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Monosporascus Root Rot and Vine Decline

An Emerging Disease of Melons Worldwide

The *Cucurbitaceae* plant family provides us with numerous edible products. The flesh is an important food source of carbohydrates and water for many people of the tropical and semiarid climates. Seeds, rich in oil and proteins, are important foods as well. Texas is a major producer of cucurbits, primarily watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai) and muskmelon (*Cucumis melo* L.). Approximately 40,000 ha of cucurbits are grown yearly in Texas. Watermelons (ca. 24,000 ha) are grown throughout Texas, but major commercial production is concentrated in the eastern, southwestern (winter garden), and southern portions of the state. Muskmelons (ca. 10,000 ha), however, are grown primarily in the Lower Rio Grande Valley of south Texas, although scattered production occurs elsewhere, notably in the Trans-Pecos area of west Texas. The average annual production value of watermelon and muskmelon alone in Texas is over $300 million. The continuous and intensive cultivation of these crops has resulted in an increase in the number and severity of soilborne diseases which, in turn, has accounted for substantial economic losses.

In recent years, a group of soilborne diseases known as vine declines has become prevalent and causes serious economic losses in melons (3,38). Vine decline is a generic term applied to a group of diseases with similar symptoms but different causal agents. General symptoms of this group of diseases include yellowing and death of the crown leaves and a gradual decline of the vine as the plant approaches maturity. A rapid collapse of the vine typically occurs just before harvest. Fruits from affected plants are more subject to sunburn and more likely to be low in sugars and to abscise from the pedicle before ripening (full slip). Vine decline diseases are most severe under conditions that tend to stress the plant, e.g., heat, drought, fruit load, and other biotic agents such as insect feeding. Examples of vine decline diseases of melons include gummy stem blight (*Didymella bryoniae* (Auersw.) Rehm), charcoal rot (*Macrophomina phaseolina* (Tassi) Goidanich), purple stem (*Diaporthe melonis* Baraha & O'Brien), Botrydiplodia decline (*Botrydiplodia theobromae*), and most recently, Monosporascus root rot and vine decline (*Monosporascus cannonballus* Pollack & Uecker).

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A New Vine Decline Disease in Texas

In the mid-1980s, a new vine decline disease of muskmelon was observed in the Lower Rio Grande Valley of Texas (5). Aboveground symptoms included stunting, yellowing, and necrosis of the inner crown leaves, followed by progressive necrosis of the leaves (Fig. 1). Approximately 10 to 14 days before harvest, the entire canopy may collapse, exposing fruit to intense solar radiation (Fig. 2). There are no distinctive lesions on runners or petioles. Belowground symptoms include root lesions, particularly at root junctions (Fig. 3), loss of secondary and tertiary feeder roots, and under wet conditions, a secondary root rot and death of the tap root (Fig. 4). *Fusarium* spp. initially were implicated in this disease (4,6). Several of the symptoms resemble those of crown and foot rot, caused by *F. solani* (Mart.) Sacc. f. sp. *cucurbitae* W.C. Snyder & H.N. Hans.; and isolations from roots of diseased plants consistently yielded *F. solani*. In addition, the discovery of *F. oxysporum* Schlechtend.:Fr. f. sp. *melonis* W.C. Snyder & H.N. Hans., causal agent of Fusarium wilt of muskmelon in the Lower Rio Grande Valley (24), coincided with the appearance of this vine decline. In a 2-year study, however, numerous isolates of *F. solani* and *F. oxysporum* recovered from diseased plants failed to reproduce all of the symptoms of this new disease, either alone or in combination (4), and *Fusarium* was ruled out as a causal agent. In 1990, the causal agent was identified as the little-known soilborne ascomycete *M. cannonballus*, and the disease was named Monosporascus root rot and vine decline (29,31).

Economically significant losses due to Monosporascus root rot and vine decline were first noticed in Texas in 1986 in the Lower Rio Grande Valley, but it is probable that losses occurred prior to this and were either undetected or attributed to other causes. The disease persists and continues to be a major constraint to melon production in some major commercial areas. Losses fluctuate year to year from approximately 10 to 25% of the crop, although individual fields may suffer 100% loss. Similar losses have been reported from southern Spain (15–17) and Israel (13,47,48).

Fig. 1. Early stages of yellowing and necrosis of the leaves of muskmelon infected with *Monosporascus cannonballus*.

Fig. 2. Late stage of Monosporascus root rot and vine decline of muskmelon showing complete collapse of the canopy and exposure of the fruit to solar radiation.

Taxonomy of *Monosporascus* spp.

The genus *Monosporascus* was described by Pollack and Uecker (46) in 1974 based on specimens obtained from necrotic muskmelon roots obtained from Troutman and Matejka (56) in Arizona. Pathogenicity was suggested based on greenhouse studies; however, no details were provided in this brief report. *Monosporascus* was described as a genus et species novus, and *M. cannonballus* was established as the holotype specimen. The main features of *M. cannonballus* were: (i) it formed only one large, spherical ascospore per ascus (rarely two) that did not germinate, and (ii) there was no known anamorph. They noted that it had some similarities to two other pyrenomycetes, *Rechingeriella eutypoides* Petr. n. spec. (45) and *Anixiella monospora* Malloch & Cain, sp. nov. (23). The same year Pollack and Uecker described *M. cannonballus*, Sivanesan et al. (52) erected another new genus and species, *Bitrimonospora indica* Sivanesan, Talde & Tilak, based on an isolate obtained from surface-sterilized roots of *Achyranthes aspera*. It was distinguished from *M. cannonballus* in having predominantly two ascospores per ascus that were slightly smaller, but which germinated readily via multiple germ tubes. Pathogenicity of this species was not determined. In 1976, von Arx (64) transferred *R. eutypoides* into *Monosporascus*, creating a second species, *M. eutypoides* (Petrak) von Arx, and also reduced *B. indica* to synonymy with *M. eutypoides*. The two species were thus separated on the basis of the number and germinability of ascospores: *M. cannonballus* had one spore that did not germinate and *M. eutypoides* had two spores that germinated readily. In 1978, Hawksworth and Ciccarone (10) transferred *Anixiella monospora* into *Monosporascus*, creating a third species, *M. monosporus* (Malloch & Cain) comb. nov.; however, no known culture of *M. monosporus* exists. This species was described from a herbarium specimen of an *Iris* sp. rhizome from Iran (23) and may not be a valid description and transfer. Since the species name *monospora* suggests only one ascospore, it actually may have been *M. cannonballus*. Most researchers who are familiar with this fungus recognize only two species, *M. cannonballus* and *M. eutypoides;* although, as we show later, they may be conspecific.

Fig. 3. Cantaloupe (muskmelon) roots infected with *Monosporascus cannonballus* showing discrete lesions and lack of secondary and tertiary roots.

Biology, Pathology, and Epidemiology

Monosporascus spp. received little attention from either mycologists or plant pathologists until recently. The only cytological study of the fungus was conducted by Uecker and Pollack on *M. cannonballus* in 1975 (58). They described it as a pyrenomycete and somewhat unique among ascomycetes. It is apparently homothallic and produces fertile perithecia in both host roots (Figs. 5 and 6) and culture (Fig. 7A). Instead of the usual eight ascospores, however, it forms only one large (38 to 50 µm diameter) ascospore per ascus (Fig. 7D). When mature, the spores are dark brown or

Fig. 4. Secondary root rot associated with Monosporascus root rot and vine decline under extended wet periods.

Fig. 5. Roots of muskmelon plant grown in *Monosporascus*-infested soil in the greenhouse showing numerous perithecia.

Fig. 6. Close-up of perithecia in muskmelon root.

black and resemble cannonballs (Fig. 7F), but as they mature they pass through several hyaline and light-refractive stages (Fig. 7B and C). Ascospores are slightly smaller when formed in vitro on laboratory media (42) and have a density of 1.23 to 1.28 in sucrose (27). The spores have two distinct walls. The heavily pigmented outer wall (epispore) is multilayered (Fig. 7B to D) and accounts for 15 to 20% of the spore diameter. It is distinguishable from the thicker hyaline inner wall. Ascospores are multinucleate and may contain 1 to 16 nuclei per spore, but typically have eight nuclei at maturity (58). They rarely germinate under laboratory conditions (Fig. 7E) (27,65).

The perithecia are essentially spherical with a short neck and are embedded in the root cortex (Fig. 6). At times, perithecial production is extremely abundant, resulting in densities of over 100 per cm of necrotic root (15). For this reason, the disease has been referred to as black pepper spot or black spot root rot in Japan (62). Perithecia may contain 200 or more asci each, and when ruptured (Fig. 8) they release the spores into the soil. No conidial (anamorph) stage is known. As described (50,51), the primary difference between *M. cannonballus* and *M. eutypoides* is in the number and germinability of the ascospores: *M. cannonballus* has one spore (rarely two) that does not germinate (or does so rarely) in the laboratory; whereas *M. eutypoides* has two spores (rarely one) that germinate via multiple germ tubes. We have examined cultures identified as both species and, with only rare exceptions, they contained one spore, which did not germinate.

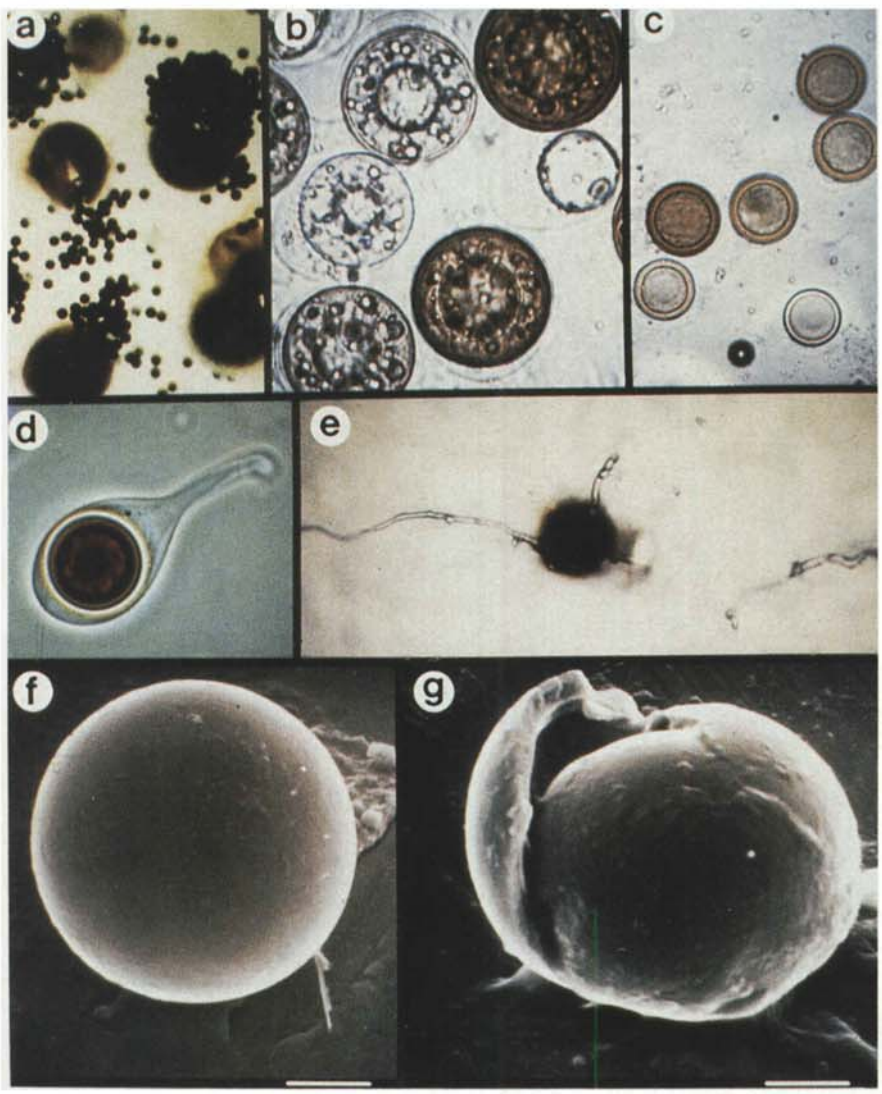

Fig. 7. *Monosporascus cannonballus* ascospores. (A) Numerous black ascospores discharged from perithecia formed on potato-dextrose agar; (B) Light microscopy (40×) of immature ascospores showing light-refractive stage; (C) Light microscopy (20×) of ascospore showing the two distinct spore walls; (D) Almost-mature ascospore within an ascus; (E) 6-month-old ascospore of *Monosporascus cannonballus* that was induced to germinate after heating to 45°C for 10 min; (F) Scanning electron micrograph of ascospore showing smooth external appearance (scale bar = 10 µm); (G) Scanning electron micrograph of a crushed ascospore showing the separation of the external wall from the inner wall (scale bar = 10 µm).

The report of *M. cannonballus* on melon (*C. melo*) roots in Japan in 1978 (65) may be the first concerted attempt to prove pathogenicity, although several earlier reports allude to its pathogenicity (10,52,56). However, in this report, pathogenicity was not demonstrated under the conditions tested. To our knowledge, the first report to confirm pathogenicity of *Monosporascus* was from Israel in 1983 in which Reuveni et al. (48) showed it to be the causal agent of a mature melon plant collapse in the hot and arid regions of Israel. The fungus was identified as *M. eutypoides* by Sivanesan (Commonwealth Mycological Institute, Kew), even though the asci contained only one ascospore (48). The disease also has been reported as serious on watermelon in Israel (13). In 1985, *M. cannonballus* was reported again from Japan (61) as causing a root rot of melons, and pathogenicity of several isolates was confirmed in artificially infested soil. In 1990, *M. cannonballus* was reported simultaneously from Spain (15), Texas (31), and California (53 and cited in 31). A progressive decline of melons known locally as "colapso" (collapse) or "muerte subita" (sudden death) has occurred in the Valencia region of Spain for the last 10 years. There is some disagreement as to the cause. Some Spanish researchers (8,9) suggest that an *Acremonium* sp. is responsible, while others (15,16,22,26) attribute the cause to *M. cannonballus*. Lobo-Ruano (15) reported that perithecia and ascospores of *M. cannonballus* were observed on the root systems of infected plants in Spain; however, pathogenicity of the isolates was not initially confirmed. Martyn and Lovic (*unpublished*) observed *M. cannonballus* perithecia on many dying melons in Spain and since then have conclusively shown that Spanish isolates of *M. cannonballus* obtained from infected plants, the Spanish culture collection (Colleccion Espanola de Cultivos Tipo, Valencia), and the International Mycological Institute (Kew, Surrey, UK) are highly aggressive and pathogenic to muskmelon (26). While other pathogens may be present and cause disease in melons, we believe that *M. cannonballus* contributes to the colapso and muerte subita disease in Spain.

The most recent reports of *M. cannonballus* are from Tunisia (25), where it

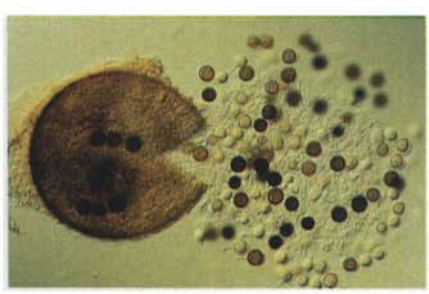

Fig. 8. Squash mount of a perithecium discharging asci and ascospores (10×).

causes a vine decline of watermelon, and from Taiwan (R.O.C.) (57), where it causes a serious vine decline and root rot disease of muskmelon. We have confirmed pathogenicity of *Monosporascus* isolates obtained from most areas of the world where it has been reported (Texas, Arizona, California, Spain, Japan, Taiwan) (Fig. 9).

Monosporascus appears to be adapted to hot, semiarid climates with soils that tend to be saline and alkaline. This is inferred from areas where the fungus has been found (Israel, Iran, India, Pakistan, southern Spain, Tunisia, Libya, the southwest United States) (Fig. 10) and by its high growth temperature optimum (30 to 35°C) (Fig. 11). These areas are noted for their relatively hot summer temperatures. In Japan, Monosporascus root rot is mainly associated with plastic mulch, plastic tunnel, or glasshouse melon culture (60). These cultural conditions may raise soil temperatures to a level conducive to growth and infection by *M. cannonballus*, thus producing an artificial niche for the fungus in an otherwise temperate zone. While vegetative mycelial growth is optimal over the range of 25 to 35°C, perithecia formation in vitro is optimal at 25 to 30°C. Isolates from Japan had a growth optimum of 28 to 32°C and were inhibited above 40°C (59,62). Similar results were observed with isolates from the United States and Israel (47), while an isolate from Libya had an optimum of 45°C (10).

We have shown also that *Monosporascus* grows optimally at pH 6 to 7 in vitro, but will grow at pH values up to at least 9.0. Growth is reduced below pH 5 and inhibited completely at pH 4. The pH of most of the soils in the muskmelon growing regions of south Texas is 7.7 or higher (31). In addition, much of the soil in south Texas approaches saline conditions, as do the soils in the African Rift Valley of eastern Israel where *Monosporascus* occurs (13). Calcium, magnesium, and sodium are typically the principal cations in the cation exchange complex of soils in arid regions as a consequence of saline irrigation water and inadequate leaching by rain. This is particularly true in soils covered with plastic mulches, which present an effective barrier for rain-induced leaching. *Monosporascus* spp. can tolerate relatively high levels of sodium and calcium chloride salts (8 to 10%) in vitro.

Little information is available on the epidemiology of *Monosporascus*. Ascospores are presumably long-lived and serve as the long-term survival structure (13,28,62). Numerous ascospores are discharged into the soil from each perithecium, but since germination rarely occurs, their role in the disease cycle and infection is unknown. We presume that Monosporascus root rot and vine decline is a monocyclic disease (28) since *M. cannonballus* has no known anamorph stage and only forms ascospores near the end of the season. Mertely et al. (33) separated ascospores from soil using differential mesh-size sieves and sucrose centrifugation (53), and they reported numbers of 3 to 15 per g of dry soil in commercial melon fields in the Lower Rio Grande Valley of Texas. Fewer ascospores per gram (1 to 2.4) were reported from commercial melon fields in Arizona (55). In Texas, ascospore numbers were highest at soil depths of 10 to 20 cm, which correlates with the region of maximum root density. Ascospores were also highest in fields cropped annually to melons compared to fields in which melons were rotated with cotton and onions. Root disease severity at harvest, however, was similar in each field, and few significant positive correlations between ascospore number and disease severity were found. Until ascospores can be germinated consistently, it will be difficult to determine their significance as an inoculum source and their role in the epidemiology of this disease. The progression of symptoms of Monosporascus root rot and vine decline is affected by a variety's fruit load and the stage of maturity. Miller (35) demonstrated that when young cantaloupe (cv. Magnum 45) fruit were removed from the vines, vine symptoms were delayed as much as 14 days compared to vines from which the fruit had not been removed. Also, late-maturing varieties of cantaloupe expressed less severe disease symptoms than early-maturing varieties when rated for vine decline symptoms at the same number of days postseeding. When disease ratings were made in respect to fruit maturity, however, early- and late-maturing varieties generally were characterized by the same level of disease. Wolff (67) also reported that muskmelon genotypes with a concentrated fruit set appeared more susceptible to vine decline. Since fruit load and maturity stage affect symptom expression, it is critical to take these factors into account when evaluating breeding lines and varieties for resistance to *M. cannonballus*.

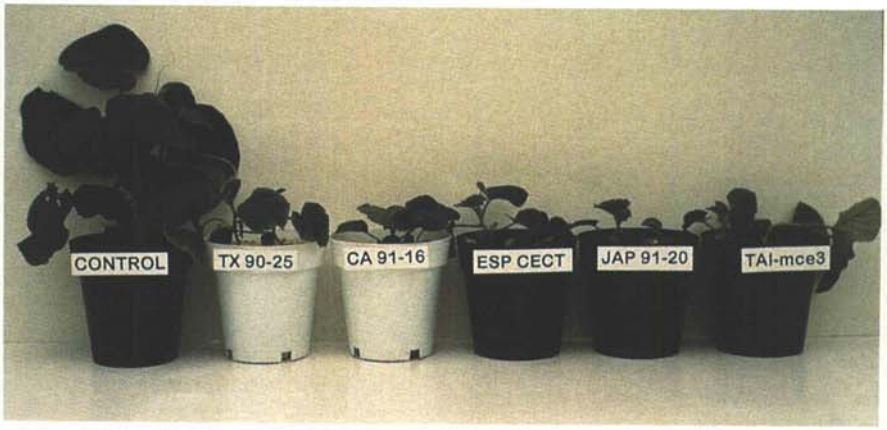

Fig. 9. Muskmelon (cv. Magnum 45) grown in soil infested with isolates of *Monosporascus cannonballus* from different geographic regions. From right to left: control, Texas isolate, California isolate, Spain isolate, Japan isolate, and Taiwan isolate.

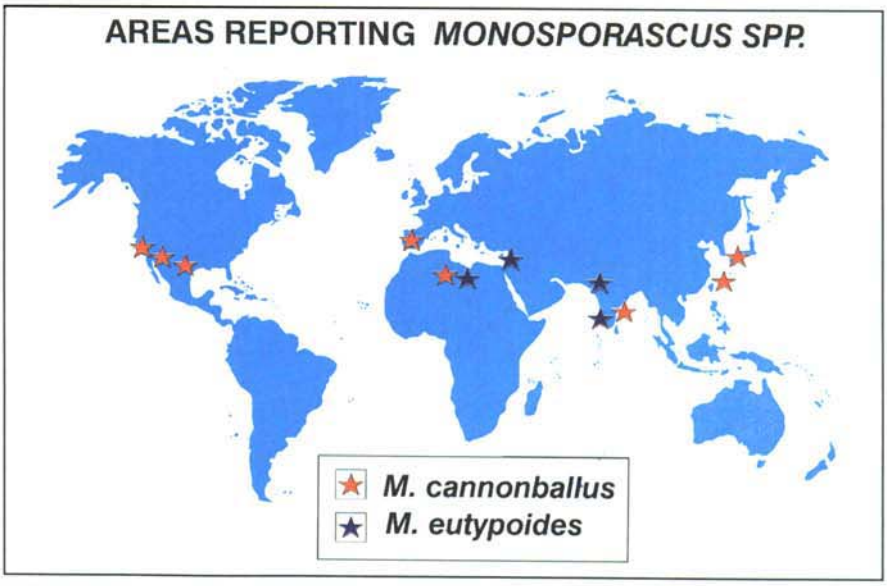

Fig. 10. World map where *Monosporascus* spp. have been reported: southern United States (Texas, Arizona, California), southern Spain, Israel, Iran, Tunisia, Libya, India, Pakistan, Japan, and Taiwan.

Host Range and Control

Monosporascus root rot and vine decline has been studied extensively in Texas (18,22,26,28,31–33,38). *M. cannonballus* was originally reported as a pathogen of muskmelon; however, muskmelon, watermelon, cucumber, summer squash, pumpkin, and several winter squashes were shown to be susceptible to the fungus in greenhouse tests (32). The disease, however, has been reported only on muskmelon and watermelon in the field, except in Japan, where it was reported on bottle gourd root stock used for grafting watermelon for control of Fusarium wilt (60).

There is some evidence that *M. cannonballus* can infect and reproduce on some noncucurbit hosts. The original report of *Monosporascus* from Arizona (56) indicated that the disease was more severe on muskmelons when planted in rotation with small grains. Similar circumstantial evidence for wheat as a host to *M. eutypoides* was provided by Krikun (13) in Israel. In addition, Hawksworth and Ciccarone (10) isolated *M. eutypoides* from the necrotic base of an unnamed *Triticum* sp. in Libya. *Monosporascus* has been observed also on *Achyranthes aspera* L. (52), *Medicago sativa* L. (46), *Trifolium pratense* L., *Iris* sp. (10,23), and *Sesamum indicum* L. (50). None of these reports, however, established pathogenicity to these plants by fulfilling Koch's postulates. Mertely et al. (32) reported that *M. cannonballus* infected and colonized roots of wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.) in greenhouse inoculation tests and caused significant reductions in plant dry weight. Perithecia were observed on roots of these plants, and *M. cannonballus* was reisolated. In addition, perithecia were observed on roots of beans and sorghum, but no significant reduction in plant growth was observed.

Short-term options for control of *M. cannonballus* are limited. All cucurbits tested to date are susceptible, and several noncucurbits may serve as hosts for the fungus (32). Ascospores are presumably long-lived and may serve as survival structures. Thus, once a field is infested with *M. cannonballus*, short-term crop rotations are of minimal value. Reuveni and Krikun (47) reported that soil solarization of melon beds did not control the collapse disease of muskmelons caused by *M. eutypoides* in Israel. Similar results have been observed in Texas (35). *Monosporascus* spp. exhibit thermophilic properties, and solarization would not be expected to reduce their populations in infested fields. Seed treatments and in-furrow soil applications of several fungicides and commercial biocontrol agents also have been ineffective in controlling the disease in Texas. Muskmelon cultivars with acceptable levels of resistance or tolerance to the fungus and acceptable horticultural characteristics have not been identified. Other than avoiding fields infested with *M. cannonballus*, preplant fumigation of muskmelon beds is the best option for controlling Monosporascus root rot and vine decline (36,37). Fumigation with 1,3-dichloropropene plus chloropicrin, chloropicrin alone, methyl bromide, and methyl bromide plus chloropicrin reduces plant stunting and increases fruit yield of muskmelon in fields infested with *M. cannonballus* (Table 1). Melon collapse (*M. eutypoides*) in Israel has been controlled commercially by fumigation with methyl bromide and metam-sodium (48).

All muskmelon and watermelon varieties tested to date are susceptible to *M. cannonballus*; however, sources of resistance in *Cucumis melo* have not been evaluated thoroughly. Mertely et al. (32) reported that honeydew varieties are more resistant than either cantaloupe or watermelon varieties. In a preliminary evaluation, Wolff (68) found that 108 of 130 muskmelon cultigens were moderately to highly susceptible to Monosporascus root rot and vine decline in the field. All cultigens tested had root lesions, and perithecia of *M. cannonballus* formed on roots of mature plants. Two Ananas-type cultivars (Deltex and Spice) and two honeydew cultivars (Morning Ice and Rocio) expressed the least amount of disease symptoms. Of the cantaloupe cultigens, only breeder line TAES 2024 was rated moderately susceptible. Stanghellini et al. (55) also reported that while there was a range in disease among melon cultivars planted in Arizona melon fields, all were highly or moderately susceptible, with the exception of an Ananas-type. Cohen et al. (7) reported that a melofon breeding line, P6a-1, did not succumb to sudden wilt in Israel; however, the pathogen responsible for sudden wilt in the field was not identified.

The progression of decline symptoms is affected by a cultivar's fruit load, maturity, and other physiological stresses. It is possible also that different plant root architectures play a significant role in the interaction of different genotypes with *M. cannonballus*. Much of the muskmelon and watermelon culture around the world has changed to growing plants under plastic mulch and drip irrigation. Plants supplied with constant water and nutrients produce large vines and small root systems, resulting in an abnormally high shoot:root ratio (13). Thus, any damage to the root, especially under the high evapotranspiration conditions late in the season, can lead to collapse of the vines because there is not enough root biomass to support the canopy and fruit.

Development of a PCR-Mediated Detection Method

The early and accurate diagnosis of plant disease is a crucial component of any sustainable crop management system. Plant diseases generally are managed most effectively if control measures are introduced at an early stage of disease development. Reliance on symptoms is often not adequate in this regard, since disease may be well under way when symptoms first appear, and symptom expression can be highly variable due to external influences. The complexity of the soil environment, with its myriad of microorganisms and interactions, makes the identification and control of many soilborne diseases difficult. This is further complicated by the fact that many vine decline and root rot diseases are actually disease complexes involving two or more causal agents acting in concert.

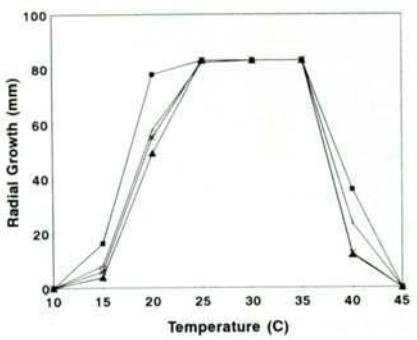

Fig. 11. In vitro temperature growth curves of four different isolates of *Monosporascus cannonballus*. Growth was on potato-dextrose agar, and each data point is the average of 10 replicate plates.

Table 1. Effect of fumigants on vine length and yield of muskmelon (cv. Mission) grown on black plastic mulch in fields infested with *Monosporascus cannonballus* in south Texas

Treatment	Rate	Vine length[x] (cm)	Yield[y] (t/ha)
Chloropicrin (96.5%)	397 kg/ha	107.7 a[z]	63.4 a
1,3 dichloropropene (DCP)	142 liters/ha	105.1 a	51.6 cd
DCP (74%) + Chloropicrin (16.5%)	142 liters/ha	101.7 a	59.5 ab
Methyl bromide (67%) + chloropicrin (33%)	397 kg/ha	98.1 a	53.3 bc
Metam-sodium	189 liters/ha	83.9 b	45.7 de
Control (no treatment)	...	72.8 b	43.6 e

[x] Each numerical entry is an average of five plants 50 days postplanting.
[y] Each numerical entry is an average of four replicates.
[z] Numbers in a column followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

Soilborne plant pathogens are notoriously difficult to detect and identify, but certain characteristics of *Monosporascus* make this problem even more pronounced. Since ascospores are the only spore stage and germination is rare, isolation and identification of vegetative mycelium on standard laboratory media are tenuous. *Monosporascus* can be isolated from infected root tissue and tentatively identified based on mycelial characteristics, but it is easily masked by other organisms present, and it requires 3 to 4 weeks to form the characteristic perithecia and ascospores in pure culture. Molecular systematics is increasingly being used to provide solutions in situations where classical taxonomic characters fail to provide convincing evidence for a consensus identification and classification (2,34). Ribosomal DNA (rDNA) has proved especially valuable in this effort, since different coding and noncoding regions of rDNA evolve at different rates and provide optimal levels of sequence variation for differentiating among a range of taxa (2,12,66). The internal transcribed spacer (ITS) regions of rDNA generally are conserved at the species level but are variable in higher taxa, making them particularly useful for species identification (2). The sensitivity of polymerase chain reaction (PCR)-based amplification and its applicability for detection from minimal amounts of tissue and complex environments such as plant roots (11) make it an ideal choice for overcoming some of the current constraints to the detection and identification of *Monosporascus*.

Species-Specific Primers for *Monosporascus*

Isolates of *Monosporascus* obtained from the United States, Spain, and Japan were identified as either *M. cannonballus* or *M. eutypoides* by us or other researchers. DNA was extracted from 12 isolates using standard protocols (14,49) from fungal mats grown in liquid culture, and the ITS regions were amplified by PCR for 25 cycles with primers 1 to 4 from the conserved areas of the 18S and 28S genes of *Saccharomyces* (66). Specific conditions of amplification have been described previously (18,22). The 610-bp amplification product from each isolate, which consisted of the ITS regions 1 and 2 plus the 5.8S gene, was subjected to restriction enzyme digestion using nine different restriction enzymes. No length polymorphisms were observed among any of the restriction products (18), suggesting sequence similarity and conservation in all isolates. The amplified products from two isolates, one from the United States (ATCC 26931) identified as *M. cannonballus* and one from Spain (IMI 345145) identified as *M. eutypoides*, were cloned and sequenced. No difference in sequence or length was detected between the two isolates ("species"): each was 599 bp long. Sequence analysis revealed 74 to 90% homology within the 18S, 5.8S, and 28S genes with those reported for these same genes in other fungi. Five sequences (primers A to E) 21 to 23 bp in length from within the ITS regions were synthesized (Table 2) and tested for their ability to amplify *Monosporascus* DNA as well as nontarget DNA from other genera (18). Each primer pair amplified the predicted size fragment from all isolates of *Monosporascus* but failed to amplify the DNA from any of a number of ecologically and taxonomically related fungi (Fig. 12). Thus, the primers were determined to be specific to the genus *Monosporascus*. These genus-specific primers and DNA probes obtained by digoxigenin-labeling the PCR-amplified ITS sequences of *Monosporascus* were used to develop a diagnostic protocol for this pathogen from both plant tissue and soil (22). With this protocol, DNA from 5- to 10-mg samples of infected root tissue is extracted and amplified (45 cycles) using *Monosporascus*-specific primers A + D and then probed with a labeled ITS-probe. In addition, ascospores can be extracted from soil by differential sieving and sucrose centrifugation, crushed, and processed for DNA extraction and PCR amplification as for tissue (14). Using this methodology, the DNA extracted from a single ascospore could be identified, thus allowing for positive identification of the fungus even from nonviable cultures or plant tissue (25). Development of the PCR-mediated detection protocol has increased the accuracy and reduced the time necessary to confirm *Monosporascus* in plant tissue and soil; what used to require 2 to 3 weeks can now be done in 2 days.

An additional benefit to having genus-specific primers and probes is that they can be used to infer taxonomic similarity (66). The ITS region of fungi has been shown to be generally conserved at the species level but variable in higher taxa; however, recently it was shown to be variable in several fungi, allowing for subspecies differentiation and phylogenetic analysis (41). In the case of *Monosporascus*, no apparent sequence difference was detected in the ITS regions among numerous isolates from around the world, even those identified as different species. Likewise, the base sequences from cloned ITS regions of two isolates identified as *M. cannonballus* (United States) and *M. eutypoides* (Spain) were identical across the entire 599-bp fragment (18). In addition, all isolates of *Monosporascus* we collected or received as *M. cannonballus* or *M. eutypoides* from other laboratories or culture collections have one ascospore per ascus, which does not germinate. They also are all pathogenic to muskmelon. Taken together, these data suggest strongly that *M. cannonballus* and *M. eutypoides* are conspecific.

Double-Stranded RNA, Culture Variability, and Hypovirulence

During the early work with *Monosporascus*, variability in virulence and aggressiveness was noticed among some

Table 2. Sequence and location of the polymerase chain reaction primers within the internal spacer region of the rDNA in *Monosporascus cannonballus*

Primer ID	Sequence	Location
A	CGCTGGCTTCTGGCCACTAAACC	501-523
B	GCTTGGTGTTGGGAGCTTATCGC	391-413
C	CCGGTGGACCATGTAAACTCTT	151-172
D	GAGTAGCCTACCCGGTAGCTAC	107-128
E	CTTACCTATGTTGCCTCGGCG	60-80

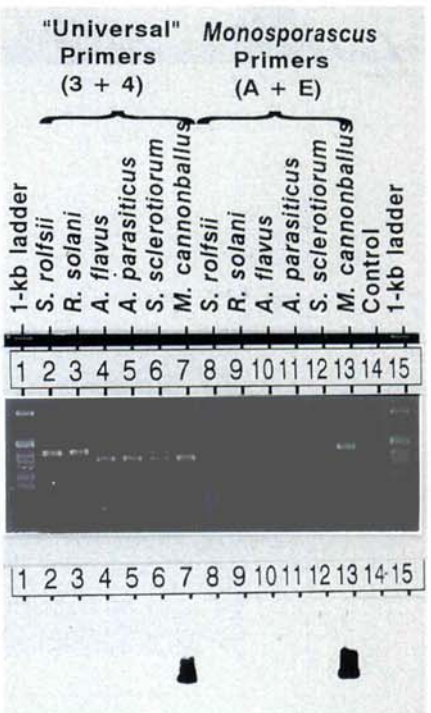

Fig. 12. Agarose gel electrophoresis and Southern hybridization of the polymerase chain reaction (PCR) amplification products using conserved (universal) internal transcribed spacer (ITS) primers 3 and 4 and *Monosporascus*-specific primers A and E. Conserved primers (1 and 4) amplified DNA from each of the different fungi; whereas the *Monosporascus*-specific primers (A and E, Table 2) only amplified DNA from *Monosporascus*. For details of the PCR and hybridization conditions, see references 18 and 22.

isolates (31). In initial screening tests, pathogenicity to muskmelon seedlings among isolates ranged from very high to nonpathogenic. These observations later were extended to many more isolates and included changes in culture morphology, perithecia production, pigmentation, and ultimately, culture degeneration and death (19,21). We were concerned initially about the degeneration and death of individual isolates under laboratory conditions. Several isolates were lost, and others became unrecognizable as *Monosporascus*. Culture degeneration initially was believed to be related to the culture medium, i.e., degeneration appeared to be quicker and more pronounced on potato-dextrose agar than on V8 juice agar. However, this was not substantiated in later tests. It was challenged also later by the fact that morphologically different isolates could be observed soon after their isolation from a single field-grown muskmelon root (19). While growth on a medium such as potato-dextrose agar exacerbates the situation, it does not induce it per se. An example of the culture aberrations that occur can be seen in Figure 13A. The first noticeable change is usually the development of a yellow pigment that darkens with each successive generation until it becomes a dark orange-brown. Concurrent with this is a reduced growth rate, sectoring, reduction in perithecia formation, and a wet, slimy appearance to the culture. Under some conditions, death of the isolate occurs. The change in phenotype can be such that the isolate is no longer recognizable as *Monosporascus*. Amplification of the DNA from these cultures with the *Monosporascus*-specific ITS primers and subsequent hybridization with the labeled probe, however, confirm that they are *Monosporascus* (Fig. 13B and C).

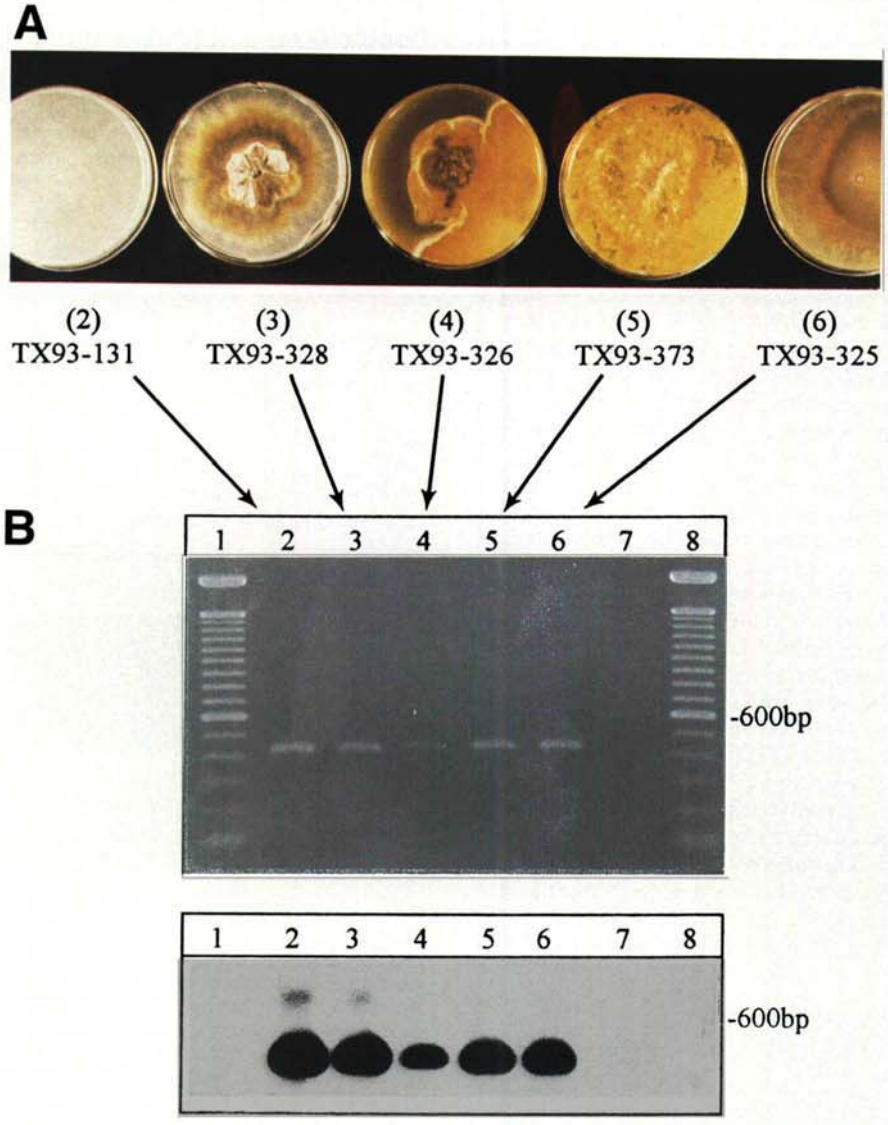

Fig. 13. Analysis of the polymerase chain reaction (PCR) amplification products using primers A + D from cultural aberrants of *Monosporascus cannonballus*. (A) Cultural aberrations in *M. cannonballus* induced by dsRNA; (B) Agarose gel electrophoresis of the PCR-amplified internal transcribed spacer (ITS) region and stained with ethidium bromide; (C) Southern hybridization of agarose gel in panel B with the *Monosporascus*-specific labeled ITS probe.

Variability in colony morphology among progeny of a single individual is a frequently observed phenomenon in fungi. In several soilborne fungal pathogens, this variability was associated with either an increase (hypervirulence) or a decrease (hypovirulence) in pathogenicity or senescence and has been correlated with the presence of double-stranded RNA (dsRNA) components (40,63). Transfer of dsRNA elements into healthy strains can result in hypovirulence and has been demonstrated experimentally in several systems (40,63). The role of dsRNA in culture degeneration (senescence) and pathogenicity of *M. cannonballus* was investigated. A subset of isolates representing a diverse geographical range (United States and Spain) and consisting of both culturally stable, wild-type, aggressive strains and culturally variable, degenerative strains was used in several comparative studies. DNA was extracted from mycelial mats of each isolate grown in liquid culture, electrophoresed, and visualized. Isolates that had the most pronounced variation in culture phenotype (i.e., dark pigmentation, reduced growth rate and perithecia) harbored one or more low-molecular-weight genetic elements that were readily visible in agarose gels stained with ethidium bromide (Fig. 14). These extra genetic elements varied in number from one to four and in size from 1.9 to 3.5 kb. In contrast, isolates that had a wild-type cultural morphology lacked these nucleic acid fragments. These fragments were digested to completion with RNase and were resistant to DNase and several restriction endonucleases, suggesting they were RNA. In addition, they were increasingly resistant to RNase A as the salt concentration was increased and were completely resistant at 0.8 M NaCl, sug-

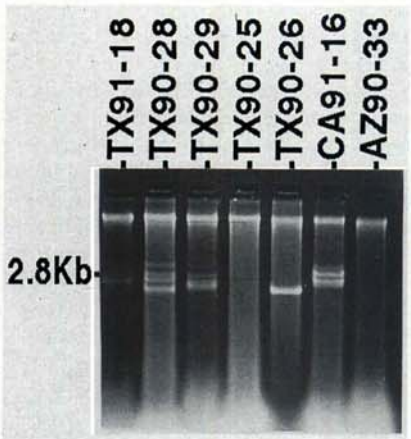

Fig. 14. dsRNA elements detected in several isolates of *Monosporascus cannonballus* showing cultural aberrations. Isolate TX90-25 and AZ90-33 have normal colony morphology and do not contain any dsRNA. All other isolates display some form of phenotypic change in cultural morphology and contain one or more dsRNAs.

gesting they were dsRNA. The double-stranded nature of these molecules was further confirmed by purification on CF-11 (39) and by their green fluorescence following staining with acridine orange (30).

Pathogenicity of the isolates was determined in two separate greenhouse tests. Magnum 45 muskmelon was grown in soil infested with each isolate for 7 weeks and rated for the following disease criteria: presence or absence of perithecia on the roots, isolation of *M. cannonballus* from root sections, vine length, and fresh and dry weight. Isolates with a wild-type phenotype and that contained no dsRNA were pathogenic in each test and caused significant reductions in each of the disease parameters. However, isolates that contained dsRNA and were culturally variable also were less aggressive and caused considerably less disease. Later tests with some of these same isolates confirmed these observations and resulted in an even greater reduction in virulence of the isolates, some of which appeared to have lost pathogenicity completely (Fig. 15) (21).

Transfer of at least one of the dsRNA fragments was shown to occur by hyphal anastomosis (20) and resulted in a concomitant change in culture phenotype. In addition, dsRNA has been detected in the ascospores of some isolates (1). Recent experiments showed that an isolate could be cured of its dsRNA by subculturing at 37°C for at least seven generations and that, once cured, it reverted to a wild-type culture morphology and virulence (43,44). Whether these dsRNA elements are mycoviruses is not yet known; nevertheless, they do appear to be responsible for the degeneration and senescence observed in some isolates of *M. cannonballus*. The dsRNAs are abundant and quite stable in *Monosporascus*. Lovic et al. (19) reported that 67% of more than 400 *M. cannonballus* isolates recovered from two commercial muskmelon fields in south Texas harbored dsRNA. Sixteen different dsRNA profiles were identified among the isolates, and up to eight isolates with different dsRNA profiles were obtained from a single plant root system. The size of the dsRNA detected among these isolates ranged from 1.7 to 15 kb. Four different dsRNA profiles were detected from a smaller set of isolates from Spain, two of which were identical to two Texas isolates. The dsRNA profiles appeared stable for a select few isolates, as no change was noted after either six successive transfers or after storage as dry soil cultures for 2 years. We are currently exploring the potential of these dsRNA elements as biocontrol agents for this disease.

Conclusions and Future Research

Monosporascus root rot and vine decline is a serious disease of muskmelons and watermelons worldwide, and it appears to be on the increase. Since its original discovery in Arizona in 1970, it has been confirmed in three states in the United States (Arizona, Texas, and California) and in eight other countries. It appears to be most severe in the southwestern United States and southern Spain. The reason for its apparent sudden appearance is not known; however, some of it can be attributed to the fact that the disease is now being recognized and accurately diagnosed. Furthermore, many of the vine decline disease problems attributed to other pathogens during the last 15 years may have been due to Monosporascus root rot and vine decline. It is likely that this disease has been present for many years at very low levels and that conditions somehow have changed resulting in an increase in disease. There is some evidence for this. A specimen label from the herbarium of the New York Botanical Garden indicates that *M. cannonballus* was isolated from roots of red clover in Kansas in 1956 (*personal communication,* photocopy obtained from the late F. A. Uecker). In addition, *Monosporascus* ascospores have been found in native, nonagricultural soils in both Texas (R. D. Martyn, *unpublished*) and Arizona (54). We showed recently that an isolate recovered from a native soil in central Texas was pathogenic to muskmelon (R. D. Martyn, *unpublished*). While it is difficult to conclude exactly what might have changed radically enough to trigger this disease increase, much of the muskmelon culture changed drastically during the early and mid-1980s to include the use of plastic mulches, drip irrigation, and hybrid cultivars. Plastic mulches and tunnels characteristically increase soil temperatures greatly, and hybrid melons typically produce earlier and bigger fruit than the older, open-pollinated varieties, placing a greater physiological stress on the plant. We believe also that tolerance to this disease may be related to root architecture. Hybrids (e.g., cv. Magnum 45) typically have a high shoot:root ratio and thus may not tolerate root damage as well as do cultivars with lower shoot:root ratios.

Future work on this disease will center around developing economical and practical control measures. Efforts to develop resistant or tolerant germ plasm will continue, as will evaluation of new experimental compounds for efficacy against *M. cannonballus*. We are also trying to exploit the use of dsRNA found in some isolates as a biocontrol of this pathogen.

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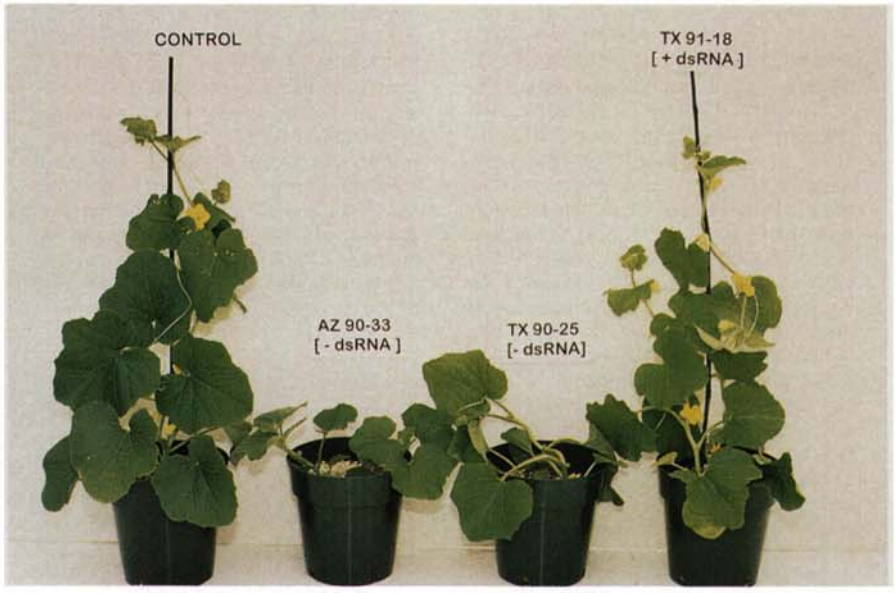

Fig. 15. Three-week-old muskmelon (cv. Magnum 45) plants growing in soil infested with dsRNA$^-$ (AZ90-33 and TX90-25) and dsRNA$^+$ (TX91-18) isolates of *Monosporascus cannonballus*. Isolates that contain dsRNA are hypovirulent; whereas isolates that do not have dsRNA are virulent.

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