INFLUENCE OF SOILS, NUTRITION, AND WATER RELATIONS UPON CHARCOAL ROT DISEASE PROCESSES IN KANSAS

by

DAVID RICARDO CRUZ JIMENEZ

B.S., Universidad Nacional de Colombia, 2006

A THESIS

Submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Plant Pathology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2011

Approved by:

Major Professor
Christopher R. Little

Abstract

Charcoal rot, caused by *Macrophomina phaseolina*, is the most important soybean disease in Kansas. Several strategies have been recommended to control this disease including crop rotation, lower plant densities, biological control, plant resistance and tolerance, and fungicide application. However, those techniques have not been completely effective and the information concerning soil texture, irrigation and micronutrient fertility (particularly manganese) upon charcoal rot disease severity and the pathogen population is limited. The objective of this study was to determine key factors that affect the biology of *M. phaseolina* and charcoal rot processes under laboratory, greenhouse and field conditions. *M. phaseolina* microsclerotia were produced from PDA pure isolate and infested Japanese millet in the laboratory and characterized by different techniques such as serial dilutions in semi selective media with the aim to produce quality inoculum to reliably infect soybean seedling roots under greenhouse conditions; production of inoculum by infesting Japanese millet was the most efficient method.

Root colonization and root infection of soybean seedlings was assessed through the use of *M. phaseolina* inoculum under controlled conditions in the greenhouse. Root infection by *M. phaseolina* and microsclerotia longevity in soil is determined by environmental factors such as soil moisture content, soil texture and source of inoculum. The objective of the greenhouse study was to determine the impact of these variables on seedling root infection at the V1 and V2 development stages. Artificial soils with different textures were infested; *M. phaseolina* microsclerotia and soybean seedlings were exposed to different soil moisture contents including pot saturation, pot (field) capacity, and permanent wilting point. Soil populations and levels of root colonization for the stages were assessed by estimating CFUs and root length. Results indicate that soil texture has a significant impact upon root morphology and root length. Root populations of *M. phaseolina* were significantly higher in sandy soil textures and lower in the fine-textured soils, suggesting an impact of soil water holding capacity in the root infection process. The effect of water stress on seedling root colonization by *M. phaseolina* indicates that early infection may be more important than previously thought.

A field study was also conducted to determine the effect of the aforementioned variables in a 2-year field experiment conducted at two Kansas locations. Pathogen colonization was assessed by measuring colony-forming units (CFUs) from ground root tissue at R2-R4 (post-flowering/early pod development) and R8 (maturity) stages. Soil populations (pre-planting and post-harvest) of *M. phaseolina*, yield parameters, and plant characteristics were obtained. Results indicated that there are complex relationships between soil physiochemical properties (pH, NPK content, exchangeable cations, and organic matter) and soil texture (sand, soil, and clay composition), which may mitigate disease severity and pathogen levels in host tissue. Results also indicated that in natural *M. phaseolina*-infested soils, cropping history and soil texture play an important role in charcoal rot processes and influence the levels of pathogen soil populations, root colonization at maturity and, more importantly, soybean yield.

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Acknowledgements

I would like to deeply thank my major advisor Dr. Christopher R. Little for his mentoring during my master at Kansas State University. His vast knowledge of microbiology and plant pathology inspired me to keep working and gave me the motivation to put all my effort on this research project. I would also like to acknowledge my committee members, Dr. Kraig Roozeboom, Dr. DeAnn Presley and Dr. Vara Prasad for their support, advice and suggestions. Lance Noll and Xin Li who helped me with the field sampling and statistical analysis respectively. My deepest gratitude goes to my family and friends.

Finally, I would like to acknowledge the Kansas soybean commission for funding this project.

CHAPTER 1 - Literature Review

Soilborne pathogens are especially challenging because their infection propagules often survive for several years in the soil and they reduce yield and quality in numerous crops. Soilborne diseases are difficult to manage, detect and predict; additionally the soil matrix is complex, making it difficult to understand the variables that govern infection processes and epidemics.

Macrophomina phaseolina (Tassi) Goid. is the causal agent of seedling blight root rot, and charcoal rot of more than 500 crop and non-crop species, including economically important crops such as soybean, corn, sorghum and cotton. Charcoal rot of soybean is gaining importance throughout the world. In the United States, charcoal rot occurs in the north central and southern regions and ranks fourth in economic impact after soybean cyst nematode, phytophthora root rot and seedling diseases (Smith and Carvil, 1997). Average yield losses due to charcoal in the United States were estimated at about of 27,000,000 bushels per year from 1996 to 2009 (Wrather and Koenning, 2010).

Charcoal rot of soybeans is more pronounced when unfavorable environmental conditions stress the plant, such as prolonged periods of drought and high temperatures (28 to 35°C). Although infection occurs at soybean seedling stages and the pathogen attacks the plant throughout the season, symptoms appear after midseason or when the plant reaches maturity (growth stages R5, R6, and R7) (Hartman et al., 1999).

Nomenclature and synonymy

Macrophomina (subdivision Deuteromycota, form-class Coelomycetes) is a genus composed only of a single species, *M. phaseolina* (Tassi) Goid. (Mihail, 1992). The fungus produces two anamorph structures: microsclerotia and pycnidiospores in both host tissues and culture media.

According to Mihail (1992), there is an unconfirmed report of a *M. phaseolina* teleomorph state named *Orbilia obscura* by Ghosh et al. (1964), but since then no reports have been done about a teleomorph state.

Historically many different synonyms have been assigned to *M. phaseolina* due to the biological characteristics of this pathogen and the difficulty of its taxonomical classification (Holliday et al., 1970).

One of the first descriptions of *M. phaseolina* was made by Halsted in 1890 causing disease on *Ipomoea batatas* observing "black nodules" (sclerotial stage) on the roots of this plant, at this time *M. phaseolina* was named as *Rhizoctonia bataticola*. Later the pycnidial state was described by Tassi (1901) and named the fungus as *Macrophoma phaseolina*. Shaw (1912) described a fungus with black sclerotia causing seedling disease on jute (*Corchorus capsularis* L.), cowpea (*Vigna unguiculata* (L.) Walp.), groundnut (*Acharis hypogaea* L.) and cotton (*Gossypium herbaceum* L.) in India (Dhingra and Sinclair, 1978). Ashby (1927) discovered the connection between the sclerotial and the pycnidial stage and proposed the binomial name of the fungus, *Rhizoctonia bataticola* (Taub.) Butler for blight and stems roots, and *Macrophomina phaseoli* (Maubl.) Ashby for the pycnidial stage. He considered *Macrophoma phaseoli* Maubl. (1905), *Sclerotium bataticola* Taub. (1913), *Macrophoma chochori* Saw. (1916), *Macrophoma cajani* Syd. & Butl. (1916). *Macrophomina philippinensis* Petr. (1923), *Rhizoctonia lamellifera* Small. (1924), *Rhizoctonia bataticola* (Taub.) Butl. (1925), *Dothiorella cajani* Syd. & Butl. (1925), and *Macrophoma sesame* Saw (1922) as synonyms (Kulkarni and Patil, 1966).

The binomial *Macrophomina phaseoli* (Maulb.) Ashby (Ashby, 1927) was changed to *Macrophomina phaseolina* (Tassi) G. Goidanich, by Goidanich in 1947. In the past the common genera referring to the sclerotial state of *M. phaseolina* were *Rhizoctonia* and *Sclerotium* while the genera that have been used to refer to the pycnidial stage have been *Macrophomina, Macrophoma,* and *Dothiorella.* However, the current binomial *Macrophomina phaseolina,* is applied to the pycnidia and sclerotial anamorph stages (Mihail, 1992).

By using morphological characteristics and 28 rDNA sequences, Corus et al. (2006) included the pathogen in the ascomycete family *Botryosphaeriace,* pointing out differences between *Tiarosporella and Macrophomina phaseolina* in which the latter may be distinguished by having persistent proliferating conidiogenous cells.

Macrophomina phaseolina diversity and pathogen populations

M. phaseolina is a soilborne pathogen with a wide range of hosts, about 5000 species of plants, and must have a good genetic variability to be able to not discriminate in its host selection (Wyllie and Scott, 1988). However, problematic to the plant pathologist and mycologist is differentiation of isolates in this species, since morphological characteristics are highly variable (Babu et al., 2010). Isolates from different hosts, soils or geographical regions can differ in their morphological characteristics, production of microsclerotia, pycnidial size, pycnidiospores and pathogenicity (Dhingra and Sinclair, 1978). Due to this variability between isolates, morphological or phenotypic criteria are often not reliable (Babu et al., 2007; Saleh et al., 2009).

According to Babu (2010) three methods have been used to classify *M. phaseolina* isolates. First, morphological and cultural characterization, by using chlorate (a nitrate analog) containing minimal medium is possible to differentiate three phenotypic groups: dense, feathery and restricted. *M. phaseolina* isolates taken from corn tissue are chlorate-resistant having a dense growth, in contrast isolates taken form soybean tissue and soil are chlorate-sensitive and vary from feathery to restricted growth. Different nitrogen composition in the host and usage of this nutrient by the pathogen could have an impact on the selection and/or specialization leading to differentiation of *M. phaseolina* strains (Pearson et al., 1986). However, recent studies show that in the case of sorghum it is possible to obtain all three of the *M. phaseolina* phenotypes mentioned before and there is no correlation between chlorate sensitivity isolates and host specificity (Das et al., 2008). In addition, supplementary evidence of *M. phaseolina* isolates with non-host specialization where found by Zazzerini and Tosi (1989) conducting pathogenicity tests.

Second, biochemical and serological methods also have been used. Even though immunological methods are highly sensitive, there are no extensive reports on the detection and quantification of *M. phaseolina*. Srivasta and Arora (1997) describe a technique using a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and demonstrate that antibodies raised from ribosomal proteins are specific at the genus level and the methodology can be useful for detection of *M. phaseolina,* but is restricted to certain *in vitro* and greenhouse environments and cannot differentiate between *M. phaseolina* isolates.

Third are polymerase chain reaction based (PCR) molecular techniques. Internal transcribed spacers (ITS) and intergenic spacer have been one of the most explored and exploited genes to elucidate fungal taxonomy (Babu et al., 2010). The first report of using and developing specific ITS primers for identification of *M. phaseolina* was given by Babu et al. (2007). Although there has not been enough evidence to suggest a separation of *formae specialis* or races within the *Macrophomina* genus, advances in molecular techniques and refined PCR-based techniques such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and amplified fragment length polymorphism (AFLP) have helped researchers understand more about *M. phaseolina* genetic and pathogenic variability. According to Jana et al. (2003), design and use of a RAPD primer (OPA-13) was able to distinguish *M. phaseolina* isolates from soybean, sesame, ground nut, chickpea, cotton, common bean, and other hosts, however the study was confined to a particular area of the United States including states like Arkansas, Texas and Alabama. Through AFLP analysis, Vandemark et al. (2000) concluded that it was impossible to obtain a correlation at the DNA polymorphism level with geographic location or host. Even with these molecular approaches, there is not sufficient evidence to suggest a *formae specialis* or subspecies within the *M. phaseolina*.

Genetic variation has always been evident among *M. phaseolina* isolates. Even isolates taken from a single host have different levels of pathogenicity. Using 114 isolates representing four host families and two continents, Mihail and Taylor (1995) were able to obtain hyphal fusions between *M. phaseolina* isolates from Somalia and Arizona, a geographical scale in which geographical isolation would appear reasonable. Their study suggested that *M. phaseolina* does not have genetic barriers for non-sexual genetic interchange. A plausible explanation of the variability in *M. phaseolina* is presented in studies conducted by Punithalingam (1983). This author reported that conidiogenous cells and hyphal cells are initially uninucleate, but a single nucleus can undergo various mitotic divisions and some conidia (pycnidiospores) possess up to 36 nuclei each. As the conidia produce germ tubes the nuclei move into the developing hyphae dividing mitotically during migration. The chromosome number most frequently observed is six, however, aneuploid, haploid and diploid nuclei have been observed in different *M. phaseolina* isolates (Punithalingam, 1983).

Even though mycelia are predominantly homokaryotic it is probable that heterokaryosis as well as heteroploidy occur after hypal fusion and this process may account for the generation of new cultural types or physiologic races (Dhingra and Sinclair, 1978; Wyllie and Scott, 1988).

Morphology and biology of *Macrophomina phaseolina*

Among *M. phaseolina* isolates, cultural morphological characteristics such as hypha pigmentation, microsclerotia size and shape, and presence or absence of pycnidia can vary. In general, colonies are gray or white with short mycelium inclined towards the growth direction, although aerial mycelium is not often produced (Reichert and Hellinger, 1947). Under the microscope, hyphae branch at right angles from the main hyphae, but later hypha bend and grow nearly parallel to the main hyphae (Hartman et al., 1999). Sometimes hyphae present dark colors from pale brown to grey. Septa width varies from approximately 2 to 11 µm and cells measure at least 46 µm in length. However, the most important characteristic regarding taxonomy and classification are the production size and composition of microsclerotia (Reichert and Hellinger, 1947).

M. phaseolina microsclerotia are black bodies composed of 50-200 hyphal cells aggregated by a melanin-like cementing agent that give its color (Gangopadhyay and Wyllie, 1974; Short and Wyllie, 1978). Microsclerotia vary in size (60-200 µm) and number depending upon the nutrient availability in the culture media or a specific host. In addition, the number of cells and germ tubes are directly related with the microsclerotia size (Dhingra and Sinclair, 1977). *M. phaseolina* microsclerotia are produced in five different ways during mycelia growth. The most frequent formation is by the spontaneous production of swollen barrel-shaped dark cells from a single hyphae measured from 4 to 23 µm, that later are self-divided or segmented forming a microsclerotium. It was also observed that barrel-shaped cells growing from different hyphae fuse to generate a microsclerotium. Normal cells from a single or various hyphae can also fuse and intertwine or normal hyphal cells fuse with swollen-barrel cells to form microsclerotia. These types of microsclerotia formation were observed among isolates from various hosts, fusion of barrel-shaped cell being the most common type of formation. Fusion of mature-formed microsclerotia also was observed (Reichert and Hellinger, 1947).

Using transmission and scanning electron microscopy, Wyllie and Brown (1970) describe in more detail the formation of microsclerotia for two other ways. Intertwining of hyphal strands and propagation or proliferation of hypal strands in a specific point on a major hyphal strand. When the hypha grows and interwaves, its mass increases and acquire a spherical form. The hyphae keep growing, the microsclerotium inner cells swell, and microsclerotia reach a size about 50-100 µm. In contrast, when the microsclerotia formation occurs by fusion on a major hyphal strand, a series of bud-like structures rise above the surface and microsclerotia start developing. During formation, microsclerotia are covered by a mucilaginous matrix and inner cells become dark.

Wyllie and Brown (1970) describe a microsclerotium cross section. They pointed out that all the inner cells have the cellular organelles necessary for germination, such as, mitochondria, endoplasmic reticulum, lomasomes, lipids, and one to three nuclei per cell. They also reported the observation of septal pores that connect the sclerotial cells and hypothesized that they allow the continuity of the cytoplasm and that some microsclerotia cells serve as a nutrient suppliers to others during the microsclerotia germination process.

A morphological description of germ tubes and hyphae from *M. phaseolina* microsclerotia germinated on diluted cornmeal agar (CMA) is given by Pratt, (2006). On this media an average of four germ tubes are produced per microsclerotium, with a length of a few micrometers to several hundred after 18 to 24 hours of exposure to this media. Pratt (2006) also reported that germ tubes were sometimes unbranched and with curly growth patterns.

M. phaseolina pycnidia are obtained by alternating the light regime by 12 hours dark and light intervals and growing the fungus in fresh plant tissue. The pycnidial stage has been reported on different bean tissues such as garden and jute bean and there are reports of the pycnidial stage on soybean (Mihail, 1992). However, Ma et al. (2010) described a culture media, peanut butter extract-saturated filter paper over soynut butter extact agar (PESEA) in which conidia and pycnidia are produced in an optimum manner.

Pycnidia are globose to fusiform, black or grayish colored. In early stages of formation they are immerse in the host tissue but erumpent at maturity. The small truncate ostiole may be inconspicuous or have a clear or hyaline opening (Hartman et al., 1999; Mihail, 1992). Pycnidiospores are hyaline, single-celled, and ellipsoid to ovoid with a length-width ratio of 3:1 (Dhingra and Sinclair, 1978).

Macrophomina phaseolina in other hosts

Reichert and Hellinger (1947), listed a wide number of plant species around the world affected by *M. phaseolina*. The most economically important are: pepper (*Capsicum annum* L.), papaya (*Carica papaya* L.), sweet orange (*Citrus × sinensis* L. Osbeck), coffee (*Coffea arabica* L.), soybean (*Glycine max* (L.) Merr.), sunflower (*Helianthus annus* L.), sweet potato (*Ipomoea batatas* (L.) Lam.), apple (*Malus domestica* Borkh.), alfalfa (*Medicago sativa* L.), plantain (*Musa paradisiaca* L.), tobacco (*Nicotiana tabacum* L.), common bean (*Phaseolus vulgaris* L.), garden pea (*Pisum sativum* L.), tomato (*Lycopersicon sculentum* Mill.), potato (*Solanum tuberosum* L.), grain sorghum (*Sorghum bicolor* (L.) Moench), cacao (*Theobroma cacao* L.), clover (*Trifolium sp.*), grape (*Vitis vinifera* L.), and corn (*Zea mays* L.). Host range is wide spread in the tropics and subtropics, and includes cereals, legumes, fruits, vegetables, herbaceous, and woody plants (Dhingra and Sinclair, 1978; Holliday et al., 1970).

The pathogen is being reported in new hosts and areas causing charcoal rot in previously reported hosts. For example, in Spring 2006, tan-brown wilted canola plants (*Brassica napus* L.) were observed in an experimental plot at Merredin, Western Australia. Longitudinal streaks along the main stem, wilting of branches, and shriveled pods were the characteristic symptoms caused by *M. phaseolina* (Khangura and Aberra, 2009). Charcoal rot of canola was also reported Argentina in 2006 (Gaetan et al., 2006). Tropical soda apple (*Solanum viarum* Dunal), considered one of the most invasive weeds in Florida, was reported showing symptoms of progressive necrosis from leaves to petiole caused by *M. phaseolina* in 2006. Production of pycnidia and pycnidiospores was also observed on infected tissues. Iriarte (2007) suggested that *M. phaseolina* could be a limiting factor for the spread of this weed. However, the fungus is a pathogen for desirable crops produced in the area and tropical soda apple may be a reservoir for the pathogen.

In 2006 in southern Spain, Aviles et al. (2008) confirmed *M. phaseolina* causing crown and root rot in several strawberry cultivars. Affected plants presented necrotic roots, dark brown necrotic areas around the crown and along the woody vascular ring. The same disease and symptoms were also reported in strawberries in Florida (United States) and Israel in 2005.

M. phaseolina is able to cause different symptoms: hollow stem rot, wilt, and pre-emergent and post-emergent damping off, depending on the plant tissue colonized. In the case of melon, *M. phaseolina* infects plant roots and later colonizes the fruit via the peduncle through the abscission zone. Once the fruit is colonized, *M. phaseolina* infects the seed coat and cotyledons, and these infected seeds give rise to diseased seedlings that can increase the inoculum potential in the soil. Melon is one of the hosts in which *M. phaseolina* serves as a soilborne and seedborne pathogen (Reuveni et al., 1983).

Charcoal rot of sunflower (*H. annuus*) was first reported in 1927 in Sri Lanka and in subsequent years in various continents around the world (Wyllie and Scott, 1988); the most recent report described by Mahmoud and Budak (2011) in Turkey. Predominant symptoms of charcoal rot on sunflower are gray to black discoloration with lesions on the stem above the soil line, black microsclerotia usually observed in the fibro-vascular system of roots and lower internodes covering an average of one-third of the plant height (Khan, 2007; Raut, 1985; Yang and Owen, 1982). Like soybean, disease severity is high dependent upon environmental conditions, specially drought and high air temperatures. Dawar and Ghaffar (1998) pointed out that there is a significant correlation between the level of inoculum in the soil and infection or colonization of sunflower roots by *M. phaseolina*.

M. phaseolina is reported to be soil, seed and stubble born in the specific case of sunflower. The pathogen has been reported to cause seedling blight, damping off, basal stem rot and early maturity of sunflower (Khan, 2007). One infected plant can have up to 44 percent infected seeds (Raut, 1985). Yang et al. (1983) reported that *M. phaseolina* also can be spread by insects in this crop. A small percentage of *Cylindrocopturus adsperus* (sunflower stem weevil) carry *M. phaseolina* as they emerge after overwintering in roots and stalks. Later, the insects spread the pathogen while feeding and ovipositioning on other plants. Presumably, *M. phaseolina* infests the egg cavity, and grows and spreads through the stalks via larval tunnels.

Distribution and biology of *Macrophomina phaseolina* in soil

Many authors have investigated the physical and chemical soil variables and their impact upon the survival and activity of *M. phaseolina* soil populations, and its relation with severity and infection in different hosts. No more than two decades ago, researchers were still working on laboratory and field techniques that allow a more precise idea of the behavior of *M. phaseolina* and other soilborne fungi (Collins et al., 1991).

Soilborne pathogens survive in the soil through such environmentally resistant structures as microsclerotia in the case of *M. phaseolina* (Raaijmakers et al., 2009). Fungal propagules have been recovered from soil in cultivated and non-cultivated soils in locations throughout the world. The highest concentration of inoculum is generally found in the top 30 cm of the soil profile (Bruton and Reuveni, 1985). On the other hand, studies of propagules and their horizontal distribution in soil not only for *M. phaseolina*, but for several other pathogens, have shown nonrandom distribution patterns in soil. The propagules of soilborne pathogens tend to be in clusters, compared to foliar pathogens. An aggregated distribution in soil has been reported with pathogens such as *Phytophthora, Verticillium,* and *Gaeumannomyces* (Raaijmakers et al., 2009). In cultivated soils, aggregated patterns of *M. phaseolina* have been documented as measured by the mean-to-variance ratio and *Morisita's index of dispersion.* (Mihail and Alcorn, 1987). In this study authors suggest that the soil sampling strategy is dependent of the expected outcome from the samples; if only the population mean is required, a collection of samples spaced evenly through a systematic path is enough. However, probabilistic site selection or random site selection is unsatisfactory, since the microsclerotia population in soil is not randomly distributed in the horizontal level.

Mihail (1989) also studied the dynamics of *M. phaseolina* populations in soil in the United States on a susceptible host, gopher plant (*Euphorbia lathyris*), in which only 1 microsclerotia per gram of soil was enough to cause 90% mortality. Microsclerotia population increased over two years of the study (from 8.9 to 98.6 microsclerotia per gram of soil), as colonized host debris was incorporated into the soil. Horizontal patterns of aggregation of inoculum were reported, as suggested above. Population size did not remain constant over the time due to constant cultivation of the study plots. The temporal dynamics of *M. phaseolina* in

soil when soybean is rotated with other crops show lower fluctuations than *E. lathyris* (Meyer et al., 1973; Short et al., 1980).

Tillage has an impact not only on the physical but biochemical and biological properties of the soil and also on the distribution and dynamics of microbial communities (Raaijmakers et al., 2009). Tillage and disking impact *M. phaseolina* propagule aggregation patterns and redistribution in the soil. The degree of aggregation can diminish after one or two consecutive tillage treatments with discs, and this phenomenon of propagule redistribution is more evident when microsclerotia populations are high (Olanya and Campbell, 1988). The development of new *M. phaseolina* foci near older foci across time can be generated by tilling practices. The generation and aggregation of *M. phaseolina* microsclerotia has been reported to have no correlation with soil chemical characteristics such as pH, Ca and Mg, cation exchange capacity (CEC), and soil macro- or micronutrients as well as other soil physical properties (Campbell and Vandergaag, 1993).

In soybean crops under no tillage management, *M. phaseolina* populations tend to be higher than in systems under tillage. *M. phaseolina* soil populations declined as the soil compaction increased (Wrather et al., 1998). As the soil bulk density increases *M. phaseolina* soil propagules declined in many of the soils studied (Gangopadhyay et al., 1982). In contrast, soybean root populations can be reduced in a non-tillage system as reported by Mengistu et al. (2007). This author also suggested that reduction in the number of colony forming units (CFU) in tissue is due to the accumulation of crop residue in the soil, which promotes higher soil water content and lower temperatures reducing disease incidence.

Impact of soil water content on *Macrophomina phaseolina* soil populations

Environmental conditions like temperature, atmospheric humidity, and soil water potential play an important role in the viability and inoculum potential of *M. phaseolina* (Khan, 2007). *M. phaseolina* is able to produce microsclerotia under relatively low water conditions; thus, survival of this inoculum is influenced by the soil matric water potentials. Viability of microsclerotia were drastically reduced at high water potentials (-30 J/Kg, field capacity), and

was virtually not affected at low water potentials (-1.500 Kg/J, permanent wilting point) in a sandy loam soil (Olaya et al., 1996).

Soil water content affects the gaseous conditions in the soil and may cause reduced microsclerotia survival by the reduction of O_2. Substances found in flooded soils such as alcohols, volatiles and increased levels of CO_2 can have a detrimental effect on the inoculum (Olaya et al., 1996; Wyllie et al., 1984). Microsclerotia germination is annulated in artificial atmospheres containing less than 16% of O_2 concentration in soil column systems; indicating that reduction in viability is not due to nutrient deprivation (Wyllie et al., 1984). This knowledge has been used to reduce propagule densities in the soil not only for *M. phaseolina* but *Sclerotinia sclerotiorum* and *Sclerotium cepivorum* under anoxic-flooded soils (Abawi et al., 1985; Banks and Edgington, 1989).

Goudarzi et al. (2008) describes *M. phaseolina* fungal growth creating different matric and osmotic potentials by using polyethylene glycol (PEG 6000) and sodium chloride respectively, on *in vitro* conditions. Microsclerotia germination and radial growth increases as the osmotic and water potentials decreases. However, there is an optimum of - 0.6 MPa for the osmotic potential and - 1.2 MPa for the matric potential, this suggest that a positive turgor is maintained in the hypha of *M. phaseolina* during growth and this adaptation to survive in low water potentials is used for the pathogen to survive in host tissue under these conditions.

Epidemiological data of the disease shows differences in mortality of *B. juncea* across three different soil textures in soils inoculated with *M. phaseolina.* Sandy soils lead plant mortality of approximately 90% in comparison to loamy and clayey soils in which plant mortality reached 77% and 52%, respectively. In addition, in low levels of soil moisture disease severity is higher and the microsclerotia population in soil increases as well (Srivastava and Dhawan, 1980).

Microsclerotia soil populations declined in soils at 60 to 100% water holding capacity amended with glucose, sucrose, starch, cellulose, and $NaNO_3$. At high soil moistures, soil bacterial populations increased and parasitized *M. phaseolina*, degrading its cell walls. Compounds such as amino acids, sugars, and organic acids have been found to stimulate microsclerotia germination in soil (Dhingra and Sinclair, 1978).

Survival of *Macrophomina phaseolina* on plant debris, and soybean roots in soil

Infected crops residues are one of the most important sources of inoculum and inoculum dispersal of *M. phaseolina* in the field and provide a mechanism of survival for long periods in the soil. Degradation of plant debris and relative longevity of *M. phaseolina* depends upon several factors, such as soil moisture and temperature (Baird et al., 2003).

In general, *M. phaseolina* populations in soybean root debris or residue in the soil tend to increase over time. Root debris at or near the soil surface increases the *M. phaseolina* population more rapidly than buried residue, but surface residues are more directly exposed to environmental variations. Fluctuations in the population are inversely related to soil depth (Short et al., 1980).

Tissue degradation is affected by soil depth and this has a significant impact on *M. phaseolina* survival. Microorganisms associated with plant tissue degradation are isolated more frequently with increasing burial depths of *M. phaseolina*-infected soybean root tissues compared with surface debris. As reported by Baird et al. (2003), cellulose degrading fungi such as *Trichoderma* spp. and basidiomycetes are more active at soil depths in which temperature and moisture are favorable and could be partially responsible for the reduced survival of *M. phaseolina* on those degrading tissues since their isolation frequency increases as that of *M. phaseolina* decreases.

Soil moisture affects microsclerotia in crop residue. Microsclerotia embedded in infected roots can survive longer under dry and hot temperature conditions than in saturated soil conditions. According to Dhingra and Sinclair (1978), recovery of microsclerotia from soybean root tissues in soils that have less than 40 to 100% moisture holding capacity is 0 to 12% after four weeks. In soils having a moisture holding capacity at 20%, the percentage of recovery from soybean root tissues was 100% after eight weeks.

In soils where soybean or susceptible hosts are planted successively year after year, *M. phaseolina* populations tend to increase. Cook et al. (1973) reported an increase in microsclerotia in a field planted on soybeans for three years from 80, 120, to 149 microsclerotia per gram of soil.

Macrophomina phaseolina disease cycle

M. phaseolina can survive in the soil as microsclerotia (resilient structure) for about two years or survive embedded in root debris for longer periods (Baird et al., 2003). Microsclerotia are black, spherical structures produced in host tissue that are released into the soil and allow the fungus to survive adverse conditions (Hartman et al., 1999). Each microsclerotium consists of a number of globose cells that can germinate independently.

Germination of microsclerotia in the soil occurs throughout the growing season when temperatures are between 28 to 32°C. Microsclerotia germinate in the presence of a susceptible host on the root surface or in proximity producing numerous germ tubes. Penetration generally occurs from swellings of mature hyphae (described as *hyphopodia*) that penetrate the epidermal cells walls or through natural openings (Bressano et al., 2010; Dhingra and Sinclair, 1977). In the first stages of pathogenesis hyphae are restricted to the intercellular spaces in the root cortex, and later intracellularly colonize the vascular tissue through the xylem (Wyllie and Scott, 1988). In this stage, penetration of the cell wall is the result of both mechanical pressure and chemical softening by producing pectolytic enzymes (Ammon et al., 1974). Infection can occur at emergence, cotyledon (VC) or even early vegetative stages 2-3 weeks after planting (V1-V2). Infection remains latent until environmentally stressful conditions overlap with plant reproductive stages (R1-R7), creating conditions that enable the fungus to further colonize the root and stem tissues of the weakened plant (Hartman et al., 1999; Wyllie and Calvert, 1969). At reproductive stages and close to maturity, infected plants show loss of vigor, chlorotic and necrotic leaves remain attached to the stem. Eventually, when levels of *M. phaseolina* in the root and stems are high, a grey or silvery discoloration can be observed in root and stem epidermal and subepidermal tissues (Ammon et al., 1974). High levels of *M. phaseolina* colonization are positively correlated with poor seed quality and lower yields. After harvesting, roots loaded with microsclerotia start decaying and release the microsclerotia into the soil (Olaya et al., 1996). Inoculum survival in the soil depends on soil moisture, temperature, and the soil microbial community (Collins et al., 1991) (Figure 1-1).

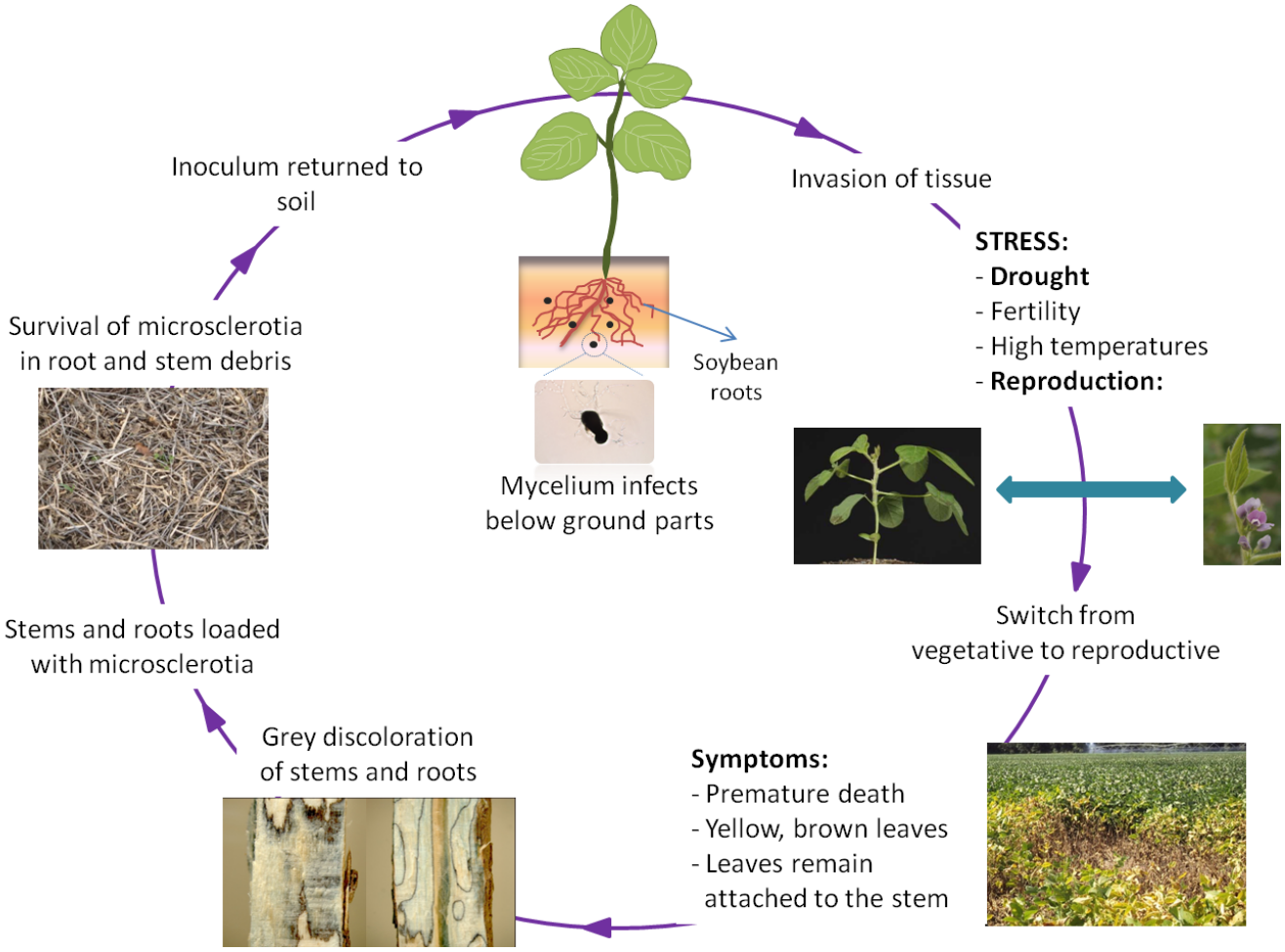

Figure 1-1. The *Macrophomina phaseolina* disease cycle in soybean.

(Pedersen, 2003; Wrather et al., 2007)

Management strategies

The primary aim of *M. phaseolina* management is to reduce pathogen propagule in soil and host roots, and avoid favorable conditions for further pathogen survival and propagation.

Impact of irrigation on microsclerotia survival in soil and roots has been studied by several researchers. Irrigation has been one of the most effective ways to deal with charcoal rot for different plant species (Kendig et al., 2000). Irrigation through the whole soybean growth season reduces the population and colonization of *M. phaseolina* on roots compared with a non-irrigated crop system, even though the propagules remain during the season in both systems and no symptoms in soybean plants were found in the irrigated field (Kendig et al., 2000).

Microsclerotia of *M. phaseolina* can be degraded and eliminated from the surface to 20 cm depth under paddy rice soil conditions. Such flooded conditions reduced the number of viable microsclerotia by 83% in two years. After two years, cotton roots only showed 20% of infection by *M. phaseolina* (Zaki and Ghaffar, 1988).

Herbicides can also have an impact on the *M. phaseolina* population. Plant stress and root injury due to high herbicides doses can lead to an indirect increase of root colonization. Herbicides such as alachlor, chloramben, and 2,4-D cause extensive proliferation of adventitious roots in the upper 5-7 cm of soil where dry conditions predominantly exist and favor *M. phaseolina* root colonization (Canaday et al., 1986).

Fungicide has been unsatisfactory for the control of *M. phaseolina* on host species such as groundnut (*Arachis hypogaea* L.) and cotton (*Gossypium arboretum* L.). Root exudates from the plant host, such as amino acids, carbohydrates, phenolics, and sulphydral compounds, play an important role on reversing the toxic effect of fungicides to control root diseases (Dhingra and Sinclair, 1978; Raaijmakers et al., 2009).

Hooda and Grover (1989) reported inefficacy of five fungicide seed treatments (Carbendazim, Thiophanate-methyl, Captafol, Thiram, and PMA) for the control of charcoal rot on five different plant species. Under *in vitro* conditions, mungbean and cotton root exudates reduced the efficacy of Carbendazim and Captafol at low concentrations in more than 50% compared to the non-treated control.

An important method for reducing the viability of microsclerotia in the soil is polyethylene mulching. Increasing soil temperatures within a range of 52-65°C for one week reduced propagule viability by 100% in the first 5 cm of soil depth and 50% in 20 cm depth of naturally infested soils (Sheikh and Ghaffar, 1984). Maintenance of high soil moisture was necessary for increasing soil thermal conduction in the mulched soil. Higher populations of bacteria and actinomycetes were found in soils that were heated compared to untreated soils (Sheikh and Ghaffar, 1984).

In Turkey, where *M. phaseolina* is becoming a serious disease on strawberry, the use of plastic covers is showing promise. Plastic covers raised the soil temperature in the first 5 cm to 56.6°C. Survival of microsclerotia in soil declined rapidly after 17 hours at 50°C and was completely reduced after 20 hours. A 66% reduction in microsclerotium viability was observed at a depth of 5 cm. However, viability was not significantly reduced at 10 to 20 cm depth where temperatures remained at about 48°C and 40°C, respectively (Yildiz et al., 2010).

Antagonistic microorganisms have been investigated for the control of charcoal rot on diverse species. Bacteria (*Bacillus, Pseudomonas,* and *Streptomyces* spp.) and fungi (*Trichoderma, penicillium, Gliocladium, Aspergillus* and *Rhizopus* spp.) are known to suppress *M. phaseolina* growth under specific conditions and temperatures (Gacitua et al., 2009).

Pseudomonas spp. may inhibit a wide range of root pathogens, including *M. phaseolina*. This is due to the production of antifungal compounds and the ability of this microorganism to create symbiotic associations with higher plants. Such antagonistic activity is attributed to the production of hydrolases, including chitinases and glucanases, which degrade the main components of the fungal cell wall (Ajit et al., 2006). *In vitro*, isolates of *Pseudomonas* showed 80% inhibition of fungal growth, mycelia grew to the interaction zone, deformed and degraded. In addition, mustard plants show no symptoms of charcoal rot 60 days after planting in soils inoculated with *M. phaseolina* when mustard seeds were inoculated with *Pseudomonas fluorescens.* Production of β-1,3-glucanase by *P. fluorescens* caused significant inhibition of *M. phaseolina* (Arora et al., 2008).

P. fluorescens also has proven to have antagonistic activity on *M. phaseolina* causing groundnut (*Arachis hypogaea* L.; peanut) root rot. A talc-based formulation of the *P. fluorescens* isolate Pf 1 which showed 86% of growth inhibition of *M. phaseolina in vitro*, was applied to the seeds and soil before planting. Bacterial treatments reduced incidence and severity of charcoal

rot and increased root nodulation as well as yield (Shanmugam et al., 2003). Kumar et al. (2007) also reported positive effects in the inhibition of *M. phaseolina* by *P. fluorescens* isolate Pf4-99 in greenhouse and field conditions on chickpea.

In vitro, Trichoderma harzianum can inhibit *M. phaseolina* microsclerotia production and growth by 82% (Elad et al., 1986). *Trichoderma* spp. are able to degrade fungal cell walls by the production of cellulase (Baird et al., 2003). In turn, cell wall components of *M. phaseolina* stimulate *T. harzianum* conidia production. Likewise, viability of microsclerotium decreased from 3400 to 100 CFU/mL when exposed to *T. harzianum* in dual culture. *T. harzianum* is able to reduce *M. phaseolina* disease severity by 37-74% on melon (*Cucumis melo* L.) when conidia are applied to soils artificially infested with *M. phaseolina*. Similarly in commercial fields, melon plants from seed treated with *T. harzianum* yielded 61% more fruit than plants from non-treated seeds in naturally infested soils (Elad et al., 1986). *Trichoderma* spp. have also been reported to be isolated in high frequency from root, stalks and stubble debris in soil. This antagonistic fungus reduces the viability and longevity of *M. phaseolina* microsclerotia on decaying crop tissues (Baird et al., 2003).

Effects of manganese on fungal pathogens and soybean plants

Studies of the effects of microelements on the growth of *M. phaseolina* under *in vitro* conditions have been conflicting. Zinc (Zn), manganese (Mn) and boron (B) increase the growth rate of *M. phaseolina* in concentrations ranging from 5 to 100 µg/ml. In contrast, copper (Cu) was toxic at concentrations above 3.5 µg/ml (Daftari, 1966).

In addition, there is no effect on the *M. phaseolina* growth rate on media having low concentrations of magnesium (Mg) and sodium (Na) (Sankhla and Mathur, 1967). *M. phaseolina* is a nutrient scavenger, especially in soil with nutrient deficiencies and at high soil temperatures (Wyllie and Scott, 1988).

The positive effects of Mn application on soybean yield have been reported by Gettier et al. (1984). Consecutive soil applications of Mn to soil during two soybean seasons can increase seed weight by 29%. Under severe Mn deficiency seed viability in soybean is reduced

significantly. Excess Mn causes root thickening, lignification of the cortex cells, and disorganization of the xylem vessels in some soybean varieties (Lavres et al., 2009).

Direct effects of Mn against pathogens include growth inhibition, reduced sporulation, and toxin production. For example, some strains of *Gaeumannomyces graminis* and *Magnaporthe grisea* that lack the ability to oxidize Mn exhibit decreased aggressiveness and severity in take-all disease of wheat and rice blast, respectively (Datnoff et al., 2007).

Soil-water relations

Soil and water undergo a series of gradual physical changes when they remain in contact. These behaviors are due to the capillarity forces generated by the soil pores and the physical properties of water such as cohesion, adhesion, and surface tension (Brady and Weil, 2004).

As water is depleted from soil, the level of tension also increases, holding water more tightly in the smallest soil pores. Porosity is a function of soil particle size distribution, which refers to the proportion of small, medium, and large particles (clay, silt and sand, respectively) and the air spaces between them (McVay et al., 2006).

Several authors have reported a correlation between soil water-holding capacity and sand, silt and clay content, organic matter and bulk density and have used those variables to accurately predict the water content of soils (Arya and Paris, 1981; Manrique et al., 1991). Soils composed mainly of small soil particles, such as clay and silt, have more porosity and surface area than those with larger soil particles. This characteristic allows clayey soils to hold more water per unit volume and retain water of higher tensions (Brady and Weil, 2004).

According to Brady and Weil (2004), a soil is considered "saturated" when all soil pores are filled with water. In a saturated state the volume of water in the soil is equal to the volume of the total soil porosity. As long as water percolates from the macropores due to gravitational forces the soil is at a maximum retentive capacity. As water moves downwards in the soil profile by capillarity and gravity. When the soil stops draining and water in the macropores is replaced with air, the soil is said to be at "field capacity".

The term field capacity does not apply to pots in a greenhouse because there is not underlying soil that pulls the water downwards. However, the term "pot capacity" can be used,

which is the amount of water remaining after irrigation and when visible drainage has stopped (Kirkham, 2005).

As the soil continues to dry, the water retained at higher tensions in the smaller pores is removed. Consequently, the amount of water leftover in the soil is held so tightly that plants are not able to take it up and will wilt. This level of available water is known as the "permanent wilting point" (Brady and Weil, 2004; Kirkham, 2005).

Available water is considered to be the water retained in the soil between field capacity and the permanent wilting point and can be impacted significantly by soil texture and organic matter (Table 1-1).

Table 1-1. Field capacity and permanent wilting points for common textural classes. Values are given in volumetric water content (ϴV), which is the relationship between a volume of water associated with a volume of dry soil (Rowell, 1994).

	Volumetric water content (ϴV)	
Soil Texture	**Permanent wilting point**	**Field capacity**
Sand	0.04	0.12
Loamy Sand	0.06	0.14
Sandy Loam	0.1	0.23
Loam	0.12	0.26
Silt Loam	0.15	0.3
Silt	0.165	0.32
Sandy Clay Loam	0.175	0.33
Silty Clay Loam	0.19	0.34
Silty Clay	0.21	0.36
Clay	0.21	0.36

Soybean irrigation

In adequate soil conditions, soybean roots can grow to a depth of 6 feet in the soil profile. However, roots are more concentrated in the upper half of the root zone. A root depth of 3 feet is commonly used to calculate the water requirements of soybean plants (Rogers, 1997).

Soybean water requirements during the season range from 18 to 24 inches per year. The higher water demand during soybean growth occurs when plants are approaching the beginning

of pod development (R2-R3) and demand decreases as plants mature. For maximum yields, water availability is critical at the beginning of pod fill (Rogers, 1997; Scott et al., 1986).

Studies have demonstrated that irrigation is also beneficial at the latter part of the reproductive stages because water requirements for vegetative growth can be supplied by rainfall and/or stored soil water. In the worst-case scenario, a 5% reduction in yield can result if soybean is under water stress during the vegetative growth stages (Stegman et al., 1990).

Once established, soybean is quite drought tolerant, sometimes the only water required to supplement rainfall is a 4-inch flood irrigation in the late bloom to early pod filling stages in medium and fine-textured soils. In contrast, sandy soils require a more frequent irrigation, about 1 to 2 inches of water every three to seven days during the critical period of soybean reproductive development (Rogers, 1997).

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CHAPTER 2 - *Macrophomina phaseolina* quantitative detection methods from soil and inoculum potential

Introduction

Numerous methods have been developed in order to understand the biology of *M. phaseolina* (Tassi) Goid. in soil and plant debris. Most of these methods involve, grinding, sieving and bleaching of soil in a variety of simple to complex protocols. In addition, the use of selective media for quantification and enumeration of microsclerotia have played an important role in the study of soil borne fungi (Pratt, 2006).

There are several methods for *M. phaseolina* microsclerotia isolation and quantification from soil and root tissue, going from microscopic counting, water flotation, microsclerotia staining, sieving, direct counting and soil serial dilutions. However, none of these methods have been compared in its efficiency of recovering of *M. phaseolina* microsclerotia in the same level of inoculum and soil conditions (Papavizas and Klag, 1975; Pratt, 2006).

Production of *M. phaseolina* inoculum has been useful to determine the effectiveness of different detection methods. Soils artificially and naturally infested ranging from one to more than one thousand microsclerotia per gram of soil has been used (Papavizas and Klag, 1975). However, details of the inoculum viability and the sources in which it is obtained from have been poorly described in the literature.

Factors that affect the survival and quality of *M. phaseolina* inoculum such as size, productioon of germtubes, percentage of germination, and number of cells per microsclerotia have been documented (Short et al., 1980), but there are other factors that may influence directly or indirectly the inoculum quality, such as source of inoculum, time, conditions of storage and nutrient availability.

The objectives of these studies were:

- Determine the impact of nutrient deprivation on microsclerotia size, production of germ tubes and percentages of microsclerotia germination.

- Characterize and evaluate *M. phaseolina* inoculum produced by two different methods and sources, infested Japanese millet and PDA pure culture extraction.
- Compare the effect of soil nutrients on *M. phaseolina* inoculum by using soil extract agar (SEA).
- Evaluate the efficiency and sensitivity of two standard methods for *M. phaseolina* quantification and recovery from soil.
- Develop a suitable method to identify and detect *M. phaseolina* inoculum and observe early stages of soybean root infection in seedlings under microscope.

Material and methods

Microsclerotia size and production of germ tubes by Macrophomina phaseolina

Two different sizes of microsclerotia were produced by growing *M. phaseolina* inoculum in two different volumes of PDA media in 100 mm-diameter petri dishes. A volume of PDA of 5 cm^3, equivalent to a media layer of about 0.8 mm thick, in which small *M. phaseolina* microsclerotia were produced after incubation for four days at 30°C. In contrast, a volume of 30 cm^3, equivalent to a layer of 5 mm thick produced larger microsclerotia after incubation for four days at 30°C. *M. phaseolina* microsclerotia were harvested from these pure cultures and serial dilutions were performed. Microsclerotia from the diluted solutions were placed over a glass depression slide containing PDA media and the slides were placed on sealed 100 mm-diameter plastic petri dishes with a drop of sterile distilled water and incubated at 30°C in the dark to promote the production of germ tubes. Microsclerotia were evaluated for germination at 10, 11, and 19 hours. Counting of germ tubes and measurement of microsclerotium size produced by two different procedures were performed under the stereoscope and microscope, respectively. Microsclerotia diameters were measured using Zeiss AxioVision (version 4.7) software.

Analysis of variance (ANOVA) was performed using SAS version 9.2, (SAS institute Inc., Cary, North Carolina, USA). The "microsclerotia size" was analyzed using SAS Proc Glm using a completely randomized design (CRD). On the other hand, the "number of germ tubes" variable was analyzed using SAS Proc Mixed in a CRD with repeated measures over time.

Results and discussion

Effect of growth media thickness on microsclerotium size

Media thickness had a significant impact on microsclerotium size when the fungus was grown at 30°C in the dark for four days. On a 0.8 mm thick media layer, the average diameter of microsclerotia was 86 µm. For a 5 mm thick media layer, the average diameter of microsclerotia was 106 µm. Differences in microsclerotia size may be attributed to a reduction in nutrients and water availability while growing over the media as reported by Dhingra and Sinclair (1977). There were not observable differences in growth rate between the two thicknesses of media set in the experiment. On the other hand, there were visual differences in colony morphology. Production of aerial mycelium was common at 0.8 mm media thickness, but not at the 5 mm thickness.

Impact of microsclerotia size and time upon the number of germ tubes

Significant differences in microsclerotium size ($p = 0.0002$) and time ($p < 0.0001$) were found in the "number of germ tubes". Small microsclerotia (86 µm diameter) produced greater numbers of germ tubes than inoculum having larger microsclerotia (106 µm diameter). In addition, the number of germ tubes per microsclerotia significantly increases over time ($p < 0.0001$). Microsclerotia start geminating after the first ten hours of incubation, producing in average one germ tube per propagule. One hour later, at eleven hours of incubation, microsclerotia have produced in average five germ tubes per microsclerotium. Between the eleventh and eighteenth hours of incubation there was not a significant increase in the number of germ tubes. However, at nineteen hours of incubation, microsclerotia have produced in average fourteen germ tubes per propagule. In the subsequent observations, after twenty hours of incubation, the numbers of germ tubes per microsclerotia were so many that it was difficult to count them reliably. For this reason, all the observations were stopped at nineteen hours of incubation.

These findings agree with those reported by Pratt (2006) who observed a high rate of microsclerotia germination at eighteen to twenty-four hours on PDA, water agar (WA) and cornmeal agar (CMA) media.

Even though small microsclerotia produced higher numbers of germ tubes, the germination rate was low compared to larger microsclerotia. Only 30% of the smaller microsclerotia germinated in the incubation conditions previously described, whereas germination rate for larger microsclerotia was about 80%. This may provide insights into inoculum survival and potential. Growing inoculum in more stressful nutrient conditions, may lead to smaller inner globose cells, which are required for the germination of microsclerotia, compromising the integrity and continuity of the entire cellular mass of the propagule as suggested by Wyllie and Brown (1970).

Inoculum potential of microsclerotia on rifampicin media

Inoculum potential of *M. phaseolina* microsclerotia was assessed from two different sources: dried microsclerotia harvested from culture media (full strength 39 g/L PDA, mg/L penicillin 40, 20mg/L streptomycin, and 20 mg/L tetracycline), and microsclerotia produced on *M. phaseolina*-infested Japanese millet seeds. Inoculum was prepared using a *M. phaseolina* isolate taken from infected soybean roots obtained from 30-year continuous soybean plots at Ashland Bottoms research farm in Riley County, Kansas.

Inoculum was passed through a 600 µm sieve and blended in a 0.5% NaOCl solution for 3 min, collected in a 45 µm sieve and rinsed with distilled water for 2 min. Inoculum was spread over sterile plastic trays and dried in the laminar flow hood for one day. Dried microsclerotia were collected from the tray surface and stored in the refrigerator in plastic bags. Inoculum from Japanese millet was kept in the refrigerator at 4°C for one and four months and inoculum obtained from PDA pure isolation was kept in the refrigerator at the same conditions for one month.

In order to assess the inoculum potential, the number of CFUs (colony forming units) and number of microsclerotia contained in the inoculum samples were assessed. A selective medium [PDA (39 g/L), penicillin (40 mg/L), streptomycin (20mg/L), tetracycline (20 mg/L), and rifampicin (20 mg/L)] was used to estimate the number of CFUs. From each source of inoculum 0.02 g samples of pure microsclerotia were added to 10 mL of sterile-distilled water. Aliquots of 1mL were taken from the 10 mL microsclerotia solution and added to 50 mL of the previously described media. The 50 ml of molten media with the microsclerotia were poured evenly into

five 100 mm-diameter petri dishes (10 mL of molten media each) and they were incubated for five days at 30°C in the dark, in order to assess the number of CFUs in the inoculum samples.

To count the number of microsclerotia extracted from the inoculum sources, 0.02 g samples of pure microsclerotia were added to 10 mL of sterile-distilled water. Aliquots of 1mL were taken from the 10 mL microsclerotia solution and placed in a nematode counting slide.

Inoculum potential was calculated as follows:

Equation 1

$$Inoculum\ potential = \frac{\text{Number of CFUs/g inoculum}}{\text{Number of microsclerotia/g of inoculum}} \times 100$$

Analysis of variance (ANOVA) was performed using SAS system version 9.2, (SAS institute Inc., Cary, North Carolina, USA). The number of CFUs, number of microsclerotia and inoculum potential were analyzed using SAS Proc Mixed in a completely randomized design (CRD) with repeated measures. The response variable "number of CFUs" was logarithmically transformed in order to meet the assumptions of the ANOVA (normal distribution of data and equal variances).

Results and discussion

Effect of inoculum source on the number of colony forming units (CFUs) and number of microsclerotia

Sources of inoculum differed significantly in the number of CFUs ($p < 0.0001$). Inoculum harvested from infested Japanese millet produced higher numbers of CFUs compared to the inoculum harvested from PDA plates. Inoculum extracted from infested Japanese millet produced 25,000 CFUs per 0.02 g of inoculum whereas inoculum extracted from PDA pure culture produced 6,008 CFUs per 0.02 g of inoculum. Inoculum from infested Japanese millet produced more than four times CFUs compared to the inoculum extracted from PDA pure culture.

In addition, storage time for the infested Japanese millet inoculum also had a significant impact on the number of CFUs. A threefold decrease in the number of CFUs was found in the infested Japanese millet inoculum due only to the storage time, going from 25,233 CFUs per

0.02 g of inoculum to 8,508 microsclerotia per 0.02 g of inoculum in only four months of storage. The number of microsclerotia contained in the different sources of inoculum also differs significantly ($p < 0.0001$). Inoculum obtained from PDA pure culture isolation had the highest number of microsclerotia approximately (67,250 microsclerotia per 0.02 g of inoculum). The number of microsclerotia found between the Japanese millet inoculums was different, (43,300 and 28,808 microsclerotia per 0.02 g of inoculum for the four and one month storage inoculum respectively). It is not completely clear why the amount of microsclerotia varied between the sources of inoculum. However, these results may indicate that inoculum growing freely over an even surface such as PDA media generates high numbers of microsclerotia after extraction. On the other hand, it seems that inoculum growing on the millet seeds surface generates a lower but more highly variable amount of microsclerotia.

Effect of inoculum source on microsclerotia viability

Inoculum potential differs significantly between the sources of inoculum ($p < 0.0001$). Inoculum extracted from infested Japanese millet presented a high inoculum potential (85.6%) after one month of harvest, and relatively low inoculum potential (20.2%) after four months of harvest. This is consistent with previous reports by Pratt (2006), who stated a significant reduction in the frequency of microsclerotia germination after four weeks of storage and completely reduced or even eliminated germination after 6 or 8 weeks; pointing out that this phenomenon may not happen on *M. phaseolina* inoculums storage in soil that can provide nutrient to microsclerotia. Inoculum potential from the PDA pure culture isolate was the lowest compared to the infested millet inoculum, having only 9% of germination (Figure 2-1).

According to these results, inoculum produced on PDA pure culture isolate result in a poor source of inoculum. Even though this inoculum has the highest number of microsclerotia its viability is low. In contrast, inoculum extracted from infested Japanese millet does not have a high amount of microsclerotia but its germination rate is higher (85%). However, storage time and low temperatures can affect microsclerotia viability.

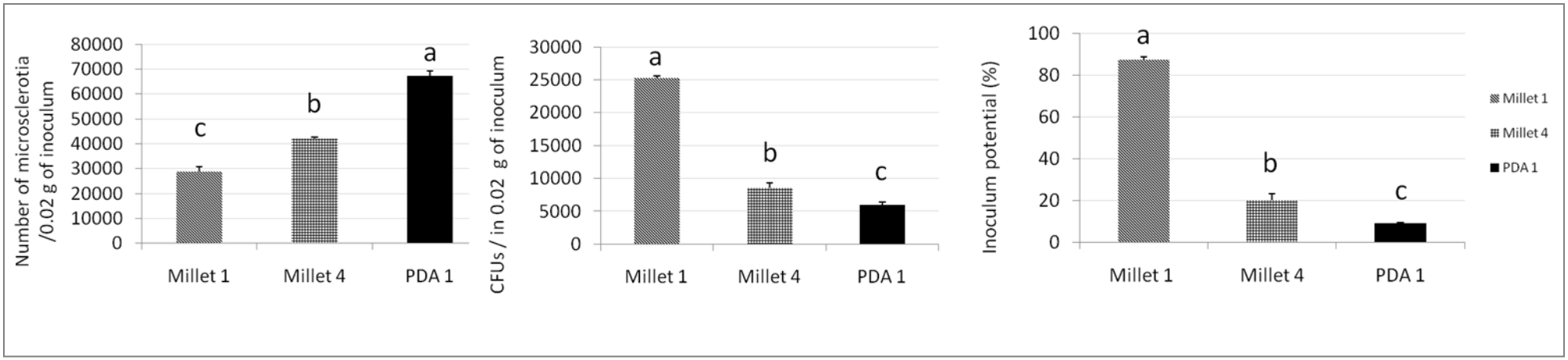

Figure 2-1. Effect of inoculum source and storage time upon inoculum potential. Means with the same letter are not significantly different ($p < 0.05$). Vertical bars denote the standard deviation. "PDA" and "Millet" denote the two different sources of inoculum; "1" and "4" denote months of storage.

Based on findings by Short et al. (1980) inoculum source was critical for *M. phaseolina* microsclerotia viability and inoculum potential. Inoculum extracted from ground infected soybean roots had a greater ability to survive than inoculum extracted from potato dextrose broth (PDB). Comparisons between sources of inoculum, indicate that a more natural growing media, such as soybean root tissues or millet seeds, provide the inoculum with better sources of nutrients and produce more vigorous microsclerotia than the standard growing media used for fungal isolates such as PDA or PDB.

In addition, inoculum potential gives a real measure of the inoculum conditions. Measurement of the inoculum potential is highly valuable since the amount of microsclerotia and number of CFUs can vary each time the inoculum is prepared (Smith and Carvil, 1997).

Effect of soil extract agar (SEA) on M. phaseolina inoculum

Soil extract agar was prepared from five artificial soils (Table 2-1). 500 g of soil were added to 1 L of tap water and autoclaved at 121°C for 1 h. Soil suspensions were decanted for 30 min and the soil water suspension was filtered through a Whatman No. 2 sterile filter paper. Glucose (2 g), yeast extract (1 g), KH_2PO_4 (0.5 g), and agar (15 g) were added to the filtered soil solution and brought to 1 L. The solution was re-autoclaved at 121°C for 30 min and antibiotics added (40 mg/L penicillin, 20mg/L streptomycin, and 20 mg/L tetracycline) when cooled. The final pH solution was 7.0.

Table 2-1. Artificial soil textures generated by mixing sand and silt loam in a weight basis.

SAND (%)	SILT LOAM (%)	TEXTURAL CLASS
100	0	Sand
75	25	Loamy sand
50	50	Sandy loam
25	75	Loam
0	100	Silt loam

M. phaseolina microsclerotia germination was assessed using two types of inoculum obtained from different methods and sources: dried microsclerotia harvested from culture media (full strength PDA (39 g/L), penicillin (40 mg/L), streptomycin (20 mg/L), and tetracycline (20 mg/L)), and microsclerotia produced on *M. phaseolina*-infested Japanese millet seeds.

Both inocula were prepared from an *M. phaseolina* isolate taken from infected soybean roots obtained from 30 year continuous soybean plots at Ashland Bottoms Research Farm in Riley County, Kansas near Manhattan.

Inoculum potential for each soil extract agar was estimated by doing serial dilutions of the two sources of inoculum. Samples of 0.02 g of microsclerotia were added to 10 mL of sterile-distilled water. Aliquots of 1 mL of the 10 mL shaken microsclerotia solution were added to 50 mL of molten soil agar and poured evenly into five 100 mm-diameter petri dishes (10 mL each). Dishes were incubated in the dark at 30°C for five days and colony forming units (CFUs) were counted.

Analysis of variance (ANOVA) was performed using SAS version 9.2 (SAS institute Inc., Cary, North Carolina, USA). The number of CFUs was analyzed using SAS Proc Mixed in a completely randomized design (CRD) with repeated measures. The response variable CFUs was logarithmically transformed in order to meet the assumptions of the ANOVA (normal distribution of data and equal variances).

Results and discussion

Effect of the inoculum sources and soil extract agar (SEA) on the number of colony forming units (CFUs)

Significant differences between sources of inoculum ($p < 0.0001$) were found in the response variable number of colony forming units (CFUs). As it was observed and discussed before in previous analysis, the inoculum extracted from the infested Japanese millet presented a significantly higher number of CFUs than the inoculum obtained from PDA pure culture isolation. According to the averages of non-logarithmic transformed data, microsclerotia extracted from infested Japanese millet had 19,430 CFUs per 0.02 g of inoculum and the microsclerotia extracted from PDA pure culture had in average 4,837 CFUs per 0.02 g of inoculum, which is almost four times higher number of CFUs for the millet inoculum and also agrees with the number of CFUs previously obtained from both inoculums.

The five different soil extract agars used had not significant effect on the number of CFUs ($p = 0.0693$). Soil constituents such as organic matter and soil nutrients present in the media did not have a suppressive or enhancing effect on the number of CFUs at the incubation conditions (30°C in the dark for four days), neither observable changes in colony morphology or

colony formation. However, it is likely that the soil extract agar media does not reflect the impact on microsclerotia viability as in the case when inoculum is incubated in soil for longer periods of time. Even though significant differences in the SEAs were not observed, trends were similar between both sources of inoculum. Sand extract agar yielded one of the lowest numbers of CFUs whereas Loamy extract agar yielded the highest number of CFUs (Figure 2-2).

Quantitative determination of two M. phaseolina soil populations detection methods

In order to determine the best method for the isolation of *M. phaseolina* from soil, two techniques were compared regarding work input and most importantly efficiency of the recovery of propagules (microsclerotia) from infested artificial soils on different soil textures and *M. phaseolina* infestation levels.

Soils were mixed and passed through a 2 mm sieve to homogenize the samples. Soils were autoclaved twice at 121°C for 20 min. After autoclaving, 1 g samples for each artificial soil were placed in 100 mm-diameter petri dishes containing PDA (39 g/L), tergitol (1mL/L), penicillin (40 mg/L), streptomycin (20 mg/L), tetracycline (20 mg/L), and placed in the incubator at 21°C. Dishes were incubated and observed for four days to detect any undesirable fungal growth and to confirm that soil was sterile.

Soils were artificially infested with dried *M. phaseolina* microsclerotia obtained from culture media (full strength PDA (39 g/L), penicillin (40 mg/L), streptomycin (20mg/L), and tetracycline (20 mg/L)). The total number of dried microsclerotia was counted and weighed in order to determine the inoculum potential.

Dilutions of infested and non-infested soils with microsclerotia were made for every artificial soil to determine which *M. phaseolina* soil population levels for each procedure will work the best. Soils were infested with 200 microsclerotia per g of soil for the direct counting technique and 100 microsclerotia per g of soil for the serial dilution technique. Afterwards, five levels of infestation were made on a weight basis: 0 % infested soil, 25% infested soil + 75% non-infested soil, 50% infested soil + 50% non-infested soil, 75% infested soil + 25% non-infested soil, and 100% infested soil. Afterwards soils were incubated for two weeks in the dark at 30°C.

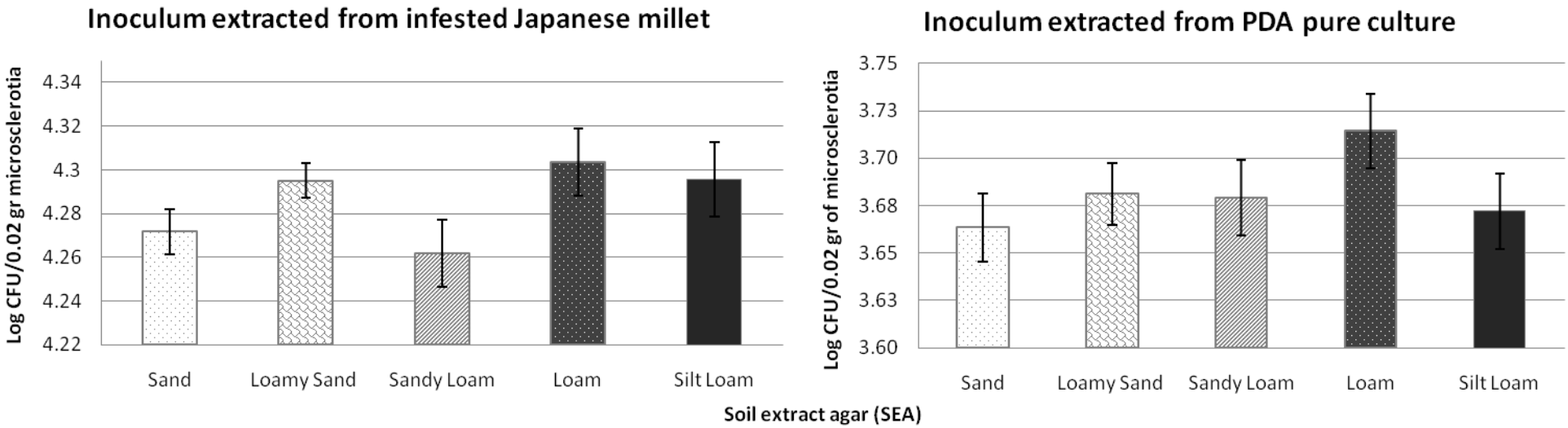

Figure 2-2. Number of CFUs for two *M. phaseolina* sources of inoculum (infested Japanese millet-left and PDA pure culture isolate-right) in different soil extract agars (SEAs). Vertical bars denote the standard deviation.

For the two methods tested, a selective media containing rifampicin was used for the isolation of *M. phaseolina*. This media consisted of PDA (39 g/L), penicillin (40 mg/L), streptomycin (20mg/L), tetracycline (20 mg/L), rifampicin (20 mg/L), and tergitol (1mL/L).

The first method used for *M. phaseolina* counting and isolation from soil is described by Cloud (1991) and it is also known as the direct counting technique. One g of soil infested with 100 microsclerotia was blended for three 30 sec intervals, alternated with 30 sec idle periods in a 0.5% NaOCl solution. The resulting soil slurry was then poured into a 45 µm sieve and rinsed with distilled water while gently shaking for 1 min. By using a squeeze bottle containing sterile-distilled water, the washed soil was concentrated in one side of the sieve and transferred to a 50 mL plastic tube that was later filled with 50 mL of the rifampicin selective media (described above). The inoculated media was dispensed into three 100 mm-diameter petri dishes (10 mL each) and incubated in the dark at 30°C for 4 days. Additional 10 g soil samples were placed in an oven at 105°C for 24 hours to determine percentage of soil moisture.

The second method for isolation and quantitative determination of *M. phaseolina* used was a modification of the serial dilution technique as described by Papavizas and Klag (1975). One g of soil infested with 200 microsclerotia was blended for 5 sec in 100 mL of distilled water. The triturate content was passed through a 600 µm sieve in tandem with a 45 µm sieve. Residues in the 600 µm sieve were discarded and the soil residue on the 45 µm sieve was transferred to a 250 mL beaker containing a solution of 100 mL of 0.5% NaOCl. After exposure of NaOCl for 8 min, the beaker content was washed with distilled water for 1 min on a 45 µm sieve. The soil slurry was resuspended in 10 mL of sterile-distilled water to produce a 1:10 dilution. One mL aliquots were taken from the dilution while shaking and pipetted on the surface of 2 day old 100 mm-diameter petri dishes containing the selective media as described above, and spread with a sterile plastic stick over the surface. Plates were incubated in the dark at 30°C for four days. Additional 10 g soil samples were placed in an oven at 105°C for 24 hours to determine percentage of soil moisture.

The experimental design was a completely randomized design (CRD) with repeated measures. The data set was composed of four replications for the direct counting technique and five replications for the serial dilution technique. Differences between methods for isolation of *M. phaseolina* from soil were evaluated using analysis of variance with SAS system version 9.2, (SAS institute Inc., Cary, North Carolina, USA) using Proc Mixed protocol.

Detection methods were analyzed independently for the response variables percentage of recovery and levels of *M. phaseolina* soil infestation, since highly significant differences between techniques ($p < 0.0001$) were found in the overall ANOVA. Infestation treatments comparisons were done with LSD multiple comparison procedures. Logarithmic transformation was used to standardize the response variable number of CFUs per gram of soil.

Percentage of recovery was calculated as follows:

Equation 1

$$Percentage\ of\ recovery = \left(\frac{\text{Number of CFUs /g soil}}{\frac{\text{Inoculum potential} \times \text{Infestation level}}{100}} \right) \times 100$$

Where inoculum potential is equal to:

Equation 2

$$Inoculum\ potential = \frac{\text{Number of CFUs /g of inoculum}}{\text{Number of microsclerotia / g of inoculum}} \times 100$$

Results and discussion

Differences between soil detection techniques on the number of CFUs

Analysis of variance (ANOVA) was used to assess any differences in detection methods, soil texture and *M. phaseolina* levels of infestation on the response variable number of CFUs. Interactions between these factors were also included in the analysis (Table 2-2).

Table 2-2. Overall and split ANOVAs including main affects and interactions for two *M. phaseolina* detection methods for the response variables soil CFUs and percentage of recovery. Overall ANOVA is split by detection methods.

	Soil CFUs			**Percentage of recovery**		
		Split ANOVA by detection method			Split ANOVA by detection method	
Effect †	Overall ANOVA	Direct counting	Serial dilutions	Overall ANOVA	Direct counting	Serial dilutions
Detection methods	***	---	---	ns	---	---
Soil types	**	ns	ns	***	***	***
Infestation levels	***	*	*	ns	ns	ns
Detection methods-Soil types	ns	ns	ns	***	ns	ns
Soil types-Infestation levels	ns	---	---	ns	---	---
Detection methods-Infestation levels	ns	---	---	ns	---	---
Detection methods-Soil type-Infestation levels	ns	---	---	ns	---	---

† Detection methods = direct counting and serial dilutions, Soil types = sand, loamy sand, sandy loam, loam, and silt loam, Infestation level = (0% infested soil, 25% infested soil, 50% infested soil, 75% infested soil, 100% infested soil). Level of significance from F-tests are indicated by the asterisks, *, **, and *** correspond to $p < 0.05$, 0.01 and 0.001 respectively, ns = no significant difference, and --- = not determined.

According to the overall ANOVA there is a differential effect of the detection method in the number of soil CFUs. On average the serial dilution technique has more CFUs per gram of soil than the direct counting technique. Serial dilution method was assessed with double amount of inoculum (200 microsclerotia per g of soil) than the direct counting method (100 microsclerotia per g of soil). When serial dilution technique was assessed with 100 microsclerotia per g of soil about 80% of the data obtained were zeros, meaning that this technique was not reliable at that level of soil infestation. When comparing the non-transformed data the serial dilution technique had almost four times higher CFUs than the direct counting technique at levels of infestation of 200 and 100 microsclerotia per gram of soil, respectively.

There were also significant differences between the soil types and the *M. phaseolina* infestation levels in the number of CFUs (Table 2-2).

In the split ANOVAs infestation level is significant for both techniques in the number of CFUs (Table 2-2). There is an increase in the number of CFUs as the levels of infested soil increases. However, soil infestation levels of 25% and 50% did not show any difference but higher levels of infestation (75% and 100%) were statistically different for both detection techniques; soil infestation levels at or higher than 75% increases significantly the number soil CFUs (Figure 2-3).

Differences between soil detection techniques on the percentage of recovery

According to the overall ANOVA there is a significant effect of the soil type in the response variable "percentage of recovery", and also a significant interaction between detection method and soil type (Table 2-2).

Overall ANOVAs split by detection method showed that percentage of recovery is highly influenced by soil type in both techniques. For the serial dilution technique the percentage of recovery varies across soil types whereas in the direct counting detection method the percentage of recovery had a gradual increase from the lightest soil textures (sand and loamy sand) to the heaviest ones (sandy loam, loam and silt loam), the direct counting detection method appeared to have good performance in clayey soils, with 65% and 85% of recovery from loam and silt loam, respectively (Figure 2-4).

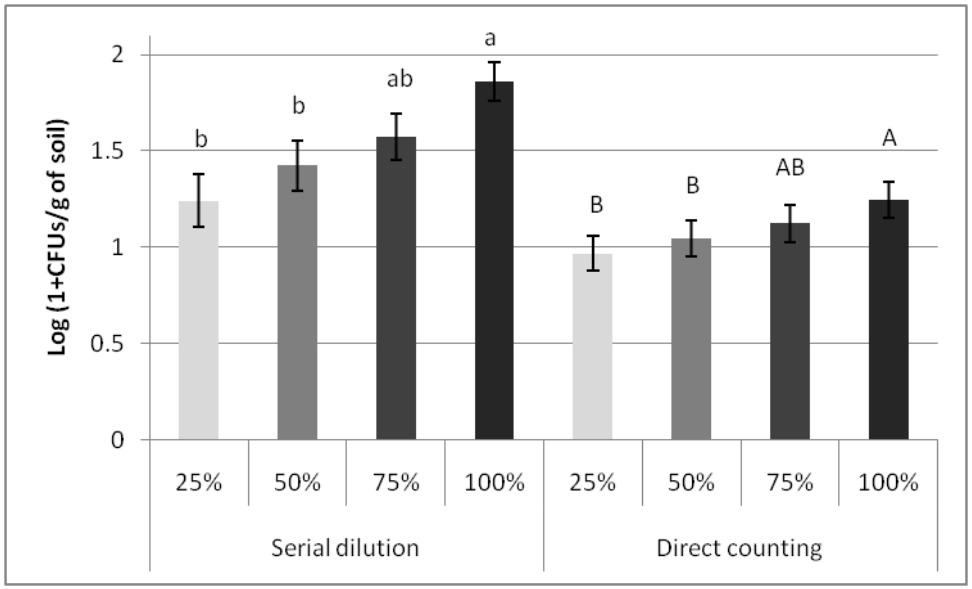

Figure 2-3. Effect of soil infestation levels in the number of soil CFUs. Non-infested soil (0% infestation level) data were not included in the graph. Vertical bars represent the standard deviation. Means with the same letter are not significantly different at $p < 0.05$. Small letters correspond to comparisons of soil infestation levels within the serial dilution technique, capital letters correspond to comparisons of soil infestation levels within the direct counting technique.

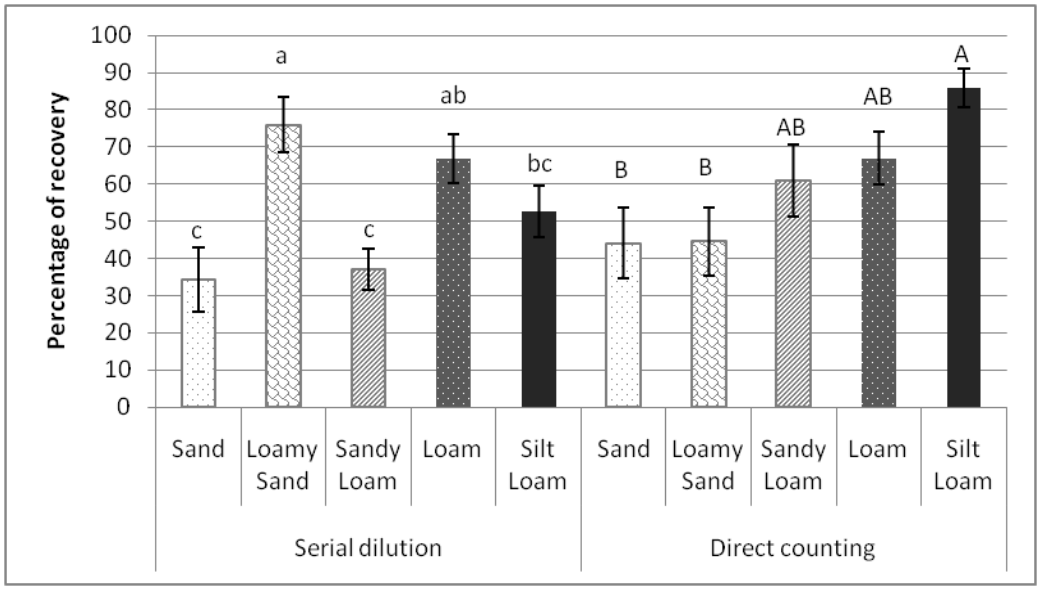

Figure 2-4. Impact of the soil type on the percentage of recovery of *M. phaseolina* for each detection method. Vertical bars represent standard deviation. Means with the same letter are not significantly different at $p < 0.05$.

Even though there is not information about the differences in percentage of recovery in these detection methods due to soil type, according to Ammon et al. (1974) there are several previously described phenomena that could explain recovery efficiency. Initial modest increases in the *M. phaseolina* soil population are frequently reported in soils that have been physically manipulated. In addition, the soil was incubated for two weeks and the impact of soil nutrient status is reflected in the viability of microsclerotia and also recovery. For example, it is likely that sand did not offer a good nutritional environment for the propagules. During the two weeks of soil incubation, *M. phaseolina* population levels decreased and that could affect the percentage of recovery indirectly.

In addition, *M. phaseolina* soil infestation levels did not have a significant impact on the percentage of recovery for both techniques (Table 2-2), meaning that the performance in the recovery of propagules from soil by using the techniques is similar even though the amount of inoculum in the soil increases.

In summary, both detection methods are useful; they recover the same relative proportion of inoculum over different levels of *M. phaseolina* soil infestation, as described by Campbell and Vandergaag (1993). However, the serial dilution technique is time consuming; an average of 8

min per soil sample is required. Another important disadvantage of this technique is its high amount of variability, which is likely due to the preparation of the serial dilutions. However, according to the results of this experiment, the serial dilution technique could be useful for small numbers of soil samples in which there is previous knowledge of high populations of *M. phaseolina* propagules present in the soil.

On the other hand, the direct counting technique provided a reliable estimate of populations even when *M. phaseolina* soil populations were low, or when a considerable number of soil samples needed to be processed, requires only 3 min per soil sample.

KOH aniline-blue fluorescence microscopy technique for the detection of M. phaseolina inoculum and root infection

In order to detect the interaction of *M. phaseolina* with soybean seedling roots at the microscopic level, a staining procedure utilizing aniline-blue fluorescence as described by Hood and Shew (1996) was used. Detection of pure inoculum also was performed by autoclaving pure dried microsclerotia at 121°C for 30 min in a 1M KOH solution. Microsclerotia were rinsed with sterile-distilled water for 2 min and stored in the refrigerator at 4°C until observed under the microscope. PDA agar cubes with a pure isolate of *M. phaseolina* on the upper surface were placed in a glass slide, stained with several drops of aniline blue solution, and after 20 min observed under the microscope.

Soybean seedlings were collected from soils infested with 0.02 g of *M. phaseolina* microsclerotia (obtained from infested Japanese millet). Seedlings were collected at the V1 and V2 soybean developmental stages. Plants were cut below the cotyledonary node and roots were washed with tap water in order to remove soil particles from the surface. Subsequently, roots were autoclaved at 121°C for 30 min in a 1M KOH solution followed by a rinse under sterile-distilled water. Roots were maintained in sterile distilled water in 50 ml plastic tubes at 4°C until they were observed in the microscope.

The aniline-blue dye solution contained 0.05% aniline-blue in 0.067 M K_2HPO_4 adjusted to a pH of 9.0, and was prepared at least two hours before use. Otherwise, the solution was maintained in the refrigerator at 4°C in a glass container covered with aluminum foil. Autoclaved root samples were mounted on glass slides in several drops of the stain solution for at least 20 min before observation under the microscope.

Microscopy was performed using a Zeiss Axioplan 2 IE MOT microscope (Carl Zeiss, Thornwood, New York). Images were acquired with an Axiocam HRc camera using the Axiovision software (release 4.6.3). Fluorescence was observed with an X-Cite® 120 (EXFO Life Sciences) mercury lamp. Filters were set as follows: (excitation 358 ± 10 nm, emission 463 ± 10 nm) at 423 microseconds exposure.

Results and discussion

The KOH-aniline blue technique for fluorescence allowed the detection of inoculum even when it was not autoclaved in the KOH solution. Germ tubes emerging from microsclerotia had enough fluorescence to contrast with dark backgrounds and also host tissue (Figure 2-5).

Microsclerotia from *M. phaseolina* inoculum infected soybean seedling roots by producing germ tubes that attached to the root surface. According to the images collected by using this technique, germ hyphae start growing intercellularly at early stages of the infection as reported by Ammon et al. (1974). Infecting hyphae was observed to grow upon and colonize the root tissue in different directions. Hyphae grew in an unrestricted manner on the epidermal root tissues, since the integrity of the hyphae appeared intact and mainly parallel to the root axis as reported by Bressano et al. (2010).

Through the use of fluorescent microscopy we were able to observe the initial stages of infection in soybean roots by *M. phaseolina.* In addition, all the observations in the fluorescence microscope were done on intact root segments placed on glass slides. This mounting technique did not allow us to explore the pathogen host interaction in more detail beyond the initial stages of epidermal root infection where the *M. phaseolina* start colonizing intercellularly.

Detection of *M. phaseolina* root infection points were more easily located in seedlings growing in sand than in other textures such as loamy sand, sandy loam, loam, and silt loam.

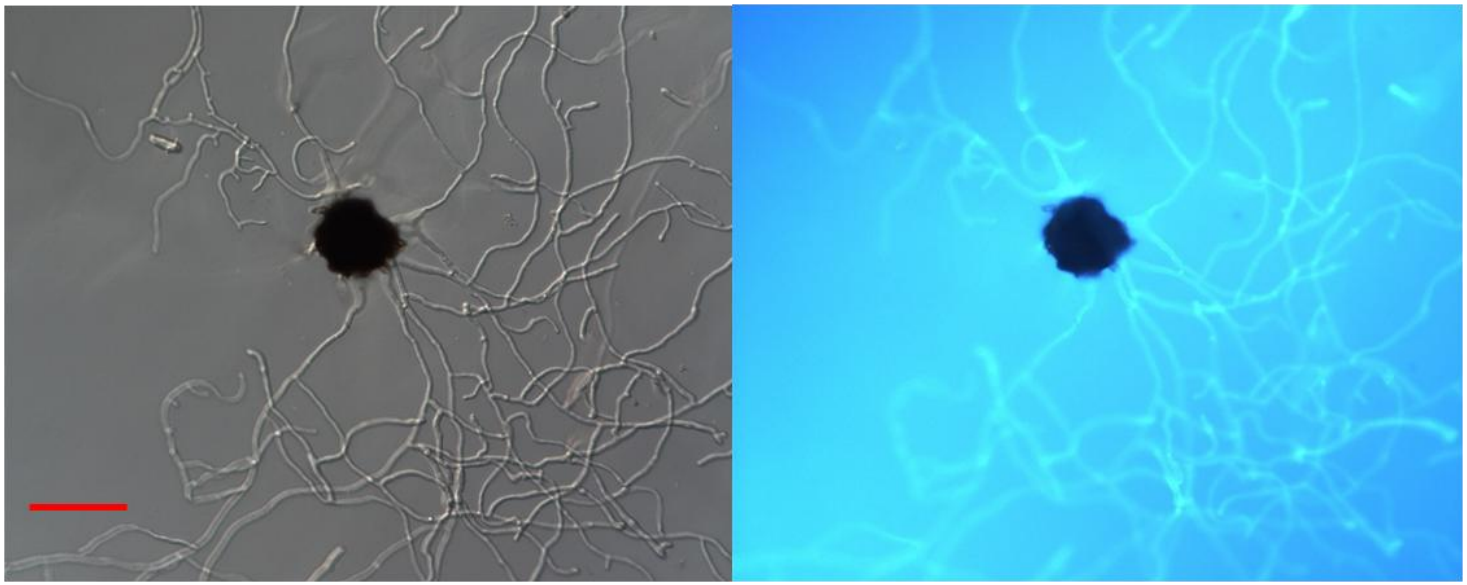

Figure 2-5. Detection of *M. phaseolina* inoculum by KOH-aniline blue fluorescence microscopy technique. Detail of microsclerotium growing on PDA media, image obtained after 20 min of aniline blue addition. Left = view under standard light microscopy, right = fluorescent image at 423 microseconds exposure, fluorescence of germ tubes (hyphae). Bar = 100 µm.

The KOH-aniline blue technique was useful to stain and detect *M. phaseolina* inoculum on infected soybean seedling roots and also to see in more detail the interactions between the pathogen and host in early stages of root infection (Figure 2-6).

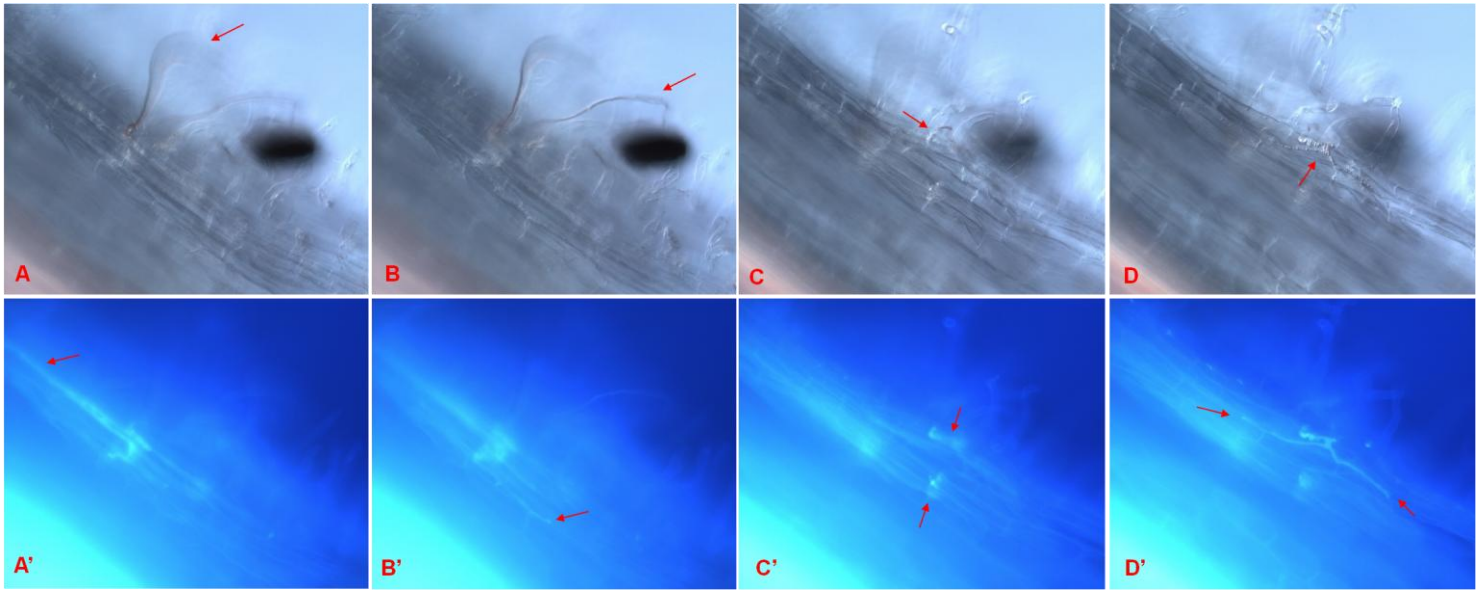

Figure 2-6. Observation of *M. phaseolina* soybean root infection. A and B, C and D, represent a point of initial root infection respectively. A´, B´, C´, D´ = fluorescent images under filter (excitation 358 ± 10 nm, emission 463 ± 10 nm). A, B, and C = arrows indicate germ tubes from microsclerotium. D = arrow indicates epidermal root infection. A´, B´ and D´ = arrows indicate intercellular hyphal proliferation on the epidermal root tissue. C´ = arrow indicates two initial infection points.

Conclusions

Nutrients availability highly impacts the survival of *M. phaseolina* inoculum. Inoculum potential, microsclerotia size and germination were reduced by a short period of nutrient deprivation. In contrast, inoculum showed an increase in the number of CFUs when incubated in soil for a period of two weeks at 30°C.

However, CFU produced on soil extract agar (SEA) may indicate that soil could have a positive or negative impact on the viability of the propagules in the long term, depending on the level of nutrients, organic matter or soil texture. Even though there were no significant differences in the SEAs used for each of the five soil types, trends indicate that soil textures such as sand and loamy sand could have a negative impact upon inoculum survival. In contrast, more clayey soils such as silt loam or loam may have a positive effect on the propagules; they would be at least maintained or increased by growing in richer nutrient conditions via higher organic matter content.

Source of inoculum was important for inoculum potential. Isolation of *M. phaseolina* on PDA media is not the most adequate method to produce inoculum. This method of extraction is time consuming and limited the amount of propagules that can be obtained from a single culture plate. Conversely, inoculum production by extracting microsclerotia from *M. phaseolina*-infested Japanese millet provides high quality inoculum in reasonable amounts and in a timely manner.

Both direct counting and serial dilution methods for quantifying *M. phaseolina* soil populations have their own advantages and disadvantages. The serial dilution detection method had good performance at high *M. phaseolina* soil populations; however, it is highly variable across soil textures and time consuming. In addition, direct counting had good performance at low *M. phaseolina* soil populations, and its variability across soil textures was lower compared to the serial dilution technique.

KOH-aniline blue technique was useful to detect *M. phaseolina* inoculum and it was adequate to observe the early stages of root colonization by this pathogen in soybean root seedlings at V1 and V2 developmental stages. However, it was only possible to observe the pathogen-host interaction at lower magnifications (20×) and only in the root epidermis when fungus colonizes intercellularly when using this technique.

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CHAPTER 3 - Influence of soil texture and soil water availability upon charcoal rot caused by *Macrophomina phaseolina* at two different soybean seedling stages.

Introduction

Nowadays more attention is given to the relationships between the soil physicochemical properties and *M. phaseolina* microsclerotia. *M. phaseolina* soil population dynamics can be explained by several soil properties such as soil moisture, texture, porosity, bulk density, and cationic exchange capacity between others (Gangopadhyay et al., 1982).

Soil moisture is also considered to be one of the most important factors affecting the survival and activity of microsclerotia in soil and root infection.

Sandy soil supports higher germination rates of microsclerotia in soil, because sandy soils retain less water compared to a clayey soil, providing an optimum range of oxygen and a reduced microbial competition in these kind of soil environments (Collins et al., 1991). Some authors have reported a higher disease severity caused by *M. phaseolina* in sandy soils across different hosts. However, in these soils conditions levels of *M. phaseolina* soil populations were not affected (Srivastava and Dhawan, 1980; Wyllie and Calvert, 1969).

Conversely, *M. phaseolina* soil populations may be compromised at high levels of soil moisture, mainly by low concentrations of soil oxygen and the proliferation of actinomycetes and bacteria that proliferates in high moisture conditions and degrade microsclerotia and germ tubes (Srivastava and Dhawan, 1980; Dhingra and Sinclair, 1975).

M. phaseolina can infect plants at seedling stages, one or two weeks after planting. Afterwards, infection stays dormant if stressful environmental conditions are not present during this period of time, once the plants reach reproductive stages physiological stress occurs and infection will progress (Hartman et al., 1999). However, information of charcoal root of soybean disease processes at seedling stages has been poorly documented.

The objective of these studies were:

- To estimate the impact of artificial soil textures and soil moisture upon *M. phaseolina* soil populations and root infection on soybean seedlings.
- To evaluate the effect of soil texture and soil water content on soybean seedling root length and its potential effect on *M. phaseolina* root infection.
- To compare the effect of *M. phaseolina* artificially infested soils and non-infested soils on soybean seedling root length.
- To assess the relationships between root length and *M. phaseolina* root infection across different artificial soil textures and irrigation regimes.

Materials and Methods

Setting variables

Charcoal rot of soybean was assessed at seedling stages V1 and V2 by the production of *M. phaseolina* inoculum from infested Japanese millet. Artificial soil textures were infested with *M. phaseolina* microsclerotia and seedlings were exposed to different soil moisture contents including pot saturation (PS), pot capacity (PC), and permanent wilting point. Ability of the inoculum to infect seedling root tissue was qualitatively confirmed by visualization with KOH-aniline blue fluorescence staining under the microscope (Hood and Shew, 1996). However, the infection quantification in seedling root tissue was determined by counting *M. phaseolina* CFU's in rifampicin media as well as the level of infestation in soil using similar procedures as described by Pearson et al. (1984) and Cloud (1991) respectively. *M. phaseolina* soil and root populations were quantified at two soybean developmental stages V1 (first trifoliate leaves unfolded) and V2 (second trifoliate leaves unfolded).

Generation of artificial soil and textures

Experiments were performed under greenhouse conditions to understand the influence of soil textural classes upon charcoal rot disease. Artificial soils were constructed by mixing sand and silt loam in different proportions (Table 3-1). Sand and silt loam soils were pasteurized in a soil steamer for 2.5 hours and dried on the greenhouse bench for two days. Mixtures were made

on weight basis to generate a range of textures such as sand, loamy sand, sandy loam, loam and silt loam. Resulting textural classes were confirmed using sodium hexametaphosphate by the hydrometer method (Bouyoucos, 1951).

Table 3-1. Textural classes generated by mixing sand and silt loam in a weight basis.

SAND (%)	SILT LOAM (%)	TEXTURAL CLASS
100	0	Sand
75	25	Loamy sand
50	50	Sandy loam
25	75	Loam
0	100	Silt loam

Calibration of soil moisture levels

Since the soil water retention properties for each soil texture differ, three levels of soil moisture including pot saturation, pot capacity, and permanent wilting point were set for each soil. Pots containing 1200 mL of dried soil were watered to saturation. One day after the pots were saturated and water drainage has stopped, each pot was weighed once a day for five days.

After obtaining, weights a calculation of volumetric water content was performed for each pot using the following equation:

Equation 2

$$\theta_V = \frac{V_w}{V_s}$$

Where θ_V is the volumetric water content of soil, V_w is the volume of water, and V_s is the volume of soil. However, to calculate θ_V, V_w was calculated as follows:

Equation 3

$$V_w = \text{weight of watered pot} - \text{soil weight}$$

A linear relationship was constructed for each soil textural class with the pot weight and volumetric water content of each soil. With this calibration it was possible to set three levels of soil moisture or irrigation regimes based on volumetric water content for each soil texture: pot saturation (PS), pot capacity (PC), and permanent wilting point (PWP) (Figure 3-1).

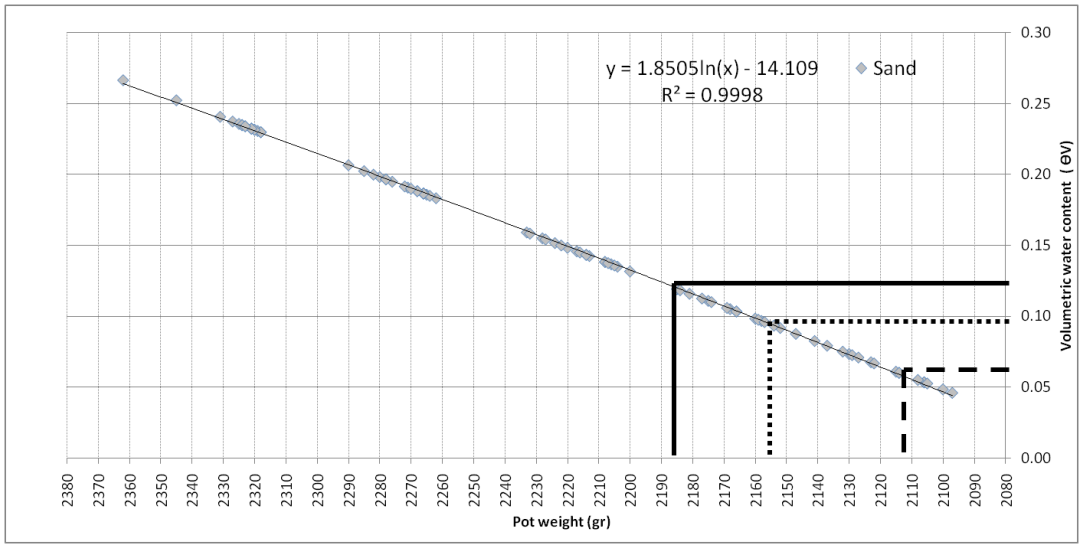

Figure 3-1. Relationship between pot weight and volumetric water content and of a sandy textured soil. Pot saturation (—PS), Pot capacity (···PC), Permanent wilting point (---PWP)

Pots were set to the three levels of soil moisture (pot saturation (PS), pot capacity (PC), and permanent wilting point (PWP)) for three days. Six pots were used for each irrigation level and the experiment was repeated with the five soil textures (Table 3-1). Every 24 hours pots were weighed and water was added to maintain the desired level of soil moisture. An electronic probe soil sensor (Stevens Hydra-probe Soil Sensor, Stevens Water Monitoring Systems Inc., Portland, Oregon) was used in preliminary experiments during the calibration phase to estimate the volumetric water content in each pot after the desired irrigation level was reached. Since the electronic probe had volumetric water content levels established for each soil texture in the settings, using this electronic soil probe served as a control for the soil moisture levels (Figure 3-2).

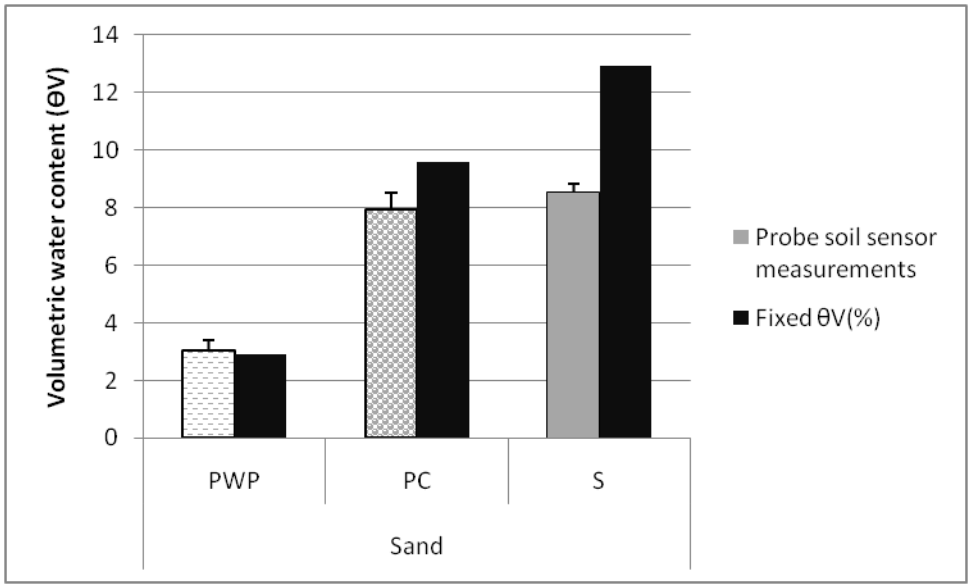

Figure 3-2. Evaluation of soil water content regimens by two methods in a sandy soil texture: manual weighing of pots (fixed θ_V, %) and an electronic probe sensor. Probe soil sensor bars are the average of three replications.

Inoculum production

M. phaseolina microsclerotia were obtained from infested Japanese millet seeds with a procedure as described by Mengistu et al. (2007). Four kg of Japanese millet (*Echinochloa frumentaceae* L.) seeds were soaked for twenty hours in a 3 L solution containing 40 g of sucrose and 1 g of tartaric acid. The solution was decanted using a 45 µm No325 sieve and seeds were divided into four autoclave bags with a 5 cm diameter and 10 cm long PVC plastic tube inserted into each autoclavable bag approximately half the length of the tube. The autoclave bags were secured around the tube, a foam plug was inserted in the top of the tube, and covered with aluminum foil. Inoculum bags were autoclaved at 121°C for 30 min. Subcultures of ~1 cm^2 of *M. phaseolina* (prepared from an *M. phaseolina* isolate taken from infected soybean roots obtained from 30-year continuous soybean plots at Ashland Bottoms research farm in Riley County, Kansas) grown on full strength PDA, were added to the Japanese millet carrier in the autoclave bags. Five petri dishes colonized with *M. phaseolina* were used per bag. Inoculum bags were stored at lab temperature (~21°C). One week after inoculation, bag contents were mixed by shaking, and left to incubate for an additional 10 days. After incubation the colonized millet was

dried on a plastic sheet and passed through a 600 µm sieve, blended in a 0.5% NaOCl solution for 3 min, collected in a 45 µm sieve and rinsed with distilled water for 2 min. Inoculum was spread over sterile plastic trays and dried in the laminar flow hood for one day. Dried microsclerotia were collected from the tray surface and stored in the refrigerator in plastic ziploc bags.

Experimental design

The experimental design was a completely randomized design (CRD), containing five factors: soil texture (sand, loamy sand, sandy loam, loam and silt loam), soil volumetric water content (ϴV) (pot saturation (PS), pot capacity (PC), and permanent wilting point (PWP)), soil infestation (inoculated soil, non-inoculated soil), soybean developmental stage (V1, V2), and three independent experiments (three replications) (Figure 3-3).

The soybean variety used for these experiments was NK S37-F7 (RR (Roundup Ready), SCN-R (soybean cyst nematode-resistant), SDS-tolerant (sudden death syndrome-tolerant) obtained from plants at the Ashland Bottoms location in 2009. Seeds with visual symptoms of fungal or bacterial diseases and viruses were discarded. Healthy seed was kept in the refrigerator for six months until the greenhouse assays were initiated.

A constant volume of soil was kept in each pot in order to maintain a regular concentration of *M. phaseolina* inoculum. 1200 mL of each soil type was measured in a graduated plastic cylinder and inoculated with 0.02 g of *M. phaseolina* microsclerotia obtained from infested Japanese millet. 1200 mL of soil and inoculum were mixed by shaking in a plastic bag and added to a 1350 mL plastic pot.

This greenhouse experiment consisted of three experiments (replications). The first experiment was planted on May 28th, 2010. The second and third experiments were planted simultaneously on September 6th, 2010. Soils for the first replication were artificially inoculated with microsclerotia collected four months before the experiment started, and for the second and third, the microsclerotia were collected one month before soil inoculation. Inoculum was kept in the refrigerator at 4°C for one and four months, these inocula were characterized for inoculum potential (see Chapter 2 – "Microsclerotia inoculum potential on rifampicin media").

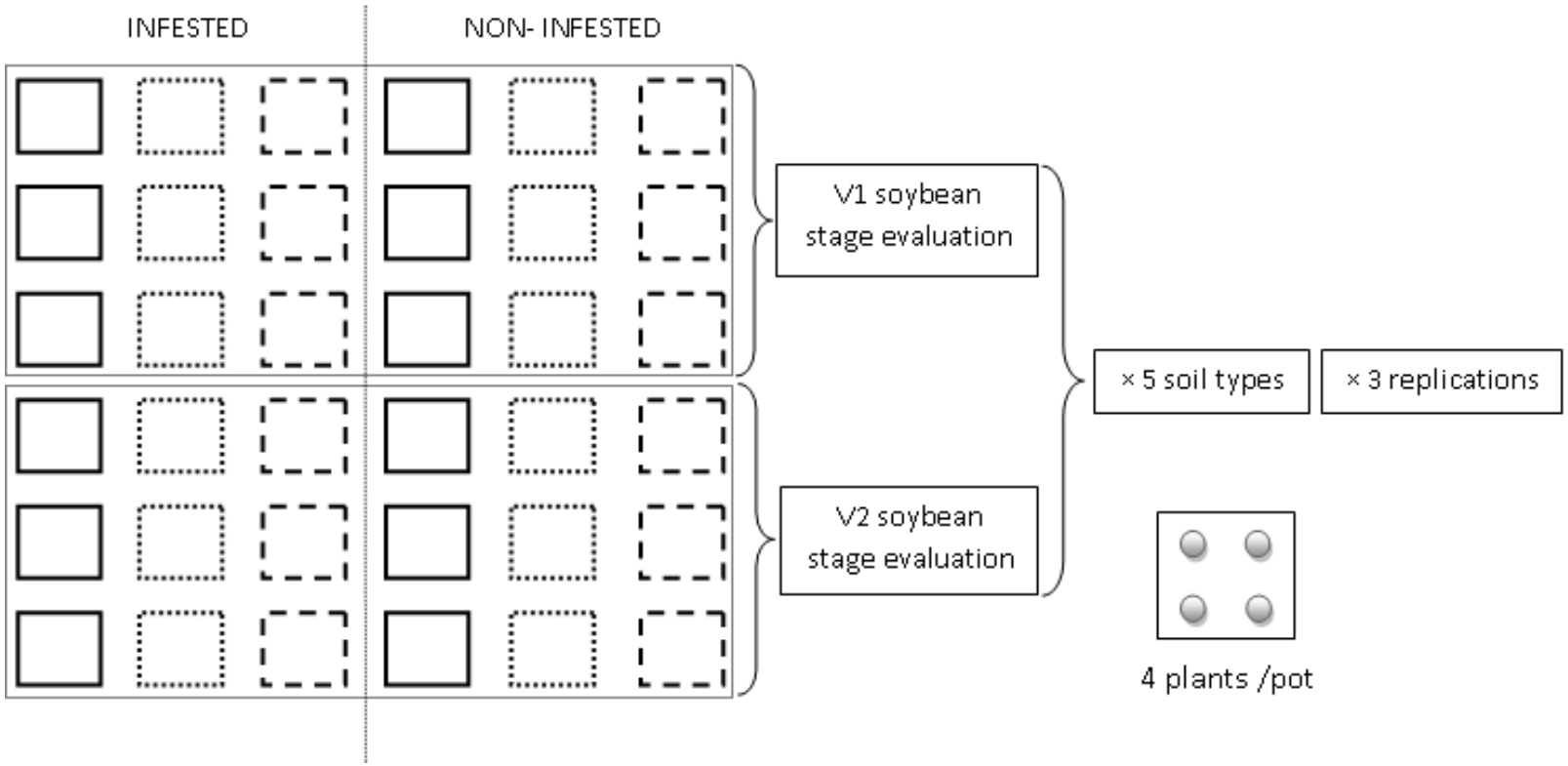

Figure 3-3. Experimental design representing one of the five soil types and two soybean developmental stages assessed in the greenhouse experiment. Volumetric water contents (θV) in each pot are represented by pot saturation (—PS), pot capacity (¨PC), and permanent wilting point (---PWP).

After inoculating soils with microsclerotia, soybean seeds were surface sterilized with a 10% bleach solution (approx. 0.5% NaOCl) for 3 min and rinsed with distilled water for 8 min. Nine seeds per pot were planted immediately after sterilization at a depth of 2 cm. Pots were watered every day until the seedlings reached the VC (cotyledon) stage. Pots were thinned to four seedlings per pot to keep a constant rate of evapotranspiration in each pot. Pot saturation (PS), pod capacity (PC), and permanent wilting point (PWP) irrigation regimes were introduced at the VC stage until the experiments were completed.

Relative humidity and greenhouse temperature were recorded during the term of the experiments using a data logger (Model HOBO U10-003, Onset Computer Corporation, Bourne, Massachusetts).

Evaluation of M. phaseolina soil populations and root infection

M. phaseolina soil and root populations were evaluated by taking soil samples from each pot and seedling roots at V1 and V2 soybean developmental stages using a destructive sampling. The soil contained in each pot was spread out upon a sterile plastic sheet on a greenhouse bench and dried for 2-3 days (depending upon the soil's water content). Once the soil was dried, it was returned to sterile plastic bags and stored in the refrigerator at 4°C until further processing. Soil samples were ground individually (by pot), and passed through a 2 mm sieve in a soil grinder (Model H-4199, Humboldt Corporation, Illinois) to remove the larger soil particles. A 1 g sub-sample from each sample was used to estimate the *M. phaseolina* soil population following a modified procedure as described by Cloud (1991).

One g of ground soil was blended in 250 mL of 0.79% NaOCl (15% household bleach solution, v/v) solution for 3 min, with 30 sec idle intervals. The resulting soil slurry was poured into a 45 µm No325 sieve and rinsed with distilled water while shaking gently. The washed soil was concentrated in on side of the sieve using a squeeze bottle containing sterile-distilled water and transferred to a 50 mL plastic tube that was later filled with 50 mL of selective media (39 g/L PDA, 1mL/L tergitol, 40 mg/L penicillin, 20 mg/L streptomycin, 20 mg/L tetracycline, and 100 mg/L rifampicin) and poured into five 100 mm-diameter petri dishes (10 ml each) (Cloud, 1991; Mengistu et al., 2007). Petri dishes were placed in an incubator at 30°C in the dark. After five days CFUs were counted and converted to CFU/g of dried soil (Figure 3-4). Another 10 g

soil sample was taken to calculate the gravimetric water content to be used in the calculation of the *M. phaseolina* soil population.

Simultaneously, soybean seedlings were collected and cut below the cotyledonary node, transported to the lab and rinsed with sterile-distilled water to remove soil particles. Roots were scanned, rinsed and dried on a paper towel for one day at room temperature (21°C). Roots were maintained in plastic bags at 4°C until processing. With a UDY cyclone sample mill (Model 0.14, UDY Corporation, Fort Collins, Colorado) containing a 600 µm No30 sieve, the root samples were ground separately, cleaning the unit with compressed air between samples. For each root, a 0.05 g sub-sample of ground tissue was obtained to calculate root CFUs using a modification of the procedure described by Mengistu et al. (2007), Mihail (1992), and Pearson et al. (1984). The crushed tissue was blended in 250 ml of a 0.5% NaOCl solution for 3 min with 30-sec idle intervals, collected in a 45 µm sieve and rinsed with distilled water for 1 min. The root tissue was concentrated in one side of the sieve using a squeeze bottle containing sterile-distilled water, and poured into a 50 mL sterile plastic tube. Afterwards, a selective media, as described above, was added to each tube, mixed, and poured in three 100 mm-diameter petri dishes. Poured dishes were placed in the incubator at 30°C in the dark for 5 days (Figure 3-4). After the incubation period, CFUs of *M. phaseolina* were counted and transformed to CFUs/g of root tissue.

Stage V1 and V2 soybean roots were rinsed to remove the soil debris from their surface. Roots were immersed in a glass tray filled with water to spread out the secondary and tertiary roots, then scanned at a 400 dpi resolution (using an EPSON Expression 10000 XL scanner, Epson America, Inc.) and images imported into Adobe Photoshop Elements 2.0. Water from the tray was frequently replaced to avoid debris or soil particles that could interfere with image quality. Assess 2.0 (The American Phytopathological Society (APS), Saint Paul, Minnesota) was used to measure root length. Recommended settings for measuring root length in the ASSESS user guide were modified to generate the appropriate contrast for imaging.

Results and discussion

Main factor effects on the number of soil CFUs

Analysis of variance (ANOVA) was performed by using the SAS system version 9.2, (SAS Institute, Inc., Cary, North Carolina, USA) using the PROC GLM to test levels of

significance of main effects for the response variable "soil CFUs". In this analysis, pots that contained non-inoculated soil were not taken in consideration (the soil infestation main factor was removed from the analysis). The effects of the three independent experiments were considered as fixed factor, since they differed in type of inoculum. Inoculum for experiment one had four months of storage after harvest and inoculum for experiments two and three had one month of storage after harvest). Linear contrast analysis was performed to assess significant differences between the three blocks. Interactions for main factors were also included in the analysis. Correlation analyses and second-degree polynomial regressions were performed between root CFUs and root length across the different soil textures and soil volumetric water contents in SAS using Proc Corr and Proc Reg respectively (Table 3-2).

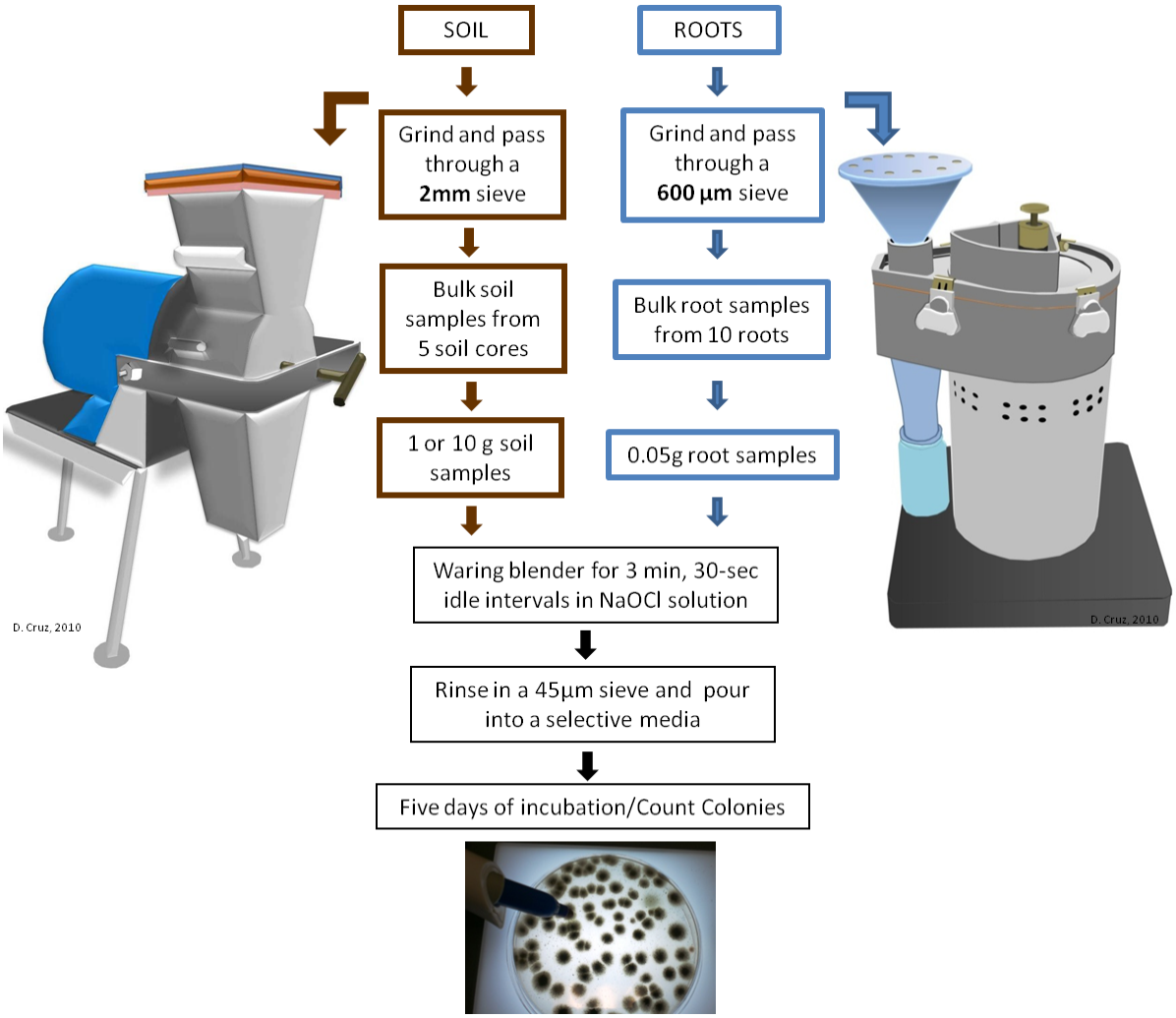

Figure 3-4. Procedure to obtain *M. phaseolina* CFUs from soil and root tissue. Modified from Smith and Carvil (1997).

Table 3-2. Significance of the main effects in the response variable "soil CFUs".

Soil CFUs				
Factor †	Overall ANOVA	Overall ANOVA split by blocks		
		Experiment 1	Experiment 2	Experiment 3
ST	***	*	***	***
ϴV	ns	ns	ns	ns
ST - ϴV	ns	ns	ns	*
Stage	ns	ns	***	ns
ST - Stage	ns	ns	ns	ns
ϴV - Stage	ns	ns	ns	ns
ST - ϴV - Stage	ns	ns	ns	ns
Block	***	---	---	---
ST - Experiment	***	---	---	---
ϴV - Experiment	*	---	---	---
ST - ϴV - Experiment	ns	---	---	---
Stage - Experiment	***	---	---	---
ST - Stage - Experiment	ns	---	---	---
ϴV - Stage - Experiment	***	---	---	---
ST - ϴV -Stage - Experiment	ns	---	---	---
Orthogonal contrast				
Experiment 1 VS 2,3	***			
Experiment 2 VS 3	ns			

† ST = soil texture (sand, loamy sand, sandy loam, loam, and silt loam); ϴV = soil volumetric water content (S, PC, PWP), Stage = Developmental soybean stage (V1, V2); Experiment = replications (3 reps.). For the SAS analysis the response variable "soil CFUs" was logarithmically transformed (Log(1+CFU/g of soil). Overall ANOVA is split by blocks. Level of significance from F-tests are indicated by the asterisks: *, **, and *** correspond to $p < 0.05$, 0.01 and 0.001, respectively; ns = no significant difference, and "---" = not determined.

Based on the overall ANOVA, results indicate a differential effect of experiments in soil CFUs. Linear contrast show a significant difference of experiment one from the other two in the number of soil CFUs. Experiment one fewer CFUs per gram of soil than experiments two and three. This effect is due mainly by differences in *M. phaseolina* inoculum potential (Figure 3-5).

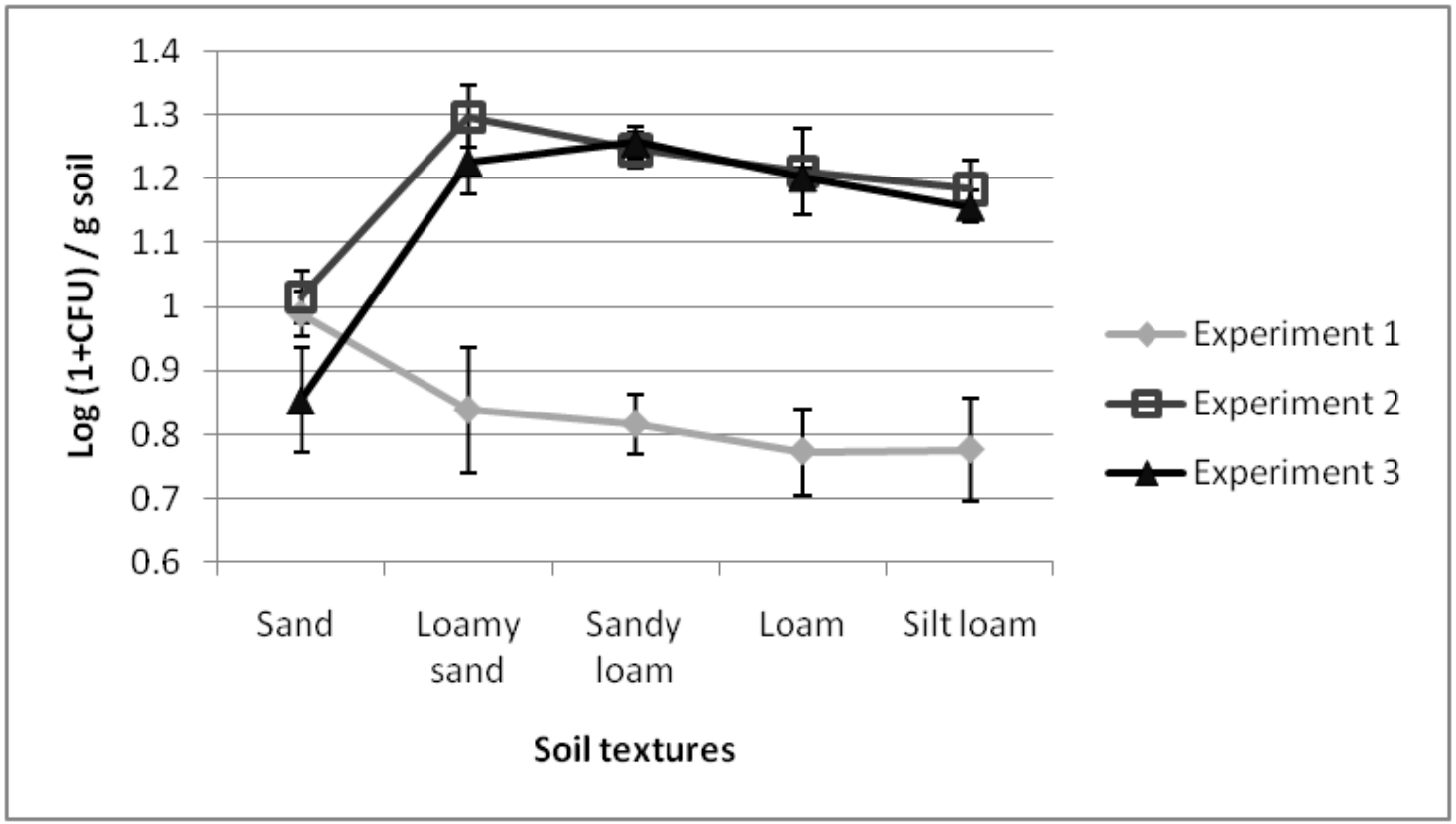

Figure 3-5. Interaction of soil type and experiments for *M. phaseolina* soil populations. Vertical bars represent the standard deviation. Averages only include *M. phaseolina* infested soils.

Inoculum for experiment one produced fewer soil CFUs and had a different trend in the artificial soil textures except for sandy soils in which the soil populations presented similar levels. When analysis was made by experiment, soil texture was significant in all in experiments meaning that this factor had a high impact on the *M. phaseolina* soil populations According to these results, soil texture has a significant impact on *M. phaseolina* soil population dynamics. There was variation in the populations across soil types but this variation was significantly different in the sandy soil texture in which populations were reduced (Figure 3-6).

Decline in the viability and germination of *M. phaseolina* propagules in sandy soils could be attributed to nutrient deprivation. In poor soil nutrient conditions, secondary microsclerotia are more likely to be smaller than primary microsclerotia and consequently a lower energy reserve. Thus, reducing propagule viability in the long term (Gagopadhyay et al., 1982).

Decreasing effects in the *M. phaseolina* populations were observed for soil textures such as silt loam, loam, sandy loam, and loamy sand. Nevertheless the soils were steamed, it was found an increasing number of colonies of other inhabiting soil fungi along with *M. phaseolina* as the amounts of silt loam in each soil were increased in order to form the artificial soil textures (Table 3-1). Saprophytic fungi from different genera such as *Fusarium, Aspergillus, Rhizopus* were identified as being part of the soil microflora, except for sandy soil texture in which only *M. phaseolina* was present. When the colonies of saprophytes increased, *M. phaseolina* soil populations decreased. This may be attributed to the amount of soil organic matter present that can sustain a diverse fungal soil population, which may compete with *M. phaseolina* in the soil and in vitro.

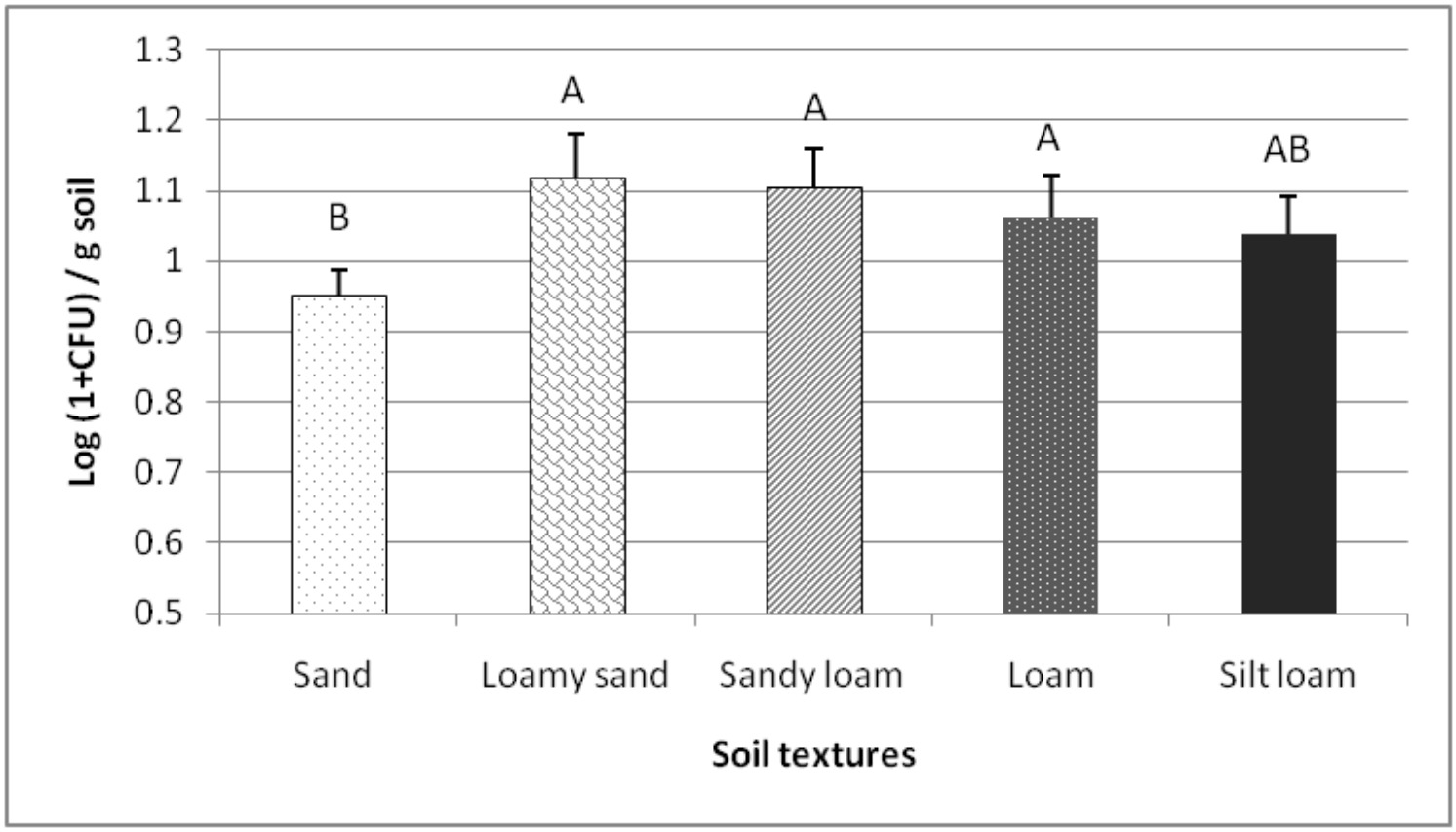

Figure 3-6. Effect of soil texture on *M. phaseolina* soil populations. Vertical bars represent standard deviation. Letters above the bars denote significant differences between soil populations at $p < 0.05$. Averages only include *M. phaseolina* infested soils.

In summary there are two phenomena that can explain the changes in *M. phaseolina* soil populations. First, deprivation of soil nutrients in textures such as sand, in which the inoculum slowly dies and in which the presence of antagonists is virtually zero, and second, soils more rich in organic matter that can sustain a diverse number of microorganisms. These assumptions are supported by findings in which different soil microorganisms such as actinomycetes or other bacterial species have been reported to act as antagonists to *M. phaseolina* (Collins et al., 1991; Dhingra and Sinclair, 1975). Furthermore, these antagonists have been observed surrounding *M. phaseolina* hyphae inhibiting microsclerotia germination under pot study conditions (Arora et al., 2008; Pratt, 2006).

Volumetric soil water content (∈V) levels did not affect the number of soil CFUs during the time the experiment was performed. However, the PWP soil water content treatment had the highest number of *M. phaseolina* CFUs per gram of soil for experiments 2 and 3 (Figure 3-7). These findings agree with Olaya (1996), who described a similar survival of microsclerotia in soil under dry conditions that affected the development of antagonistic microflora.

M. phaseolina soil populations decreased between the V1 and V2 evaluation times. Although this reduction in the soil population was significant only in experiment two, and experiment three maintained a similar trend, whereas experiment one had a completely different soil population dynamic in which only loamy sand and sandy loam soils exhibited a reduction in the soil population. The effect of time upon the *M. phaseolina* soil population is difficult to explain. However, the reduction in *M. phaseolina* populations from V1 to V2 across the soil textures indicate that inoculum in these artificially infested soils was decaying (Figure 3-8). Similar findings were found by Gangopadhyay et al., (1982), who reported reduction in the viability of *M. phaseolina* propagules in soil after 120 days of incubation at 30°C in the dark at different soil moisture levels under laboratory conditions. In addition, soil microflora may play an important role in the *M. phaseolina* soil population dynamics. A laboratory study conducted by Collins et al. (1991) reported a maximum of 50% microsclerotia germination for unpasteurized soil and a maximum of 80% to 90% germination for pasteurized soils at constant temperatures of 25°C and 33°C, respectively.

It is important to consider that there was also a high fluctuation in the daily greenhouse air temperature that may have affected germination of propagules in the soil (Table 3-3).

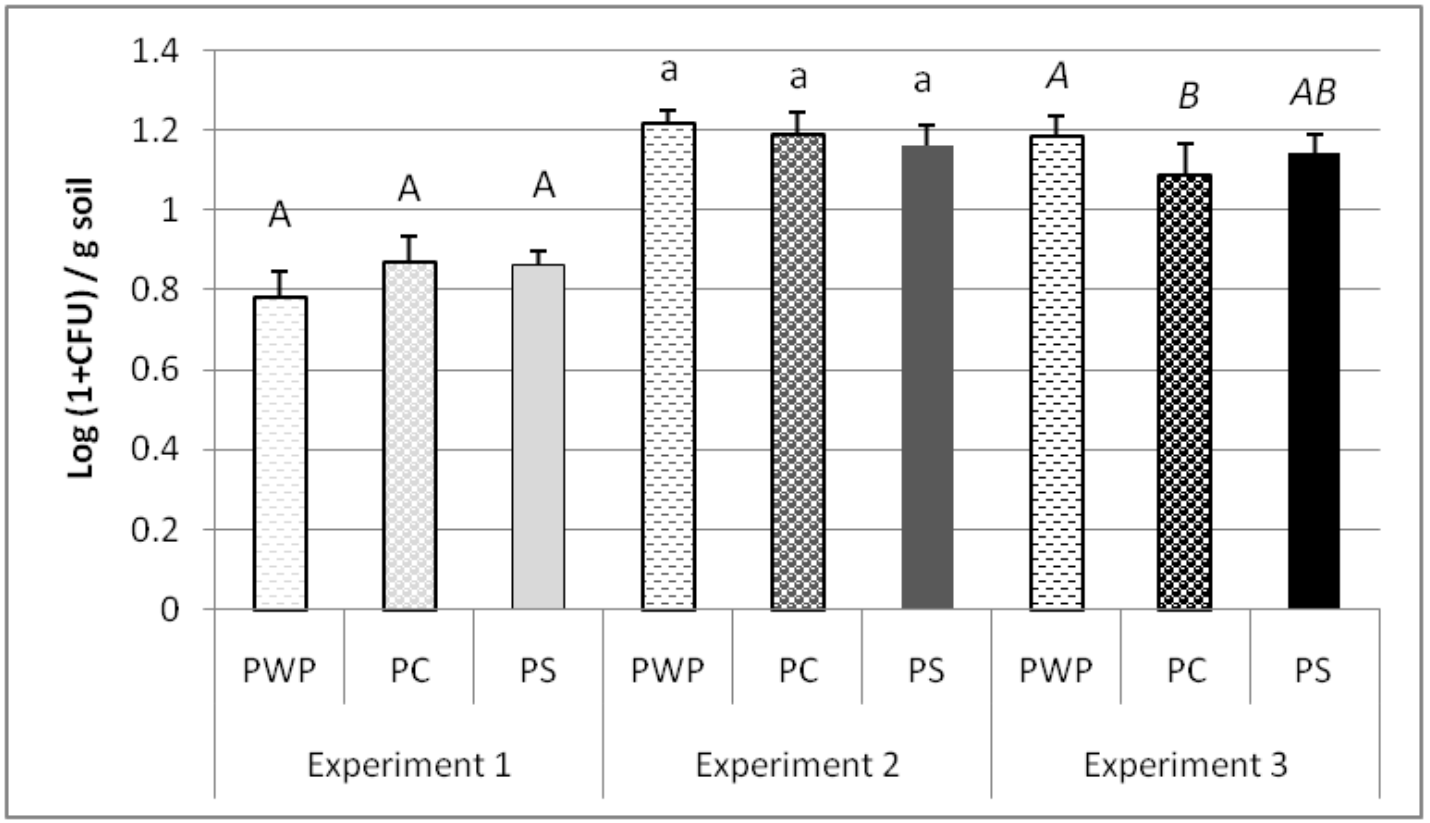

Figure 3-7. Effect of soil volumetric water content (θV) on *M. phaseolina* soil populations. PWP = permanent wilting point, PC = pot capacity and PS = pot saturation. Experiments 1, 2, and 3 denote replications. Vertical bars represent standard deviation. Letters above the bars denote significant differences between soil populations at $p < 0.05$. Averages only include *M. phaseolina* infested soils.

Table 3-3. Temperature and relative humidity data recorded during the greenhouse study.

	Temperature (°C)			Relative humidity (%)		
	Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
Average	29 A	26 B	26 B	63 a	54 b	52 b
Max	31	30	28	72	70	70
Min	25	22	22	54	40	38

Capital letters denote levels of significance for temperatures and small letters denote levels of significance for relative humidity, LSD test $p < 0.05$. Max = Maximum, Min = minimum. Averages were obtained by using a data logger.

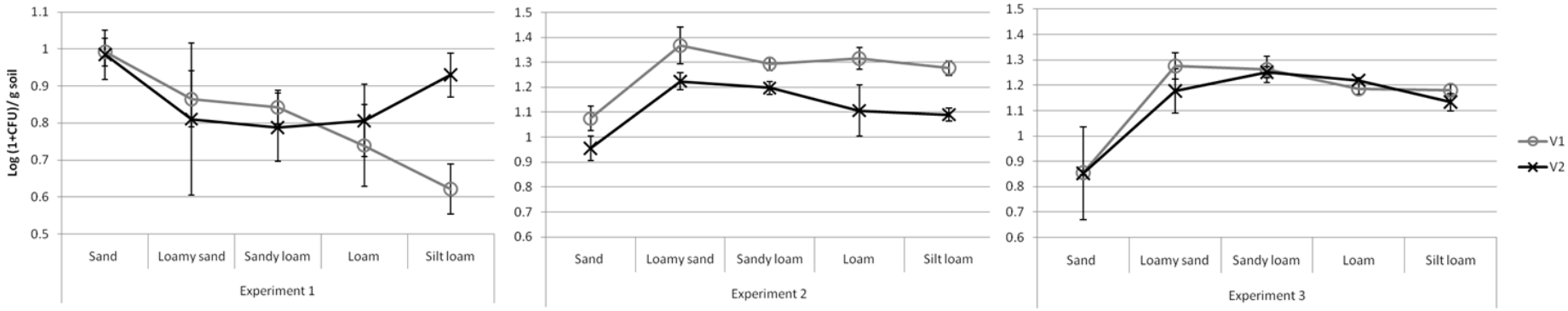

Figure 3-8. Effects of soil texture and soybean developmental stage on *M. phaseolina* soil populations. Vertical bars represent standard deviation. Experiments 1, 2, and 3 denote replications. Averages only include *M. phaseolina* infested soils.

Main factors effects in the number of root CFUs

Analysis of variance (ANOVA) was performed by using Proc Glm (SAS version 9.2) (SAS institute Inc., Cary, North Carolina, USA). For the statistical analysis only plants grown on *M. phaseolina* infested soils were taken in consideration for the analysis (i.e., the soil infestation factor was removed from the analysis). An average of the root CFUs from the four plants contained in each pod was obtained for the statistical analysis and also to meet assumptions of normality and equal variances.

Main factors effects and their possible interaction were considered in the analysis. Linear contrast analyses were performed in order to identify differences between experiments (replications). Finally, overall ANOVA was split by experiments (replications) and soybean developmental stages in order to elucidate their impact in the root infection (Table 3-4).

According to the overall ANOVA all the main factors had significant effects on root CFUs (Table 3-3). Similarly to *M. phaseolina* soil populations, root infection presented significant differences between experiments; there is an appreciable reduction in the *M. phaseolina* root populations in experiment one compared to experiments two and three. This reduction in the number of root CFUs is mainly due to the quality of the inoculum used in each of the blocks. For experiment one the inoculum potential was 20% and for experiments two and three, 85% (Figure 3-6).

Inoculum potential plays an important role in soybean root infection. According to the non-transformed data, *M. phaseolina* inoculum with 85% microsclerotia viability averaged 6.6 CFUs per g of root tissue, whereas inoculum with 20% viability averaged 1.2 CFUs per g of root tissue, this is a 5.5-fold increase in the number of root CFUs when the inoculum potential was high.

Soil texture had a highly significant impact on seedling root infection. Plants growing in sand resulted in the highest *M. phaseolina* root populations, followed by seedlings planted in loamy sand and loam soil textures (Figure 3-10). Likewise, Collins (1991) and Dhingra and Sinclair (1975) described increasing *M. phaseolina* root populations and root severity in sandy soils for corn and sorghum.

Table 3-4. Significance of the main effects and interactions for the response variable "root CFUs".

Root Infection (root CFUs)						
Factor †	Overall ANOVA	Overall ANOVA split by blocks			Overall ANOVA split by stages	
		Experiment 1	Experiment 2	Experiment 3	Stage V1	Stage V2
ST	***	***	***	***	***	***
ϴV	***	***	***	***	ns	***
ST - ϴV	**	***	***	ns	ns	**
Stage	***	***	***	ns	---	---
ST - Stage	ns	ns	ns	ns	---	---
ϴV - Stage	***	***	***	ns	---	---
ST - ϴV - Stage	ns	ns	ns	ns	---	---
Experiment	***	---	---	---	***	***
ST - Experiment	***	---	---	---	ns	***
ϴV - Experiment	***	---	---	---	*	*
ST - ϴV - Experiment	ns	---	---	---	ns	ns
Stage - Experiment	*	---	---	---	---	---
ST - Stage - Experiment	ns	---	---	---	---	---
ϴV - Stage - Experiment	ns	---	---	---	---	---
ST - ϴV -Stage - Experiment	ns	---	---	---	---	---
Orthogonal contrasts						
Experiment 1 VS 2,3	***					
Experiment 2 VS 3	ns					

† ST = soil texture (sand, loamy sand, sandy loam, loam, and silt loam); ϴV = soil volumetric water content (S, PC, PWP); Stage = soybean developmental stage (V1, V2); Experiment = replications (3 reps.). For the SAS analysis the response variable "root CFUs" was averaged and logarithmically transformed (Log(1+CFU)/g of root tissue). Overall ANOVA is split by blocks and soybean developmental stages. Level of significance from F-tests are indicated by the asterisks: *, **, and *** correspond to $p < 0.05$, 0.01 and 0.001, respectively; ns = no significant difference and "---" = not determined.

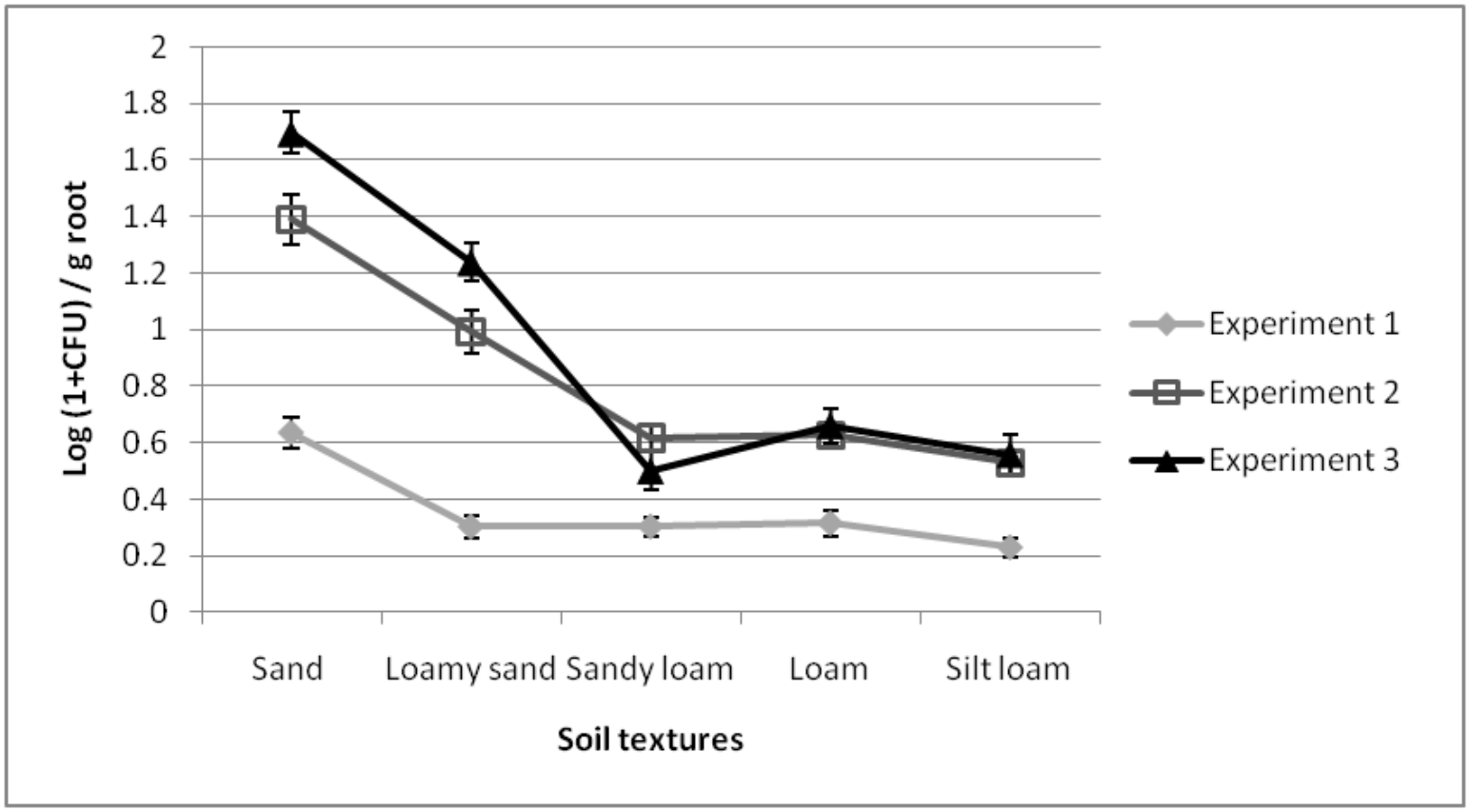

Figure 3-9. Effects of soil texture on *M. phaseolina* root populations. Experiments 1, 2, and 3 denote biological replications. Vertical bars represent standard deviation. Letters above the bars denote significant differences between root populations $p < 0.05$. Averages only include both soybean stages (V1 and V2), and all volumetric soil water contents (PS, PC, PWP).

Results suggest that *M. phaseolina* colonized the root surface more freely in sandy soils compared to other soil textures in which there was an antagonistic effect by other soil fungi. It may that both inoculum and seedlings were under nutrient stress in sand but this particular stressful condition favors root infection. *M. phaseolina* is a soilborne pathogen that takes advantage of stress, as reported by Kendig et al. (2000).

M. phaseolina root populations responded differently across levels of soil volumetric water content (∈V) (Table 3-3). Exposure of the soybean seedlings to a water deficit (PWP) regime resulted in high numbers of root CFUs. In contrast, PS was detrimental to root infection and possessed the lowest numbers of *M. phaseolina* root CFUs, except for a few exceptions in loam soil, in which the lowest populations were present under the PC water content regime (Figure 3-10).

Soil water holding capacity also caused variations in *M. phaseolina* root populations across soil textures. Soils with low percentages of sand such as sandy loam, loam and silt loam soils retain water more strongly compared to soils with higher contents of sand such as sand and sandy loam. These differences in water holding capacity are mainly due to differences in the soil pore size and hygroscopic forces. Small pore sizes, such as those in clayey soils, generate high hygroscopic forces that retain water more strongly than sandy soils, which have larger pores. For this reason, levels of moisture remain more constant in a clayey soil across time. Even at soil saturation, sandy soils lose water content faster than clayey soil at the same temperature. This results in higher levels of stress for the plant and consequently greater root infection (Brady and Weil, 2004).

Seedlings grown in sand had the highest levels of root infection and silt loam and loam soils reported the lowest number of root CFUs. Similar findings about root infection at low levels of soil water content have been reported by Kendig et al. (2000) and Olaya et al. (1996). These authors describe strong effects of water stress at R2 soybean stages in *M. phaseolina* root populations and high rates of colonization in soybean root segments buried in the soil at low water potentials.

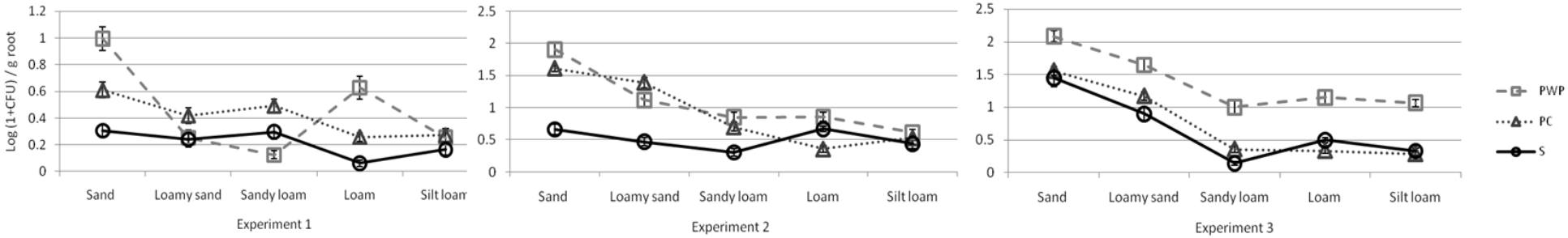

Figure 3-10. Effects of soil texture and soil volumetric water content (θV) upon root infection by *M. phaseolina*. Experiments 1, 2, and 3 denote biological replications. PWP = permanent wilting point, PC = pot capacity, and S = pot saturation. Vertical bars represent the standard deviation. Averages includes both soybean stages (V1 and V2), and all volumetric soil water contents (PS, PC, PWP).

Colonization and infection of roots by *M. phaseolina* does occur at early soybean seedling stages. If environmental stressful conditions for the plant are avoided soybean root infection remains latent and progresses only at the reproductive stages (Hartman et al., 1999). Evidence presented here shows increasing numbers of root CFUs from V1 to V2 soybean stages, which indicates that *M. phaseolina* root colonization is in progress throughout vegetative growth.

Significant effects of the volumetric soil water contents were also observed in soybean stage V2 in addition to the changes in *M. phaseolina* root populations from stage V1 to V2 (Table 3-4) (Figure 3-12).

Trends remained similar when comparing V1 and V2, but differences were only appreciable when plants reached V2 stage, implying a cumulative effect of volumetric soil water content treatments upon *M. phaseolina* root populations. Root infection increased when plants were under water stress (PWP) across all soil textures, but this effect was more evident at V2. In contrast, soil saturation (PS) had a significant detrimental effect on root populations in all soil types, being significantly different from the PWP treatment at V2. Volumetric water content in saturated soils (PS) showed good control of the *M. phaseolina* root population, even in saturated (PS) sandy loam soils where zero root CFUs were obtained at V2. This indicates that it is likely for plants to produce new root tissue without further infection if moisture remains relatively constant.

This information relates to the impact irrigations or rain events at during the early vegetative stages in order to prevent root colonization by *M. phaseolina*. Previous studies have also documented the strong effect of water stress early in the season on field conditions (Kendig, 2000). Optimum water conditions are an integrated management strategy for the disease when soils are sandier. These lighter soils lead to dry conditions predisposing the plant to stress and subsequent *M. phaseolina* colonization.

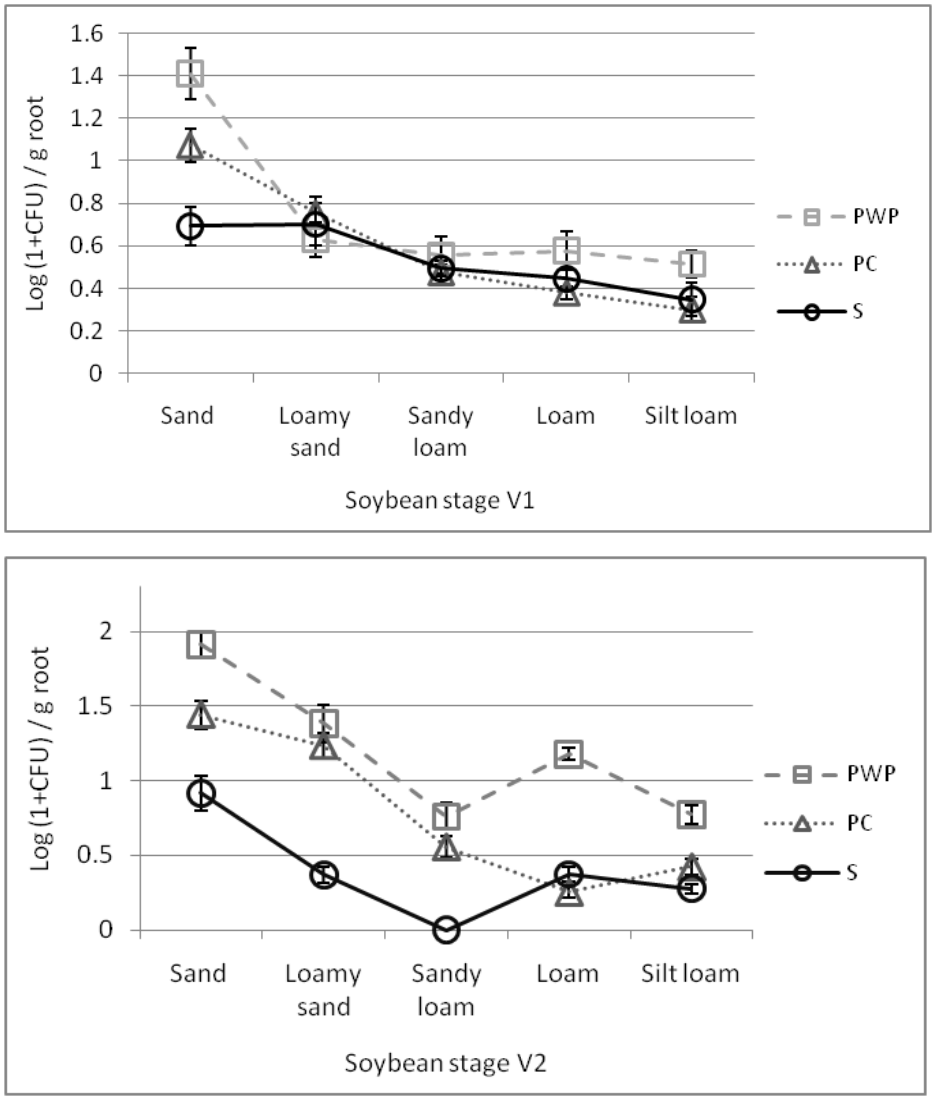

Figure 3-11. Comparisons of *M. phaseolina* root populations by soybean developmental stage V1 and V2 in five artificial soils. PWP = permanent wilting point, PC = pot capacity, and S = pot saturation. Vertical bars represent standard deviation.

Main factors affecting root length

For the statistical analysis all plants were taken in consideration (infested and non-infested soils), except for plants in experiment one, since they did not have homogeneous distribution and equal variance. Additionally all four plants in each pot were averaged and the averages were considered for the statistical analysis. Analysis of variance (ANOVA) was performed to test the impact of the soil texture, soil volumetric water content, soil infestation and soybean developmental stages and their interactions on the response variable "root length". SAS system software version 9.2 (SAS institute Inc., Cary, North Carolina, USA) was used to construct the ANOVAs using Proc Glm. All the main factors and some interactions were highly significant (Table 3-3).

Soil texture and soil volumetric water content impacted root morphology and length. Soil texture influenced the vertical and horizontal distribution of roots. It was observed that root length increased when the content of sand in each soil type increased. In sandy soil, root systems were able to grow profusely. This "loosen" soil optimized the capabilities of soybean roots to explore the soil because the relatively big pore size in the sandier soil textures decreased the resistance to root penetration. In contrast, soil textures with lower contents of sand augmented the soil strength in a manner that had a negative effect in root penetration of the soil and caused a negative impact on the root length. Soybean seedlings growing in sandy soils got longer roots compared to seedling that grown on more heavy clayey soils such as silt loam and sandy loam. (Figure 3-13).

This is consistent with previous reports that have shown significant impacts of soil texture on root morphology and root length in grapevines under irrigation. Vines had a deeper root system growing in coarse soils than moderately coarse and fine soils (Nagarajah, 1987; Soar and Loveys, 2007).

Even though root diameter (thickness) was not measured, an appreciable thickening of roots was observed in soils with high percentages of sand such as sand and loamy sand. On the other hand, thin roots were observed in soil with higher contents of clay such as silt loam and loam. This phenomenon of root thickening may be explained by the arrangement of soil particles micro and macro pores. Plants have to modify its root thickness to the soil pore sizes to grow and penetrate the soil (Drew, 1991).

Table 3-5. Significance of main effects and interactions on the response variable "root length".

	Root length								
Factor †	Overall ANOVA	Overall ANOVA split by block		Overall ANOVA split by stages		Overall ANOVA split by Infestation and soybean stages			
		Experiment 2	Experiment 3	Stage V1	Stage V2	V1 Inoculated	V1 Non-inoculated	V2 Inoculated	V2 Non-inoculated
ST	***	***	***	***	***	***	***	***	***
∈V	***	***	***	***	***	***	***	***	***
ST × ∈V	***	***	***	***	***	***	***	***	***
Stage	***	***	***	---	---	---	---	---	---
ST × Stage	***	***	***	---	---	---	---	---	---
∈V × Stage	***	***	ns	---	---	---	---	---	---
ST × ∈V × Stage	ns	ns	ns	---	---	---	---	---	---
Infest	***	***	***	***	***	---	---	---	---
ST × Infest	***	***	***	***	***	---	---	---	---
∈V-Infest	***	*	***	***	**	---	---	---	---
ST-∈V-Infest	***	***	***	***	***	---	---	---	---
Stage-Infest	ns	ns	ns	---	---	---	---	---	---
ST-Stage-Infest	ns	ns	ns	---	---	---	---	---	---
∈V-Stage-Infest	ns	---	---	---	---	---	---	---	---
Soil-∈V-Stage-Infest	*	---	---	---	---	---	---	---	---
Experiment	***	---	---	***	ns	***	***	*	ns
ST - Experiment	**	---	---	***	ns	ns	*	ns	ns
∈V - Experiment	**	---	---	***	ns	ns	ns	ns	ns

ST - ϴV - Experiment	ns	---	---	ns	ns	ns	ns	ns	ns
Stage - Experiment	***	---	---	---	---	---	---	---	---
ST - Stage - Experiment	ns	---	---	---	---	---	---	---	---
ϴV - Stage - Experiment	*	---	---	---	---	---	---	---	---
ST - ϴV -Stage - Experiment	ns	---	---	---	---	---	---	---	---
Infest-Experiment	**	---	---	**	ns	---	---	---	---
ST-Infest-Experiment	ns	---	---	ns	ns	---	---	---	---
ϴV-Infest-Experiment	ns	---	---	ns	ns	---	---	---	---
ST-ϴV-Infest-Experiment	**	---	---	ns	ns	---	---	---	---
Stage-Infest-Experiment	ns	---	---	---	---	---	---	---	---
ST-Stage-Infest-Experiment	ns	---	---	---	---	---	---	---	---
ϴV-Stage-Infest-Experiment	ns	---	---	---	---	---	---	---	---
ST-ϴV-Stage-Infest-Experiment	ns	---	---	---	---	---	---	---	---

† ST = soil texture (sand, loamy sand, sandy loam, loam, and silt loam); ϴV = soil volumetric water content (S, PC, PWP); Stage = Soybean development stage (V1, V2); Experiments = replications (3 reps.); Infest=infestation (Inoculated and non-inoculated soils). For the SAS analysis the response variable "root length" was averaged and logarithmically transformed. Overall ANOVA is split by blocks, soybean developmental stages, and infestation. Level of significance from F-tests are indicated by the asterisks, *, **, and ***, which correspond to $p < 0.05$, 0.01 and 0.001, respectively; ns = no significant difference and "---" = not determined.

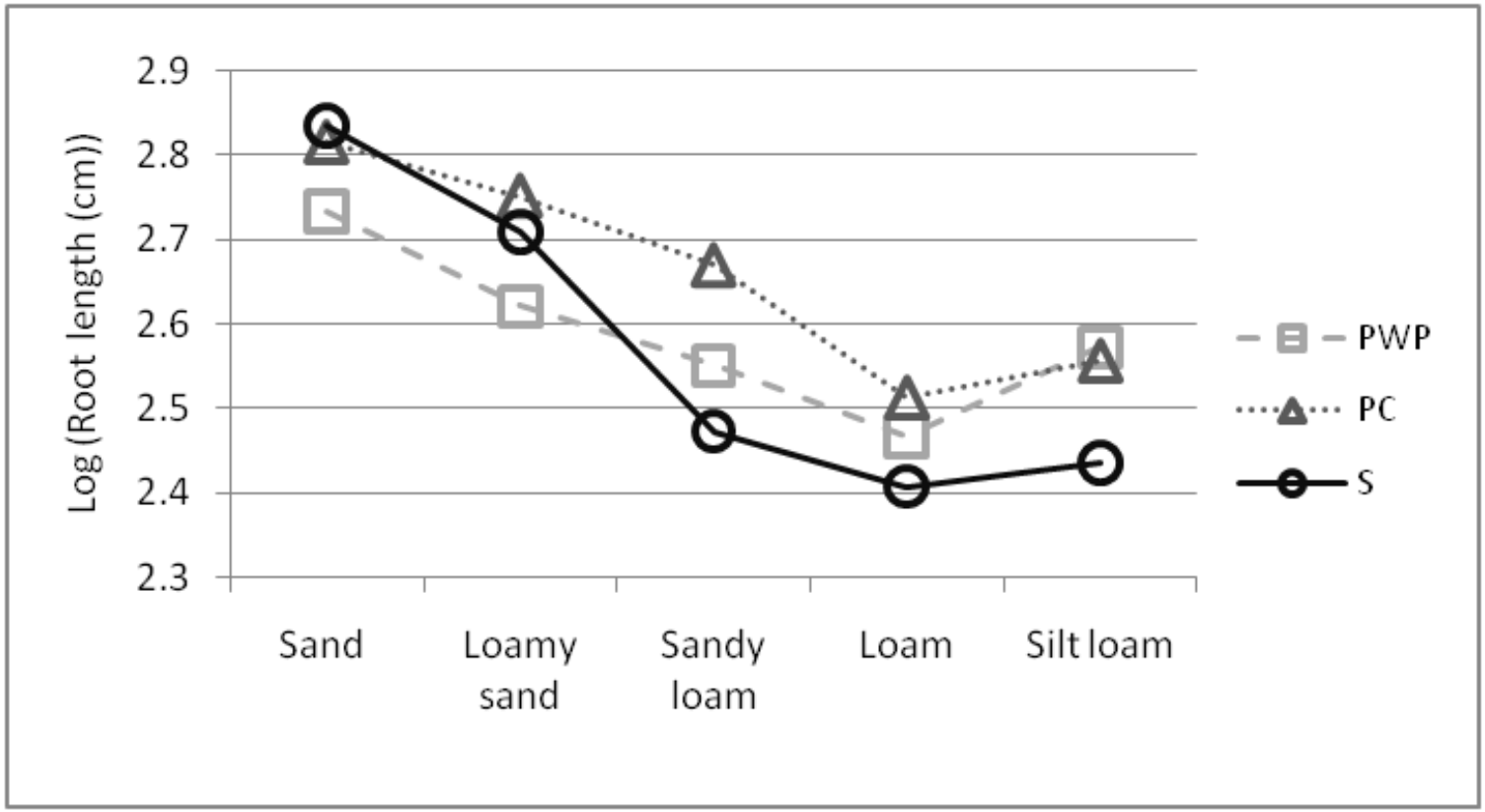

Figure 3-12. Effect of soil texture and soil volumetric water content (θV) on root length. Averages include only experiments 2 and 3, infested and non-infested soils, soil volumetric water contents (PS, PC, and PWP) and seedling stages V1 and V2.

Regarding volumetric soil water content, PC presented the largest roots across all five soil types tested in this experiment. Conversely, PWP had a negative impact on root length across the sandiest soils (sand and sandy loam). However, plants growing under saturated soil conditions PS had short roots in soils with higher contents of clay such as loam, silt loam and sandy loam (Figure 3-13).

Water holding capacity is critical in root development and root length. For example, the lowest values in root length in the PWP treatment were present in sand. As previously described, these kinds of soils lose water content faster than any other soil and expose plants to drought for longer periods of time compared to heavier soil types. According to Wang and Yamauchi (2006), one of the most common causes for reduced root growth is drought at water potentials lower than -1.5 MPa (i.e., PWP). However, other important responses to drought may include increased branching, deep rooting and enhanced geotropism, but this is dependent upon plant species and soil conditions.

Saturated soils (PS) also resulted in decreased root length in silt loam and loam textures. Other authors have reported similar results about hypoxic conditions in soils; oxygen diffusion is lower in water than in air, in flooded soil conditions root growth is limited in length and mass by absence of O_2. It may also be associated with the production of ethylene by root apices and accumulation of CO_2 produced by the roots and also soil microorganisms (Jackson and Ricard, 2003).

Saturated conditions in silt loam and loam soils may also promote activities of facultative anaerobes that diminish the nitrate availability to the roots by soil denitrification (Wang and Yamauchi, 2006). Since the soils have higher water holding capacity, the PWP treatment was not detrimental for the roots (Figure 3-13).

The effects of the volumetric soil water content were more evident at V2 in which roots from the PWP regime in sandy soils did not show a significant increase in root length from V1 to V2. This was also true for the PS water regime in the heavier silt loam, loam and sandy loam soils. These had a greater impact on the V2 stage because the water demand for the plant increases with its growth and development, resulting in more severe water deficit-stress in sandy soils, whereas PS produces the same stress in heavier soils by generating anoxic soil conditions (Figure 3-14).

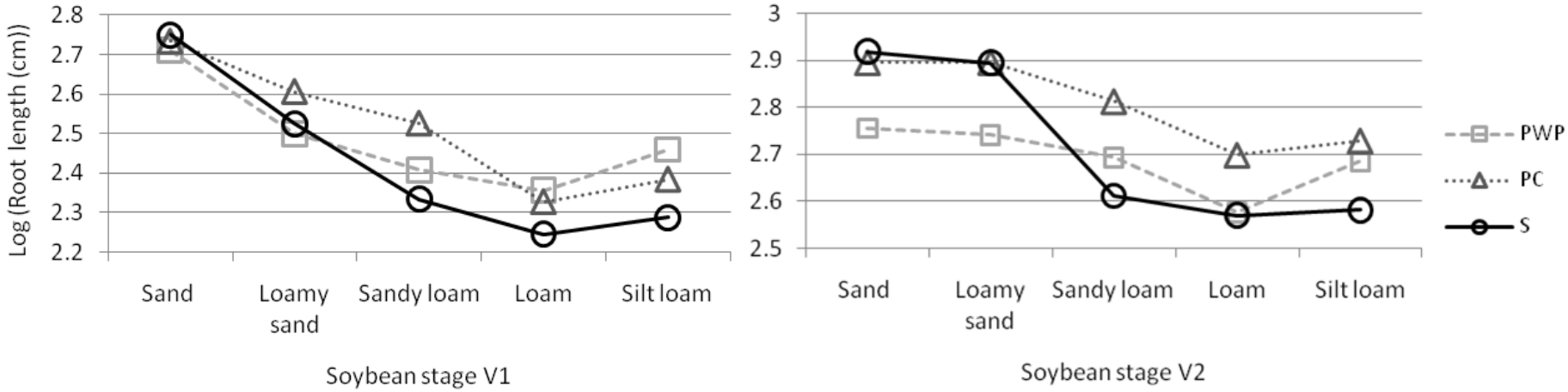

Figure 3-13. Impact of *M. phaseolina* upon root length by soybean developmental stages V1 and V2. Averages include only experiments 2 and 3 and *M. phaseolina* infested soils.

Inoculum also had a detrimental effect on root length in both experiment one and experiment two and soybean developmental stages V1 and V2. Differences in root length from inoculated and non-inoculated soils were evident in all soil types except for sand, in which plants from inoculated and non-inoculated soil had virtually the same root length (Figure 3-15).

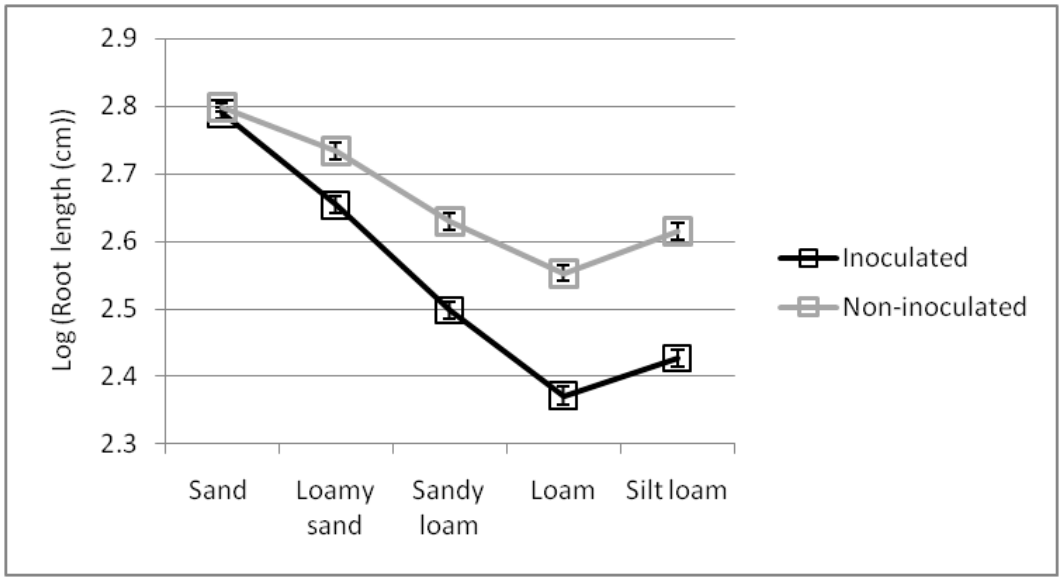

Figure 3-14. Impact of *M. phaseolina* inoculum upon root length in five artificial soils. Vertical bars represent standard deviation. Averages include only experiments 2 and 3 and seedling stages V1 and V2.

Results suggest that *M. phaseolina* inoculum had a suppressive effect on the growth of soybean root seedlings regardless the level of volumetric soil water content. The detrimental impact on root growth is more evident in fine-textured soils with higher percentages of silt.

There was no impact of inoculum on root length in sandy soils, even though *M. phaseolina* root infection did occur, root infection was no detrimental for the plant as it can freely produce more roots due to a lower resistance in coarse textured soil. In contrast, for clayey soils the production of new roots was lower and the effect of the inoculum was more obvious. For example, roots in silt loam soils exhibited a lower level of root infection, but they were also shorter in length.

Relationships between root length and root infection

Complex relationships were observed between root length and root infection. Linear regression and polynomial regression analyses were performed with these variables across the soil types and the soil volumetric water contents (ϴV). The relationship between root length and root infection differed across the soil types on its level of significance, being highly significant in sand and less or not significant in the other artificial soil textures (Table 3-6)

Significant correlations were obtained in sandy textured soils; this trend was kept after splitting the correlation analysis by volumetric soil water content treatment and by soybean developmental stages. These high correlations may be explained by the high levels of root populations obtained in roots grown in sandy soils and the overall frequency of root infection. In addition, root infection tended to decrease as the levels of clay increased as well as the level of significance in the correlations in fine-textured soils such as silt loam, loam and sandy loam.

Relationships seem to be more correlated in V2 than in V1 soybean stage except for loamy sand soil texture; however, these relationships are not highly significant except for plants at V2 stage in sandy soils.

Table 3-6. Correlation coefficients for root length and *M. phaseolina* root populations

Soil textures	† OVERALL		Volumetric soil water content (∈V)						Soybean developmental stage			
			PWP		PC		PS		V1		V2	
	R^2	*P* > *F*	R^2	*P* > *F*	R^2	*P* > *F*	R^2	*P* > *F*	R^2	*P* > *F*	R^2	*P* > *F*
Sand	0.3094	0.004	0.5035	0.0052	0.5467	0.023	0.4736	0.004	0.3612	0.0049	0.4471	0.0046
Loamy sand	0.0721	0.2729	0.4372	0.1852	0.2204	0.0568	0.3826	0.3109	0.1632	0.7341	0.0052	0.8647
Sandy loam	0.0314	0.7963	0.0695	0.9848	0.1151	0.4665	0.5835	0.0776	0.0967	0.9816	0.3478	0.6848
Loam	0.0108	0.4116	0.5641	0.1909	0.418	0.2133	0.1715	0.064	0.0049	0.837	0.6298	0.7745
Silt loam	0.126	0.1545	0.228	0.4253	0.1674	0.7227	0.8497	0.0423	0.0355	0.7417	0.0467	0.1948

† Overall = corresponds to correlation analysis including the entire data set obtained in the respective soil texture. S, PC, and PWP correspond to "pot saturation", "pot capacity", and "permanent wilting point", respectively. Overall correlation analysis on each soil texture is split by volumetric soil water content and soybean developmental stage. Coefficients in the table correspond to a polynomial second grade regression. "Experiment 1" was not included in the correlation analyses.

Conclusions

In summary, the *M. phaseolina* soil population dynamics, root infection, root length and root morphology are highly influenced by soil texture and soil water content. Evaluation times and soybean developmental stages (V1 and V2) also played an important role in the charcoal rot disease processes.

Findings in the soil and root populations, suggest that there is a slight decrease in the *M. phaseolina* soil populations across time (from V1 to V2), but root colonization levels increased in this period of time.

In the soil *M. phaseolina* populations may be indirectly influenced by clay content. There is some evidence to suggest that increasing levels of organic matter contained in the silt loam soil used to form artificial soil textures can support antagonistic microorganisms that compete with microsclerotia in the soil having a negative impact on soil populations. Decreasing soil populations in sandy soils also suggest that propagules require a certain level of soil nutrients to remain viable and active in soil. Even though soil water regimes did not have a significant effect on *M. phaseolina* soil populations there was a positive effect of (PWP) in the average soil population.

Seedling root infection is impacted by the level of water stress to which the plants were exposed and the water-holding capacity of each soil. Sandy soils resulted in higher root populations due to low water-holding capacity, which exposes plants to a constant water deficit that predisposes plants to root infection.

In sandy soil textures, levels of root colonization may be higher due to the fact that root length was longer and got more in contact with the inoculated soil, increasing the sites of root infection. In contrast, soils with high clay content can retain water for a longer period of time diminishing the impact of water stress and decreasing the possibility of *M. phaseolina* root colonization. Moreover, adequate volumetric soil water content in soybean seedling such as PS in most sandy soils and PC in heavy clayey soils can suppress but not prevent *M. phaseolina* root colonization or root infection.

Dynamics of root infection were observed in the worst and the best-case scenarios. Sandy soil texture at PWP soil water content generated the highest levels of root colonization, contrasting to silt loam and loam soils at PS having the lowest levels of root colonization.

However, PS in fine textured soils can produce a level of water stress by anoxic conditions that impact negatively plant growth and development.

Relationship between root length and root infection resulted to be complex and it could not be expressed by a simple linear regression. However, the most significant relationships were found in the coarsest soil textures where root infection and root length were the high. It may imply that root architecture and root development play an important role in the charcoal rot disease progression. In poor nutrient and in stressful soils water conditions soybean seedlings may produce more roots to find more water and nutrient acquisition, which lead to higher levels of root infection.

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CHAPTER 4 - Influence of soils and water relations upon charcoal rot of soybean caused by *M. phaseolina* in two different field locations in Kansas.

Introduction

Several strategies for the suppression of charcoal rot of soybean have been tested, crop rotation, plant populations, moderate tolerant varieties, soybean late maturity cultivars, compost amendment, biological control, irrigation and soil fertilizers.

Mn plays an important role in the suppression of root diseases on different hosts. Mn participates in photosynthesis and consequently the production of root exudates that can affect the soil microflora. Mn is also involved in the synthesis of lignin and phenols and activates enzymes involved in the shikimic acid pathway. High concentrations of Mn inhibit the production of pectin-methylases by fungal pathogens (Graham and Rovira, 1984). Beneficial effects of Mn in charcoal rot of soybean have never been measured.

Between other important cultural practices, irrigation may be the most important for the control of charcoal root. Soybean irrigation may be necessary at any stage of soybean in order to obtain the maximum yield a single irrigation during the early reproductive stages is enough to reduce the inoculum survival by 25 to 42% in bare soil (Lodha, 1995; Rogers, 1997). Soybean irrigation at R2-R4 full bloom and beginning of pot reduces *M. phaseolina* root colonization and favored soybean yield (Kendig et al., 2000).

However, other factors may affect the *M. phaseolina* soil populations and root infection such as crop rotation and soil texture. One of the few options to control this disease is to crop a non-host in order to reduce the levels of inoculum in the soil (Short et al., 1980). In the case of soybeans, low microsclerotia densities in the soil generally resulted in less root infection and consequently more yield (Francl et al., 1988).

The objectives of these studies were:

- To determine the effects of soil-applied manganese fertilization on *M. phaseolina* soil populations, soybean root colonization and yield.
- To estimate the impacts of soil texture and cropping history on *M. phaseolina* soil populations, soybean root colonization and yield.
- To evaluate the effect of irrigation regimes at different soybean developmental stages on *M. phaseolina* soil populations, soybean root colonization and yield.
- To assess the relationships between physicochemical soil components and *M. phaseolina* soil and root populations.

Materials and methods

Description of the field location

Experiments were conducted at two different Kansas State University's experiment fields in 2008-2009: the Ashland Bottoms research farm near Manhattan in Riley County, and the Kansas River Valley Experimental Field located 3.5 miles east of Silver Lake at the Paramore research site in Shawnee County. The predominant soil series for the locations were Bismarckgrove-Kimo Complex (fine-silty, smectic, mesic Aquertic Hapludolls), and Eudora series for each location respectively. The area of experimentation within each location was 146.3 m wide by 36.5 m long with a previous crop (2007) of soybean and wheat at Ashland Bottoms and 146.3 m wide by 27.4 m long with a preceding crop (2007) of corn at Paramore.

Soil samples were taken across the field locations at the beginning of each year of the study prior to planting (14 May 2008 and 20 May 2009 at Paramore; 15 May 200 and 22 May 2009 at Ashland Bottoms), and analyzed for pH, Phosphorus (P), Manganese (Mn), Amonium (NH4), Nitrate (NO3), Electric Conductivity (EC), Cation Exchange Capacity (CEC), Organic Matter (O.M.), total N, total P and texture (Table 2-1). pH was measured directly using a 1:1 slurry of 5 g of prepared soil with deionized water and also with the SMP buffer technique (Schofield and Taylor, 1955). Soil phosphorus (P) content was measured by Melich III extraction (Mehlich, 1984). Mn (manganese) was extracted by the DTPA method and assessed by flame atomic absorption spectrometry (Lindsay and Norvell, 1978). NH_4 and NO_3 were analyzed by 1M KCl and cadmium reduction (Huffman and Barbarick, 1981). EC (electric conductivity) was determined with a conductivity meter. CEC (cationic exchange capacity) was assessed by the

saturating ammonium acetate method (Rhoades, 1982). Organic matter (O.M.) was determined by the Wakly-Black procedure (Walkley, 1947). Total nitrogen and phosphorus were assessed with a modified Kjeldahl digestion technique. Texture was determined using sodium hexameta-phosphate by the hydrometer method.

Soil variables including Mn, P and pH that were considered to change the most under the prevalent field conditions were reassessed with a second set of soil samples taken before planting in the second year of study.

Experimental design, soil and root sampling

The experimental design used in both locations was a split plot design with four replications, where main plots consisted of four irrigation regimes conducted at different soybean growth stages as follows: non-, pre-flowering, R4-, full season irrigation. The subplots consist of two factors: amended and non-amended with a source of manganese (5% manganese glucoheptonate solution; ClawEL Manganese – Gold Label, Brandt Consolidated, Pleasant Plains, Illinois) applied at a rate of 5 lbs/A mixed with starter fertilizer (10-34-0; 10 lbs/A), before planting in each year of the field experiment (Figure 2-2).

Some irrigation treatments were not conducted imposed because of excess precipitation in parts of both growing seasons. In the year 2008, the pre-flowering irrigation treatment could not be imposed in either location. Thus, for data analysis, non-irrigated and pre-flowering irrigation treatments were combined. Only the R4 irrigation treatment was imposed at the Ashland Bottoms location and the Paramore field location remained constantly flooded for most of the season. During the field experiment, disease symptoms were not observed, because the prevalent weather conditions did not favor disease manifestation, although *M. phaseolina* was present in the soil and root infection did occur.

Table 4-1. Averages of soil physicochemical properties for the two locations in two years (pre-planting soil samples).

Year	Location	pH	P	Mn	NH_4	NO_3	Total N	Total P	EC	CEC	OM
			ppm						mS/cm	meq/100g	(%)
2008	Ashland Bottoms	6.4	27.8	30.5	4.7	6.2	1000.4	398.6	0.2	16.9	2.2
	Paramore	6.2	37.1	19.4	3.6	6.7	770.8	362.9	0.2	10.7	1.4
2009	Ashland Bottoms	6.4	23.3	17.4	---	---	---	---	---	---	---
	Paramore	6.1	35.3	20.5	---	---	---	---	---	---	---

--- Correspond to soil variables not assessed in 2009

P = corresponds to phosphorus, Mn = corresponds to manganese, NH_4 = corresponds to ammonium, NO_3 = corresponds to nitrate, N = corresponds to nitrogen; P = corresponds to phosphorus; EC = corresponds to electric conductivity; CEC = corresponds to cation exchange capacity, OM = corresponds to organic matter.

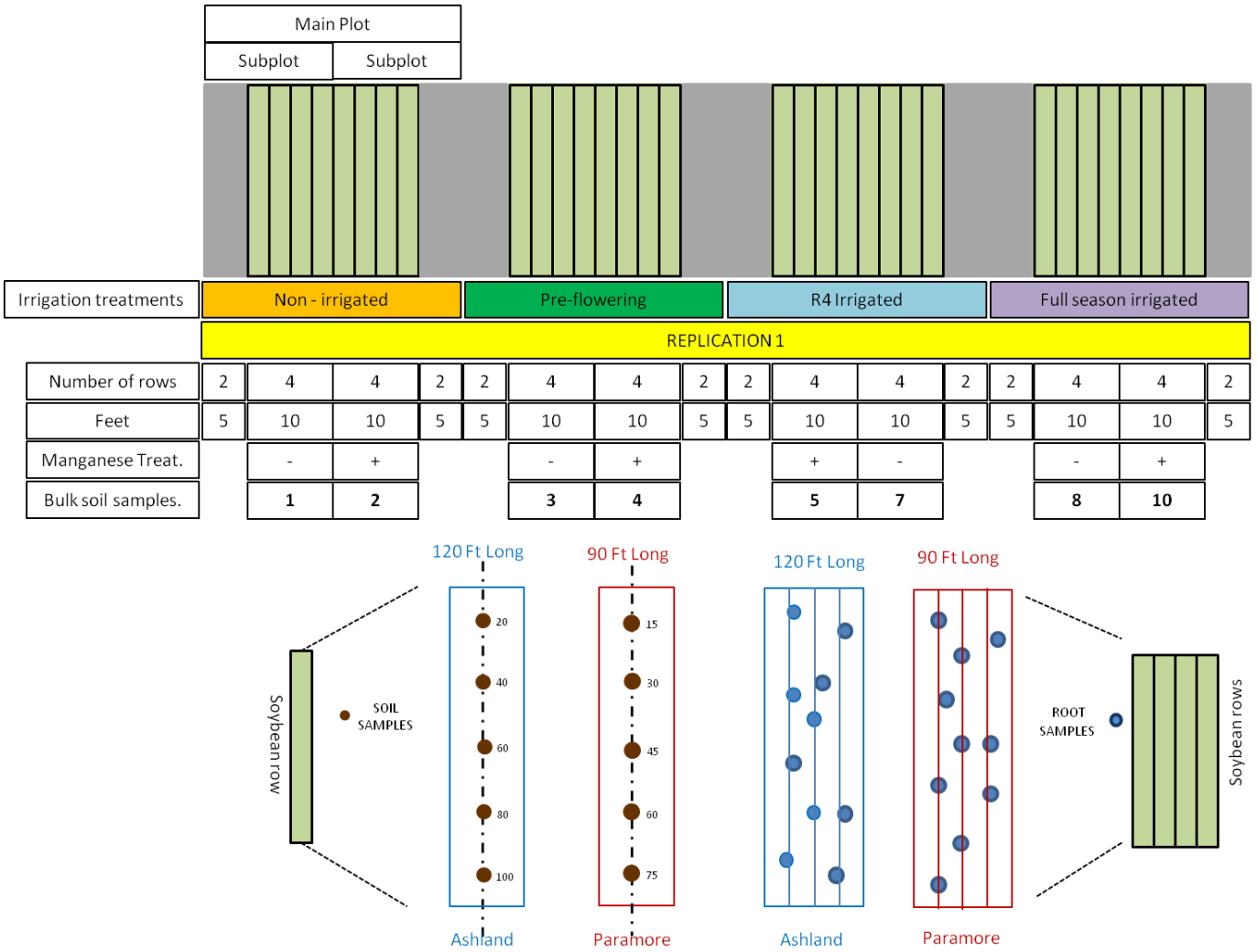

Figure 4-1. Plot layout and design of the field experiment (only showing replication 1). "+" = amended with manganese, "-" = non-amended with manganese at 5 lbs/A. Irrigation and manganese treatments were randomly assigned in the four replications across the field.

Table 4-2. Summary of weather data from the field locations Ashland Bottoms and Paramore in 2008 and 2009.

	Precipitation (mm)				Soil temperature (°C)				Air temperature (°C)				Relative Humidity (%)			
Location	Ashland		Paramore		Ashland		Paramore		Ashland		Paramore		Ashland		Paramore	
Year	2008	2009	2008	2009	2008	2009	2008	2009	2008	2009	2008	2009	2008	2009	2008	2009
May	4	0	2	1	19	20	17	19	17	18	18	19	68	65	68	66
June	10	7	3	5	25	25	24	25	23	24	24	24	73	73	74	75
July	4	4	2	5	27	26	27	26	26	23	26	23	75	76	75	77
August	4	4	1	3	27	25	26	25	24	23	24	23	74	76	75	76
September	5	2	5	1	22	21	21	20	19	18	19	19	77	79	79	79
October	2	2	3	1	16	12	15	12	13	9	13	10	71	77	73	77
Average	5	3	3	3	23	22	22	21	20	19	21	20	73	74	74	75

Weather data were collected from the nearest meteorological station from each plot:

- Weather data from Ashland Bottoms were collected from the Kansas State University Meteorological Station at Manhattan (KS)
- Weather data from Paramore were collected from the Kansas State University Meteorological Station at Silver Lake (KS)

The soybean variety used for these experiments was NK S37-F7 (RR, SCN-R, SDS-tolerant). Seed was treated with Apron XL, active ingredient mefenoxam (ISO-name: metalaxyl-M), before planting to prevent seedling diseases and to provide a good germination rate. Seeds were planted at a density of 9 seeds/foot and at a row spaced at 30 inches for both locations. Additionally, a border space of four rows (120 inches) separated the main plots (e.g., irrigation treatments) (Figure 4-1).

Samples were taken over time during two-year field study to examine *M. phaseolina* soil and root population dynamics within the experimental field locations. *M. phaseolina* soil populations were evaluated from a bulk soil samples that consisted of 5 cores removed with a steel probe to a depth of 20 cm. The first 4 cm of soil from each core sample were removed to discard the stubble; the probe was cleaned between each plot sampling (Figure 4-1).

Soil sampling was done at pre-planting and post-harvest for the two locations in both years. Soil samples were transported to the greenhouse and dried at about 25-28°C for 2-3 days and refrigerated at 4°C for further processing.

Ten soybean plants were uprooted arbitrarily across each subplot (consisting of four soybean rows per plot) at R2-R4 (full bloom to full pod, respectively) and R8 (full maturity) soybean plant development stages following the criteria established by Fehr et al. (1971). Entire plants were transported to the lab and the roots were cut off below the cotyledonary node. Each root was rinsed with tap water to eliminate soil residues. Plants collected at (R8) were also used for evaluation of plant yield.

Sample processing, obtaining colony forming units (CFUs)

M. phaseolina soil populations were measured from bulk soil samples composed of five 20 cm cores collected within one of four subplot soybean rows. Each of the five soil cores were dried at room temperature, ground separately, and passed through a 2 mm sieve to remove the larger soil particles. Once the soil was sieved, a 10 g sub-sample from each of the five cores was taken to make a 50 g bulk soil sample. From this 50 g bulk soil sample *M. phaseolina* soil populations were evaluated. There were a total of 32 bulked soil samples replicated three times for each field location.

M. phaseolina soil populations were estimated using the procedure described by Cloud (1991). Ten g of ground soil samples were blended in 250 ml of 0.5% NaOCl solution for 3 min,

with 30-sec idle intervals. The resulting soil slurry was poured into a 45 µm No325 sieve and rinsed with distilled water while gently shaking. The washed soil was concentrated in on side of the sieve using a squeeze bottle containing sterile-distilled water and transferred to a 50 ml plastic tube that was later filled with 50 ml of selective media (39 g/L PDA, 1mL/L tergitol, 40 mg/L penicillin, 20 mg/L streptomycin, 20 mg/L tetracycline, 100mg/L rifampicin) and poured into five 100 mm-diameter petri dishes (Cloud, 1991; Mengistu et al., 2007). Petri dishes were placed in an incubator at 30°C in the dark. After five days CFUs were counted and converted to CFU/g of dried soil. Additionally, 10 g samples were taken from the soil bulk samples and placed in an oven at 105°C for 24 hours to estimate the soil gravimetric water content (Figure 3-4).

The level of *M. phaseolina* root severity was evaluated by splitting the taproot of each sample, and rating root discoloration caused by microsclerotia at the soybean growth stages previously mentioned using the root and stem severity scale by Mengistu et al., (2007) and Paris et al., (2006).

Root colonization and *M. phaseolina* root population levels were estimated by grinding the split roots remaining after the severity evaluation. Ten soybean roots were ground with a UDY cyclone sample mill (Model 0.14, UDY Corporation, Fort Collins, Colorado), passing the samples through a 600 µm No30 sieve. All ten subplot roots were ground together to create a bulk sample. From the bulk sample three 0.05 g root tissue subsamples were used to estimate CFUs following a modification of the procedure described by Mengistu et al. (2007), Mihail (1992), and Pearson et al. (1984). The crushed tissue was blended in 250 mL of a 0.5% NaOCl solution for 3 min with 30-sec idle intervals, collected in a 45 µm sieve and rinsed with distilled water for 1 min. Using a squeeze bottle containing sterile-distilled water. The root tissue was concentrated in one side of the sieve and poured into a 50 mL sterile plastic tube. Afterwards, a rifampicin selective media, as described above, was added to each tube and this was poured in five 100 mm-diameter petri dishes, and placed in the incubator at 30°C in the dark for five days. After incubation, CFUs of *M. phaseolina* were counted and transformed to CFUs per gram of root tissue (Figure 3-4).

Data analysis

Analysis of variance ANOVA was conducted using SAS version 9.2, (SAS institute Inc., Cary, North Carolina, USA) under the Proc Mixed. Response variables (*M. phaseolina* initial and final soil populations, root populations at R4 and R8 soybean reproductive stages, root severity at R8, secondary roots at R8, mechanical harvest and manual harvest) were logarithmically transformed to meet the assumptions of equal variances and normal distribution of data.

Because the irrigation treatments were performed only in 2008 and did not have a significant impact on the response variables and the soil textures randomly distributed in the locations, years (2008 and 2009) and locations (Ashland Bottoms and Silver Lake) were considered as random factors and soil texture and manganese were consider as fixed factors in the ANOVA analysis using Proc Mixed protocol.

Because plots at Ashland Bottoms location differed in cropping history (soybean and wheat), analysis of variance was also performed in order to find the possible effects of this factor on the response variables since the *M. phaseolina* soil and root populations can increase or decrease depending upon the previous crop.

Correlations between the physicochemical variables obtained from the pre-planting soil samples in 2008 and 2009 and the response variables (*M. phaseolina* initial and final soil populations, root populations at R4 and R8 soybean reproductive stages, root severity at R8, secondary roots at R8, mechanical harvest and manual harvest) were performed using SAS system version 9.2, (SAS institute Inc., Cary, North Carolina, USA). Linear and second order polynomial regressions were developed in order to explore the relationships of *M. phaseolina* with soil physicochemical parameters.

Results and discussion

Effect of manganese on M. phaseolina soil and root populations and yield

Manganese amendment did not have any impact on the soil and root *M. phaseolina* population or soybean yield (Table 4-3). Even though manganese was applied at pre-planting for two years, there was not a significant cumulative effect of this element in the soil; on the contrary, there was a decrease in the average Mn in the year 2009 in Ashland Bottoms. The average Mn content at Paramore soil remained constant (Table 4-1; Figure 4-2).

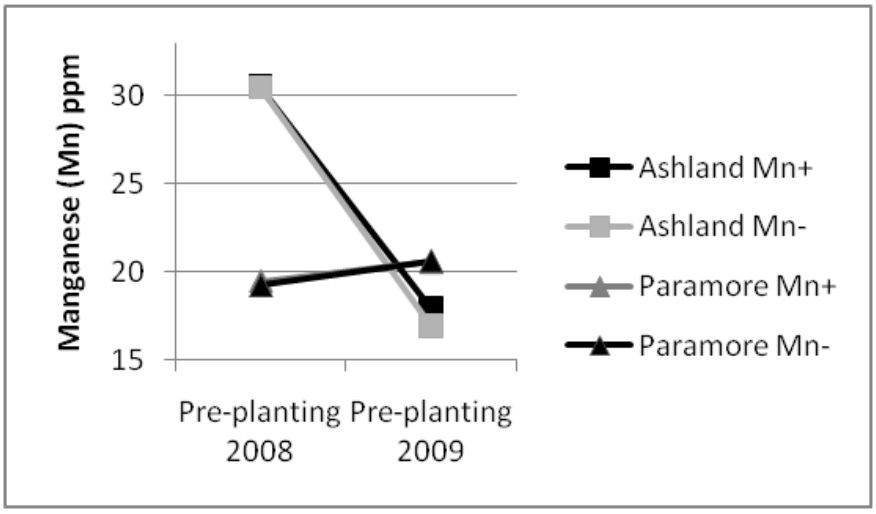

Figure 4-2. Soil manganese content at Ashland Bottoms and Paramore compared from 2008 to 2009.

These trends may be explained by the fact that Mn is less available in soils with higher contents of clay (Heitholt et al., 2002). The majority of plots at the Ashland Bottoms location contained clayey soils such as silty clay and clay loam and for the Paramore location soils possessed loam and sandy loam textures. There were not significant differences in the Mn content from amended and non-amended plots (Table 4-3).

Effect of soil texture on M. phaseolina soil populations under field conditions

Analysis from 2008 pre-planting soil samples indicated that there was variability in soil texture across the experimental plots for both locations. At Ashland Bottoms soil texture was diverse across the experimental plots including soil textures such as silty clay loam, loam, and silty clay. On the other hand, soil texture was more uniform across the experimental plots at Paramore, having only loam and sandy loam textures. However, the average soil composition for 32 bulked soil samples (160 total samples) taken in the field was 20% sand, 52% silt, and 28% clay for Ashland Bottoms; and 34% sand, 50% silt, and 16% clay for Paramore (Figure 4-3).

Table 4-3. Significance of main effects and interactions for *M. phaseolina* soil and root populations and yield parameters.

RESPONSE VARIABLES †	OVERALL ANOVA			OVERALL ANOVA (Split by location)			
	Effects			Ashland Bottoms		Paramore	
	Mn	Soil texture	Mn-Soil Texture	Mn	Soil Texture	Mn	Soil Texture
Initial soil population	ns	**	ns	ns	***	ns	ns
Final soil population	ns	*	ns	ns	ns	ns	*
Root population (R2-R4)	ns	ns	ns	ns	ns	ns	ns
Root population (R8)	ns	***	ns	ns	***	ns	ns
Root severity (R8)	ns	***	ns	ns	***	ns	*
Secondary roots (R8)	ns	ns	ns	ns	ns	ns	ns
Mechanical harvest	ns	ns	ns	ns	ns	ns	ns
Plant yield	ns	***	ns	ns	***	ns	ns

† Initial soil population = *M. phaseolina* soil population at pre-planting; Final soil population = *M. phaseolina* soil population at post-harvest; Root population (R2-R4) = *M. phaseolina* root population ("full bloom" to "full pod"), respectively; Root population (R8) = *M. phaseolina* root population at full maturity; Root severity (R8) = Root severity evaluated by a split-stem severity scale from 1 to 5 where 1 = no discoloration and 5 = highly discolored at full maturity soybean stage (see Mengistu et al., 2007); Secondary roots (R8) = Number of secondary roots at full maturity; Mechanical harvest = weight of soybean seeds obtained by harvesting whole plots with the combine; Yield = weight of soybean seeds obtained from ten plants arbitrarily selected on each plot. Response variables were logarithmically transformed. Manganese-soil texture interactions are not significant for all response variables in the ANOVAs split by location. Level of significance from F-tests are indicated by the asterisks: *, **, and *** correspond to $p < 0.05$, 0.01 and 0.001 respectively; ns = no significant difference.

The overall analysis of variance showed a significant effect of soil texture for most of the field variables determined. However there are some differences in the level of significance when the analysis was made by location, probably due to the differences in the soil textures between field locations as previously mentioned (Table 4-3).

In general *M. phaseolina* soil populations remained steady during the two years of the field study. However, population sizes varied across soil textures. For Ashland Bottoms initial soil populations were higher in loam and silty clay loam soils. Loam soils in Paramore possessed higher densities of *M. phaseolina* than sandy loam soil; however, this difference was not significant (Figure 4-4).

Overall, the coarser soil texture (loam) presented the highest densities of *M. phaseolina*. This effect may be attributed to the soil water holding capacity, which is negatively correlated with the percentage of sand; the higher the percentage of coarse sand, the lower the soil water holding capacity (Salter et al., 1966). For this reason, the lower soil water content supported higher germination percentages and production of secondary microsclerotia providing more optimum soil water content for *M. phaseolina* survival as reported by Collins et al. (1991) and Dhingra and Sinclair (1975).

Reductions in *M. phaseolina* soil populations were observed between pre-planting (May) and post-harvest soil populations (October) for both locations and years. Similar findings were reported by Short et al. (1980) working with *M. phaseolina*-inoculated soils in a soybean field in Missouri. Numbers of germinable microsclerotia increased from January to April and latter declined on late October in a similar manner during two years.

These fluctuations are mainly due to prolonged rainy periods; the greatesest drop in the population was observed at Ashland bottoms in the year 2008 from pre-planting to post-harvest. May and June above-average monthly precipitation: 4 mm and 10 mm, respectively (Table 4-2) (Figure 4-4).

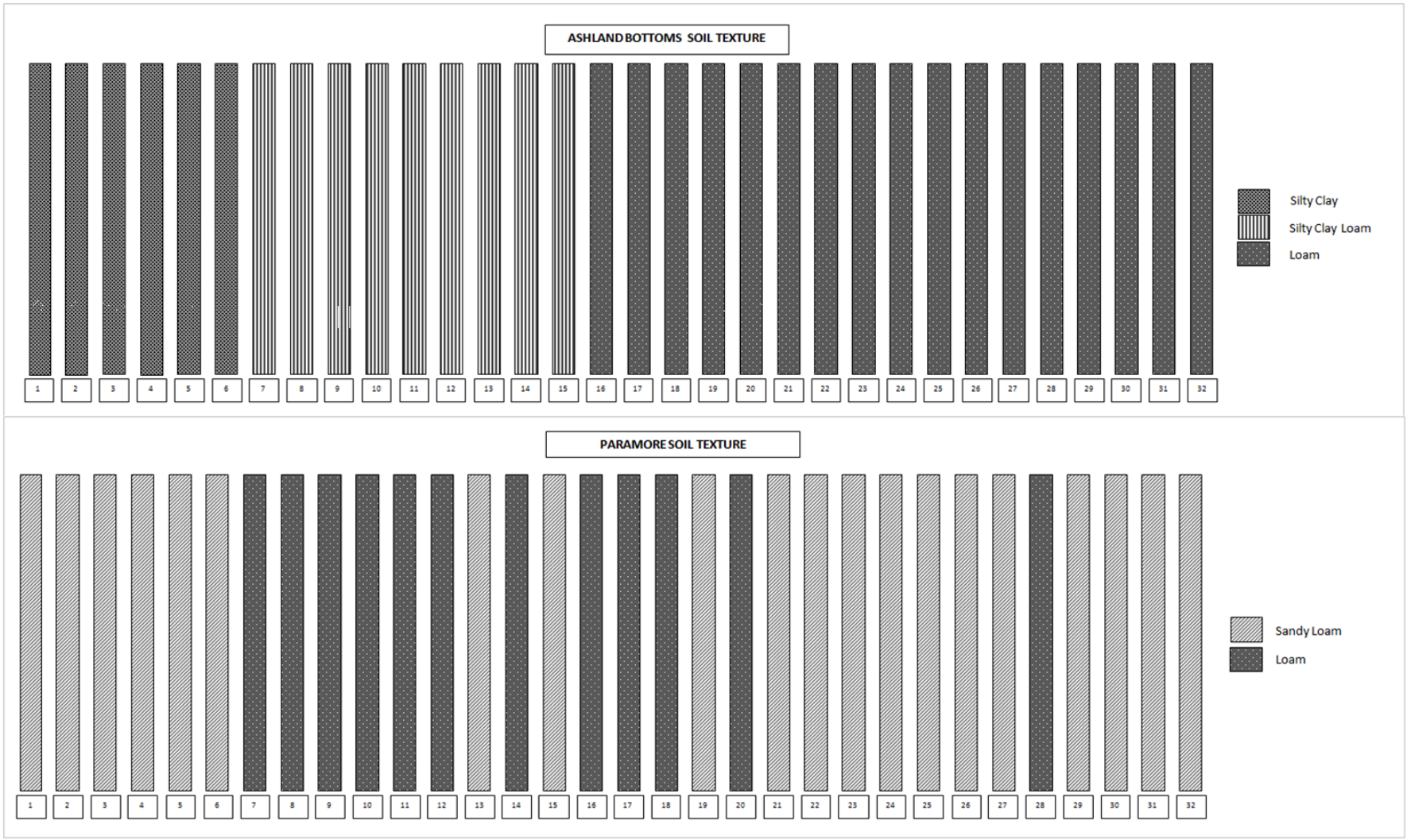

Figure 4-3. Distributions of soil textures in each plot at the field locations in Kansas. Bars and numbers correspond to each plot (from 1 to 32). Soil texture was determined using sodium hexametaphosphate analysis of the 2008 pre-planting soil samples.

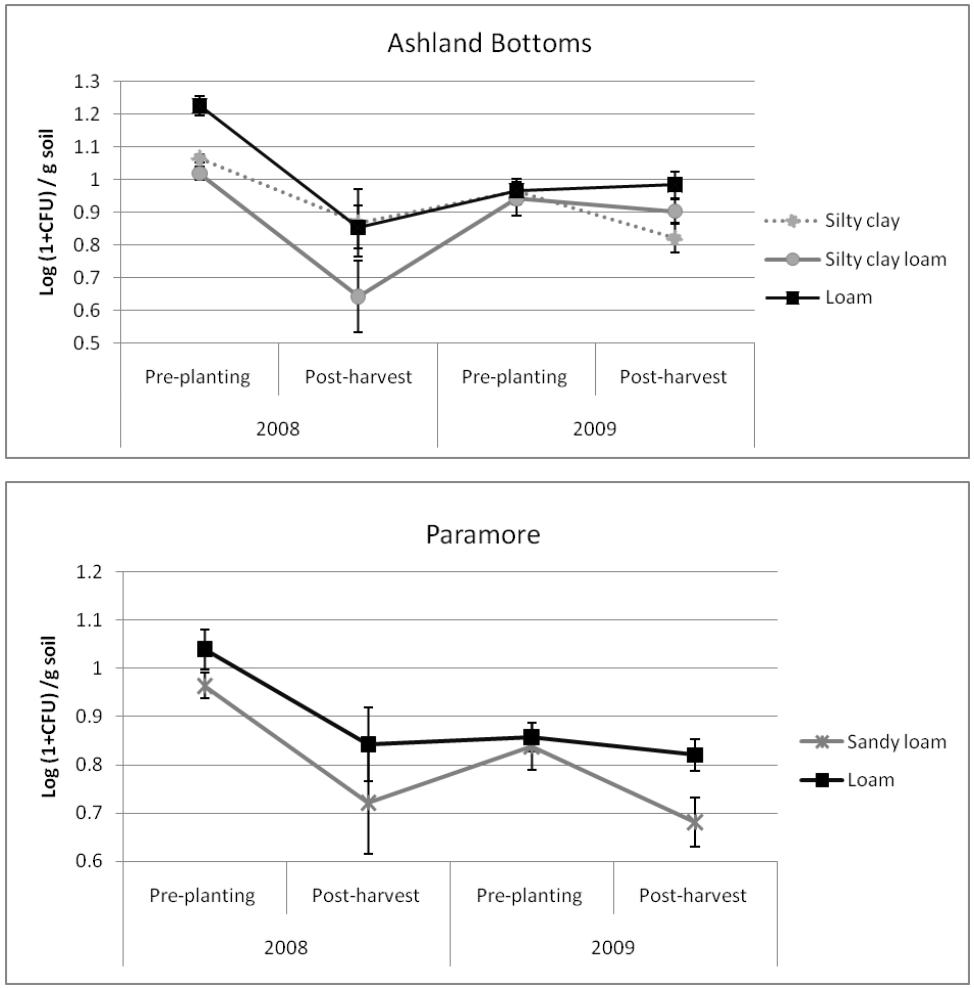

Figure 4-4. Effects of soil texture on *M. phaseolina* soil populations at two locations. Vertical bars represent the standard deviation of the mean.

Effect of soil texture on M. phaseolina root populations and soybean yield

Even though disease symptoms were not observable during the crop season, root infection did occur by the R2-R4 and R8 soybean stages when *M. phaseolina* was assessed. However, root populations at R2-R4 were lower than root populations close to the end of the season, near plant maturity (R8) (Figure 4-5).

In the same manner, there was not a significant difference in root populations across soil textures at R2-R4 stages, but *M. phaseolina* root populations increased at R8 with significant differences across soils textures (Figure 4-5).

According to the results, plant stress associated with low water-holding capacity soils has a great impact on *M. phaseolina* root populations from R2-R4 to R8. Low water holding capacity soils such as loam and sandy loam exhibited the greatest increase in the root population from R2-R4 to R8. On the contrary, clayey soils with high water holding capacity such as silty clay and silty clay loam resulted in the smallest increase in root populations (Figure 4-5).

These results may be attributed to higher water deficits presented in loam and sandy loam soils, producing higher root populations at the end of the crop season. In comparison, plants growing in finer textured soils, such as silty loam and silty clay had lower levels of *M. phaseolina* root infection since these soils offered more favorable soil moisture conditions. Kendig et al. (2008) described a gradual increase in the number of root CFUs under drought conditions from R1 to R8 in which root populations reach a maximum colonization at plant maturity (R8).

Plant yield was impacted significantly by soil texture. The average seed weight obtained from soybean plants used for assessment of root populations and root severity at R8, indicated that soybeans on clayey soil textures yielded more seeds than those on sandy soil textures such as loam and sandy loam soils (Figure 4-6).

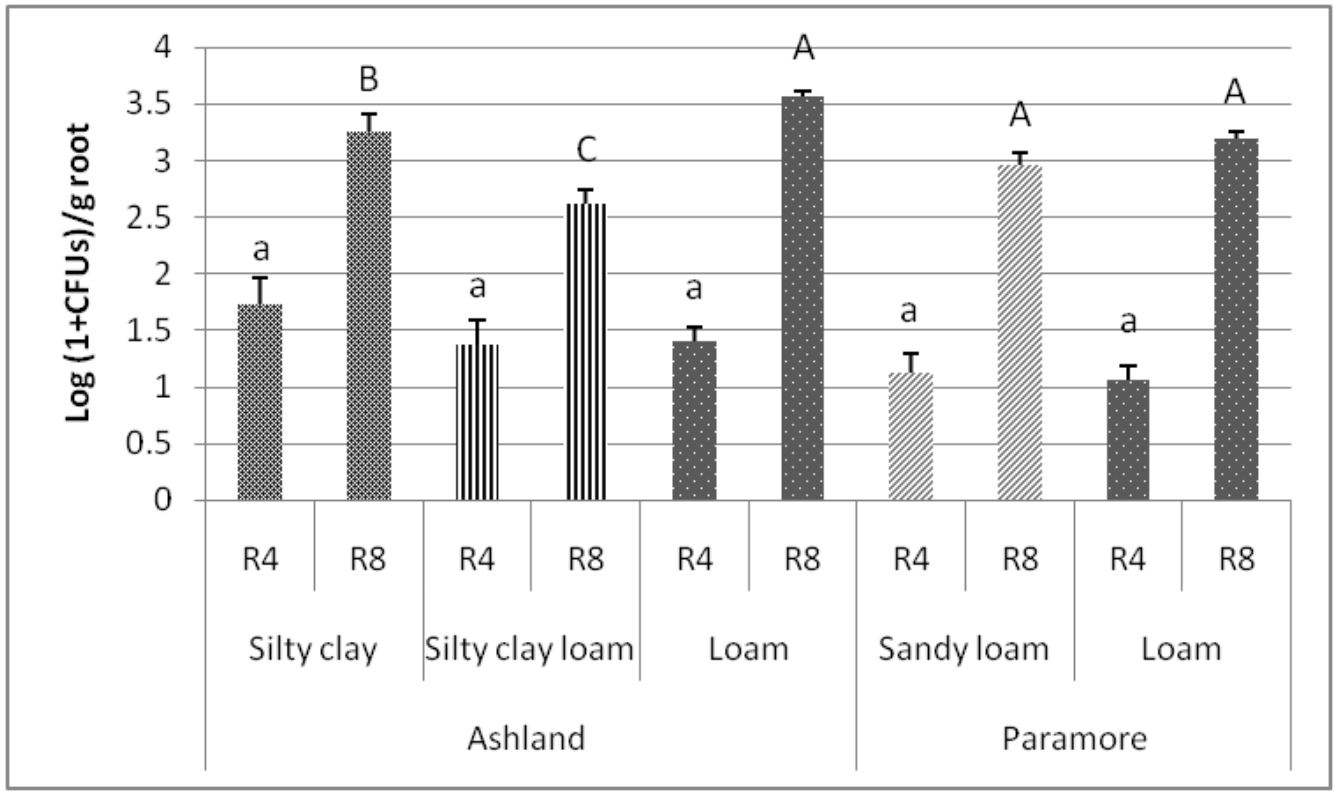

Figure 4-5. Impact of soil type on *M. phaseolina* root populations at R2-R4 ("R4") and R8 soybean stages. Vertical bars represent the standard deviation. Means with the same letter, for a soybean stage, are not significantly different at $p < 0.05$.

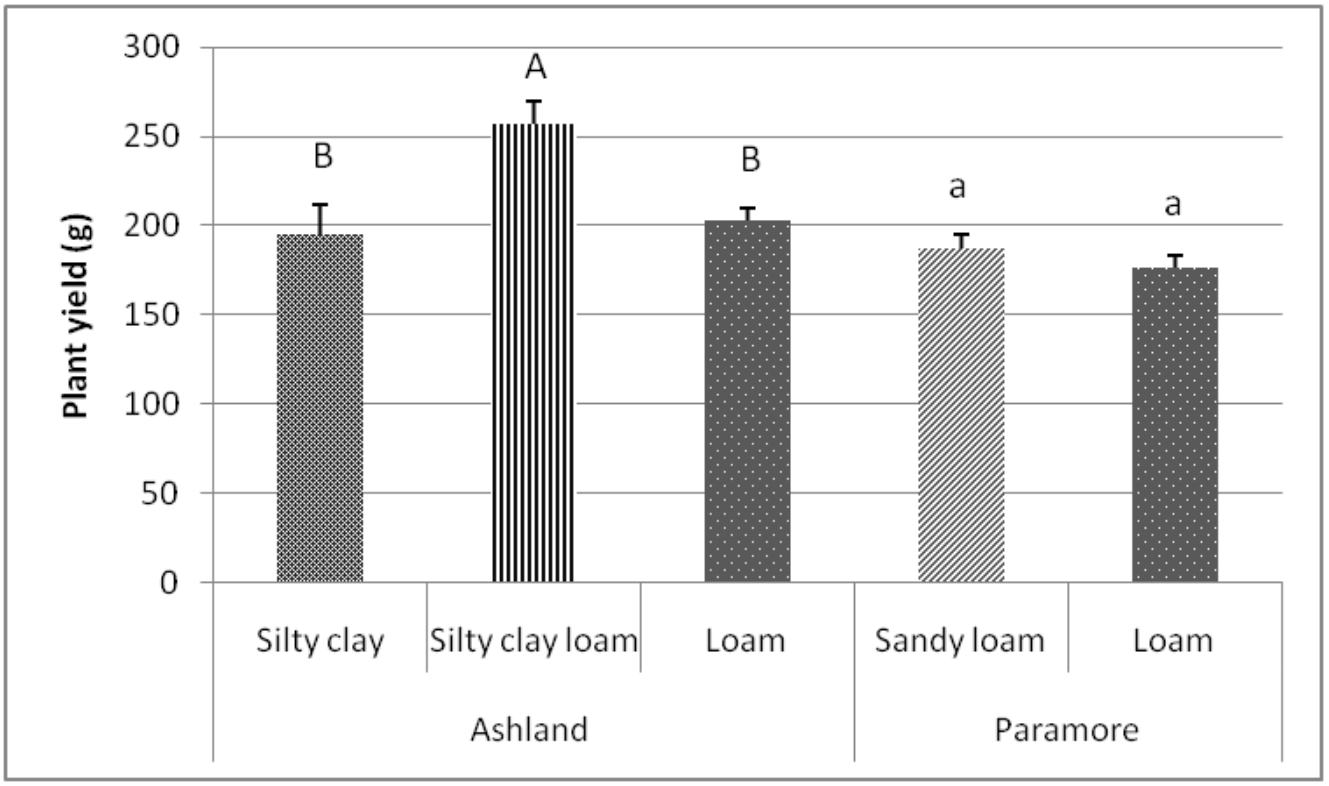

Figure 4-6. Effect of soil texture on soybean plant yield. Plant yield averages were obtained from the seed weight of ten arbitrarily selected soybean plants per plot. Vertical bars denote standard deviation. Means within a location, with the same letter, are not significantly different $p < 0.05$.

A closer view of *M. phaseolina* root populations at R8 and yield indicates a negative relationship. Increases in root populations at R8 lead to a decline in soybean yield, suggesting that yield is affected not only by water stress, but also by root colonization during water deficit (Short et al., 1980).

Effect of irrigation on M. phaseolina root population and yield

The nature of our experiment and the high level of rainfall present in the 2009 did not allow obtaining clear conclusions about the effect of irrigation treatments at different soybean stages on *M. phaseolina* root infection. However, interesting trends could be observed for both locations from yield averages in 2008, in which the following irrigation treatments were performed: non-irrigated, irrigation at R2-R4, and full-season irrigation) (Figure 4-7).

Soybean yields were lower at Paramore than at Ashland Bottoms. This could be due, in part, to soil texture. Coarse-textured soils with low water holding capacity were predominant at the Paramore location, reducing the water availability to the plants and negatively impacting overall yield. In contrast, clayey fine-textured soils, present at Ashland Bottoms, have greater optimum water availability and a reduced water stress impact.

The highest soybean plant yields were obtained from the full-season irrigated treatments in both locations. However, in Ashland Bottoms, non-irrigated plots also showed high yields, suggesting a low effect of water stress on the plants and higher levels of soil moisture during the cropping season. This is due the relatively high amount of moisture and moderate temperatures observed during both the 2008 and 2009 seasons (Table 4-2).

Cropping history effect on M. phaseolina soil and root populations

Plots at the Ashland Bottoms location had a different cropping history (wheat was planted in plots 1 - 20, and soybean was planted in plots 25 - 32) in the previous year (2007) where field experiments were performed (Figure 4 -2). Analysis of variance (ANOVA) showed significant effects of cropping history in the response variables evaluated at Ashland Bottoms (Table 4-4).

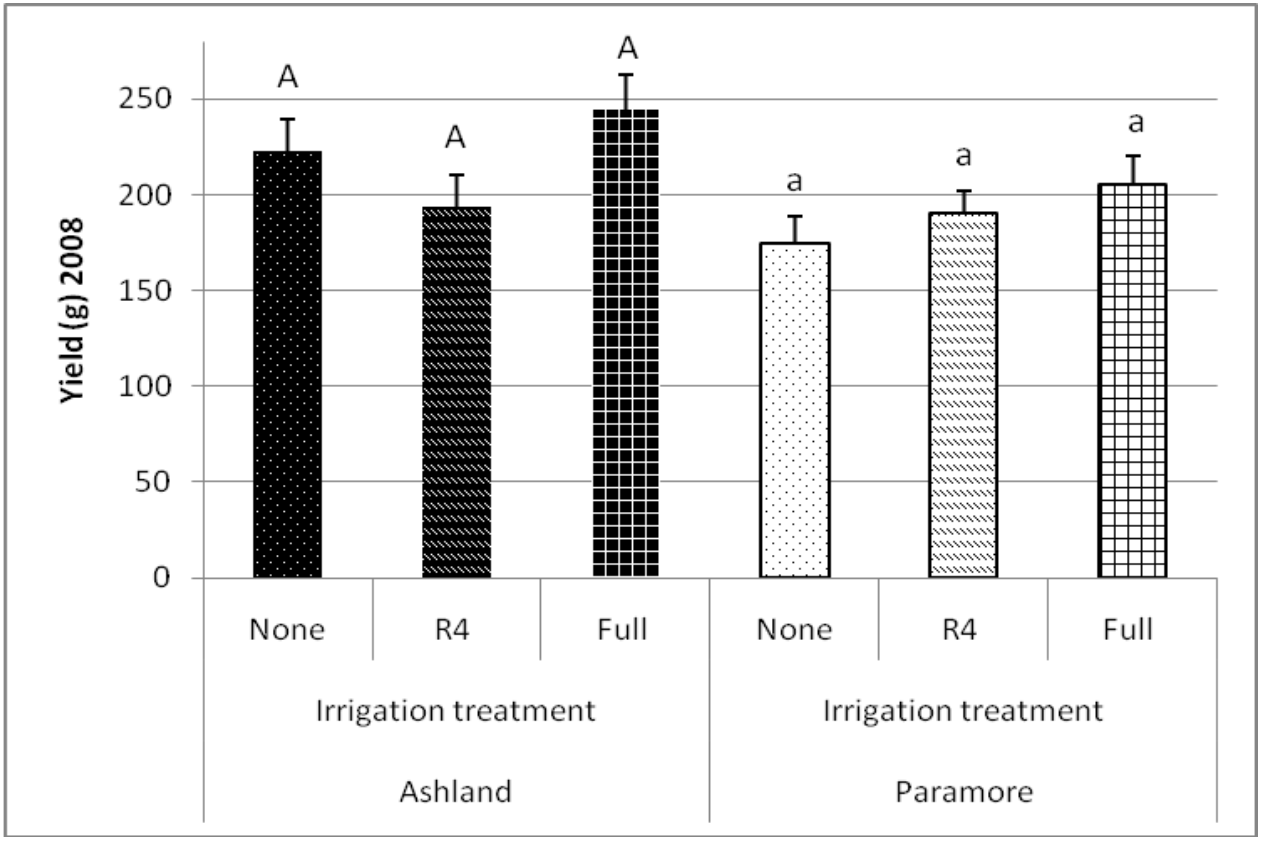

Figure 4-7. Effect of irrigation on plant yield (2008). Plant yield averages were obtained from the seed weight of ten arbitrarily selected soybean plants per plot. Vertical bars denote standard deviation.

Table 4-4. Effect of cropping history on *M. phaseolina* soil and root populations at Ashland Bottoms.

RESPONSE VARIABLES†	Level of significance F - tests	Previous crops (2007)	
		Soybean	Wheat
Initial soil population	***	1.170 a	1.007 b
Final soil population	ns	0.910 a	0.848 a
Root population (R2-R4)	ns	1.449 a	1.456 a
Root population (R8)	***	3.728 a	3.078 b
Root severity (R8)	***	0.554 a	0.462 b
Secondary roots (R8)	ns	1.122 a	1.127 a
Mechanical harvest	ns	1.641 a	1.652 a
Plant yield	*	2.270 a	2.339 b

† Initial soil population = *M. phaseolina* soil population at pre-planting (Log (1+CFUs / g of soil)); Final soil population = *M. phaseolina* soil population at post-harvest (Log (1+CFUs / g of soil)); Root population (R2-R4) = *M. phaseolina* root population (full bloom - full pod) respectively(Log (1+CFUs / g of root)), Root population (R8) = *M. phaseolina* root population at full maturity (1+CFUs / g of root)); Root severity (R8) = Root severity evaluated by scoring intensity of root discoloration from split roots, scales from 1 to 5, where 1 is no microsclerotia visible in the root tissue and 5 is highly darkened the root due to the microsclerotia embedded inside and outside of the root tissue (Log (severity sclale)); Secondary roots (R8) = Number of secondary roots at full maturity (Log(number of roots / plant)); Mechanical harvest = weight of soybean seeds obtained by harvesting with combine in whole plots (Log (seed weight (g))) ; Plant yield = weight of soybean seeds obtained from 10 plants arbitrarily selected on each plot (Log (seed weight (g))). Level of significance from F-tests are indicated by the asterisks, *, **, and ***, which correspond to $p < 0.05$, 0.01 and 0.001, respectively; ns = no significant difference. Different letters denote significant differences between previous crops.

M. phaseolina soil populations were affected by cropping history at the experimental sites. Higher soil populations were found in plots where soybeans were grown previously, compared to soil populations in previously planted wheat. The fact that a *M. phaseolina* non-host was planted during the previous year likely reduced the inoculum survival of *M. phaseolina* in soil.

Soil populations from the non-transformed data indicated a difference of 5 CFUs/g of soil more in the plots where soybeans were planted previously compared to plots previously planted with wheat. *M. phaseolina* survives well in soybean residues in soil. The presence of such residues increase *M. phaseolina* soil populations when microsclerotia are released into the soil as residue is decomposed (Short et al., 1980).

Higher microsclerotia counts were found in soybean monoculture than when soybean was grown every other year or in rotation win a non-host. Further, *M. phaseolina* soil and root

populations increase as soybeans appear more frequently in rotations as reported by Francl et al. (1988).

Soybean crop residues from 2007 may have increased the soil inoculum level in these plots, leading to a higher level of infection that were more evident in later stages of soybean development when soybean have passed through more stress during the crop season This may help explain the fact that there was no effect of previous crops at the R2-R4 soybean stages (Table 4-4).

Detrimental effects on yield were observed in plots where soybeans were planted in previous years. High levels of inoculum and conditions of drought stress due to the nature of the soil texture (loam) in these particular plots predisposed the plants to a higher level of infection compared to the more favorable conditions in the plots where wheat was planted. In addition, some authors have found a direct relation between soil populations and yield reduction (Short et al., 1980).

Conclusions

There was not any effect of manganese soil amendment at pre-planting on suppressing *M. phaseolina* soil and root populations or a positive impact on soybean yield. There was no an accumulative effect of the manganese in soil after the subsequent amendments done at pre-planting in 2008 and 2009, on the contrary, there was a decrease in the manganese content in the soil at Ashland Bottoms location.

Results of *M. phaseolina* soil population dynamics were different for greenhouse experiments and field experiments. In the greenhouse, sandy soils had a negative impact on the fungal population; conversely, in the field studies it was observed that sandier soils had a positive effect on soil populations of the pathogen. Loamy soils (the sandiest soil texture found in the experimental plots) contained the highest populations. However, the cause of this contrary result is likely due to the cropping history present at Ashland Bottoms, which caused significant differences in the soil population.

It is important to take into consideration many variables, including cropping history, that influence the behavior of *M. phaseolina* under field conditions. Soybean planted as a previous crop in Ashland affected the *M. phaseolina* soil populations and consequently the root infection

of soybean plant grown on these plots previously planted on soybeans. On the contrary, a previous crop such as wheat, a *M. phaseolina* non-host, reduced the soil populations and root infection in the soybeans planted the subsequent years. These are important factors that must be taken into consideration as they may affect the analysis or treatment performance of following experiments.

It was not possible to estimate a clear effect of the irrigation treatments planned in the field experiments due to the rainfall presented during the two years of study. However, it seems that the full season irrigation resulted beneficial for the total average yield and also reduced the *M. phaseolina* soil and root populations compared to the non-irrigated treatment.

M. phaseolina root populations and root severity at maturity (R8) presented a highly significant correlation ($p < 0.0001$); high root populations were obtained in roots with high severity by visually rating the scale of discoloration. In summary, the root severity scale (Mengistu et al., 2007) can give a good idea of the level of root population at least in the stage in which the evaluations were performed.

Recommendations

Manganese has been reported to have positive effects on yield and also to suppress root infection by different soil borne pathogens. It was shown in our experiments that manganese soil amendments did not have any impact on yield or *M. phaseolina* soil and root populations. However, it remains the question whether or not other sources of manganese instead of glucoheptonate such as manganese sulfate would have a different outcome for helping in the control of charcoal rot of soybean.

Natural soil populations of *M. phaseolina* vary in the horizontal and vertical soil profile and this could add some complexity to the data analysis in field experiments. To avoid this natural variation that interferes with the proper data analysis of this soil borne pathogen, future experiments may include *M. phaseolina* artificial soil inoculation. Infested Japanese millet is a good quality source of *M. phaseolina* inoculum that can be produced in large quantities and can be incorporated into the soil at pre-planting. This approach may allow the study of charcoal rot of soybean at different levels of inoculum across experimental field.

M. phaseolina soil and root populations estimates and the physicochemical soil data collected in the field experiments during 2008 and 2009 in both locations resulted to be complex and the analysis of variance and correlations may be not enough to have the most proper statistical analysis. However, statistical methodologies such as spatiotemporal modeling or spatial statistics could serve as a better approach for data analysis since data were collected over time in a spatial pattern. In addition, natural soil conditions can vary enough in an experimental field, as it was the case for the soil texture in the locations where the field experiments were performed, in which conventional statistics (ANOVAs) are not a proper way to analyze this level of complexity.

Future experiments should explore charcoal rot of soybean under controlled conditions in later vegetative stages to estimate the effect of soil volumetric water content and different soil textures on *M. phaseolina* soil populations and root infection in longer periods of time. It may also be important to establish amendments of manganese fertilizer under greenhouse conditions in pots with soils infested with *M. phaseolina* to observe its impact on the fungal soil populations and root infection and probably elucidate what are the best environmental conditions in which manganese could be an option for the control of charcoal rot of soybean.

Soil steaming is not enough to get rid of all soil inhabiting fungi, for this reason when studying *M. phaseolina* soil populations it is going to be imperative for future experiments to assess the soil populations of other soil microorganisms that may suppress the viability of microsclerotia in soil; this may help to get a more clear interpretation of *M. phaseolina* soil population dynamics.

M. phaseolina soil populations and root infection of soybean should be evaluated in a more broad range of soil textures. Including soil textures such as clay, sandy clay and clay loam in the experiments may allow to assess the impact of other physicochemical soil properties linked to soil texture such as organic matter, cation exchange capacity and water holding capacity that affect the levels of root infection and soil populations. Other soil properties that impact charcoal rot diseased processes such as soil bulk density and level of soil oxygen should be taken in consideration in future experiments since *M. phaseolina* is considered to be a facultative anaerobe and its survival in soil is affected by oxygen concentrations.

Since we were able to set variables such as *M. phaseolina* inoculum and soil volumetric water content accurately in the experiments, it should be good idea to explore the response of

different soybeans varieties to charcoal rot in these experimental conditions. A screening of soybean varieties resistant to drought with some degree of moderate resistance to charcoal rot may be of interest to growers who struggle with the consequences of climatic change, high temperatures and drought.

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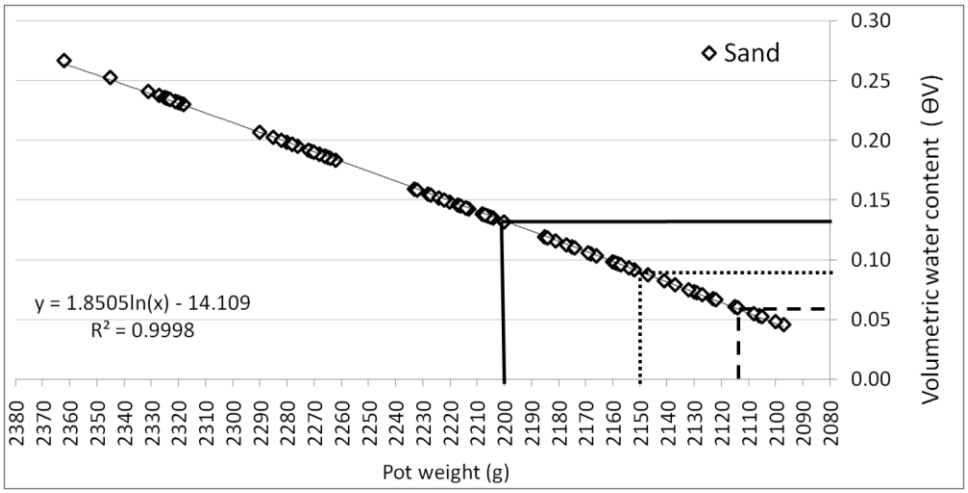
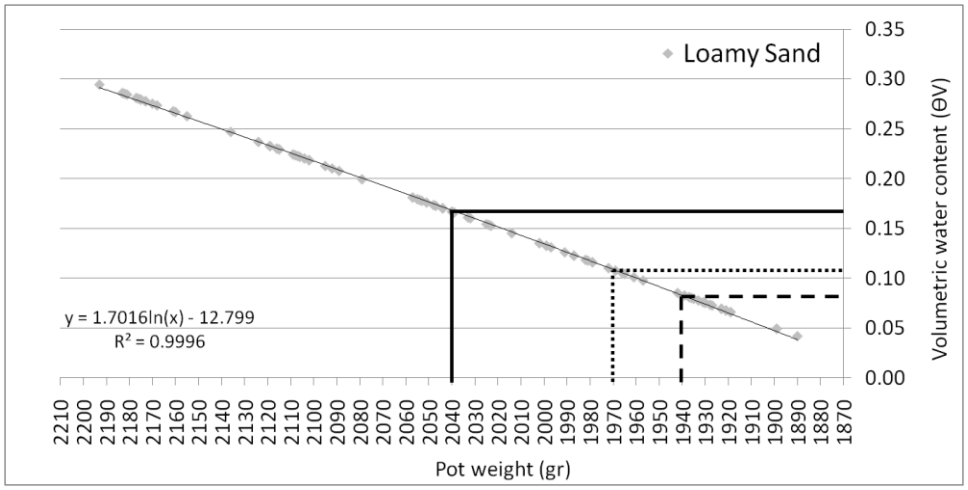
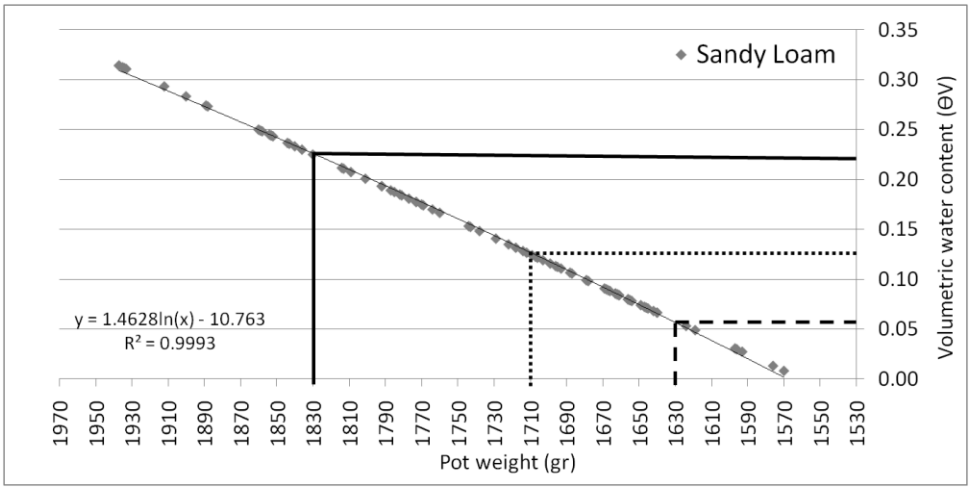
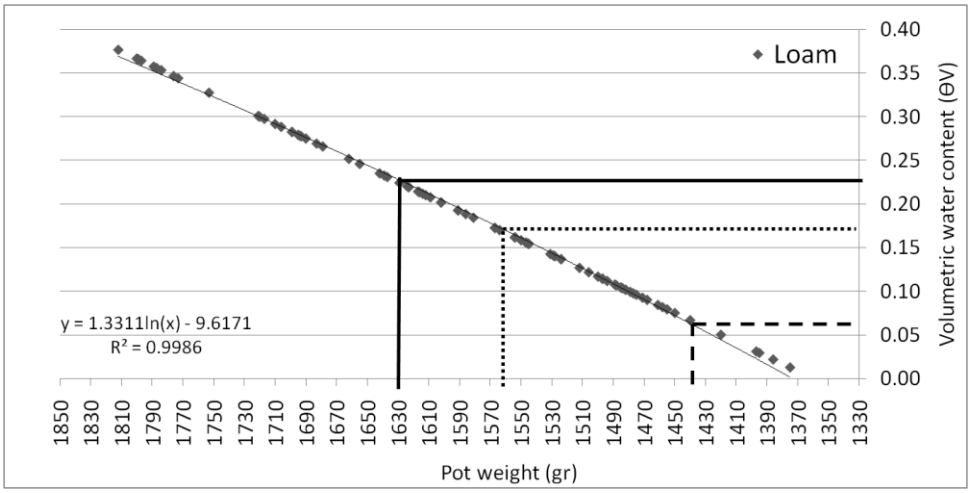
Appendix A - Calibration curves of soil volumetric water content in different soil textures in pots.

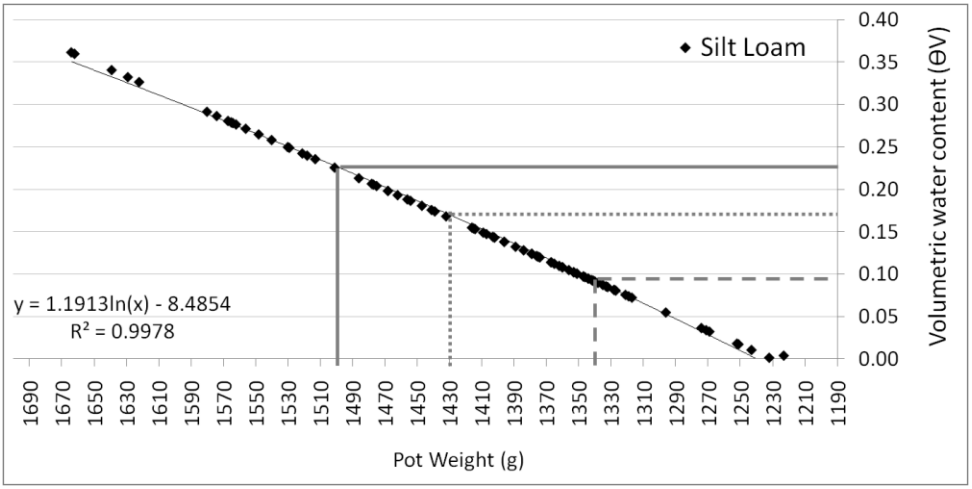
Calibration curves were constructed to reach tree different soil volumetric water contents pot saturation (—PS), pot capacity (···PC), permanent wilting point (---PWP) in five soil textures (sand, loamy sand, sandy loam, loam and silt loam).





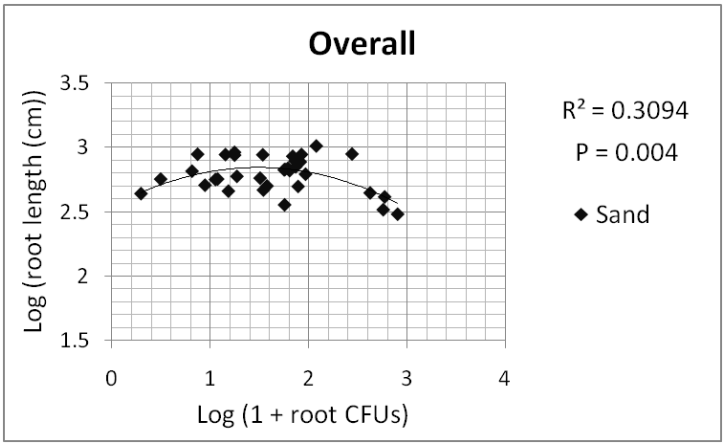

Sandy Loam
y = 1.4628ln(x) - 10.763
R² = 0.9993
Volumetric water content (ΘV)
Pot weight (gr)


Loam
y = 1.3311ln(x) - 9.6171
R² = 0.9986
Volumetric water content (ΘV)
Pot weight (gr)

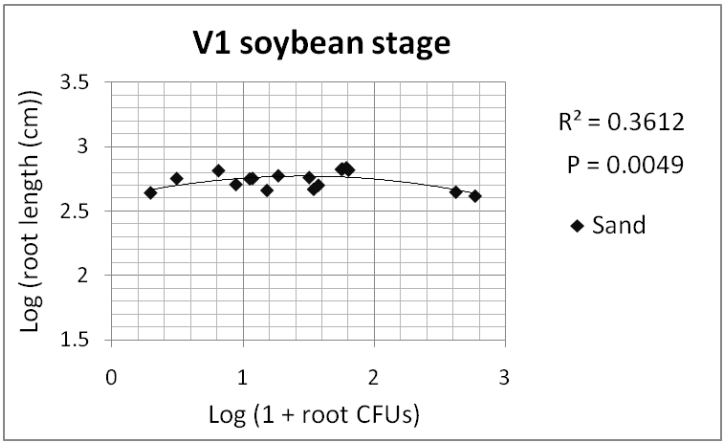

Silt Loam
y = 1.1913ln(x) - 8.4854
R² = 0.9978
Volumetric water content (ΘV)
Pot Weight (g)

Appendix B - Relationships between root length and *M. phaseolina* root infection

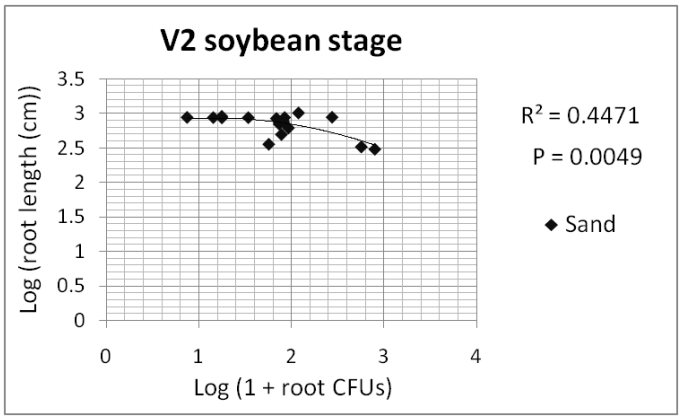
Second grade polynomial regressions between soybean root length and root infection split by soybean stages V1 and V2 and soil volumetric water contents in sand texture.



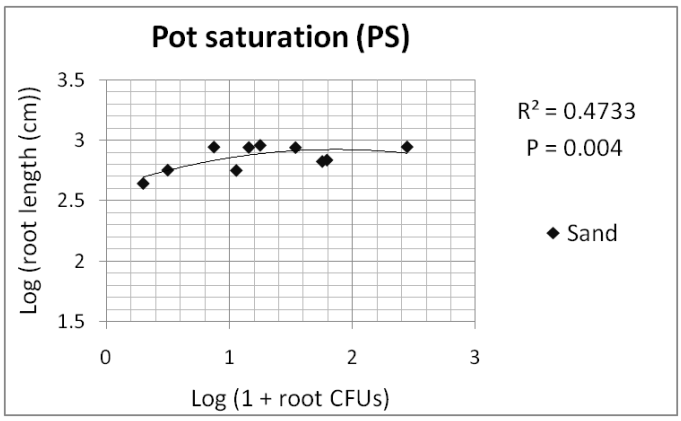
Overall regression includes data from V1 and V2 soybean stages, soil volumetric water contents pot saturation (—PS), pot capacity (···PC), permanent wilting point (---PWP) on *M. phaseolina* infested soils.



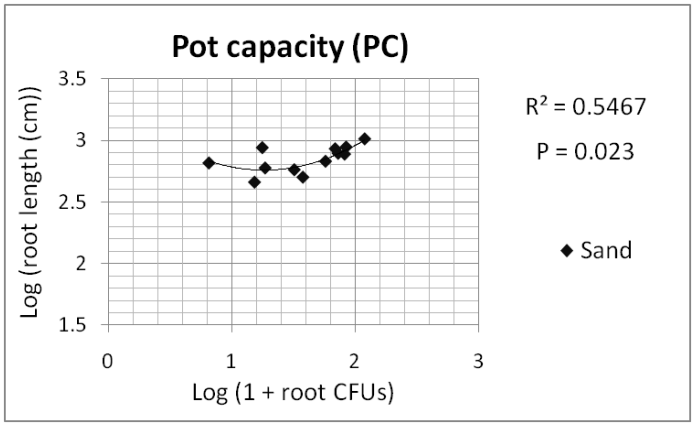
V1 soybean stage polynomial regression includes data from soil volumetric water contents pot saturation (—PS), pot capacity (···PC), permanent wilting point (---PWP) on *M. phaseolina* infested soils.



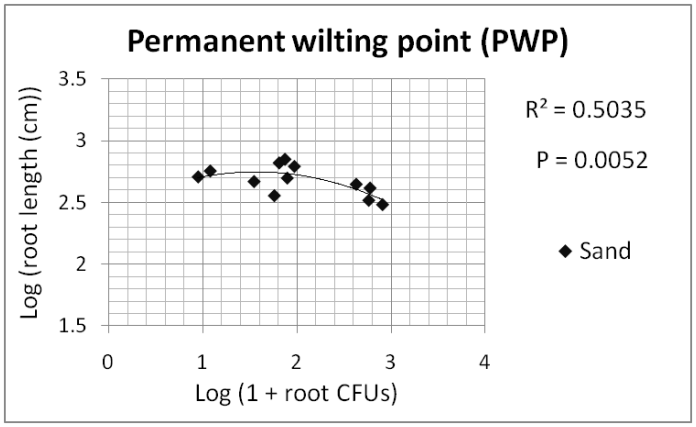
V2 soybean stage polynomial regression includes data from soil volumetric water contents pot saturation (—PS), pot capacity (···PC), permanent wilting point (---PWP) on *M. phaseolina* infested soils.



Pot saturation (PS) polynomial regression includes data from soybean stages V1 and V2 on *M. phaseolina* infested soils.



Pot capacity (PC) polynomial regression includes data from soybean stages V1 and V2 on *M. phaseolina* infested soils.



Permanent wilting point (PWP) polynomial regression includes data from soybean stages V1 and V2 on *M. phaseolina* infested soils.