Fusarium oxysporum f. sp. dianthi virus 1 affects the virulence and other phenotypic traits of its fungal host

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ABSTRACT

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Fusarium oxysporum f. sp. dianthi virus 1 (FodV1) has been detected infecting isolate *Fod* 116 (*Fod* 116V^{+}) of *Fusarium oxysporum* f. sp. *dianthi* (*Fod*), reaching a high accumulation level. FodV1 consists of four double-stranded RNA segments, that correspond to a new "chryso-like" mycovirus inside the *Chrysoviridae* family. We have obtained a version of isolate *Fod* 116 (*Fod* 116V^{-}) with only a residual level of FodV1

by selecting single conidia. Comparison of the growth rate and colony morphology on solid medium, the conidiation rate in liquid medium, and the virulence on susceptible carnation cultivars, using both V^+ and V^- versions of isolate *Fod* 116, showed significant alterations in all these phenotypic traits when high amounts of FodV1 were infecting the fungal host. FodV1 significantly reduced the growth and altered the morphology of the colonies on solid medium, and diminished the conidiation in liquid medium. Inoculation of four susceptible carnation cultivars showed that FodV1 significantly reduced the virulence of its fungal host. All these results place FodV1 in the group of hypovirulent mycoviruses, and identify it as a potential biocontrol agent against Fusarium wilt of carnation. This is the first report of a mycovirus that causes hypovirulence in the species *Fusarium oxysporum*.

Additional keywords: carnation, hypovirulence, mycovirus.

INTRODUCTION

Fungal viruses (mycoviruses) have been described infecting all major groups of fungi, including plant-pathogenic fungi. Estimates of mycovirus incidence suggest that 30-80% of fungal species may be infected (Ghabrial and Suzuki 2009). They are not known to have natural vectors, and are transmitted intercellularly by hyphal anastomosis (horizontal transmission) and during sporogenesis (vertical transmission). Fungal viruses are often associated with latent infections of their hosts, but in some cases alterations in growth, sporulation, pigmentation, and colony morphology, have been described as a consequence of virus infection. More interestingly, a number of

mycoviruses have been reported to attenuate fungal virulence (hypovirulence). Hence, interest in mycoviruses has increased because their potential contribution to sustainable agriculture as biological control agents of their fungal hosts (reviewed in: Ghabrial and Suzuki 2009; Ghabrial et al. 2015; Pearson et al. 2009; Xie and Jiang 2014).

The genus *Fusarium* contains important plant-pathogens of many economically important crops. *Fusarium*-infecting mycoviruses have been detected in a number of *Fusarium* species as *Fusarium graminearum* (Aminiam et al. 2011; Chu et al. 2002, 2004; Darissa et al. 2011; Yu et al. 2009, 2011; Wang et al. 2013; Li et al. 2015, 2016), *Fusarium solani* (Nogawa et al. 1993), *Fusarium proliferatum* (Heaton and Leslie 2004), *Fusarium poae* (Fekete et al. 1995; Wang et al. 2016), *Fusarium virguliforme* (Marvelli et al. 2014), and *Fusarium oxysporum* (Kilic and Griffin 1998; Sharzei et al. 2007), but association of these viruses with hypovirulence of their fungal hosts has been reported only for a few isolates. That is the case of three viruses identified in the *Fusarium graminearum* species complex: Fusarium graminearum virus China 9 (FgV-ch9) (Darissa et al. 2012), and Fusarium graminearum virus 1 and 2 (FgV1 and FgV2) (Lee et al. 2014).

Recently, a new double-stranded RNA (dsRNA) mycovirus, designated Fusarium oxysporum f. sp. dianthi virus 1 (FodV1), has been reported (Lemus-Minor et al. 2015). FodV1 was found with an unusual high level of accumulation in strain *Fod* 116 of *Fusarium oxysporum* f. sp. *dianthi*, the forma specialis of *Fusarium oxysporum* that infects carnation (*Dianthus caryophillus*) causing the most devastating carnation disease worldwide. Genome of FodV1 has been fully sequenced, and it consists of four dsRNA segments, ranging from 3555 bp to 2646 bp in length. Analysis of its genomic structure, homology searches of the deduced amino acid sequences, and phylogenetic analysis, all

indicated that FodV1 is a new member of the family *Chrysoviridae*, closely related to other "chryso-like" viruses previously reported (Lemus-Minor et al. 2015). Interestingly, phylogenetic analyses of FodV1 shows the highest similarity to FgV-ch9 and FgV2, two of the *Fusarium graminearum*-infecting mycoviruses that have been associated with the induction of phenotypic changes in their fungal host, including hypovirulence (Darissa et al. 2012; Lee et al. 2014).

On the basis of these precedents, in this study we have considered the hypothesis that the presence of mycovirus FodV1 could induce phenotypic changes associated to hypovirulence in *F. oxysporum* f. sp. *dianthi*. To test this hypothesis, we have compared the fungal mycelium growth rate, the conidiation rate, and the virulence to carnation, using two versions of isolate *Fod* 116, the original one infected with a very high titer of mycovirus FodV1, and another one obtained in this work with a very low titer of the mycovirus. Results obtained demonstrate that FodV1 reduced fungal mycelium growth rate, conidiation rate, and virulence on carnation plants. This is the first report of a mycovirus that causes hypovirulence in the species *Fusarium oxysporum*.

MATERIALS AND METHODS

Fungal isolates and culture conditions. The strain *Fod* 116 of *Fusarium oxysporum* f. sp. *dianthi* was isolated in 2008 from a diseased carnation plant collected in the Cádiz province of Spain (Gómez-Lama Cabanás et al. 2012). The presence of mycovirus FodV1 in isolate *Fod* 116, and its molecular characterization, has been recently reported (Lemus-Minor et al. 2015). This isolate, with a very high level of accumulation of viral dsRNA molecules, was designated as *Fod* 116V^{+}. Otherwise, a version with a very low

level of accumulation of the mycovirus was obtained by single conidia analyses (see screening of monoconidial cultures, below); this new version was named *Fod* 116V^{-}. These two versions were used for all phenotypic analyses. Isolates *Fod* 116V^{+} and *Fod* 116V^{-} were stored at -80 ºC in glycerol, and propagated on Potato Dextrose Agar (PDA) medium at 25°C in the dark.

Total nucleic acid extractions, dsRNA purifications, and total RNA extractions. Total nucleic acids were extracted from lyophilized mycelium using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), as previously described (Gómez–Lama Cabanás et al. 2012). DsRNA purifications were performed by cellulose column chromatography as described by Valverde et al. (1990).The total RNA extractions were performed from 100 mg of frozen grounded mycelium using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich), following the manufacturer's instructions. Total nucleic acid extracts and the purified dsRNAs extracts were analyzed by electrophoresis on 0.8% agarose gels stained with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Seongnam-si Gyeonggi-do, Korea). Total RNA extracts were visualized by electrophoresis on 1% agarose gels with 1% v/v household bleach (Aranda et al. 2012) to check for RNA integrity.

Obtention of a virus-free version of isolate *Fod* 116. In a first attempt to obtain a virus-cured version, isolate *Fod* 116V^{+} was grown on PDA plates containing different concentrations (25 µg·mL^{-1} to 100 µg·mL^{-1}) of cycloheximide (Sigma-Aldrich), as described by Aminian et al. (2011). A second attempt to obtain a virus-free version was done by selecting single conidia and analyzing the obtained monoconidial cultures for the presence of mycoviral dsRNA. For that, thirty single conidia were obtained from isolate *Fod* 116V^{+} and cultured on PDA plates at 25 ºC for 4 days. Mycelial plugs from

each monoconidial culture were used to inoculate PDA plates with cellophane, and the grown mycelia were used for dsRNA purifications as previously described.

Detection and relative quantification of FodV1 dsRNA. Total RNA extracts from isolates *Fod* 116V^{+} and *Fod* 116V^{-} were used to detect and quantify FodV1 dsRNA using primers directed to the RNA-dependent RNA polymerase (RdRp) sequence of FodV1 (see Supplemental Table 1). The presence of FodV1 dsRNA in the total RNA extracts was detected using primers FodV1RT, for the reverse transcription, and FodV1F/FodV1R, for the PCR. The absence of reverse transcription inhibitors was confirmed using primer pair Actin-1/Actin-2, directed to the fungal actin beta/gamma 1 gene (Lopez-Fernandez et al. 2013). Reverse transcription reactions were performed from 2 µL of total RNA extract using the enzyme NZY M-MuLV Reverse Transcriptase (NZYTech). PCR amplifications were performed using 2 µL of the cDNA synthesized, and the enzyme GoTaq® DNA Polymerase (Promega Corporation, Madison, WI USA). Products of the RT-PCR amplifications were analyzed by electrophoresis on 2.5% agarose gels.

To assess the relative quantity of FodV1 dsRNA, total RNA extracts from three replicates of each *Fod* 116V^{+} and *Fod* 116V^{-} isolate were used. Reverse transcription reactions were performed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™, Thermo Fisher Scientific,Waltham, MA USA), using 2 µg of each total RNA extract, and applying the "Random Primers" system following the manufacturer's instructions. The synthesized cDNA was used for the qPCR using primer pair FodV1qF/FodV1qR. To determine the relative amount of FodV1 dsRNA, primers targeting the fungal genes actin beta/gamma 1 (FoAcF/FoAcR), and β-tubulin (FoTubF/FoTubR) (fungal housekeeping genes) were used. Q-PCR reactions with each primer pair were carried out using the GoTaq qPCR SYBR Mastermix (Promega)

following the manufacturer's instructions. Reactions were performed in a StepOne™ Real-Time PCR System (Applied Biosystems). Cycle threshold (Ct) values obtained from each fungal reference gene, and Ct values obtained from the RdRp viral gene, were used to calculate the relative quantity of the RdRp viral gene in both *Fod* 116V$^-$ and *Fod* 116V$^+$ isolates, expressed as the Fold Change between these isolates, using the comparative C_T method ($\Delta\Delta$Ct) (Tomas and Keneth 2008). Data analysis was done with the StepOne™ Software v2.2.2 (Applied Biosystems).

Estimation of mycelial growth rate on solid medium and conidiation rate in liquid medium. To determine the effect of FodV1 on the mycelial growth rate, 100 conidia of each isolate *Fod* 116V$^+$ and *Fod* 116V$^-$ were placed in the center of a PDA plate (9 cm diameter, three replicates), and incubated at 25°C in the dark for 8 days. The radial growth of the four cardinal points was measured every day, and the growth area ($A= \pi \cdot r^2$) was calculated for each radius (four measures) in each plate (three replicates), and used to do a completely randomized analysis of variance (ANOVA). Significant differences among means for daily growth area values were determined using the Tukey's Honest Significant Difference (HSD $P \leq 0.01$) test. ANOVA analysis was performed using the Statistix program (Version 10.0 for Windows. Analytical software1985–2013). To estimate the effect of FodV1 on the conidiation rate in liquid medium, 200 conidia$\cdot$mL^{-1} of each isolate *Fod* 116V$^+$ and *Fod* 116V$^-$ were added to 5 mL (in a 50 mL conical tube) of casein hydrolyzed medium with AZ solution, and cultures were incubated with shaking (120 rpm) at 25 ºC. Conidia were counted at 2, 3, 4, and 6 days of growth, using three replicates for each sampling time. Number of conidia was expressed as conidia$\cdot$mL^{-1}, transformed with the logarithm function to normalized data, and used to do a two factors (day and isolate) analysis of variance (ANOVA). Significant differences among means for daily conidia count values with

each isolate were determined using the Tukey's Honest Significant Difference (HSD P ≤0.01) test. ANOVA analysis was performed using the Statistix 10.0 program (Analytical software).

Pathogenicity tests. Isolates *Fod* 116V^{+} and *Fod* 116V^{-} were inoculated on 5 different carnation cultivars, one resistant (Galaxia) and four susceptible (Candela, Baltico, Pink Bijou, and Master) to Fusarium wilt. Obtention of fungal inoculum, inoculation of carnation cuttings, and greenhouse conditions, were as described by Gomez-Lama Cabanás et al. (2012). Tests were performed at two different dates, August 2014 and March 2015. Two additional tests were done only with cultivar Candela at June 2015 and November 2015. Eight plants (replicates) for the 2014 test, and 16 plants for the March 2015 test, were inoculated for each cultivar/isolate combination. In the tests of June and November 2015, 16 plants of cultivar Candela were inoculated with each *Fod* 116 version. In all tests, 8 non-inoculated plants of each cultivar were used as controls. Fusarium wilt symptoms were evaluated every 2 days for approximately 3 months using a scale of disease from 0 (no symptoms) to 5 (dead plant). The area under the disease progress curve (AUDPC) was calculated by the trapezoidal integration method, and the standardized AUDPC (sAUDPC) was calculated using the corresponding time span for each test (Madden, 2007) and expressed as a percentage of the maximum sAUDPC for each pathogenicity test. A factorial analysis of variance (ANOVA) was used to analyze sAUDPC percentage of tests performed in August 2014 and March 2015, and a completely randomized design ANOVA was used to analyze sAUDPC percentage of tests performed in June and November 2015. Significant differences among means for disease severity values with each isolate were determined using the Fisher's Least significant difference (LSD P ≤0.05). ANOVA analyses were performed using the Statistix 10.0 program (Analytical software).

Two to three weeks before the end of each pathogenicity test, representatives of each cultivar/isolate combination were used to perform fungal isolation assays. The selected carnation plants were cut at the stem base, and the leaves were excised. After superficial disinfestation of the entire stem, stem sections corresponding to 1-3 cm, 5-10 cm, 15-20 cm, and 25-30 cm of the stem height, were placed on V8 agar plates, and incubated at 25 ºC in the dark.

RESULTS

Identification of FodV1 and obtention of a "virus-free" strain of *Fod* 116. "Chryso-like" mycovirus FodV1 was found in isolate *Fod* 116 (*Fod* 116V^{+}) of *F. oxysporum* f. sp. *dianthi* (Lemus-Minor et al. 2015), with such a high level of accumulation that it was detected while carrying out routine extractions of fungal genomic DNA (Fig. 1A). To assess the effect of FodV1 on its fungal host, a virus free strain was required. With the aim of obtaining a virus-free version of *Fod* 116, the virus-infected isolate was treated with cycloheximide. After several trials to obtain a cured version using cycloheximide, none of the concentrations applied resulted in a virus-free version. As a matter of fact, the final versions obtained after the cycloheximide treatments had similar high amounts of FodV1 dsRNA than the original *Fod* 116V^{+} isolate (not shown). A further attempt to obtain a virus-free version was performed by selecting single conidia from isolate *Fod* 116V^{+}. Total nucleic acid extracts were obtained from the monoconidial cultures and then the dsRNAs were purified by cellulose chromatography. After agarose gel electrophoresis of the dsRNA extracts, one of the *Fod* 116 monoconidial cultures apparently lacking viral dsRNA was selected randomly, and named *Fod* 116V^{-}. Comparison of agarose gel electrophoresis of the total nucleic acids

extracts (Fig. 1A) and the cellulose-purified dsRNA extracts (Fig. 1B), corresponding to both *Fod* 116V^{+} and *Fod* 116V^{-}, showed that FodV1 was present with a very high level of accumulation in isolate *Fod* 116V^{+}, but apparently absent in isolate *Fod* 116V^{-}. To confirm this observation, a reverse transcription followed by a PCR, targeting the RdRp segment of FodV1, was applied to both *Fod* 116V^{-} and *Fod* 116V^{+} total RNA extracts (Fig.2). Results from the RT-PCR showed that mycovirus FodV1 was still present in isolate Fod 116V^{-}, but probably as a residual infection (Fig. 2B). In order to probe this, we determined the relative quantity of FodV1 dsRNA in total RNA extracts from each *Fod* 116 version, by doing a RT-qPCR targeting the RdRp segment. The relative amount of the RdRp segment was calculated as a proportion of the fungal genes actin beta/gamma 1 and β-tubulin. Results showed that the quantity of FodV1 dsRNA in isolate *Fod* 116V^{-} was extremely low compared to that in isolate *Fod* 116V^{+}. The proportional quantity of FodV1 dsRNA in the version *Fod* 116V^{-} was assessed as $\approx 2.86 \times 10^{-5}$ (mean of both reference genes), when the quantity in *Fod* 116V^{+} was equal to 1 (Table 1). That is, the quantity of the RdRp segment found in the version *Fod* 116 V^{+} was ≈34,962 times higher than the quantity found in the version *Fod* 116V^{-}.

Effect of FodV1 on mycelial growth and conidiation. To study if the high accumulation level of FodV1 had any effect on selected phenotypic traits of its fungal host, a comparison of both *Fod* 116 versions, the one with a high titer of FodV1 dsRNA (*Fod* 116V^{+}), and the other with a residual titer of FodV1 dsRNA (*Fod* 116V^{-}), was done. To estimate differences in the growth on solid medium, including colony appearance and growth rate, the radius of the four cardinal points of colonies grown on PDA was measured. Results showed that mycovirus FodV1 significantly reduced the growth rate of isolate *Fod* 116, and modified the fluffy appearance of the colonies (Fig.

3). The significant differences in growth rate between both isolates were evident from days 5 to 8, showing a ratio of ~5:3 (V^- : V^+) on the day 8 of growth.

To estimate the influence of FodV1 on the conidiation rate, the two *Fod* 116 versions (V^+ and V^-) were inoculated in casein hydrolyzed liquid medium, and conidia counted at different days of growth. Results obtained showed that the presence of high amounts of FodV1 dsRNA in isolate *Fod* 116 led to a significantly lower conidiation rate (Fig. 4). It was observed that differences in the number of conidia produced by each isolate were more evident in the last days of growth, reaching a proportional difference of ≈2:1 (V^- : V^+).

Effect of FodV1 on the virulence. To determine the effect of FodV1 on the virulence of isolate *Fod* 116, two pathogenicity tests were performed. For all pathogenicity tests, disease severity values were used to calculate the percentage of sAUDPC. Factorial analysis of the data put in evidence a significant difference ($P \leq 0.05$) between the sAUDPC percentages obtained with the two inoculated *Fod* isolates, showing a significant reduction in the disease severity values obtained with all carnation cultivars when the fungal isolate was infected with high levels of FodV1 dsRNA (Table 2). There was only one exception in the test performed in 2014, where cultivar Candela showed no significant differences in the severity of the symptoms obtained after inoculation with either *Fod* 116V^+ or *Fod* 116V^-. To clarify this discrepancy, two additional tests were performed in 2015 with this cultivar. Results obtained in both tests showed a significant reduction of the sAUDPC percentage when Candela was inoculated with isolate *Fod*116V^+ compared to isolate *Fod* 116V^- (Table 2). Fungal isolation assays performed with representatives of all the carnation cultivars inoculated with each *Fod* 116V^+ and *Fod* 116V^- showed that while isolate *Fod* 116V^- was recovered from all the

stem sections of all the plants analyzed, isolate *Fod* 116V^{+} was just recovered from a few plants with disease symptoms values ≥ 3, and only from the sections corresponding to the lower part of the stem (Fig. 5).

DISCUSSION

Chrysovirus FodV1 was identified in isolate *Fod* 116 of *Fusarium oxysporum* f. sp. *dianthi* while performing routine DNA genomic extractions (Lemus-Minor et al. 2015). In this study we have tested the hypothesis that the presence of high amounts of mycovirus FodV1 in isolate *Fod* 116 (*Fod* 116V^{+} version), could affect particular phenotypic traits of its fungal host, including the virulence. To conduct the comparative phenotypic assays, attempts were made to obtain a cured (without virus) version of *Fod* 116 using cycloheximide. Unfortunately, although this treatment has been reported to cure some particular mycovirus infections in other fungal hosts (Aminian et al. 2011), in the case of *F. oxysporum* f. sp. *dianthi* the cycloheximide treatment was not suitable to cure the FodV1 mycovirus infection. Similar results have also been reported for other *Fusarium oxysporum* formae speciales infected with other dsRNA mycoviruses (Kilic & Griffin 1998; Sharzei et al. 2004); therefore, it seems that the cycloheximide treatment may not be appropriate to cure mycoviral infections in some particular fungal species. Another attempt to obtain a virus-free version was performed by selecting single conidia from isolate *Fod* 116V^{+}, and doing a screening for the presence of FodV1 dsRNA in the resultant monoconidial cultures. In this way, we could finally select a version of *Fod* 116 apparently free from virus infection (*Fod* 116V^{-} version). Isolate *Fod* 116V^{-} showed absence of FodV1 dsRNA when total nucleic acids or cellulose

purified dsRNA extracts were visualized, but RT-PCR revealed that isolate *Fod* 116V$^-$ was still infected with FodV1, although with such a very low titer of the mycovirus that it was only detectable by this technique. Quantitative RT-PCR analysis showed that the quantity of FodV1 dsRNA contained in isolate *Fod* 116V$^-$ was ≈34,962 times lower compared to isolate *Fod* 116V$^+$, and could, therefore, be considered as a residual infection. These two *Fod* 116 versions, V$^+$ and V$^-$, were used to study the influence of the mycovirus on particular phenotypic traits of the fungal host. Results obtained evidenced differences between both isolates, thus demonstrating a clear dose-dependent effect of mycovirus FodV1 on the phenotypic traits analysed. This observation is comparable to that previously reported for Fusarium graminearum mycovirus China 9 infecting *Fusarium graminearum* (Darissa et al. 2012), where the studies were also performed with a low titer and a high titer virus-infected version of *F. graminearum*, and suggests that alteration of the phenotypic traits analyzed requires a particular level of viral infection. In agreement with this, a higher ratio of accumulation of a Rosellinia necatrix partitivirus 2 strain in the heterologous fungus *Cryphonectria parasitica* has been linked to an induction of phenotypic alterations (Chiba et al. 2013).

Comparison of the growth rate and colony morphology on solid medium, the conidiation rate in liquid medium, and the virulence on a set of susceptible carnation cultivars, using both V$^+$ and V$^-$ versions of *Fod* 116, showed significant alterations in all these phenotypic traits when high amounts of FodV1 were infecting the fungal isolate. The presence of high amounts of mycovirus FodV1 clearly altered the morphology, and reduced the growth rate on solid medium and the conidiation rate in liquid medium, of the mycovirus-infected *F. oxysporum* f. sp. *dianthi* isolate. Similar phenotypic alterations have been reported for other mycoviruses that have been found infecting different fungal species. That is the case of mycoviruses FgCh9 (Darissa et al.

2012) and FgV2 (Lee et al. 2014) in *F. graminearum*; of mycoviruses MoCV1-A (Urayama et al. 2010) and MoCV1-B (Urayama et al. 2014) in *Magnaporthe oryzae*; and of mycovirus BdCV1 (Wang et al. 2014) in *Botryosphaeria dothidea*. Results obtained from phylogenetic analyses group all these above mentioned mycoviruses in clade I of the *Chrysoviridae* family. Mycoviruses in this clade have been proposed to constitute a new genus in that family (Darissa et al. 2011), but they have not yet been assigned to a distinct taxa by the ICTV. To distinguish them from the rest of mycoviruses in the family *Chrysoviridae*, recently they have been designated as "chryso-like" mycoviruses (Liu et al. 2012; Ghabrial et al. 2015). That is also the case of the virus analyzed in this work. Mycovirus FodV1, found infecting *F. oxysporum* f. sp. *dianthi*, has also been classified as a "chryso-like" mycovirus (Lemus-Minor et al. 2015). The fact that almost all "chryso-like" mycoviruses reported so far induce similar changes in colony morphology and growth rate and conidiation of their fungal host, suggest that this group of viruses could be specifically involved in the alteration of metabolic routes related to the normal growth and asexual sporulation in fungi.

In addition to the alterations in growth and conidiation mentioned above, FodV1 induces hypovirulence in his fungal host. Severity of the disease symptoms produced on a set of susceptible carnation cultivars were significantly lower when the plants were inoculated with the high titer FodV1-infected isolate (*Fod* 116V^{+}), compared to the low titer FodV1- infected isolate (*Fod* 116V^{-}). The effect of mycovirus FodV1 on the virulence of isolate *Fod* 116 was tested at two different moments, August 2014 and March 2015, with four susceptible carnation cultivars. Although FodV1 induced a significant reduction of the sAUDPC percentage on all carnation cultivars in the test performed in 2015, in the test performed in 2014 results obtained with cultivar Candela showed no significant differences in the sAUDPC percentage when the plants where

inoculated with either isolate *Fod* 116V^{+} or isolate *Fod* 116V^{-}. This different behavior of cultivar Candela in comparison with the rest of susceptible carnation cultivars tested could be associated to a different response of this particular cultivar to the fungal infection depending on changes in external factors such as the temperature or the artificial light provided to the plants. Even though the bioassays were performed in a greenhouse with controlled temperature and artificial light supply when needed, conditions of the two bioassays differed in the length of the natural sunlight, and the temperatures that they were exposed to. Whereas August (2014) had ≈12 h·day^{-1} of natural sunlight, and temperatures rather hot than cold (always between 25 and 30°C), March (2015) had ≈8 h·day^{-1} of natural sunlight, and temperatures rather cold than hot (always between 20 and 25 °C). To discard the possibility that differences in external factors related to the season could be contributing to the different results obtained with cultivar Candela, two new bioassays were performed in June and November 2015 using only that carnation cultivar. Results obtained supported those of the test of March 2015, thus confirming that the presence of high levels of mycovirus FodV1 induced a significant reduction of the severity of the disease symptoms in all carnation cultivars tested. In the case of *F. oxysporum* f. sp. *dianthi*, a vascular pathogen, this mycovirus-associated reduction of virulence could be related with the significant reduction of the mycelial growth and the decrease in conidiation produced by the viral infection. Both alterations could affect the efficiency in the colonization of the vascular system of the plant, thus contributing to the decrease in the virulence. In fact, results obtained from reisolation assays with the inoculated carnation plants demonstrated that, in most of the cases, hypovirulent strain *Fod*116V^{+} could not be isolated from the aerial part of the plant.

All the results obtained in this work confirm the initial hypothesis, indicating a significant alteration of the hypovirulence-associated traits studied induced by the presence of high levels of mycovirus FodV1. Our results are similar to those reported for other closely related "chryso-like" mycoviruses, as is the case of FgV-ch9 (Darissa et al. 2012), MoCV1-A (Urayama et al. 2012), and MoCV1-B (Urayama et al. 2014), and places FodV1 in the group of hypovirulent mycoviruses, with potential to be used as a biological control agent in the control of Fusarium wilt of carnation.

Mycovirus-associated hypovirulence is largely unknown in the genus *Fusarium*. Although mycoviral dsRNA have been reported for several *Fusarium* species, the association of these viruses with host-hypovirulent traits has been suggested only in the case of *F. graminearum* (Chu et al. 2002; Darissa et al. 2012). Thus, this is the first report of a case of mycovirus-associated hypovirulence in the important species *Fusarium oxysporum*. Even though mycovirus FodV1 has been found in the forma specialis *dianthi*, its use as a biological control agent could be extended by transfection of other formae speciales of *F. oxysporum.* Although transmission of hypovirulence-associated double-stranded RNA (dsRNA) viruses between different formae speciales can be prevented by the vegetative incompatibility barrier, this barrier could be overcome in the laboratory by using protoplast fusion (Lee et al. 2011). Success in transfection of other formae speciales would open the possibility of using FodV1 not only for the biological control of Fusarium wilt of carnation but also for the control of other agronomically important Fusarium wilts. Therefore, results contained in this work constitute the basis for further research on the application of mycovirus FodV1 to the control of Fusarium wilt diseases.

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LITERATURE CITED

Aminian, P., Azizollah, A., Abbas, S., and Naser, S. 2011. Effect of double-stranded RNAS on virulence and deoxynivalenol production of *Fusarium graminearum* isolates. J. Plant Prot. Res. 51(1):29-37.

Aranda, P. S., LaJoie, D. M., and Jorcyk, C. L. 2012. Bleach gel: A simple agarose gel for analyzing RNA quality. Electrophoresis 33(2):366-369.

Cao, Y.-f., Zhu, X.-w., Xiang, Y., Li, D.-q., Yang, J.-r., Mao, Q.-z., and Chen, J.-s. 2011. Genomic characterization of a novel dsRNA virus detected in the phytopathogenic fungus *Verticillium dahliae* Kleb. Virus Res. 159(1):73-78.

Chiba, S., Lin, Y.-H., Kondo, H., Kanematsu, S., and Suzuki, N. 2013. Effects of defective interfering RNA on symptom induction by, and replication of, a novel partitivirus from a phytopathogenic fungus, *Rosellinia necatrix*. J. of Virol. 87(4):2330-2341.

Chu, Y.M., Lim, W.S., Yea, S.J., Cho, J.D., Lee, Y.W., and Kim, K.H. 2004. Complexity of dsRNA mycovirus isolated from *Fusarium graminearum*. Virus Genes 28(1):135-143.

Chu, Y.-M., Jeon, J.-J., Yea, S.-J., Kim, Y.-H., Yun, S.-H., Lee, Y.-W., and Kim, K.-H. 2002. Double-stranded RNA mycovirus from *Fusarium graminearum*. Appl. Environ. Microbiol. 68(5):2529-2534.

Darissa, O., Adam, G., and Schafer, W. 2012. A dsRNA mycovirus causes hypovirulence of *Fusarium graminearum* to wheat and maize. Eur. J. Plant Pathol. 134(1):181-189.

Darissa, O., Willingmann, P., Schafer, W., and Adam, G. 2011. A novel double-stranded RNA mycovirus from *Fusarium graminearum*: nucleic acid sequence and genomic structure. Arch. Virol. 156(4):647-658.

Fekete, C., Giczey, G., Papp, I., Szabo, L., and Hornok, L. 1995. High-frequency ocurrence of virus-like particles with double-stranded-RNA genome in *Fusarium poae*. Fems Microbiol. Lett. 131(3):295-299.

Ghabrial, S. A., and Suzuki, N. 2009. Viruses of plant pathogenic fungi. Annu. Rev. Phytopathol. 47:353-84.

Ghabrial, S. A., Castón, J. R., Jiang, D., Nibert, M. L., and Suzuki, N. 2015. 50-plus years of fungal viruses. J. Virol. 479–480(0):356-368.

Gómez-Lama Cabanás, C., Valverde-Corredor, A., and Perez-Artes, E. 2012. Molecular analysis of Spanish populations of *Fusarium oxysporum* f. sp *dianthi* demonstrates a high genetic diversity and identifies virulence groups in races 1 and 2 of the pathogen. Eur. J. Plant Pathol. 132(4):561-576.

Heaton, L., and Leslie, J. 2004. Double-stranded RNAs associated with *Fusarium proliferatum* mitochondria. Mycol. Progress 3(3):193-198.

Ikeda, K.I., Nakamura, H., Arakawa, M., and Matsumoto, N. 2004. Diversity and vertical transmission of double-stranded RNA elements in root rot pathogens of

trees, *Helicobasidium mompa* and *Rosellinia necatrix*. Mycol. Res. 108(6):626-634.

Kilic, O., and Griffin, G. J. 1998. Effect of dsRNA-containing and dsRNA-free hypovirulent isolates of *Fusarium oxysporum* on severity of Fusarium seedling disease of soybean in naturally infested soil. Plant Soil 201(1):125-135.

Lee K.M., Yu J., Son M., Lee Y.W., Kim K.H. 2011. Transmission of *Fusarium boothii* mycovirus via protoplast fusion causes hypovirulence in other phytopathogenic fungi. PLoS One 6 (6): e21629.

Lee, K. M., Cho, W. K., Yu, J., Son, M., Choi, H., Min, K., Lee, Y. W., and Kim, K. H. 2014. A comparison of transcriptional patterns and mycological phenotypes following infection of *Fusarium graminearum* by four mycoviruses. Plos One 9(6): e100989.

Lemus-Minor, C. G., Cañizares, M. C., García-Pedrajas, M. D., and Pérez-Artés, E. 2015. Complete genome sequence of a novel dsRNA mycovirus isolated from the phytopathogenic fungus *Fusarium oxysporum* f. sp. *dianthi*. Arch. Virol. 160(9):2375-2379.

Li, P., Zhang, H., Chen, X., Qiu, D., Guo, L. 2015. Molecular characterization of a novel hypovirus from the plant pathogenic fungus *Fusarium graminearum*. Virology 481: 151-160.

Li P, Lin Y, Zhang H, Wang S, Qiu D, Guo L. 2016. Molecular characterization of a novel mycovirus of the family *Tymoviridae* isolated from the plant pathogenic fungus *Fusarium graminearum*. Virology 489, 86-94. .

Liu, H., Fu, Y., Xie, J., Cheng, J., Ghabrial, S. A., Li, G., Peng, Y., Yi, X., and Jiang, D. 2012. Evolutionary genomics of mycovirus-related dsRNA viruses reveals

cross-family horizontal gene transfer and evolution of diverse viral lineages. BMC Evol. Biol. 12:91.

Lopez-Fernandez, L., Ruiz-Roldan, C., Pareja-Jaime, Y., Prieto, A., Khraiwesh, H., and Roncero, M. I. G. 2013. The Fusarium oxysporum gnt2, encoding a putative N-acetylglucosamine transferase, is involved in cell wall architecture and virulence. PLoS One 8(12): e84690.

Madden, L. V., Hughes, G., and van den Bosch, F. 2007. The study of plant disease epidemics. American Phytopathological Society Press, St. Paul, MN.

Marvelli, R.A., Hobbs, H.A., Li, S., McCoppin, N.K., Domier, L.L., Hartman, G.L., Eastburn, D.M. 2014. Identification of novel double stranded RNA mycoviruses of *Fusarium virguliforme* and evidence of their effects on virulence. Arch. Virol. 159(2): 349-352.

Nogawa, M., Shimosaka, M., Kageyama, T., and Okazaki, M. 1993. A double-stranded RNA mycovirus from the plant pathogenic fungus *Fusarium solani* f. sp. *robiniae*. FEMS Microbol. Lett. 110(2):153-157.

Pearson, M. N., Beever, R. E., Boine, B., and Arthur, K. 2009. Mycoviruses of filamentous fungi and their relevance to plant pathology. Mol. Plant. Pathol. 10(1):115-128.

Robinson, H. L., and Deacon, J. W. 2002. Double-stranded RNA elements in *Rhizoctonia solani* AG 3. Mycol. Res. 106:12-22.

Schmittgen, T. D., and Livak, K. J. 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protocols 3(6):1101-1108.

Sharzei, B., Afsharifar. 2007. Detection and characterization of a double-stranded RNA mycovirus in *Fusarium oxysporum* f. sp. *melonis* Iran J. Plant Path. 43:9-26.

Urayama, S.-i., Sakoda, H., Takai, R., Katoh, Y., Tuong Minh, L., Fukuhara, T., Arie, T., Teraoka, T., and Moriyama, H. 2014. A dsRNA mycovirus, Magnaporthe oryzae chrysovirus 1-B, suppresses vegetative growth and development of the rice blast fungus. Virology 448:265-273.

Urayama, S., Ohta, T., Onozuka, N., Sakoda, H., Fukuhara, T., Arie, T., Teraoka, T., and Moriyama, H. 2012. Characterization of Magnaporthe oryzae chrysovirus 1 structural proteins and their expression in *Saccharomyces cerevisiae*. J. Virol. 86(15):8287-8295.

Urayama, S., Kato, S., Suzuki, Y., Aoki, N., Le, M. T., Arie, T., Teraoka, T., Fukuhara, T., and Moriyama, H. 2010. Mycoviruses related to chrysovirus affect vegetative growth in the rice blast fungus *Magnaporthe oryzae*. J. Gen. Virol. 91:3085-3094.

Valverde, R. A., Nameth, S. T., and Jordan, R. L. 1990. Analysis of double-stranded-RNA for plant-virus diagnosis. Plant Dis. 74(3):255-258.

Wang, S., Kondo, H., Liu, L., Guo, L., and Qiu, D. 2013. A novel virus in the family *Hypoviridae* from the plant pathogenic fungus *Fusarium graminearum*. Virus Res. 174(1–2):69-77.

Wang, L. P., Jiang, J. J., Wang, Y. F., Hong, N., Zhang, F. P., Xu, W. X., and Wang, G. P. 2014. Hypovirulence of the phytopathogenic fungus *Botryosphaeria dothidea*: association with a coinfecting Chrysovirus and a Partitivirus. J. Virol. 88(13):7517-7527.

Wang, L., Zhang, J., Zhang, H., Qiu, D., Guo, L. 2016. Two novel relative double stranded RNA mycoviruses infecting *Fusarium poae* strain SX63. Int. J. Mol. Sci. 17(5): 641.

Xie, J., and Jiang, D. 2014. New Insights into mycoviruses and exploration for the biological control of crop fungal diseases. Annu Rev Phytopathol 52(1):45-68.

Yu, J., Kwon, S.J., Lee, K.M., Son, M., Kim, K.H., 2009. Complete nucleotide sequence of double-stranded RNA viruses from *Fusarium graminearum* strain DK3. Arch. Virol. 154, 1855–1858.

Yu, J., Lee, K. M., Son, M., and Kim, K. H. 2011. Molecular characterization of Fusarium graminearum virus 2 isolated from *Fusarium graminearum* strain 98-8-60. Plant Pathol. J. 27(3):285-290.

TABLES

TABLE 1. Relative quantification of the viral RdRp segment in isolates *Fod* 116 V^- and *Fod* 116 V^+ [a]

Reference genes	Mean fold changes				*t* test P value[e]
	$\Delta Ct_{(V+)}$ [b]	$\Delta Ct_{(V-)}$ [b]	$\Delta\Delta Ct$ [c] ($\Delta Ct_{(V+)} - \Delta Ct_{(V-)}$)	Fold Change[d] ($2^{-\Delta\Delta Ct}$)	
Actin beta/ gamma 1	-14.06	1.60	-15.66	$1.92x10^{-5}$	0.0057
β-tubulin	-8.95	5.72	-14.68	$3.80x10^{-5}$	0.0097

[a] Comparison assessed with the data obtained from qPCR of both *Fod* 116 V^- and *Fod* 116V^+ versions. Ct (Cycle threshold) values of actin beta/gamma 1 and β-tubulin, used as reference genes, and of viral RdRp segment, where used to establish the Fold Change.
[b] $\Delta Ct_{(V+ \text{ and } V-)}$ was obtained from the subtraction of the Ct mean value of each reference gene from the Ct mean value of the viral RdRp segment of each *Fod* 116 V^+ and *Fod* 116 V^- version.
[c] $\Delta\Delta Ct$ was obtained from the subtraction of ΔCt_{V-} from ΔCt_{V+}.
[d] Fold Change was expressed as the proportional reduction of the viral RdRp segment amount in *Fod* 116 V^- when the quantity of the viral RdRp segment in *Fod* 116V^+ is equal to 1.
[e] *t* test applied to Ct mean values

TABLE 2. Percentage of the standardized area under the disease progress curve (sAUDPC)[y]

Test date	Isolate inoculated	Carnation cultivar			
		Candela	Baltico	Pink Bijou	Master
August 2014	116 V$^-$	0.548^{bc}	0.713^{ab}	0.845^{a}	0.533^{bc}
	116 V$^+$	0.492^{bc}	0.063^{e}	0.337^{cd}	0.163^{de}
March 2015	116 V$^-$	0.880^{ab}	0.786^{c}	0.927^{a}	0.826^{bc}
	116 V$^+$	0.059^{d}	0.008^{d}	0.073^{d}	0.072^{d}
June 2015	116 V$^-$	0.779^{a}	N/T	N/T	N/T
	116 V$^+$	0.222^{b}	N/T	N/T	N/T
November 2015	116 V$^-$	0.386^{a}	N/T	N/T	N/T
	116 V$^+$	0.001^{b}	N/T	N/T	N/T

[y]Means followed by the same letter in each test date are not significantly different according to Fisher's Least significant difference (LSD) ($P \leq 0.05$).
N/T: Not tested.

CAPTIONS FOR FIGURES

Fig. 1. Agarose gel electrophoresis of **A**, total nucleic acid extracts, and **B**, cellulose-purified dsRNA extracts, from isolates *Fod* 116V^{+} and *Fod* 116V^{-}. **M**: molecular weight marker λ HindIII. **V^{+}**: high titer virus infected version of isolate *Fod* 116. **V^{-}**: low titer virus infected version of isolate *Fod* 116.

Fig. 2. Agarose gel electrophoresis of **A**, total RNA extracts, and **B**, specific RT-PCR amplifications of the FodV1 RdRp segment, of isolates *Fod* 116V^{-} and *Fod* 116V^{+}. **M**: molecular weight marker λ HindIII. **V^{-}**: low titer virus infected version of isolate *Fod* 116. **V^{+}**: high titer virus infected version of isolate *Fod* 116.

Fig. 3. Effect of FodV1 on the mycelial growth and colony morphology on potato dextrose agar medium. **A,** Colony morphology of isolates *Fod* 116V^{+} and *Fod* 116V^{-} after 8 days of growth. **B,** Two dimensional colony growth rate of isolates *Fod* 116V^{+} and *Fod* 116V^{-}. The fungus was grown at 25 °C in the dark. Values are the average area of 3 colonies; different letters indicate statistically significant differences according to Tukey's Honest Significant Difference (HSD $P \leq 0.01$) test.

Fig. 4. Effect of FodV1 on the conidiation rate. Isolates *Fod* 116V^{-} and *Fod* 116V^{+} were cultured in casein hydrolyzed medium, and conidia counted at 2, 3, 4 and 6 days of growth (three replicates per time). Number of conidia was expressed as conidia·mL^{-1}, and used to do a two factors (day and isolate) analysis of variance (ANOVA). Significant differences among means for daily conidia count values with each isolate were determined using the Tukey's Honest Significant Difference (HSD $P \leq 0.01$) test. ANOVA analysis was performed using the Statistix 10.0 program (Analytical software). Vertical lines in each point represent the standard error.

Fig. 5. A, Disease symptoms, and **B**, fungal isolation from stem sections, of carnation plants inoculated with *Fod* 116V^{+} and *Fod* 116V^{-}. **A,** Difference in disease severity symptoms showed by two plants of cultivar Baltico, 8 weeks after the inoculation. **B**, Stem sections from plants of carnation cultivar Baltico inoculated with isolate *Fod* 116V^{-} or *Fod* 116V^{+} cultured on V8 agar plates. Result show the positive isolation from the plant inoculated with isolate *Fod* 116V^{-}, and the failure in the case of isolate *Fod* 116V^{+}.

***e*-Xtra Supplemental Table 1.** Specific primers used to detect and quantify mycovirus FodV1.