Genetic and biological characterization of *Macrophomina phaseolina* (Tassi) Goid. causing crown and root rot of strawberry

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ABSTRACT

In recent years, crown and root rot of strawberry (*Fragaria ×ananassa* Duchesne ex Rozier) caused by *Macrophomina phaseolina* (Tassi) Goid. has affected strawberry production areas worldwide, and in developed countries its emergency has been attributed to the replacement of methyl bromide. The disease was reported in strawberry crop in Chile in 2013, in fields without fumigation. The use of resistant cultivars rises as an alternative to the management of this disease. The objective of this study was to perform a biological and molecular characterization of isolates obtained from two growing regions in Chile and Spain. A total of 35 isolates were characterized for mycelial growth at different temperatures and for chlorate sensitivity. Seven simple sequence repeat loci were used for genetic characterization. Differences were found between Chilean and Spanish isolates in both characterizations. The optimal temperature for mycelial growth was lower in Chilean than in Spanish isolates (30 and 35 °C, respectively). Meanwhile, Chilean isolates were more sensitive to chlorate. In terms of genetic characterization, Polymorphism Information Content (PIC) ranged from 0.38 to 0.85, two main groups were identified, the first group included Spanish isolates and the second group corresponded to Chilean isolates, results were supported by a population structure analysis. This study determined clear differences between two populations of Chilean and Spanish *M. phaseolina* isolates as causal agent of crown and root rot of strawberry.

Key words: Charcoal rot, chlorate sensitivity, *Fragaria ×ananassa*, genetic diversity, microsatellite marker, mycelial growth, optimal temperature, SSR.

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INTRODUCTION

Macrophomina phaseolina (Tassi) Goid. is a pathogenic fungus that attacks more than 500 plant species, including crops such as soybean, sorghum, corn, cotton, sunflower, sesame, common bean, among others (Dinakaran and Mohammed, 2001; Mayék-Pérez et al., 2001; Khan, 2007). Diseases caused by *M. phaseolina* are typically associated with high temperature and drought (Fang et al., 2011). Nevertheless, the severity can fluctuate depending on the environmental conditions and geographic areas (Mihail and Taylor, 1995).

Macrophomina phaseolina has a wide host range and the ability to adapt to different agro-ecological conditions, suggesting a great genetic diversity within this species. Some physiological characteristics that can differ between populations have been reported, such as response to different temperatures (Fang et al., 2011) and resistance to chlorate (Saleh et al., 2010; Mahdizadeh et al., 2011). Despite its wide variability, it has not been possible to determine races or subspecies of this pathogen. Several molecular studies with different molecular markers have been conducted to genetically characterize *M. phaseolina* populations from different geographic areas and hosts. These studies have mainly focused on isolates obtained from common bean, soybean, cotton, sorghum, and sunflower (Mayék-Pérez et al., 2001; Jana et al., 2005; Das et al., 2008; Baird et al., 2010; Mahdizadeh et al., 2011).

In strawberry (*Fragaria ×ananassa* Duchesne ex Rozier), an increased incidence of root and crown rot caused by *M. phaseolina* has been detected in recent decade, seriously damaging strawberry fields in different countries, including Spain (Avilés et al., 2008), Argentina (Baino et al., 2011), Iran (Sharifi and Mahdavi, 2012), Australia (Hutton et al., 2013), and Chile (Sánchez et al., 2013).

In several studies, the increase of the disease has been attributed to the recent elimination of methyl bromide as a pre-plant fumigant and its replacement mostly by 1,3-dichloropropene plus chloropicrin for the control of soilborne pathogens and weeds (Avilés et al., 2008).

In Chile, the strawberry crop has approximately 1000 ha (INE, 2015) distributed in a wide area between parallels 29° and 36° S lat. Strawberry production is mainly for the domestic market as fresh fruit and for exportation as frozen product with good commercial perspectives. One of the largest growing areas in the country is San Pedro-Melipilla due to its proximity to the main markets located in Santiago. In this region, the growers are small producers, and in contrast to the main producing countries, most

of them do not apply pre-transplant chemical fumigants. Curiously, an explosive increase in the crown and root rot of strawberry caused by *M. phaseolina* has been detected (Sánchez et al., 2013).

In this context, the search for resistant cultivars certainly rises as an alternative for efficient disease management. In a study screening 11 commercial genotypes used both in Spain and in Chile for resistance to the disease, only one showed a degree of resistance (Sánchez et al., 2016). On the other hand, the genetic and biological diversity reported in *M. phaseolina* isolates in others host, indicate that the same may occur in strawberry making necessary to have a better understanding of the biology and population dynamics of this fungus, with the aim to obtain cultivars with a durable and stable resistance to this disease.

Actually, there are few studies of the diversity of *M. phaseolina* as the causal agent of the crown and root rot of strawberry regarding biological and genetic characteristics. The objective of this study was to perform a complete characterization of *M. phaseolina* isolates obtained from strawberry crops from two growing regions: San Pedro-Melipilla (Chile) and Huelva (Spain).

MATERIALS AND METHODS

Collection of isolates

Twenty-two isolates were recovered from strawberry plants showing symptoms of crown and root rot from San Pedro-Melipilla (33°53'40" S, 71°27'21" W, 130 m a.s.l.), Chile, and 13 isolates from Huelva (37°15'0" N, 6°57'0" W, 54 m a.s.l.), Spain.

The crowns from symptomatic plants were superficially disinfected with 0.5% NaOCl for 3 min. Longitudinal sections were made to observe the lesions, and the affected area was disinfected with 70% ethanol for 30 s. Small pieces of necrotic tissue were placed on potato dextrose agar (PDA) amended with 200 µL mL^{-1} streptomycin sulfate. Plates were incubated at 25 ± 2 °C for 7 d, after which hyphal tips from dark gray colonies with abundant dark oblong sclerotia were transferred to PDA and incubated for 7 d at 25 ± 2 °C to obtain pure cultures. Isolates were identified to the species level using the species-specific primers MpKFI and MpKRI (Babu et al., 2007).

Growth temperature and chlorate sensitivity

To assess the optimum temperature for mycelial growth of the 23 Chilean isolates and the 12 Spanish isolates, a 5 mm mycelia disc of each isolate was transferred to PDA in a 90 mm Petri dish. The cultures were incubated at 25.0, 30.0, 32.5, 35.0 and 37.5 °C under dark conditions. Colony diameters were measured at 48 h after inoculation, and the data were used to calculate the mycelial growth. The sensitivity to chlorate of the same isolates was evaluated using the minimum medium described by Pearson et al. (1987). A 5 mm mycelia disk of a 5 d culture of each isolate was placed in the center of a 90 mm Petri dish containing 20 mL minimal medium supplemented with 120 mM potassium chlorate. The cultures were incubated at 22 °C for 7 d. The chlorate phenotype of each isolate was determined according to the characteristics of the colony and the density of the microsclerotia (Das et al., 2008). As a control, a set of isolates was grown in minimal medium without the supplementation of potassium chlorate. In both trials, a completely randomized design with three replicates for each isolate using a Petri dish as the experimental unit was used.

Genetic diversity

For genetic diversity, the same strawberry isolates used for biological characterization were assessed. Additionally, three isolates from melon (*Cucumis melo* L.), and two isolates from pine (*Pinus radiata* D. Don) from Chile, were also included. Mono-sclerotic fungus cultures were obtained, transferred to 8 mL 2% potato dextrose broth, and incubated for 7 d at 24 °C. To extract genomic DNA, a modification of the protocol proposed by Lodhi et al. (1994) was used, basically a second phenol-chloroform-isoamyl alcohol (25:24:1; v/v/v) extraction was added. DNA concentrations were determined by spectrophotometry (ND-1000 Spectrophotometer; NanoDrop Technologies, Wilmington, Delaware, USA).

Twelve SSR primers pairs previously described by Baird et al. (2009) were initially tested using the genomic DNA of four isolates: two from Chile and two from Spain. A successful amplification was obtained with 9 of the 12 primers (Table 1). These primers were selected to genotype the 40 isolates of *M. phaseolina* used in this study. The amplification and genotyping of SSRs were performed according to Schuelke (2000) using each SSR forward primer with an M13 (-21) tail at its 5' end, the reverse primers without modifications, and the universal fluorescent-labeled M13 primer. The PCR conditions were: 4 ng genomic DNA, 2.5 mM $MgCl_2$, 1x PCR buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.2 mM dNTPs, 0.6 U Taq (Thermo Fisher Scientific), 8 µM fluorescent-labeled M13 primer, 2 µM M13 forward primer, and 8 µM reverse primer. For the amplification, an initial denaturation for 5 min at 94 °C was performed, followed by 30 cycles of 30 s at 94 °C, 45 s at 56 °C, and 45 s at 72 °C; 8 cycles of 30 s at 94 °C, 45 s at 53 °C, and 45 s at 72 °C; and a final extension of 10 min at 72 °C. The PCR products were separated and measured at the Macrogen capillary electrophoresis facility (Macrogen, Seoul, South Korea). When the isolates did not amplify for a particular locus, the PCR was repeated. If there was no reaction, the presence of a null allele for that locus was assumed.

To estimate the genetic diversity of the isolates of *M. phaseolina*, the Polymorphism Information Content (PIC) of the nine SSR loci was calculated using the Excel microsatellite tool kit v.3.1 (Park, 2001) and analyzed using the program POPULATIONS v.1.2.30 (Langella, 2000), calculating the genetic distances between individuals and creating individual matrices for each isolate according to Nei's minimum genetic distance (Nei, 1972). Corresponding

Table 1. Loci SSR of the 35 *Macrophomina* isolates from strawberry and five outgroups.

Locus	Primer sequences (5'–3')	Repeated sequence	Number of alleles (nr unique alleles)	Allele size (bp)	PIC value
MP04	F: GTGCGAGAAAAATCCGTAAAC R: CCACACAATTACGAAACCTCA	(AG)24	7 (1)	114-125	0.6173
MP05	F: AGTCAAAAGTAGAACAAG R: GACGGGCGCATACATCTC	(GA)11TA(GA)4	6 (3)	132-141	0.5819
MP11	F: AGCCCATGAAGTGGAAAGCTC R: GAAAGAGGTAACCCGCGTTGT	(TC)9CC(TC)8	7 (3)	217-280	0.7274
MP16	F: CACCACATTCGCTGCAAG R: AAGAGGTAACCCGCGTTGT	(CT)7CC(CT)8	5 (1)	209-215	0.5158
MP25	F: TTTCTAGGCTTCTACCCTAC R: CTGCTGCTGCTGCTGCTGT	(CAA)24	NA	NA	NA
MP26	F: CGCTGCCGAACTTACAC R: GTTGGAAGCGGGTGATACTG	(CA)3TA(CA)17	NA	NA	NA
MP27	F: TGACAGGCGTCTGATTG R: CGAGAGGGTTGGTTGGTTTA	(CT)8TT(GT)10	14 (6)	135-153	0.8452
MP29	F: TAGTGCTGAAGCCAGAAG R: CTTAACCTACCCCGATTG	(GT)5	4 (2)	106-109	0
MP31	F: CTTAACCTACCCCGATTG R: TAGTGCTGAAGCCAGAAGTC	(CA)6(GA)16	3 (0)	104-108	0.375
MP32	F: TAGTGCTGAAGCCAGAAGTC R: CCCACAGAAAGAAGCTTAAC	(TG)6	2 (0)	162-163	0
MP34	F: ACGGTGGAAGAGAGCGA R: CCCTTTACAGGCGCAGAG	(AG)3CG(AG)18	NA	NA	NA
MP35	F: AACGGGAAAAATTAATGACACG R: AGGGCAAAAGAAGTAAGAGTGC	(AC)18	6 (3)	147-163	0.5828

PIC: Polymorphism information content; NA: amplification not observed.

dendrograms were constructed with the unweighted pair group method with arithmetic mean (UPGMA), and the matrix was bootstrapped 1000 times to measure the quality of the resulting groups using the program Past v3.01 (Hammer et al., 2001). Furthermore, the original matrix was subjected to a principal coordinates analysis to determine the relationship between isolates. Moreover, Bayesian inference was used to confirm the population structure of Chilean and Spanish *M. phaseolina* isolates using the program STRUCTURE 2.3.4 (Pritchard et al., 2000); 10 tests were conducted (K = 1 to K = 10), where K is the possible number of populations, with a burn-in period length of 100 000 and 50 000 Markov chain Monte Carlo repetitions assuming an admixture model.

RESULTS

Growth temperature and chlorate sensitivity

The optimal temperature for most of the Chilean isolates was 30.0 °C, while that for the Spanish isolates ranged from 32.5 to 35.0 °C (Table 2). However, Chilean and Spanish isolates had similar growth at 37.5 °C (3.37 and 3.56 mm^2, respectively) after 48 h.

In general, Chilean isolates showed a higher mean growth than Spanish isolates at different temperatures (Figure 1). The differences in mycelial growth within Chilean isolates were significant at 25.0, 30.0 and 32.5 °C. However, at 25.0 °C, major differences between isolates were detected.

Four different mycelial phenotypes for chlorate sensitivity were observed in these experiments; A = dense growth, B = medium dense growth, C = feathery growth, and D = restricted growth (Figure 2). Most of the Chilean isolates were chlorate-sensitive (90%), meaning that they

Table 2. Origin of *Macrophomina phaseolina* isolates from strawberry used in the study. Optimal temperature for mycelial growth and chlorate sensitivity of isolates.

Isolate	Origin	Optimal temperature (°C)	Chlorate phenotype*
MpCh1str	Quilamuta (San Pedro, Chile)	35.0	D
MpCh2str	Loica arriba (San Pedro, Chile)	30.0	C
MpCh3str	Quilamuta (San Pedro, Chile)	30.0	C
MpCh4str	Quilamuta (San Pedro, Chile)	30.0	D
MpCh5str	San Pedro (San Pedro, Chile)	30.0	D
MpCh6str	San Pedro (San Pedro, Chile)	30.0	-
MpCh7str	Quilamuta (San Pedro, Chile)	30.0	D
MpCh8str	Quilamuta (San Pedro, Chile)	30.0	C
MpCh9str	Las Pataguas (San Pedro, Chile)	30.0	D
MpCh10str	Quilamuta (San Pedro, Chile)	30.0	C
MpCh11str	Quilamuta (San Pedro, Chile)	35.0	D
MpCh12str	Quilamuta (San Pedro, Chile)	30.0	C
MpCh13str	Quilamuta (San Pedro, Chile)	30.0	C
MpCh14str	Quilamuta (San Pedro, Chile)	35.0	C
MpCh15str	Quilamuta (San Pedro, Chile)	30.0	D
MpCh16str	San Pedro (San Pedro, Chile)	30.0	B
MpCh17str	Quilamuta (San Pedro, Chile)	30.0	C
MpCh18str	San Pedro (San Pedro, Chile)	30.0	D
MpCh19str	Quilamuta (San Pedro, Chile)	35.0	D
MpCh20str	Quilamuta (San Pedro, Chile)	30.0	A
MpCh21str	Quilamuta (San Pedro, Chile)	30.0	C
MpCh22str	Quilamuta (San Pedro, Chile)	30.0	-
MpEsp1str	Huelva (Spain)	30.0	B
MpEsp2str	Huelva (Spain)	32.5	B
MpEsp3str	Huelva (Spain)	32.5	D
MpEsp4str	Huelva (Spain)	35.0	B
MpEsp5str	Huelva (Spain)	35.0	D
MpEsp6str	Huelva (Spain)	35.0	A
MpEsp7str	Huelva (Spain)	35.0	A
MpEsp8str	Huelva (Spain)	35.0	B
MpEsp9str	Huelva (Spain)	35.0	A
MpEsp10str	Huelva (Spain)	32.5	C
MpEsp11str	Huelva (Spain)	32.5	B
MpEsp12str	Huelva (Spain)	35.0	C
MpEsp13str	Huelva (Spain)	35.0	A

*A and B correspond to chlorate-resistant phenotypes, while C and D correspond to chlorate-sensitive phenotype.

Figure 1. Mycelial growth of the 22 Chilean *Macrophomina phaseolina* isolates and the 13 Spanish *M. phaseolina* isolates from strawberry at different temperatures after 48 h inoculation.

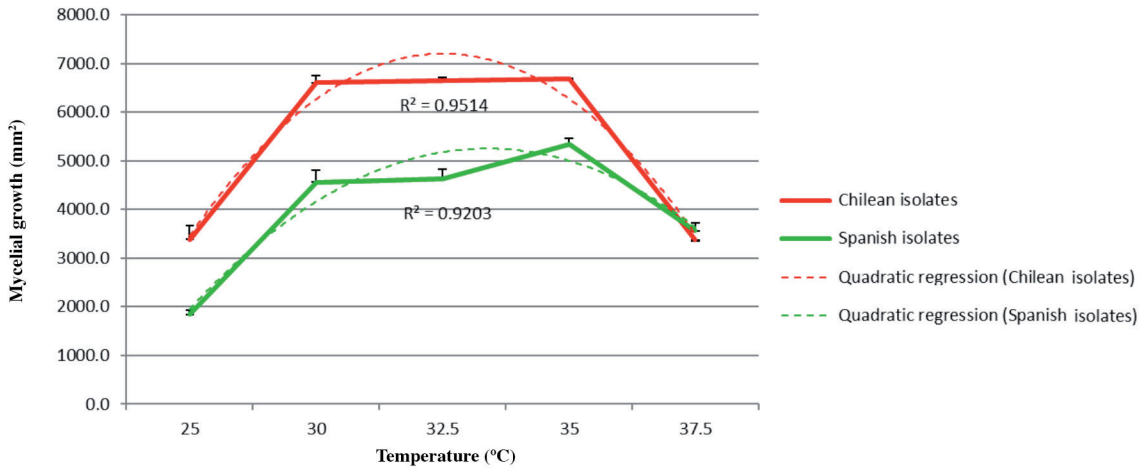
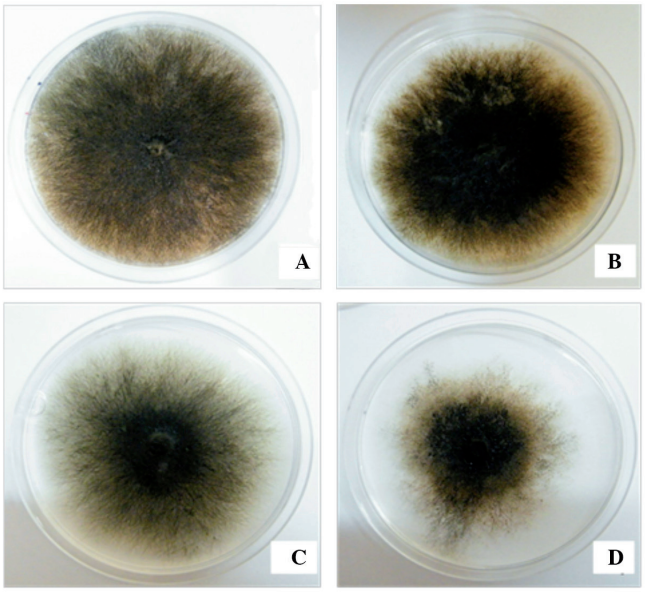


Figure 2. Chlorate phenotypes of *Macrophomina phaseolina* isolates from strawberry used in this study. A and B correspond to chlorate-resistant phenotypes, while C and D correspond to chlorate-sensitive phenotype.



were cataloged with C (45%) and D (45%) phenotypes, while 10% were resistant A (5%) and B (5%). In contrast, in the Spanish isolates only a 30% were chlorate-sensitive, C (15%) and D (15%). While, 70% were chlorate-resistant cataloged with A (31%) and B (39%) phenotypes (Table 2).

Genetic diversity

Seven of the nine successfully amplified SSR loci were polymorphic for the 40 isolates of *M. phaseolina* that were included in this study. A range of 2 to 14 alleles per locus, with a total of 54 alleles, were detected. PIC values were between 0.38 (MP31) and 0.85 (MP27). Among the seven polymorphic loci, six had a PIC value greater than 0.5, being highly informative (Table 1).

Using data obtained for the 40 isolates and the seven selected primers, a dendrogram was created based on the matrix of genetic distance pairs (Figure 3). Two main groups were identified. Group I corresponded to the 22 Chilean isolates from strawberry plants grown in the San Pedro-Melipilla area. While, Group II contained 16 isolates, including the 12 isolates obtained from strawberry fields in Huelva (Spain), two isolates from pine and two from melon of Chilean origin. A separated group was detected that included two isolates: one from Chilean melon and one from Spanish strawberry.

A principal coordinates analysis (Figure 4) confirmed the existence of two major groups in which strawberry isolates from Chile and Spain were clearly distinguished, while isolates from melon and pine were genetically closer to Spanish isolates.

The analysis of the population structure using Bayesian inference permitted the identification of two populations (Figure 5). The first population contained the 22 Chilean strawberry isolates plus one isolate from Spain. The second population was formed by Spanish strawberry isolates plus Chilean pine and melon isolates, ruling out the existence of a third group (Figure 5).

The two major groups obtained through different analyses generated by the SSRs indicate that the genetic distances observed are consistent and allow the establishment of significant differences among the studied populations.

DISCUSSION

In this study, differences were found between two populations of *M. phaseolina* related to biological and genetic characteristics. The results obtained in this study from comparing *M. phaseolina* isolates from strawberries grown in Chile and Spain are consistent with the findings of other authors reporting optimal temperature for mycelial growth between 30 and 35 °C (Csöndes et al., 2012; Sexton et al., 2016). We detected differences between Spanish and Chilean *M. phaseolina* isolates. Spanish isolates reached the maximum mycelial growth with the highest temperatures. However, Chilean *M. phaseolina* isolates had a lower

Figure 3. Dendrogram constructed by clustering method for 40 isolates of *Macrophomina phaseolina* based on Nei's minimum genetic distance matrix on seven SSR loci.

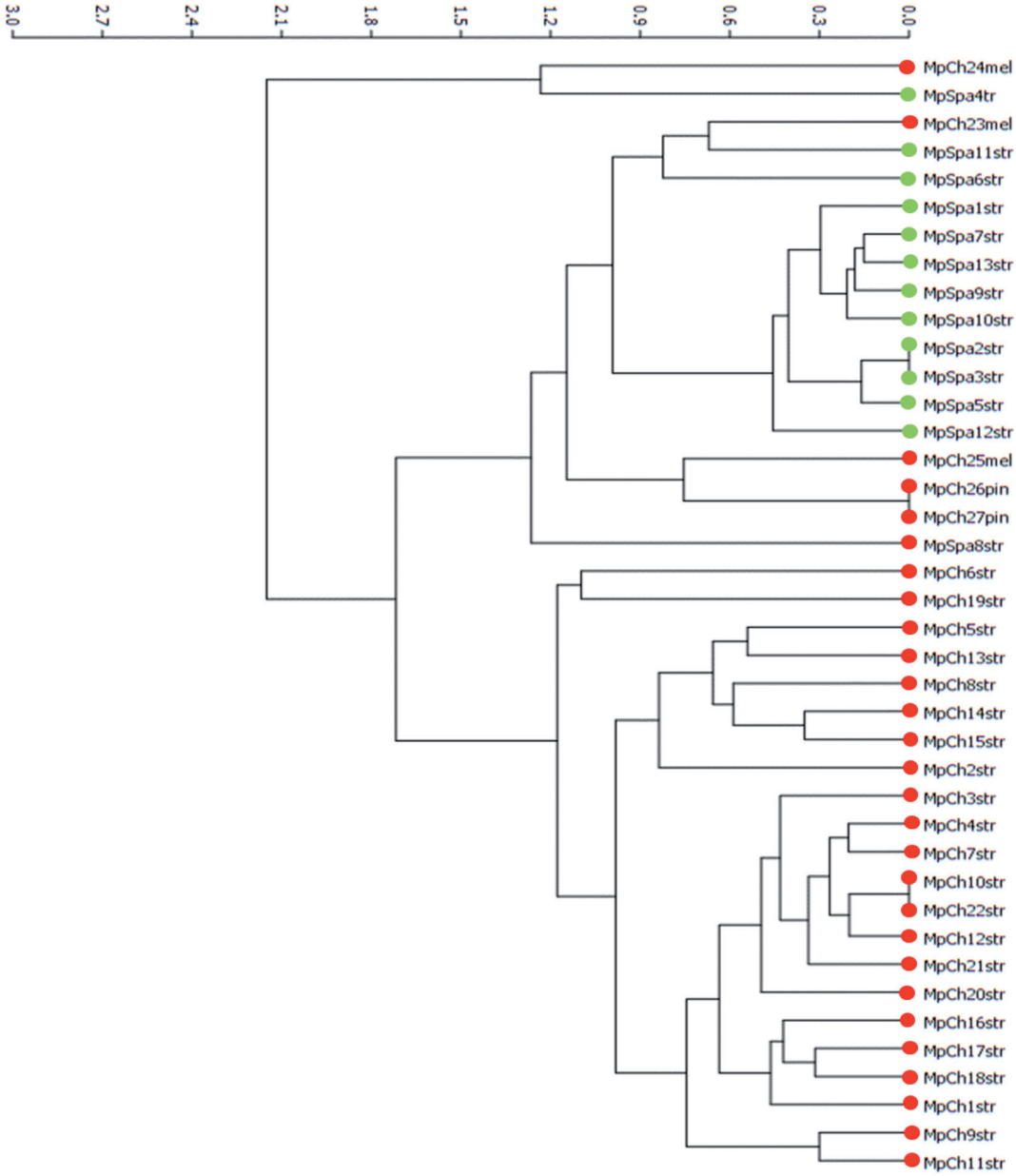
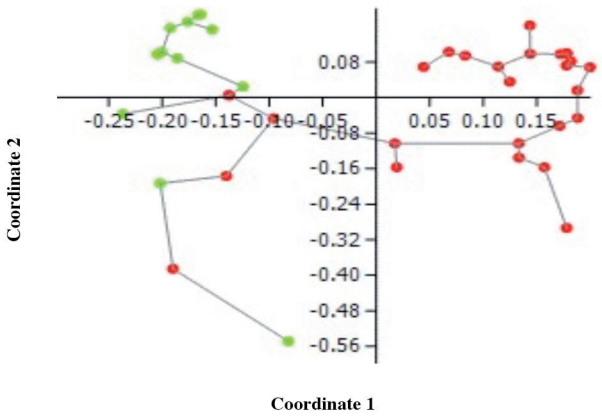


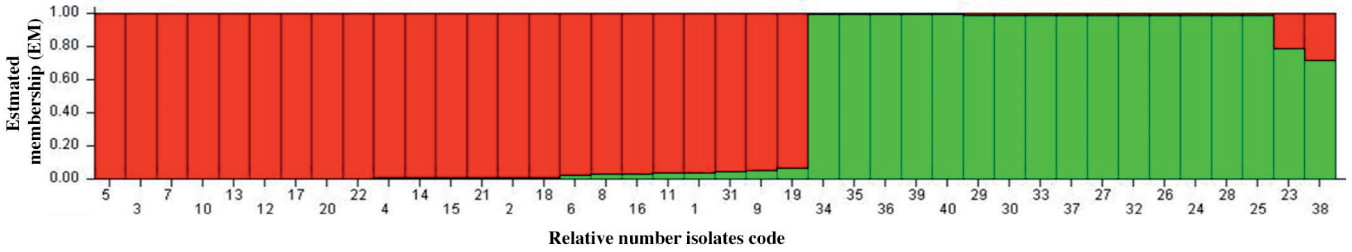
Figure 4. Principal coordinates analysis showing grouping of 40 *Macrophomina phaseolina* isolates based on seven SSR markers.



optimal temperature. These variations may be explained by the geographic origin (Csöndes et al., 2012; Sexton et al., 2016), as soil temperatures may have led to the selection of individuals who were adapted to local conditions. In Chile, the average soil temperature is between 27 and 29 °C at the peak of production during the growing season (www.agromet.cl). In contrast, in Huelva, the average soil temperature is greater than 30 °C (Medina et al., 2004), which could explain the differences between the isolates from these origins.

In this study, we found four phenotypes related to chlorate sensitivity. These results agree with the findings of Das et al. (2008) in sorghum isolates. Chlorate-sensitive isolates showed either feathery or restricted growth; these phenotypes were predominant in Chilean isolates, while

Figure 5. Estimated populations structure of 40 isolates of *Macrophomina phaseolina* from Chilean and Spanish populations (with possible numbers of population K = 1 to K = 10).



the Spanish isolates were mostly chlorate-resistant from medium to dense growth. Chlorate is an analog of nitrate, and the reduction of chlorate via nitrate reductase can result in toxicity to fungi, among other organisms. Chlorate-sensitive isolates can reduce chlorate to chlorite, which can restrict the growth when the nitrate reductase pathway is active. Consequently, unrestricted growth is a result of the inactivity of one or more enzymes in the nitrate reductase pathway (Pearson et al., 1987).

In terms of genetic characterization, SSRs effectively determined the genetic diversity of Chilean and Spanish *M. phaseolina* isolated from symptomatic strawberry plants. Through an analysis of nine SSR loci, it was found that there was a moderate to high genetic diversity for the loci analyzed. Previous studies have been able to successfully establish genetic diversity of *M. phaseolina* isolates, mostly from soybean, using SSR markers (Jana et al., 2005; Baird et al., 2010).

Since *M. phaseolina* is an anamorphic species, part of its variability has been linked to the abundant presence of transposons and repeated DNA sequences in its genome. Its genome contains 2.84% repeated sequences and 3.98% mobile transposable elements, the latter of which are associated with an increased range of virulence because mobile transposable elements are responsible for the inactivation of genes that activate the immune system of plants (Islam et al., 2012).

The results obtained in the present study using SSR markers showed a clear tendency in isolates to cluster by origin, making evident a genetic distance between Chilean and Spanish strawberry isolates. An analysis performed with different molecular techniques has produced similar results in *M. phaseolina* isolates from other species. An AFLP study on common bean roots reported a high genetic diversity among *M. phaseolina* isolates from different regions of Mexico (Mayék-Pérez et al., 2001). Other studies based on RAPD have also shown significant genetic distances between isolates from different geographic origins in crops such as soybean, sesame, bean, chickpea, jute, and cotton (Jana et al., 2003), in addition to sorghum (Das et al., 2008). Meanwhile, using SSRs, Jana et al. (2005) determined the genetic relationships between Indian and American *M. phaseolina* isolates mainly from soybean that were grouped according to host and origin.

Interestingly, two isolates from pine that were included in this study showed a greater genetic proximity to Spanish isolates, clustering outside the clade in which the Chilean strawberry isolates were found. This result may indicate that strawberry isolates from central Chile were not necessarily related to pathogenic populations that affect pine plantations in the southern region of the country, which coincides with the strawberry plants production area. However, it is necessary to include a larger number of isolates to prove this hypothesis.

CONCLUSIONS

This study showed clear differences between two populations of Chilean and Spanish *Macrophomina phaseolina* isolates. This biological and genetic diversity must be considered in breeding programs for resistance to crown and root rot disease of strawberry caused by this soilborne pathogen, even though differences between cultivar susceptibility have been previously determinate.

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