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VEGETATIVE COMPATIBILITY OF *FUSARIUM OXYSPORUM* ISOLATED FROM WEEDS IN EASTERN CROATIA

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SUMMARY

Different formae speciales of Fusarium oxysporum are the main causal agent of Fusarium wilts. In 2008 and 2009 we collected F. oxysporum samples from symptomless Abutilon theophrasti, Xanthium strumarium, Chenopodium album, Matricaria perforata, Ambrosia artemisiifolia, Polygonum lapathifolium, Sonchus arvensis, Amaranthus blitoides, Amaranthus retroflexus, Datura stramonium, Sorghum halepense and Hibiscus trionum. Only 16 out of 41 isolates of F. oxysporum yielded nit mutants. The frequency of nit3 mutants was higher (43%) than the frequency of nit1 (35%) and NitM (22%) mutants. Two vegetative compatibility groups (VCGs) of F. oxysporum were determined in the complementation tests. These results stress out the problem with isolation of nit mutants and show a high genetic diversity of F. oxysporum isolated from weeds.

Key-words: weed, Fusarium oxysporum, vegetative compatibility

INTRODUCTION

Fusarium species are widespread and play an important role in disease etiology of many cultivated plants (Parađiković et al., 2000, Ćosić et al., 2004). Many weed species are also important hosts for numerous fungal species (Vrandečić et al., 2010) including *Fusarium oxysporum.*

Fusarium oxysporum Schlecht. *emend*. Snyder et Hansen, is a common soil fungi distributed worldwide, which consists of both pathogenic and nonpathogenic strains (Gordon and Martyn, 1997). *F. oxysporum* causes vascular wilts, damping-off problems and crown and root rots (Nelson, 1981; Summerell and Rugg, 1992). Large genetic diversity among isolates has led to the realization that *F. oxysporum* represents a complex of species. Members of *F. oxysporum* complex cause a vascular disease in a broad range of host plants commonly known as wilt, root rot, or yellows. *F*. *oxysporum* is pathogenic to the variety of hosts and is considered to be the most economically important *Fusarium* species. However, individual isolates within this fungus normally have a narrow host range, and the species is classified into *forma specialis* based on specific pathogenicity on a host plant. According to Fourie et al. (2011) pathogenicity tests can be used to distinguish pathogenic and nonpathogenic strains from each other, but the same can not be done with morphological analysis or sexual compatibility studies. The plant pathogenic strains are divided into *formae speciales* according to the plant species on which they cause disease, and into races according to crop cultivar specificity. Isolates that attack the same crop are included in the same *forma specialis*. Most *formae speciales* are pathogenic to a single crop (e.g. *F. oxysporum* f. sp. *dianthi* on carnation). Some, however, attack more than one crop (*F. oxysporum* f. sp. *cucumerinum)*. Currently, 150 host-specific *formae speciales* have been identified (Baayen et al., 2000). Use of DNA sequencing has shown that *F*. *oxysporum* is comprised of number of distinct lineages (Baayen *et al.*, 2000). Why is *F*. *oxysporum* so diverse? It is a reproductive strategy, because natural selection favours genotypes with a reproductive advantage.

According to Kuhn et al. (1995) as an asexual organism *F*. *oxysporum* is thought to evolve by means of mutations only and potentially through the process of parasexual recombination (i.e. a non-sexual mechanism for creating new genetic combinations). Parasexual recombination, however, would be limited to fungi that

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are vegetatively compatible. Vegetative compatibility has often been used in fungal diversity studies. It relies on heterokaryon formation and may be determined with complementation assays between auxotrophic nutritional mutants. Puhalla (1985) developed an efficient technique for determining compatibility that utilizes nitrate-non utilizing auxotrophic (*nit*) mutants being readily recovered and stable. In order to form a stable heterokaryon two isolates need to share a common allele at each *vic* locus (Correll, 1991). Therefore it could be expected that the rest of the genomes of isolate in one VCG of an asexual species would also be very similar (Leslie, 1990; Leslie, 1993). It means that a mutation at a single *vic* locus would place closely related individuals in different VCGs (Bentley et al., 1998). In accordance to that VCGs represent good phenotypic characters for assessing diversity within populations, but genetic relationships among VCGs must be assessed by other means (Fourie et al., 2011). The relationship between VCG and race is even more complex, with a single race being associated with multiple VCGs (Katan et al., 1993). In this paper we present our research of vegetative compatibility of *F. oxysporum* isolates from weeds.

MATERIAL AND METHODS

Samples of weeds were collected from 4 locations in eastern Slavonia, Croatia in 2008 and 2009. Sampled weed species included: *Abutilon theophrasti* L., *Xantium stramonium* L., *Chenopodium album* L., *Matricaria perforata* Merat., *Ambrosia artemisiifolia* L*., Polygonum lapathifolium L., Sonchus arvensis* L*.*, *Amaranthus blitoides* S. Watson*, Amaranthus retroflexus* L., *Datura stramonium L., Sorghum halepense* L. Pers. and *Hibiscus trionum* L. Roots and lower stalk parts of weeds were cut into pieces 2-3 cm long and 0.5-1 cm wide. Pieces were surface sterilized with 95% ethanol and placed on sterile filter paper in 9 cm Petri dishes. Samples contaminated with bacteria or unwanted fungal species were discarded. After seven days mycelia appeared to belong to a *Fusarium* spp. were transferred to PDA (Difco, Detroit, Michigan) plates and incubated (Leslie and Summerell, 2006) for seven days under fluorescent lights (Philips TL 36W/80 RS F40 BLB) on a 12/12 day/night schedule. After seven days mycelia were microscopically examined and only strains containing macro- or microconidia were retained. Samples that did not sporulate were transferred to plates with CLA media (Leslie and Summerell, 2006) and incubated at 20-25°C for fourteen days on 12/12 day/night schedule. Morphological identification was based on mycelial characters of the mycelia from the PDA culture, whereas the presence and morphology of micro- and macroconidia and chlamydospores from the CLA culture. Mycelia growth rate, color and density of mycelia growing on PDA were also recorded. Micro- and macroconidia were photographed by Olympus camera and characterized as described by Leslie and Summerell (2006). First step in determining vegetative compatibility is to obtain *nit* mutants on chlorate media containing ClO_3. The amount of Cl varies from 0.5% all the way to 6%, and most frequently is 2.5%. It is necessary to grow isolates on PDA or minimal media to obtain good mycelial growth. After the colony starts to grow rapidly, usually after 2-3 days, small mycelial parts (1-2 mm^3) from the colony edge are transferred to minimal media with ClO_3. Isolates obtained on ClO_3 media are transferred to minimal media with $NaNO_3$. Isolates that grow thinly on minimal media are *nit* mutants and are kept for later analysis and isolates with dense growth on minimal media with $NaNO_3$ should be discarded. Ability of nit mutants to use different forms of nitrogen are used for their phenotyping. Basically, media which contains NH_4 is used as positive control and media with NO_3 as negative control. *Nit* mutant phenotyping begins with cultures grown on PDA or minimal media for 3-4 days on 25ºC. The following media with different forms of nitrogen is used: MM (minimal media) + $NaNO_3$, MM + $NaNO_2$, MM + hypoxantin and MM +NH_4. In each phenotyping Petri dish we also put controls for *nit1*, *nit3* and NitM controls. On the remaining place in Petri dish we place a certain number of *nit* mutants whose phenotype needs to be determined. Isolates are grown for 3-4 days on 25°C. The results of phenotyping media should place mutants in one category presented in the Table 1.

Table 1. Determination of nit mutants according to nitrogen source

Tablica 1. Određivanje kategorije nit mutanata prema iskorištavanju izvora dušika

Isolate/*Izolat*	Media/*Podloga*				
	NH_4	NO_3	NO_2	Hypoxantine	ClO_3
Wild isolate/*Divlji izolat*	+	+	+	+	-
nit1	+	-	+	+	+
nit3	+	-	-	+	+
NitM	+	-	+	-	+
Crn	+	+	+	+	+

Pairings are done on MM + $NaNO_3$ media with NO_3 as the only nitrogen source. *Nit* mutants will grow thinly on this media unless it forms a heterokaryon upon contact with another mutant. Tests can be done in Petri dishes and 24 well tissue culture plates (1.7 ml minimal agar/well). Wells are inoculated with small pieces of isolate grown on minimal media (< 1mm^3). Inoculated plates are grown on 25ºC. Colonies are usually met after 3–4 days and heterokaryon can be observed 1-2 days after that. Heterokaryon formation is usally finalized

after 14 days. Usually two isolates are tested in this way. 24 well tissue culture plates (1.7 minimal agar/well) are inoculated with spore suspension, prepared by adding 1 ml 2% Tween 60 into the small tubes. Pasteur pipete is used to add several drops of each isolate into the well in accordance with the prepared pairing scheme. After inoculation plates should be left open for a while so the liquid can get dry. Plates are incubated on 25°C, light is not necessary. Positive reaction appears as a line across the well or as air mycelium. VCG recording can start 4 days after inoculation and usually ends 7 days after inoculation.

RESULTS AND DISCUSSION

Collected weed yielded the following number of *F. oxysporum* isolates: 9 from *A. theophrasti*, 3 from *X. stramonium*, 7 from *C. album*, 1 from *M. perforata*, 3 from *A. artemisiifolia,* 3 from *P. lapathifolium,* 2 from *S. arvensis*, 1 from *A. blitoides,* 2 from *A. retroflexus*, 1 from *D. stramonium,* 8 from *S. halepense* and 1 *H. trionum.* The *nit* mutants were generated from only sixteen out of 41 *F. oxysporum* strains after several trials. Out of all mutants isolated, for 10 isolates of *F. oxysporum* only single members of *nit* mutants were identified (for example, only *nit3* or NitM) and we were not able to pair them. No *nit* mutants were generated from 25 strain of *F. oxysporum* through several trials. The common problem was that mutants growing very thinly on chlorate media reverted when transfered to minimal media. All of the *nit* mutants isolated were characterized by their phenotypes (*nit1*, *nit3*, or NitM) with prevalence of *nit3* (43%) over *nit1* (35%) and NitM (22%) mutants. In all, 230 *nit* mutants were obtained from all isolates: 99 *nit3* mutants, 81 *nit1* mutants and 50 NitM mutants. We do not know why so few isolates yielded *nit* mutants. Probably it has something to do with the fact that isolates originate from weeds. It is certain that additional research should be done in order to investigate this issue. Most isolates produced complementary *nit* mutants at 3% chlorate media. At the end we were able to test compatibility of only six *F. oxysporum* isolates. They all proved to be self-compatible. Based on the results of compatibility tests among mutants of 6 isolates in all possible combinations two vegetative groups (VCGs) of *F. oxysporum* were determined.

Vegetative compatibility is usually used in two cases. Firstly, in the case of new population, for which we have no information, that would be our case. Secondly, in case when we believe that our isolates belong to a certain VCG group. In this research we have a new population of *F. oxysporum* isolated from symptomless weeds and plant debris. Vegetative compatibility of *Fusarium* spp. has been studied by several authors. Classification of *F. oxysporum* isolated from Paris daisy (*Argyanthemum frutescens* L.) was done by Pasquali *et al*. (2004) and their isolates belonged to a new group of *F. oxysporum* f. sp. *chrysanthemi*. They were classified as a new VC group (0052). Catti *et al*. (2007) have studied *F. oxysporum* isolated from wilted plants of rocket (*Eruca vesicaria* and *Diplotaxis* spp.). They determined two VC groups and classified them as *formae speciales conglutinans* and *raphani*. According to some authors *forma specialis* concept in the *F. oxysporum* is restricted to causing vascular wilt of a specific host (Corell, 1991). Harveson and Rush (1997) collected *F. oxysporum* isolates from wilted sugar beet (*Beta vulgaris* L.) and pigweed plants (*A. retroflexus*). Seven VC groups were identified. Corell (1991) stated that limitations of VC technique depend on the particular *forma specialis* or group of strains being examined. According to him specific limitations include difficulty in recovering *nit* mutants from certain isolates on chlorate media, weak heterokaryon reactions between nit mutants of some strains, cross-compatibility reactions between some isolates and different VCGs and self-incompatible isolates. In most of the mentioned research *forma specialis* of *F. oxysporum* can be assumed based on the host from which it was isolated. In our research we were not able to do so because our hosts were weeds. VCGs are usually tested with already registered testers of VCG groups and *formae speciales* of *F. oxysporum*. Weed hosts collected in this research did not exibit disease symptoms, therefore *F. oxysporum* isolated from them can be considered non-pathogenic and/or endophytic. According to Gordon and Okamoto (1992) nonpathogenic strains of *F. oxysporum* are widespread and genetically more diverse than their pathogenic counterparts and according to Edel et al. (2001) they are not studied either. Due to the fact that there was a very low compatibility between our isolates and that we managed to differentiate only two VCG groups, we were not able to determine *forma specialis* of our isolates and therefore did not test them with already registered VCG testers. We would probably have to sequence each of our isolates of *F. oxysporum* and obtain more details on possible *forma specialis*. The problem is that, for example in NCBI database, not all *F. oxysporum* that are registered have their *forma specialis* determined. The best approach would be to test our isolates with all existing and reported VCG representatives, that would be rather costly and can be envisaged as the whole new research.

CONCLUSION

The aim of this research was to determine vegetative compatibility of *F. oxysporum* isolates from symptomless weeds. Three types of *nit* mutants, *nit3*, *nit1* and NitM were obtained through use of vegetative compatibility method. The frequency of *nit3* mutants was higher (43%) than frequency of *nit1* (35%) and NitM (22%) mutants. Complementation tests determined two vegetative compatibility groups. The results indicate high genetic diversity of *F. oxysporum* isolated from weeds and clearly reveal the determination problem of *formae speciales* of *F. oxysporum* isolated from weeds. Additional research needs to be conducted in order to pair our isolates with the existing representatives of different *F. oxysporum formae speciales* and their VCG

groups. Isolates should also be evaluated by molecular methods and compared with the established VCGs, to thoroughly determine possible phylogenetic relationships.

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VEGETATIVNA KOMPATIBILNOST *FUSARIUM OXYSPORUM* IZOLIRANOGA S KOROVA U ISTOČNOJ HRVATSKOJ

SAŽETAK

Različite formae speciales Fusarium oxysporum glavni su uzročnik fuzarijskih venuća. U 2008. i 2009. godini prikupili smo F. oxysporum uzorke sa sljedećih korova bez simptoma bolesti: Abutilon theophrasti, Xanthium strumarium, Chenopodium album, Matricaria perforata, Ambrosia artemisiifolia, Polygonum lapathifolium, Sonchus arvensis, Amaranthus blitoides, Amaranthus retroflexus, Datura stramonium, Sorghum halepense i Hibiscus trionum. Izolirali smo nit mutante za 16 od 41 izolata F. oxysporum. Učestalost nit3 mutanata bila je veća (43%) od učestalosti nit1(35%) i NitM (22%) mutanata. Testovima uparivanja utvrđene su dvije vegetativne grupe (VCG) F. oxysporum. Ti rezultati ukazuju na problem izolacije nit mutanata i upućuju na veliku genetsku raznolikost F. oxysporum izoliranoga s korova.

Ključne riječi: korovi, Fusarium oxysporum, vegetativna kompatibilnost

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