Soybean - *Macrophomina phaseolina* specific interactions and identification of a novel source of resistance

Running title: Soybean - *Macrophomina phaseolina* specific interactions

Sebastián Reznikov[1*], María A. Chiesa[2*], Esteban M. Pardo[1], Vicente De Lisi[1], Noelia Bogado[3], Victoria González[1], Fernando Ledesma[1], Eligio N. Morandi[2], L. Daniel Ploper[1#] and Atilio P. Castagnaro[1#]

[1]Instituto de Tecnología Agroindustrial del Noroeste Argentino (ITANOA), Estación Experimental Agroindustrial Obispo Colombres (EEAOC) - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. William Cross 3150, C.P. T4101XAC Las Talitas, Tucumán, Argentina.

[2]Laboratorio de Fisiología Vegetal, Instituto de Investigaciones en Ciencias Agrarias de Rosario (IICAR), Universidad Nacional de Rosario (UNR) - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Parque Villarino S/N, 2125 Zavalla, Santa Fe, Argentina.

[3]Instituto Paraguayo de Tecnología Agraria (IPTA), Centro de Investigación Capitán Miranda, Ruta VI, Km 16, C.P. 6990 Capitán Miranda, Itapúa, Paraguay.

[*]S. Reznikov and M.A. Chiesa contributed equally to this work.

[#]Corresponding authors: L.D. Ploper, E-mail address: dt@eeaoc.org.ar; A.P. Castagnaro, E-mail address: atiliocastagnaro@gmail.com and atilio@eeaoc.org.ar.

ABSTRACT

Charcoal rot, caused by the fungus *Macrophomina phaseolina,* is an economically important disease of soybean (*Glycine max*) worldwide. Objectives of the present research were to (i) study the genetic and pathogenic diversity in a collection of *M. phaseolina* isolates from Argentina and Paraguay and (ii) develop an improved *in vitro* phenotyping method to evaluate disease response of soybean genotypes to *M. phaseolina* isolates. Cluster analysis showed no clear association among simple sequence repeats (SSR) profiles, year of collection, pathogenicity and geographical origin of the isolates from Argentina and Paraguay. Subsequently, the response of four soybean genotypes against seven *M. phaseolina* isolates was evaluated in the field and the results were confirmed using the *in vitro* assay developed. This assay, which is based on root disease development on soybean seedlings, allowed the detection of a differential level of aggressiveness among the isolates on four soybean genotypes. The results suggest the existence of specific interactions among soybean genotypes and *M. phaseolina* isolates. In addition, cultivar Munasqa RR showed a superior response against *M. phaseolina* compared with DT 97-4290 (moderately resistant), thus becoming a novel source of resistance to charcoal rot.

Additional keywords: charcoal rot; genetic and pathogenic diversity; *in vitro* phenotyping.

INTRODUCTION

Macrophomina phaseolina (Tassi) Goid. is a polyphagous fungus, infecting nearly 500 species in more than 100 plant families worldwide (Mihail and Taylor 1995) including cereals, legumes, vegetables, fruits and fiber crops (Dhingra and Sinclair 1978). In soybean [*Glycine max* (L.) Merr.], *M. phaseolina* is the causal agent of charcoal rot, an economically serious and potentially destructive disease; in 2006, it was estimated to be responsible for yield losses of around 4% worldwide (Wrather et al. 2010). Measurements in experimental field plots recorded losses between 18 and 30% caused by this fungus (Mengistu et al. 2011). In South America, charcoal rot has caused severe problems in soybean crops in Paraguay, where the prevalence of the disease was 100% in 48 localities evaluated from April to August 2008 (Orrego Fuente et al. 2009). In Argentina, the hot and dry weather that prevailed in the northwestern region (NW) during the 2000-2001, 2011-2012 and 2012-2013 growing seasons favored charcoal rot development in soybean crops. This affected the production areas in the provinces of Catamarca, Salta, Santiago del Estero and Tucumán in the NW, resulting in varying levels of yield losses, and even total losses in some fields (Ploper et al. 2001; Reznikov 2016).

The use of resistant cultivars provides an effective approach for disease control, particularly for charcoal rot (Romero-Luna et al. 2017). In addition, genetic resistance is a key strategy that minimizes the use of fungicides, reduces crop losses and supports sustainable production management (Bowen and Schapaugh 1989; Bristow and Wyllie 1984; Smith and Carvil 1997). However, to date, the germplasm line DT 97-4290 is the only soybean genotype registered as moderately resistant to *M. phaseolina* (Paris et al. 2006).

Genetic and pathogenic diversity in the species *M. phaseolina* was previously observed (Su et al. 2001). Recently, genetic diversity among isolates of *M. phaseolina* collected mainly from soybean fields in the United States was determined using simple sequence repeats (SSR) markers (Baird et al. 2010; Baird et al. 2009); and an association between *M. phaseolina* and plant-host origin of the isolates was found (Arias et al. 2011). Similar results were reported with the use of universal rice primers (URP) for polymerase chain reaction (PCR) fragment amplification, when analyzing the diversity of isolates from three different crop species (Jana et al. 2005). Saleh et al. (2010) reached the same conclusion through random amplification of genomic fragments by using oligo repeats comparing crops with wild host species.

In order to screen the disease reaction of soybean genotypes to this pathogen accurately, a precise and reproducible phenotyping method is required. To this day, the best method to evaluate host resistance to charcoal rot has been the analysis of root and stem severity at the R7 growth stage based on a colony-forming unit (CFU) index in field conditions (Mengistu et al. 2007). However, this method is not only time-consuming and expensive, but also requires artificial inoculation of the soil with *M. phaseolina*. These limitations make this method difficult to be used consistently when evaluating the reaction of large numbers of soybean genotypes against charcoal rot.

The underlying hypothesis of the current study was the existence of genotype-genotype specific interactions between soybean germplasm and *M. phaseolina* isolates. In order to validate these interactions, the specific aims of this research were to (i) characterize the genetic diversity and aggressiveness of *M. phaseolina* isolates collected from soybean fields in Paraguay and Argentina, and (ii) develop a rapid and reliable *in vitro*

assay to evaluate and confirm specific responses of soybean genotypes to *M. phaseolina* isolates.

MATERIALS AND METHODS

Fungal isolates and growth conditions. Thirty-nine isolates of *M. phaseolina* were obtained from roots and stems of soybean plants showing characteristic symptoms of charcoal rot in fields from Argentina and Paraguay in 2008, 2009, 2010 and 2013 (Table 1). The geographic areas were selected based on their importance regarding soybean production in each region (Fig. 1).

All plant samples were rinsed with deionized water, and 0.5-cm tissue sections were surface-disinfested with 70% (v/v) ethanol for 30 s, followed by 5% (v/v) NaClO for 1 min, rinsed with sterile water, and then air-dried in sterile conditions. Samples were placed on potato dextrose agar (PDA; Difco, Detroit, MI) dishes, acidified with 0.2% (v/v) lactic acid and incubated at 28 ± 2ºC for 4 days. A single microsclerotium of each isolate was removed with a sterile needle under the stereoscopic microscope and transferred to a new acidified PDA dish. Pure cultures of each isolate were obtained in 24 to 48 h and preserved at -20°C on filter paper.

Nucleic acid purification. Fungal DNA was extracted by employing the CTAB method for total nucleic acid extraction (Murray and Thompson 1980). First, mycelium was grown in 100 mL of potato-glucose broth for 2 weeks in darkness at 28 ± 2°C. Then, it was harvested by filtration through a layer of metal filter (1 mm), washed twice with sterile water, and dried at room temperature in sterile conditions for 24 h. Dry mycelium was ground with liquid nitrogen using a mortar and pestle, and 100 mg of each sample was used for total nucleic acid extraction.

Polymerase chain reaction amplification of SSR markers. Genotypic analysis of 39 *M. phaseolina* isolates was performed using the 28 SSR primers reported by Arias et al. (2011): StvMPh_209a, StvMPh_213a, StvMPh_329a, StvMPh_415b, StvMPh_114a, StvMPh_146a, StvMPh_100a, StvMPh_102a, StvMPh_144a, StvMPh_162a, StvMPh_173a, StvMPh_190a, StvMPh_19b, StvMPh_20a, StvMPh_34a, StvMPh_132a, StvMPh_49a, StvMPh_63a, StvMPh_182a, StvMPh_197a, StvMPh_310a, StvMPh_461a, StvMPh_484a, StvMPh_562c, StvMPh_109b, StvMPh_116a, StvMPh_123a, and StvMPh_137a. Primers labelled with 6-carboxy-fluorescein (FAM) (IDT Technologies, Coralville, IA) were used for amplification of 10 ng of fungal DNA using Titanium Taq DNA Polymerase (Clontech, Fremont, CA) in 5-µL reactions in a thermal cycler using the following amplification scheme: 95°C for 1 min, 60°C for 1 min (2 cycles), 95°C for 30 s, 60°C for 30 s, 68°C for 30 s (27 cycles) and a final extension cycle at 68°C for 4 min. Fluorescently-labelled amplified PCR fragments were analyzed on an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA).

Genetic cluster analysis. For SSR markers, amplicons were screened for length polymorphisms and transformed into binary data for each locus based on the presence (= 1) or absence (= 0) of alleles. For each SSR marker, the number of amplified alleles, the size range (bp), the number of polymorphic alleles, and the polymorphism information content (PIC) were calculated (Milbourne et al. 1997). In addition, Info-Gen software (Balzarini and Di Rienzo 2013) was used to estimate the percentage of polymorphism (band or locus), the average number of alleles per primer set or per locus, the effective number of alleles, and Nei's genetic diversity (Nei 1973). Cluster analysis of *M. phaseolina* isolates was performed using the Unweighted Pair-Group Method with Arithmetic Averages (UPGMA) with the SSR data in Info-Gen. To evaluate the robustness of the diversity analysis and the

clusters formed, the binary data set was subjected to 1,000 bootstrapping replicates using the WINBOOT program (Yap and Nelson 1996), and confidence values higher than 50% were indicated (Highton 1993).

Pathogenicity of *M. phaseolina* isolates in field conditions. A field test to evaluate the pathogenicity of *M. phaseolina* isolates was conducted at the Capitán Miranda Research Center (CICM) within Instituto Paraguayo de Tecnología Agraria (IPTA), Capitán Miranda, Itapúa, Paraguay (27° 11' 59.92" S and 55° 47' 28.90" W). The trial was planted by hand on 16 January 2013 with four soybean genotypes from different maturity groups (MG): DT 97-4290 and CRIA 4 (MG IV), DM 6.2i RR (MG VI) and Munasqa RR (MG VIII). Soybean genotype DT 97-4290 was the moderately resistant control (Paris et al. 2006) and DM 6.2i RR was the susceptible control. The experimental design was a blocked split plot with three replicates, in which *M. phaseolina* isolates were assigned to the whole plots and soybean genotypes to the subplots, each of which consisted of four 1-m rows, spaced 0.5 m apart, and planted at a density of 23 seeds/m. As inoculum, seven isolates of *M. phaseolina* (Mp15, Mp17 and Mp18 from Argentina, and Mp32, Mp37, Mp42 and Mp48 from Paraguay) (Table 1) were used to inoculate sterile millet, which was then incubated for 20 days at 30°C in darkness to stimulate the development of microsclerotia. When soybean seeds were planted, 5 g of millet colonized with each isolate of *M. phaseolina* was applied by hand per linear meter. At the R7 growth stage (Fehr et al. 1971), disease severity was estimated on 10 plants per plot using the scale established by Paris et al. (2006): 1 = no discoloration and no microsclerotia visible; 2 = no discoloration of vascular tissue, with very few microsclerotia visible in the pith, vascular tissue or under the epidermis; 3 = partially discolored vascular tissue, with microsclerotia partially covering the tissue; 4 = discolored vascular tissue, with numerous microsclerotia visible in

136 the tissue under the outer epidermis, in stem and root sections; and 5 = vascular tissue with
137 numerous microsclerotia producing a dark color inside and outside of the stem and root
138 tissue. In addition, CFU/g of tissue was determined on the same 10 plants, according to
139 Mengistu et al. (2007). Briefly, plant samples were obtained by cutting 10 cm above and
140 below the soil line including root and stem tissue. Three rinses with tap water were
141 performed to remove traces of soil from the samples, which were then dried at room
142 temperature and ground. From each sample, 5 mg of ground tissue was placed in a test
143 tube and disinfested with 5% (v/v) NaClO for 1 min, followed by three 1-min washes using
144 sterile distilled water. Subsequently, 5 mL of 60°C sterile PDA was added and samples
145 were poured into sterile Petri dishes. Total CFU was quantified after incubation at 28°C for
146 3-5 days, and expressed as CFU per gram of dry tissue, CFU/g. Disease severity at R7
147 (log-transformed) and CFU/g (square root-transformed) were analyzed by generalized
148 linear mixed models followed by mean comparison (LSD, $P = 0.05$) with InfoStat software
149 (Di Rienzo et al. 2011).

150 ***In vitro* method of soybean root infection with *M. phaseolina*.** Soybean seeds were
151 disinfested with 5% (v/v) NaClO for 1 min, followed by 70% (v/v) ethanol for 30 s and
152 three 1-min rinses with sterile distilled water. Disinfected seeds were placed in a Petri dish
153 containing a layer of sterile filter paper, to which 15 mL of sterile distilled water was
154 added. Seeds were incubated for 48 h at 28°C in darkness in order to induce germination.
155 Next, five healthy germinated seeds were placed in a sterilized glass flasks (15 cm high and
156 10 cm diameter) containing a 3 cm layer of cotton and filter paper and 50 mL of sterile
157 distilled water. Three toothpick pieces (2 cm long) colonized with a *M. phaseolina* isolate
158 derived from a single microsclerotium were added to each flask in a sterile flow chamber.

159 The flasks containing the germinated and inoculated soybean seeds were kept in a growth
160 chamber under a 16-h light (600 μE $m^{-2}s^{-1}$)/8-h dark regime and a temperature of 30°C.
161 The *in vitro* infection method was conducted at the Plant Physiology Laboratory of
162 the Instituto de Investigaciones en Ciencias Agrarias de Rosario (IICAR), Universidad
163 Nacional de Rosario (UNR) and Consejo Nacional de Investigaciones Científicas y
164 Tecnológicas (CONICET), located in Zavalla, Santa Fe, Argentina. The root infection
165 severity of four soybean cultivars: DT 97-4290 and CRIA 4 (MG IV), DM 6.2i RR (MG
166 VI) and Munasqa RR (MG VIII) was tested with seven single-microsclerotium isolates of
167 *M. phaseolina* (Mp15, Mp17, Mp18, Mp32, Mp37, Mp42 and Mp48) (Table 1). Root
168 disease severity was evaluated every 48 h for 12 days; to do this, images were acquired
169 with a digital camera (Nikon D50) and the length of necrosis in the root system was
170 measured for each seedling with an image processing program (ImageJ; NIH, Bethesda,
171 MD). Disease severity values were expressed as a percentage of necrosis of the root
172 system.
173 The experimental design was a randomized complete block with three replicates, and
174 was repeated twice. Each replication consisted of three flasks, each containing five
175 germinated seeds. Two flasks contained the germinated seeds inoculated with *M.*
176 *phaseolina* (10 experimental units) and the control sample flask contained the non-
177 inoculated germinated seeds (5 experimental units). The area under the disease progress
178 curve (AUDPC) was calculated based on disease severity data (Madden et al. 2007) and
179 analyzed by generalized linear mixed models followed by mean comparison (LSD, P =
180 0.05) with InfoStat software (Di Rienzo et al. 2011). To determine the predictive ability of
181 the *in vitro* assay, Spearman´s rank correlation coefficients were calculated between *in vitro*

AUDPC value and field disease severity or field CFU/g values using InfoStat software (Di Rienzo et al. 2011).

RESULTS

Genetic relationships among *M. phaseolina* isolates. For each SSR marker, information on the number of amplified alleles (1 to 14), the size range (bp) (91 to 270 bp), the number of polymorphic alleles (0 to 14) and PIC (-0.710 to 0.841) are presented in Table 2. When the 39 *M. phaseolina* isolates were genotypically analyzed with 28 SSR markers, a total of 213 amplicons were obtained, of which 155 (72.8%) were polymorphic. The value of Nei´s genetic diversity obtained was 0.13.

A single-linkage dendrogram was obtained from the SSR markers data. Genetic relationships among isolates were determined by the Jaccard's similarity coefficient (Fig. 2). The cluster analysis showed that isolate Mp18 from Argentina was clearly differentiated from the rest of the isolates at a genetic distance of 0.90. The rest of the isolates were separated into two major clusters with a 0.80 dissimilarity value. Cluster I harbored the majority of *M. phaseolina* isolates, including all isolates from Paraguay and 11 from Argentina, whereas cluster II included 10 *M. phaseolina* isolates from Argentina (Fig. 2), seven from the NW and the remaining three (Mp06, Mp08 and Mp12) from the central region of the country (Table 1 and Fig. 1).

Pathogenicity of *M. phaseolina* isolates in field conditions. Seven isolates of *M. phaseolina* from the collection (Table 1) were selected for the pathogenicity tests. These isolates originated from both countries (Mp15, Mp17 and Mp18 from Argentina and Mp32, Mp37, Mp42 and Mp48 from Paraguay) and belonged to the two main clusters in the dendrogram, except for isolate Mp18 from Argentina, which, as stated above, was

differentiated from the rest of the isolates (Fig. 2). In addition, the selected isolates showed differences in cultural and growth characteristics (data not shown).

When the seven isolates of *M. phaseolina* were inoculated on the four soybean genotypes in field conditions, no significant differences in aggressiveness were observed among isolates considering the results of disease severity and CFU/g, for each one as an average in the four soybean genotypes (Table 3). Disease severity values ranged from 1.9 to 2.2 ($P = 0.6203$) and CFU/g values ranged from 233.3 to 611.6 ($P = 0.5015$). However, when the effect of the soybean genotypes were tested and the disease reaction of each of the four genotypes (DM 6.2i RR, CRIA 4, DT 97-4290, and Munasqa RR) was considered as an average against the seven *M. phaseolina* isolates, significant differences were found in disease severity ($P = 0.0001$) and also in CFU/g values ($P = 0.0001$) (Table 3).

Additionally, the analysis of the soybean genotype x *M. phaseolina* isolate interactions showed significant effects for both disease severity ($P = 0.0277$) and CFU/g ($P = 0.0025$) (Table 3). As shown in Fig. 3A, contrasting responses were detected in field conditions when the specific interactions between each soybean cultivar inoculated with the different isolates of the pathogen were analyzed in detail. The lowest value of CFU/g was obtained in the Munasqa RR x Mp15 combination (33.3), whereas the highest value of CFU/g was obtained in the DM6.2i RR x Mp48 combination (2,366.7) (Fig. 3A).

Since the significance of the soybean cultivar x *M. phaseolina* isolate interaction for disease severity in field conditions was relatively marginal ($P = 0.0277$), the main effects of the cultivars were analyzed (Table 4). Munasqa RR displayed the highest level of resistance among the four genotypes evaluated, although it was not significantly different in disease severity values from DT 97-4290, which was classified as moderately resistant.

Both genotypes showed the lowest values of disease severity and differed from CRIA 4 and DM 6.2i RR (P = 0.0001). However, DM 6.2i RR was the most susceptible to *M. phaseolina* and was significantly different from CRIA 4 that was classified as moderately susceptible (Table 4).

Pathogenicity of *M. phaseolina* isolates *in vitro*. Initially, the *in vitro* root infection assay was optimized with isolate Mp17, and reproducible results were obtained after a 2-week incubation period. At day 2 post inoculation (dpi), no symptoms of necrosis on the radicle of either resistant or susceptible genotypes were observed (Fig. 4). At 5 dpi, initial symptoms of necrosis were observed in 60% of the germinated seeds of the susceptible genotype DM 6.2i RR, whereas in 40% of the initially germinated seeds the germination process was arrested, compared with the uninfected germinated seeds control. At 9 dpi, all seedlings of the two susceptible genotypes (DM 6.2i RR and CRIA 4) showed symptoms of necrosis on the root system, whereas the moderately resistant genotype DT 97-4290 presented initial symptoms of necrosis in 60% of the seedlings. It is interesting to note that none of the Munasqa RR seedlings showed signs of necrosis in the root system, demonstrating that also in controlled conditions this genotype displayed the highest level of resistance. At 12 dpi, clear and reproducible differences were observed between the susceptible genotypes and the moderately resistant genotype DT 97-4290 when compared with Munasqa RR inoculated with Mp17 isolate (Fig. 4). Disease severity, measured as a percentage of necrosis on the root system, was evaluated every 48 h during 12 dpi, allowing the disease progress to be determined in each interaction.

By applying the *in vitro* phenotyping method previously described, the root disease severity of the same four soybean genotypes (Munasqa RR, DT 97-4290, CRIA 4 and DM 6.2i RR) previously inoculated in field conditions with seven isolates of *M. phaseolina*

253 (Mp15, Mp17, Mp18, Mp32, Mp37, Mp42, and Mp48) was monitored in controlled
254 conditions. The results obtained in the pathogenicity test are summarized in Table 3 and in
255 Figs. 3B and 5. Statistical differences were found in the specific interactions among the
256 four soybean genotypes and the seven *M. phaseolina* isolates, evaluated in controlled
257 conditions ($P = 0.0004$), in accordance with the field trial (Table 3). As shown in Fig. 3B,
258 contrasting responses among the soybean cultivar were also detected in controlled
259 conditions when inoculated with the different isolates of the pathogen. Again, Munasqa
260 RR showed the highest level of resistance among the four genotypes evaluated, displaying
261 the lowest values of AUDPC against all the inoculated isolates (Fig. 3B). In controlled
262 conditions, the lowest value of AUDPC was obtained in the Munasqa RR x Mp17
263 combination (4.2), whereas the highest value was obtained in the DM6.2i RR x Mp37
264 combination (233.3) (Fig. 3B). The contrasting phenotypes observed in some
265 representative specific interactions by applying the *in vitro* root infection method are shown
266 in Fig. 5. Indeed, the *in vitro* assay data (AUDPC) correlated with the square root-
267 transformed CFU/g field data with a Spearman´s rank correlation coefficient value of 0.62,
268 $P = 0.0004$ (Fig. 6A). In addition, the AUDPC data obtained in controlled conditions
269 correlated with the ln-transformed severity field data with a rank correlation coefficient of
270 0.59, $P = 0.0009$ (Fig. 6B), validating the *in vitro* phenotyping method developed and
271 presented in this work.
272

DISCUSSION

In this study, the genetic diversity in a collection of 39 isolates of *M. phaseolina,* an important fungal pathogen that seriously affects soybean crops worldwide, was analyzed. Previous genetic studies using molecular markers and subsequent cluster analysis showed a high degree of global genetic diversity among isolates of this pathogen (Arias et al. 2011; Muñoz-Cabañas et al. 2005). Our cluster analysis based on 28 SSR markers revealed two distinct genetic groups and one ungrouped isolate, Mp18, at a genetic distance threshold of 0.80 in the dendrogram. Nevertheless, all the isolates from different geographical origin and year of collection had a unique and monomorphic allele for SSR marker StvMPh_162a, which has a significant sequence homology to an endoglucanase or a cellulase gene (E-value: 2×10^{-40}) (Arias et al. 2011). Further studies are needed to confirm whether isolate Mp18 might belong to another species, such as the novel species *Macrophomina pseudophaseolina*, reported in Senegal (Sarr et al. 2014).

Our results suggest a certain degree of genetic association between geographical region and isolates, which could be explained by the movement of infected seed throughout the different soybean-producing regions included in this study. In this sense, all the *M. phaseolina* isolates from Paraguay grouped together in cluster I but the isolates from Argentina were separated in both clusters. No clear associations were observed between the year of collection and the SSR profiles. Previously, genetic association among isolates from the same geographical region, where two groups with different genetic profiles and pathogenicity had been isolated from two different geographical regions, was reported from México (Mayék-Pérez et al. 2001). In contrast, other studies have found only a very low

association between the genetic profile and geographical origin of *M. phaseolina* isolates (Baird et al. 2010; Baird et al. 2009; Muñoz-Cabañas et al. 2005).

In the pathogenicity assays in field conditions, disease variables were first analyzed for each inoculated isolate averaging the data obtained interacting with the four tested soybean genotypes. No significant differences in aggressiveness were observed between the isolates from Paraguay and Argentina. However, when the results obtained in the same field assay were analyzed for each soybean genotype tested averaging the data obtained interacting with the seven inoculated *M. phaseolina* isolates, significant differences were observed. The results obtained in the present study demonstrated that the local elite genotype Munasqa RR exhibited lower values of the disease parameters than the moderately resistant DT 97-4290 in field conditions. Furthermore, specific interactions among the four soybean genotypes and the seven *M. phaseolina* isolates evaluated in field conditions were found.

The use of resistant soybean germplasm is the most effective strategy for charcoal rot management (Mengistu et al. 2013b; Romero-Luna et al. 2017). However, although new sources of genetic resistance need to be identified, very few genotypes were accurately characterized as resistant to *M. phaseolina* in both field and controlled conditions. Previously, the plant introduction genotypes PI594302, PI567562A, PI506764 and PI567334 were reported as markedly more resistant to *M. phaseolina* infection in field conditions than DT 97-4290, the first genotype described by Paris et al. (2006) as moderately resistant (Mengistu et al. 2013a). Later, three other genotypes, PI548302, PI548414 and PI548178, were also characterized as more resistant against *M. phaseolina* than DT97-4290, using a cut-stem inoculation technique in semi-controlled greenhouse

318 conditions (Pawlowski et al., 2015). However, it is important to note that none of these
319 genotypes is a commercial cultivar with good agronomic traits.

320 Consequently, in order to corroborate the best performance of the local elite cultivar
321 Munasqa RR and to evaluate the specific interactions observed in the field trial, an
322 improved method for phenotyping the disease response in controlled conditions was
323 developed. The new *in vitro* method proved to be an effective and reliable technique to
324 assess the disease reaction of multiple soybean genotypes against several *M. phaseolina*
325 isolates. It is also simple to perform; inexpensive since does not require culture medium,
326 reproducible, and quick, since the whole process takes no longer than 2 weeks (inoculation
327 of 2-day germinated soybean seeds and evaluation after 12 days). In this context, Bressano
328 et al. (2010) used an *in vitro* method to evaluate the *M. phaseolina* infection process in
329 soybean seedlings (in growth stage V1) by using culture medium and Petri dishes.
330 However, this approach has not been used to evaluate the response of different soybean
331 genotypes to charcoal rot. In addition, the *in vitro* method developed in this work allows
332 quantifying the progress of the disease without damaging the infected tissue (non-
333 destructive). Furthermore, it is considerably faster than the cut-stem inoculation technique
334 described previously (Twizeyimana et al. 2012), in which each cycle of evaluation takes
335 about 8 weeks, not to mention the field evaluation, which requires a full crop season to
336 complete the process (Mengistu et al. 2013b). Moreover, this approach can be used to
337 screen a large number of soybean genotypes, searching for alternative sources of genetic
338 resistance to charcoal rot. Most importantly, environmental variation, which can contribute
339 to inconsistent results between field tests (Mengistu et al. 2013b), can be controlled by
340 using this *in vitro* approach in growth chambers, further reducing experimental error and
341 improving reliability in the evaluation of resistance to *M. phaseolina*. It is noted that, the

results obtained by this method in controlled conditions were correlated with those obtained in field conditions. Finally, this *in vitro* infection method evaluates necrosis on the root system, the natural tissue infected by *M. phaseolina*, a soilborne pathogen. Other methods previously described infect leaf (Mayék-Pérez et al. 2001) or stem (Twizeyimana et al. 2012) tissues. Consequently, the method presented here should be useful to perform more advanced studies, like transcriptomic and metabolomics analysis, of this specific plant-pathogen interaction.

In addition to characterizing a novel soybean genotype resistant to charcoal rot (Munasqa RR) and specific interactions between soybean genotypes and different *M. phaseolina* isolates in field conditions; these specific interactions were also observed and analyzed in controlled conditions. Except for Munasqa RR which showed significantly lower levels of disease response with most of the isolates, the other three analyzed genotypes developed contrasting responses to the different isolates (Fig. 3B). Moreover, crossed specific interactions were observed among DT97-4290 and CRIA4 genotypes with isolates Mp15 and Mp17 (Fig. 3B). On the other hand, isolate Mp37, which showed the same disease response when interacting with DT97-4290 and CRIA4, was also the most aggressive on DM 6.2i RR while was one of the least aggressive on Munasqa RR (Fig.3B). In addition, although isolate Mp17 was the least aggressive on all genotypes when compared with the other isolates, it showed different degrees of aggressiveness in the genotypes tested, being significantly more aggressive when interacting with DM 6.2i RR than when interacting with Munasqa RR.

Macrophomina phaseolina is a polyphagous pathogen and there is currently no evidence of host specificity (Gupta et al. 2012). The *in vitro* infection method revealed significant differences in AUDPC values between the *M. phaseolina* isolates when

366 interacting with the same soybean genotype. Also, these results showed that *M. phaseolina*
367 isolates from Paraguay were more aggressive than the ones collected in Argentina, in
368 controlled conditions. In addition, it was demonstrated that the cultivar Munasqa RR
369 showed a better response against all the *M. phaseolina* isolates tested than DT 97-4290
370 (moderately resistant), as previously shown in the field trial, thus rendering the results
371 obtained by this method more robust.

372 Based on our results, we suggest the existence of specific interactions between
373 soybean genotypes and *M. phaseolina* genotypes in field and controlled conditions, a result
374 which had not been demonstrated empirically before. These specific interactions between
375 soybean elite cultivars and the local genotypes of the pathogen should be considered when
376 developing improved charcoal rot management programs. Other studies have previously
377 recommended that soybean genotypes in general adapted to a particular region should be
378 tested with local isolates when screening genotypes resistant to charcoal rot (Sexton et al.
379 2016).

380 In conclusion, a better understanding of the genetic and pathogenic diversity and the
381 specific interactions between soybean genotypes and *M. phaseolina* isolates will be useful
382 to develop new technologies that would favor the sustainability of this crop. The present
383 results indicate that the local elite cultivar Munasqa RR has the best performance against
384 the seven *M. phaseolina* isolates tested, not only local ones, but also those from Paraguay,
385 in both field and controlled conditions. Resistance levels in Munasqa RR were even higher
386 than in the moderately resistant genotype DT 97-4290. Therefore, Munasqa RR should be
387 regarded as a new source of resistance, available to soybean breeders, a finding which will
388 help in charcoal rot management in northwestern Argentina and in other soybean-producing
389 countries. Furthermore, a new simple, reproducible and reliable method is now available to

rapidly screen the disease response of soybean genotypes to *M. phaseolina* infection in controlled conditions and would be useful for other studies aims to understand this complex plant-pathogen interaction. Finally, we demonstrated the existence of specific interactions between soybean and *M. phaseolina* genotypes.

ACKNOWLEDGMENTS

We thank Renee Arias (USDA-ARS National Peanut Research Laboratory, Dawson, GA) for her technical support in SSR analysis. This study was supported by grants from EEAOC; Ministerio de Ciencia, Tecnología e Innovación Productiva (MinCyT), Plataforma BiotecSur-UE, Proyecto BiotecSojaSur I N°: 127119; Proyecto Investigación Regional MERCOSUR BiotecSojaSur II-Convenio MinCyT Fundación InnovaT (2012)-Acta Comp. Nº 52; PICTO 35332 EEAOC- Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and PICT 2012-2261 from ANPCyT. S. Reznikov was awarded a scholarship by CONICET; M. A. Chiesa, E. N. Morandi, L. D. Ploper and A. P. Castagnaro are CONICET Career Researchers.

LITERATURE CITED

Arias, R. S., Ray, J. D., Mengistu, A., and Scheffler, B. E. 2011. Discriminating microsatellites from *Macrophomina phaseolina* and their potential association to biological functions. Plant Pathol. 60:709-718.

Baird, R. E., Wadl, P. A., Allen, T., McNeill, D., Wang, X., Moulton, J. K., Rinehart, T. A., Abbas, H. K., Shier, T., and Trigiano, R. N. 2010. Variability of United States isolates of *Macrophomina phaseolina* based on simple sequence repeats and cross

genus transferability to related genera within Botryosphaeriaceae. Mycopathologia 170:169-180.

Baird, R. E., Wadl, P. A., Wang, X., Johnson, D. H., Rinehart, T. A., Abbas, H. K., Shier, T., and Trigiano, R. N. 2009. Microsatellites from the charcoal rot fungus (*Macrophomina phaseolina*). Mol. Ecol. Resour. 9:946-948.

Balzarini, M. G., and Di Rienzo, J. A. 2013. Info-Gen: Software para análisis estadístico de datos genéticos. Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina. https://www.info-gen.com.ar.

Bowen, C., and Schapaugh, W. 1989. Relationships among charcoal rot infection, yield, and stability estimates in soybean blends. Crop Sci. 29:42-46.

Bressano, M., Giachero, M. L., Luna, C. M., and Ducasse, D. A. 2010. An *in vitro* method for examining infection of soybean roots by *Macrophomina phaseolina*. Physiol Mol. Plant Pathol. 74:201-204.

Bristow, P., and Wyllie, T. 1984. Reaction of soybean cultivars to *Macrophomina phaseolina* as seedlings in the growth chamber and field. Transactions of the Missouri Academy of Science (USA) 18:5-10.

Dhingra, O. D., and Sinclair, J. B. 1978. Biology and pathology of *Macrophomina phaseolina*. Universidade Federal de Vicosa, Vicosa, Brazil, 166 pp.

Di Rienzo, J., Casanoves, F., Balzarini, M., Gonzalez, L., Tablada, M., and Robledo, Y. C. 2011. InfoStat versión 2011. Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina. http://www.infostat.com.ar.

Fehr, W. R., Caviness, C. E., Burmood, D. T., and Pennington, J. S. 1971. Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. Crop Sci. 11:929-931.

437 Gupta, G. K., Sharma, S. K., and Ramteke, R. 2012. Biology, epidemiology and
438 management of the pathogenic fungus *Macrophomina phaseolina* (Tassi) Goid with
439 special reference to charcoal rot of soybean (*Glycine max* (L.) Merrill). J.
440 Phytopathol. 160:167-180.
441 Highton, R. 1993. The relationship between the number of loci and the statistical support
442 for the topology of UPGMA trees obtained from genetic distance data. Mol.
443 Phylogenet. Evol. 2:337-343.
444 Jana, T. K., Singh, N. K., Koundal, K. R., and Sharma, T. R. 2005. Genetic differentiation
445 of charcoal rot pathogen, *Macrophomina phaseolina*, into specific groups using URP-
446 PCR. Can. J. Microbiol. 51:159-64.
447 Madden, L. V., Hughes, G., and van den Bosch, F. 2007. The study of plant disease
448 epidemics. APS Press St. Paul, MN.
449 Mayék-Pérez, N., López-Castañeda, C., González-Chavira, M., Garcia-Espinosa, R.,
450 Acosta-Gallegos, J., de la Vega, O. M., and Simpson, J. 2001. Variability of Mexican
451 isolates of *Macrophomina phaseolina* based on pathogenesis and AFLP genotype.
452 Physiol. Mol. Plant Pathol. 59:257-264.
453 Mengistu, A., Ray, J. D., Smith, J. R., and Paris, R. L. 2007. Charcoal rot disease
454 assessment of soybean genotypes using a colony-forming unit index. Crop Sci.
455 47:2453-2461.
456 Mengistu, A., Smith, J. R., Ray, J. D., and Bellaloui, N. 2011. Seasonal progress of
457 charcoal rot and its impact on soybean productivity. Plant Dis. 95:1159-1166.
458 Mengistu, A., Bond, J., Nelson, R., Rupe, J., Shannon, G., Arelli, P., and Wrather, A.
459 2013a. Identification of soybean accessions resistant to *Macrophomina phaseolina*
460 by field screening and laboratory validation. Plant Health Prog. 10:1094.

461 Mengistu, A., Reddy, K. N., Bellaloui, N., Walker, E. R., and Kelly, H. M. 2013b. Effect
462 of glyphosate on *Macrophomina phaseolina in vitro* and its effect on disease severity
463 of soybean in the field. Crop Prot. 54:23-28.
464 Mihail, J. D., and Taylor, S. J. 1995. Interpreting variability among isolates of
465 *Macrophomina phaseolina* in pathogenicity, pycnidium production, and chlorate
466 utilization. Can. J. Botany 73:1596-603.
467 Milbourne, D., Meyer, R., Bradshaw, J. E., Baird, E., Bonar, N., Provan, J., Powell, W.,
468 and Waugh, R. 1997. Comparison of PCR-based marker systems for the analysis of
469 genetic relationships in cultivated potato. Mol. Breed. 3:127-136.
470 Muñoz-Cabañas, R. M., Hernández–Delgado, S., and Mayek–Pérez, N. 2005. Análisis
471 patogénico y genético de *Macrophomina phaseolina* (Tassi) Goid. en diferentes
472 hospedantes. Rev. Mex. Fitopat. 23:11-18.
473 Murray, M., and Thompson, W. F. 1980. Rapid isolation of high molecular weight plant
474 DNA. Nucleic Acids Res. 8:4321-4326.
475 Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci.
476 U.S.A. 70:3321-3323.
477 Orrego Fuente, A. L., Grabowski, C., Soilan, L., Ferreira, L., and Del Valle, C. 2009.
478 Distribución geográfica de *Macrophomina phaseolina* en los cultivos de soja, sésamo
479 y maní. Pages 27-34 in: *Macrophomina phaseolina*, hongo causante de la pudrición
480 carbonosa del tallo. AL Orrego Fuente, ed. Alamo, S.A., FCA, UNA/INBIO, San
481 Lorenzo PY.
482 Paris, R. L., Mengistu, A., Tyler, J., and Smith, J. 2006. Registration of soybean
483 germplasm line DT 97–4290 with moderate resistance to charcoal rot. Crop Sci.
484 46:2324-2325.

485 Pawlowski, M. L., Hill C. B., and Hartman G. L. 2015. Resistance to charcoal rot
486 identified in ancestral soybean germplasm. Crop Sci. 55:1230-1235.
487 Ploper, L., González, V., Vázquez de Ramallo, N., Gálvez, M., and Devani, M. 2001.
488 Presencia de la podredumbre carbonosa del tallo de la soja en el centro y noroeste
489 argentino. Avance Agroind. 22:30-34.
490 Reznikov, S. 2016. Evaluación de la diversidad genética de *Macrophomina phaseolina* en
491 el Norte argentino y búsqueda de alternativas de manejo sostenible de la
492 podredumbre carbonosa en soja. Tesis Doctoral, Facultad de Bioquímica, Química y
493 Farmacia., Universidad Nacional de Tucumán. Tucumán, Argentina.
494 Romero Luna, M. P., Mueller, D., Mengistu, A., Singh A. K., Hartman, G. L. and Awise K.
495 2017. Advancing our understanding of charcoal rot in soybeans. J. Integr. Pest
496 Manag. 8:1-8.
497 Saleh, A. A., Ahmed, H. U., Todd, T. C., Travers, S. E., Zeller, K. A., Leslie, J. F., and
498 Garret, K. A. 2010. Relatedness of *Macrophomina phaseolina* isolates from tallgrass
499 prairie, maize, soybean and sorghum. Mol. Ecol. 19:79-91.
500 Sarr, M. P., Ndiaye, M. B., Groenewald, J. Z., and Crous, P. W. 2014. Genetic diversity in
501 *Macrophomina phaseolina*, the causal agent of charcoal rot. Phytopathol. Mediterr.
502 53:250-268.
503 Sexton, Z. F., Hughes, T. J., and Wise, K. A. 2016. Analyzing isolate variability of
504 *Macrophomina phaseolina* from a regional perspective. Crop Prot. 81:9-13.
505 Su, G., Suh, S. O., Schneider, R. W., and Russin, J. S. 2001. Host specialization in the
506 charcoal rot fungus, *Macrophomina phaseolina*. Phytopathology 91:120-126.

Twizeyimana, M., Hill, C., Pawlowski, M., Paul, C., and Hartman, G. 2012. A cut-stem inoculation technique to evaluate soybean for resistance to *Macrophomina phaseolina.* Plant Dis. 96:1210-1215.

Wrather, A., Shannon, G., Balardin, R., Carregal, L., Escobar, R., Gupta, G., Ma, Z., Morel, W., Ploper, D., and Tenuta, A. 2010. Effect of diseases on soybean yield in the top eight producing countries in 2006. Plant Health Progress. doi:10.1094/PHP-2010-0125-01-RS.

Yap, I., and Nelson, R. J. 1996. Winboot: a program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. IRRI Discussion Paper Series No. 14. International Rice Research Institute, Manila, Philippines.

CAPTIONS

Fig. 1. Location of the sampling regions of the 39 *Macrophomina phaseolina* isolates during 2008, 2009, 2010 and 2013 cropping seasons in Argentina and Paraguay.

Fig. 2. Genotypic diversity analysis of 39 *Macrophomina phaseolina* isolates from Argentina (ARG) and Paraguay (PY). Dendrogram constructed using the unweighted pair-group method using arithmetic averages of Jaccard´s similarity coefficient and 28 SSR marker profiles. Numbers at the node for each cluster represent the bootstrap values (> 50) obtained from 1,000 replicates. Boxed text indicates the *M. phaseolina* isolates used in the pathogenicity assays.

Fig. 3. Evaluation of disease response of four soybean genotypes (DM 6i RR, CRIA 4, DT 97-4290 and Munasqa RR) against seven *Macrophomina phaseolina* isolates (Mp15, Mp17, Mp18, Mp32, Mp37, Mp42 and Mp48). **A,** Soybean – *M. phaseolina* interactions in

field conditions in Capitán Miranda, Itapúa, Paraguay in the 2013 cropping season. Disease response presented as colony-forming units of the pathogen per gram of root tissue (CFU/g). **B,** Soybean – *M. phaseolina* interactions in controlled conditions using the *in vitro* phenotyping method on germinated seeds. Disease response presented as area under the disease progress curve (AUDPC) during the 12 days post-inoculation period. (*) Means followed by the same letters are not significantly different ($P < 0.05$) from each other based on two-way mixed model analysis of variance followed by means separation (LSD).

Fig. 4. Progress of symptoms on four soybean genotypes inoculated with isolate Mp17 of *Macrophomina phaseolina*. The artificial inoculation was made on 2-day germinated seeds in controlled conditions. Disease development was measured as percentage of necrosis on the root system of the seedlings, and its progress recorded every 48 h for 12 days post-inoculation (dpi).

Fig. 5. Differential disease response of four soybean genotypes interacting with four representative *Macrophomina phaseolina* (Mp) isolates in controlled conditions at 12 days post-inoculation (dpi). For each soybean genotype - *M. phaseolina* isolate interaction, one representative flask with five inoculated individuals is shown.

Fig. 6. Scatter plot showing the relationship between the area under the disease progress curve (AUDPC) data obtained for each combination of soybean genotypes and *Macrophomina phaseolina* isolates in controlled conditions, and **A,** the square root-transformed colony-forming units per gram of root (CFU/g) and **B,** ln-transformed disease severity data from the field trial.

554 **Table headers:**

555 **TABLE 1.** Isolates of *Macrophomina phaseolina* from Argentina and Paraguay
556 characterized in this study.

557 **TABLE 2.** Summary of genotyping results of 39 isolates of *Macrophomina phaseolina*
558 from Argentina and Paraguay using 28 single sequence repeats (SSR) markers.

559

560 **TABLE 3.** Results of mixed-model analyses of variance to determine the main effects and
561 interactions of soybean cultivar and *Macrophomina phaseolina* (Mp) isolate on colony-
562 forming units per gram of tissue (CFU/g) and disease severity data obtained in the field and
563 on area under the disease progress curve (AUDPC) obtained in the *in vitro* assay.

564

565 Footnote:

566 [a] Field conditions in Capitán Miranda, Itapúa, Paraguay, during the 2013 growing season.

567 [b] Disease severity at R7 stage.

568 *Data of CFU/g were transformed to $\sqrt{x}$ and data of disease severity to ln(x) before the
569 statistical analysis (LSD, $P = 0.05$).

570

571 **TABLE 4.** Assessment of averaged disease response of four soybean genotypes inoculated
572 with seven *Macrophomina phaseolina* isolates in field conditions in Capitán Miranda,
573 Itapúa, Paraguay during the 2013 cropping season.

574

575 Footnote:

576 [a] Resistance according to Paris et al. (2006). S: susceptible, MS: moderately susceptible,
577 MR: moderately resistant.

578 [b] The severity of the disease at R7 stage is the average for each genotype inoculated with
579 each of the seven evaluated isolates of *M. phaseolina* (Mp15, Mp17, Mp18, Mp32, Mp37,
580 Mp42 and Mp48).

581 *Statistically significant differences (LSD, $P \leq 0.05$) in each column are shown in different
582 capital letters. Data of disease severity were transformed to ln(x) before the statistical
583 analysis.

TABLE 1. Isolates of *Macrophomina phaseolina* from Argentina and Paraguay characterized in this study.

Isolate	Locality	Municipality[b]	Coordinates	Country	Year
Mp01	San Agustín	Tucumán	26°49'24.2"S 64°51'00.9"W	Argentina	2008
Mp02	San Agustín	Tucumán	26°49'30.1"S 64°51'00.5"W	Argentina	2008
Mp03	San Agustín	Tucumán	26°49'33.6"S 64°51'09.0"W	Argentina	2008
Mp05	San Agustín	Tucumán	26°49'36.6"S 64°51'01.8"W	Argentina	2009
Mp06	Reconquista	Santa Fe	29°12'36.9"S 59°56'23.6"W	Argentina	2009
Mp07	San Agustín	Tucumán	26°49'22.8"S 64°51'36.2"W	Argentina	2009
Mp08	Tres Arroyos	Buenos Aires	38°22'38.6"S 60°20'24.5"W	Argentina	2009
Mp10	Arenales	Santiago del Estero	26°41'37.1"S 64°30'47.9"W	Argentina	2009
Mp12	Juan N. Fernández	Buenos Aires	37°59'34.6"S 59°15'39.7"W	Argentina	2009
Mp13	Santa Teresita	Tucumán	26°40'20.0"S 64°30'46.7"W	Argentina	2010
Mp14	Piedra Buena	Tucumán	26°44'15.1"S 64°39'26.6"W	Argentina	2010
Mp15	San Agustín	Tucumán	26°49'15.1"S 64°51'33.8"W	Argentina	2010
Mp16	San Agustín	Tucumán	26°49'41.9"S 64°51'31.4"W	Argentina	2010
Mp17	Piedra Buena	Tucumán	26°44'05.6"S 64°40'19.4"W	Argentina	2010
Mp18	San Agustín	Tucumán	26°49'24.5"S 64°51'06.3"W	Argentina	2010
Mp19	Arenales	Santiago del Estero	26°41'35.3"S 64°31'41.5"W	Argentina	2010
Mp20	Arenales	Santiago del Estero	26°41'20.2"S 64°33'02.8"W	Argentina	2010
Mp23	Arenales	Santiago del Estero	26°40'52.1"S 64°32'06.6"W	Argentina	2010
Mp24	El Palomar	Santiago del Estero	26°51'06.9"S 64°42'23.7"W	Argentina	2010
Mp25	Piedra Blanca	Tucumán	26°43'39.2"S 64°47'44.1"W	Argentina	2010
Mp26	Piedra Blanca	Tucumán	26°44'02.5"S 64°47'12.0"W	Argentina	2010
Mp27	San Agustín	Tucumán	26°49'54.3"S 64°51'29.8"W	Argentina	2010
Mp32	Chore	Chore	24°10'24.7"S 56°34'47.1"W	Paraguay	2013
Mp33	Chore	Chore	24°11'39.9"S 56°34'54.1"W	Paraguay	2013
Mp34	Chore	Chore	24°11'17.3"S 56°37'05.4"W	Paraguay	2013
Mp35	Chore	Chore	24°11'38.6"S 56°37'45.9"W	Paraguay	2013
Mp36	Chore	Chore	24°11'41.3"S 56°34'16.6"W	Paraguay	2013
Mp37	Capitán Miranda	Capitán Miranda	27°13'04.1"S 55°46'49.2"W	Paraguay	2013
Mp39	Capitán Miranda	Capitán Miranda	27°13'01.4"S 55°46'21.6"W	Paraguay	2013
Mp40	Yhovy	Yhovy	24°17'52.0"S 54°58'47.9"W	Paraguay	2013
Mp41	Yhovy	Yhovy	24°17'47.7"S 54°58'57.7"W	Paraguay	2013
Mp42	Yhovy	Yhovy	24°18'02.7"S 55°00'15.1"W	Paraguay	2013
Mp43	Yhovy	Yhovy	24°17'52.4"S 55°00'10.1"W	Paraguay	2013
Mp44	Yhovy	Yhovy	24°18'22.9"S 55°00'05.5"W	Paraguay	2013
Mp45	San Juan Bautista	San Juan Bautista	26°40'14.7"S 57°07'24.8"W	Paraguay	2013
Mp46	San Juan Bautista	San Juan Bautista	26°39'43.3"S 57°07'18.9"W	Paraguay	2013
Mp47	San Juan Bautista	San Juan Bautista	26°40'22.6"S 57°09'58.2"W	Paraguay	2013
Mp48	San Juan Bautista	San Juan Bautista	26°40'52.0"S 57°05'45.7"W	Paraguay	2013
Mp49	San Juan Bautista	San Juan Bautista	26°40'47.2"S 57°06'02.6"W	Paraguay	2013

[b] Provinces of Argentina and districts of Paraguay, respectively.
Boxed text indicates the *M. phaseolina* isolates tested in the pathogenicity assays.

TABLE 2. Summary of genotyping results of 39 isolates of *Macrophomina phaseolina* from Argentina and Paraguay using 28 single sequences repeats (SSR) markers.

Locus	N° Alleles	Size range (bp)	N° polymorphic alleles	PIC*
StvMPh_209a	5	162-176	5	0.7
StvMPh_213a	6	137-166	6	0.5
StvMPh_329a	9	96-181	9	0.7
StvMPh_415b	4	164-235	4	0.2
StvMPh_114a	7	168-203	7	0.7
StvMPh_146a	4	91-120	4	0.5
StvMPh_100a	2	174-176	2	-0.7
StvMPh_102a	3	179-183	3	0.6
StvMPh_144a	14	147-219	14	0.6
StvMPh_162a	1	129	0	0.0
StvMPh_173a	6	122-157	6	0.8
StvMPh_190a	5	136-169	5	0.5
StvMPh_19b	5	172-185	5	0.5
StvMPh_20a	3	165-173	3	0.4
StvMPh_34a	4	169-185	4	0.4
StvMPh_132a	10	120-166	10	0.7
StvMPh_49a	7	117-190	7	0.8
StvMPh_63a	5	159-178	5	0.2
StvMPh_182a	7	102-122	7	0.7
StvMPh_197a	5	133-142	5	0.7
StvMPh_310a	4	165-173	4	0.6
StvMPh_461a	6	99-173	6	0.7
StvMPh_484a	13	124-270	13	0.8
StvMPh_562c	5	116-184	5	0.7
StvMPh_109b	3	133-169	3	0.1
StvMPh_116a	7	103-138	7	0.5
StvMPh_123a	3	159-164	3	0.4
StvMPh_137a	3	172-176	3	0.5

* Polymorphism information content for each SSR

TABLE 3. Results of mixed-model analyses of variance to determine the main effects and interactions of soybean cultivar and *Macrophomina phaseolina* (Mp) isolate on colony-forming units per gram of tissue (CFU/g) and disease severity in the field and on area under the disease progress curve (AUDPC) in the *in vitro* assay.

	Field test[a] [CFU/g]			Field test [disease severity][b]			*In vitro* assay [AUDPC]		
Source	df	*F*	*P**	df	*F*	*P*	df	*F*	*P*
Main effects:									
Cultivar	3	60.82	<0.0001	3	74.01	<0.0001	3	84.79	<0.0001
Mp Isolate	6	0.94	0.5015	6	0.75	0.6203	6	26.96	<0.0001
Interactions:									
Cultivar X Mp isolate	18	2.40	0.0025	18	1.77	0.0277	18	3.23	0.0004

[a] Field conditions in Capitán Miranda, Itapúa, Paraguay, during the 2013 growing season.
[b] Disease severity at R7 stage.
*Data of CFU/g were transformed to √x and data of disease severity to ln(x) before the statistical analysis (LSD, $P = 0.05$).

TABLE 4. Assessment of averaged disease response of four soybean genotypes inoculated with seven *Macrophomina phaseolina* isolates independently and in field conditions in Capitán Miranda, Itapúa, Paraguay during the 2013 growing season.

Genotype	Resistance reaction [a]	Severity at R7 [b]
DM 6.2i RR	S	3.1 A*
CRIA 4	MS	2.5 B
DT 97-4290	MR	1.6 C
Munasqa RR	MR	1.4 C
P =		<0.0001

[a] Resistance according to Paris et al. (2006). S: susceptible, MS: moderately susceptible, MR: moderately resistant.
[b] The severity of the disease at R7 stage is the average for each genotype inoculated with each of the seven evaluated isolates of *M. phaseolina* (Mp15, Mp17, Mp18, Mp32, Mp37, Mp42 and Mp48).
*Statistically significant differences (LSD, $P \leq 0.05$) in each column are shown in different capital letters.
Data of disease severity were transformed to ln(x) before the statistical analysis.

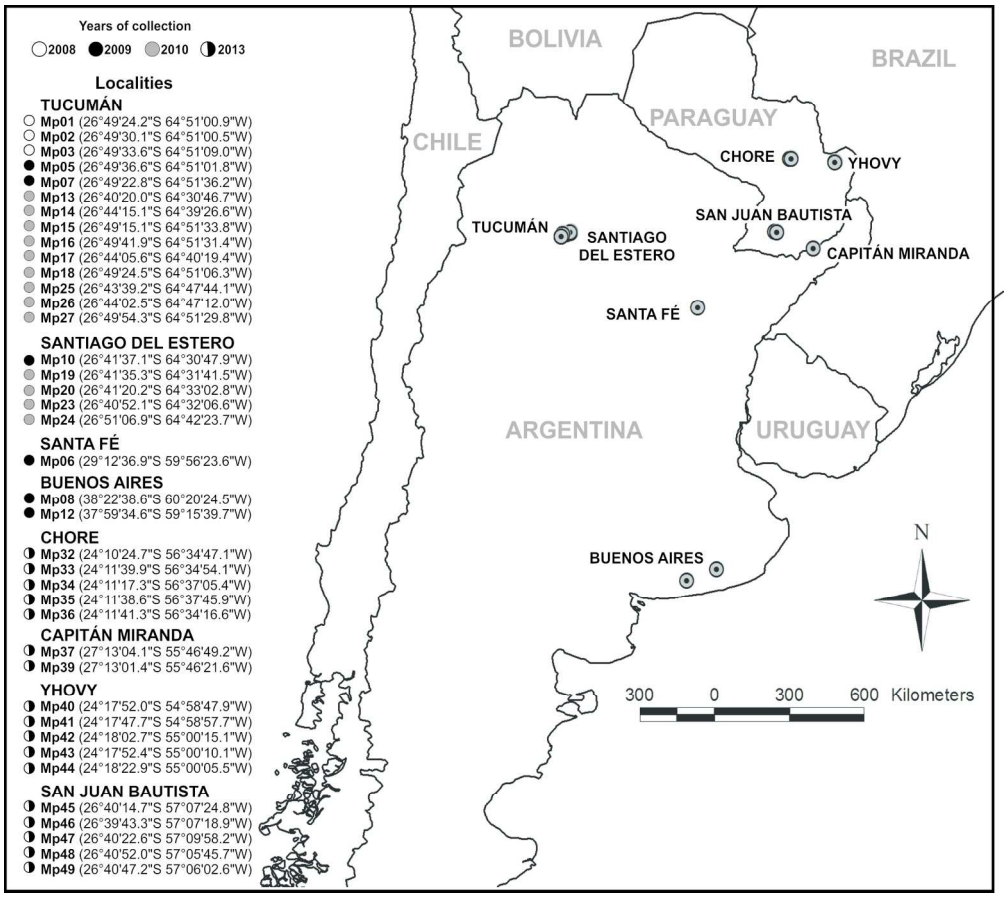

Fig. 1. Location of the sampling regions of the 39 Macrophomina phaseolina isolates during 2008, 2009, 2010 and 2013 cropping seasons in Argentina and Paraguay.

175x155mm (300 x 300 DPI)

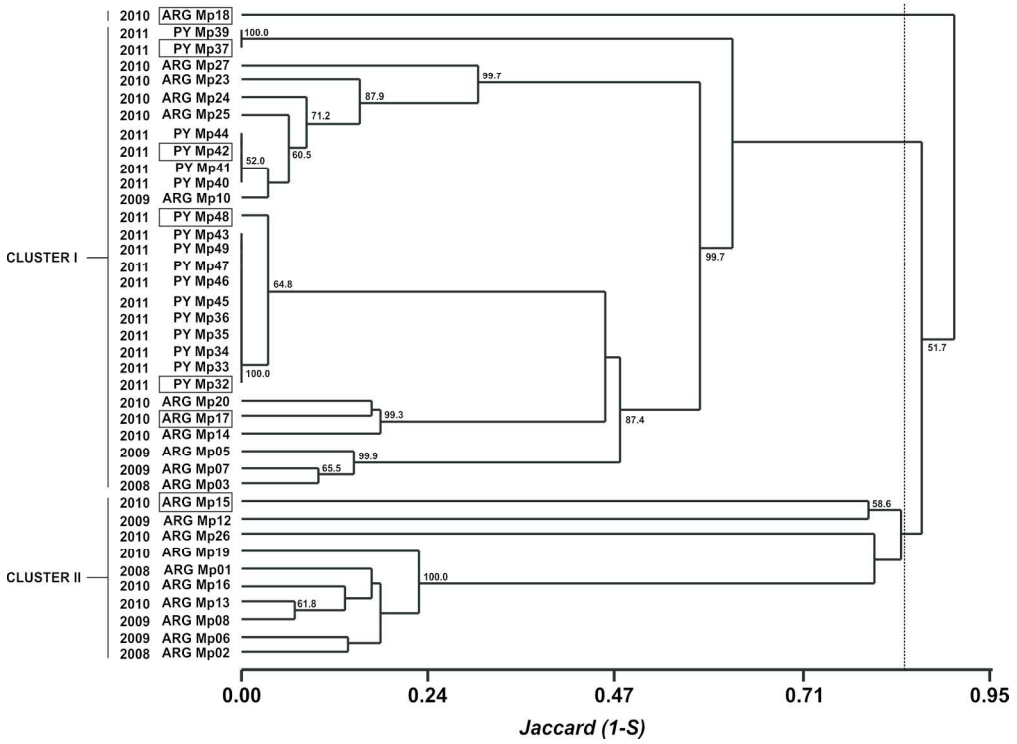

Fig. 2. Genotypic diversity analysis of 39 Macrophomina phaseolina isolates from Argentina (ARG) and Paraguay (PY). Dendrogram constructed using the unweighted pair-group method using arithmetic averages of Jaccard´s similarity coefficient and 28 SSR markers profiles. Numbers at the node for each cluster represent the bootstrap values (> 50) obtained from 1,000 replicates. Boxed text indicates the M. phaseolina isolates used in the pathogenicity assays.

184x140mm (300 x 300 DPI)

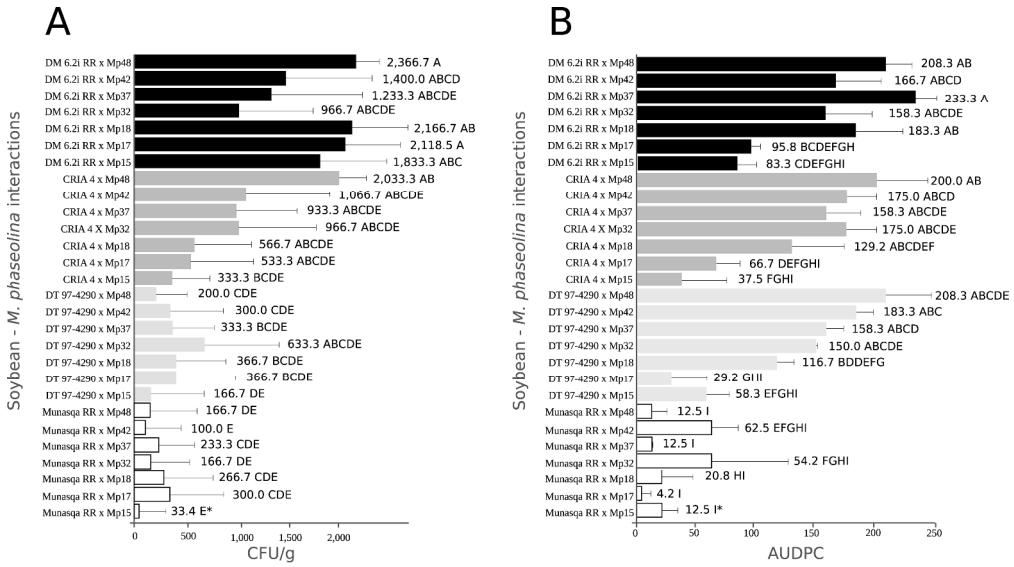

Fig. 3. Evaluation of disease response of four soybean genotypes (DM 6i RR, CRIA 4, DT 97-4290 and Munasqa RR) against seven Macrophomina phaseolina isolates (Mp15, Mp17, Mp18, Mp32, Mp37, Mp42 and Mp48). A, Soybean – M. phaseolina interactions in field conditions in Capitán Miranda, Itapúa, Paraguay in the 2013 cropping season. Disease response presented as colony-forming units of the pathogen per gram of root tissue (CFU/g). B, Soybean – M. phaseolina interactions in controlled conditions using the in vitro phenotyping method on germinated seeds. Disease response presented as area under the disease progress curve (AUDPC) during the 12 days post-inoculation period. (*) Means followed by the same letters are not significantly different ($P < 0.05$) from each other based on two-way mixed model analysis of variance followed by means separation (LSD).

283x157mm (300 x 300 DPI)

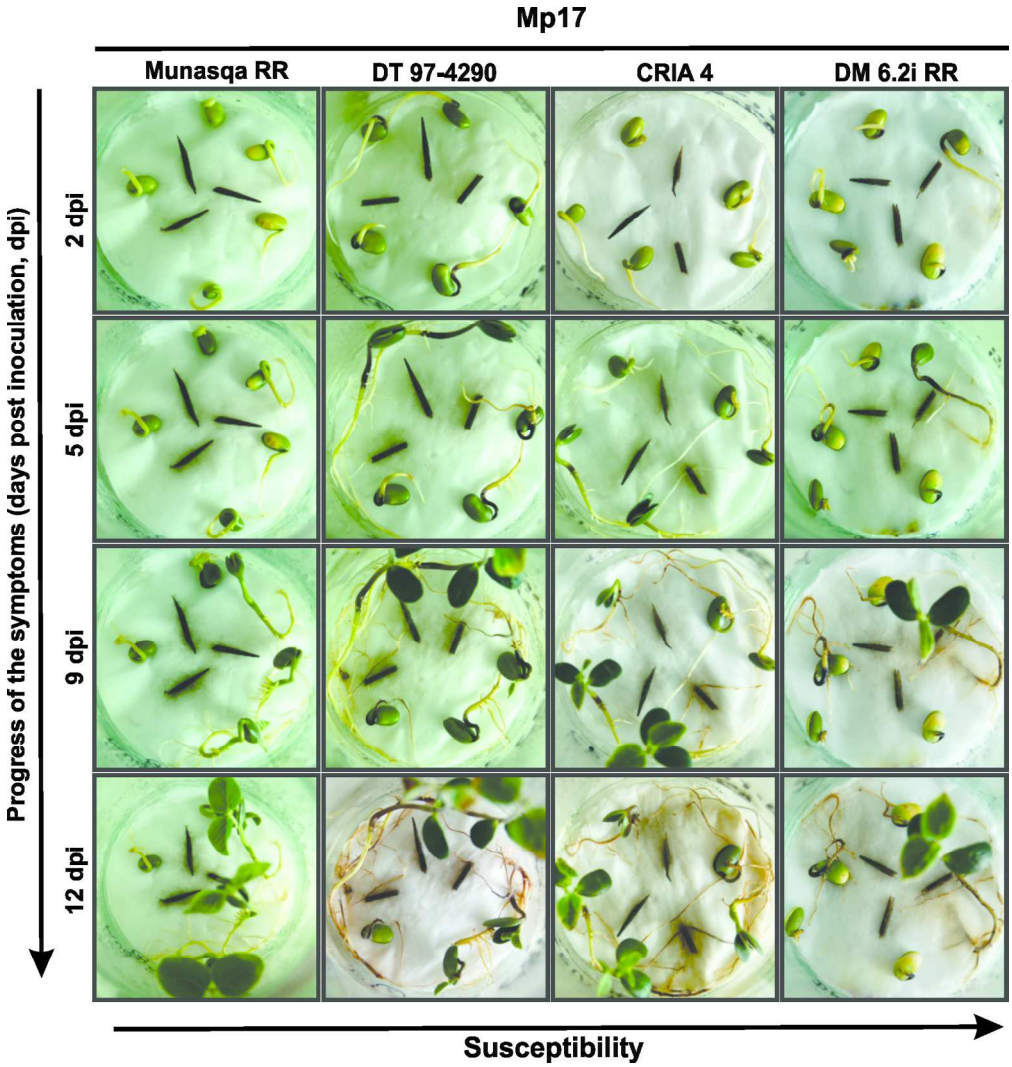

Fig. 4. Progress of symptoms on four soybean genotypes inoculated with isolate Mp17 of Macrophomina phaseolina. The artificial inoculation was made on 2-day germinated seeds in controlled conditions. Disease development was measured as percentage of necrosis on the root system of the seedlings, and its progress recorded every 48 h for 12 days post-inoculation (dpi).

177x187mm (300 x 300 DPI)

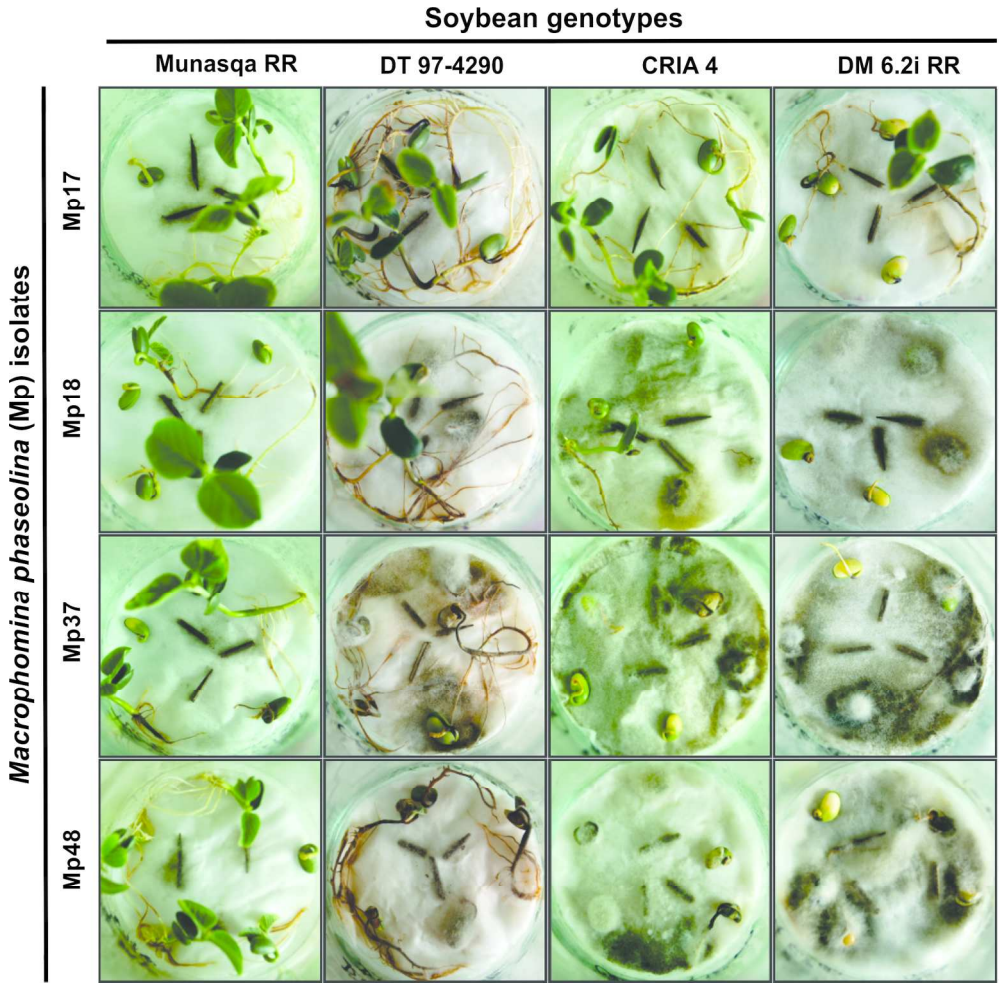

Fig. 5. Differential disease response of four soybean genotypes interacting with four representative Macrophomina phaseolina (Mp) isolates in controlled conditions at 12 days post-inoculation (dpi). For each soybean genotype - M. phaseolina isolate interaction, one representative flask with five inoculated individuals is shown.

178x174mm (299 x 299 DPI)

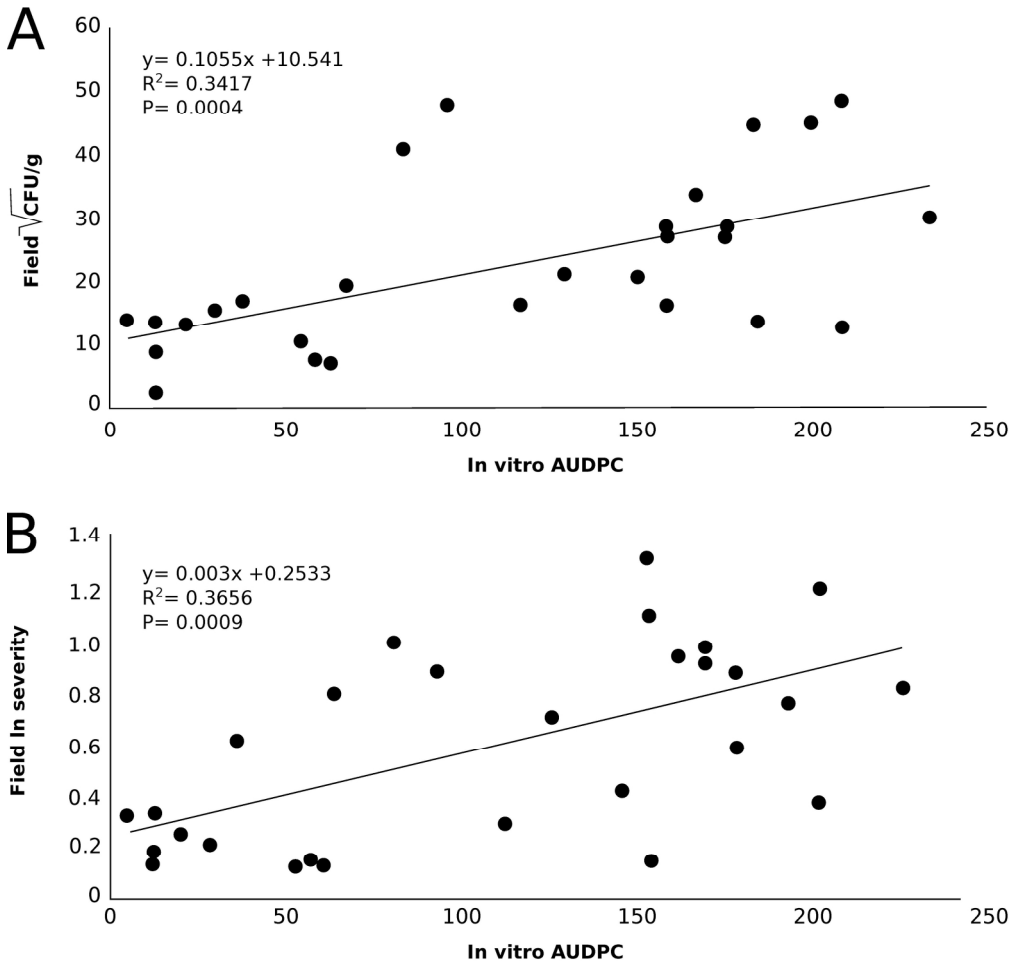

Fig. 6. Scatter plot showing the relationship between the area under the disease progress curve (AUDPC) data obtained for each combination of soybean genotypes and Macrophomina phaseolina isolates in controlled conditions, and A, the square root-transformed colony-forming units per gram of root (CFU/g) and B, ln-transformed disease severity data from the field trial.

192x183mm (300 x 300 DPI)