Protection to Tomato Wilt Disease Conferred by the Nonpathogen *Fusarium oxysporum* Fo47 is More Effective Than that Conferred by Avirulent Strains

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ABSTRACT

Although the vascular pathogen *Fusarium oxysporum* is notorious for being the causal agent of Fusarium wilt disease, the vast majority of *F. oxysporum* strains are harmless soil and root colonizers. The latter *F. oxysporum*'s are often endophytes colonizing roots intracellularly without negatively affecting plant fitness. Actually, most of them, like Fo47, are beneficial providing biological control to various root pathogens. Interestingly, also pathogenic *F. oxysporum* inoculated on a resistant host (i.e., avirulent *F. oxysporum* f. sp. *lycopersici*) can reduce susceptibility to virulent *F. oxysporum* strains via a mechanism called “cross protection.” It has been hypothesized that cross protection is based on activation of a resistance protein of the host upon recognition of a cognate avirulence (Avr) protein of the pathogen. Currently, it is unknown whether the biocontrol activity of *F. oxysporum* endophytes utilizes

similar mechanisms as cross protection conferred by avirulent pathogens, and whether both provide a quantitative similar level of protection. Here, we show that in tomato biocontrol activity of the Fo47 endophyte to the pathogen *F. oxysporum* f. sp. *lycopersici* is more effective than cross protection induced by avirulent *F. oxysporum* f. sp. *lycopersici* strains activating either I, I-2, or both resistance proteins upon recognition of Avr1 or the Avr2/Six5 pair, respectively. These findings imply that cross protection and biological control utilize different mechanisms to reduce susceptibility of the host to subsequent infections.

Keywords: *Fusarium oxysporum*, biological control, cross protection, disease control and pest management, endophyte-mediated resistance, genetics and resistance, tomato

Fusarium oxysporum, a filamentous ascomycete, is the causal agent of Fusarium wilt disease (Michielse and Rep 2009). This soilborne vascular fungus ranks among the top 10 major fungal plant pathogens (Dean et al. 2012). Each *F. oxysporum* pathogen harbors a specific set of effector genes required for pathogenicity on a specific host (van Dam et al. 2016). Nevertheless, the ample majority of *F. oxysporum* strains are nonpathogenic saprotrophs able to endophytically colonize plant roots (Bao et al. 2004). It has been well established that wilt disease-suppressive soils contain beneficial *F. oxysporum* endophytes that are responsible for protecting a susceptible host to pathogenic *F. oxysporum* strains (Alabouvette 1986; Tamietti et al. 1993). These *F. oxysporum* endophytes exert biological control by directly affecting the invading pathogen and/or by inducing plant immune responses. The latter response, also referred to as endophyte-mediated resistance (EMR), results in induction of a systemic immune response halting a wide variety of root-infecting pathogens, but typically not shoot-infecting pathogens (de Lamo and Takken 2020). EMR appears distinct from the well-established systemic acquired resistance (SAR) and induced systemic resistance (ISR) responses as, at least in tomato (*Solanum lycopersicum*), it is independent of the defense hormones salicylic acid, jasmonate, and ethylene (Constantin et al. 2019). Furthermore, whereas EMR and ISR are typically induced by nonpathogenic root colonizers, SAR can be triggered in incompatible interactions in which an avirulent pathogen infects a resistant host. Infection by a pathogen producing

an avirulence (Avr) protein that is perceived by a matching host resistance (R) protein results in activation of effector triggered immunity (ETI) (Pieterse et al. 2014). Resistant plants infected with such an avirulent *F. oxysporum* pathogen show a reduction in disease symptoms upon co-, or subsequent, inoculation with a virulent *F. oxysporum* strain (Biles and Martyn 1989; Huertas-Gonzalez et al. 1999). The observed resistance response, called cross protection, has been found to be effective in reducing Fusarium wilt symptoms in crops such as watermelon co-inoculated with an avirulent and a virulent *F. oxysporum* f. sp. *niveum* (pathogen of watermelon) isolate (Biles and Martyn 1989). Likewise, co-inoculation of tomato with a virulent and an avirulent *F. oxysporum* f. sp. *lycopersici* race 1 (pathogen of tomato) isolate resulted in cross protection (Huertas-Gonzalez et al. 1999). In the latter study, cross protection was also observed in melon upon co-inoculation with virulent and avirulent strains of *F. oxysporum* f. sp. *melonis* (pathogen of melon) (Huertas-Gonzalez et al. 1999).

Whereas endophytic *F. oxysporum* strains trigger EMR, cross protection induced by avirulent *F. oxysporum* strains could be a combination of EMR and ETI-triggered SAR, or only involve the latter. To study whether EMR- and cross protection-signaling pathways interact and affect each other, we compared cross protection induced by avirulent *F. oxysporum* pathogens with biological control exerted by *F. oxysporum* endophytes. Thereto, we used tomato cultivar Motelle carrying the R genes *I* and *I-2* that confer resistance to *F. oxysporum* f. sp. *lycopersici* races 1 and 2 (Laterrot 1993; Takken and Rep 2010). *I* encodes a receptor-like protein (RLP) detecting the presence of extracellular *F. oxysporum* f. sp. *lycopersici* Avr1 (Catanzariti et al. 2017), while *I-2* is a nucleotide-binding leucine-rich-repeat-type receptor (NLR) detecting intracellular Avr2 that requires Six5 for its translocation through the host symplast and perception by *I-2* (Cao et al. 2018; Houterman et al. 2009; Ma et al. 2015; Simons et al. 1998). A *F. oxysporum* f. sp. *lycopersici* isolate carrying only Avr2 or Six5 is therefore virulent on *I-2* containing tomato varieties (Ma et al. 2015). The use of the Motelle cultivar allows us to quantify and compare the

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level of wilt disease resistance induced by either endophytic *F. oxysporum* (=EMR) or avirulent *F. oxysporum* f. sp. *lycopersici* strains triggering either I- or I-2-mediated immunity (=ETI-triggered SAR and maybe EMR).

By using various host–pathogen combinations, we examined whether cross protection triggered by the RLP I is equivalent to, or different from, that activated by the NLR I-2. The following four avirulent *F. oxysporum* f. sp. *lycopersici* strains were used to activate I and/or I-2: Fol004 (race 1 carrying *Avr1* and *Avr2/Six5*), Fol004Δ*Six5* (race 1 carries *Avr1* and *Avr2* but lacks *Six5*), Fol004Δ*Avr1* (race 2 carries *Avr2/Six5* but not *Avr1*), and Fol007 (race 2 carrying *Avr2/Six5* but not *Avr1*) (Table 1). As virulent control Fol029 (race 3) lacks *Avr1* and carries a single amino acid substitution in the *Avr2* protein allowing it to evade I and I-2 mediated recognition (Houterman et al. 2009). By using tomato cultivar Motelle (carrying I and I-2), these strains allow us to study whether cross protection against the virulent race 3 Fol029 isolate triggered by the I cell surface immune receptor is quantitatively different from that triggered by the intracellular I-2 receptor.

As expected, bipartite interactions of tomato cultivar Motelle with either avirulent *F. oxysporum* f. sp. *lycopersici* pathogens or with Fo47 (Fig. 1A, upper row panels) did not result in disease symptoms, such as a reduction of plant fresh weight (FW) compared with the mock (water-inoculated) control (Fig. 1B). In contrast, and as anticipated, inoculation with the virulent Fol029 (race 3) strain resulted in severe disease symptoms such as stunting and wilting (Fig. 1A, bottom left panel), concomitant with a strongly reduced FW of the infected plants (Fig. 1B). The disease index (DI) of tomato inoculated with Fol029 differed significantly from seedlings inoculated with either of the four avirulent *F. oxysporum* f. sp. *lycopersici* pathogens or Fo47 (Fig. 1C). These results are as predicted based on the combination of plant and fungal genotypes employed; no disease symptoms occurred when plants were inoculated with avirulent *F. oxysporum* f. sp. *lycopersici* strains while the virulent strain caused disease.

The virulent Fol029 strain was subsequently co-inoculated with each of the four avirulent *F. oxysporum* f. sp. *lycopersici* strains separately to assess their potential to induce cross protection and reduce disease symptoms caused by the pathogen. When all treatments are compared using a Kruskal-Wallis test (Dunn's multiple comparison test), then plant weight following all inoculation involving Fol029 differed significantly ($P < 0.0001$) from the mock, except when Fol029 was co-inoculated with Fo47 (47:029). The nonsignificant P value ($P = 0.06$) for the latter pair shows that Fo47 confers protection against the pathogenic Fol029 strain as plant weight did not significantly differ from the mock. Notably, in single comparisons (using a Mann-Whitney test), cross protection was triggered in tomato by each of the avirulent strains, as co-inoculation with Fol029 led to a significant higher FW (Fig. 1B) and a lower DI (Fig. 1C) than tomato plants infected solely with Fol029.

No differences in the extent of cross protection were found when Fol029 was co-inoculated with any of the avirulent *F. oxysporum* f. sp. *lycopersici* strains that activate either I or I-2 (Fig. 1) as no significant differences were found in FW and DI (Fig. 1B and C, compare treatments 004:029, 004Δ*Six5*:029, 004Δ*Avr1*:029, and 007:029-). As I has been reported to restrict host colonization by race 1 *F. oxysporum* f. sp. *lycopersici* strains to a higher extent than I-2 halting race 2 isolates (van der Does et al. 2018), one would anticipate I to confer a stronger containment toward Fol029 than I-2. Indeed, some tomato plants inoculated with either Fol004Δ*Avr1* or Fol007 (both races 2 only triggering I-2) exhibited brown vessels at stem cross sections at the height of the cotyledons indicating the presence of *F. oxysporum* f. sp. *lycopersici* (i.e., DI 2, Fig. 1C), while this was not observed in seedlings inoculated with the I-activating race 1 Fol004Δ*Six5* strain. Notwithstanding this difference, susceptibility to Fol029 was equally reduced following either I or I-2 activation upon co-inoculation as DI and FW were not significantly different between these bioassays.

Co-inoculation of Fol004, which carries *Avr1* and *Avr2/Six5*, with Fol029 allowed us to check whether cross protection induced by I and I-2 is additive and confers stronger protection than that induced by a single R protein. Comparing FW and DI of Fol004:Fol029 co-inoculated plants (Fol004 activates I and I-2) with plants co-inoculated with Fol029 and Fol004Δ*Six5* (activate solely I) or with Fol004Δ*Avr1* or Fol007 (both only activate I-2), showed that simultaneous activation of both I and I-2 does not result in enhanced disease reduction compared with co-inoculation with a strain that activates a single immune receptor (Fig. 1, compare treatment 004:029 with 004Δ*Six5*:029, 004Δ*Avr1*:029, and 007:029-). The non-additive nature of the response could indicate that both receptors alone can trigger the maximal cross protection response. Alternatively, if different responses are triggered, they are apparently not additive.

To test whether the endophyte Fo47 confers EMR via a defense mechanism similar as used in cross protection, the extent of disease reduction in tomato plants co-inoculated with Fo47 or an avirulent *F. oxysporum* f. sp. *lycopersici* strain with the Fol029 isolate was compared. The FW of Fo47:Fol029 co-inoculated plants was significantly higher than of plants inoculated solely with Fol029 (Fig. 1B), and not different from the mock as mentioned before. In correspondence, the DI of the co-inoculated plants was lower than that of Fol029-inoculated tomato (Fig. 1C). These data confirm that Fo47-based EMR also reduces susceptibility to Fol029 in this tomato cultivar. Notably, the FW of plants co-inoculated with Fo47:Fol029 was at least fivefold higher than of plants co-inoculated with any of the avirulent pathogenic strains and Fol029 (Fig. 1B, brown comparison). In correspondence, the DI in less than half of the Fo47:Fol029-co-inoculated tomato reached a DI of 4, whereas the majority of the plants co-inoculated with the avirulent *F. oxysporum* f. sp. *lycopersici* strains showed a DI of at least 4 (Fig. 1C, brown

TABLE 1. *Fusarium oxysporum* strains that were used to (co)inoculate tomato roots

Fungal strain	Tomato pathogen	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> race	Relevant genotype	Avirulent in plants containing the following	Reference
Fo47	No	–	–	–	(Alabouvette 1986)
Fol004	Yes	1	<i>Avr1</i> <i>Avr2/Six5</i>	<i>III-2</i>	(Rep et al. 2005)
Fol004Δ <i>Six5</i>	Yes	1	<i>Avr1</i> <i>Avr2/–</i>	<i>II–</i>	(Ma et al. 2015)
Fol004Δ <i>Avr1</i>	Yes	1 ^a	– <i>Avr2/Six5</i>	<i>–II-2</i>	(Houterman et al. 2008)
Fol007	Yes	2	– <i>Avr2/Six5</i>	<i>–II-2</i>	(Rep et al. 2005)
Fol029	Yes	3	– <i>Avr2^b/Six5</i>	–	(Rep et al. 2005)

^a Deletion of *Avr1* converts this race 1 strain into a race 2 isolate.

^b *Avr2* of race 3 *F. oxysporum* f. sp. *lycopersici* harbors a mutation that prevents detection by I-2 but retains the virulence function.

A

Strain:	(Mock)	Fo47	Fol004	Fol004 Δ Six5	Fol004 Δ Avr1	Fol007
Avr1:	-	-	+	+	-	-
Avr2/ Six5:	-	-	+	-	+	+

B **C**

Fig. 1. Co-inoculation of Fo47 with the virulent Fol029 pathogen results in less severe disease symptoms than co-inoculation of Fol029 with avirulent strains. **A**, Ten-day-old seedlings of tomato cultivar Motelle (carries *I* and *I-2*) were root dip-inoculated for 5 min with either Fo47, Fol004, Fol004 Δ Six5, Fol004 Δ Avr1, Fol007, and virulent Fol029, or co-inoculated with Fol029 and each of the previously-mentioned strains in a 1:1 ratio using 10^7 spores/ml (de Lamo et al. 2018; Di et al. 2016). The heading above the photographs (taken 3 weeks postinoculation [wpi]) depicts presence (+) or absence (–) of the cognate *Fusarium oxysporum* f. sp. *lycopersici* effectors (Avr1 and Avr2/Six5, respectively) triggering *I* or *I-2*, respectively. **B**, Fresh weight and **C**, disease index (DI) of inoculated plants were scored 3 wpi. Colors depict the different DIs as indicated in the legend on the right, ranging from 0 (green) being not diseased to 5 (aubergine) plants being dead (de Lamo et al. 2018). The experiment was repeated twice using 20 plants per inoculation and similar results were obtained. Plants were grown in soil within a climate-controlled greenhouse at 25°C under a relative humidity of 65% and with a photoperiod of 16 h. Microconidial spores were isolated from a 5-day-old shake-incubated (150 rpm) culture grown in the dark at room temperature in minimal medium (de Lamo et al. 2018; Di et al. 2016). Data were analyzed using a Mann-Whitney test where * indicates *P* value < 0.05 using PRISM 7.0 (GraphPad). The purple line shows single comparisons of every co-inoculated treatment versus solely Fol029 treatment, while the brown line compares Fo47:Fol029 co-inoculation with every single co-inoculated treatment where avirulent *F. oxysporum* f. sp. *lycopersici* strains were employed.

comparison). Taken together, both Fo47-based EMR and cross protection reduce wilt disease symptoms. The reduction of disease symptoms upon co-inoculation with the pathogenic FoI029 strain, however, was much more prominent for the Fo47 endophyte than for the avirulent *F. oxysporum* f. sp. *lycopersici* isolates.

Based on previous studies, an overall higher level of cross protection was anticipated than the one observed in the current study (Fig. 1). For instance, avirulent *F. oxysporum* f. sp. *niveum* reduces watermelon susceptibility to virulent *F. oxysporum* f. sp. *niveum* (Biles and Martyn 1989) by at least twofold while co-inoculations of tomato with avirulent and virulent *F. oxysporum* f. sp. *lycopersici* reduces symptom development by 30% (Huertas-Gonzalez et al. 1999). In the latter case, even co-inoculation ratios 0.1:1 of avirulent/virulent *F. oxysporum* f. sp. *lycopersici* resulted in a significant disease reduction. We had therefore expected avirulent *F. oxysporum* f. sp. *lycopersici* strains to trigger a stronger reduction of susceptibility than the endophyte, as they not only trigger ETI, but possibly also EMR following root colonization. However, since cross protection is much weaker than EMR, either EMR is not triggered by avirulent strains or it is to a much lower extent not able to significantly contribute to resistance.

One possible explanation for the poor protection conferred by avirulent *F. oxysporum* f. sp. *lycopersici* pathogens might be their ability to compromise (parts of) the tomato immune system using their host-specific effectors (Di et al. 2016, 2017; Gawehns et al. 2014; van Dam et al. 2016). Absence of these effectors in Fo47 explains its inability to cause disease in tomato (van Dam et al. 2016), as transfer of the *F. oxysporum* f. sp. *lycopersici* pathogenicity chromosome to Fo47 can turn it into a pathogen (Ma et al. 2010; van Dam et al. 2017). The observation that stem colonization by Fo47 is increased in the presence of *F. oxysporum* f. sp. *lycopersici* (Constantin et al. 2020) supports the idea that, unlike Fo47, avirulent pathogens compromise immunity notwithstanding their inability to cause disease. Indeed, pathogenic *F. oxysporum* strains suppress early host responses like reactive oxygen species accumulation and host cell death more effectively than Fo47 (de Lamo and Takken 2020; Humbert et al. 2015; Olivain et al. 2003). In summary, compared with the endophyte Fo47, avirulent pathogens only weakly reduce susceptibility to *F. oxysporum* f. sp. *lycopersici*. A possible explanation for this observation is that the Fo47 endophyte is less, or even unable to suppress host immune responses. Presumably, once immune responses are triggered by an endophyte they cannot be suppressed by the effectors present in a virulent strain, or it is to a much lower extent not able to significantly contribute to resistance.

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