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POLYMORPHISM OF TEN NEW MINISATELLITE MARKERS IN SUBPOPULATIONS OF PHYTOPATHOGENIC FUNGUS *LEPTOSPHAERIA MACULANS* DIFFERING WITH METCONAZOLE TREATMENT

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Abstract: Stem canker of brassicas is one of the most damaging diseases of oilseed rape worldwide. The disease is caused by two related *Leptosphaeria* species, and *L. maculans* is regarded as the more damaging one. Being an ascomycete, the pathogen is able to quickly create new variants that can overcome new resistance genes introduced by researchers and breeding companies. The aim of this work was to study polymorphism of *L. maculans* populations using 10 recently developed minisatellite markers. The studied subpopulations differed with metconazole treatment.

Seven minisatellite markers showed polymorphisms and formed alleles varying from 2 to 10 different core motifs, with 5 alleles on average. In total 36 alleles were found. The majority of alleles (72%) were found in both studied subpopulations of *L. maculans*. There were 28 alleles in the group of *L. maculans* isolates originating from plants not treated with any fungicide and 32 in the subpopulation treated with metconazole. Ten unique alleles and imbalanced ratios between some alleles contributed to differences between *L. maculans* subpopulations. The minisatellites *MinLm555, MinLm935-2, MinLm939, MinLm1139* and *MinLm2451* showed 6 new variants as compared to the isolates described so far.

Key words: genetic polymorphism, *Leptosphaeria maculans*, minisatellite marker, VNTR, oilseed rape, PCR, stem canker of brassicas

INTRODUCTION

Stem canker of brassicas is one of the most damaging diseases of oilseed rape in Australia, Canada and Europe, including Poland (Zhou *et al.* 1999; Khangura and Barbetti 2001; Fitt *et al.* 2006; Jędryczka 2007).

The disease is caused by *Leptosphaeria maculans* and *L. biglobosa*, two related species differing in harmfulness to oilseed rape. The first species is regarded as more damaging to rapeseed plants. It forms disease symptoms at stem base and root neck, leading to blockage of vessels or even breakage of a whole stem. The disease causes great economic losses and it is a high concern to oilseed rape growers (West *et al.* 2001; Mrówczyński and Pruszyński 2008).

Being the ascomycete, *Leptosphaeria* sp. pathogen is able to quickly create new variants that can overcome new resistance genes introduced by researchers and breeding companies. Numerous techniques, first of all based on genetic and chemical methods, allow to describe polymorphism of living organisms, including fungi. Populations of *L. maculans* were up to date typed using PCR-based methods such as RFLP (Johnson and Lewis 1990; Hassan *et al*. 1991; Patterson and Kappoor 1995; Voigt *et al.* 2001), AFLP (Pongam *et al.* 1991; Purwantara *et al.* 2000), rep-PCR (Jędryczka *et al.* 1999), as well as molecular karyotyping (Taylor *et al*. 1991; Morales *et al.* 1993), sequencing of DNA fragments (Mendes-Pereira *et al.* 2003; Voigt *et al.* 2005) and chemotaxonomical methods (Pedras and Seguin Swartz 1992; Kachlicki and Jędryczka 1994; Pedras and Biesenthal 2000). However, these methods were mainly used to show differences between two subpopulations, subsequently separated by Shoemaker and Brun (2001) in two species: *L. maculans* and *L. biglobosa*.

Minisatellite markers allowing to fingerprint numerous living organisms were recently used to characterise population structures of different species (Jeffreys *et al.* 1985). Minisatellites are usually defined as tandemly repeated short (6 to 100 bp) motifs spanning 0.5 to several kilobases (Vergnaud and Denoeud 2000). Variation in the number of core fragments and the ability of these arrays to cross-hybridize with numerous similar loci throughout the genome made minisatellites a powerful tool in genetic analyses. Minisatellite markers were also implemented in genetic studies of plant pathogens and they are successfully used to describe differences within subpopulations or even individual isolates of fungi. The first tandemly repeated core motif was described in *Saccharomyces cervisiae* (Horowitz and Haber 1984). Since then minisatellite fragments were found in several fungal species, including human, animal and plant pathogens.

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Phytopathogenic *L. maculans* is currently one of the fungal species well recognized with minisatellite markers. Sequencing of its genome allowed to find numerous tandemly repeated fragments, of which 39 showed polymorphisms what led to study them in detail (Attard *et al.* 2001; Eckert *et al.* 2005; Jędryczka 2006; Stachowiak 2008). Some of these minisatellites, such as *MinLm2*, contained numerous core motifs. Recent experiments showed that *L. maculans* populations in Europe have at least 20 alleles of this minisatellite, of which 14 were found in Poland (Stachowiak 2008). High pathogen variation was demonstrated, showing that as many as 12 alleles could be detected within one oilseed rape field containing plants infected with *L. maculans* (Jędryczka *et al.* 2009).

The aim of this work was to characterize genetic polymorphism of *L. maculans* using 10 new minisatellite markers, which have never been used for population studies of this patogen in Poland. Variability of minisatellite alleles concerned plants not treated with any fungicides and the sub-population that received a single autumn spray with metconazole.

MATERIALS AND METHODS

Fungal isolates of *L. maculans* originated from winter oilseed rape cultivar Bosman (Plant Breeding Strzelce, Poland). The experiment was done in the vegetative season 2006/2007 at the Agricultural Experimental Station of IPP-NRI in Winna Góra (central-west Poland). Field experiment was designed in standard randomized blocks with individual plots of 21 m^2 in 4 replicates. Fungicide treatment was done in autumn 2006, according to SPEC reccommendation (www.spec.edu.pl). Metconazole (triazole fungicide) was applied using the solution of 60 g of the active ingredient per 1 litre (Caramba 60 SL).

The studied population contained 40 hyphal tip isolates of *L. maculans* originating from control and sprayed plots. DNA isolation procedure was done according to Irzykowski *et al.* (2005). PCR protocol followed the methods described by Stachowiak (2008) with further modifications. The primers ranged from 18 to 23 bp, with annealing temperatures from 54°C to 60°C (Table 1). Product amplification was done using the thermal cycler MJ Research PTC-200. PCR reaction profiles consisted of initial DNA denaturation for 2 min at 94°C, followed by 45 cycles: 30 s – 94°C, 30 s – 58–60°C, 60 s – 72°C. For the convenience of experiment performance the annealing temperature for *MinLm1139* was raised to 58°C. The final elongation step lasted 5 min at 72°C.

Separation of PCR amplicons was done at 180 V for 1.5 to 4 hours. The gels contained 2% agarose. Sample DNA was separated in the presence of Gene Ruler 100 bp DNA Ladder (Fermentas) and internal markers varying from 68 bp to 471 bp, which helped to precisely evaluate the size of the studied products. Gel documentation was done using Scion IMAGE for Windows system (Scion Corporation).

At first, 8 randomly selected isolates – 4 per each subpopulation of *L. maculans* – were used. The further study of the whole isolate collection was done only in case of polymorphisms found in the preliminary experiment.

Table 1. Characterization of new minisatellite markers used in this study

No.	Primer symbol	Primer sequence 5′ – 3′	Annealing temperature [°C]	Core sequence [bp]
1	*MinLm555F*	CACTGTCATTCCTCCTCCTGGTT	60	63
	MinLm555R	TGCAGCCGTTTAGTTCTCCATTT		
2	*MinLm585F*	GTCCAAGAGGGGTCTAATG	60	26
	MinLm585R	TGCAATACCTATCAACTATGCTA		
3	*MinLm935-2F*	AGTAGGCAACACAACAGCACACA	58	39
	MinLm935-2R	CCCTCTCTGCCATTTTCCATTAG		
4	*MinLm939F*	ACCTCTTCCTTGCATGCAAACCC	60	16
	MinLm939R	CGAGAGTGGCGAGTTGGAGTTGA		
5	*MinLm1139F*	ACGACGCGGAAGGGTTTT	54	17
	MinLm1139R	ACCATCTACCTCATGCCCTGAAC		
6	*MinLm1188F*	GCGCCTTCCTGGTACTTCA	60	30
	MinLm1188R	CAATTCTCAGAGACTGCCAAGAC		
7	*MinLm2448F*	TTGAGCCTACTTGGGGAACA	60	90
	MinLm2448R	AAGTGGCTAGTGGATTGGAAGAT		
8	*MinLm2448-1F*	GCAACATGCCTTGAGCCTACT	58	90
	MinLm2448-1R	CAAGTGGCTAGTGGATTGGAAGA		
9	*MinLm2451F*	GGGGCGAATGGTATGTTTATAGT	58	24
	MinLm2451R	CGGACACAATACTCACCACCTC		
10	*MinLm2452F*	GTACATGGGCGGACAGGC	60	21
	MinLm2452R	CATTTACACTGCACACCTGCTCA		

RESULTS

In the preliminary experiment performed to check whether the new markers generate polymorphic PCR products, 7 minisatellites showed differences among randomly chosen 8 isolates of *L. maculans*. These markers were selected for further experiments. The markers *MinLm585, MinLm1188* and *MinLm2448* did not generate polymorphic products. The remaining 7 minisatellite markers generated from 2 to 10 alleles (Fig. 1). On average there were 5 alleles. In total 36 alleles were found. The majority of alleles (72%) were found in both studied subpopulations of *L. maculans*. There were 28 variants in the group of *L. maculans* isolates originating from plants not treated with any fungicides and 32 in the subpopulation treated with metconazole.

Ten alleles were unique; they were found in 5 out of 7 polymorphic minisatellites, excluding *MinLm2448-1* and *MinLm2452* (Fig. 1d, g). In most cases the percent of isolates with the same variant of the core motif was comparable. The exception, with the difference exceeding 30% were the following 3 alleles: *MinLm939* 5x (51.7% difference), *MinLm939* 8x (39.1%) and *Min935-2* 2x (30.2%) (Fig. 1b, c). Considerable imbalance between the isolates of *L. maculans* originating from untreated oilseed rape plants and from plants sprayed with metconazole was also found for *MinLm935-2* 3x and *MinLm1139* 3x; in both cases the difference was 24.8% (Fig. 1e).

Out of 10 alleles unique for one of the isolate subgroups, 8 alleles were unique for the cultures of *L. maculans* obtained from plants treated with metconazole; these were the following core motifs: *MinLm555* 7x (Fig. 1a), *MinLm939* 11x (Fig. 1b), *MinLm935-2* 5x and 9x (Fig. 1c), *MinLm1139* 12x and 14x (Fig. 1e), *MinLm2451* 2x and 6x (Fig. 1f). Only two alleles: *MinLm935-2* 7x (Fig. 1c) and *MinLm2451* 10x (Fig. 1f) represented the opposite situation, when unique variants were found in *L. maculans* isolates originating from untreated plants.

The obtained PCR products greatly differed in size. The shortest was 85 bp allele of *MinLm2452* consisting of 2 core motifs of 21 bp each supplemented with 43 bp flanking sites. The longest product (478 bp) was generated by *MinLm555* minisatellite, it consisted of 7 replicates having 63 bp core motifs and 37 bp flanking sites. Apart from a size marker, internal markers were also used and selected in the way allowing to evaluate products' size with maximal precision. In most cases sizes of obtained products did not differ from the expected sizes by more than a few base pairs.

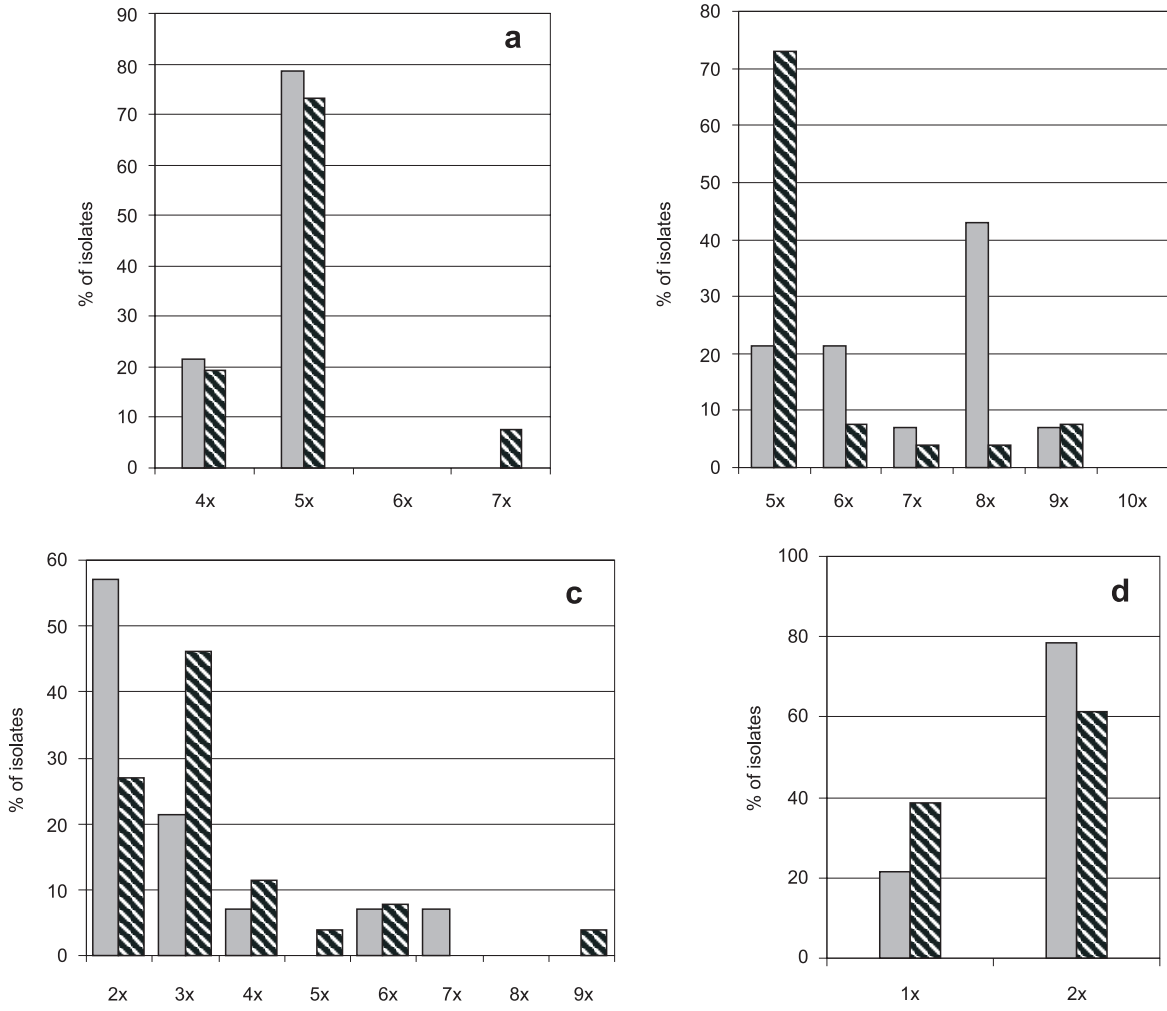

Fig. 1. Frequency of minisatellite alleles in populations of the phytopathogenic fungus *L. maculans* isolated from oilseed rape plants untreated (grey bars) and treated with metconazole (black striped bars): a) *MinLm555*; b) *MinLm939*; c) *MinLm935-2*; d) *MinLm2248-1*

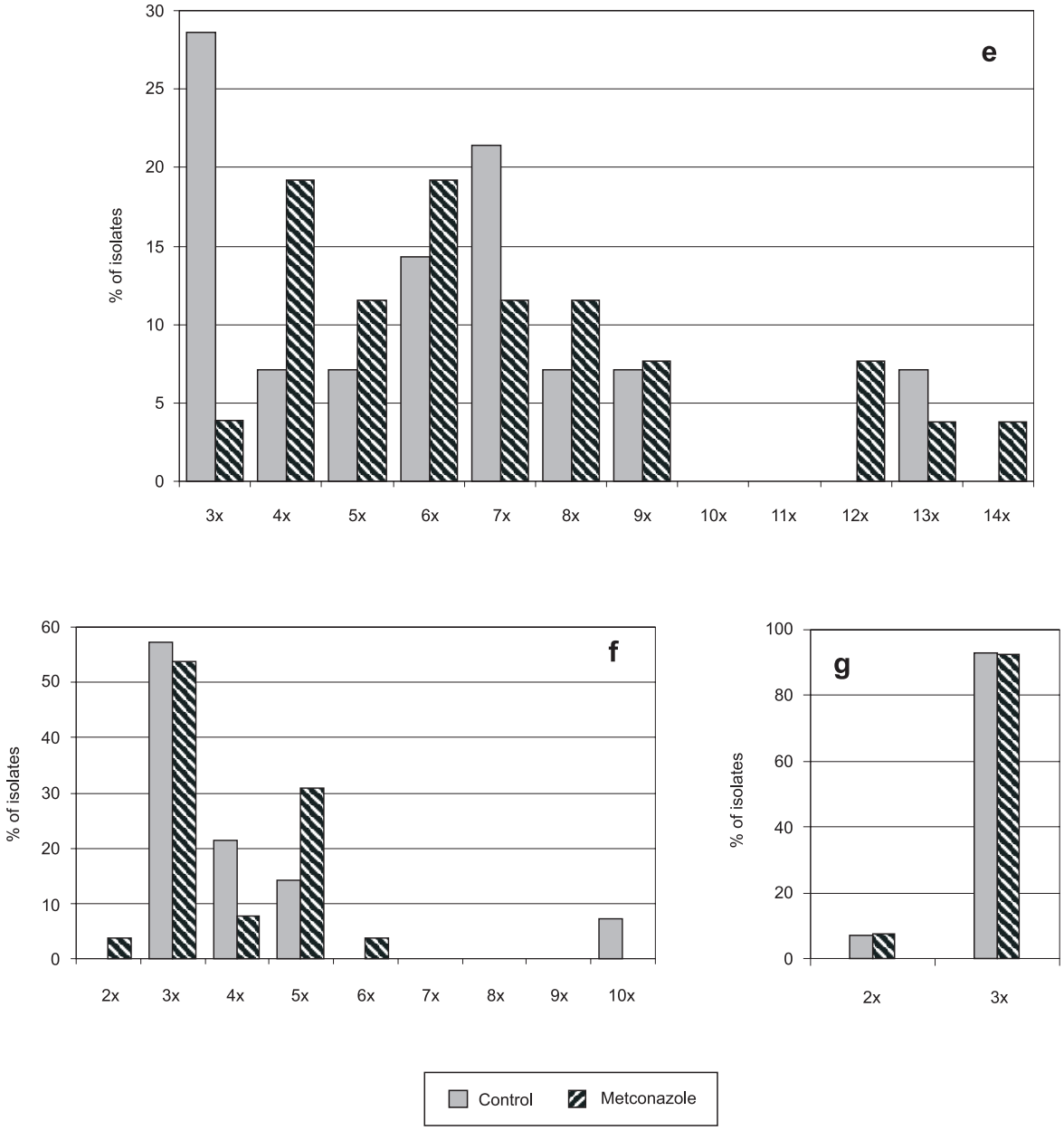

Fig. 1. Frequency of minisatellite alleles in populations of the phytopathogenic fungus *L. maculans* isolated from oilseed rape plants untreated (grey bars) and treated with metconazole (black striped bars): e) *MinLm1139*; f) *MinLm2451;* g) *MinLm2452*

The selection of internal markers was based on knowledge concerning sizes of PCR products obtained in previous experiments (Stachowiak 2008). However, in a few cases the PCR products obtained in this study were different than expected, what sometimes resulted in products shorter or longer than the internal markers (Fig. 2, 3). In this case the evaluation of the exact size of the amplicon could be done with a relatively bigger error.

Frequency of alleles depends on their number, selection pressure exerted on particular isolate variants as well as the length of time when the allele was created. The most frequent variants are usually detected when the number of alleles is small, although the situations with imbalanced ratios among numerous alleles can also be found. The most popular allele among the studied ones was *MinLm2452* 3x with frequency exceeding 90%, both in *L. maculans* isolates obtained from treated and untreated plants (Fig. 1g). The other usually found variant was 5x core motif of *MinLm555* minisatellite, found in 78.6% and 73.1% fungal strains isolated from plants that were respectively untreated and sprayed with metconazole (Fig. 1a). Minisatellite *MinLm2248-1* 2x core motif was also very popular; it was found in 78.6% and 61.5% of the respective isolates (Fig. 1d). Minisatellite fragment *MinLm939* repeated 5 times (5x) was also frequent (73.1%), but only in the subpopulation of *L. maculans* obtained from metconazole-treated plots (Fig. 1b). Core fragment of *MinLm2451* replicated 3 times (3x) was found in more than half of the studied isolates, but its frequency was equal in both subpopulations of *L. maculans* (Fig. 1f).

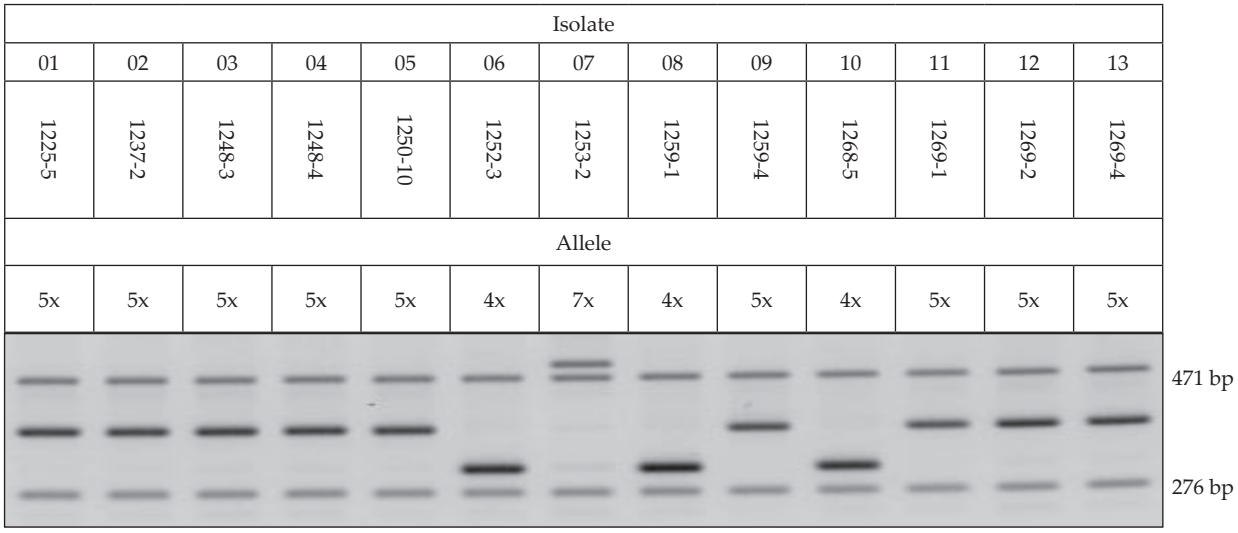

Fig. 2. The alleles of *MinLm555* minisatellite sequence of exemplary isolates of the phytopathogenic fungus *L. maculans* originating from infected oilseed rape plants; 276 bp and 471 bp – internal markers

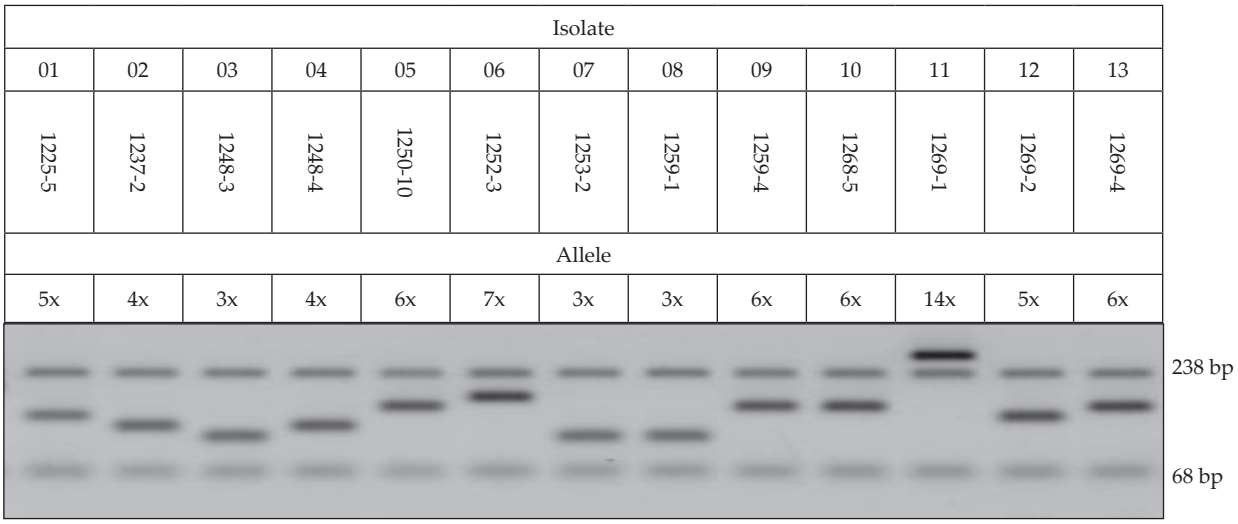

Fig. 3. The alleles of *MinLm1139* minisatellite sequence of exemplary isolates of the phytopathogenic fungus *L. maculans* originating from infected plants of oilseed rape; 68 bp and 238 bp – internal markers

In contrast, some alleles were rare and did not reach 10% frequency. Such phenomenon concerned several alleles of minisatellites with high DNA polymorphism, but it was also true for 2x core motif of the minisatellite *MinLm2452,* which produced only two variants among the studied isolates of *L. maculans* (Fig. 1g).

Six alleles, including *MinLm555* 7x, *MinLm935-2* 9x, *MinLm939* 11x, *MinLm1139* 13x, *MinLm1139* 14x and *MinLm2451* 10x, were found for the first time. Their identity must be confirmed by sequencing.

DISCUSSION

High demand for plant oil used for human consumption, high energy cake and meal for animal feed and technical oil for biofuel led to the great success of oilseed rape cultivation worldwide. However, intensification of its production, connected with greater areas of oilseed rape cultivation increased damage caused by insect pests and fungal pathogens. Stem canker of brassicas, regarded as one of the most important factors decreasing yields of this crop plant is currently one of the biggest concerns and key topic for research and breeding activities. One of the most advanced scientific programmes is the *L. maculans* genome initiative launched in 2004 by INRA (France) and the University of Melbourne (Australia). By now, the pathogen's genome has been sequenced and assembled. At present the structural and functional annotations are in progress (http://urgi.versailles.inra.fr/projects/lmaculans). In such way, *L. maculans* entered the genomic era (Rouxel and Balesdent 2005) and the first genetic linkage map and genome organization data were recognized (Kühn *et al.* 2006).

The access to sequences of *L. maculans* genome allowed to find out numerous tandemly repeated DNA fragments, known as VNTRs (Variable Number of Tandem Repeats) or minisatellites. It was proved that minisatellites could be associated with many interesting features of human genome biology and evolution, usually revealed by pathologies of genetic origin (Vergnaud and

Denoeud 2000). Hence, these fragments were also found interesting for plant pathologists and geneticists researching on *L. maculans.*

The first studies on polymorphisms in *L. maculans* isolates from Poland using 4 minisatellite markers from *MinLm1* to *MinLm4* showed 6, 9, 3 and 2 alleles respectively, with *MinLm1* 2x, *MinLm2* 7x, *MinLm3* 3x and *MinLm4* 3x as the most frequent variants in particular minisatellites (Jędryczka 2007). The studied population composed of 103 *L. maculans* isolates gathered from infected leaves of oilseed rape collected in 3 subsequent autumn seasons, starting in 2001. Further evaluation of DNA polymorphisms in Polish isolates of *L. maculans* using *MinLm1-MinLm3* and *MinLm5* minisatellite markers, using twice as big number of isolates as compared to the former study, showed 6, 14, 17 and 6 alleles respectively (Stachowiak 2008).

Following the detection of 20 variants of core motif numbers in *MinLm2*, this highly polymorphic marker was used to study variation in field populations of *L. maculans* isolates originating from plants differing by fungicide treatments (Jędryczka *et al.* 2009). Unexpectedly, 11 out of 14 alleles previously detected in Polish isolates of *L. maculans* could be found within one field of oilseed rape. Moreover, a novel 28x allele of *MinLm2* was additionally found. Only 3 alleles (6x, 9x and 12x) of this marker were found in both fungal subpopulations, whereas 5 and 4 alleles respectively were unique for fungal strains originating from control plants and the plants treated with metconazole. The most frequent product of 296 bp (*MinLm2* 9x) was present in 30.8% of *L. maculans* isolates from untreated plants and it was the second top product (23.1%) in the subgroup of isolates obtained from plants treated with metconazole. In this case the number of unique alleles was comparable for both subpopulations of *L. maculans*.

In the present study using 10 new minisatellite markers, 7 were found polymorphic. Eight out of 10 unique alleles were found in *L. maculans* subpopulation originating from plants treated with the pesticide. It suggests that the fungus population of *L. maculans* surviving on metconazole treated plants is very diverse and polymorphic. The hypothesized decrease of the number of *L. maculans* genetic variants that could survive in unfavourable conditions was not observed. In contrast, the number of alleles unique for this subpopulation was high. Additionally, 4 out of 6 newly found minisatellite variants were also detected uniquely in *L. maculans* isolates from plants sprayed with the fungicide. Conversely, only one allele was characteristic for unsprayed plants, and one was found in both subgroups of the pathogen. The results obtained in this study do not support any proof of the reduction of genetic diversity of *L. maculans* subpopulation originating from pesticide treated oilseed rape plants. However, it must be emphasized that the comparisons presented in this study concern the subpopulations that are equal in size, whereas in field conditions populations originating from untreated plants always greatly outnumbers populations from plants subjected to a fungicide treatment.

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POLISH SUMMARY

POLIMORFIZM DZIESIĘCIU NOWYCH MARKERÓW MINISATELITARNYCH CHOROBOTWÓRCZEGO GRZYBA *LEPTOSPHAERIA MACULANS* W SUBPOPULACJACH ZRÓŻNICOWANYCH POD WZGLĘDEM TRAKTOWANIA METKONAZOLEM

Sucha zgnilizna kapustnych stanowi jedną z najgroźniejszych chorób rzepaku na świecie. Jej przyczyną są dwa spokrewnione gatunki rodzaju *Leptosphaeria*, przy czym *L. maculans* powoduje większe straty plonu nasion. Przynależność do workowców umożliwia szybkie tworzenie nowych wariantów patogena, zdolnych do przełamania genów odporności.

Celem pracy było oznaczenie polimorfizmu w obrębie populacji *L. maculans*, przy zastosowaniu dziesięciu nowych markerów minisatelitarnych. Badane subpopulacje patogena różniły się pod względem ich traktowania preparatem grzybobójczym zawierającym metkonazol.

W zestawie badanych markerów minisatelitarnych 7 wykazało polimorfizm i tworzyło od 2 do 10 alleli – średnio 5 alleli. Sumaryczna liczba wariantów wynosiła 36, przy czym 28 alleli znaleziono w grupie izolatów *L. maculans* wyodrębnionych z roślin nie traktowanych żadnymi fungicydami, natomiast w subpopulacji z rzepaku traktowanego metkonazolem stwierdzono występowanie 32 alleli. Do zróżnicowania badanych subpopulacji przyczyniło się 10 unikalnych alleli obecnych wyłącznie w jednej grupie izolatów oraz zróżnicowanie procentowego udziału niektórych alleli. Wśród produktów PCR otrzymanych dla markerów minisatelitarnych *MinLm555*, *MinLm935-2*, *MinLm939*, *MinLm1139* oraz *MinLm2451* stwierdzono występowanie sześciu nowych wariantów, nie znalezionych w dotychczas badanych izolatach grzyba *L. maculans*.