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Potential for biocontrol of Monosporascus root rot/vine decline under greenhouse conditions using hypovirulent isolates of *Monosporascus cannonballus*

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Abstract

Monosporascus root rot/vine decline (MRR/VD) causes root necrosis and severe stunting of muskmelon and watermelon plants in several countries around the world. MRR/VD is caused by the soilborne ascomycete fungus, *Monosporascus cannonballus.* Currently, there are few options available for control of MRR/VD. This research describes experiments to test the possibility of using naturally occurring *M. cannonballus* isolates containing double-stranded RNA (dsRNA) for the biological control of MRR/VD. These isolates often develop a degenerate phenotype characterized by slow growth and reduced ascospore production. In addition, these degenerate isolates are hypovirulent on muskmelon. Plants co-inoculated with a hypovirulent, dsRNA$^+$ isolate (Tx93-449$^+$) and a virulent, dsRNA$^-$ isolate (Az90-33$^-$) at an inoculum ratio of 10 : 1 (hypovirulent : virulent) were indistinguishable from the uninoculated plants in greenhouse pathogenicity trials. *In vitro* infection assays using fluorescence microscopy on aniline-stained muskmelon roots suggested that although the hypovirulent dsRNA$^+$ isolate Tx93-449$^+$ penetrated and partially colonized roots of the seedlings, it was not as efficient in colonizing the roots as the virulent, dsRNA$^-$ isolate Az90-33$^-$. While more extensive experiments are needed, these data suggest that hypovirulent dsRNA$^+$ isolates of *M. cannonballus* have potential for development as biological control agents to reduce disease pressure associated with MRR/VD.

Introduction

During the past 30 years, double-stranded RNAs (ds RNAs) have been reported from numerous fungal species (Buck, 1986; Ghabrial, 1998; Wickner, 1996). Several fungal phenotypes have been associated with the presence of dsRNA, including the killer phenomenon in *Ustilago maydis* (Koltin, 1988), culture degeneration in *Agaricus bisporus* (Wach et al., 1987), hypervirulence in *Phytophthora infestans* and *Rhizoctonia solani* (Finkler et al., 1985; Jian et al., 1998; Tooley et al., 1989), and hypovirulence in *Cryphonectria parasitica* (Elliston, 1985; Van Alfen et al., 1975). Hypovirulent isolates of *C. parasitica* have been used to control chestnut blight in Europe and in parts of the United States (Anagnostakis, 1982; Heiniger and Rigling, 1994). The success using transmissible hypovirulence to control chestnut blight suggests that debilitating dsRNAs associated with economically important soilborne fungi might be

useful for biological control of other fungal pathogens (Anagnostakis et al., 1998; Nuss, 1996).

Monosporascus root rot/vine decline (MRR/VD) (Mertely et al., 1991) of muskmelon (*Cucumis melo*) and watermelon (*Citrullus lanatus*), caused by the soilborne ascomycete *Monosporascus cannonballus* Pollack and Uecker (1974), has been responsible for significant yield losses throughout the world (Martyn and Miller, 1996). Melon losses due to *M. cannonballus* have been reported in Israel (Reuveni et al., 1983), Tunisia (Martyn et al., 1994), Taiwan (Tsay and Tung, 1995), Japan (Uematsu et al., 1985), and Mexico (Martyn et al., 1996). Recently, *M. cannonballus* was reported in Saudi Arabia (Karlatti et al., 1997), Guatemala (Bruton and Miller, 1997a), and Honduras (Bruton and Miller, 1997b). Melon losses also have been reported in the Valencia region of Spain due to a disease known as 'colapso' (collapse) or 'muerte subita' (sudden death), which appears to be due to *M. cannonballus* infections (Lobo-Ruano, 1990; Martyn and Miller, 1996). In Texas, MRR/VD accounts for annual losses in muskmelon of approximately 15%, with up to 100% loss in individual fields (Martyn and Miller, 1996).

Currently, there are no effective control measures against MRR/VD other than soil fumigation, which is expensive and may be prohibited in the near future due to increasing regulation of pesticide use in the US. Therefore, alternative measures are under investigation to control this disease on melons. In 1993, a survey of two commercial muskmelon fields in the Lower Rio Grande Valley of Texas revealed that approximately 65% of the *M. cannonballus* isolates recovered from diseased plants harbored dsRNAs (Lovic, 1994). A diverse assortment of different sizes and number of dsRNAs, ranging in size from ca. 1.5 to 12 kbp, based upon agarose gel electrophoresis, were associated with the isolates. Some isolates harbored only a single dsRNA, while others harbored as many as 13 dsRNAs (data not shown). After laboratory maintenance, most dsRNA$^+$ isolates developed a degenerate culture phenotype characterized by slow growth, yellow to orange pigment accumulation, and reduced ascospore production in culture (Lovic, 1994; Martyn and Miller, 1996). In addition, these dsRNA$^+$ isolates were hypovirulent on muskmelon in greenhouse pathogenicity trials.

Several experiments involving curing dsRNA$^+$ isolates and transferring dsRNAs to wildtype dsRNA$^-$ isolates suggested that some dsRNAs were causing the debilitating phenotypes (Park, 1996). More extensive curing and re-infection indicated that the dsRNAs associated with isolate Tx93-449$^+$ were responsible for the degenerative and hypovirulent phenotypes experiments (Park, 1996; Park et al., unpublished).

Interestingly, other isolates that harbored dsRNA maintained a phenotype, resembling wildtype dsRNA$^-$ *M. cannonballus* isolates. The purpose of this research was to sample *M. cannonballus* isolates from our collection to determine if there was a correlation between fungal culture phenotype, virulence to muskmelon, and the presence of specific dsRNA species. Three degenerate, hypovirulent dsRNA$^+$ isolates were tested further for their ability to provide protection to muskmelon plants against virulent isolates of *M. cannonballus* under greenhouse conditions. Using an *in vitro* pathogenicity assay and fluorescence microscopy, the infection process of a virulent dsRNA$^-$ isolate was compared with that of a hypovirulent dsRNA$^+$ isolate. Hypovirulent dsRNA$^+$ isolates may have the potential to be developed into biological control agents against virulent isolates of *M. cannonballus*.

Materials and methods

Fungal isolates

Eighteen *M. cannonballus* isolates from different geographic regions were used (Table 1). Six isolates, representing several different dsRNA profiles typically found in *M. cannonballus*, were used in greenhouse pathogenicity trials from 1993 to 1996 (Figure 1; Table 1). Isolate Ca91-17^{96+} is a subculture of the original Ca91-17$^+$ isolate that contains dsRNAs, but which has not yet developed the degenerate phenotype or hypovirulence. Actively growing cultures of each isolate were maintained at 22–25 °C on either V8 juice agar (Miller, 1955) or on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) plates. Cultures were stored as dormant mycelia at either room temperature or at −20 °C in an autoclaved and hydrated mixture of sand, oat, and wheat hulls (1 : 1 : 1; Lovic et al., 1995; Mertely et al., 1993).

Nucleic acid extraction and dsRNA isolation

Bulk mycelia for nucleic acid extractions were obtained by inoculating 100 ml of Monosporascus liquid culture (MLC) medium with a mycelial plug of *M. cannonballus* followed by incubatation in shake culture for 2–4 weeks at room temperature. MLC medium consists of 20 g sorbitol, 0.25 g $MgSO_4 \cdot 7H_2O$, 1.5 g

Table 1. Features of *M. cannonballus* isolates used for greenhouse pathogenicity trials and growth rate studies

Isolate[1]	dsRNA[2]	Pigmentation	Growth rate	Virulence
Tx90-25$^-$	–	–	Fast	High
Az90-33^{-**}[3]	–	–	Fast	High
Jpn91-20$^-$	–	–	Fast	High
Jpn91-21$^-$	–	–	Fast	High
Tx90-30$^-$	–	–	Fast	High
Esp91-1$^-$	–	–	Fast	High
Tx90-23$^-$	–	–	Fast	High
Tx91-19$^-$	–	–	Fast	High
Ca91-16$^+$	M	–/+[5]	Fast	High/ moderate[5]
Tx93-314^{+**}	L?	–	Fast	High
Tx93-447$^+$	L, M	–	Fast	High
Ca91-17^{96+**}	M	–	Fast	High
Ca91-17^{95+}	M	+	Slow	Low
Tx93-529^{+**}	L, S	–	Fast/ medium	Moderate
Tx90-26$^+$	L, S	–	Slow (non-radial)[4]	Low
Tai95-2$^+$	L?, M, S	+	Slow	Low
Tx93-449^{+**}	L, M, S?	+	Slow	Low
Tx91-18^{+**}	L, M, S	+	Slow	Low

[1]Isolates were obtained from the following locations: Tx = Texas, Az = Arizona, Ca = California, Jpn = Japan, Esp = Spain, Tai = Taiwan (R.O.C). Negative superscript (−) = no detectable dsRNA; positive superscript (+) = dsRNA detected.
[2]dsRNA sizes: L = large (>12 kbp), M = medium (~2.7–4.0 kbp), S = small (< 2.7 kbp), ? = band occasionally present in dsRNA preparations.
[3]Isolate marked with '**' were used in several repeated trials from 1993 to 1996.
[4]Although isolate Tx90-26$^+$ does not develop the yellow-brown pigment, its growth pattern is non-radial and slow compared to dsRNA$^-$ isolates.
[5]Pigmentation and hypovirulence developed as the isolate was in continuous culture from 1992 to 1995.

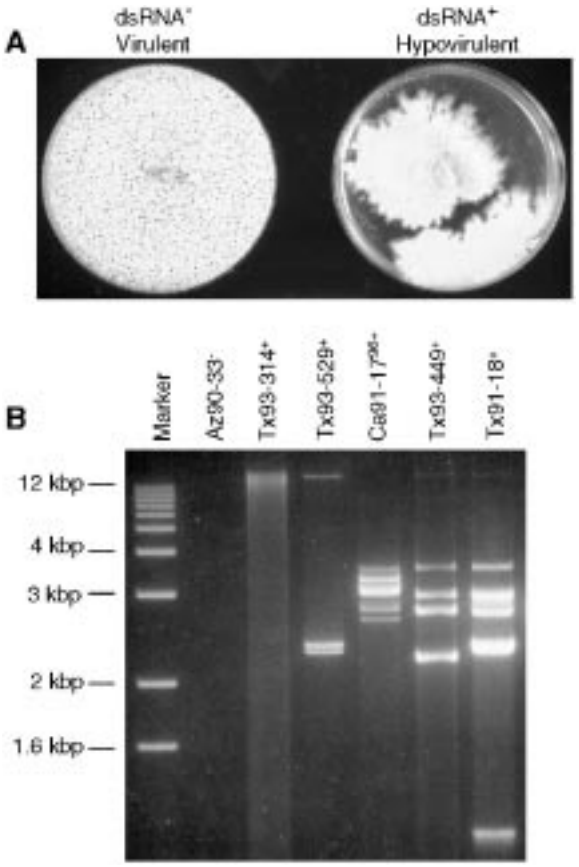

Figure 1. *M. cannonballus* cultures and agarose gel of dsRNAs. (A) A virulent dsRNA$^-$ isolate (left) and a degenerate, hypovirulent dsRNA$^+$ isolate (right) grown on potato dextrose agar plates. (B) Six isolates were used in repeated experiments from 1995 to 1996. The dsRNAs were purified by CF11 chromatography, treated with DNase, and separated on a 1.0% agarose gel stained with ethidium bromide. dsRNAs were not detected in the virulent wildtype isolate, Az90-33$^-$. The DNA ladder in kilobase pairs (kbp, marker) was run for relative size comparison.

KH_2PO_4, 2 g KNO_3, 1 g yeast extract, and 1 ml trace elements per liter of ddH_2O (Esposito and Fletcher, 1961; Lovic et al., 1995; Puhalla, 1985). The dsRNAs were preferentially isolated by CF11 chromatography (Hansen et al., 1985; Morris and Dodds, 1979) and separated by electrophoresis in native TBE (90 mM Tris–borate, 2 mM EDTA) agarose gels.

In vitro *growth and pigmentation assay*

Test isolates of *M. cannonballus* were evaluated for their radial growth and pigment accumulation on PDA. A 5-mm plug from an actively growing culture of each isolate was plated onto PDA and incubated between 22 °C and 25 °C. Radial growth measurements were taken daily for 1 week from five replicated plates per isolate. Cultures were grown for 4 weeks on PDA and observed for the accumulation of a yellow to orange pigment, often associated with dsRNA$^+$ isolates. Wildtype dsRNA$^-$ isolates produce white mycelia embedded with black perithecia.

Greenhouse pathogenicity trials

Virulence of *M. cannonballus* isolates was tested on the susceptible muskmelon cultivar 'Magnum 45' (Petoseed Co., Saticoy, CA). Four hundred grams of

red milo sorghum (*Sorghum bicolor*) seed was imbibed for at least 1 h with 300 ml of ddH_2O in 1.1 L mason jars and autoclaved for 20 min (121 °C, 15 psi) once a day for 3 consecutive days. The sterile sorghum seed was infested with five agar plugs from actively growing cultures of *M. cannonballus* and incubated for at least 2 weeks at room temperature to allow for complete colonization of the sorghum seed. One hundred grams of the colonized sorghum seed was mixed with 3 liters of pasteurized sand/perlite/vermiculite soil (1 : 1 : 1), 10 g of Sierra 17–6–12 N–P–K plus minor elements slow release fertilizer (Sierra-Scott, Marysville, OH), and distributed to 15-cm-diameter pots such that each pot received an equivalent of 20 g of inoculum. 'Magnum 45' muskmelon seeds were surface sterilized in 0.525% $NaClO^-$ for 1 min, rinsed with sterile ddH_2O, planted four per pot, and later thinned to two plants per pot. A 2 cm top-layer of non-infested soil mixture was added to prevent cross contamination between pots during watering. The pots were spaced ca. 30 cm apart and carefully watered to prevent soil splashing. Each treatment consisted of five replicated pots and a control treatment consisting of 20 g per pot of non-infested, sterile sorghum seed. At the conclusion of each trial, plants were harvested and graded for disease using vine length and root weight as disease criteria. Root weight was obtained by drying the muskmelon roots at 50 °C for two days followed by weighing of the dried roots. Additionally, roots were graded for disease severity on a scale of 0 (healthy) to 4 (extensive necrosis and lesion development of both taproot and lateral roots; Mertely et al., 1991; 1993). Disease criteria were subjected to variance analysis (LSD-T) using Statistix 4.0 computer software (Analytical Software, Tallahassee, FL). Roots from all treatments were surface sterilized for 1 min in 0.525% $NaClO^-$, washed with ddH_2O, and then plated onto 2% water agar amended with 200 mg/ml streptomycin and 200 mg/ml ampicillin (Sigma Chemical Co., St. Louis, MO) for re-isolation of *M. cannonballus.*

Biocontrol greenhouse trials

Two separate greenhouse trials were conducted during October 1995 and 1996. Inoculum preparation and planting were as described for the greenhouse pathogenicity trials. Each treatment consisted of five replicate pots with two 'Magnum 45' muskmelon plants per pot. Mixtures of a virulent, $dsRNA^-$ isolate ($Az90\text{-}33^-$) and a hypovirulent, $dsRNA^+$ isolate (either $Tx91\text{-}18^+$ or $Ca91\text{-}17^{95+}$) were tested in October 1995. Each isolate mixture was made by combining infested sorghum seed in either a 1 : 1, 5 : 1, or 10 : 1 ratio of $dsRNA^+ : dsRNA^-$ for a total inoculum of 20 g per pot. To determine if the 10 : 1 inoculum ratio with 2 g of $Az90\text{-}33^-$ inoculum per pot was sufficient to cause disease and stunting in muskmelon, a separate experiment was conducted in which 2 g of inoculum per pot was compared with the standard of 20 g of inoculum per pot. Plants were allowed to grow for at least 4 weeks at which time vine length and root weight were measured to assess disease.

In the second trial (October 1996), isolate $Tx93\text{-}449^+$ was substituted for $Ca91\text{-}17^{95+}$ which was lost in December 1995 due to extensive degeneration. For this trial, an inoculum mixture of 10 : 1 ($dsRNA^+ : dsRNA^-$) was tested using 20 g per 20-cm-diameter pot of the $dsRNA^-$ isolate ($Az90\text{-}33^-$) and 200 g of one of the two $dsRNA^+$ isolates (either $Tx93\text{-}449^+$ or $Tx91\text{-}18^+$). Control plants were mock inoculated with 200 g of sterile sorghum seed.

Fluorescence microscopy of M. cannonballus *infections*

The *in vitro* infection process of a wildtype $dsRNA^-$ isolate ($Az90\text{-}33^-$) and a hypovirulent, $dsRNA^+$ isolate ($Tx93\text{-}449^+$) was examined using a fluorescence staining protocol (Hood and Shew, 1996) on the susceptible muskmelon cultivar, 'Magnum 45'. Muskmelon seeds were surface sterilized and plated onto 2% water agar amended with 200 mg/ml of streptomycin and ampicillin (Sigma Chemical Co., St. Louis, MO). After approximately 4 days, a mycelial plug of either $Az90\text{-}33^-$ or $Tx93\text{-}449^+$ (Table 1) was added to the petri dish approximately 10 mm from the germinated seedling. Once contact between the fungal mycelia and the seedling occurred, points of infection were collected at three time intervals: early (1–3 days), middle (4–7 days), and late (2–4 weeks). Roots were observed for symptoms of infection, cut into 5-mm sections, submerged in 1 M KOH, and autoclaved for 20 min (121 °C, 15 psi). Autoclaved root sections were washed three times with ddH_2O and then mounted on glass microscope slides in several drops of 0.05% aniline blue (color index #42755, Sigma Chemical Co., St. Louis, MO) in 0.067 M K_2HPO_4 (Hood and Shew, 1996). Aniline-stained roots were observed with an Olympus BH2-RFC microscope equipped with a mercury burner and an Olympus UG1 fluorescence cube (365 nm excitation, LP420 nm emission; Olympus

America, Inc., Melville, NY). Photographs were taken with an Olympus PM-10ADS Photomicrographic system using Kodak Elite 400 ASA color slide film (Eastman Kodak Co., Rochester, NY) with 8- to 12-s exposures. Whole root images were captured using a Sony CCD-IRIS color video camera attached to an Olympus SZ60 stereo microscope and processed using Image-Pro Plus, version 1.3 (Media Cybernetics, Silver Spring, MD).

Results

Hypovirulence and culture degeneration associated with the presence of dsRNA

Eighteen *M. cannonballus* isolates from different geographic regions of the world were screened for virulence to muskmelon in greenhouse pathogenicity trials. These isolates were selected because they represent all dsRNA profiles type identified thus far, including eight isolates apparently lacking dsRNAs (Table 1). The dsRNA$^-$ isolates had a wildtype (normal) phenotype characterized by rapid growth of white mycelia and formation of numerous black perithecia (Figure 1A, left). In contrast, dsRNA$^+$ isolates exhibited a range of phenotypes (Table 1). While some dsRNA$^+$ isolates, such as Ca91-17^{96+}, initially had a wildtype phenotype, others such as Tx93-449$^+$ or Tx90-26$^+$ exhibited differing degrees of culture degeneration including slow and/or sectored growth and reduced or erratic sporulation (Figure 1A, right; Table 1). These isolates often accumulate a yellow to orange pigment that is not observed in wildtype dsRNA$^-$ isolates. These degenerate symptoms became more severe as these dsRNA$^+$ cultures were maintained through laboratory transfer.

Using muskmelon vine length as a disease parameter, data from several greenhouse pathogenicity trials conducted in 1992, 1995, and 1996 were used to compare the virulence of the eighteen *M. cannonballus* isolates. Mean vine lengths for each isolate were normalized using the virulent dsRNA$^-$ isolate, Az90-33$^-$, which was used in each trial as a diseased positive control. This comparison indicates the increased melon vine length relative to that observed in plants inoculated with Az90-33$^-$. The dsRNA$^-$isolates (Figure 2, clear bars) Tx91-19$^-$, Jpn91-20$^-$, Tx90-30$^-$, Esp92-1$^-$, and Tx90-25$^-$, and Tx90-23$^-$ caused severe stunting of muskmelon vines as indicated by values of -7%, 0%, 2%, 5%, 13%, 17%, and 19% respectively, compared to the virulent isolate Az90-33$^-$ (0%, Figure 2). In contrast, dsRNA$^+$ isolates (Figure 2, shaded bars) such as Tx90-26$^+$, Tx93-529$^+$, Tx91-18$^+$ and Tx93-449$^+$ had vine lengths of 29%, 60%, 70%, and 113%, respectively, longer than for plants inoculated with Az90-33$^-$. As a group, these dsRNA$^+$ isolates were associated with less stunting than the dsRNA$^-$ isolates. Analysis of variance showed that the latter three dsRNA$^+$ isolates (Tx93-529$^+$, Tx91-18$^+$ and Tx93-449$^+$) caused significantly less stunting than most of the dsRNA$^-$ isolates tested (Figure 2, $P = 0.1$ LSD).

While the tendency is for dsRNA$^+$ isolates to be less virulent than dsRNA$^-$ isolates in greenhouse trials, two dsRNA$^+$ isolates, Tx93-314$^+$ and Ca91-17$^+$, were mildly pathogenic on muskmelon (Figure 2). Unexpectedly, Ca91-16$^+$ caused severe stunting in the first pathogenicity trial in June 1992 as indicated by 66% reduction in average vine length compared to uninoculated control plants (data not shown). At the time of this trial Ca91-16$^+$ harbored several medium-sized dsRNAs ($\sim$2.7–4.0 kbp), but exhibited a virulence and culture phenotype similar to dsRNA$^-$ wildtype *M. cannonballus* isolates. In the July 1992 greenhouse pathogenicity trial, Ca91-16$^+$ caused a 38% reduction in vine lengths compared to uninoculated control plants (data not shown). However, by October 1995 Ca91-16$^+$ had a distinct degenerate phenotype characterized by pigment accumulation and caused only a minimal reduction (8%) in muskmelon vine length when retested in a greenhouse pathogenicity trial. Therefore, isolate Ca91-16$^+$ became increasingly degenerate and hypovirulent with repeated laboratory transfers even though the amount and profile of the dsRNAs remained unchanged.

The dsRNA$^-$ isolates were uniformly virulent to muskmelon, while the dsRNA$^+$ isolates exhibited variability in virulence as indicated by large standard deviations compared to wildtype isolates (data not shown). This variability might be due to the particular dsRNA species, rather than the mere presence of dsRNA. As dsRNA$^+$ isolates were repeatedly transferred on artificial media in the laboratory, most developed a degenerate phenotype (i.e., Ca91-16$^+$ and Ca91-17^{95+}). In contrast, wildtype isolates lacking dsRNA did not degenerate or lose virulence, even after several years of repeated transfer. Repeated laboratory transfer and greenhouse trials with dsRNA$^+$ isolates revealed that the sustained presence of dsRNA resulted in hypovirulence and culture degeneration.

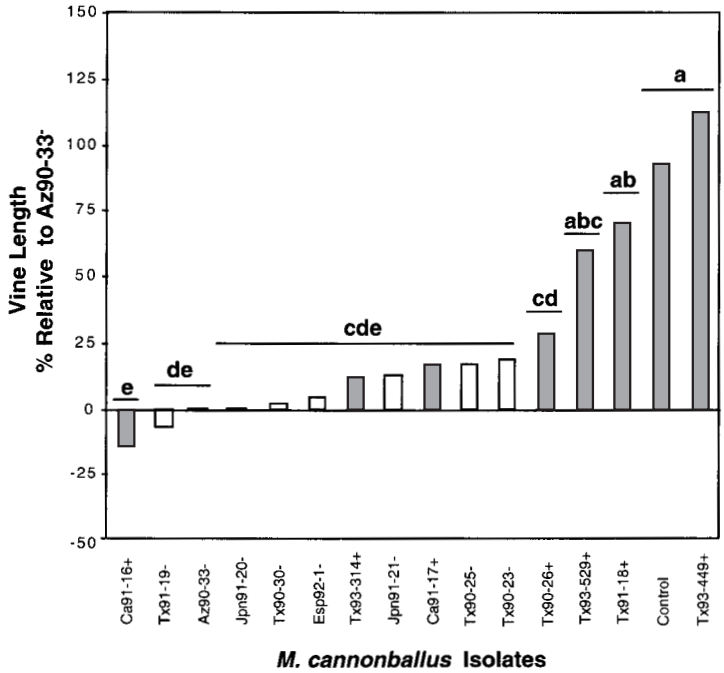

Figure 2. Pooled greenhouse pathogenicity data (mean vine length). Mean vine length data from plants inoculated with the virulent, dsRNA$^-$ isolate Az90-33$^-$ was used to normalize the data from individual treatments. The isolates defined as virulent had vine length percentages near 0% (relative stunting compared to Az90-33$^-$). Hypovirulent dsRNA$^+$isolates caused less stunting therefore the comparison values were greater than 0% and approached levels observed for uninoculated control plants. Isolates harboring dsRNAs are indicated with the dark bars. Means for each isolate with the same letter are not significantly different (LSD(t), $P = 0.1$).

Greenhouse pathogenicity trials with M. cannonballus *isolates possessing different dsRNA profiles*

Based upon greenhouse pathogenicity trials, growth rate studies, and culture phenotype, degenerate, dsRNA$^+$ isolates such as Tx93-449$^+$ were the most hypovirulent (Table 1, Figure 2). These degenerate isolates grew slowly, produced few mature perithecia, and often produced sectors or had irregular growth patterns (Figure 1A, right). These isolates also accumulated a yellow to dark orange pigment, regardless of the culture medium (data not shown). However, not all of the dsRNA$^+$ isolates were hypovirulent or degenerate. To determine if certain sized dsRNAs were linked to hypovirulence and/or degeneracy, five *M. cannonballus* isolates with different dsRNA profiles, including one dsRNA$^-$ isolate, were examined in greenhouse pathogenicity trials between 1995 and 1996 (Figure 1B, Table 1'**'). The pooled data from four greenhouse trials is summarized in Table 2, as a comparison of average vine length or root weight percentages between each treatment to Az90-33$^-$ (dsRNA$^-$, wildtype). As explained in the previous section, isolates with comparison values close to 0% caused similar stunting or reduction in root mass to that induced by virulent isolate, Az90-33$^-$.

Isolates Tx93-314$^+$ and Ca91-17^{96+} caused some stunting and root damage, but not as severe compared to muskmelon plants inoculated with Az90-33$^-$ (Table 2). In contrast, dsRNA$^+$ isolates Tx93-529$^+$ and Tx93-449$^+$ caused little or no stunting or root destruction as indicated by vine and root comparison values between ca. 50% and greater than 100% compared to that caused by Az90-33$^-$, and were similar to uninoculated control plants (Table 2).

Evaluation of hypovirulent isolates as potential biocontrol agents of MMR/VD

Based upon the initial results from the greenhouse pathogenicity trials, three hypovirulent isolates (Ca91-17^{95+}, Tx93-449$^+$, or Tx91-18$^+$) were evaluated

Table 2. Comparisons of mean vine length and root weight of muskmelon plants from greenhouse pathogenicity trials conducted in 1995 and 1996

Treatment	% Comparison to Az90-33-[1]	
	Vine length	Root weight
Tx93-449^{+}	87.95 a[2]	194.71 a[2]
Control^{na}	70.17 a	138.59 ab
Tx93-529^{+}	48.27 ab	109.63 abc
Tx93-314^{+}	25.50 bc	61.98 bc
Ca91-17^{96+}	20.03 bc	18.07 bc
Az90-33^{-}	0.00 c	0.00 c

[1]% Comparison represents the reduction in vine stunting compared to plants inoculated with Az90-33^{-}, and was calculated as follows: ((vine length Az90-33^{-} − vine length treatment)/vine length Az90-33^{-}) × 100 Root weight data was compared in a similar manner.
[2]Means in each column with the same letter are not significantly different (LSD(t), $P = 0.1$).

Table 3. Mean vine length of 7-week-old muskmelon plants inoculated with various ratio mixtures of dsRNA^{+} and dsRNA^{-} isolates of *M. cannonballus*

Treatment	dsRNA	Vine length (cm)	% Comparison[1]
Control^{na} (sterile sorghum)	na	55.4 bc[2]	59
Az90-33^{-}	−	35.4 cd	0
Tx91-18^{+}	+	64.8 ab	83
Tx93-449^{+}	+	74.5 ab	110
Tx91-18^{+} : Az90-33^{-} (10 : 1)[3]	− and +	31.9 d	−10
Tx93-449^{+} : Az90-33^{-} (10 : 1)	− and +	84.1 a	138

[1]% Comparison represents the reduction in vine stunting compared to plants inoculated with Az90-33^{-}, and was calculated as follows: ((vine length treatment−vine length Az90-33^{-})/vine length Az90-33^{-}) × 100.
[2]Means in each column with the same letter are not significantly different (LSD (t), $P = 0.1$)
[3]Plants were co-inoculated with a hypovirulent isolate (Tx91-18^{+} or Ca91-17^{95+}) and a virulent isolate (Az90-33^{-}) in a 10 : 1 ratio.

for their ability to reduce disease incidence by the virulent isolate, Az90-33^{-}. A 5 week greenhouse trial in 1995 indicated that when inoculum from a degenerate, dsRNA^{+}, hypovirulent isolate was combined in a 10 : 1 ratio with that of a virulent, dsRNA^{-} isolate, vine stunting was reduced. Muskmelon plants grown in a 10 : 1 inoculum ratio of Tx91-18^{+} or Ca91-7^{95+} and Az90-33^{-} tended to exhibit reduced stunting as opposed to plants inoculated with Az90-33^{-} alone (data not shown). A 5 : 1 ratio of hypovirulent (dsRNA^{+}) to virulent (dsRNA^{-}) isolates resulted in some reduced stunting compared to the virulent isolate alone (data not shown). While the mean vine lengths between treatments were not significantly different ($P = 0.1$, LSD), the observed trends warranted further trials.

A similar pathogenicity trial was conducted in Fall 1996 using the virulent isolate, Az90-33^{-}, co-inoculated with one of two hypovirulent isolates, either Tx93-449^{+} or Tx91-18^{+} (Table 3). Muskmelon vine stunting was reduced by 138% in plants co-inoculated with Tx93-449^{+} and Az90-33^{-} at a ratio of 10 : 1 ($P = 0.1$, LSD; Table 3, Figure 3). Overall, plants inoculated with Tx93-449^{+} alone, or in combination with Az90-33^{-}, did not show any indications of disease (Figure 3). Plants co-inoculated with Tx91-18^{+} and Az90-33^{-} (10 : 1) did not benefit from the presence of the hypovirulent isolate and were severely stunted, similar to plants inoculated with Az90-33^{-} alone (Figure 3, Table 3). The active culture of Tx91-18^{+} used for the Fall 1996 greenhouse trial had degenerated to the point that most of the infested sorghum inoculum was not viable. This indicated that the fitness of the hypovirulent isolate is an important consideration for its effectiveness as a biocontrol agent. Although isolate

Figure 3. Seven-week-old muskmelon plants from a greenhouse pathogenicity trial in the Fall, 1996. Plants inoculated with the virulent, non-dsRNA isolate Az90-33^{-} alone (far right) were severely stunted compared to uninoculated control plants (far left). In contrast, plants inoculated with hypovirulent, dsRNA^{+}isolate Tx93-449^{+} (middle left), or co-inoculated with Tx93-449^{+} and Az90-33^{-} (10 : 1 ratio; middle right) were healthy and indistinguishable from uninoculated control plants.

Tx93-449$^+$ also was degenerate, at a 10 : 1 inoculum ratio it appeared to affect the ability of a virulent isolate (Az90-33$^-$) to cause disease on muskmelon.

Fluorescence microscopy of M. cannonballus *infections*

To further examine the nature of the hypovirulence associated with Tx93-449$^+$, muskmelon seedlings were subjected to an *in vitro* pathogenicity assay (Figure 4). Roots were analyzed at three different time intervals: during the first 3 days of infection (early), 1 week post-infection (middle), and between 2 and 4 weeks post-infection (late). Isolate Az90-33$^-$ grew rapidly, contacting the seedling roots in 1 to 2 days; whereas, Tx93-449$^+$ grew more slowly and generally took 4–6 days before it contacted the roots. Visually, the roots infected by both isolates appeared healthy even though aniline blue staining revealed that Az90-33$^-$ rapidly penetrated the epidermis immediately after root contact (Figure 4A). Colonization of the roots by Az90-33$^-$ occurred rapidly within 1 week following contact (Figure 4A). In contrast, the hypovirulent isolate Tx93-449$^+$ was able to penetrate and colonize the root tissue, although, not as quickly nor as effectively as Az90-33$^-$ (Figure 4B). Infections by Az90-33$^-$ appeared to degrade the tissue as evidenced by the lack of root cytoplasm hence making visualization of the mycelia easy (Figure 4A). The cytoplasm appeared to be maintained in those roots infected by Tx93-449$^+$, making visualization of the mycelia difficult (Figure 4B). These differences between the virulent and hypovirulent isolates may suggest that enzymatic degradation might be a part of the infection process of virulent isolates.

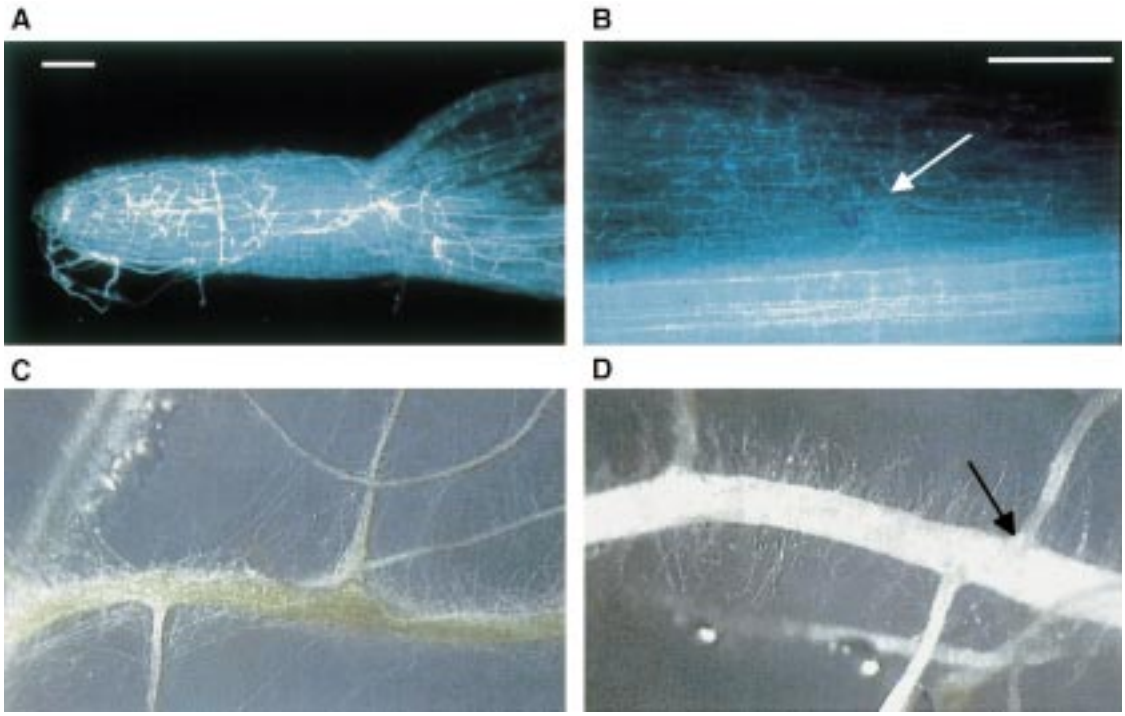

Figure 4. Fluorescence microscopy of *in vitro* pathogenicity assays of 'Magnum 45' muskmelon seedlings infected with *Monosporascus cannonballus*. Roots were stained with aniline blue as described in the text. (A) Colonization of the root tip by virulent, isolate Az90-33$^-$, 1 week post-contact. Magnification bar equals 100 μm. (B) Colonization of the root by hypovirulent, dsRNA$^+$isolate Tx93-449$^+$, 2–4 weeks post-contact. Unlike roots infected with Az90-33$^-$, the plant tissue infected with Tx93-449$^+$ does not appear to be degraded, and thus, it is difficult to see the mycelia (white arrow). Magnification bar equals 50 μm. (C) Extensive necrosis on lateral roots of a seedling colonized by Az90-33$^-$, 2 weeks post-contact. (D) Tx93-449$^+$ did not colonize the outside of the roots as readily as Az90-33$^-$ and only caused a very slight necrosis at root junctions (black arrow).

After 2 weeks, Az90-33$^-$ colonized the outside of the root, followed by development of necrotic areas initiating at root junctions and extending along the axis of the root (Figure 4C). In contrast, Tx93-449$^+$ took 1 month to partially colonize the outside of the roots, and in these cases, the mycelia only covered portions of the root (Figure 4D). For roots inoculated with Tx93-449$^+$, very minor necrosis was observed only around root junctions, and it did not progress to the rest of the root (Figure 4D, arrow). Both isolates formed perithecia within the cortex of the root after 1 month. However, the perithecia produced by Tx93-449$^+$ rarely progressed beyond initial stages of development and did not contain mature ascospores (data not shown). These results suggest that the basis for hypovirulence in Tx93-449$^+$ may be due in part to slow growth and an inability to rapidly colonize the plant roots. In contrast, the virulent isolate Az90-33$^-$ easily penetrated and rapidly colonized the root tissue, produced perithecia with mature ascospores, and caused severe necrosis in the muskmelon roots, confirming the virulent nature of this isolate.

Discussion

Monosporascus root rot/vine decline (MRR/VD) continues to result in significant losses for melon growers in at least 13 countries around the world (Aegerter et al., 2000; Martyn and Miller, 1996). The presence of dsRNA in a high percentage of *M. cannonballus* isolates and a correlation with culture degeneracy prompted us to investigate if degeneracy and hypovirulence were correlated with the presence of specific dsRNAs.

The first observation drawn from these data is that there is not a strong correlation between dsRNA profile and virulence or culture degeneration. The wide range of dsRNA species observed in *M. cannonballus* isolates initially suggested to us that such differences in phenotype might be due to differences in specific dsRNA, not to the presence of dsRNA *per se*. It is clear that isolates containing dsRNAs have a strong tendency to have variable levels of virulence on muskmelon and exhibit some degree of degenerate culture phenotypes (Figure 2, Table 1). Upon repeated transfer of dsRNA$^+$ isolates under laboratory conditions, symptoms of dsRNA$^+$ infection were manifested as increased pigmentation, reduced growth and sporulation, and reduced virulence to muskmelon. However, there appears to be a correlation between culture degeneration and hypovirulence. Three different virulence phenotypes: dsRNA$^-$/virulent, dsRNA$^+$/moderate virulence, and dsRNA$^+$/hypovirulent, were identified. It was not possible to associate a single dsRNA species, either by relative size or using dsRNA-specific probes (Batten et al., 2000), with hypovirulence or degeneration.

Three hypovirulent isolates were tested for their ability to act as biocontrol agents against virulent isolates in greenhouse pathogenicity trials. Although the degenerate isolates tended to grow more slowly than wildtype isolates, it appears that this problem may be overcome by adding more of the hypovirulent isolate to the soil mixture. Commercial muskmelon plants are often raised in the greenhouse and later transplanted into the field therefore, muskmelon seeds could be germinated in soil containing a hypovirulent isolate such as Tx93-449$^+$ in order to help protect the plants when they are transplanted into the field.

The basis for hypovirulence of dsRNA$^+$ *M. cannonballus* was explored by comparing the *in vitro* infection process of a dsRNA$^+$ isolate to that of a virulent dsRNA$^-$ isolate. The fluorescence microscopy data show that the hypovirulent isolate, Tx93-449$^+$, was able to penetrate muskmelon roots, but that it did so at a slow rate and was not able to fully colonize the roots or cause necrosis compared to the virulent isolate, Az90-33$^-$ (Figure 4).

While these data do not clearly identify a mechanism for the observed protection against a virulent isolate, we can suggest several possibilities including competition for infection and colonization sites, triggering plant defenses, and finally dsRNA transfer via anastomosis to the virulent isolate. The dsRNAs in *M. cannonballus* can be transferred from a dsRNA$^+$ to a dsRNA$^-$ isolate by anastomosis *in vitro*, and this transfer results in degeneration and hypovirulence in the recipient isolate (Lovic, 1994; Park, 1996 et al., unpublished). *In planta* dsRNA transmission has been demonstrated for several fungi suggesting that this may be a possible mechanism in our system (Anagnostakis et al., 1998; Lawrence et al., 1988; Melzer and Boland, 1996).

In conclusion, a subset of *M. cannonballus* isolates in our collection were screened and isolates were found that were degenerate, hypovirulent, and harbored dsRNAs ranging in size from approximately 1.5–12 kbp. The sustained presence of dsRNAs, and perhaps specific dsRNA species, rather than the mere presence of dsRNAs *per se*, lead to development of culture degeneration and onset of hypovirulence to muskmelon. *In vitro* fluorescence microscopy

suggested that the basis for hypovirulence was an inability to successfully colonize muskmelon root tissue. Hypovirulent (dsRNA$^+$) isolates may have the potential to be developed into biocontrol agents to reduce losses caused by virulent (dsRNA$^-$) isolates of *M. cannonballus*.

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