CHARCOAL ROT OR ASHY STEM BLIGHT

Charcoal rot or Ashy stem blight, caused by *Macrophomina phaseolina* (Tassi) Goid, is favored by warm dry growing conditions and is often associated with drought stress although charcoal rot disease has been found under humid tropical conditions. *M. phaseolina* can infect a broad array of major crops including common bean, maize, sorghum, soybean, sesame and cotton (Mayek-Pérez et al. 1997a; 1997b; 2002a, 2002c). The common name of the disease caused by *M. phaseolina* derives from the symptoms present on adult plants where stem tissues show the growth of numerous microsclerotia and pycnidia (Fig. 1). Ashy stem blight exhibits significant morphological, physiological, pathogenic and genetic variability which makes the pathogen more capable of adapting and attacking susceptible hosts in diverse environments. Diversity in *M. phaseolina* is due to the heterokaryotic condition of mycelium as well as the presence of two asexual sub-phases, one saprophytic (*R. bataticola*) where microsclerotia and mycelia are mainly produced and another pathogenic (Beas-Fernández et al., 2006; Reyes-Franco et al., 2006) where microsclerotia, mycelia and pycnidia are produced in host tissues (Fig. 2).

Figure 1. Typical symptoms of 'charcoal rot' or 'ashy stem blight' exhibited by adult bean plants infected by *M. phaseolina*. (Photographs provided by H. F. Schwartz; AgImage - Colorado State University).

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Centro de Biotecnología Genómica-Instituto Politécnico Nacional. Reynosa, México.
E-mail: nmayek@ipn.mx]

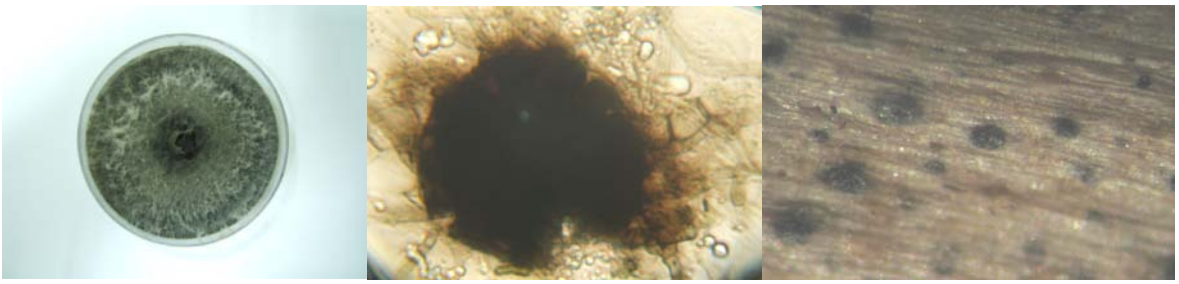

Figure 2. Growth of *M. phaseolina* on PDA plates (left), microsclerotium (center) and microsclerotia and picnydium at common bean tissues (right) (Photographs provided by S. Hernández-Delgado).

Special emphasis has been given to the study of the charcoal rot disease due to its great capability to grow and attack crops grown under arid and water-stressed conditions. Cultural, chemical or biological strategies for disease management are not adequate to control the disease efficiently or economically. Genetic resistance appears promising for reducing damage and yield losses caused by the pathogen. Research on this disease in common bean has focused on i) improving screening methodologies for evaluating reaction of diverse germplasm; ii) identifying resistant parents; iii) studying plant-pathogen interactions to unveil pathogenicity mechanisms as well as genetic resistance factors which block pathogen infections; and iv) the molecular characterization of genetic diversity and disease resistance, and development of PCR-based-diagnostic tools.

Charcoal rot inoculation and screening methods

Abawi and Pastor-Corrales (1989; 1990) described screening procedures for evaluating common bean reaction to ashy stem blight. Dried sclerotia are an effective form of inoculum to establish a high and uniform incidence of ashy stem blight. Sclerotia can be produced on an artificial medium consisting of 10 g peptone, 15 g dextrose, 0.25 g $MgSO_4$ and 0.5 g K_2HPO_4 per liter of water. The medium should be placed in a 1 cm layer in Petri plates. A 4 mm diameter disk of agar colonized with *M. phaseolina* was placed in the center of the Petri plate. After incubation for 15 days at 30^0 C, the mycelial-sclerotial mats of the pathogen were blended with distilled water, followed by centrifuging at 3000-5000 rpm. The pellets should be re-suspended in water and centrifuged again. The washed pellets should be spread on filter paper and allowed to dry for 48 hr at 30^0 C. The dried sclerotial masses were ground in a mortar and pestle and mixed in sterilized soil at a rate of 2 g of sclerotia per kg of soil. Greenhouse inoculations can be conducted by placing bean seed in pots on top of clean soil and then covering the seed with a layer of 2-3 cm of infested soil. Seedlings of a susceptible line will either fail to emerge or exhibit lesions after emergence. Lesions soon spread to the stem and can kill the seedling in 2-3 weeks.

Whole rice or sugar-beet seeds can also be used to prepare inoculum (Olaya et al., 1996; Pastor-Corrales and Abawi, 1988). For example, after autoclaving the rice seed with water (1 g rice seed to 1 ml of water), 7-day-old mycelial disks of agar infected with the pathogen are placed into containers of rice seed (Abawi and Pastor-Corrales, 1990). The seed should be incubated at 30^0 C for 15 days. In the greenhouse, 2 to 3 rice seeds infected with *M. phaseolina* are placed around each bean seed and covered with clean soil. Field plots should be inoculated at a rate of 2 or more g of infected rice seeds per meter of row (Pastor-Corrales and Abawi, 1988). At planting, the infected rice seed and the bean seed should be placed in the open furrows and covered with soil. Most of the susceptible seedlings will show aboveground symptoms at emergence and can be evaluated using a 1-9 scale (Table 1). Spearman rank correlations between percentage germination and percentage of infection with ashy stem blight near senescence were positive and significant (Beaver et al., 1990; Mayek-Pérez et al., 2001b). Echávez-Badel and Beaver (1987) inoculated bean plants in the greenhouse by inserting toothpicks infected with *M. phaseolina* into the stem just below the cotyledonary node. Inoculations were made 30 days after planting and the length of the lesion on the main stem was evaluated at 10, 20 and 30 days after inoculation. In Puerto Rico, uniform and severe levels of ashy stem blight infection were obtained when bean nurseries were planted after sorghum [*Sorghum bicolor* (L.) Moench].

Table 1. Rating[†] scale (1-9) used to evaluate beans for aboveground infections caused by *Macrophomina phaseolina*.

Disease score	Evaluation of stem rot caused by *Macrophomina phaseolina*
1	No visible symptoms.
3	Lesions are limited to cotyledonal tissues.
5	Lesions have progressed from cotyledons to about 2 cm of stem tissues.
7	Lesions are extensive on stems and branches. The foliage exhibits chlorosis and necrosis.
9	Most of the stem, petioles and growing point are infected. A considerable amount of pycnidia and sclerotia is produced.

[†]Abawi and Pastor-Corrales (1990).

Adaptations to other methods to assess pathogenicity or reaction to charcoal rot isolates have been published recently. For example, good correlations among seed (Manici et al., 1995) or seedling inoculation and detached-leaf method have been found (Bañuelos-Balandrán and Mayek-Pérez, 2008) as well as the use of toothpicks for assessing disease under controlled drought-stressed conditions or soil infestations by using colonized PDA or microsclerotia (De la Peña-Devesa et al., 2009).

Figure 3. Post-emergence seedling death under controlled conditions by using colonized rice seeds (left) or infested toothpicks (center); and adult plant death after water-stress under naturally infested soils at Rio Bravo, México (Photographs provided by S. Hernández-Delgado).

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Pathogenic and genetic variability in *M. phaseolina*

In order to establish a method for the characterization of pathogenicity patterns of *M. phaseolina*, Mayek-Pérez et al. (2001c) proposed a set of bean differential cultivars and assigned a binary value to each cultivar, similar to the system used for classifying races of anthracnose. The 12 cultivars and their binary value are described on Table 2.

Table 2. List of bean cultivars used for pathogenicity tests and their assigned binary value[†]. The sum of the assigned numbers of each cultivar infected by an isolate of *M. phaseolina* gives a unique number which describes the pathotype of the isolate.

Cultivar	Race	Assigned value
Bayo Durango	Durango	1
Pinto UI-114	Durango	2
Pinto Villa	Durango	4
Bayo Mecentral	Jalisco	8
G 4523	Nueva Granada	16
Rio Tibagí	Mesoamerica	32
Azufrado Tapatío	Jalisco	64
G 19428	Perú	128
SEQ 12	Mesoamerica	256
BAT 477	Mesoamerica	512
Negro 8025	Mesoamerica	1024
TLP 19	Mesoamerica	2048

[†]Mayek-Pérez et al. (2001c)

Mihail and Taylor (1995), Mayek-Pérez et al. (1997c) and Mayek-Pérez et al. (2001c) found a high level of pathogenic variability among isolates of *M. phaseolina,* although isolates from the same location and species had related pathotypes. The most aggressive isolates come from tropical areas (Mayek-Pérez et al., 2001c). Consistent tolerance to drought-stressed conditions have been demonstrated in *M. phaseolina* (Cervantes-Garcia et al., 2003) which appears to be associated to the synthesis of active osmolytes such as glycerol and arabitol during osmotic stress (Tijerina-Ramírez et al., 2008; Ramírez-Benavides et al., 2009)

Jones et al. (1998), Mayek-Pérez et al. (2001c), Vandemark et al. (2000); Su et al. (2001) and Almeida et al. (2003) confirmed the high genetic diversity of *M. phaseolina* when isolates from different host or geographical origins were compared using AFLPs, RAPDs or RFLPs marker methodologies. Reyes-Franco et al. (2006) found Mexican isolates of *M. phaseolina* are more aggressive in common bean that those from other countries such as Japan, Brazil, Australia, USA or Italy. In addition, they demonstrated that Mexican isolates are genetically different than isolates from Asia, per example, but similar to American (USA, Brazil, Argentina, Colombia) isolates. Recent studies have demonstrated the genetic diversity among *M. phaseolina* isolates (Beas-Fernandez *et al.*, 2004; 2006; Muñoz-Cabañas et al., 2005; Das *et al.*, 2006, Purkayastha *et al.*, 2006, Aboshosha *et al.*, 2007, Csondes *et al.*, 2007, Omar *et al.*, 2007; Babu et al., 2010; Baird et al., 2010; Saleh et al., 2010). Saleh et al. (2010) used AFLP markers and rDNA-internal transcribed spacer (ITS) region sequences to assess the genetic diversity and relationships of *M. phaseolina* isolates from four hosts (tallgrass prairie, maize, sorghum, and soybean). Fungal populations from wild species were more diverse than from crop species. Using Bayesian cluster analysis based on the estimation of co-ancestry coefficients they demonstrated the incomplete specialization by host in charcoal rot fungus, inversely to results reported by Su et al. (2001) and Almeida et al. (2008). In addition, the authors suggested the generation of 'hybrids' with novel genetic profiles and pathogenic capabilities after the interaction among crop and wild host species.

Resistance to charcoal rot in common beans

Reactions to charcoal rot disease in bean germplasm have been conducted in different countries such as Colombia (Pastor-Corrales and Abawi, 1988); México (Mayek-Pérez et al., 2001b; 2002b) and Kenya (Songa et al., 1997). Mayek-Pérez et al. (2001b; 2002b) showed that Mesoamerica germplasm (black beans) exhibited resistance to charcoal rot while Durango and Jalisco (pinto, bayo and flor de mayo beans) races exhibited susceptibility (Table 3). Mayek-Pérez et al. (2001b, 2002b) and Pastor-Corrales and Abawi (1988) demonstrated that BAT 477 expresses resistance to *M. phaseolina* under both greenhouse and field conditions. Close relationship between charcoal rot and drought resistance in bean has been found (Mayek-Pérez et al., 1997a, 1997b; Hernández-Delgado et al., 2009a). BAT 477, Negro 8025 and TLP 19 had resistance to the greatest number (≥ 70%) of the isolates from Mexico (Mayek-Pérez et al., 2001c).

Table 3. Sources of resistance to ashy stem blight in different seed classes.

Name or number	Seed color / type	Resistance genes	Reference
A 300, BAT 85, BAT 332, BAT 1385, BAT 1651, IPA 1, San Cristóbal 83, EMP 86, G 5059	Miscellaneous	*Unknown*	Pastor-Corrales and Abawi (1988)
XAN 176	9 / Black	*Four QTL (B4 and B7)*	Miklas et al. (1998); Miklas et al. (2000)
TLP 19, TLP20 Negro Tacaná Negro Perla Jamapa, Negro 8025			Mayek-Pérez et al. (2001b, 2002b); Abawi and Pastor-Corrales (1989; 1990)
BAT 477	2 / Cream	*Mp-1*, *Mp-2*	Mayek-Pérez et al. (2001b, 2002b), Olaya et al. (1996), Abawi and Pastor-Corrales (1988)
SEQ 12	2/ Cream	*Derived from BAT 477 x Negro 8025 (probably Mp1 and Mp2)*	Mayek-Pérez et al. (2001b, 2002b)
Manzano, Bayo Zacatecas, Bayo Baranda			
PT 91084	2M / Pinto		Mayek-Pérez et al. (2002b)
ICA Palmar (G 4523)	6M / Red mottled		Mayek-Pérez et al. (2002b)
Carioca	"Ojo de cabra"		Mayek-Pérez et al. (2001b)
Amarillo de Calpan	Yellow		Mayek-Pérez et al. (2002b)
Landraces ("Pastilla de Teocaltiche", Michoacán 9-1-A, "Colorados" from Teopisca, Chiapas) and *P. coccineus* ("ballacote" from Querétaro, México)	Miscellaneous		Mayek-Pérez et al. (2002b)Mayek-Pérez et al. (2002b)
B98311, VAX5	9 / Black	*Unknown*	Frahm et al. (2004)
Negro Veracruz, A 774, TLP20/NT81, NGO 99165		*Unknown*	Mayek et al. (2004)

Resistance to a highly virulent strain of *M. phaseolina* was reported to be conferred by two complementary dominant genes in TLP 19 and BAT 477 (Olaya et al., 1996; Mayek-Pérez et al., 2001a). Mayek-Pérez et al. (2009) reported from two to nine genes conferring resistance to charcoal rot in BAT 477 growing under field conditions in Veracruz, Mexico; but when reactions of 137 recombinant inbred lines (RILs) from BAT 477 x Pinto UI-114 were re-classified as resistant/susceptible reactions, only two dominant genes with double-recessive epistatic effects were detected in Cotaxtla, México. Under controlled conditions drought stress shows higher negative effects than *M. phaseolina* on water relations, vegetative growth and histopathology in *P. vulgaris*.

Drought stress decreases transpiration rate, water potential, osmotic potential, turgor potential, relative water content, leaf area and dry weight of all vegetative structures of *P. vulgaris* as well as increases charcoal rot development and stomatal resistance and the association among physiological and growth characteristics and charcoal rot development. *M. phaseolina* invaded between epidermal cells of BAT 477 and Pinto UI-114 hypocotyls. The fungus infected cortex tissues, vascular cylinder, and pith cells of Pinto UI-114, but only epidermal and parenchyma cells of BAT 477 (Mayek-Pérez et al. 2002a, c). BAT 477, compared with susceptible germplasm such as Pinto UI-114, shows some traits which could be closely associated to charcoal rot resistance: higher early vigor based on high dry biomass accumulation during vegetative growth (Mayek-Pérez et al., 2002d); low negative effects on water relations (transpiration rate; stomatal resistance; water, osmotic and turgor potentials under water stress) during vegetative and reproductive phases; and restricted growth of the pathogen to parenchyma and epidermal cells (Mayek-Pérez et al. 2002a, c). Further studies that clarify associations of other morphological, physiological or biochemical traits in resistant bean germplasm will be needed, to determine the genetic basis of resistance for each characteristic.

Regardless of the previous work and interest to detect bean germplasm with resistance to charcoal rot under both controlled and field conditions, current interest has been focused to measure disease resistance and yield stability in resistant genotypes. Frahm et al. (2004) analyzed two black bean RIL populations with resistance to terminal drought, developed from crosses between a drought resistant line, B98311 from Michigan, with TLP 19 and VAX 5, two lines from CIAT with improved disease resistance and adaptation to growing conditions in Latin America. The RIL populations were evaluated in experiments conducted in Zamorano, Honduras and Veracruz, Mexico under drought stress and well-watered (non-stress) treatments. Yields were reduced in each experiment by drought and the fungal pathogen, *M. phaseolina*. One RIL, L88-63 ranked first in GM yield at both locations. Subsequent testing in Honduras and Michigan confirmed the high yield potential and broad adaptation of L88-63. Although the association of charcoal rot reaction and yield stability is unclear, yield stability appears to be more frequent in resistant germplasm. Yield estimations under contrasting conditions of evaluation (drought-irrigated; inoculated-non inoculated) calculated as ‘intensity indexes’ or ‘susceptibility’ indexes’ could be a good choice to detect bean germplasm with combined resistance to charcoal rot and drought stresses (Mayek-Pérez et al., 2003; Mayek-Pérez et al., 2004; García-Olivares et al., 2009).

Molecular markers linked with resistance to charcoal rot have been identified in BAT 477 (Olaya et al., 1996) (Table 2). Miklas et al. (1998; 2000) identified QTL conditioning resistance to charcoal rot in XAN 176 under field conditions in Puerto Rico. Hernández-Delgado et al. (2009b) reported one QTL associated to charcoal rot resistance in BAT 477. Unfortunately, neither RAPD or AFLP markers linked or associated to *M. phaseolina* resistance have not been cloned, sequenced and SCAR markers developed. The RAPD markers linked to ashy stem blight resistance in BAT 477 by Olaya et al. (1996) appear to be un-reproducible under laboratory conditions (personal communications by S. Hernández-Delgado and Tim Porch, USDA-ARS, Mayagüez, Puerto Rico). SCARs should be developed for markers associated with

resistance to charcoal rot to implement MAS for bean breeding (Miklas and Singh, 2007).

Charcoal rot diagnostics

Molecular methods have been used for the detection and differentiation of *M. phaseolina*. Strategies including RFLPs or rDNA ITS regions; RAPDs and AFLPs (Vandemark et al., 2000; Mayek-Pérez et al., 2001c; Pecina-Quintero et al., 2001; Su et al., 2001; Almeida et al., 2003; Jana et al., 2003; Das et al., 2006; Meena et al., 2006; Purkayastha et al., 2006; Reyes-Franco et al., 2006; Aboshosha et al., 2007; Omar et al., 2007; Rajkumar and Kuruvinashetti, 2007; Almeida et al., 2008; Brooker et al., 2008; Aghakhani and Dubey, 2009; Jalali et al., 2009a; Zade et al., 2009; Babu et al., 2010; Saleh et al., 2010). In addition new strategies have been used in the diagnostics of charcoal rot pathogen such as Universal Rice-Primer PCR (URP-PCR) (Jana et al., 2005b); ISSRs (Jana et al., 2005a; Purkayastha et al., 2008) and Rep-PCR (Purkayastha et al., 2008). So far none of these methods have been able to clearly differentiate *M. phaseolina* isolates according to host or geographic origin although the efficient detection or 'diagnosis' of charcoal rot pathogen has been achieved.

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