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THE PATHOGENICITY AND DNA POLYMORPHISM OF *FUSARIUM OXYSPORUM* ORIGINATING FROM *DIANTHUS CARYOPHYLLUS*, *GYPSOPHILA* SPP. AND SOIL

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Abstract

A number of *Fusarium oxysporum* pathogenic isolates originating from *Dianthus caryophyllus*, *Gypsophila paniculata*, *G. repens* and non-pathogenic strains obtained from soil was screened for pathogenicity and genetic variation. RAPD analysis conducted with arbitrary 10-mer primers gave 23 RAPD markers resulted from the DNA polymorphism. Clustering analysis based on RAPD fingerprint data revealed several distinct groups within *F. oxysporum* which often correspond to the origin of isolate. Very high level of DNA polymorphism in all isolates made subdivision within groups possible. The analysis let to distinguish *F. oxysporum* isolates pathogenic and non-pathogenic to carnation and babies'-breath plants.

Key words: *Dianthus caryophyllus*, *Fusarium oxysporum*, *Gypsophila* spp., molecular markers, pathogenicity, RAPD

Introduction

Formae speciales of *Fusarium oxysporum*, causing vascular diseases of numerous plant species, are defined on the basis of their specific pathogenicity towards their hosts (Snyder and Hansen 1949, Armstrong and Armstrong 1968, 1975, Rataj-Guranowska and Pieczul 2002, Rataj-Guranowska et al. 2007).

One of the several dozen *formae speciales* described to date is *F. oxysporum* f.sp. *dianthi*. This fungal pathogen causes a serious disease of cultivated carnation species (*Dianthus caryophyllus*). Within *F. oxysporum* f.sp. *dianthi* (FOD) several strains were distinguished on the basis of their specific pathogenicity against different cultivars of greenhouse carnations (Armstrong and Armstrong 1968, Garibaldi 1977, Baayen et al. 1988). Moreover, it was found in infection greenhouse tests

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that *F. oxysporum* f.sp. *dianthi* may, apart from *D. caryophyllus*, infects plants of other species from the *Caryophyllaceae* family and the pathogenicity of various *F. oxysporum* f.sp. *dianthi* isolates towards host plants is varied (Armstrong and Armstrong 1954, Ben-Yephet et al. 1996, Werner 2003).

The vascular disease of carnations, caused by *F. oxysporum* f.sp. *dianthi*, was described in Poland over 30 years ago (Tatarynowicz 1973, Orlikowski 1978). Fusarium wilt spreading fast in greenhouse cultivations caused considerable economic losses in 1985 and 1986 (Mynett et al. 1989). Later *F. oxysporum* was isolated from wilting plants of *Gypsophila paniculata* grown in various greenhouses. Wilt caused by *F. oxysporum* was also observed on *G. paniculata* and *G. repens* growing outdoors (Werner and Szpanbriker 1997, Werner and Antkowiak 2002, 2003).

Considerable losses caused by vascular wilt showed the necessity to search for an effective plant protection method. Apart from other methods, in the protection of carnation against *F. oxysporum* f.sp. *dianthi* non-pathogenic isolates of the fungal species were applied. However, the identification of isolates is difficult and is based on the assessment of their pathogenicity towards host plants (Armstrong and Armstrong 1975, Komada 1975, Rataj-Guranowska and Pieczul 2002). Currently, molecular diagnostics based on an analysis of subtle differences in DNA sequence offer sensitive means for identification and characterization of fungi (Irzykowska 2006). Molecular analyses are independent from environmental conditions, medium contents and developmental stage of the studied organism. Different methods have been used to assess inter- and intraspecific variation in *Fusarium* and to find markers for their precise classification. One of the common and useful methods is random amplified polymorphic DNA (RAPD). RAPD has provided a suitable tool for distinguishing species and detecting polymorphism within single species (Williams et al. 1991). Apart from *F. oxysporum* RAPD have been used to analyse genetic variation of phytopathogenic *F. avenaceum*, *F. chlamydosporum*, *F. graminearum*, *F. moniliforme* and *F. solani* (Ouellet and Seifert 1993, Khalil et al. 2003). RAPD was also applied to determine mating groups within several members of *Fusarium* in *Liseola* section (Amoah et al. 1996).

The aims of the study were (i) to evaluate pathogenicity of *F. oxysporum* isolates towards carnation and babies'-breath under greenhouse conditions, (ii) to evaluate effectiveness of the RAPD analysis for determination of intraspecific variation and for differentiation between pathogenic and non-pathogenic strains of the studied *F. oxysporum* isolates.

Material and methods

Fungal isolates, culture maintenance and pathogenicity tests

Pathogenic isolates of *F. oxysporum*, selected for the study, were collected in 1978–2003 (Werner 1991–1992, Werner and Szpanbriker 1997, Werner and

Antkowiak 2002, 2003). They were obtained from plants with symptoms of vascular wilt and came from greenhouse carnations and from *G. paniculata* and *G. repens*:

– isolates FOD 1, F.ox. 1, F.ox. 2, F.ox. 3, FOD 2 were isolated from carnations of various cultivars of the "Sim" group, grown in 1978–1987 in different greenhouses in the Wielkopolska region,

– isolates F.ox. 6 and F.ox. 7 were obtained in 1992 from dying *G. paniculata* plants grown in greenhouses,

– isolates F.ox. 5 and F.ox. 8 to F.ox. 14 were obtained in 2001 and 2002 from dying plants of *G. repens* (F.ox. 5) and *G. paniculata* (F.ox. 8 to F.ox. 14) growing outdoors,

– isolate IMI 141130 of *F. oxysporum* f.sp. *dianthi* (FOD 30) comes from the International Mycological Institute (IMI), Egham, England; the isolate was obtained from *Dianthus* sp. by Armstrong (1969); geographic origin USA, South Carolina,

– non-pathogenic isolates of *F. oxysporum* (F.ox. 15 – Pz 71, F.ox. 16 – Pz 72 and F.ox. 17 – Z 54).

Non-pathogenic isolates of *F. oxysporum* were isolated according to Mańka (1974) from fields in Złotniki (F.ox. 17 – Z 54), where in successive years wheat, rape and rye were cultivated and in Poznań (F.ox. 15 – Pz 71 and F.ox. 16 – Pz 72) where rye was grown with no crop rotation applied. Both the applied isolation method and macro- and microscopic characteristics of pathogenic *F. oxysporum* isolates were described previously by Werner (1990). Pathogenicity of selected isolates towards greenhouse carnation cv. 'Pink Dona', *G. paniculata* and *G. repens* was assessed in greenhouse infection tests, following the method given by Werner (1991–1992) and Werner and Wyrwa (1994). The effect of non-pathogenic *F. oxysporum* isolates on greenhouse carnation cv. 'Pink Dona' and *G. paniculata* was assessed using a method described by Werner and Andrzejak (2004).

DNA preparations

Mycelia from seven-day-old single-spore cultures grown on liquid medium (5 g/l of glucose, 1 g/l of yeast extract) were collected by vacuum filtration using a Büchner funnel. DNA was extracted and purified using a DNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations with slight modifications (mycelium was ground with carborundum instead of disruption in liquid nitrogen).

RAPD assays

The RAPD reactions were carried out using a *Taq* PCR Core Kit (Qiagen). Six random 10-mer primers were tested in polymerase chain reaction. Two of them, OPJ-15 and OPJ-20 (Sigma-Genosys), at last were used to screen the isolates for DNA polymorphism. The reaction assay and PCR conditions were described earlier (Irzykowska et al. 2005 a, b, Irzykowska and Bocianowski 2008). Amplification was carried out in a Biometra T*personal* 48 thermocycler. RAPD assay was repeated twice to verify reaction reproducibility.

The electrophoresis conditions

The PCR products were separated by electrophoresis (6 V/cm) in 1.5% agarose gels with 1× TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.0) and visualised under UV light following ethidium bromide staining. A Gene Ruler ™ 100 bp DNA Ladder Plus (Fermentas) was used as a molecular size standard for PCR products.

Statistical analysis

Polymorphic bands were scored and analyzed by Treecon for Windows version 1.3b software (Van de Peer and de Wachter 1994). The coefficients of genetic similarity (GS) of the investigated isolates were calculated according to the formula given by Nei and Li (1979):

$$GS_{ij} = 2N_{ij}/(N_i + N_j)$$

where N_{ij} is the number of alleles present at the *i*-th and the *j*-th isolates, N_i – the number of alleles present at the *i*-th isolate, N_j – the number of alleles present at the *j*-th isolate, $i, j = 1, 2, \ldots, 19$. The coefficients were used to group the isolates hierarchically using the unweighted pair group method of arithmetic means (UPGMA).

Results

Pathogenicity test

Collected isolates did not differ in terms of their macroscopic and microscopic characteristics. In infection tests it was shown that all isolates coming from carnations caused wilting of plants, although their pathogenicity varied. The highest pathogenicity was found for isolate FOD 1 and FOD 2 – in a substrate infected with this isolate 100% plants showed vascular wilt.

Wilting of carnations during the infection experiment was caused by only one of the isolates obtained from *G. paniculata* – F.ox. 7 (Gp 2) – 6.3% of plants. Both *F. oxysporum* isolates obtained from *G. paniculata* infested carnations and on the day the experiment was completed a total of 18.8% carnation plants were infected (F.ox. 7 – Gp 2) and in the case of the other isolate of F.ox. 6 (Gp 1) it was 53.1%. Pathogenicity of these isolates towards *G. paniculata* was higher and in the case of isolate F.ox. 6 (Gp 1) a total of 35.7% wilting plants were found during the experiment and 64.3% were infected on the date it was completed, whereas in the case of F.ox. 7 (Gp 2) it was 71.4% and 25.0%, respectively (Fig. 1).

Non-pathogenic isolates F.ox. 15, F.ox. 16 and F.ox. 17 included in the study, although they did not cause symptoms of vascular disease, were capable of colonizing different percentages of plants – they were reisolated from 65, 50 and 40% plants in case of babies'-breath plants and 33, 38 and 12% plants in case of carnations (Fig. 2).

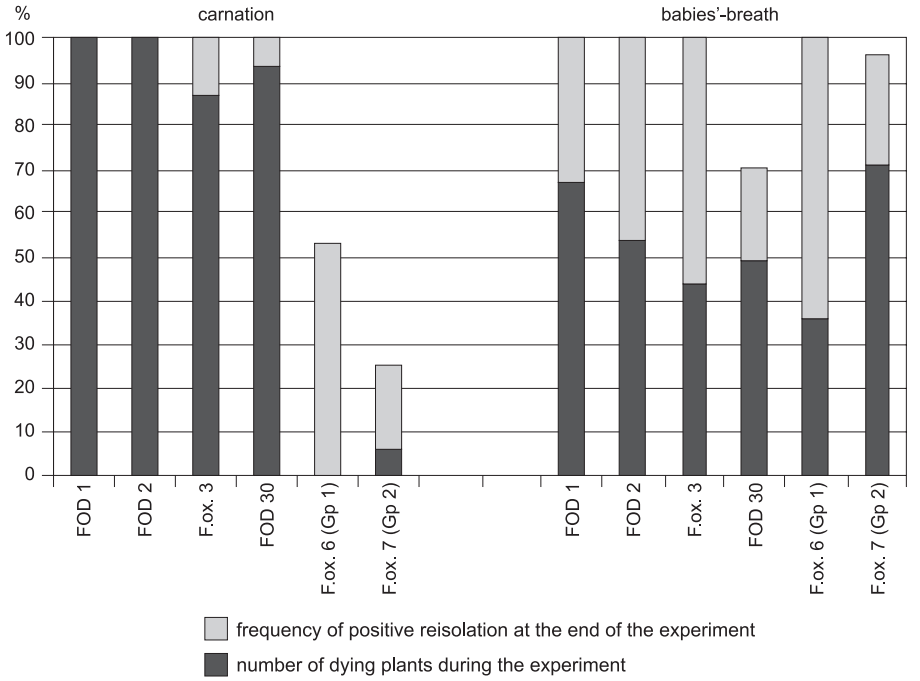


Fig. 1. Pathogenicity of different *Fusarium oxysporum* isolates towards carnation and babies'-breath plants

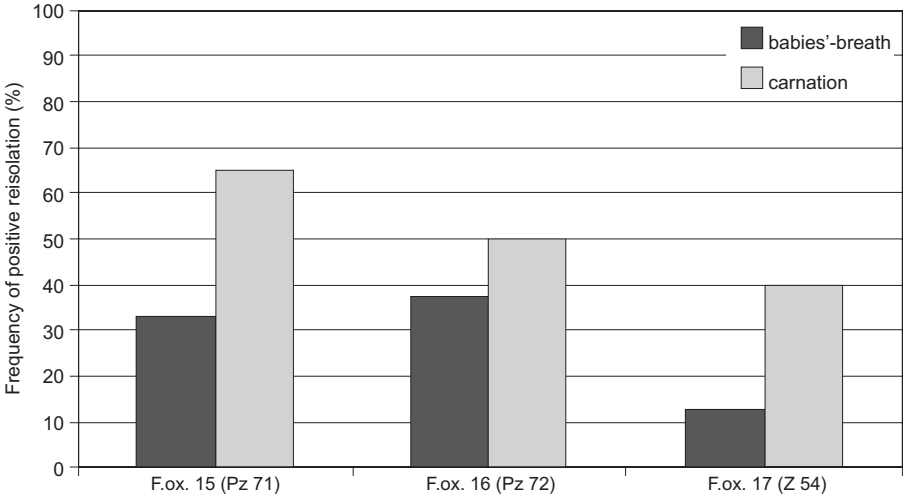
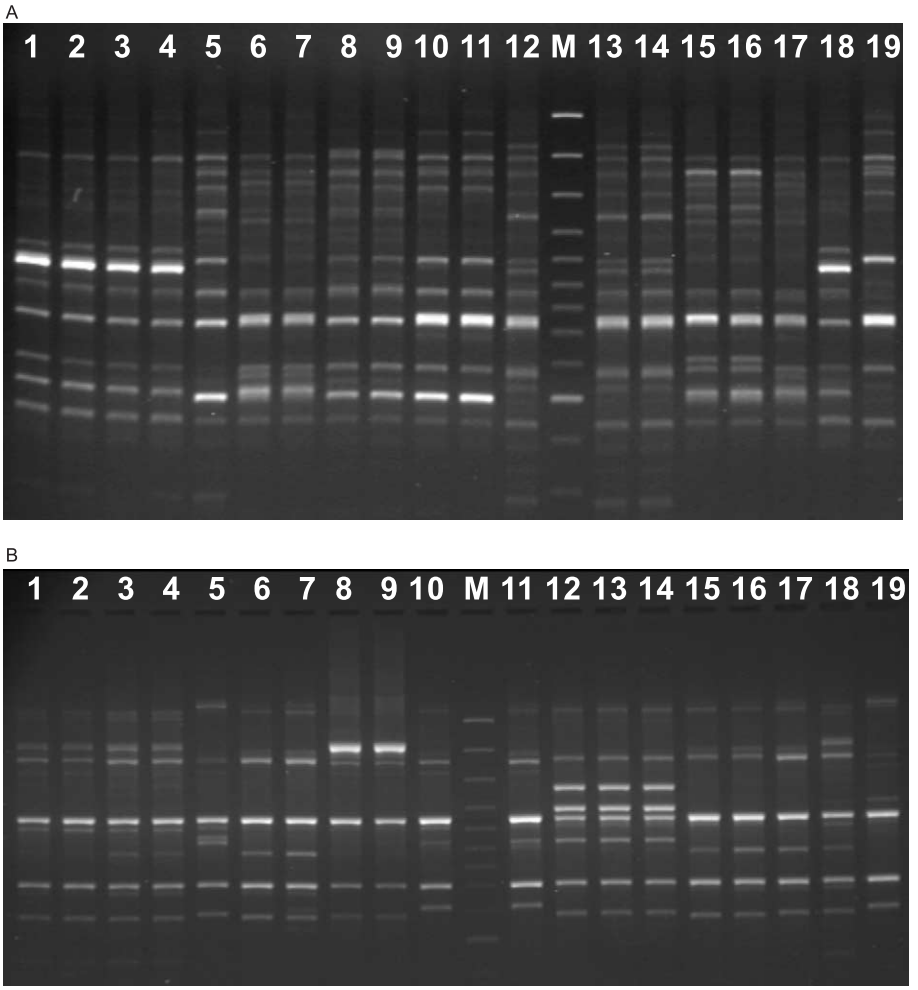


Fig. 2. Results of reisolation of *Fusarium oxysporum* non-pathogenic isolates from plants growing in infested peat

Molecular characterization

RAPD analysis enabled determination of genetic variability among 19 isolates of *F. oxysporum*. Both RAPD primers generated polymorphic PCR products. Only reproducible and exhibiting sufficient intensity bands were scored. Seven and 16 polymorphic PCR products were obtained with OPJ-20 and OPJ-15 primers, respectively (Phot. 1). The PCR products size ranged from 0.1 to 3.0 kb.

The comparison of each profile for each primer was done on the basis of presence (1) *versus* absence (0) of RAPD products of the same length. Each band was



Phot. 1. RAPD-PCR patterns of *Fusarium oxysporum* isolates amplified with (A) OPJ-15 and (B) OPJ-20 primers. Lanes 1–3: F.ox. 1 – F.ox. 3, lane 4: FOD 2, lanes 5–17: F.ox. 5 – F.ox. 17, lane 18: FOD 1, lane 19: FOD 30, M – Gene Ruler ™ 100 bp DNA Ladder Plus (photo by L. Irzykowska)

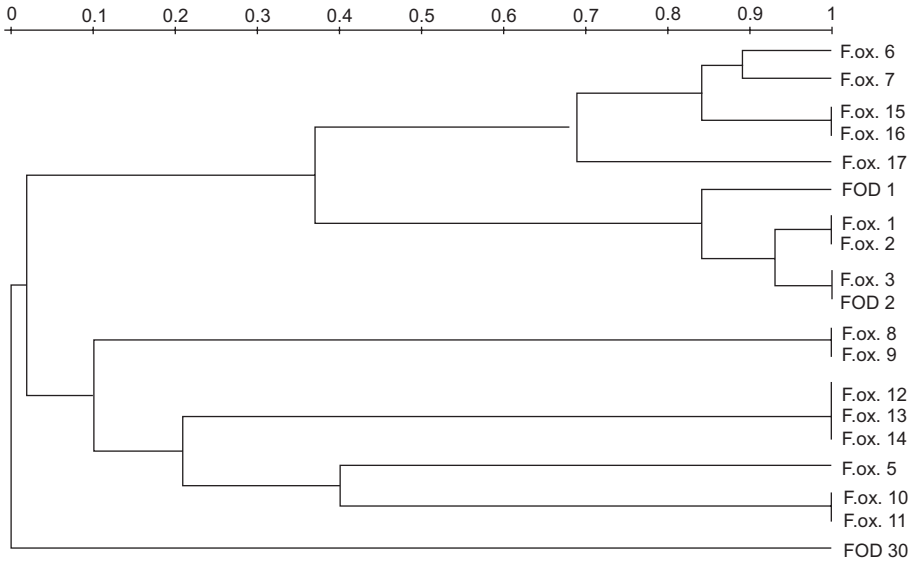


Fig. 3. Genetic analysis of *Fusarium oxysporum* isolates. Genetic distances were obtained by RAPD analysis

assumed to represent a single genetic locus. Results were used to group of isolates by the UPGMA method. The relationship among isolates is presented in the form of a dendrogram (Fig. 3).

Clustering based on RAPD fingerprint data revealed several distinct groups corresponding in some cases to the origin of *F. oxysporum* isolates. Two main groups of isolates were resolved at 3% similarity level. One of them included only isolates originating from *Gypsophila* spp. growing outdoors. The other grouped isolates originating from *D. caryophyllus,* two isolates from *G. paniculata* and non-pathogenic isolates. All examined isolates exhibit a very high level of DNA polymorphism making a subdivision of main groups possible.

Discussion

Variation in morphological traits of *F. oxysporum* analyzed in this study was slight and could not constitute a significant criterion in their identification. For this reason pathogenicity of these isolates was assessed in infection tests. In a similar manner pathogenicity of *F. oxysporum* towards different plant species was successfully evaluated in earlier studies. In these experiments, although infection tests were performed under identical conditions in a greenhouse, a considerable variation was recorded in the analyzed *F. oxysporum* f.sp. *dianthi* isolates in terms of their pathogenicity towards carnations and babies'-breath.

Although assessment of *F. oxysporum* pathogenicity in cross-infection tests is laborious, it still remains the basic criterion for their classification to an appropriate

forma specialis. Excellent tools supplementing the characterization of different isolates of the same fungus include serological analyses, analyses of vegetative compatibility and also molecular diagnostics (Postma 1992, Manicom and Baayen 1993, Woo et al. 1996).

Important problems being addressed by genetic diversity studies are the degree of genetic diversity that exists among subspecific correlation between pathogenic genotype and phenotype (Kistler 1997). Molecular methods based on DNA analysis help to determine a genetic variability of fungi (Martin et al. 2000, Taylor et al. 2001). Application of molecular markers eliminates doubts, which appear during classical research based on morphology evaluation. Markers are especially useful in the case of fungi never undergoing sexual reproduction what narrows down the set of traits available for study (Irzykowska 2006).

Molecular studies conducted with different techniques show that there are interspecific variation within *Fusarium* genus and also intraspecific differences within *F. oxysporum* (Kistler 1997). Using amplified fragment length polymorphism (AFLP) analysis several *formae speciales* of *F. oxysporum* were distinguished (Baayen et al. 2000). Chiocchetti et al. (1999) distinguished isolates of *F. oxysporum* f.sp. *dianthi* using the fungal transposable elements *Fot 1* and *impala* as probes for Southern hybridization. They obtained DNA fingerprints patterns corresponding to three groups of isolates: the first group – isolates of races 1 and 8; the second group – isolates of races 2, 5 and 6; and the third group – isolates of race 4. Similar grouping of isolates was obtained when restriction fragment length polymorphism (RFLP) was applied to determine genetic variation in *F. oxysporum* f.sp. *dianthi* from wilting carnations (Manicom et al. 1990, Manicom and Baayen 1993, Baayen et al. 1997). Isolate groups derived from RFLP analysis were consistent with existing vegetative compatibility groups (VCGs) in *F. oxysporum* f.sp. *dianthi*, but not in all cases with races.

It was reported before that also RAPD analysis could differentiate reliably between non-pathogenic and pathogenic isolates of *F. oxysporum* (Freeman and Maymon 2000, Tantaoui et al. 1996). Khalil et al. (2003) used RAPD in combination with pathogenicity assays to study the taxonomic kinships among five *Fusarium* species. Unfortunately, there was no clear-cut relationship between clustering in the RAPD dendrogram, pathogenicity test and geographic origin of tested isolates.

The present study confirms that the RAPD technique is an effective tool in the search for intraspecific polymorphism within *F. oxysporum* f.sp. *dianthi*. DNA polymorphisms recorded with RAPD markers revealed that isolates of *F. oxysporum* isolated from carnation and babies'-breath plants, though morphologically indistinguishable, exhibit a high level of genetic variation. Two main groups of isolates, revealed by clustering, were subdivided. This is in agreement with the results of Zamani et al. (2004), who described a high level of genetic variability within *F. oxysporum* originating from chickpea.

Isolate FOD 30 originating from South Carolina (USA) varied from Polish isolates in the screened part of the genome and was represented by distinct multiple band RAPD pattern. Considering that FOD 30 isolate was derived from distinct

gene pool, such significant differences may seem understandable. Assigbetse et al. (1994) also reported an association between genetic variation and geographic origin of *F. oxysporum* isolates. Similarly, Migheli et al. (1997) using RAPD fingerprinting procedure to characterize *F. oxysporum* f.sp. *dianthi* isolates of different geographical origin, obtained a few genetically distinct groups. Moreover, it seems to be probable that FOD 30 and the studied Polish isolates can belong to different races. Manicom and Baayen (1993) proved that isolate groups derived from analysis of RFLP were consistent in most cases with races and VCG.

RAPD fingerprints let to discriminate pathogenic and non-pathogenic *F. oxysporum* isolates, but more extensive studies are necessary to confirm correlation between pathogenicity toward hosts and molecular markers.

Streszczenie

PATOGENICZNOŚĆ I POLIMORFIZM DNA *FUSARIUM OXYSPORUM* POCHODZĄCEGO Z *DIANTHUS CARYOPHYLLUS*, *GYPSOPHILA* SPP. I GLEBY

Badano izolaty *Fusarium oxysporum* uzyskane z roślin z objawami fuzaryjnego więdnięcia. Izolaty FOD 1, F.ox. 1, F.ox. 2, F.ox. 3, FOD 2 izolowano w latach 1978–1990 z goździków różnych odmian grupy „Sim" uprawianych w szklarniach na terenie Wielkopolski. Odrębną grupę stanowiły izolaty F.ox. 5 oraz 8 do 14, które uzyskano z uprawianych w gruncie roślin *Gypsophila repens* (F.ox. 5) i *G. paniculata* (pozostałe izolaty), oraz F.ox. 6 i 7, które uzyskano w 1992 roku z uprawianych w szklarniach zamierających roślin *G. paniculata*.

Do badań włączono również izolat IMI 141130 *F. oxysporum* f.sp. *dianthi* (FOD 30) pochodzący z Międzynarodowego Instytutu Mikologicznego (IMI) w Egham, Anglia. W testach infekcyjnych wykazano, że wszystkie te izolaty powodują objawy charakterystyczne dla choroby naczyń, jednak ich patogeniczność wobec goździków i łyszczca jest zróżnicowana.

Włączone do badań niepatogeniczne izolaty F.ox. 15, F.ox. 16, F.ox. 17, choć nie powodowały objawów choroby naczyń, zdolne były do zasiedlania roślin w różnym procencie: reizolowano je odpowiednio z 65, 50 i 40% roślin łyszczca oraz 33, 38 i 12% goździków.

Stosowane obecnie molekularne metody analizy genomu umożliwiają ocenę zróżnicowania genetycznego izolatów w obrębie poszczególnych gatunków. W celu określenia zróżnicowania genetycznego badanych izolatów *F. oxysporum* zastosowano metodę RAPD. Uzyskane 23 polimorficzne markery RAPD posłużyły do skonstruowania dendrogramu obrazującego wewnątrzgatunkowe zróżnicowanie badanych izolatów. Niektóre z utworzonych na dendrogramie grup korespondują z pochodzeniem izolatów. Całkowicie odrębny w badanych obszarach genomu był izolat FOD 30, pochodzący z kolekcji CABI IMI, co wydaje się zrozumiałe przy uwzględnieniu odrębności puli genowej, z jakiej się wywodził. Ponadto analiza RAPD umożliwiła odróżnienie saprotroficznych i pasożytniczych izolatów *F. oxysporum*.

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