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## Population structure of *Monosporascus cannonballus* isolated from melons produced in Northeastern Brazil based on mycelial compatibility groups

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**ABSTRACT.** The population structure of *Monosporascus cannonballus*, which causes vine decline in melons, was assessed based on the determination of mycelial compatibility groups (MCGs) in a collection of 58 isolates obtained from seven melon fields in three municipalities of Northeastern Brazil. For comparison, an additional 11 isolates of *M. cannonballus* from Spain were included in the analysis. MCGs were determined through comparisons of paired isolates growing on PDA culture media in the dark at 30°C in various combinations. The Brazilian isolates were assigned into four MCGs: MCG-1 (n = 35 isolates), MCG-2 (n = 20), MCG-3 (n = 2), and MCG-4 (n = 1). MCG-1 and MCG-2 included isolates from all surveyed areas. The Spanish isolates were assigned into six different MCGs, and none of them were compatible with the Brazilian isolates. The genetic structure was determined using the frequencies of MCGs and genotypic diversity indices. The maximum genotypic diversity was 6.9 and 54.5% for the Brazilian and Spanish populations, respectively. The low level of genetic diversity in the *M. cannonballus* population from Northeastern Brazil suggests that breeding melons for disease resistance may be a promising strategy for the region.

**Keywords:** *Cucumis melo*, melon, vine decline, soilborne pathogen, genetic diversity.

## Estrutura populacional de *Monosporascus cannonballus* isolados de melão produzido no Nordeste brasileiro com base em grupos de compatibilidade micelial

**RESUMO.** A estrutura populacional de *Monosporascus cannonballus*, que causa o declínio das ramas do meloeiro, foi avaliada com base em grupos de compatibilidade micelial (MCGs) determinados numa coleção de 58 isolados obtidos de sete áreas de cultivo de meloeiro em três municípios do Nordeste brasileiro. Adicionalmente, 11 isolados de *M. cannonballus* da Espanha foram incluídos para comparação. Os MCGs foram determinados pelo pareamento das culturas em todas as combinações possíveis em meio BDA no escuro a 30°C. Os isolados brasileiros foram distribuídos em quatro MCGs: MCG-1 com 35 isolados, MCG-2 com 20 isolados, MCG-3 com dois isolados e MCG-4 com um isolado. Os MCG-1 e MCG-2 incluíram isolados brasileiros de todas as áreas de meloeiro. Os isolados espanhóis foram distribuídos em seis diferentes MCGs e nenhum destes foi compatível com os isolados brasileiros. A estrutura genética foi estimada com base na frequência de MCGs e em índices de diversidade genotípica. A porcentagem máxima de diversidade genotípica da população brasileira foi de 6,9% comparada com 54,5% da população espanhola. A pequena diversidade genética na população de *M. cannonballus* no Nordeste brasileiro indica que um programa de melhoramento de cultivares resistentes tem grande chance de sucesso no controle da doença na região.

**Palavras-chave:** *Cucumis melo*, melão, declínio das ramas, patógeno habitante do solo, diversidade genética.

### Introduction

*Monosporascus cannonballus* Pollack and Uecker (Ascomycota: Sordariales) is a destructive root pathogen that causes vine decline in melons (*Cucumis melo* L.) and watermelon (*Citrullus lanatus* (Thunb.) Matsum. and Nakai) in arid, semi-arid and subtropical regions worldwide (ARMENGOL et al.,

2011; COHEN et al., 2000; MARTYN; MILLER, 1996; PIVONIA et al., 2010). The disease symptoms include yellowing, the death of the crown leaves and a gradual decline of the vine as the plant approaches maturity. As the disease progresses, the root system becomes necrotic, with numerous discrete lesions, and lacks most of

the secondary and tertiary feeder roots. *Monoporascus cannonballus* produces perithecia with ascospores that are present on the affected roots by the end of the cropping season (MARTYN; MILLER, 1996).

In 2002, *M. cannonballus* was first detected in Northeastern Brazil, causing root rot and vine decline in melon plants (SALES JÚNIOR et al., 2004). Currently, the disease is widespread in this region (SILVA et al., 2010), which accounts for 85% of Brazilian melon production and covers approximately 14,900 ha. The primary producing areas are located in Ceará State (CE), which covers 4,880 ha and produces 124,000 ton, and Rio Grande do Norte State (RN), which covers 6,806 ha and produces 192,100 ton. (IBGE, 2010).

The success of melon breeding programs for resistance to soil-borne diseases depends on the breadth of the knowledge concerning pathogen variability; thus, this aspect should be investigated before selecting sources of resistance in the host (BRUTON, 1998). Understanding the genetic diversity of the pathogen and the resulting spatiotemporal changes in the population structure is of primary importance to the success of any breeding program for the optimization of disease resistance (McDONALD; LINDE, 2002).

The genetic structure of a pathogen can be assessed using morphological, molecular, selective, or neutral markers (BURDON, 1993). Mycelial incompatibility can be considered as a neutral marker that provides information for the analysis of the genetic diversity of the fungal population (LESLIE, 1993).

Mycelial incompatibility is a common phenomenon in filamentous fungi, including ascomycetes, which has been a useful tool in studies to identify intraspecific diversity within populations of fungal plant pathogens (ARMENGOL et al., 2010; CILLIERS et al., 2000; IKEDA et al., 2011; KAUSERUD, 2004; KOHN et al., 1991; KULL et al., 2004; LESLIE, 1993; WU; SUBBARAO, 2006). A subset of the vegetative incompatibility reactions includes events that require hyphal fusion and heterokaryon formation, whereby genetically different nuclei coexist in a common cytoplasm. Non-self recognition leading to the rejection of heterokaryon formation is referred as to 'heterokaryon incompatibility', which is a genetically regulated process and most often results in the death of the hyphal fusion cell (GLASS; KANEKO, 2003; GLASS et al., 2000).

To date, only one study has been conducted to determine mycelial incompatibility in *M. cannonballus*. Chilosi et al. (2008) analyzed 14 isolates from Italy, Spain and the United States. These authors used 'nit' mutants to determine vegetative compatibility among the isolates. Given the self-incompatibility of these isolates, it was impossible to ascertain vegetative compatibility groups, and consequently, genetic relatedness was not determined in that study.

Although the importance and spread of *Monoporascus* root rot and vine decline has increased within the melon growing regions of Northeastern Brazil in the last decade, there is no information concerning the genetic variation within the populations of *M. cannonballus*. Therefore, the aim of this study was to determine the population structure of the regional populations of *M. cannonballus* that cause vine decline in the melons of Northeastern Brazil using MCG analysis.

## Material and methods

### Fungal isolates

Fifty-eight isolates of *M. cannonballus* were obtained from diseased melon plants collected in 2009 across seven melon fields in two states in the Northeastern region of Brazil. Two areas were located in the municipalities of Icapuí and Quixeré in Ceará State (indicated as BR-1 and BR-2) (Table 1), and five areas were located in the municipality of Mossoró in Rio Grande do Norte State (indicated as BR-3 to BR-7) (Table 1). In addition, eleven Spanish isolates of *M. cannonballus* were included in the analysis for comparison purposes. Each one of the Spanish isolates was collected from a different field in the Mediterranean cucurbit growing areas of the provinces of Castellón, Ciudad Real, Murcia and Valencia (indicated as SP-1 to SP-11) (Table 1). The isolates were obtained from the diseased roots of melons, watermelons and cucurbit rootstocks (*Cucurbita maxima* x *C. moschata*) supplied by the Fungal Culture Collection of the Instituto Agroforestal Mediterráneo (IAM-UPV, Valencia, Spain).

The stock cultures were maintained on V8 agar slants [20 mL of V8 juice (Campbell Soup Company, Toronto, ON, Canada), 3 g of CaCO<sub>3</sub>, 20 g of agar, and 1000 mL of H<sub>2</sub>O] at 25°C in darkness. All isolates were deposited in the Culture Collection of Phytopathogenic Fungi 'Prof. Maria Menezes' (CMM) of the Universidade Federal Rural de Pernambuco (Recife, Pernambuco State Brazil).

**Table 1.** Information for the *Monosporascus cannonballus* isolates from Brazil and Spain used to determine the mycelial compatibility groups (MCGs).

Population code	<i>n</i> <sup>a</sup>	Year	Country	State/Province	Location/Municipality	Host
BR-1	11	2009	Brazil	Ceará	Icapuí	melon
BR-2	10	2009	Brazil	Ceará	Quixeré	melon
BR-3	5	2009	Brazil	Rio Grande do Norte	Mossoró	melon
BR-4	7	2009	Brazil	Rio Grande do Norte	Mossoró	melon
BR-5	9	2009	Brazil	Rio Grande do Norte	Mossoró	melon
BR-6	6	2009	Brazil	Rio Grande do Norte	Mossoró	melon
BR-7	10	2009	Brazil	Rio Grande do Norte	Mossoró	melon
SP-1	1	1998	Spain	Murcia	Fuente Álamo	melon
SP-2	1	1998	Spain	Ciudad Real	Argamasilla de Alba	melon
SP-3	1	2003	Spain	Castellón	Almenara	cucurbit rootstock
SP-4	1	2003	Spain	Castellón	Almenara	melon
SP-5	1	2003	Spain	Castellón	Almenara	watermelon
SP-6	1	2004	Spain	Valencia	Alboraia	cucurbit rootstock
SP-7	1	2003	Spain	Valencia	Alboraia	melon
SP-8	1	2004	Spain	Valencia	Alboraia	melon
SP-9	1	2004	Spain	Valencia	Alboraia	watermelon
SP-10	1	2003	Spain	Valencia	Picassent	watermelon
SP-11	1	2004	Spain	Valencia	Silla	watermelon

<sup>a</sup>Number of isolates.

### Mycelial compatibility grouping

Before initiating the experiments, *M. cannonballus* isolates were transferred onto PDA culture media (Acumedia Co., Lansing, USA) plates and grown at 25°C in the dark for 10 days. Mycelial incompatibility among the isolates was determined using cultures grown in pairwise combinations as previously described (BURGESS et al., 2009). The isolates were initially paired in all possible combinations for a single location. Six mycelial plugs (6-mm-diameter) from the margin of the colony, each representing a single isolate, were inoculated onto PDA at 2 cm apart in a predetermined order using a template on a 9-cm Petri dish (Figure 1). The cultures were incubated at 30°C in the dark for three weeks and observed at 15 and 21 days to detect the barrage zone, which occurs for vegetative incompatible reactions. The isolates that did not form a barrage zone were considered compatible. After the initial comparisons were completed, the representative MCGs from each population were paired in all possible combinations. All pairings were conducted at least twice.

### Analysis of genotypic diversity

The isolates within each population of *M. cannonballus* belonging to the same MCG were considered as having the same phenotype because the genetic background was unknown. Each MCG represented a genotype. A genotype diversity analysis was performed for each population to distinguish total diversity, richness and evenness. The total diversity indices were calculated as Hill's index  $N_1$  (HILL, 1973) and Stoddart and Taylor's index  $G$  (STODDART; TAYLOR, 1988) according to Grünwald et al. (2003). In the formula for Hill's index  $N_1 = e^{H'}$ ,  $H'$  refers to Shannon-Wiener's

index  $H' = \{-\sum [p_i \times \ln(p_i)]\}$  (SHANNON; WEAVER, 1949), in which  $p_i$  is the frequency of the  $i$ th genotype, and  $N_1$  represents the number of equally common genotypes that would produce the same diversity. The  $G$  index is calculated using the formula  $G = 1/\sum p_i^2$ . The indices  $G$  and  $N_1$  measure the effective distribution of the proportional abundances among the different genotypes. The difference between the two indices is that  $N_1$  strongly considers the number of rare genotypes, whereas  $G$  strongly considers the number of abundant genotypes (GRÜNWARD et al., 2003).

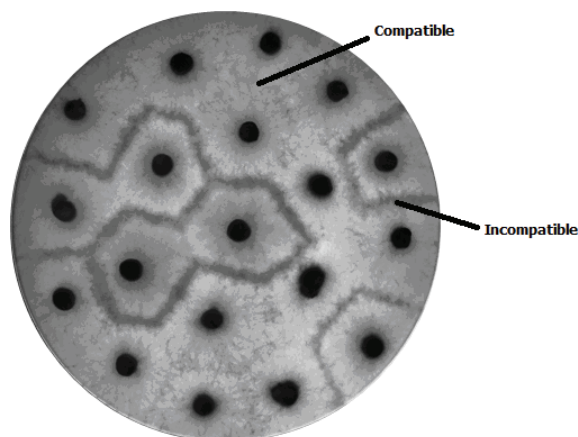
Differences in the  $N_1$  and  $G$  among populations were tested based on a 90% confidence interval estimated with 1,000 resamples using the accelerated bootstrap method (GRÜNWARD et al., 2003). The genotypic richness was estimated using the rarefaction method (GRÜNWARD et al., 2003), which assumes that the number of expected genotypes, or  $E(g_n)$ , in a random sample of  $n$  individuals out of a total sample of  $N$  individuals, where  $n_i$  corresponds to the number of individuals per genotype is. Therefore, the  $E(g_n)$  is based on the sum of the probabilities that each genotype will be included in the sample. To compare the richness data between regional populations (agricultural poles), the expected number of MCGs  $E(g_n)$  was estimated for samples of sizes  $N = 10$ , which was the smallest sample size of the population. The rarefaction estimates were obtained after compiling the algorithm <Rarefac.c> (GRÜNWARD et al., 2003). We calculated the Ludwig and Reynolds's index  $E_5$  to estimate evenness, which is the ratio of the number of abundant genotypes to the number of rare genotypes given as  $E_5 = G-1/N_1-1$  (LUDWIG; REYNOLDS, 1988). All analyses were conducted using the R software package (R DEVELOPMENT CORE TEAM, 2010).

The analyses were conducted in two steps: (i) the 58 Brazilian isolates, irrespective of the regional populations, were compared with all of the isolates from Spain; and (ii) all of the isolates from a single regional population were compared among themselves. The indices of genotypic diversity were used to measure and statistically compare the variety of phenotypes within different populations. Thus, the null hypothesis is that no significant differences in genotypic diversity exist between the Brazilian and Spanish populations of *M. cannonballus* and within the regional populations of Brazil.

## Results and discussion

### Mycelial compatibility grouping

After pairing the *M. cannonballus* isolates on PDA, two types of mycelial interactions were observed: intermingling mycelia, where the two colonies grew together with a uniform surface, which was typical of a vegetative compatibility reaction; and a barrage zone formed as a result of the interaction between the colonies, considered a vegetative incompatibility reaction (Figure 1). Both intermingling mycelia and barrage zones were observed when scoring reactions for paired isolates. The barrage zones varied from slight to strong, that is, from narrow lines of mycelia formed at the point of contact among the paired isolates to wide strips of dense, light to dark brown mycelia.



**Figure 1.** Mycelial compatible and incompatible reactions among *Monosporascus cannonballus* isolates in PDA culture media.

Among the 69 *M. cannonballus* isolates, 10 MCGs were identified (Table 2). The Brazilian isolates were assigned into four MCGs: MCG-1 with 35 isolates, MCG-2 with 20 isolates, MCG-3 with two isolates and MCG-4 with one isolate. MCG-1 and MCG-2 included isolates from all regional populations in Northeastern Brazil. The maximum number of MCGs observed within an individual

field was three: MCGs 1, 2 and 4 in BR-1 and MCGs 1, 2 and 3 in BR-2 and BR-6. The isolates from the Quixeré and Mossoró populations belonged to MCG-1, MCG-2 and MCG-3, while the isolates from Icapuí belonged to MCG-1, MCG-2 and MCG-4. The Spanish isolates were assigned into six different MCGs and were not grouped according to the province of origin or the host from which they were isolated. No Spanish isolates were compatible with Brazilian isolates (Table 2).

**Table 2.** Mycelial compatibility groups (MCGs) of *Monosporascus cannonballus* isolates from Brazil and Spain.

MCG group	Population (n = number of isolates)
1	BR-1 (4), BR-2 (7), BR-3 (3), BR-4 (6), BR-5 (4), BR-6 (3), BR-7 (9)
2	BR-1 (6), BR-2 (2), BR-3 (3), BR-4 (1), BR-5 (5), BR-6 (2), BR-7 (1)
3	BR-2 (1), BR-6 (1)
4	BR-1 (4)
5	ES-1 (1)
6	ES-5 (1), ES-11 (1)
7	ES-4 (1), ES-8 (1), ES-10 (1)
8	ES-7 (1)
9	ES-3 (1), ES-6 (1), ES-9 (1)
10	ES-10 (1)

This study was the first to determine the genotypic diversity of the *M. cannonballus* isolates in the melon growing areas in Northeastern Brazil. The MCG analysis showed that 94.8% of the Brazilian isolates belonged to two major MCGs, regardless of the production region of origin.

The relative number of MCGs in the populations of ascomycetes has previously been used to indicate whether a pathogen has recently been introduced into an area or has been present for a longer time (ADAMS et al., 1990). The number of MCGs not only depends on the length of time that the pathogen was introduced but also on the diversity of the source population, the frequency with which the pathogen was introduced and the ability to outcross (MILGROOM; CORTESI, 1999). The emergence of new genotypes within an individual field might also be an indication of MCGs or clones that are adapted to specific microclimates or hosts that unlikely resulted from genetic exchange and recombination (KULL et al., 2004).

### Genetic structure based in MCG analysis

Four distinct genotypes of *M. cannonballus* were observed in the Brazilian population. The genotypic diversity for the total population was 6.9% of the possible maximum, where every isolate had a unique genotype. A total of 3 (75%)

of the 4 genotypes had frequencies greater than one and represented 57 of the 58 isolates studied. Two genotypes were found in the three regional populations, one genotype was found only in Icapuí, and another genotype was found in Quixeré and Mossoró.

The indices  $N_1$  and  $G$  were similar for the Icapuí, Quixeré and Mossoró populations. The estimated confidence intervals for the indices of all three agricultural poles overlapped, suggesting the lack of significant differences between them (Table 2). When the  $G$  diversity index was scaled to the number of expected genotypes [ $E(g_n)$ ], a lower richness was estimated for the Mossoró population (2.27), while a higher richness was estimated for the Icapuí (2.83) and Quixeré (3.00) populations. Regarding the evenness index, the Quixeré population had the higher value (0.96), while the Icapuí population showed a lower value (0.60) (Table 3).

In the Spanish population, six different genotypes were found, with 54.5% of the possible genotypic diversity maximum. Three genotypes were observed only once in the total population, while the two most frequent genotypes were present three times. No common genotypes were observed between the Brazilian and Spanish populations. When the populations of these countries were compared, the bootstrapped confidence intervals of the diversity indices  $N_1$  and  $G$  did not overlap. Lower values for the total genotypic diversity, richness and evenness were obtained for the Brazilian population (Table 2).

The *M. cannonballus* regional populations from Brazil had low levels of genetic diversity because the bootstrapped confidence intervals of diversity overlapped for all indices assayed between these

populations, confirming the null hypothesis that no significant difference in diversity exists among the Icapuí, Quixeré and Mossoró regional populations.

The homogeneity within the *M. cannonballus* regional populations derived from different melon growing areas in Brazil, which were more than 90 km apart in some cases (Icapuí and Quixeré), suggests that *M. cannonballus* populations have undergone little genetic change since the disease was first detected in 2002 (SALES JÚNIOR et al., 2004). This result is consistent with studies conducted in Italy where this pathogen was first reported in 2001 (INFANTINO et al., 2002). Chilosi et al. (2008) reported little variation among the *M. cannonballus* isolates from Italy based on RFLP studies. Conversely, in Spain, where the disease was first described in 1990 (LOBO-RUANO, 1990), a higher genotypic diversity was detected.

The current low diversity in the Brazilian population of *M. cannonballus* could have resulted from a founder effect. The evidence suggests that *M. cannonballus* was introduced into Brazil through propagation material or other potential means of dispersal; thus, this pathogen might be a native of the Caatinga biome soils, characteristic of the Northeastern semiarid regions of Brazil (MEDEIROS et al., 2006). In the United States, the fungus was considered as an indigenous soil inhabitant (STANGHELLINI et al., 1996). The Brazilian *M. cannonballus* population that is pathogenic to melons potentially emerged from a native population through selection pressure as a consequence of intensive melon cultivation. This selection pressure could have led to a new population of individuals more adapted to melon crops, which spread easily within a cultivated area.

**Table 3.** Genetic diversity indices for *Monosporascus cannonballus* isolates grouped into three regional populations (Icapuí, Quixeré and Mossoró) according to the growing region in Brazil and into two country populations (Brazil and Spain).

Indices	Populations			Country	
	Icapuí	Quixeré	Mossoró	Brazil	Spain
$n^a$	11	10	37	58	11
Diversity					
$N_1^b$	2.43 (1.67-3.18)	2.23 (1.34-3.11)	2.12 (1.72-2.51)	2.37 (1.97-2.77)	5.33 (3.80-6.85)
$G^c$	1.85 (0.98-2.72) <sup>e</sup>	2.18 (1.44-2.91)	1.92 (1.54-2.29)	2.10 (1.78-2.41)	4.84 (3.33-6.34)
Richness					
$g_{obs}^d$	3	3	3	4	6
$E(g_n)^e$	2.83	3.00	2.27	2.53	6.00
Evenness					
$E_s^f$	0.60	0.96	0.82	0.80	0.89

<sup>a</sup>Number of isolates. <sup>b</sup>Hill's diversity index (HILL, 1973). <sup>c</sup>Stoddart and Taylor's genotypic diversity index (STODDART; TAYLOR, 1988). <sup>d</sup>Number of observed genotypes (MCG). <sup>e</sup>Expected number of genotypes (MCGs) estimated by the rarefaction method (GRÜNWARD et al., 2003). <sup>f</sup>Ludwig and Reynolds's evenness index (LUDWIG; REYNOLDS, 1988). <sup>g</sup>Numbers in parentheses indicate 90% confidence interval calculated by bootstrapping (1,000 resamples) using the accelerated bootstrap method (GRÜNWARD et al., 2003).

In contrast, we observed significant genetic variation between the Brazilian and Spanish populations. All isolates from Brazil were incompatible with isolates from Spain. Similarly, *M. cannonballus* isolates from the United States were not compatible with any isolate from Israel and Spain. Geographical isolation, differential soil environments and cultural practices could potentially explain the incompatibility of these isolates.

## Conclusion

The population of *M. cannonballus* from melon growing areas in Northeastern Brazil showed little genetic variation, which suggests that a breeding program for resistant cultivars may be successful in this region.

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