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Mini-review

Recent developments in the molecular discrimination of *formae speciales* of *Fusarium oxysporum*

Bart Lievens,[1] Martijn Rep[2] and Bart PHJ Thomma[3]*

[1]*Scientia Terrae Research Institute, Fortsesteenweg 30A, 2860 Sint-Katelijne-Waver, Belgium*
[2]*Plant Pathology, Swammerdam Institute for Life Sciences, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands*
[3]*Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, The Netherlands*

Abstract: Rapid and reliable detection and identification of potential plant pathogens is required for taking appropriate and timely disease management measures. For many microbial species of which all strains generally are plant pathogens on a known host range, this has become quite straightforward. However, for some fungal species this is quite a challenge. One of these is *Fusarium oxysporum* Schlechtend:Fr., which, as a species, has a very broad host range, while individual strains are usually highly host-specific. Moreover, many strains of this fungus are non-pathogenic soil inhabitants. Thus, with regard to effective disease management, identification below the species level is highly desirable. So far, the genetic basis of host specificity in *F. oxysporum* is poorly understood. Furthermore, strains that infect a particular plant species are not necessarily more closely related to each other than to strains that infect other hosts. Despite these difficulties, recently an increasing number of studies have reported the successful development of molecular markers to discriminate *F. oxysporum* strains below the species level.
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1 INTRODUCTION

Fusarium oxysporum Schlechtend:Fr. is an asexual fungus that occurs in soils worldwide. As a species, *F. oxysporum* includes morphologically indistinguishable pathogenic as well as non-pathogenic strains. The latter are defined as strains for which no host plants have been identified (yet). Pathogenic *F. oxysporum* strains can cause vascular wilt or root rot in over 100 plant species, among which are several economically important crops including banana, bulb flowers, cucumber, cutting flowers, date palm, melon and tomato.[1] In spite of the broad host range of the species as a whole, individual strains usually infect only a single or a few plant species. Therefore, pathogenic strains have been assigned to *formae speciales* based on host specificity, and presently over 70 *formae speciales* have been described.[2] For example, *F. oxysporum* f. sp. *cucumerinum* and *F. oxysporum* f. sp. *lycopersici* only cause disease on cucumber and tomato respectively.[3,4] Some *formae speciales* have broader host ranges, such as *F. oxysporum* f. sp. *radicis-cucumerinum* and *F. oxysporum* f. sp. *radicis-lycopersici* which, apart from infecting cucumber and tomato respectively, can cause root and stem rot on multiple hosts from different plant families.[4–6] Several *formae speciales* have been further subdivided into races based on cultivar specificity.[2] To define genetic relationships within *formae speciales, F. oxysporum* strains have been grouped into vegetative compatibility groups (VCGs)[7] based on the ability of strains to form heterokaryons. Strains that belong to the same VCG normally have identical alleles at their compatibility loci, enabling the exchange of nuclear material.[8] Therefore, isolates of the same VCG are usually clonally related, although exceptions have been reported.[9] Many *formae speciales*, and even some races, comprise strains that belong to multiple VCGs,[10,11] suggesting independent origins within the *F. oxysporum* species complex. Indeed, recent molecular and genetic studies suggest a polyphyletic nature for most (if not all) *formae speciales* that harbour multiple VCGs, including the f. sp. *asparagi, cubense, cucumerinum, dianthi, gladioli, lini, lactucae, lycopersici, melonis, opuntiarum, phaseoli, radicis-lycopersici* and *vasinfectum*.[12–19]

Currently, no effective curative treatments to control *F. oxysporum* exist, and infected plants should be removed quickly to prevent spread of the disease. Therefore, most efforts are directed towards prevention of the disease. In general, effective means of disease control prior to infection include soil fumigation, disinfestation of plant material or, if available, the use of resistant plant cultivars.[20]

* Correspondence to: Bart PHJ Thomma, Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, The Netherlands
E-mail: bart.thomma@wur.nl
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However, breeding for resistance is complicated when no dominant resistance locus has been identified (e.g. for carnation and cyclamen) or with dioecious host species (e.g. date palm). In addition, new races may develop that can overcome host resistance.[21] Alternatively, fungal presence in the soil can be controlled by manipulation of the environment in such a way that growth, sporulation and virulence of the pathogen decrease, or by reducing its presence through crop rotation with non-hosts.[22] In addition, biocontrol strategies are being developed using either non-pathogenic *F. oxysporum* strains or other antagonistic microorganisms,[20] although for most of them the efficacy under field conditions is still unknown. With all these approaches, implementation of appropriate disease management measures requires timely detection and reliable identification of the pathogen. In addition, pathogen quantification is becoming more and more important, since it serves as a basis for establishing damage thresholds at which a pathogen causes disease, and action thresholds that determine when measures should be taken to limit or prevent losses.

2 IDENTIFICATION OF PATHOGENIC *FUSARIUM OXYSPORUM* ISOLATES

Classically, plant pathogens have been identified on the basis of morphological features and growth characteristics on specific media. However, because of their specific limitations, these techniques are increasingly being complemented or replaced by molecular technologies, of which those based on detection of pathogen DNA or RNA are the most predominant.[23] In general, the molecular techniques are faster, more specific, more sensitive and more accurate than the traditional methods, and can be performed and interpreted by personnel with no specialized taxonomical expertise. In addition, and perhaps even more importantly, these techniques allow detection of microorganisms that cannot be cultivated *in vitro*.[23] The number of pathogens that can be identified by molecular methods has now expanded to the extent that only few cannot be accurately identified. These few mainly comprise subspecies of a species that, as a whole, is harmless to the crop of interest, such as *F. oxyporum*. For these species, pathogenic and non-pathogenic strains can usually not be discriminated by targeting the generally used conserved household genes, which severely complicates the development of reliable molecular markers.[23,24]

Currently, identification of pathogenic *F. oxysporum* isolates is mainly based on pathogenicity testing.[24] Determination of the *forma specialis* is generally done by testing the fungus for pathogenicity on various plant species, while races are determined by pathogenicity assays on different cultivars of a single plant species. Although these bioassays are very effective, they are highly time consuming and laborious. In addition, because of the abundance of *formae speciales*, for correct identification, strains must be inoculated onto a vast number of plant species and cultivars.[20] Therefore, attempts are being made to replace these methods with molecular identification techniques. Ideally, molecular identification of pathogenic strains is based on diagnostic traits that are directly linked to pathogenicity.[25,26] However, so far the genetic basis of host specificity in *F. oxysporum* is poorly understood. Furthermore, molecular discrimination of *F. oxysporum* isolates is complicated by the polyphyletic nature of many *forma specialis*.[27] Currently, several approaches are being developed in order to differentiate *formae speciales* and races of *F. oxysporum*. The most important approaches are based on the use of molecular markers identified by genotyping or polymerase chain reaction (PCR) amplification of transposon insertions (Table 1).

2.1 Genotyping: sequence-unbiased approaches for molecular identification of pathogenic strains

Multiple genotyping techniques have been used to identify random sequence differences between subspecific groups of *F. oxysporum*,[27] including restriction fragment length polymorphism (RFLP),[28] random amplified polymorphic DNA (RAPD)[29] and amplified fragment length polymorphism (AFLP)[30] assays. A major drawback of RFLP analysis is its labour-intensive nature and the need for relatively high amounts of DNA.[31] A major disadvantage often associated with RAPD assays is poor interlaboratory reproducibility,[32] while AFLP is relatively costly and a rather complicated technical procedure.[31] For these reasons, diagnostic DNA fragments identified with these approaches are often converted into more simple and reliable molecular markers. Sequence-characterized amplified region (SCAR) primers[33] are then designed specifically to amplify the selected markers. This approach has proven to be effective for the identification of several *formae speciales* and races of *F. oxysporum*.[15,34–40] However, as these markers can be localized anywhere in the genome, there often is little sequence data available in public databases for comparison with other sequences. Therefore, extensive screening using a large collection of strains is necessary to validate the robustness of the marker.

For *F. oxysporum* it appears that the anonymous markers generated by these techniques often correspond to mobile elements. For example, the markers used by Jiménez-Gasco and Jiménez-Díaz[38] to identify *F. oxysporum* f. sp. *ciceris* race 0, 1A and 6 contain a fragment that is identical to the *Impala* transposon,[41] while the *F. oxyporum* f. sp. *radicis-cucumerinum* marker developed by Lievens *et al.*[15] shows strong similarity with *Folyt1*, a transposable element identified in *F. oxysporum* f. sp. *lycopersici*.[42]

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Table 1. PCR primers[a] for the detection and identification of *formae speciales* and races of *Fusarium oxysporum* in environmental samples

Target organism	Forward primer (5′-3′)		Reverse primer (5′-3′)		Target gene[b]	Specificity[c]	Reference
F. oxysporum f. sp. *albedinis*	BIO3	GGCGATCTTGATTGTATTGT-GGTG	FOA1	CAGTTTATTAGAAATGCCGCC	*Fot1* transposon	270/286; 0/113	48
F. oxysporum f. sp. *albedinis*	TL3	GGTCGTCCGCAGAGTATAC-CGGC	FOA28	ATCCCCGTAAAGCCCTGAAGC	*Fot1* transposon	286/286; 0/113	48
F. oxysporum f. sp. *basilici*	Bik1	ATTCAAGAGCTAAAGGTCC	Bik4	TTTGACCAAGATAGATGCC	RAPD-derived SCAR marker	35/35; 0/36	35
F. oxysporum f. sp. *basilici*	Bal3	GTCGAATGTCAAGGAAAGG-CTC	Bar3	CGGCTCATCAGTTGATGGTAC	RAPD-derived SCAR marker	11/11; 0/9	40[d]
F. oxysporum f. sp. *chrysantemi*	Mg5	GGGGTCGGTTACATGGGTG	Mg6	CAACAACAAGGCGAAGAGGG	*Fot1* transposon	9/9; 0/26	49
F. oxysporum f. sp. *chrysantemi*	Mg5	GGGGTCGGTTACATGGGTG	Mg6	CAACAACAAGGCGAAGAGGG	*Fot1* transposon	10/12; 0/5	50[e]
F. oxysporum f. sp. *ciceris* (wilt-inducing isolates)	Wilt-2	TATCAGAGCATCTCCCTCCC	Wilt-1	TGATGTGAGGACGGCCAGG	RAPD-derived SCAR marker	29/29; 0/50	39
F. oxysporum f. sp. *ciceris* (wilt-inducing isolates)	WiltNF-2	TTGTATGGCGTTGGAGAGGG	WiltNR-2	TTGTTCAGATCGGAATCGGG	RAPD-derived SCAR marker	4/4; 0/2	37
F. oxysporum f. sp. *ciceris*	Foc0-12f	GGCGTTTCGCAGCCTTACAA-TGAAG	Foc0-12r	GACTCCTTTTTCCCGAGGTAG-GTCAGAT	RAPD-derived SCAR marker	76/76; 0/93	38
F. oxysporum f. sp. *ciceris* race 0	FocR0-M15f	GGAGAGCAGGACAGCAAAG-ACTA	FocR0-M15r	GGAGAGCAGCTACCCTAGAT-ACACC	RAPD-derived SCAR marker	36/36; 0/133	38
F. oxysporum f. sp. *ciceris* race 1 B/C	FocR1B/C-N5f	GAGAGCAGGGTCAGCGTAG-ATAG	FocR1B/C-N5r	GCAGCAGAAGAGGAAGAAA-ATGTA	RAPD-derived SCAR marker	2/9; 0/160	38
F. oxysporum f. sp. *ciceris* race 5	FocR5-L10f	GGAAGCTTGGCATGACATAC	FocR5-L10r	AAGCTTGGGCACCCTCTT	RAPD-derived SCAR marker	10/10; 0/159	38
F. oxysporum f. sp. *ciceris* race 6	FocR6-O2f	GAGCAGTCAATGGCAATGG	FocR6-O2r	AGAGCAGGGTCAGCGTAGAT-A	RAPD-derived SCAR marker	13/13; 0/156	38
F. oxysporum f. sp. *ciceris* race 1A and 6	FocR6-P18f	GGAGAGCAGTAGAGTTACA-GCAGTATT	FocR0-M15r	GGAGAGCAGCTACCCTAGA-TACACC	RAPD-derived SCAR marker	16/16; 0/153	38
F. oxysporum f. sp. *cucumerinum*	FocF1	TCAACGGGACACTTTATGTTC	FocR2	TCAACGGGACTCCCTTCG	RAPD-derived SCAR marker	46/46; 2/227	15

Table 1. Continued

Target organism		Forward primer (5′-3′)		Reverse primer (5′-3′)	Target gene[b]	Specificity[c]	Reference
F. oxysporum f. sp. *dianthi* race 1 and 8	Ft3	GGCGATCTTGATTGTATTGT-GGTG	R8.1	CGATGAAGTCGGTTTGCGATT	*Fot1* transposon	29/29; 0/61	51
F. oxysporum f. sp. *dianthi* race 2	Ft3	GGCGATCTTGATTGTATTGT-GGTG	R2.1	CTTGTTTCTCGATTTCTGTCTC-ACG	*Fot1* transposon	22/22; 2/68	51
F. oxysporum f. sp. *dianthi* race 4	IMP2	AATCCTATAGAGAATCTGTGG	R4.2	GGTGATTGGAGGAGGAATACC	*Impala* transposon	19/19; 0/71	51
F. oxysporum f. sp. *gladioli* race 1	E	CAGCTCACGACCTGTAGT	F	CAGCTCACGATGGGAATC	RAPD-derived SCAR marker	33/36; 3/23	36
F. oxysporum f. sp. *lactucae* race 1	Hani3′	CCCTCCAAC ATTCAACAACTG	Hanilatt3rev	ATTCACTGTACACCAACCTTTT	Inter-retrotransposon-derived SCAR marker	69/69; 0/63	55
F. oxysporum f. sp. *lycopersici* (race 1, 2 and 3)	P12-F2	GTATCCCTCCGGATTTTGAGC	P12-R1	AATAGAGCCTGCAAAGCATG	Secreted in xylem 1 (*SIX1*)	4/4; 0/8[f]	64
F. oxysporum f. sp. *lycopersici* (race 2 and 3)	Sp23f	CCTCTTGTCTTTGTCTCACGA	Sp23r	GCAACAGGTCGTGGGGAAAA	Endo polygalacturonase (*pg1*)	16/16; 2/53	57
F. oxysporum f. sp. *phaseoli* (highly virulent isolates)	B310	CAGCCATTCATGGATGACAT-AACGAATTTC	A280	TATACCGGACGGGCGTAGTG-ACGATGG	RAPD-derived SCAR marker	15/15; 0/19	34
F. oxysporum f. sp. *radicis-cucumerinum*	ForcF1	GGTGACGCAGCAGTCTAGA	ForcR2	GTGACGCAGGGTAGGCAT	RAPD-derived SCAR marker	28/28; 0/245	15
F. oxysporum f. sp. *radicis-lycopersici*	sprlf	GATGGTGGAACGGTATGACC	sprlr	CCATCACACAAGAACACAGGA	Exo polygalacturonase (*pgx4*)	9/9; 1/60	57

[a] Unless otherwise indicated, PCR primers were used in conventional PCR assays.
[b] RAPD, random amplified polymorphic DNA; SCAR, sequence characterized amplified region.
[c] Specificity of the primers is represented by the ratio of correctly identified target strains versus the total number of tested target strains and by the ratio of wrongly identified strains versus the total number of non-target strains.
[d] Primers were combined with Taqman® probe 'Basil' (5′-CCAATTCTCCCGAGTGTATCAAGGA-3′) in a real-time PCR assay.
[e] Primers were combined with Taqman® probe 'Marge' (5′-TAAGCAGGCGAGTCCTTGATTTGAT-3′) in a real-time PCR assay.
[f] Specificity of the assay was recently confirmed by an extensive screening on 287 *F. oxysporum* isolates from various *formae speciales*. All of 77 *F. oxysporum* f. sp. *lycopersici* isolates were correctly identified. No cross-reaction with other isolates was observed (Lievens *et al*., unpublished results).

2.2 Transposons: sequence-biased targets for molecular identification of pathogenic strains

Transposons are discrete DNA segments that are able to jump or replicate to other locations within a genome. They are ubiquitous in virtually all organisms examined, and are a common cause of spontaneous genetic changes that can affect the biology of the organism.[43] Although the genome of *F. oxysporum* is still largely uncharacterized, at least 5% is estimated to be composed of transposons.[44] Among these are both class-I and class-II transposons, the first mobilizing via retroposition (via an RNA intermediate) and the second via a DNA cut-and-paste mechanism.[45,46] Class-II transposons are reported to play an important role in the evolution of fungal genomes[47] and have been used for various research purposes, including knockout mutagenesis and variability analysis.[46] In some cases, pathogenicity of *F. oxysporum* isolates could be linked to the presence of certain transposons. For example, a specific insertion of the *Fot1* transposon has been exploited to develop specific markers for *F. oxysporum* f. sp. *albedinis*,[48] as well as for a new VCG of *F. oxysporum* f. sp. *chrysanthemi* highly pathogenic on Paris daisy (VCG 0052).[49,50] In addition, this transposon provided the source of target sequences to discriminate certain races of *F. oxyporum dianthi* (races 1, 2 and 8),[51] whereas a copy of the transposon *Impala* was used to identify race 4 strains of this *forma specialis*.[51] Furthermore, the transposable element *Palm* displayed suitable variability for population analysis of *F. oxysporum* f. sp. *elaeidis*.[52] Apart from these class-II transposons, class-I transposons such as *Foxy* have also been proposed for the discrimination of *F. oxysporum formae speciales* and races.[26,53] This abundant retrotransposon (200–300 copies per genome) was used to construct the first mitotic linkage map of *F. oxysporum*.[54] In addition, the genomic regions between the insertions of long terminal repeat retrotransposon copies were used to develop a diagnostic assay for *F. oxysporum* f. sp. *lactucae* race 1 strains based on inter-retrotransposon amplified polymorphisms.[55]

Because class-II transposons can move around in the genome through complete excision,[46] inactivity of the transposable element should be verified in order to be used as a molecular marker for reliable pathogen diagnosis.[15,48,51] Analysis of a large collection of strains, preferably isolated from different geographic areas and at different time points, should reduce the risk of selecting an instable transposon.[51] Apart from this, incomplete copies, either truncated or containing internal deletions, are often inactive.[56]

2.3 VIRULENCE GENES: IDEAL TARGETS FOR MOLECULAR IDENTIFICATION

Virulence of a plant pathogen may be attributed to subtle nucleotide differences in a specific gene.[57,58] Alternatively, virulence of a pathogen may be determined by the unique presence of a specific gene or a set of genes that confer a specific trait to the pathogen, such as the production of a host-specific toxin.[59,60] Pathogenicity of *Nectria haematococca* Berk. & Broome [anamorph *Fusarium solani* (Mart.) Sacc.] mating population VI, which is pathogenic to garden pea, depends on a set of virulence genes that include the *pea pathogenicity* (*PEP*)*1*, *PEP2*, and *PEP5* genes, and the *pisatin demethylase* (*PDA*)*1* gene, which are all present on a conditionally dispensable chromosome.[61] These genes are absent in related species, except in *Neocosmospora boniensis* and some *formae speciales* of *F. oxysporum*.[62] In *N. boniensis*, homologues of all genes were found, whereas *PDA1* was present in only some isolates of *F. oxysporum*. Further analysis revealed that *N. boniensis* strains and one of the *PDA1*-containing *F. oxysporum* isolates were indeed able to infect pea,[62] suggesting a link between the presence of *PDA1* and virulence on pea.

A similar observation has been reported for *F. oxysporum* f. sp. *lycopersici*. In this polyphyletic *forma specialis*, the *secreted in xylem* (*SIX*)*1* gene, encoding a small cysteine-rich secreted protein, contributes to full virulence on tomato.[63,64] Apart from SIX1, additional fungal proteins were identified from xylem sap of infected tomato plants, of which most of the corresponding (*SIX*) genes are present on the same chromosome.[60,65] The unique presence of these genes in *F. oxysporum* f. sp. *lycopersici* was recently determined when screening 287 *F. oxysporum* isolates from various *formae speciales* (Lievens *et al.*, unpublished results). Most of the *SIX* genes were found to be present in all isolates that belong to the *forma specialis lycopersici* irrespective of race and clonal lineage, but not in other *formae speciales* or non-pathogenic isolates[63,64] (Lievens *et al.*, unpublished results). The strong link between this group of genes and pathogenicity on tomato makes them excellent markers for host-specific pathogenicity.

The presence of this gene cluster also sheds new light on the possible origin of host specificity. Since *F. oxysporum* f. sp. *lycopersici* does not have a monophyletic origin,[17] the ability to cause tomato wilt disease must have evolved multiple times. The finding that all *F. oxysporum* f. sp. *lycopersici* isolates, but not those that belong to closely related subspecies, contain most of the *SIX* genes that are located on the same chromosome suggests spread of this chromosome from one clonal line to another, possibly through horizontal gene transfer.[60] The importance of this mechanism for the exchange of virulence traits between plant pathogenic fungi has recently been demonstrated.[59] In addition, the fact that the *SIX* genes are absent in closely related subspecies suggests that they are dispensable for saprophytic growth and for infection of other hosts.[64] Furthermore, selection against such virulence genes may occur as well, especially since virulence factors are often secreted proteins that are known for their predisposition to trigger resistance in plants through gene-for-gene recognition mechanisms.[66] Indeed, SIX1 is the

avirulence factor that matches the *I-3* resistance gene in tomato.[64] Because the same protein can act as a virulence factor in one host plant and as an avirulence factor in another,[66,67] not only host cultivar-specific disease resistance but possibly also resistance of non-host plants can be achieved by recognition of secreted proteins.

The clustering of genes involved in virulence on a specific host resembles the situation that occurs with pathogenicity islands, chromosomal regions that typically contain a high number of repetitive elements interspersed between virulence genes.[61] Indeed, the *F. oxysporum* f. sp. *lycopersici* chromosome containing the *SIX* genes is likewise found to be rich in transposon sequences,[64] which may explain why specific transposons are linked to pathogenicity. This also implies that transposon-based markers can be used to identify a polyphyletic *forma specialis* if they are localized on such 'pathogenicity chromosomes'.

3 CONCLUDING REMARKS

Although molecular methods provide many advantages over traditional plant pathogen detection and identification methods,[23] nucleic acid-based methods are not yet widely implemented for routine plant pathogen diagnosis. Several reasons may explain this slow uptake, including the lack of pathogen quantification and multiplexing capabilities of most assays.[25,68] However, increasingly novel molecular methods are being developed that can meet these demands, of which real-time PCR and DNA array technology are currently the most suitable accurately to quantify pathogen densities and detect a large variety of microorganisms respectively.[23]

As discussed in this review, another important limitation in molecular plant pathogen diagnosis is the inability to differentiate pathogenic from non-pathogenic strains that belong to the same microbial species. This applies not only to *F. oxysporum* but also to fungi like *F. solani* (Martius) Sacc. and *Rhizoctonia solani* Kuhn.[24] As long as no molecular markers are available to discriminate pathogenic subspecies, pathogenicity tests remain the only means to determine whether or not a given isolate is pathogenic on a specific crop. The increasing number of studies reporting the successful development of molecular markers to discriminate *F. oxysporum* strains below the species level (Table 1) is therefore highly important. A DNA array containing genus-, species- and *forma specialis*-specific detector oligonucleotides has recently been developed for the detection and identification of *F. oxysporum* f. sp. *cucumerinum* and *F. oxysporum* f. sp. *radicis-cucumerinum*.[15] While the genus- and species-specific oligonucleotides were derived from the ubiquitous ribosomal RNA gene cluster, robust RAPD-derived SCAR markers were implemented in the assay to specifically identify the different *formae speciales*. Taking into account the almost unlimited expanding possibilities of DNA arrays, a comprehensive DNA array for the identification of all *formae speciales* (and possibly even races) of *F. oxysporum* may ultimately be realized. In addition, since hybridization signals are proportional to the quantity of target DNA,[69] this technique is suitable for decision-making in plant disease management.

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