Phenotypic Variability and Genetic Diversity of the Pathogenic Fungus *Macrophomina phaseolina* from Several Hosts and Host Specialization in Strawberry

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Abstract

Macrophomina phaseolina, is a pathogenic soil-borne fungus that affects more than 500 plant species, causing various types of disease to several crops, among which is the crown and root rot disease in strawberry. Its wide variability has been characterized reiteratively in the literature, but little is known about its virulence mechanisms. Morphological, physiological, genetic and phytopathogenic parameters were evaluated among 32 isolates of *Macrophomina* from different hosts occurring in Argentina and Spain. Colony characteristics, average size of microsclerotia, chlorate phenotype and mycelial growth at different temperatures (5º–40 °C), and pH (3.0–8.0) were recorded. The morphological and physiological traits were heterogeneous and did not show any association with the genetic structure nor with their pathogenicity. Most of the isolates (71.9%) exhibited chlorate-sensitive phenotype. The optimal growth temperature range was between 25 °C and 35 °C, and the optimal pH varied between 4.0 and 6.0. The genetic structure analyzed with four DNA markers (EF-1α, ITS, CAL and TUB) showed little diversity among isolates of *M. phaseolina*, with no clear association with the country of origin, but a significant association with the host. Based on their phylogenetic affinity, one isolate was reclassified as *M. pseudophaseolina* and another one as *M. tecta*. It is the first report of *M. pseudophaseolina* causing charcoal rot on beans, in Argentina, and the first report of *M. tecta* outside Australia. Pathogenicity tests on strawberry plants revealed marked host specialization, being the isolates obtained from strawberry more virulent than those from other hosts.

Introduction

The soil-borne pathogenic fungus *Macrophomina phaseolina* (Tassi) Goidanich (Botryosphaeriales) is widely distributed across the world, causing severe damage to more than 500 plant species worldwide [1], with their respective economic losses [2, 3]. Until 2013, it was considered a monospecific genus, but currently five species are described: *M.*

phaseolina, *M. pseudophaseolina* [4], *M. euphorbiicola* [5], *M. vaccinia* [6] and *M. tecta* [7]. Isolates are easily identified on potato dextrose agar (PDA), from a characteristic dark mycelium and black microsclerotia. *Macrophomina phaseolina* shows large unstructured variation in physiology, pathogenicity, chlorate phenotypes and growing conditions which hinders the classification of the different isolates into races [8–14], despite initial attempts to do so [15]. Isolates of *M. phaseolina* can also vary significantly in pathogenicity, even between those obtained from a single host species or within the same plant [11, 16]. Its high phenotypic variation and genetic diversity could be important to explain its prevalence across a wide geographical and biological spectra [17].

The severe economic impact of *M. phaseolina* on crops together with its high variability has encouraged researchers to explore the association of virulence with phenotypic traits and possible host specificity and/or host specialization. While host specificity refers to the interactions of the pathogen on a set of host species- i.e., if the isolates can infect a species or not-; host specialization relates to the

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aggressiveness of infection between host and pathogen-i.e., if isolates from one host are more virulent/aggressive on that host than isolates from other hosts [18]. Across the literature, the association of morphological and physiological variation to pathogenicity is inconsistent [17, 19, 20]. Pathogenic assays of *M. phaseolina* isolates on different hosts report mixed results, from both host-specificity and host-specialization specialization [10, 11, 21] to non-specificity [11, 22]. A deeper knowledge of the specificity and specialization of this fungi across species of hosts is important to set the basis for future research on the molecular mechanisms involved in its pathogenicity and the implementation of agricultural practice, for instance, crop rotation [23] that minimize economical losses.

Strawberry (*Fragaria* x *ananassa* Duch.) is one of the crops most severely impacted by *M. phaseolina* and has served as a host model to describe much of the variability and biology from this fungus [21, 22, 24–26]. Crown and root rot symptoms in strawberry plants include wilting of the foliage, drying and death of older leaves, and the collapse and death of plants [27]. It is considered an emerging disease with big impact on strawberry crops in Spain and Argentina [28, 29], where it has important consequences given the vast agricultural lands dedicated to strawberry: Argentina is the fifth country in production in South America, with ~ 1300 cultivated ha producing ~ 45,500 t annually [30], while Spain ranks sixth worldwide, with 7260 cultivated ha producing more than 351,900 t (FAO, 2019). However, the control of this disease in strawberry plantations continues to be a challenge in agricultural research [31, 32].

The aim of this study was to investigate the diversity and variability of *Macrophomina* isolates obtained from multiple crops (strawberry, sunflower, soybean, bean, blueberry, corn, and hemp) occurring in Argentina and Spain, by morphological, physiological, molecular and pathogenic characterization. We also tested its possible host specialization in strawberry. This knowledge could be useful to design mitigation strategies for *M. phaseolina* diseases.

Materials and Methods

Origin and Isolation of Fungal Isolates

We characterized 32 *Macrophomina* isolates from Argentina (21) and Spain (11), obtained from opportunistic sampling of plants from experimental plantations with typical symptoms of the charcoal rot disease, or the soil where these plants were grown. The 21 Argentine isolates were obtained from strawberry, sunflower (*Helianthus annuus* L.), soybeans (*Glycine max* L.), bean (*Phaseolus vulgaris* L.) and blueberry (*Vaccinum corymbosum* L.) grown in the provinces of Tucumán, Salta and Santa Fe. The 11 Spanish isolates came from strawberry, hemp (*Cannabis sativa* L.), corn (*Zea mays* L.) and blueberry grown in the provinces of Huelva and Seville (Table 1). Plants were brought to the laboratory and rinsed thoroughly with tap water to remove soil. Affected tissue was cut into small pieces (5−10 mm), surface sterilized with 2% sodium hypochlorite for 30 s and then rinsed three times with sterilized distilled water. The pieces were drained on sterile absorbent paper for a few minutes and placed on PDA medium (potato, 200 g; dextrose, 20 g; agarose, 17 g; distilled water, 1 L) on 90-mm Petri plates and incubated in the dark for five days at the optimal growth temperature of 30 °C [9, 33, 34]. A small portion of the fastest growing mycelia were taken and placed onto Petri plates containing PDA and incubated in darkness at 30 °C for five days.

Soil samples were taken from the first 20 cm depth using a vertical calibrated drill, in the space between symptomatic plants. The isolations were done as in Papavizas and Klag [35]. Briefly, 10 g of dry soil were mixed with 250 ml of 0.5% NaClO solution. The mix was centrifuged at 2,000 rpm for 30 s and allowed to stand for 3 min, repeating this cycle three times. Then the mixture was sieved, the soil retained in the mesh was washed with sterile distilled water to remove traces of NaClO, and resuspended in 100 ml of sterile distilled water. Aliquots of 1 ml of the final dilution were spread on the surface of Petri plates containing PDA-DOPCNB medium (basal medium containing p-(dimethylamino) benzenediazo sodium sulfonate (DASS, Oxgall) and pentachloronitrobenzene (PCNB) at 50, 2000 and 100 mg l^{-1}, respectively). Inoculated Petri plates were incubated in darkness at 30 °C for 7 days.

Microsclerotia were separated from the mycelia of all grown cultures using a sterile needle, with the help of a magnifying glass, transferred to Petri plates containing PDA and incubated in darkness at 30 °C. Colonies appearing from single sclerotium were transferred to PDA medium in Petri plates and incubated as mentioned above. These isolates were used for morphological, physiological, molecular and pathogenic characterization.

Morphological and Physiological Characterization

Morphological and physiological variability among the 32 isolates of *Macrophomina* collected from symptomatic samples of Argentina and Spain was studied using the following parameters: type, shape and color of the colonies, chlorate phenotype, growth at different temperatures and pH, and sclerotium size. In each assay, isolates were examined in triplicate using a 5-mm diameter mycelial disc cut from the periphery of 7-day-old cultures grown on PDA at 30 °C in darkness.

Table 1 Host, variety, source, province and country of origin of the *Macrophomina* isolates used, and year of isolation

Isolate	Host	Variety	Isolation source	Province	Coordinates	Country	Year
Fru-SWA	Strawberry	'Sweet Ann'	Root	Tucumán	65° 30' O; 27° 0' S	Argentina	2013
Fru-Ely	Strawberry	'Elyana'	Crown	Tucumán	65° 30' O; 27° 0' S	Argentina	2015
Fru-Paj	Strawberry	'Pájaro'	crown	Tucumán	65° 30' O; 27° 0' S	Argentina	2011
Fru-CR	Strawberry	'Camino Real'	Crown	Tucumán	65° 30' O; 27° 0' S	Argentina	2011
Fru-Car	Strawberry	'Carmela'	Crown	Tucumán	65° 30' O; 27° 0' S	Argentina	2011
Fru-Sab	Strawberry	'Sabrina'	Root	Tucumán	65° 30' O; 27° 0' S	Argentina	2013
Fru-Alb	Strawberry	'Albion'	Crown	Tucumán	65° 30' O; 27° 0' S	Argentina	2011
Fru-Cam	Strawberry	'Camarosa'	Crown	Tucumán	65° 30' O; 27° 0' S	Argentina	2011
Fru-Erli	Strawberry	'Erlibrite'	Crown	Tucumán	65° 30' O; 27° 0' S	Argentina	2015
Fru-Trea	Strawberry	'Treasure'	Crown	Tucumán	65° 30' O; 27° 0' S	Argentina	2011
Fru-For	Strawberry	'Fortuna'	Crown	Tucumán	65° 30' O; 27° 0' S	Argentina	2013
Fru-Mac	Strawberry	'Macarena'	Crown	Tucumán	65° 30' O; 27° 0' S	Argentina	2011
Fru-Fes	Strawberry	'Festival'	Crown	Tucumán	65° 30' O; 27° 0' S	Argentina	2015
Gi-ACA885	Sunflower	Gi-ACA 885	Root	Santa Fe	60° 41' O; 31° 02' S	Argentina	2011
Gi-P65A25	Sunflower	Gi-P65A25	Root	Santa Fe	60° 41' O; 31° 02' S	Argentina	2011
Gi-A963	Sunflower	Gi-A963	Root	Santa Fe	60° 41' O; 31° 02' S	Argentina	2011
Gi-AG4	Sunflower	Aguará 4	Root	Santa Fe	60° 41' O; 31° 02' S	Argentina	2011
Gi-PAN1031	Sunflower	Gi-PAN 1031	Root	Santa Fe	60° 41' O; 31° 02' S	Argentina	2011
Por-Lea	Bean	Alubia	Seed	Tucumán	65° 30' O; 27° 0' S	Argentina	2011
Soj-Bal	Soybean	A8002	Root	Salta	65° 24' O; 24° 47' S	Argentina	2011
Ar-SB	Blueberry	'Southern Bell'	Root	Tucumán	65° 30' O; 27° 0' S	Argentina	2011
TOR-102	Strawberry	'Camarosa'	Soil	Huelva	6° 56' O; 37° 15' N	Spain	2011
TOR-840	Hemp	HÍBRIDO-H3	Root	Seville	5° 58' O; 37° 22' N	Spain	2016
TOR-854	Strawberry	'Fortuna-Florida'	Soil	Huelva	6° 56' O; 37° 15' N	Spain	2017
TOR-855	Strawberry	'Calenda'	Soil	Huelva	6° 56' O; 37° 15' N	Spain	2017
TOR-860	Corn	nd[a]	Cane	Seville	5° 58' O; 37° 22' N	Spain	2017
TOR-861	Blueberry	'Star'	Root	Huelva	6° 56' O; 37° 15' N	Spain	2017
TOR-862	Blueberry	'Star'	Stem	Huelva	6° 56' O; 37° 15' N	Spain	2017
TOR-872	Blueberry	'Ventura'	Petiole	Huelva	6° 56' O; 37° 15' N	Spain	2017
TOR-876	Strawberry	'Rociera'	Crown	Huelva	6° 56' O; 37° 15' N	Spain	2017
TOR-878	Strawberry	'Rociera'	Root	Huelva	6° 56' O; 37° 15' N	Spain	2017
TOR-888	Hemp	'Sara'	Petiole	Seville	5° 58' O; 37° 22' N	Spain	2017

[a]nd, not defined

Type, Shape and Color of the Colonies

For studying variability in type, shape and color of the colonies, Petri plates (90-mm) with PDA medium were inoculated in the center with a mycelial disc of each isolate. Petri plates were incubated at 30°C in darkness for 5 days. At that time, the growth type was recorded as "regular", when the growth of the isolate was even and with a defined edge, or "irregular", when the edge was irregular. The type of mycelium was classified as "arborescent", when it presented aerial growth, or "resupinate", when hyphae remained compact and adhered to the culture medium, without aerial growth.

Chlorate Phenotype

Sensitivity to chlorate was assessed as in Pearson et al. [15]. A mycelial disc of each isolate of the fungus was placed on the center of 90-mm Petri plates containing LBA medium amended with 120 mM potassium chlorate. The Petri plates were then incubated at 30 °C in darkness. Three growth patterns were recorded: dense, feathery and restricted. A dense growth indicated resistance to chlorate, while a feathery or a restricted growth indicated sensitivity to chlorate. Colony phenotypes were recorded after 6 days by comparison with cultures grown on the same medium without chlorate.

Sclerotial Size

The surface of each of the three replicas of *Macrophomina* isolates grown in PDA at 30 ºC in darkness for 3 days were gently and superficially scraped with a scalpel. The scraping of each replica was placed on a different slide over a drop of sterile distilled water, dispersing well. Ten sclerotia were selected randomly per each of three replicas (n = 30) and size (length) was measured in an optical microscope (20X).

Effect of Temperature and pH on the Growth of Mycelia

For studying the growth at different temperatures, a mycelial disc of each isolate was transferred to the center of 90-mm Petri plates filled with PDA medium. The Petri plates were then placed in incubators with temperatures adjusted to 5, 10, 15, 20, 25, 30, 35 and 40 °C, in the dark.

To assess the effect of pH, PDA media with pH fixed at 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 were prepared. The pH of the medium was adjusted using 1 N HCl or KOH solutions, checked with a pH-meter, after autoclave sterilization and plated in 90-mm Petri plates. Mycelial discs were placed in the center of the plates and incubated at 30 °C in darkness.

On both assays, total area covered by the colony ($A = \pi*R*r$, where: R is the major radius and r is the minor radius) was measured at 2, 3 and 6 days after inoculation.

Molecular Characterization

DNA Extraction

Isolates were grown on Petri plates with PDA for 7 days at 30 ºC in darkness. The surface of the culture was scraped to extract mycelium. Then, samples were frozen in liquid nitrogen and ground to powder with a mortar. For each sample, 0.1 g was digested in 700 µl of lysis buffer (2% PVP, 2% CTAB, 1 M Tris-HCl at pH 8.0, 0,5 M EDTA, 0,5 M NaCl) combined with 2 µl of β-mercaptoethanol, 20 µl of proteinase K (20 mg ml^{-1}) and 10 µl of RNAse (10 mg ml^{-1}). Samples were incubated for 60 min at 65 °C.

Then, a phenol–chloroform-isopropanol (25/24/1; v/v/v) DNA extraction was carried out, followed by ethanol precipitation. DNA was quantified in ND-100 NanoDrop spectrophotometer and stored at − 20 °C.

PCR Amplification and DNA Sequencing

We targeted the elongation factor 1 alpha (EF-1α) (∼ 350 bp), calmodulin (CAL) (∼ 500 bp), tubulin (TUB) (∼ 600 bp) and internal transcribed spacer (ITS) regions (∼ 650 bp) (Table 2). PCRs were carried out in final volumes of 50 µl containing 1X of PCR buffer, 2 mM of MgCl$_2$, 20 µM of each dNTP, 0.4 mM of each primer, 1.25U of Taq Polymerase (Thermo Scientific) and 100 ng of template DNA. Each PCR consisted on an initial denaturation of 5 min at 94 °C, followed by 40 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 2 min of extension at 72 °C, ended by a final extension at 72 °C. A negative control with no DNA template was included in each set of reactions. The amplification products were run in 1% agarose gels, stained with RedSafe (0.025 µlml^{-1}) and visualized under UV. Amplifications and gel visualization were repeated twice with each primer. After confirming the amplification, the PCR products were sent to StabVida (Caparica, Portugal) for their purification and sequencing.

Sequence Editing and Alignment

The chromatograms from Sanger sequencing were converted to FASTAQ files with Seqret tool from EMBOSS 6.6.0 (http://emboss.open-bio.org/). Low-quality terminal sequences were trimmed using cutadapt1.18 [36]. The quality thresholds for cutadapt were empirically determined (-q 40,40) by comparing the trimmed sequences with the original chromatograms in Geneious R8 (https://www.geneious.com). Sequences below 50 nucleotides were discarded. Multiple sequence alignments for each marker were made with MAFFT v7.244 [37] and DECIPHER [38] using defaults parameters.

Table 2 Sequences of forward (F) and reverse (R) primers used

Gene/region	Primers	Nucleotide sequence (5′–3′)	References
Elongation factor (EF-1α)	EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn [80]
	EF1-986R	TACTTGAAGGAACCCTTACC	
Calmodulin (CAL)	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC	Carbone and Kohn [80]
	CAL-737R	CATCTTTCTGGCCATCATGG	
ITS1 + 5.8S rRNA + ITS2 + part of 28S rRNA (ITS)	ITS5F	GGAAGTAAAAGTCGTAACAAGG	White et al. [81]
	ITS4R	TCCTCCGCTTATTGATATGC	
Tubulin (TUB)	T1F	AACATGCGTGAGATTGTAAGT	O´Donnell and Cigelnik [82]
	T22R	TCTGGATGTTGTTGGGAATCC	

Phylogenetic Analysis

The information from the four markers was then integrated by concatenating them with AMAS [39] for increased phylogenetic resolution [40]. We included sequences for the same markers from reference strains of all described species in *Macrophomina* from Poudel et al. [7], and the sequence of the related fungus *Lasiodiplodia pseudotheobromae*, from the same family of *M. phaseolina*, was included as an external group. The GenBank accession numbers of the four markers of each isolate is on the Supplementary Material 1. We excluded 59 samples from GenBank which had identical sequences in the concatenated alignment to other samples in the dataset. The phylogenetic reconstruction was performed in a Maximum Likelihood framework with RAxML 7.2.8 [41], using the GTR + G evolution model with one partition for each marker and the "rapid hill-climbing" algorithm. Bootstrap support was calculated over 100 replicates. To further support the evolutionary affinities between isolates, a Bayesian phylogenic tree was inferred on the same dataset in BEAST 2.1.2 [42]. Since BEAST uses a coalescent approach, the outgroup was excluded. PartitionFinder2 [43] was used to determine the best partition scheme out from the four DNA regions considered, proposing a unique partition under a HKY model of evolution. Thus, the four markers were concatenated and HKY was selected as a site model in BEAST. We assumed a coalescent constant population size, since most samples belonged to the same or sister species. Two independent chains were run for 10 million iterations and convergence was checked with Tracer 1.6 [44]. Trees from both chains were merged with LogCombiner 2.4.4 [42] and a maximum clade credibility tree was generated after a 10% burn-in.

Genetic Structure and Diversity

Haplotype networks were performed with the sequences of the four concatenated markers. To maximize the number of positions to be compared, isolates TOR-861 and TOR-862 were excluded since they presented a higher proportion of missing data, and indels were treated as a fifth state. Haplotype networks were built with PopArt [45] using TCS algorithm [46]. This program was also used to make an Analysis of Molecular Variance (AMOVA) and to estimate genetic diversity parameters: nucleotide diversity, polymorphic sites and parsimoniously informative sites. The purpose of AMOVA was to find out if there was an association between the genetic structure and the country (Spain or Argentina) or host (strawberry, sunflower, soybean, bean, blueberry, corn or hemp) of origin. The significance ($P \leq 0.05$) of the observed structure (ϕst) was obtained from 1000 permutations.

Genetic Distance

Pairwise Kimura-2 genetic distances were computed with MEGA 7 program [47] using the concatenated sequences.

Pathogenic Characterization

We run two pathogenic assays in strawberry plants to evaluate the virulence of *Macrophomina* isolates and their possible host specialization. Around half ($n = 18$) of the isolates used had been isolated from the same species of host, strawberry, while the others ($n = 14$) came from different host species (Table 1). *Macrophomina phaseolina* exhibits an ample range of virulence, from very low to very high, depending on the experimental conditions and isolates [8, 48, 49]. To encompass this high range of virulence, we run the two independent assays with different inoculation methods described in the literature to cause different rates of severity. In the first one, we used the toothpick method, described in previous studies to cause higher virulence in shorter times of around one month [21, 50, 51]. In the second one, we induced infection by adding a suspension of microsclerotia. The latter, simulates better a natural infection but requires more time to detect the pathogenic effects of *Macrophomina*, of around 6 months [25] (details in Supplementary Material 2).

In both assays, a week before inoculation with *Macrophomina* isolates, plants were transferred to an infection chamber at 28 °C and 16-h photoperiod (100.5 µmolm^{-2} s^{-1}) and subjected to hydric stress to generate favorable conditions for the infection of the fungus. Plants were watered three days a week with 50 ml of water per pot. The experimental design of each experiment was completely randomized. At the end of the assays, susceptibility to crown and root rot was evaluated as disease severity, using a disease severity rating (DSR) according to Fang et al. [52]: 0 = plant well developed, no disease symptoms; 1 = plant slightly stunted; 2 = plant stunted and yellowing; 3 = plant severely stunted and/or wilting; 4 = majority of leaves of the plant wilted or dead; 5 = plant death. Re-isolations were made from segments of freshly harvested diseased root and/or crown onto PDA to confirm the infection.

Statistical Analyses

Statistical analysis of data from morphological and physiological characterizations were performed in Statistix 9.1 software (http://www.statistix.com/). Means comparison between treatments of a same assay were evaluated using analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) tests at $\alpha = 0.05$.

Host specialization of the isolates was evaluated by comparing DSR in strawberry isolates versus isolates from other hosts. The statistical significance of the medians between

both groups in each assay was evaluated with Mann Whitney U tests in R 4.0 (R Core Team 2020).

Results

Morphological and Cultural Characterization

All the isolates exhibited regular growth, except TOR-876 and TOR-855 with irregular growth. Colonies were black on the agar surface, except TOR-854 and TOR-861, which were brown. Regarding the type of the colony, ten isolates had resupinate mycelium and 22 an arborescent mycelium (Table 3).

After six days of incubation, nine isolates were resistant to chlorate (28.1%), whereas 23 were sensitive (71.9%). The bean (Por-Lea) and corn (TOR-860) isolates showed a dense phenotype. The soybean (Soj-Bal) isolate had a feathery phenotype, and blueberry and hemp isolates showed a restricted phenotype. Isolates obtained from strawberry and sunflower presented the three phenotypes (Table 3).

Microsclerotia sizes ranged from 50.3 μm to 114.3 μm. Isolates Fru-Fes (strawberry), TOR-840 (hemp) and TOR-872 (blueberry) had significantly bigger microsclerotia ($P \leq 0.05$) to the rest. Isolate Gi-AG4 (sunflower) showed significantly smaller ($P \leq 0.05$) microsclerotia than all others, except Por-Lea, Gi-PAN1031 and Fru-CR (Table 3).

Effect of Temperature on the Mycelial Growth

The optimal growth temperature range of the analyzed isolates varied between 25 °C and 35 °C, although the isolates could still grow relatively well at 20 °C. At 5 °C, no mycelial growth was observed even with the longest incubation times. Growth was very slow at 10 ºC (mean $\pm$ SE for the 32 isolates: 5.4 ± 5.1 cm^2) and 15 °C (12.8 ± 8.1 cm^2). At 20 °C most of the isolates completely covered the 90-mm Petri plates (56.7 cm^2) on the 6th day (52.0 ± 13.1 cm^2). Between 25 and 35 °C, mycelial growth was maximal, covering completely the plates after the 3rd day in most cases. At 40 °C, a steep decrease on the mycelial growth was observed, with great differences of mycelium growth extension on the 6th day, ranging from 56.7 cm^2 for Gi-ACA885 and Gi-A963 (both from sunflower) to 0.6 cm^2 for TOR-878 (strawberry) (Fig. 1a; Supplementary Material 3).

Effect of pH on the Growth of Mycelia

The optimal pH range for the development of *M. phaseolina* isolates was between 4.0 and 6.0. On the 2nd and 3rd days, maximum growth was observed at pH 5.0 (mean $\pm$ SE for the 32 isolates: 36.3 ± 7.4 and 54.9 ± 6.1 cm^2, respectively). On the 6th day, the average area of mycelium growth was higher than 52.0 cm^2 overall all pHs tested (3.0–8.0). A pH 3.0 was the least favorable for the pathogen. At pH 4.0, 5.0, 6.0, 7.0 and 8.0, the mycelia covered completely the Petri-dish area (56.7 cm^2) on the 6th day, except the isolates TOR-855 (strawberry) and TOR-888 (hemp) (Fig. 1b; Supplementary Material 4).

Phylogenetic Analysis

The DNA alignment used in the phylogenetic analysis contained the four concatenated DNA markers, with a total of 71 isolates, 2359 positions and 9.8% of missing data. Both, the Maximum Likelihood and Bayesian phylogenetic trees supported well the different species described in the current taxonomy, except for the position of *M. vaccini*. The latter was a sister species of *M. phaseolina* in the Maximum Likelihood tree with low bootstrap support of 67%, whereas in the Bayesian tree grouped together with *M. tecta* with a posterior probability of 0.94 (Fig. 2). All isolates from this study grouped with *M. phaseolina* except for the isolates Por-Lea that showed higher affinity with *M. pseudophaseolina* and TOR-860 with *M. tecta*. A very small genetic variability was observed among the rest of the isolates grouped together, without any apparent association by country or host of origin.

Genetic Structure and Diversity

The concatenated alignment used in the haplotype network represented 30 isolates and 1,313 nucleotide positions. Eleven different haplotypes were identified (Fig. 3; Supplementary Material 5). The bean isolate (Por-Lea) was the most differentiated one, with more than 27 mutation steps with the closest isolate, followed by the corn isolate (TOR-860), with at least 10 mutations steps. This agrees with the phylogenetic tree, where these isolates grouped with reference strains of *M. pseudophaseolina* and *M. tecta*, respectively.

The AMOVA showed that the genetic structure of the isolates was not associated with their country of origin (ϕst $= -0.012$, $P = 0.543$). However, there was some association between the genetic structure of the isolates and their host of origin (ϕst $= 0.810$, $P = 0.001$). Nucleotide diversity was 0.0039, and there were 43 polymorphic sites and 13 parsimoniously informative sites.

Genetic Distance

Genetic distance between isolates of *M. phaseolina* was low, varying from 0 to 1.1% (Supplementary Material 6). The Por-Lea isolate, identified as *M. pseudophaseolina*, differed the most, with distances between 2.1 and 3.0%

Table 3 Shape, color and type of colony, chlorate phenotype and microsclerotia length of the studied *Macrophomina* isolates

| Isolate	Host	Shape[a]	Color[b]	Type[c]	Chlo. pheno.[d]	Chlo. sens.[e]	Microsclerotia length (μm)[f]
Fru-SWA	Strawberry	R	B	A	Res	+	78.95 fghi
Fru-Ely	Strawberry	R	B	A	F	+	94.15 bcde
Fru-Paj	Strawberry	R	B	R	Res	+	94.18 bcde
Fru-CR	Strawberry	R	B	R	Res	+	64.82 ijk
Fru-Car	Strawberry	R	B	R	Res	+	94.72 bcde
Fru-Sab	Strawberry	R	B	A	Res	+	86.60 cdef
Fru-Alb	Strawberry	R	B	R	F	+	94.32 bcde
Fru-Cam	Strawberry	R	B	A	D	−	91.57 bcdef
Fru-Erli	Strawberry	R	B	A	F	+	100.60 abc
Fru-Trea	Strawberry	R	B	R	D	−	90.24 bcdef
Fru-For	Strawberry	R	B	A	F	+	99.46 abc
Fru-Mac	Strawberry	R	B	R	F	+	87.36 cdef
Fru-Fes	Strawberry	R	B	A	F	+	111.90 a
Gi-ACA885	Sunflower	R	B	A	D	−	82.82 defg
Gi-P65A25	Sunflower	R	B	A	F	+	65.75 hij
Gi-A963	Sunflower	R	B	A	D	−	100.24 abc
Gi-AG4	Sunflower	R	B	A	D	−	50.28 k
Gi-PAN1031	Sunflower	R	B	A	Res	+	63.73 jk
Por-Lea	Bean	R	B	A	D	−	57.74 jk
Soj-Bal	Soybean	R	B	A	F	+	90.04 bcdef
Ar-SB	Blueberry	R	B	A	Res	+	80.56 efgh
TOR-102	Strawberry	R	B	A	Res	+	88.43 cdef
TOR-840	Hemp	R	B	A	Res	+	114.26 a
TOR-854	Strawberry	R	Br	A	D	−	95.61 bcd
TOR-855	Strawberry	I	B	R	Res	+	104.38 ab
TOR-860	Corn	R	B	R	D	−	89.09 cdef
TOR-861	Blueberry	R	Br	A	Res	+	88.65 cdef
TOR-862	Blueberry	R	B	A	Res	+	99.38 abc
TOR-872	Blueberry	R	B	A	Res	+	111.81 a
TOR-876	Strawberry	I	B	R	Res	+	85.92 cdef
TOR-878	Strawberry	R	B	A	D	−	83.49 defg
TOR-888	Hemp	R	B	R	Res	+	68.59 ghij

[a]R, regular; I, irregular

[b]B, black; Br, brown

[c]A, arborescent; R, resupinate

[d]Chlorate phenotype. Res, restricted; F, feathery; D, dense

[e]Chlorate sensitivity; + sensitive; − resistant

[f]Different letters represent groups which are significantly different (LSD, $\alpha = 0.05$)

from the isolates of *M. phaseolina*. The TOR-860 isolate, identified as *M. tecta*, differed with distances between 0.7 and 1.3% from the isolates of *M. phaseolina*. The greatest pairwise distance corresponded to 3.0% between Por-Lea (bean, Argentina) and TOR-861 (blueberry, Spain), while many isolates shared the same haplotype across the markers studied (distance of 0%; Supplementary Material 6).

Virulence of *M. phaseolina* Isolates

In both assays, all the isolates induced symptoms of crown and root rot except Por-Lea (*M. pseudophaseolina*, bean) and Soj-Bal (soybean) on strawberry plants cv. 'Candonga'. The more virulent isolates were always strawberry isolates: Fru-SWA, Fru-Sab, Fru-Alb, Fru-For and Fru-Fes on cv. 'Pájaro'; and TOR-854 and TOR-878 on cv. 'Candonga'.

Fig. 1 Effect of temperature (**a**) and pH (**b**) and incubation time on the mycelial growth of the 32 *Macrophomina* isolates expressed as average colony area
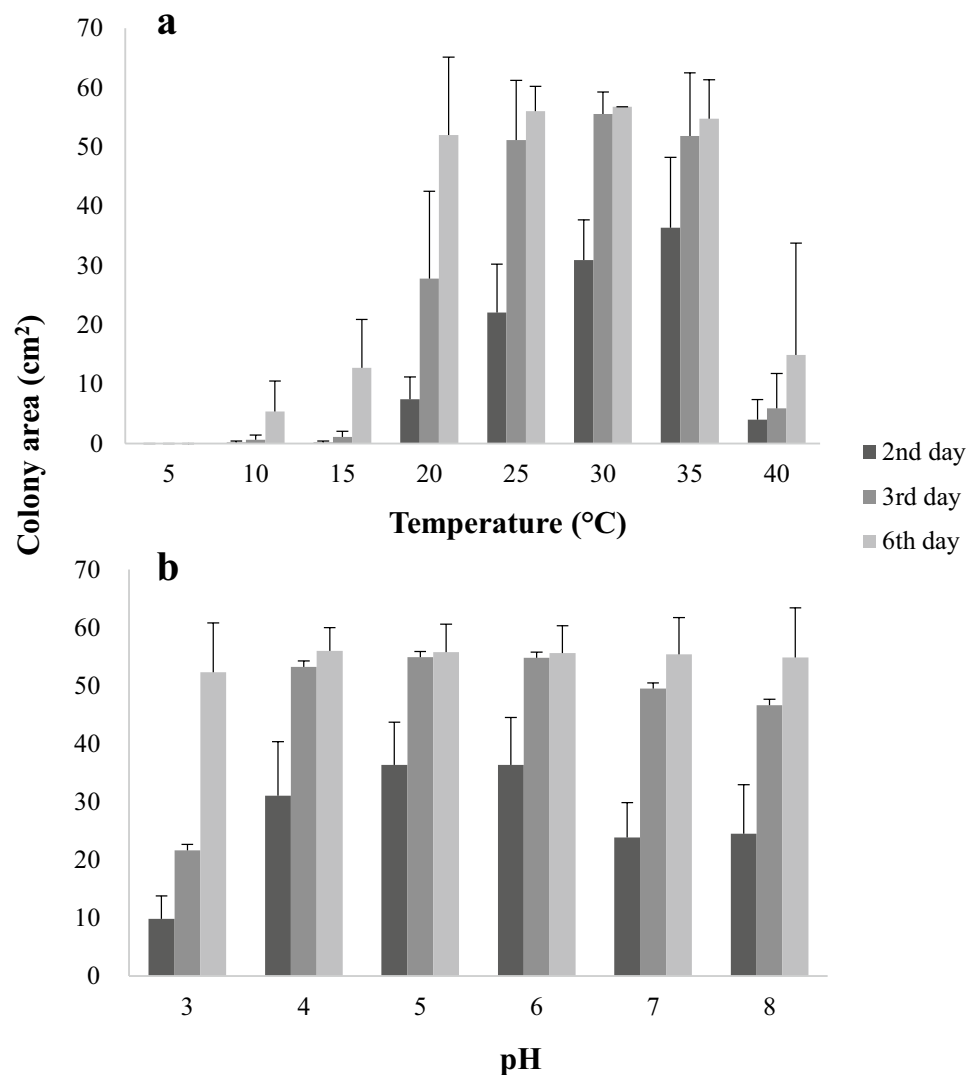
The least virulent isolates on strawberry plants cv. 'Pájaro' were Gi-ACA885 and Gi-P65A25, both coming from sunflower (Table 3). According to the Mann Whitney U tests, the virulence of isolates coming from strawberry, as measure in the Fang scale, was 2.44 higher in assay 1 ($W = 214$, *P* value < 0.001) and 2.39 higher in assay 2 ($W = 269.5$, *P* value < 0.001 (Fig. 4).

Discussion

In this study, we report high morphological and physiological variability, and describe the genetic structure between 32 isolates of *Macrophomina* from diverse economically important crops in Argentina and Spain. We also found a high host specialization: isolates coming from strawberry were more virulent on strawberry plants than isolates coming from other plant hosts.

Previous studies also described high variability in morphology and physiology of *M. phaseolina*. Indeed, type of growth can be very different, from light dense to dense and colony color can vary between grey, dark green, black or brown [14, 19, 53]; microsclerotia can have large variation in size, from 50 to 150 µm [54, 55], and sensitivity to chlorate vary from around 30% [53, 56] to 90% [57]. Despite morphological or phenotypic variations have not been associated to country or host of origin of the isolates [19], for chlorate sensitivity some authors affirm isolates from the same host generally share phenotype [11, 56, 57] and others report mixed results regarding its association with virulence [20, 58]. The optimal growth temperature is in agreement with previous studies [9, 33, 34]. Acidity of the soil affects the survival of *M. phaseolina* and influence charcoal rot incidences in crops, although there is a high variance in the optimal pH conditions, from 3.6 to 6, between isolates, as we and previous authors have described [33, 59–61]. The wide
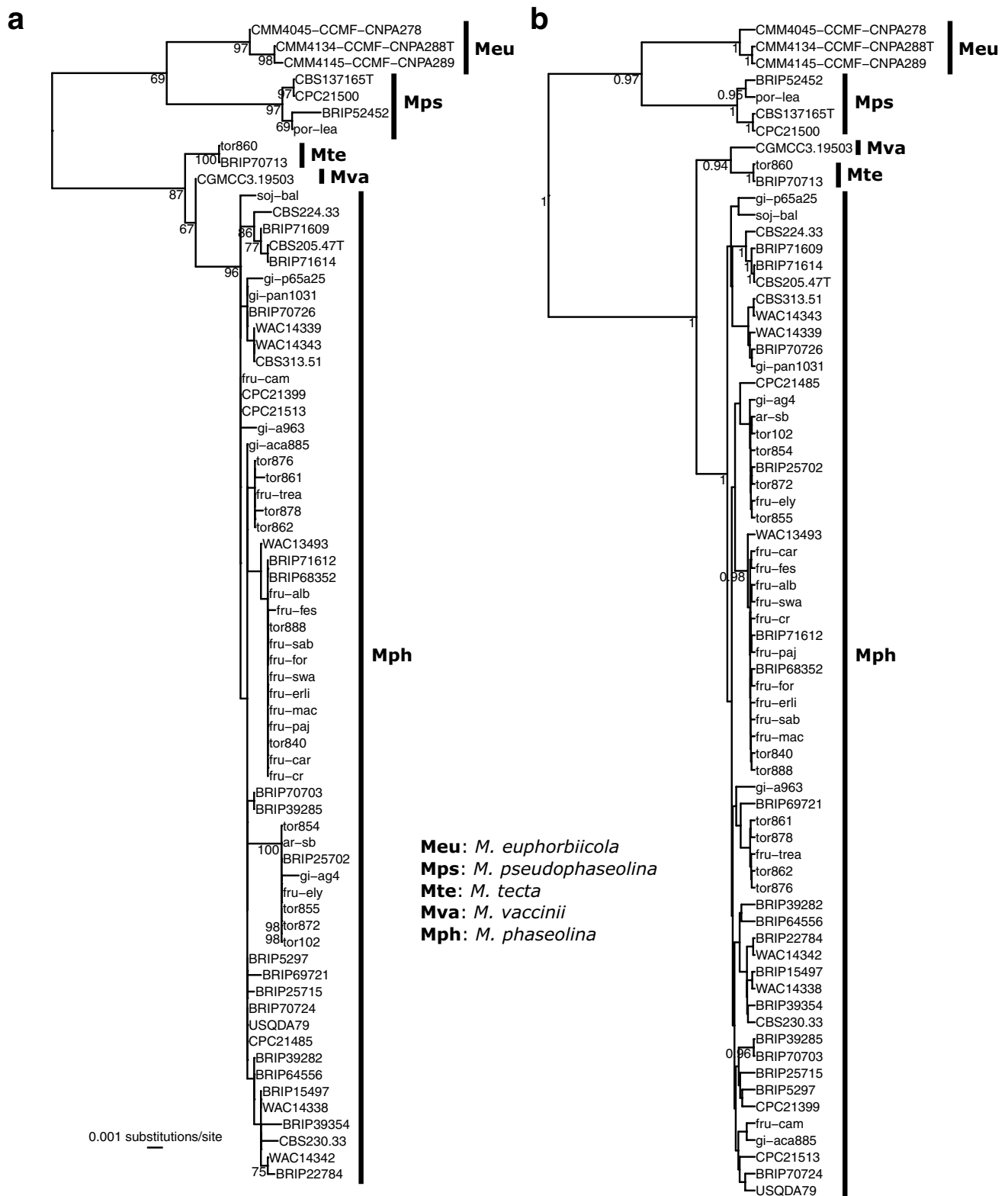
Fig. 2 Maximum Likelihood (**a**) and Bayesian (**b**) phylogenetic trees from the four concatenated DNA markers. The numbers on the nodes indicate the bootstrap support above 65% (**a**) and posterior probabilities above 0.9 (**b**). The outgroup *Lasiodiplodia pseudotheobromae* has been removed from the depiction

Fig. 3 TCS Haplotype networks obtained from the four concatenated DNA markers, represented according to the country (**a**) and host (**b**) of origin of the isolates. Isolates that have the same haplotype (same sequence) are grouped in the same circle. Its size varies according to the number of isolates with the same haplotype. Each transversal line represents a mutational step between haplotypes. The small black circles represent missing haplotypes (may exist but were not sampled)
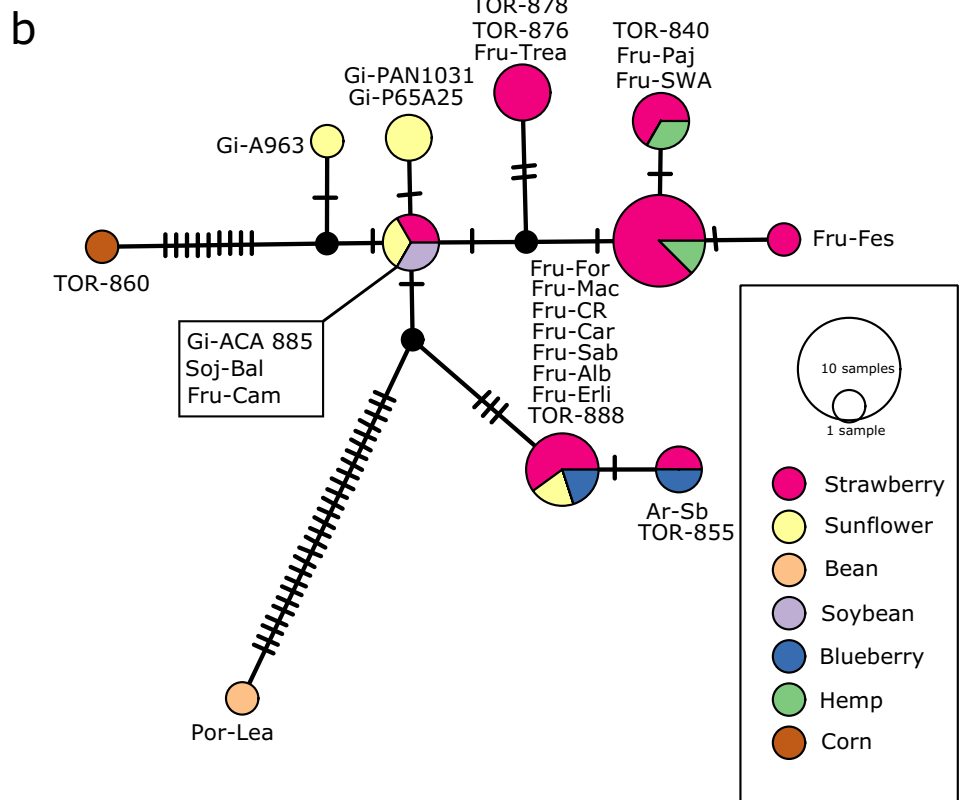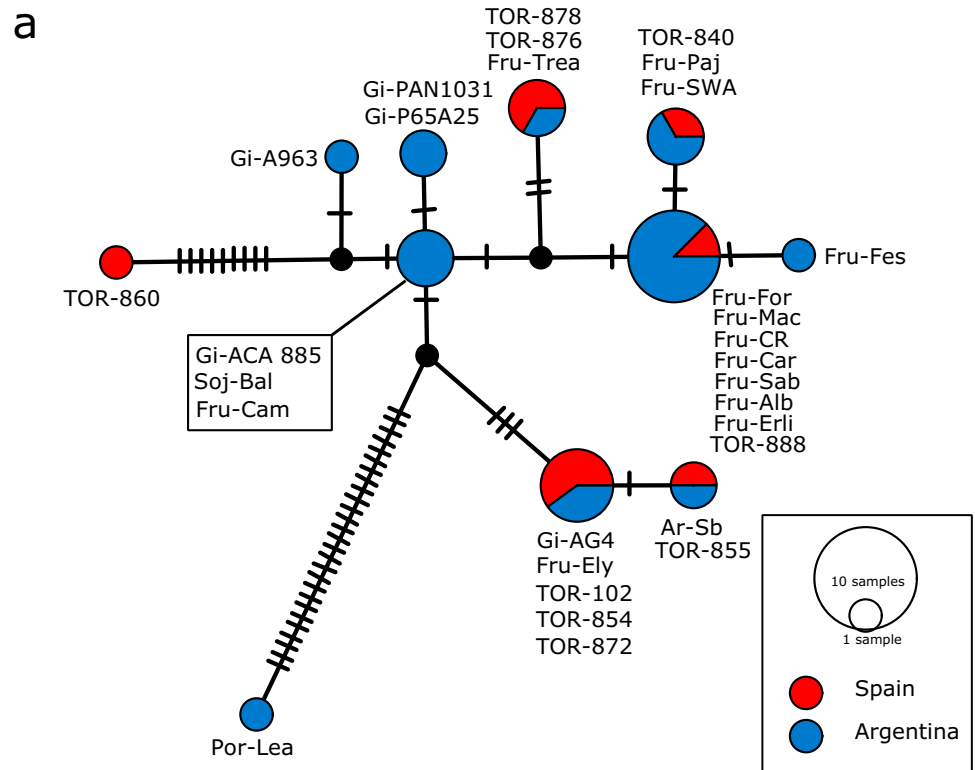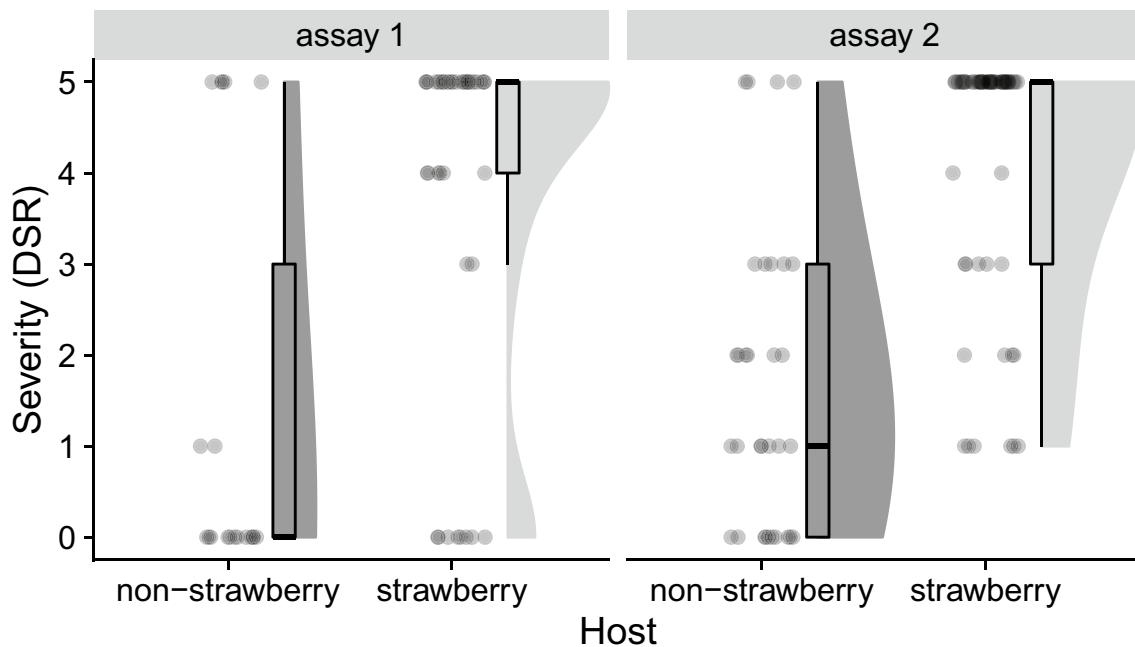
Fig. 4 Host specialization of the isolates. DSR between isolates coming from strawberry (right half) compared to other hosts (left half). Data points are depicted, together with boxes representing the 25% and 75% quantiles, and kernel density areas

morphological and physiological variability observed among isolates, together with the lack of associations between phenotypes and the country or host of origin, makes it difficult to classify them into subspecies [2, 19].

We observed little genetic variation among isolates of *M. phaseolina* (Figs. 2 and 3). Some genetic structure was detected between isolates and the host of origin, but not with the country (Fig. 3). These results must be interpreted cautiously as the number of isolates evaluated was low (30) relative to the number of hosts ($n = 7$). Most studies do not observe associations between isolates of *M. phaseolina* and their geographical origin or host [4, 12, 62]. However, with highly polymorphic DNA markers, Jana et al. [63] and Purkayastha et al. [56] use RAPDs to correlate DNA polymorphisms with the host of origin, while Jana et al. [64] and Sánchez et al. [34] used SSRs to group *M. phaseolina* isolates according to their geographic origin.

The bean (Por-Lea) and corn (TOR-860) isolates classified together with reference samples of *M. pseudophaseolina* [4] and *M. tecta* [7], respectively, in the phylogenetic tree. Despite being different species, Por-Lea and TOR-860 did not exhibit noticeable differences in phenotype compared to the other isolates of *M. phaseolina* (Table 3, Supplementary Material 3 and 4). This is the first report of *M. pseudophaseolina* causing charcoal rot on beans, in Argentina. This recently-described *Macrophomina* species has been only reported in different host species in Senegal, Brazil, India and Australia [4, 5, 7, 65–67]. This is also the first report of *M. tecta* causing charcoal rot on corn, in Spain. This

new species was found on plants of both *Sorghum bicolor* and *Vigna radiata* with symptoms of charcoal rot, in Australia [7]. So far, there are no more records of this species anywhere.

In the pathogenicity tests, strawberry isolates scored an average of 2.4 points higher on the Fang scale, suggesting specialization between the isolates and their host (Fig. 4), disregarding their phenotypic background (Table 3). Many studies have attempted, with unclear results, to relate the morphological and molecular characterizations of *M. phaseolina* isolates with their pathogenicity in diverse hosts [10–12, 19, 20, 53, 56, 68]. Das et al. [20] suggested a possible relation between the sensitivity to chlorate of *M. phaseolina* isolates and their virulence on sorghum. However, we report resistance to chlorate in isolates with large variation in virulence: e.g., Gi-PAN1031 was barely virulent while chlorate-resistant isolates such as TOR-878, TOR-854 and Fru-Trea were highly virulent (Table 3). When looking at host specificity and host specialization in *M. phaseolina*, previous studies show mixed evidence. Su et al. [11] and Mayek-Pérez et al. [10], reported isolates from corn and beans, respectively, to be more virulent when infecting the same host than those from other hosts. Koike et al. [21] also found host specialization of *M. phaseolina* in strawberry. However, other authors reported no host specificity and/or host specialization in several host species [22, 69–71]. Virulence factors and host-specificity/specialization factors play a fundamental role in pathogenicity [72, 73]. A pioneer study suggested

an adaptive mechanism behind host specialization laying on the ability of adapted *M. phaseolina* isolates to use nitrogenous compounds of a specific host [15]. However, the mechanisms involved in host-isolate specificity/specialization of *M. phaseolina* have not been studied so far. The lack of a clear genetic structure or phenotypic traits associated to virulence in this study, contrasted with the effect of host specialization. This suggests a heritable non-measured variation responsible for this differential virulence between hosts. The combined effect of fast-evolving adaptive mechanisms of virulence with certain heritability, such as epigenetics marks [74–76], could be at the base of the success of this versatile fungi to invade new species of hosts across variable environmental conditions. For instance, mechanisms of DNA methylation, modification of histone proteins and sRNA could be involved [77–79]. Further studies on the molecular mechanisms and on the epigenetic variation are required to understand the mechanisms of host specificity and host specialization in *Macrophomina*.

Conclusion

In this work we described a high diversity of *Macrophomina* in its morphological, genetic and pathogenic characteristics. We clearly differentiate isolates of *M. phaseolina* from its sister species *M. pseudophaseolina* and *M. tecta*, and found some association between the genetic structures of the isolates and their host of origin. One of the important aspects of this study reside in the finding of host specialization: isolates that had previously infested the same plant species (strawberry) showed greater virulence in strawberry plants. This contributes to the general knowledge of the behavior of this phytopathogen, but the molecular mechanisms leading to this host specialization are still little addressed. These latter considerations raise interesting directions for future research on interactions between pathogenic fungi and crops.

Declarations

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