Association of dsRNA to Down-Regulation of Perithecial Synthesis in *Monosporascus cannonballus*

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Abstract: *Monosporascus cannonballus* is a soilborne fungal pathogen that causes vine-decline of muskmelon which results in reduced yield in many melon growing areas around the world. *M. cannonballus* isolates were collected from different provinces in Spain and experiments conducted to determine pigmentation, perithecial formation, and the presence of cellular dsRNA. Thirty-one isolates were grouped based on dsRNA fragment sizes using cluster analysis and Euclidean distances. Three distinct dsRNA groupings were observed. Group 2 isolates containing 2, 3, and 3.5 kb dsRNA appeared to exhibit a decrease in perithecia production compared to the other groups. Group 1 isolates exhibited yellow pigmentation only, while Group 3 isolates expressed grey (wild-type) and yellow (degenerate) pigmentation. Isolates that did not contain dsRNA (Group 4) exhibited wild-type pigmentation. Down-regulation and variation in phenotype are hypothesized to be due to dsRNA/iRNA interactions with the fungal transcription mechanisms. Future research will focus on elucidating the mechanisms of regulation by the dsRNA of *M. cannonballus*.

Keywords: Double-stranded RNA, vine-decline, pigmentation, hypovirulence, cucurbits.

INTRODUCTION

In the last twenty-five years, soilborne diseases have become the yield-limiting factor in many cucurbit production areas around the world [1]. *Monosporascus cannonballus* (Pollack and Uecker) is one of the most important soilborne pathogens causing vine decline of muskmelon (*Cucumis melo* L.) and watermelon (*Citrullus lanatus* [Thunb.] Matsum. & Nakai). Mertely *et al.* [2] found that *M. cannonballus* was pathogenic on a broad range of cucurbits. Although the melon disease was first described in Israel, *M. cannonballus* has been reported in United States, Libya, Iran, Japan, Israel, Spain, India, Pakistan, Tunisia, Taiwan, Guatemala, Honduras, Mexico, Saudi Arabia [1], Italy [3], Korea [4], Brazil [5], and Egypt [6]. The disease complex in which *M. cannonballus* is thought to be the primary pathogen is referred to as vine decline, Monosporascus wilt, melon collapse, root rot, or sudden wilt. Melons and watermelons are grown in hot semi-arid to arid regions as well as subtropical environments around the world. High temperatures play a major role in the incidence of vine decline, caused by *M. cannonballus* [7]. As the disease name implies, the wilt of the cucurbit vines occurs suddenly just prior to harvest. Symptoms of the disease include the yellowing and death of the crown leaves that gradually radiates out and kills the vine as the fruit approach maturity (Fig. **1**).

Spain is a major producer of watermelon and melons for the European markets. In 2006, Spain produced 1.04 million metric tons of watermelon and 0.72 million tons of melons [8]. However, since *M. cannonballus* was first identified in Spain [9, 10], melon production has decreased from 61,500 ha in 1990 to 38,873 ha in 2006 [8]. The decrease in melon production has been largely due to various vine decline diseases, especially *M. cannonballus* [10, 11]. Waugh *et al.* [12] noted that the root system of a single mature cantaloupe plant is capable of supporting the production of approximately 400,000 ascospores. Consequently, the fungus has great potential to maintain and/or increase in affected melon fields. Asci with a single ascospore are produced individually within the numerous perithecia on the root (Figs. **2**, **3**) and in culture media. *M. cannonballus* has no other reproductive spore [13]. Initial investigations [14] noted great difficulty in germinating ascospores in the laboratory, therefore, making it difficult to establish the ascospore as the primary inoculum. Staghellini *et al.* [13] were able to demonstrate the germination of ascospores obtained from native soils and plate cultures, which established the role of the ascospore in the etiology of the disease.

Since there is no effective genetic resistance, control of Monosporascus vine-decline is often related to crop rotation thereby reducing ascospore populations and spread in the field. Over the years, numerous other control methods have been attempted with varying degrees of success. These include methyl bromide fumigation [14, 15], soil fungicide [15], biocontrol [16, 17], grafting [15], and plant breeding [14, 15]. The presence of double stranded RNA (dsRNA) in *M. cannonballus* isolates from muskmelon fields in South Texas was found by Lovic *et al.* [18], and was correlated with reduced aggressiveness, degeneration of hyphae, yellow

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Fig. (1). Vine decline of cantaloupe crop caused by *Monosporascus cannonballus*.

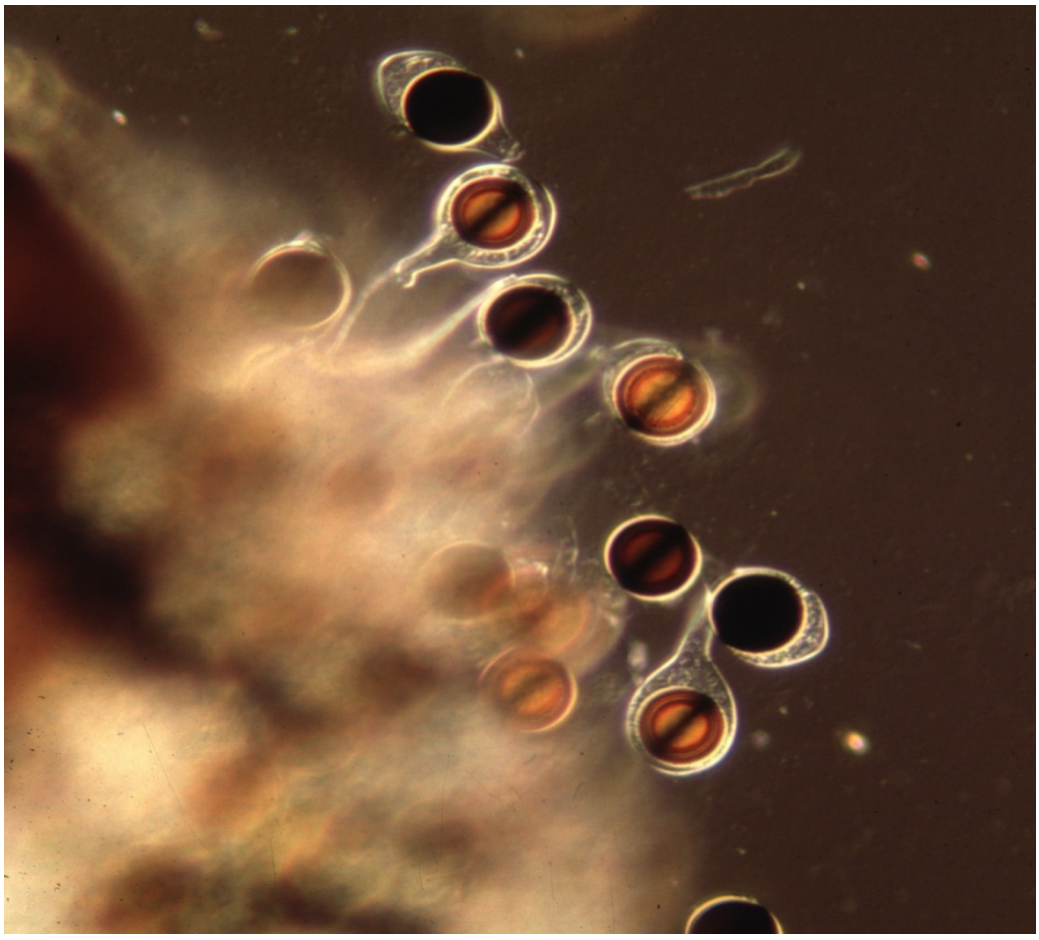

Fig. (2). Crushed perithecium of *Monosporascus cannonballus.* Note that there is a single dark ascospore in each ascus. Each ascospore is approximately 32 microns in diameter. The ascus is 42 microns by 72 microns.

Fig. (3). Perithecia of *Monosporascus cannonballus* on the roots of cantaloupe viewed under a dissecting microscope at approximately 5x magnification. Washed cantaloupe roots reveal an infection of *M. cannonballus* with perithecia showing as black dots on the tan roots.

pigmentation, and reduced perithecial production. The dsRNA profiles among the isolates were diverse among the population, yet stable within an isolate. Wheeler *et al.* [19] reported that degenerate isolates from California, Texas, Honduras, Israel, and Spain did not produce melanin as opposed to wild-type isolates. They further postulated that the loss of fungal melanization in *M. cannonballus* may be associated with loss of virulence in the pigmented isolates. The degenerative effects of dsRNA in *M. cannonballus* have been explored as a potential biocontrol strategy by Batten *et al.* [17].

The purpose of this study was to ascertain the frequency of dsRNA in Spanish isolates of *M. cannonballus* and to compare the presence or absence of dsRNA with production of perithecia and pigment formation. The variation in dsRNA banding patterns was used to group the Spanish *M. cannonballus* isolates into statistically independent dendrogram clusters.

MATERIALS AND METHODOLOGY

Fungal strains, Growth Conditions, Perithecia, and Pigmentation

In 1996, melon fields showing symptoms of vine decline were surveyed in the Spanish Provinces of Castellón and Valencia. Plants were collected from affected fields and taken to the laboratory for isolation. The plants were washed and small sections (~2 mm) of the root exhibiting decay were transferred to Potato Dextrose Agar (PDA) containing 0.5 mg mL^{-1} streptomycin sulphate (PDAS) following one-minute disinfestation in 1% NaOCl. All fungal colonies originating from the roots were hyphal-tipped and transferred to PDA and allowed to grow and/or sporulate for subsequent identification. All *M. cannonballus* isolates were collected and stored in glass vials containing sterilized artificial soil (Terra-Lite, Scotts-Sierra Hort. Products Co., Marysville, OH). The isolates were maintained in the laboratory until further use. In order to recover the fungus, a small portion of the colonized artificial soil mix from each glass vial was transferred to PDA and allowed to grow 5 days under laboratory conditions. These studies were performed in 1998, after 2 years in storage. Another set of experiments were repeated in 1999 with some of the same isolates. The isolates used in 1999 experiments are designated with the original isolate number followed by an "a" (Table **1**). Colonized agar using a #6 corkborer was removed from the leading edge of the mycelial mat, transferred to the center of a PDA plate, and incubated at 25° C. Mycelial color and/or pigmentation of agar was recorded 10 days after hyphal plugs were placed on the agar and designated as either yellow or grey (Fig. **4**). After 30 days, 3 plugs (12 mm diameter) were randomly selected and removed from each of 3 replicates (agar plates) and each plug was considered a replicate (9 plugs per isolate). Each plug was placed between two clear plastic sheets and flattened in order to observe and count the perithecia. Perithecia were observed under a low power stereoscope (Fig. **2**). Each plug consisted of a volume of 0.395 cm^3 and the number of perithecia was expressed in cm^3.

Table 1. Spain Monosporascus cannonballus Isolates Tested for Pigment Production, Perithecia Formation, and dsRNA Bands

Isolate Number	Province	Location	10-Day Pigment	30-Day Perithecia	±	SE*	dsRNA Band Length (kb)	Group
SP96-0287	Castellón	Almenara	Yellow	115.7	±	8.6	11.7, 9	1
SP96-0289	Castellón	Almenara	Yellow	46.1	±	17.2	2.8, 2.3	1
SP96-0333	Valencia	Valencia	Yellow	73.9	±	28.6	2.3, 1.8	1
SP96-0199	Castellón	Almenara	Yellow	48.1	±	17.5	3.5, 2.8, 2.6, 2.3, 1.8	2
SP96-0199a**	Castellón	Almenara	Yellow		0		3.5, 2.8, 2.6, 2.3, 1.8	2
SP96-0234	Castellón	Chilches	Grey		0		9, 5.5, 3.5, 2.8, 2.6	2
SP96-0234a	Castellón	Chilches	Yellow		0		9, 5.5, 3.5, 2.8, 2.6	2
SP96-382	Valencia	El Romani	Yellow		0		9, 5.5, 3.5, 2.8, 2.6, 1.8	2
SP96-0329	Castellón	Almenara	Yellow	30.6	±	10.6	3.5, 2.8, 2.6, 2.3	2
SP96-0186	Castellón	Almenara	Yellow		0		9, 3.5, 2.8	2
SP96-0240	Castellón	Chilches	Grey	10.9	±	5.3	3.5, 2.8, 1.8	2
SP96-0240a	Castellón	Chilches	Yellow		0		3.5, 2.8, 1.8	2
SP96-0340	Valencia	Valencia	Yellow		0		3.5, 2.8, 2.6	2
SP96-0248	Castellón	Almenara	Grey	20.8	±	12.4	3.5, 2.8, 2.6	2
SP96-0260	Castellón	Almenara	Grey	6.1	±	6.1	3.5, 2.8, 2.6	2
SP96-0436	Valencia	Silla	Yellow	85	±	29.6	9, 3.5, 2.8, 2.6	2
SP96-0352	Valencia	El Romani	Yellow	11.6	±	11.6	3.5, 2.8, 2.6	2
SP96-0232	Castellón	Chilches	Yellow	2.27	±	1.8	9, 5.5, 3.5, 2.8, 2.6, 2.3, 1.8	3
SP96-0232a	Castellón	Chilches	Yellow	1	±	0.75	9, 5.5, 3.5, 2.8, 2.6, 2.3, 1.8	3
SP96-0274	Castellón	Almenara	Grey	191.9	±	22.5	9, 3.5, 2.8, 2.6, 2.3, 1.8, 1.5, 1.3	3
SP96-0311	Castellón	Almenara	Grey	74.1	±	7.7	9, 2.8, 2.6, 2.3, 1.8, 1.5	3
SP96-0311a	Castellón	Almenara	Yellow		0		9, 2.8, 2.6, 2.3, 1.8, 1.5	3
SP96-0335	Valencia	Valencia	Grey	1.39	±	1.39	3.5, 2.8, 2.6, 2.3, 1.8, 1.5	3
SP96-0335a	Valencia	Valencia	Yellow	19.4	±	7.4	3.5, 2.8, 2.6, 2.3, 1.8, 1.5	3
SP96-0182	Castellón	Almenara	Grey	26.3	±	26.3	3.5,2.8,2.6,2.3,1.8,1.5	3
SP96-0182a	Castellón	Almenara	Yellow	130.4	±	26.8	3.5,2.8,2.6,2.3,1.8,1.5	3
SP96-0188	Castellón	Almenara	Grey		0		9, 3.5, 2.8, 2.6, 2.3, 1.8	3
SP96-0192	Castellón	Almenara	Yellow	102	±	40.7	3.5, 2.8, 2.6, 2.3, 1.8, 1.5	3
SP96-0210	Castellón	Almenara	Grey	0.25	±	0.25	3.5, 2.8, 2.6, 2.3, 1.5	3
SP96-0210 a	Castellón	Almenara	Yellow	44.8	±	24.3	3.5, 2.8, 2.6, 2.3, 1.5	3
SP96-0323	Castellón	Almenara	Grey	146.8	±	46.8	3.5, 2.6, 2.3, 1.8, 1.5	3
SP96-0244	Castellón	Almenara	Yellow	22.2	±	12.4	9, 3.5, 2.8, 2.6, 2.3, 1.8	3
SP96-0325	Castellón	Almenara	Grey	216.7	±	22.7	9, 2.8, 2.3, 1.8, 1.5	3
SP96-0313	Castellón	Almenara	Yellow	45.1	±	17.4	3.5, 2.8, 2.6, 1.8, 1.5	3
SP96-0313a	Castellón	Almenara	Grey	334.4	±	9.6	3.5, 2.8, 2.6, 1.8, 1.5	3
SP96-0220	Castellón	Chilches	Yellow	43	±	16.7	9, 3.5, 2.86, 2.6, 2.3, 1.8	3
SP96-0236	Castellón	Chilches	Grey	17	±	6.8	9, 3.5, 2.8, 2.6, 2.3, 1.8, 1.5	3
SP96-0222	Castellón	Chilches	Grey	38	±	15.7	9,5.5,3.5,2.8, 2.3, 1.8, 1.5	3
SP96-0374	Valencia	El Romani	Grey	89.1	±	6.6	3.5, 2.8, 2.6, 2.3, 1.8, 1.5	3
SP96-0206	Castellón	Almenara	Grey	89.9	±	6.6	ND****	4***
SP96-0246	Castellón	Almenara	Grey	17	±	9.1	ND	4
SP96-0224	Castellón	Chilches	Grey	21.3	±	11.4	ND	4

*The mean perithecia count per cm^3 is followed by the standard error of the mean.

**Isolates with the letter "a" represent isolates stored in soil tubes for 6 months after the original set of experiments. Experiments were repeated with the older isolates and were included as additional isolates due to variation observed in pigmentation and perithecia production.

*** Group 4 perithecia data was included in Fig. (**7**). This group did not have detectable dsRNA.

****ND= not detected.

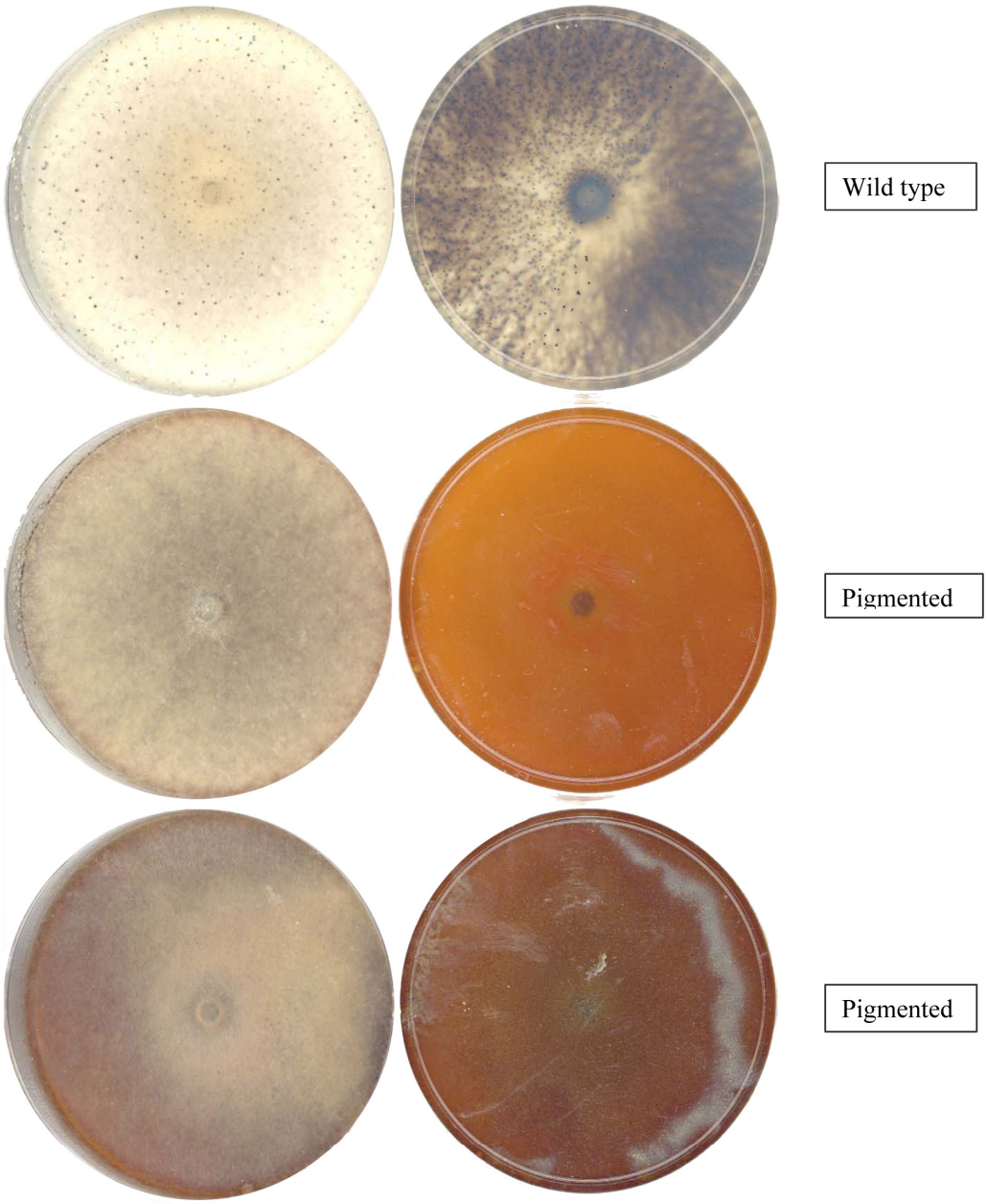

Fig. (4). Pigmentation and perithecial differences among wild-type (top two plates) and degenerate cultures (bottom four plates) of *Monosporascus cannonballus*. Agar plates on the left are topical views while the plates on the right are views from the bottom of the Petri dish.

dsRNA Isolation and Separation

In order to identify and characterize banding patterns of dsRNA, 4-5 plugs (12 mm diameter) of 4-day old fungal cultures were placed in 300 mL of minimal salts media (10 g glucose, 10 g sorbitol, 0.25 g $MgSO_4$ x $7H_2O$, 1.5 g KH_2PO_4, 2 g KNO_3, 1 g yeast extract and 1 L distilled H_2O). The 500 mL flasks containing the individual fungal isolates and broth were placed on a shaker rotating at 60 rpm at 25°C. After 31 days, mycelial mats were drained, pressed dry using absorbent towels, weighed, and stored at -20° C. Two grams of mycelia were ground in 4.5 ml of STE (100 mM NaCl, 50 mM TRIS base, 1 mM EDTA, 500 µl of 10% SDS) buffer, pH 8.0. All isolates exhibited grey wild-type growth habit when originally isolated from plant material. Other workers have observed pigmentation and degeneration in culture over time (Bruton, personal communication). As mentioned above, in order to test whether isolates degenerated in soil tube cultures, several isolates were re-tested in 1999. These isolates are designated with the original number but have an "a" added.

Nucleic acids were purified by adding a 6 ml aliquot of phenol:chloroform:isoamyl alcohol (25:24:1) to the ground fungus. The solution was mixed and centrifuged for 5 min at 6000 *g*. Chloroform:isoamyl alcohol (24:1) was added to the aqueous phase. Cold (-20°C) isopropyl alcohol was added to the aqueous phase of this solution resulting in precipitation of nucleic acids. The solution was centrifuged 15 minutes at 10,000 *g* and the supernatant decanted. The pellet was suspended in 70% ethanol and centrifuged for 10 min at 10,000 *g*. The nucleic acid pellet was dried at 25°C and re-suspended

in 4.25 ml STE buffer. Enough absolute ethanol was added to make a 15% ethanol solution. The suspension was applied to a CF-11 cellulose column that had been saturated with 15% ethanol in STE buffer. Excess nucleic acid was eluted with 4 washes of 15% ethanol in STE buffer. The dsRNA that was retained by the column matrix was eluted with 2 ml aliquots of absolute ethanol. DsRNA was precipitated using 9 ml of absolute ethanol and 0.45 ml of 3 M Na acetate, then centrifuged for 15 min at 10,000 *g*. The pellet was rinsed in 1 ml of 70% ethanol and centrifuged 10 min at 10,000 *g*. The dried pellet was re-suspended in 60 µl of 0.1 M, TE buffer, pH 8. Fractions that contained dsRNA were applied to 1% agarose gels in STE running buffer and electrophoresis was conducted for 3 hr at 60 V. Standards used were λ cut with HIND and a 1 kb DNA ladder. Gels were immediately stained with ethidium bromide (2 µl per gel) and visualized with UV light. Polaroid film was used to record band migration patterns in each gel.

Statistics

All experiments were conducted at least 2 times. Tukey-Kramer multiple comparison tests, unpaired t-tests, and One-way ANOVA were conducted using INstat Graphpad. A factor analysis using principle components analysis with a varimax rotation method (SPSS, 15.0) was performed to reveal independent grouping of banding patterns (dsRNA) based on underlying relationships.

RESULTS

Presence of dsRNA in Spanish Isolates

Thirty-nine of 42 *M. cannonballus* isolates (93%) from Spain had dsRNA (Table **1**). However, the 39 isolates varied considerably in their dsRNA banding patterns (Figs. **5**, **6**). Nine distinct band locations, ranging from 1.3 to 11.7 kb, were observed among the isolates, with 19 different patterns (Fig. **6**). The factor analysis gave four Eigen values greater than 1. The Scree plot indicated a three or four factor solution. A hierarchical cluster analysis for binary data using simple matching indicated that a four factor solution would place isolate SP96-0287 by itself. Based on this information, a three factor model which explained 62% of the variation was selected. The initial split with respect to the cluster analysis removes isolate SP96-0289 (2.3 and 2.8 kb dsRNA bands), isolate SP96-0333 (1.8 and 2.3 kb bands), and isolate SP96-0287 (9 and 11.7 kb bands) (Group 1, Fig. **7**, Table **1**). This group's shared characteristics are the total lack of certain bands, such as 2.6 kb. A second group shows more commonality since all of the isolates except one (SP96-0240) contain 2.3, 2.8 and 3.5 kb dsRNA bands. The isolate SP96-0240 has 1.8, 2.8 and 3.5 kb bands. The characteristic that separates this group from the final group is the lack of additional bands (Group 2, Fig. **7**, Table **1**). The final group shares 3.5, 2.8 and 2.6 kb bands, although SP96-0311 lacks a 3.5 kb band and SP96-0325 lacks a 3.5 kb band (Group 3, Fig. **7**, Table **1**). In addition, all but four isolates, SP96-0220, SP96-0244, SP96-0188, and SP96-0232 have a 1.5 kb band; all but one, SP96-0210, has a 1.8 kb band; and all but one (SP96-0313) has a 2.3 kb band. The commonality in Group 3 is the presence of at least two of the 1.5, 1.8, or 2.3 kb bands. Most of the isolates in this group have all three. A major subgroup of the final group has a 9 kb band appearing. The 9 kb band was present in isolates SP96-0236, SP96-0303, SP96-0222, SP96-0220, SP96-0244, SP96-0188, SP96-0274, SP96-0232, SP96-0311, and SP96-0325. The 9 kb dsRNA does not appear in isolates SP96-0335, SP96-0182, SP96-0192, SP96-0323, SP96-0313, and SP96-0210. Since the factor analysis indicated that a simpler classification was possible, a cluster analysis was used to determine groupings. The cluster analysis indicated three groups of banding patterns based on squared Euclidean distances. A hierarchical cluster analysis shown in the dendrogram (Fig. **7**) indicates the relatedness among the isolates based upon the similarity of their dsRNA banding patterns with distances used as an indication of degree of difference. Groups 1, 2, and 3 are shown on the dendrogram (Fig. **7**), and they correspond to the same groups in the cluster analysis. Group 4 (Table **1** and Fig. **8**) includes the *M. cannonballus* isolates that did not have any detectable dsRNA.

Initial experiments on all isolates were conducted in 1998. Approximately, 6 months later, 9 of the isolates were again grown from soil tube cultures on PDA. Experiments were repeated on these isolates, and the dsRNA banding patterns appeared to be consistent with the initial set of experiments. However, perithecia production and pigmentation varied from the initial experiments (Table **1**). Six of the 9 isolates changed from grey to yellow pigmentation after the storage interval. Perithecial production increased or decreased in individual isolates over time; however, perithecial production among the stored isolates did not show any significant difference ($P \leq 0.05$) when compared to initial experiments. These experiments suggest that pigmentation may be a function of length of storage and not necessarily associated with reduced perithecial production or the presence of dsRNA.

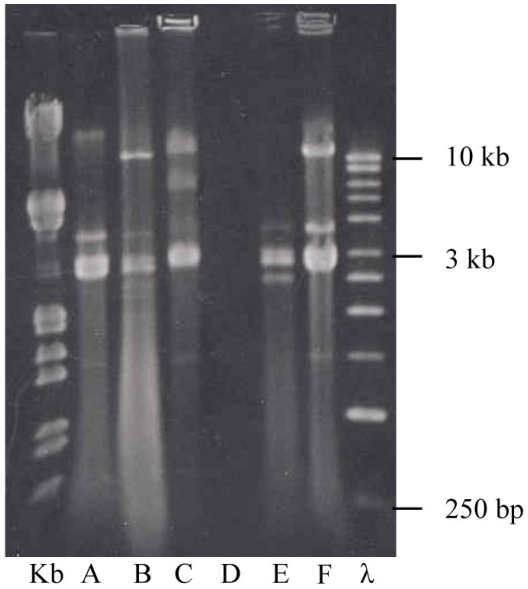

Fig. (5). dsRNA of representative *Monosporascus cannonballus* isolates from Spain. The letters represent the isolates as follows: A = SP96-0 186; B = SP96-0 190; C = SP96-0 206; D = SP96-0 234; E = SP96-0 244; F = SP96-0 248.

Pigmentation and Perithecia Production

Pigment (yellow) formation varied among and within the four groups of isolates (Table **1**, Fig. **4**). In Group 1, all 3

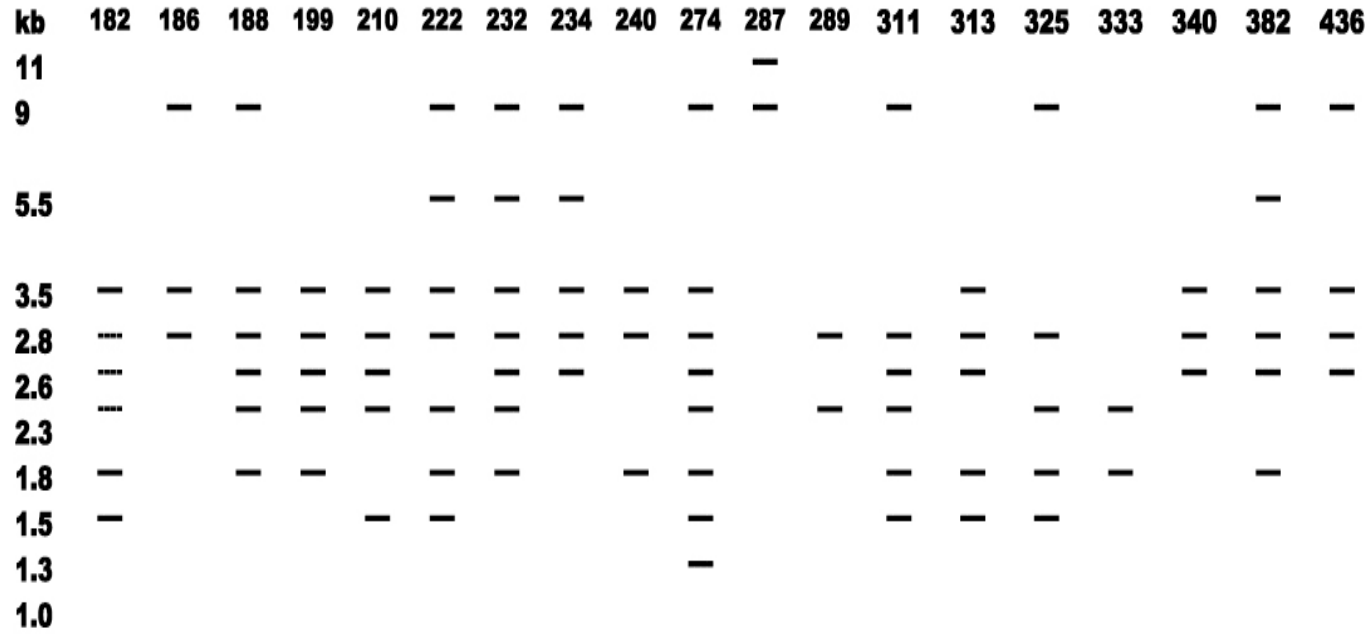

Fig. (6). dsRNA banding patterns of *Monosporascus cannonballus* from Spain. Numbers across the top represent last three digits of isolates in Table **1**.

isolates produced yellow pigment. In Group 2, four isolates produced wild-type grey pigment and 10 produced yellow pigment. Group 3 contained 12 isolates with grey wild-type pigmentation and 10 with yellow pigmentation. Group 4 contained 3 grey isolates. Perithecia production also varied among the four groups of isolates (Fig. **8**). All group 1 isolates produced perithecia and all were yellow pigmented as previously stated. In group 2, 7 isolates produced perithecia while 7 did not. Twenty isolates from group 3 produced perithecia while 2 did not. In Group 4 (no dsRNA detected) all isolates produced perithecia. The average number of perithecia for Group 1 was 79, Group 2 was 15, and Group 3 was 70, and Group 4 was 43 perithecia/cm^3 after 30 days incubation (Fig. **8**, Table **1**). Unpaired t-test between the individual groups indicated that Group 1 and 3 produced more perithecia than Group 2. Group 4 was not significantly different than Group 2 in perithecia production ($P \leq 0.05$).

The Group 2 banding patterns appeared to be associated with reduced perithecia production. The mere presence of dsRNA in the Spanish isolates of *M. cannonballus* investigated in this study was not necessarily associated with pigmentation or perithecia production. The isolate that was dark grey (no yellow pigmentation) had the highest numbers of perithecia, however, the other lighter grey isolates produced similar numbers of perithecia as the yellow pigmented isolates.

DISCUSSION

Based on results of this study, there is no relationship between the mere presence of dsRNA in Spanish *M. cannonballus* isolates and yellow pigmentation or perithecial production. Ninety-three percent of the Spanish isolates harbored dsRNA, with 2 to 8 bands each and a size range of 1.3 to 11.7 kb. However, certain dsRNAs did appear to be associated with perithecia production. Whereas two dsRNA banding patterns (Groups 1 and 3) had no apparent effect on perithecial production in the Spanish isolates, Group 2 *M. cannonballus* isolates did exhibit reduced perithecial production. Consequently, the dsRNA bands 3.5, 2.8 and 2.6 kb are implicated in quelling (down-regulation) of perithecial production. Interestingly, 37% of the isolates harboring dsRNA were Group 2 isolates consisting of both grey (wild-type) and yellow-pigmented strains. In a 1995 study [18], 300 *M. cannonballus* isolates were collected from a 6-hectare muskmelon field in Texas. Sixty-five percent of the isolates contained from 1 to 13 dsRNA bands with a size range of 1.7 to 15 kb. DsRNA was associated with hyphal degeneration and reduction in perithecia production. Martyn and Miller [14] concluded that isolates with the most pronounced variation in pigmentation, reduced growth rate, and reduced perithecial production harbored from one to four low molecular weight genetic elements that ranged in size from 1.9 to 3.5 kb. Batten *et al.* [20] reported that 60% of their *M. cannonballus* collection from the United States, Israel, Mexico, Japan and Spain tested positive for dsRNA with a size range for the dsRNA segments of 1.5 to 12 kb. The size range and the occurrence of *M. cannonballus* dsRNA was very similar when comparing isolates from the United States, Israel, Mexico, Japan [20] and the Spanish *M. cannonballus* isolates used in the present study. As in *Rhizoctonia solani* [21], dsRNA appears to be ubiquitous within the *M. cannonballus* population. The variation in phenotypes that occurs when the dsRNAs are carried by *M. cannonballus* suggests a range in up- or down-regulation as seen in other pathogen systems [22, 23].

In regard to pigmentation, grey wild-type pigment was observed in 41% of the isolates with the presence of dsRNA, indicating that the dsRNA was not the sole determinant in pigment synthesis in the present study. In addition, yellow pigmented and grey wild-type isolates harbored dsRNA. Although dsRNA has been found in several fungal species, it

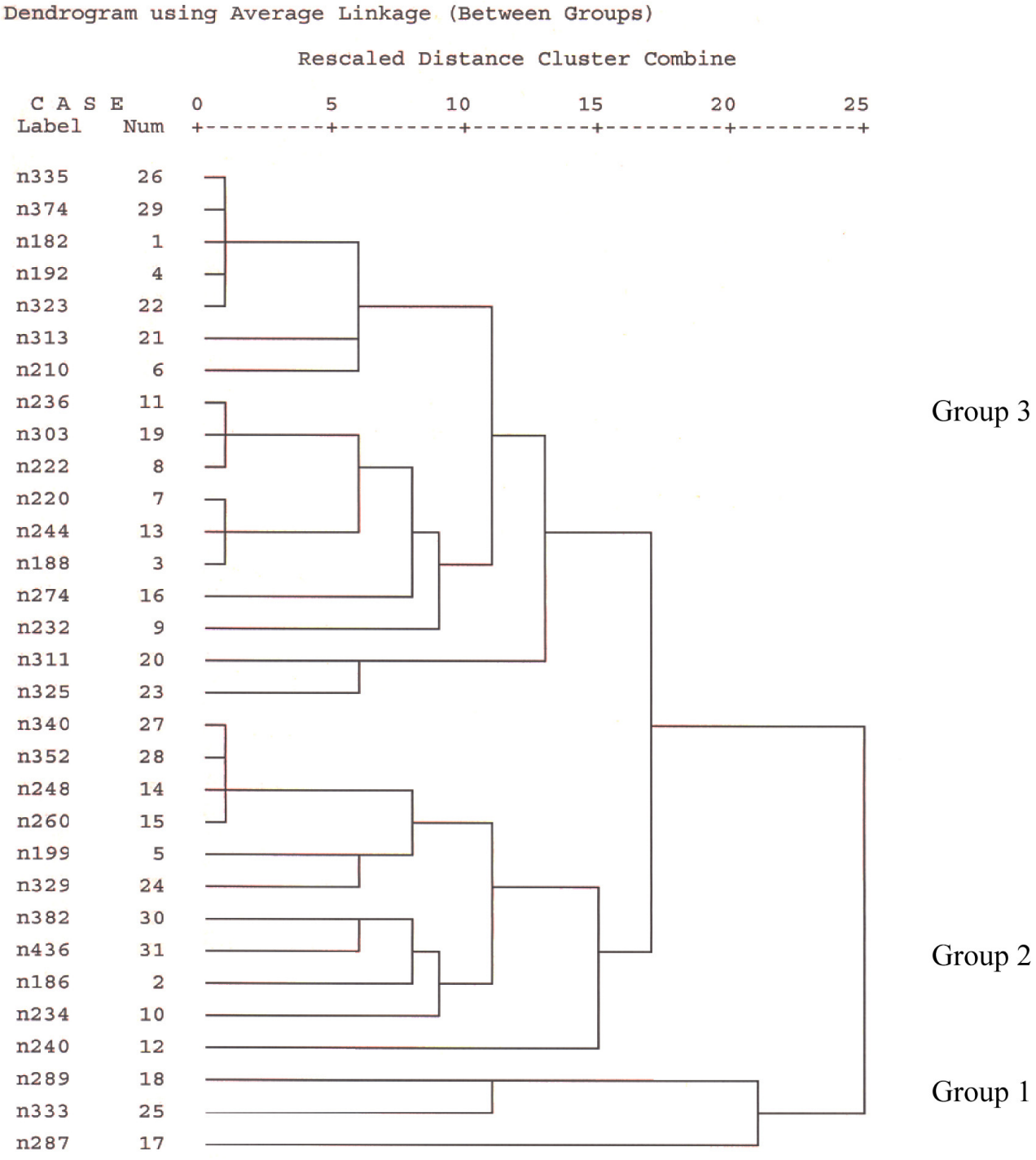

Fig. (7). Dendrogram of hierarchical cluster analysis based on squared Euclidean distances dsRNA banding patterns observed in the *Monosporascus cannonballus* Spain population. Isolates with the "a" designation (Table **1**) were not included in the cluster analysis or the dendogram.

usually does not affect the fungal phenotype [24]. In contrast, dsRNA does regulate phenotype in some fungi. DsRNA was horizontally transferred in *Beauveria bassina* and resulted in reduced virulence and conidial production [25]. The fungal pathogen *Cryphonectria parasitica*, causal agent of chestnut blight, has exhibited reduced virulence when found in association with dsRNA, also referred to as hypoviruses [26]. Texas strains of *M. cannonballus* with dsRNA were found to be associated with aberration of cultural phenotypes and hypovirulence [27]. In that study, cultures were cured of the virus (dsRNA) and compared with wild-type and dsRNA infected strains. There was no difference in the level of disease on muskmelon when comparing dsRNA-cured and a wild-type isolate without dsRNA, whereas, a strain with dsRNA did not cause significant levels of disease [27]. Wheeler *et al.* [19] suggested that the loss of fungal melanization in *M. cannonballus* may be associated with hypovirulence in the pigmented isolates.

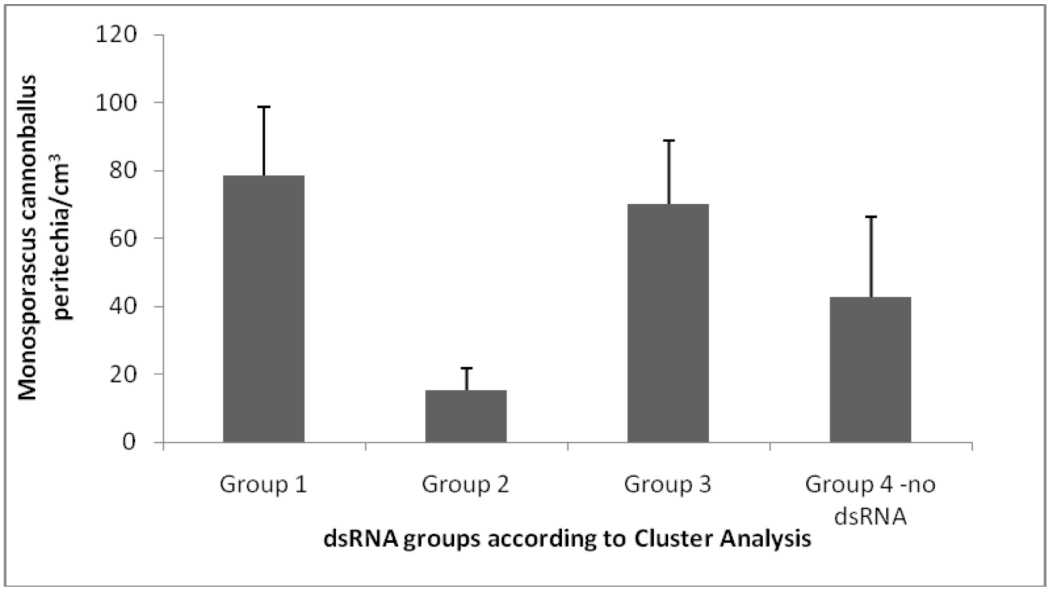

Fig. (8). Average perithecial production of the isolates according to dendrogram groups (Fig. **7**). Vertical bars represent standard error of the means.

The degenerative effects of dsRNA in *M. cannonballus* were exploited as a biocontrol strategy by Batten *et al.* [17]. However, they did not find a strong correlation between hypovirulence and cultural degeneration or virulence in *M. cannonballus* isolates. Although we did not test the virulence of the isolates used in this study, we also found no relationship between the presence of dsRNA or pigment production. In Group 2, the specific dsRNA bands appeared to be associated with lower perithecial production when compared to the other dsRNA groups.

DsRNA plays a major role in gene silencing within all known groups of organisms [28]. High prevalence of dsRNA in *M. cannonballus* populations and the variation in fungal response to dsRNA suggest that siRNA cleaved from the dsRNA may be playing a regulatory role in *M. cannonballus* growth and development. In the last 10 years, much research has been devoted to exploring the role of dsRNA cleaved products referred to as interference RNA (RNAi) or small interfering RNAs (siRNAs). The dsRNA is cleaved into small 21- to 26-nt interfering RNAs by a ribonuclease III-like enzyme commonly called Dicer [29]. The short interfering RNAs (siRNA) are then incorporated into a protein complex (RNA-inducing silencing complex [RISC)]) that uses the siRNA to guide and bind to homologous mRNA. When bound to the mRNA transcript, RNAi down-regulates the translation by cleaving the mRNA [30]. The silencing of the message by RNAi occurs by inhibiting translation of mRNA, destruction of mRNA or interfering with the promoter of that particular mRNA [31]. A group of RNA silencing proteins, including Dicers, Argonautes, and RNA-dependent RNA polymerases (RdRPs), are present throughout the eukaryote domain and are highly conserved [32]. The RNA silencing proteins are similar in most fungi, however, comparative genomics did not find RNA silencing proteins in *Saccharomyces cerevisiae, Candida guilliermondii*, and *C. lusitaniae* and the basidiomycete *Ustilago maydis* [28]. The range of roles these siRNA's play in the cell is still unknown, but in eukaryotes siRNA regulates development, protects the genome, modifies chromatin, and protects against viruses [33-35]. Small RNAs are distinguished based on their origins. The two most recognized are microRNA (mi-RNA) and si-RNA. Both are segments from a longer dsRNA. The si-RNA is artificially made or produced *in vivo*, by the general procedure described above. The mi-RNAs are encoded by genes within the cell and play regulatory roles. Over 5,000 miRNA sequences from eukaryotic genes have been identified [36]. However, miRNA-directed silencing pathways have not been identified in fungi [28].

Several fungi have been used to elucidate genetic information such as *Neurospora crassa, Saccharromyces cerevisiae, Schizosaccharomyces pombe*, and *Aspergillus nidulans* [28]. The gene silencing phenomenon was first observed in *N. crassa* in 1992 [37]. Recent work with *A. nidulans* shows that mycoviruses can inhibit RNA silencing or be converted to siRNA that inhibits mycovirus activity in the cell [38]. RNA silencing has also been demonstrated in *Magnaporthe oryzae* [39] and the human pathogen *Cryptococcus neoformans* [40]. Perhaps the variation in *M. cannonballus* pigmentation, perithecia production, and virulence [18] is because of dsRNA/RNAi post-transcriptional regulation. Molecular characterization of the dsRNA of *M. cannonballus* and cDNA probes to detect the mycoviruses has been accomplished [20]. Recently, quantitative detection of *M. cannonballus* using RT-PCR was developed [41]. The molecular tools are now available to determine if siRNA plays a role in the hypovirulence of *M. cannonballus*, and whether *M. cannonballus* has different gene silencing mechanisms that result in the variable responses of the fungus in the presence of dsRNA. Exogenous application of hypoviruses or viral cDNA has resulted in gene silencing of the complementary sequence of the hypovirus [42]. Insertion of the *M. cannonballus* hypovirus sequences in the plant genome holds the potential for a new resistance mechanism against plant pathogens. Applications for biocontrol and gene regulation in plants, fungi, and animals using dsRNA/RNAi technologies are already being realized [39, 43].

CONCLUSIONS

DsRNA appears to be ubiquitous in the *M. cannonballus* populations. As observed in other fungal species, the presence of dsRNA does not always cause degeneration of the isolate, reduce perithecia production, or alter virulence. The Spanish *M. cannonballus* isolates investigated in this study shows similar band numbers and size range as other researchers. When considering perithecial production, the cluster group 2 isolates had less perithecia produced than the other groups. Further work is needed to determine whether this combination of dsRNAs plays a definitive role in perithecia down-regulation. In light of the variation in phenotypes within a population of *M. cannonballus* and the prevalence of dsRNA, we hypothesize that dsRNA may be cleaved into si-RNAs that match mRNA specific sequences, therefore, interrupting post-transcriptional interactions. Currently there are no resistant cultivars to *M. cannonballus* and effective soil fumigants are restricted or banned in many melon producing countries. An understanding of factors that influence perithecia/ascospore formation could be useful in the development of successful control strategies for *M. cannonballus.* RNAi technology has the potential to enhance resistance of plants to *M. cannonballus,* reduce perithecial production, and therefore, disease potential of the pathogen. Future research will focus on elucidating the mechanisms of regulation by the dsRNA of *M. cannonballus*.

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