Brothers in arms

Fusarium oxysporum vs. Fusarium oxysporum

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CHAPTER 4

Protection to wilt disease conferred by non-pathogenic *Fusarium* strains is stronger than that conferred by avirulent strains

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Abstract

Although the vascular pathogen *Fusarium oxysporum* (Fo) is notorious for being the causal agent of Fusarium wilt disease, the vast majority of Fo strains are harmless soil and root colonisers. The latter Fo's are often endophytes colonising roots without negatively affecting plant fitness. Actually, some of them, like Fo47, are beneficial for the plant providing biocontrol to various root pathogens. The ability to exert biocontrol to wilt diseases appears to be a generic feature of Fo. Interestingly, also avirulent Fo inoculated on a resistant host can reduce susceptibility of a plant to virulent Fo strains via a mechanism called "cross protection". It has been hypothesised that cross protection is based on the activation of resistance proteins of the host upon recognition of a cognate Avirulence protein of the pathogenic strain. Currently, it is unknown whether biocontrol exerted by Fo 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 exerted by the endophytic strain Fo47 to the pathogen Fo f.sp. *lycopersici* (Fol) is more effective than cross protection induced by avirulent Fol strains activating either I, I-2 or both resistance proteins. These findings imply that cross protection and biocontrol utilize different mechanisms to reduce susceptibility of the host to subsequent infections.

Introduction

Fusarium oxysporum (Fo), a filamentous ascomycete, is the causal agent of Fusarium wilt disease (Michielse and Rep 2009). This soil-borne vascular fungus ranks among the top 10 major fungal plant pathogens (Dean et al. 2012). Each Fo pathogen harbours a specific set of effector genes required for pathogenicity on a specific host (van Dam et al. 2016). Nevertheless, the ample majority of Fo strains are saprotrophs able to colonise plant roots endophytically (Bao et al. 2004). It has been well established that wilt disease-suppressive soils host beneficial Fo endophytes that are responsible for protecting a susceptible host to pathogenic Fo strains (Alabouvette 1986; Tamietti et al. 1993). These Fo endophytes exert biocontrol by directly affecting the invading pathogen, or by inducing plant immune responses. The latter, also referred to as endophyte-mediated resistance (EMR), results in induction of a systemic immune response halting a wide variety of root-, 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 defence hormones salicylic acid, jasmonate and ethylene (Constantin et al. 2019). Furthermore, whereas EMR and ISR are typically induced by non-pathogenic root colonisers, SAR can be triggered by an avirulent pathogen producing an avirulence protein whose presence or action is perceived by a host resistance (R) protein resulting in the activation of effector triggered immunity (ETI) (Pieterse et al. 2014). Plants infected with an avirulent Fo pathogen show a reduction in disease symptoms upon subsequent- or co-inoculation with a virulent Fo 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 a crop such as watermelon co-inoculated with an avirulent and a virulent Fo f.sp. *niveum* (Fon) (pathogen of watermelon) isolate (Biles and Martyn 1989). Likewise, co-inoculation of tomato with a virulent and an avirulent Fo f.sp. *lycopersici* (Fol) 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 Fo f.sp. *melonis* (Fom) (pathogen of melon) (Huertas-Gonzalez et al. 1999).

Whereas endophytic Fo strains trigger EMR, avirulent Fo strains induce cross protection that could be a combination of EMR and ETI-triggered SAR, or involve only the latter response. To study whether EMR- and cross protection-signalling pathways interact and affect each other, we compared cross protection induced by avirulent Fo pathogens with biocontrol exerted by Fo endophytes. Thereto, we used tomato cultivar Motelle carrying the *R* genes *I* and *I-2* that confer resistance to Fol races 1 and 2 (Takken and Rep 2010). *I* encodes a receptor-like protein (RLP) detecting the presence of extracellular Fol Avr1 (Catanzariti et al. 2017), while I-2 is a nucleotide-binding leucine-rich-repeat-type receptor (NLR) detecting intracellular Avr2 (Houterman et al. 2009; Simons et al. 1998). The use of this cultivar allows us to quantify and compare the level of wilt-disease resistance induced by either endophytic Fo (=EMR) or avirulent Fol strains triggering either *I* or *I-2* -mediated immunity (=ETI-triggered SAR and maybe EMR). We observed that cross protection triggered by avirulent Fol strains by activation of I or I-2 similarly reduced susceptibility to Fol. Notably, a race 1 strain activating both *I*- and *I-2*-mediated resistance did not trigger a quantitatively stronger

cross protection than strains activating a single resistance protein. EMR triggered by Fo47 resulted in a stronger protection to wilt disease than cross protection.

Results & Discussion

Activation of either I or I-2 confers similar, not additive levels of cross protection towards Fusarium wilt disease

By using various host-pathogen combinations we wanted to examine whether cross protection triggered by the RLP I is equivalent to, or different from, that activated by the NLR I-2. Whereas I perceives Avr1, activation of the I-2 immune receptor requires the combined presence of two effector proteins, notably Avr2 and Six5 (Ma et al. 2015). A Fol isolate carrying only *Avr2* or *Six5* is therefore virulent on *I-2* containing tomato varieties. The following four avirulent Fol 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) was used that lacks Avr1 and carries a single aminoacidic substitution in the Avr2 protein allowing it to evade I and I-2 mediated recognition (Houterman et al. 2009). By using tomato cv. 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, bi-partite interactions of tomato cv. Motelle with either avirulent Fol pathogens or with Fo47 (Fig. 1A – upper row panels) did not result in disease symptoms, such as a reduction of plant fresh weight (FW), as compared to 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 Fol pathogens and 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 Fol strains while the virulent strain caused disease.

The virulent Fol029 strain was subsequently co-inoculated with each of the four avirulent Fol strains separately to assess their potential to induce cross protection and reduce disease symptoms caused by the pathogen. Cross protection was triggered in tomato by each of the avirulent strains, as co-inoculation with Fol029 led to a 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 Fol strains that activate either I or I-2 (Fig. 1). No significant differences were found in FW and DI (Fig. 1B and 1C -compare treatments 004:029, 004Δ*Six5*:029, 004Δ*Avr1*:029 and 007:029-). As I has been reported to restrict host colonisation by race 1 Fol strains to a higher extent than I-2 halts race 2 isolates (van der Does et al. 2018), one would anticipate I to confer a stronger

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

Fungal strain	Tomato pathogen	Fol race	Relevant genotype	Avirulent in plants containing:	Reference
Fo47	No	-	-	-	(Alabouvette 1986)
Fol004	Yes	1	*Avr1 Avr2/Six5*	*I / I-2*	(Rep et al. 2005)
Fol004*ΔSix5*	Yes	1	*Avr1 Avr2/-*	*I* / -	(Ma et al. 2015)
Fol004*ΔAvr1*	Yes	1[a]	- *Avr2/Six5*	- / *I-2*	(Houterman et al. 2008)
Fol007	Yes	2	- *Avr2/Six5*	- / *I-2*	(Rep et al. 2005)
Fol029	Yes	3	- *Avr2*[b]*/Six5*	-	(Rep et al. 2005)

[a] Deletion of Avr1 converts this race 1 strain into a race 2 isolate.
[b]Avr2 of race 3 Fol harbours a mutation that prevents detection by I-2 but retains the virulence function.

containment toward Fol029 than I-2. We indeed confirmed that some tomato plants inoculated with either Fol004Δ*Avr1* or Fol007 (both races 2 only triggering I-2) had brown vessels at the cotyledon level (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 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 as compared to 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.

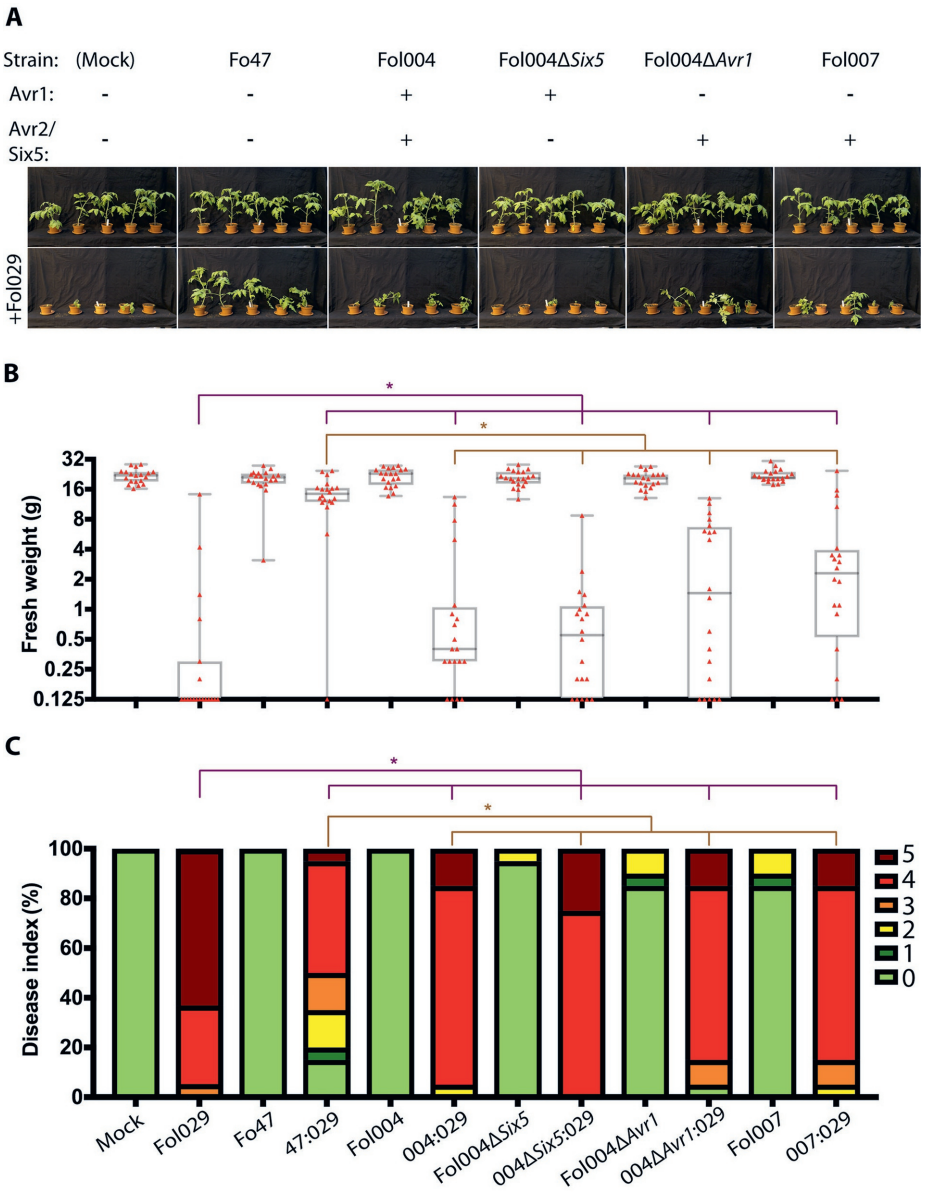

Figure 1. Co-inoculation of Fo47 with the virulent Fol029 pathogen results in less severe disease symptoms than co-inoculation of Fol029 with avirulent strains. **(A)**, Ten-days-old seedlings of tomato cv. Motelle (carries *I* and *I-2*) were inoculated with either Fo47, Fol004, Fol004Δ*Six5*, Fol004Δ*Avr1*, Fol007 and virulent Fol029, or co-inoculated with Fol029 and each of the previously-mentioned strains. The heading above the photographs (taken three weeks-post-inoculation) depicts presence (+) or absence (-) of the cognate Fol effectors (Avr1 and Avr2/Six5 respectively) triggering I or I-2, respectively. **(B)**, FW and **(C)**, DI of inoculated plants (See Materials and Methods). The experiment was repeated twice using 20 plants per inoculation and similar results were obtained. Data was analysed using a Mann-Whitney test where **Pval* < 0.05.

Endophyte-mediated resistance confers stronger protection against wilt disease than cross protection

To test whether the endophyte Fo47 confers EMR via a defence mechanism similar as used in cross protection, the extent of disease reduction in tomato plants co-inoculated with Fo47 or an avirulent Fol 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). 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 five-fold 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 Fol strains showed a DI of at least 4 (Fig. 1C, brown 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 Fol029 strain, however, was much more prominent for the Fo47 endophyte than for the avirulent Fol 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 Fo f.sp. *niveum* (Fon) reduces watermelon susceptibility to virulent Fon (Biles and Martyn 1989) by at least 2-fold while co-inoculations of tomato with avirulent and virulent Fol reduces symptom development by 30% (Huertas-Gonzalez et al. 1999). In the latter case, even co-inoculation ratios 0.1:1 of avirulent:virulent Fol resulted in a significant disease reduction. We had therefore expected avirulent Fol strains to trigger a higher reduction of susceptibility than the endophyte, as they not only trigger ETI, but possibly also EMR following root colonisation. However, since cross protection is much weaker than EMR, either EMR is not triggered by avirulent strains, or it is too a much lower extent not being able to significantly contribute to resistance.

One possible explanation for the poor protection conferred by avirulent Fol pathogens might be their ability to compromise (parts of) the tomato immune system using their host-specific effectors (Di et al. 2017; Di et al. 2016; Gawehns et al. 2014; van Dam et al. 2016). Absence of these effectors in Fo47 explain its inability to cause disease in tomato (van Dam et al. 2016), since transfer of the Fol pathogenicity chromosome to Fo47 can turn it into a pathogen (Ma et al. 2010; van Dam et al. 2017). The observation that stem colonisation by Fo47 is increased in the presence of Fol (Constantin et al. 2020), supports the idea that, unlike Fo47, avirulent pathogens compromise immunity notwithstanding their inability to cause disease. Indeed, pathogenic Fo strains suppress early host responses like ROS 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 to the endophyte Fo47, avirulent pathogens only weakly reduce susceptibility to Fol possibly because the endophyte is less- or even unable to suppress host immune responses. Presumably, once immune

responses are triggered by an endophyte they can no longer be suppressed by effectors present in a virulent strain.

Materials and Methods

Plant and fungal materials and cultivation conditions

Tomato cultivar (cv.) Motelle was used in this study. This variety carries the *R* genes *I* and *I-2* (Laterrot 1993) conferring resistance to Fol race 1 and 2 respectively. The Fo strains employed are depicted in Table 1. In brief, Fo47 was selected as a biocontrol-exerting endophyte and different tomato Fol race 1 and race 2 strains activating I and/or I-2-mediated immunity were selected (Table 1). Plants were grown in a climate-controlled greenhouse at 25 ºC under a relative humidity of 65% and with a photoperiod of 16 h.

Fusarium inoculation assays

The Fo strains depicted in Table 1 were inoculated from glycerol stocks to potato dextrose agar (PDA) plates. After at least five days of cultivation at 25 ºC in darkness, agar plugs were used to inoculate 100 ml minimal medium (0.17 % Yeast Nitrogen Base without amino acids or ammonium sulphate, 3 % sucrose and 100 mM KNO3). Liquid cultures were shake-incubated in the dark for five days at 150 rpm and filtered through Miracloth (Calbiochem). The resulting microconidial suspension was diluted to 10^7 spores/ml (de Lamo et al. 2018; Di et al. 2016). Co-inoculum of the different Fo endophyte/avirulent strains was prepared in a 1:1 ratio. Ten-days-old tomato cv. Motelle seedlings were uprooted and roots were clipped leaving a root length of approx. 1cm. Subsequently, seedlings were root dip-inoculated for at least five minutes in water (mock) or in the microconidial spore suspension. Seedlings were re-potted and three weeks-post-inoculation (wpi) the fresh weight (FW) and disease index (DI) were scored (de Lamo et al. 2018). A Mann-Whitney U statistical test was applied on FW and DI data using PRISM 7.0 (GraphPad).

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