

Identification of Soybean Genotypes Resistant to Charcoal Rot by Seed Inoculation With *Macrophomina phaseolina*

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Abstract

Soybean charcoal rot is a widespread root disease caused by the fungus *Macrophomina phaseolina*, a natural soil inhabitant that has great pathogenic variability and high survival capacity under adverse conditions. There are no registered fungicides or genotypes genetically resistant to this disease, although differences in susceptibility have been observed. As the fungus is a seed-borne pathogen, screening methods based on seed inoculation are quick and efficient. The objective of this work was to assess the efficiency of soybean seed infestation by incubation for 48 h with colonies of *M. phaseolina*, comparing two germination environment methods (germination paper or in pots with substrate), and to correlate the relative germination of genotypes of both methods with the severity of charcoal rot observed in a field test. The results showed that 48 h were sufficient to infest seeds and reduce the germination. The germination in paper was higher at 5 days after sowing (DAS) than that in pots at 8 DAS in a greenhouse. Both environments exhibited a highly negative correlation between seed germination and field disease severity ($r = -0.775$ in germination paper and $r = -0.779$ in pots with substrate). It is recommended that the germination test be performed in germination paper due to practicality and economy of space, material, and labor besides the better control of the environment.

Keywords: germination, *Glycine max*, Summer wilt

1. Introduction

Among the more than 40 soybean diseases that have been reported in Brazil, 13 occur in the root system and are caused by soil pathogens, including charcoal rot caused by *Macrophomina phaseolina* (Tass.) Goidanish (Almeida et al., 2001). Also known as dry-weather wilt or summer wilt, this root disease occurs widely in all soybean-producing regions worldwide.

The fungus *M. phaseolina* is polyphagous and cosmopolitan, and it attacks numerous crop species, including corn, soybean, sorghum, peanut, cowpea, sesame, and common bean, among others (Machado, 1980). In almost all these crop species, the fungus is efficiently transmitted through the seeds (Santos, Athayde, & Dan, 1984). The fungus is characterized by the production of microsclerotia, which are responsible for the pathogen survival in adverse conditions or in the absence of a susceptible host, being the main source of primary inoculum since they remain in the soil, seeds, and crop residues (Dhingra & Sinclair, 1978; Viana, 1996; Ndiaye, 2007; Gupta, Sharma, & Ramteke, 2012). Microsclerotia are formed by the compaction of hyphae that are dark in color due to the presence of melanin and highly abundant in infected root tissues, giving a charcoal-like or gray appearance to the infected roots.

Macrophomina phaseolina is a natural soil pathogen that is capable of infecting soybean plants at different stages of development, but symptoms mainly appear as patches on the crop in the flowering and grain-filling stages (Gupta et al., 2012). The fungus is favored by high temperature and low soil moisture conditions, *i.e.*, hot weather and dry periods.

The symptoms of infection are usually evident at the end of the crop cycle and can be confused with the plant senescence stage, so the disease may go unnoticed in crops (Almeida et al., 2001). Symptomatic plants that do not die form patches or bands with yellowish leaves and, later, fallen branches with wilted leaves attached to the stems (Dhingra & Sinclair, 1978; Ndiaye, 2007; Almeida, 2001). Because infection and the early development of

diseases caused by root pathogens occur below the ground, symptoms usually only become apparent when they reach advanced stages, which limits the control options.

According to Kimati and Bergamin Filho (2011), the use of resistant cultivars is one of the most important measures in disease management. It is always the most economical measure since it does not entail a significant increase in production costs and is compatible with other types of control. However, no source of genetic resistance to this disease has been found (Gupta et al., 2012). Almeida et al. (2003) observed genetic variability among Brazilian isolates of *M. phaseolina*, indicating that it would be difficult to create commercial soybean cultivars with resistance to root infection in Brazil. However, the occurrence of less susceptible varieties has been reported by several studies, including those by Mengistu et al. (2011, 2013).

The development of plant pathogen-resistant materials requires reliable techniques for disease assessment and genotype selection. Most studies evaluating soybean germplasm for *M. phaseolina* resistance have been conducted in the field and rely on infestation by artificial inoculation or study areas with a history of the disease. However, field experiments may produce inconsistent results because several factors inherent to the pathological system may vary between fields and harvests. To minimize this variability, tests under controlled environmental conditions in greenhouses or growth chambers with standardized inoculation techniques are recommended.

Soybean genotypes were tested for their resistance to charcoal rot under controlled environments using both seedlings (Bristow & Wyllie, 1984) and adult plants (Surette, Meints, & Trevathan, 2006; Twizeymana, Hill, Pawlowski, & Paul, 2012). Because the fungus is a seed-borne pathogen, studies of methods to infest seeds with *M. phaseolina* to select resistant materials have been conducted for other crops, such as pigeon pea (*Cajanus cajan*) (Rosa, 2006).

The simplest seed inoculation methods are immersion in a spore suspension (Tanaka & Menten, 1991) and placing the seeds in contact with a fungal colony grown in conventional culture media. Seed inoculation with fungi or bacteria has been performed for different crops (Costa, Machado, Guimarães, Pozza, & Oride, 2003; Machado, Oliveira, Vieira, & Alves, 2004; Machado, Machado, Vieira, Cassetari Neto, & Souza, 2007; Rosa, 2006; Sousa et al., 2008; Farias, Del Ponte, Corrêa, Afonso, & Pierobom, 2010; Rodrigues et al., 2016).

The objective of this work was to assess the efficiency of soybean seed infestation by contact with colonies of *M. phaseolina* for 48 h, to compare two germination methods (in germination paper or in pots in a greenhouse), and to correlate the relative germination of seven soybean genotypes in both environments with the severity of charcoal rot observed in a field experiment conducted in Sertaneja, Paraná State, Brazil.

2. Material and Methods

2.1 Field Experiment

The field experiment was conducted in Sertaneja county, Paraná State (PR), Brazil (22°52'54.7" S and 50°52'50.6" W) during the 2015/2016 harvest under a no-tillage system without irrigation. The experiment was conducted using a randomized block design with three replicates. Thirty-one soybean genotypes were sown in plots containing seven rows of 25 meters in length spaced 45 cm apart. At sowing, 40 kg/ha of inoculum produced in sorghum seeds was distributed in the planting furrows along with the soybean seeds.

The *M. phaseolina* isolate used in the experiments was obtained from symptomatic plants from the Brazilian cerrado (savannah) region. To produce the inoculum, sorghum seeds were washed, placed in 1-L white plastic bottles in an amount corresponding to approximately 2/5 of their volume, and soaked with enough water to cover the seeds. The next day, the seeds were autoclaved for 30 min at 121 °C. After cooling, six mycelial discs were removed from the margin of the fungal colonies, which had been cultivated in potato-dextrose-agar (PDA) medium at 28 °C for 5 d and inoculated into each bottle.

The bottles were incubated at 28 °C in the dark. From the third day on, the bottles were stirred daily to standardize the colonization and avoid aggregate formation. After approximately 20 d, the grains were fully colonized, showing a black color and were ready for use in the field.

Charcoal rot was evaluated between stages R5 and R6 by observing the incidence of diseased plants in relation to the total number of plants in the plot. The observed symptoms were uneven maturation, leaf wilting and retention, and deficient grain filling. The incidence data (%) were tested for the normality and homogeneity of the residuals, and the means were compared by the Scott-Knott test at the 1% level of probability.

2.2 Greenhouse Experiments

Experiments under controlled environmental conditions were conducted in a greenhouse and laboratory at the GDM Seeds research station in the city of Cambé-PR. The inoculation was performed by infesting the seed with

the same *M. phaseolina* isolate used for field inoculation based on the methodology of Rosa (2006) for inoculation of pigeon pea seed. Seven cultivars used in the field were selected for the controlled environment tests: BMX Elite IPRO, BMX Apolo RR, GDM 15I029, Vmax, BMX Potência RR, NA 5909, and BMX Tornado RR.

The *M. phaseolina* isolate was seeded in 9-mm-diameter Petri dishes containing PDA culture medium and incubated at 28 °C for 5 d.

The surfaces of the soybean seeds were disinfected by immersion in a 70% alcohol solution for 30 s and 1% hypochlorite solution for 60 s, followed by three washes in sterile distilled water. The seeds were then placed on sterile filter paper to dry at room temperature. A total of 21 Petri dishes, one for each replicate, were prepared with pathogen culture.

On each Petri dish, a single layer of 40 properly disinfected and dried soybean seeds was placed over the fungal mycelium. The Petri dishes with seeds were sealed with parafilm and lightly stirred in a circular motion to promote uniform contact of the seed surfaces with the mycelium. The experiment employed a completely randomized design in a 2 × 7 factorial arrangement (environment × genotype), with three replicates plus an uninoculated control consisting of disinfected and dried seeds.

The Petri dishes with seeds were maintained at 25 °C under a 12 h/12 h photoperiod for 48 h. Fifteen seeds from each replicate were selected to germinate under two conditions: a) in germination paper maintained in a germinator at 25 °C: the seeds were distributed on two sheets of germination paper, moistened with an amount of water corresponding to 2.5 times the weight of the non-moistened paper, covered with a third sheet, and then rolled; b) pots containing soil and sand maintained in a greenhouse: the seeds were deposited on an autoclaved mixture of soil and sand (1:3 v/v) and covered with an approximately 2-cm layer of sand in pots and watered as needed. The greenhouse temperature was set to between 28 and 30 °C.

The evaluations consisted of determining the percentage of germination of inoculated seeds relative to the uninoculated control for each genotype at 5 and 8 days after sowing (DAS) for germination paper and pots, respectively. The data were checked for normality and homogeneity of the residuals, and the means were compared by Tukey's test at the 1% level of probability. Pearson correlation analysis was performed between the germination percentage under the two controlled conditions and the disease severity in the field.

3. Results and Discussion

Seven of the 31 genotypes were selected to evaluate the seed infestation method in the greenhouse and laboratory based on resistance levels observed in the field. The incidences of charcoal rot in the seven genotypes selected in the field experiment are presented in Table 1.

Table 1. Incidence of charcoal rot and resistance/susceptibility level of soybean genotypes in a field experiment in Sertaneja-PR in -2016harvest season

Genotype	Incidence (%)	Resistance level
NA 5909	56.7 a*	S
BMX Tornado RR	53.3 a	S
BMX Apolo RR	36.7 a	S
Vmax	20.0 b	MR
BMX Potência RR	16.7 b	MR
GDM15I029	10.0 c	R
BMX Elite IPRO	8.3 c	R
CV (%)	12.1	

Note. *Values followed by the same letters do not differ by the Scott-Knott test at the 1% level of probability for ln(x)-transformed data.

It is possible to separate the varieties into three groups according to their sensitivity to *M. phaseolina*. BMX Apolo, NA5909 and BMX Tornado were more susceptible to the disease with mean incidences of 36.7% to 56.7%; BMX Potência and Vmax presented intermediate incidences (16.7% and 20%); and the materials exhibiting better performance were BMX Elite and GDM15I029, which had mean incidences of 8.3 and 10%, respectively.

Most studies evaluating resistance to charcoal rot are carried out in fields with a history of the disease, and most find differences among genotypes, such as Smith and Carvil (1997) and Mengistu et al. (2011, 2013). The problem

with work carried out in naturally infested fields is the lack of uniformity in the spatial distribution of the inoculum. In this study, although the area had a history of the disease, the inoculum was applied in the planting row to promote a greater uniformity of infection and, thus, more reliable results.

Pastor-Corrales and Abawi (1988) observed more severe root rot caused by *M. phaseolina* in bean plants in a field that had been artificially inoculated with 4 g of colonized rice grains per m of planting row compared to a naturally infested field.

The region where our experiment was performed is characterized by high temperatures, with the maximum average reaching 34 °C. In addition, the area underwent a drought during the harvest under study, causing water stress in the plants during the first stages of grain filling, which led to the development of severe charcoal rot symptoms in the most susceptible varieties.

Concerning the experiments conducted under controlled conditions, the relative germination (germinated inoculated seeds in relation to uninoculated seeds for each genotype) of seeds infested by *M. phaseolina* showed no interaction among germination, environment and genotype, indicating that the genotypes respond in a similar way regardless of the germination method used. The germination was significantly higher (Tukey's test, $p < 0.001$) when the seeds were sowed in germination paper soaked in water for 5 d than when sowed in pots. The mean germination percentage in the germination paper was 38.1% compared to 26.5% in pots kept under greenhouse conditions.

The use of germination paper leads to faster and, consequently, higher germination due to the uniformity of water, light, and temperature conditions as well as the absence of a physical barrier to get through as the germination in the soil. Because germination is slower in pots, sick seeds and seeds with low vigor suffer greater damage and may not germinate.

Genotypes were analyzed separately to study the efficiency of the methods used to discriminate their susceptibility to the pathogen, (Table 2). For germination in germination paper, Vmax was more resistant than BMX Apolo RR and BMX Tornado RR, which showed the same resistance. When evaluated in pots, it was only possible to differentiate the cultivar Vmax as more resistant than NA 5909 and BMX Tornado RR, which showed equal resistance.

Rosa (2006) also studied the efficacy of the inoculation method by exposing seeds to *M. phaseolina*, and by varying the exposure period, the author observed that exposing scarified pigeon pea seeds for 24 h is enough to detect differences in resistance to the pathogen. However, when testing other exposure periods in a second assessment, the author indicated that 32 h of exposure resulted in the best differentiation between genotypes.

In turn, Rodrigues et al. (2016) inoculated bean seeds with *Fusarium oxysporum* by exposing the seeds to the pathogen colony for 48 h under water restriction and observed a difference from the uninoculated control when evaluating the emergence at 10 d. However, there was no difference from the other inoculation methods, including substrate infestation with PDA disks containing pathogen mycelium and seed infestation by spore suspension.

When observing the germination percentage by the two methods used in this study, it is possible to confirm the low performance of the more susceptible genotypes, as occurred in the field. Thus, the methods can be considered efficient for selecting and discarding more susceptible materials. The behavior of the Vmax variety, such as its highest relative germination rate, was unexpected and may have occurred due to some characteristic inherent to the physiological quality of the seed lot.

Some authors have observed that a higher level of pathogen infection in seeds by longer exposure to the fungus results in a higher number of dead seeds and a consequent decrease in the germination and emergence percentage of the seedlings, especially due to the death of the embryo (Costa et al., 2003; Galli, Panizzi, & Vieira, 2007; Botelho, Zancan, Machado, & Barrocas, 2013; Reis, Bacchi, Gavassoni, Hirata, & Pontim, 2014). In the present study, the lower germination of the seeds in some cultivars may have been caused by greater susceptibility to the fungus since the exposure time did not vary.

We also note that the smallest difference in germination between the methods was observed for the susceptible materials BMX Apolo RR and BMX Tornado RR, indicating that seed infestation drastically affects germination capacity when the material is very susceptible to infection.

Table 2. Relative germination percentage in germination paper (germinator) and in pots (greenhouse) of seeds previously inoculated with *Macrophomina phaseolina*

Genotype	% corrected relative germination	
	Germination paper	Pot in greenhouse
Vmax	74.1 a ¹	59.0 a
GDM 15I029	64.4 ab	48.9 ab
BMX Potência RR	51.5 ab	21.4 ab
BMX Elite IPRO	42.1 ab	35.6 ab
NA 5909	22.2 abc	7.1 b
BMX Apolo RR	7.8 c	8.9 ab
BMX Tornado	4.4 c	4.8 b
Mean	38.1	26.5
CV (%)	40.7	69.8

Note. ¹Means followed by the same letter in the columns do not differ by Tukey's test at the 1% level of probability.

The consistent performance of the genotypes under field conditions and in a controlled environment is evident from the correlation analysis (Table 3). For both environments evaluated, there was a significant negative correlation between the relative germination and the disease severity observed in the field. The negative correlations mean that the magnitudes were inversely proportional, that is, the greater severity in the field was, the lower the relative germination percentage was in both the germinator and the greenhouse.

The correlation values were high and very similar (77.5% in the germinator and 77.9% in the greenhouse), which indicated that both could be used to select soybean genotypes resistant to *M. phaseolina* in a controlled environment. However, considering the practicality and economy of space, materials, and labor as well as the lower environmental variation, we recommend germinating seeds infested with *M. phaseolina* in germination paper as the most suitable method for selecting charcoal rot-resistant genotypes.

Table 3. Correlation coefficients (r) and p-values of Pearson correlations between the relative germination percentage in germination paper or in pots in a greenhouse of seeds infested with *Macrophomina phaseolina* and charcoal rot severity in a field experiment carried out in Sertaneja-PR

Germination method	Pearson correlation	
	r	p-value
Germination paper in germinator	-0.775	0.04063
Pot in greenhouse	-0.779	0.03909

Research by the same authors also found a positive correlation between charcoal rot severity observed in the field and after plant inoculation with *M. phaseolina* by the mycelium disk method on the cut stem in a controlled environment with and without a humid chamber ($r_s = 0.84$ and $r_s = 0.80$, respectively) (Ishikawa, Ribeiro, Oliveira, Almeida, & Balbi-Peña, 2018).

4. Conclusions

The methods of inoculating soybean seeds by contact with an *M. phaseolina* colony for 48 h followed by either a) evaluation of the relative germination percentage 5 DAS in germination paper in a germinator or b) evaluation of germination at 8 DAS in pots in a greenhouse are effective for discriminating genotypes regarding their resistance to charcoal rot.

In both methods, germination was highly and negatively correlated with the severity of charcoal rot observed in the field. It is recommended that the germination test be performed in germination paper due to practicality and economy of space, material, and labor besides the better control of the environment.

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