolates obtained by NJ method using RAPD markers

The first cluster contains exclusively genotypes of B type, the second one only W genotypes and the third-four genotypes of K type and one of W genotype. Within W genotypes, the isolates W5 and W3 do not cluster together. Likewise, the isolate K13 is more similar to B and W genotypes than to other K type isolates. W13 clustered together with isolates of K type. The most distinct differentiation between groups of the isolates was observed by amplification using primers G4, H20 and J13 (Fig. 3, 4, 5).

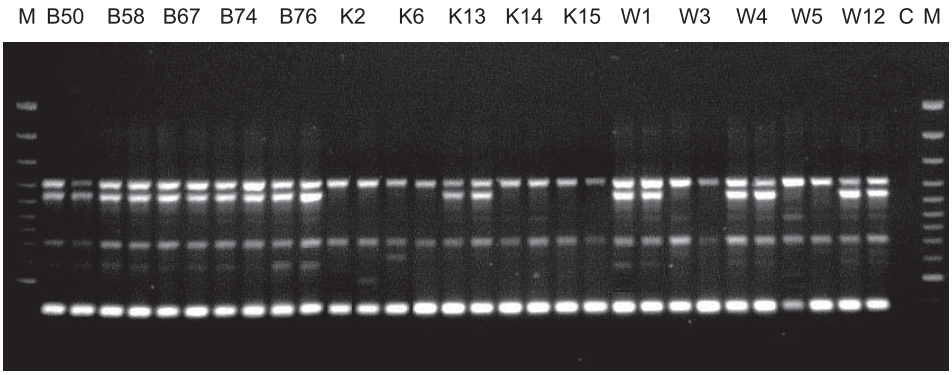

Fig. 3. RAPD profiles of 15 *B. tulipae* isolates from 3 groups (B, K, W) obtained using G4 primer (lane M shows a DNA size marker GeneRuler™ 100 bp DNA Ladder Plus (Fermentas Lithuania) and lane C – the control without DNA matrix)

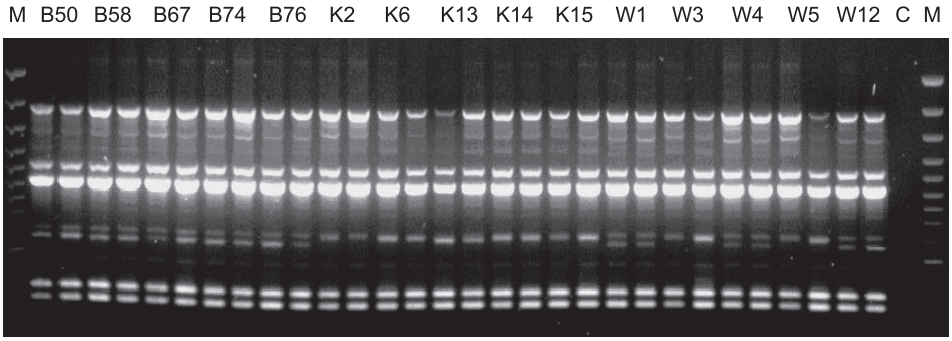

Fig. 4. RAPD profiles of 15 *B. tulipae* isolates from 3 groups (B, K, W) obtained using H20 primer (lane M shows a DNA size marker GeneRuler™ 100 bp DNA Ladder Plus (Fermentas Lithuania) and lane C – the control without DNA matrix)

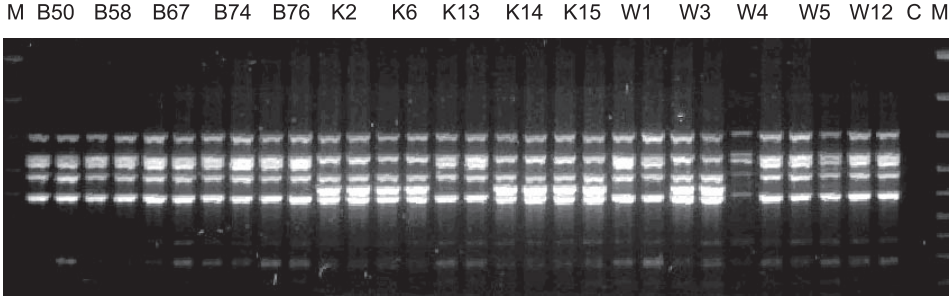

Fig. 5. RAPD profiles of 15 *B. tulipae* isolates from 3 groups (B, K, W) obtained using J13 primer (lane M shows a DNA size marker GeneRuler™ 100 bp DNA Ladder Plus (Fermentas Lithuania) and lane C – the control without DNA matrix)

DISCUSSION

The method of RAPD markers has been widely applied in studies of genetic structure of *Botrytis cinerea* populations depending on the origin of isolates, sampling data or pathogenicity (Kerssies at al. 1997; Alfonso at al. 2000) as well as the variation of sensitivity to the applied fungicides, mostly benzimidazole and dicarboximides (Yourman et. al. 2000; Paplomatas et al. 2004; Moyano et al. 2003). The comparison of the ecological and parasitic fitness of fungicide-resistant and sensitive isolates of *Botrytis tulipae* was reported by Chastagner (1984, 1986), Chastagner and Vassey (1979), Hsiang and Chastagner (1991), but the tests were conducted without molecular genetic methods. In the present research, genetic variations between the analysed groups of isolates as well as within the groups were observed. A mean similarity of genotypes within groups indicates the highest uniformity of B type genotypes and the highest diversity of the W type genotypes. The differentiation between the isolates belonging to W and B groups was lower than within W group. Both groups of isolates were obtained from plantations protected with benzimidazole and dicarboximide fungicides. In the previous study (Piwoni 2001), a decreased susceptibility of

B. tulipae isolates (obtained from the plantations mentioned above) to iprodione, procymidone and dichlofluanide in comparison with the isolates obtained from home garden without chemical protection was demonstrated and investigated isolates were not totally inhibited even by the concentration of 1000 μg a.i./ml.

The value of genetic similarity equaling one between some pairs of the isolates belonging to the groups B and K indicates that the applied method of DNA polymorphism estimation did not prove differences between some pairs of genotypes. However, it should be mentioned that in the course of reaction only a few parts of genom were amplified. Therefore, the existence of differences between DNA of these genotypes cannot be excluded.

On the basis of the dendrogram analysis it should be stated that B group of genotypes differed most from K genotypes and was most similar to W genotypes. Within the B group of isolates, the highest degree of pathogenicity to tulip cv. Apeldoorn bulbs in flower pots tests and in the leaf disks test was noticed (Piwoni 2005). The K13 isolate clustering with W and B groups showed the greater values of necrosis of bulbs and roots of tulips and the largest necrosis size on leaf disks in the entire group of K isolates. In the previous study on pathogenicity of these groups of isolates (Piwoni 2005) the coincidence between the degree of roots necrosis and the inhibition of plant growth was observed. In particular, this trend was established within the isolates belonging to the B group. The results of this study revealed genetic similarity between isolates belonging to B and W groups, which were obtained from chemically protected plantations and demonstrated a higher degree of pathogenicity in comparison to the isolates of K group obtained from unprotected plants and showing a lower degree of pathogenicity.

In the research work of Alfonso et al. (2000) and Moyano et al. (2003), the investigation of genetic diversity of *B. cinerea* populations originating from vegetables grown in greenhouses revealed a high level of genetic diversity within subpopulations originating from particular greenhouses, and the lack of differentiation of *B. cinerea* between the populations and regions. In the first case, the authors explained this phenomenon by a possibly high level of sexual reproduction in populations of the fungus, heterocaryosis, spontaneous mutations for dicarboximide fungicides resistance and a high selection pressure due to the occurrence of severe epidemics every year and the use of fungicides. In the second case a lack of differentiation of *B. cinerea* between regions could be the effect of the lack of host specialization of pathogen and a continuous gene flow occurring between greenhouses, which prevents the differentiation between populations. Also in other *B. cinera* population study, carried out by Kerssies et al. (1997), no aggregation of isolates was obtained in relation to pathogenicity, inside – outside greenhouses, or with regard to the sampling time.

In the case of *B. tulipae*, these factors are less important because of the lack of sexual reproduction and the presence of specialization of the pathogen to the host plant. However, the gene flow between greenhouses and field plantations may also occur, especially on flower bulbs plantations, where rotten plant debris is deposited outside the greenhouse and the bulbs, after forcing, are stored in the same storage room with the bulbs harvested from the field. The use of different management practices in different plantations could be expected to evoke a selection that has a significant impact on the population structure of *B. tulipae* subpopulations. A tendency towards higher pathogenicity level of B group of isolates obtained from a plantation extensively pro-

tected by fungicides may be alarming. The previous study (Piwoni 2001) revealed lowered sensitivity of *B. tulipae* isolates obtained from plantations protected by iprodione, procymidone and dichlofluanide to these fungicides.

In comparison to *B. cinerea*, the studied species *B. tulipae* causing tulip fire have much shorter exposure time to fungicides during the growth period, because tulip bulbs are sometimes harvested already at the end of June. However, by chemical treatment of bulbs before planting, the time of subsequent fungus contact with fungicides is longer. Because of specialization of pathogen to tulips, the probability of ingress of sensitive forms from neighbourring crops is low.

In 1979, Chastagner and Vassey observed the occurrence of dicarboximide resistant isolates of *B. tulipae* under laboratory conditions for the first time. Pommer and Lorenz (1987) suggested that obtaining of *B. cinerea* strains resistant to dicarboximide cultured in laboratory conditions with sub-lethal concentrations of a fungicide-amended media is due to the selection of homocaryotic hyphae with nuclei containing resistance alleles from heterocaryotic hyphae containing both the nuclei with sensibility alleles and the mutant nuclei with resistance alleles.

Unlike resistance to benzimidazole fungicides, the risk of resistance of *B. tulipae* to dicarboximides is lower and the control of tulip fire with iprodione was still effective after four years of applications (Chastagner 1984). It can be explained by a reduced stability of resistant strains and their lower pathogenicity and fitness *in vivo* (Lorenz and Pommer 1985), and by a reduced growth rate and sporulation in comparison to fungicide sensitive strains (Hsiang and Chastagner 1991). However, some stable dicarboximide – resistant *Botrytis* strains with fitness and pathogenicity similar to sensitive isolates were obtained by Maraite et al. (1980) and by Gullino and Garibaldi (1981). In greenhouse trials, dicarboximide resistant strains of *B. tulipae* were pathogenic and caused the disease on flowers (Chastagner 1986).

In the presented study, obtaining of high pathogenic *B. tulipae* isolates from plantations before the first fungicide application, in spring, may indicate the possibility of isolates to survive and contribute to a lower effectiveness of the disease control in conditions of intensive application of fungicides during the growing season.

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POLISH SUMMARY

BADANIA METODĄ RAPD POLIMORFIZMU IZOLATÓW *BOTRYTIS TULIPAE* UZYSKANYCH Z PLANTACJI TULIPANA O RÓŻNYCH WARIANTACH OCHRONY CHEMICZNEJ

Celem badań było określenie przy użyciu markerów RAPD, zróżnicowania pomiędzy 15 izolatami *B. tulipae* należącymi do trzech grup izolatów uzyskanych: z plantacji tulipana, gdzie cebule zaprawiano chemicznie przed sadzeniem, a rośliny opryskiwano fungicydami w okresie wegetacji, z plantacji gdzie rośliny jedynie opryskiwano fungicydami w okresie wegetacji oraz z ogródka przydomowego, gdzie rośliny nie były poddane zabiegom ochrony. W poprzedzających badaniach, grupa izolatów uzyskanych z plantacji intensywnie chronionej fungicydami wykazała podwyższony stopień patogeniczności mierzony zahamowaniem wzrostu roślin, odsetkiem znekrotyzowania bulw i korzeni w testach patogeniczności w warunkach pędzenia oraz wielkością nekroz w testach na krążkach z liści.

W przeprowadzonych badaniach, wartość indeksów podobieństwa genetycznego pomiędzy izolatami wahała się od 0,872 do 1, średnio wynosiła 0,933. Średnie podobieństwo genotypów w obrębie grup wskazuje na największe wyrównanie grupy genotypów izolatów uzyskanych z plantacji intensywnie chronionej chemicznie.

Na uzyskanych dendrogramach wyróżniono 3 grupy skupień. Pierwsza z nich obejmuje wyłącznie genotypy izolatów uzyskanych z plantacji intensywnie chronionej chemicznie poprzez zaprawianie cebul i opryskiwanie roślin, druga – genotypy izolatów uzyskanych z plantacji jedynie opryskiwanej fungicydami w okresie wegetacji, natomiast w skład trzeciej, wchodzi jeden genotyp izolatu uzyskanego z w/w plantacji oraz cztery genotypy izolatów pochodzących z tulipanów uprawianych w ogródku przydomowym.

W przeprowadzonych badaniach zaobserwowano zróżnicowanie genetyczne zarówno pomiędzy badanymi grupami izolatów jak również w obrębie grup. Średnie podobieństwo genotypów w obrębie grup wskazuje na największe wyrównanie genotypów izolatów uzyskanych z plantacji gdzie stosowano zaprawianie cebul oraz opryskiwanie fungicydami w okresie wegetacji i największe zróżnicowanie genotypów izolatów uzyskanych z plantacji opryskiwanej fungicydami w okresie wegetacji. Najbardziej wyraźne zróżnicowanie zaobserwowano pomiędzy grupami izolatów przy amplifikacji starterami G4, H20 oraz J13.

Wyniki badań wskazują na genetyczne podobieństwo pomiędzy izolatami uzyskanymi z plantacji chronionych fungicydami, w odróżnieniu od grupy izolatów uzyskanych z roślin nie chronionych chemicznie i wykazujących niższy stopień patogeniczności.