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Histopathology of charcoal rot disease (*Macrophomina phaseolina*) in resistant and susceptible cultivars of soybean

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ABSTRACT

Charcoal rot caused by the fungus, *Macrophomina phaseolina,* is one of the most important fungal diseases affecting soybean (*Glycine max* (L.) Merr.) production worldwide, especially under climate change scenarios. The infection events including microsclerotia germination, hyphal penetration, and pathogen colonization into the infected root tissues of two soybean cultivars, Williams (susceptible) and Hadgeston (resistant), with reported differences in the level of resistance to *M. phaseolina*, were investigated by histological analyses. The soybean plants were infected by immersing the roots in microsclerotia suspension (1 g of microsclerotes in 300 ml of agarose 0.015%). Pathogen penetration took place through the roots epidermal cells 3 days after inoculation. The observations revealed that pathogen's pre-penetration steps, including microsclerotia germination and hyphae development, are not linked to resistance as these events occurred as the same in both cultivars. However, in the post-penetration steps, there was a significant difference between two cultivars in terms of root colonization by *M. phaseolina* and disintegration of root tissues. The appearance of adventitious roots in the resistant cultivar in response to the pathogen, and infection of secondary roots at the cell differentiation stage as well as the inability of the pathogen to complete its life cycle in the resistant cultivar, are the most significant findings of this study.

1. Introduction

Charcoal rot, caused by the fungus *Macrophomina phaseolina* (Tassi) Goidanich, is a root and stems disease of soybean that develops in tropical and semi-tropical regions during the mid to late summer when plants are under heat and drought stress (Gupta et al., 2012). *M. phaseolina* has a wide host range and in addition to soybean (*Glycine max* (L.) Merrill) it is responsible for causing economic losses on sesame (*Sesamum indicum* L.), safflower (*Carthamus tinctorius* L.), sunflower (*Helianthus annuus* L.) and more than 500 cultivated and wild plant species in 75 families (Islam et al., 2012).

M. phaseolina is a monotypic seed- and soil-borne pathogen and is able to infect soybean plants at different growth stages during the growing season (Saleh et al., 2010). It mainly produces either microsclerotia (primary source of inoculum) or pycnidia (Purkayastha et al., 2006). The pathogen life cycle begins with microsclerotia germination into the soil (Fig. 1). The previous studies on the effect of temperature on microsclerotia germination showed that the maximum germination occurs at 30–33 °C (Viana and De Souza, 2002). Under favorable environmental conditions (low water potential and high soil temperature) and in the presence of the host plants, microsclerotia germinate and produce a mass of hyphal threads. The hyphae grow towards the host's roots and colonize the seedlings roots during the first weeks of seed germination (Reyes-Franco et al., 2006). These initial stages of pathogen invasion have no visible symptoms in plants aerial organs as the pathogen remains latent (Pratt, 2006). When plants approach the end of the growing season and pathogen enters into its necrotrophic phase, plants show symptoms like wilting and necrosis due to blockage of vascular bundles with microsclerotia, enzymes activity and secretion of pathogenic toxins (Gupta et al., 2012). The most diagnostic symptom of charcoal rot in invaded soybean plants is the black and dusty speckled appearance of microsclerotia on stems, pods and seeds as well as interior tissues like vascular, cortical, and pith tissues (Mengistu et al., 2011).

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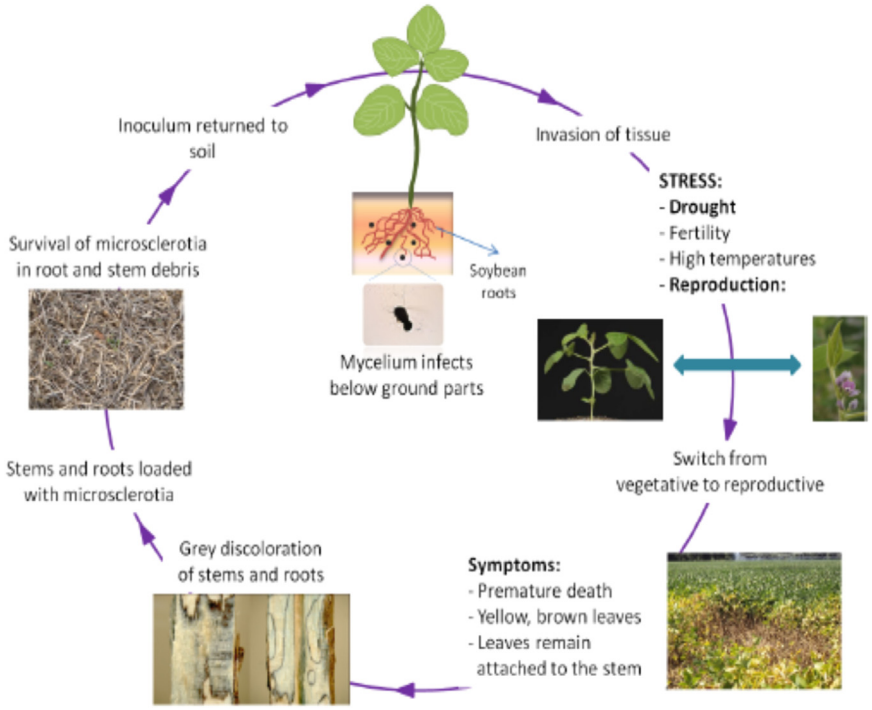

Fig. 1. : Life cycle of *Macrophomina phaseolina* on soybean plant (Wrather et al., 2007).

There are limitations in controlling the disease due to being caused by a soil-borne pathogen that is able to produce too much microsclerotia in the soil (Naseri, 2008). Moreover, excessive use of chemical pesticides not only can control the disease but also may lead to the appearance of resistance genes and causes environmental pollution (Muthomi et al., 2007). Therefore, production and cultivation of resistant cultivars are generally regarded as the most appropriate and feasible approach for controlling charcoal rot.

Understanding the relationships between host and pathogen during the development of the disease will help breeders to identify varieties with promising genetic composition and disease resistance for their breeding programs. Therefore, histopathological and histochemical studies on *Macrophomina*-induced diseases, or, in other words, understanding host-pathogen interaction at cellular or tissues level, is necessary before scheduling any breeding program. Although several techniques have been used in the study of plant-microbe interactions, histopathological analyses still remains efficient in examining infection events of pathogenic fungi in plants as they contribute information of the pathogen (e.g., stages present, their location and establishment in the cells) and its influence on host plants development (Bhuiyan et al., 2015). To date, numerous studies have been conducted on *M. phaseolina* (Mengistu et al., 2007; Mengistu et al., 2011), and its variations in morphology and pathogenicity (Purkayastha et al., 2003), however, a few studies have examined its infection process in soybean roots. Moreover, little studies have been performed with the aim of histopathological comparison between resistant and susceptible soybean cultivars. The most recent study carried out by Bressano et al. (2010) have developed a new *in vitro* method to examine the pre-penetration and the penetration phases of *M. phaseolina*. Thus, no other comprehensive studies have tried to do histological analyses to characterize microsclerotia germination, hyphal penetration, and pathogen colonization in soybean plants since 1970s. In the current study the resistant and susceptible soybean cultivars were compared at the cellular level. The aim of this study was to use light microscopy and histopathology to determine the infection process and life cycle of *M. phaseolina* in soybean roots to provide a better understanding of the cause and mechanism of resistance or susceptibility in susceptible and resistant soybean cultivars.

2. Materials and methods

2.1. Plant material and growing conditions

In this study, two soybean cultivars (Williams and Hadgeston) were selected based on their different reactions to the disease from our previous study (Hemati et al., 2013). The soybean seeds were surface-sterilized using 5% sodium hypochlorite for 5 min and rinsed several times with sterile double distilled water for the next 5 min. The seeds were sown in plastic pots containing sterile potting mix (BVB Peat Moss, sterilizing with steam, 30 min at 82 °C (Griesbach et al., 2012)). Plants were grown in greenhouse under natural light supplemented with a daily 12 h:12 h light:dark photoperiod from metal halide and high pressure sodium lamps providing 250 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at canopy height. Supplemental lighting was provided during the first and last 3 h of the daily photoperiod. Greenhouse temperature was maintained at 27 °C (days) and 14 °C (nights) with 40–50% relative humidity (Riedell and Catangui, 2006). The plants were hand irrigated daily until field capacity was reached. The field capacity was determined by slowly saturating four soil-filled pots until water started to drip from the bottom (Gholamhoseini et al., 2018).

2.2. Fungal materials and inoculation

Different isolates of *M. phaseolina* were isolated from naturally infected soybean plants collected from major soybean fields of Iran, and used for inoculations. In this study, So.3 isolate of soybean was chosen for root inoculation of soybean plants, according to the previous work (Hemati et al., 2013). Infected stems were cut into 5–10 mm pieces and then surface-disinfected in 2% sodium hypochlorite for 2 min, rinsed several times with sterile double distilled water, and then placed on potato-dextrose agar (PDA; AoBoXing, Bio-tech, Beijing, China) acidified with lactic acid and supplemented with 50 mg l^{-1} streptomycin sulfate to inhibit bacterial growth. All the plates were incubated at

25 °C for 4 days. A single colony was sub-cultured on glucose-agar media for 6 days, and in darkness at 30 °C. A small piece of a colony containing microsclerotia was re-suspended in 50 µl of sterile double distilled water to separate microsclerotia from mycelia and them grown on PDA media at 30 °C. Finally, microsclerotia were collected by placing autoclaved toothpicks on *M. phaseolina* colonies growing on PDA media for 6 days. The toothpicks containing microsclerotia were placed in microtubes and kept in the fridge at 4 °C (Edmunds, 1964). In order to prepare inoculum 1 g of microsclerotia was suspended in 300 ml 0.01% agarose solution (Reyas Gaige et al., 2010).

The root immersion inoculation method was used to inoculate seedlings of soybean plants (Reyas Gaige et al., 2010). Sixty-five days after seed sowing at the V6 growth stage, soybean seedlings were extracted from the pots, and then potting mix was gently removed from the roots by washing with deionized water, avoiding any damage to the roots. Then roots were immersed for 1 h in the microsclerotia suspension before the seedlings were transplanted in new pots. To ensure the microsclerotia will grow, while placing the roots in the suspension, a sample of microsclerotia was grown on the PDA media and microsclerotia was monitored daily by placing a drop of suspension on a slide and examined under an optical microscope.

2.3. Sampling and microscopic studies

On the second day, the microsclerotia started to germinate, so sampling was started 3 days after inoculation to ensure that the pathogen uniformly penetrated the roots. Ten plants of each cultivar were harvested every day for two weeks for root microscopic studies. For this purpose, the plants were extracted from the pots and the potting mix was removed from the roots by washing with deionized water. Then ten roots were cut into 2–3 mm pieces and soaked in 0.5 M sodium hydroxide to soften the tissues for 3 min. Lactophenol cotton blue was used to stain the fungal mycelia for easier microscopic identification. Root segments were placed on a microscope slide and then a coverslip was placed over the root sections and pressed firmly to facilitate analysis at high magnification. The specimens were examined using an optical microscope (Eclipse 50i, Nikon Instruments Inc., magnification × 100 to × 400) equipped with a digital camera connected to a computer.

3. Results

3.1. Microsclerotia germination

Early observations indicated that before germination, microsclerotia were attached firmly to the surface of the roots. Attachment of microsclerotia is essential for the successful establishment of the pathogenesis of *M. phaseolina*. The observations indicated that there was no noticeable difference between susceptible and resistant cultivars in terms of microsclerotia germination time. In addition, number and density of mycelia produced by microsclerotia during the initial stage of microsclerotia germination were found to be the same in both cultivars (Figs. 2a and 2b). From these findings, it appears that the initial stages of infection including microsclerotia attachment to the roots surface, germination and germ tube formation happen in the same way in both susceptible and resistant cultivars. Therefore, it can be concluded that these events are not linked to resistance or susceptibility.

3.2. Infection and hyphal penetration

Despite the similar reaction of both cultivars to the pre-penetration stages of the pathogen into the roots tissue, the subsequent stages of penetration or infection stage showed an obvious difference between two cultivars. The infection process initiated with the development of hyphae around the roots surface during the first four days of inoculation. Although microsclerotia germination, hyphae formation, and

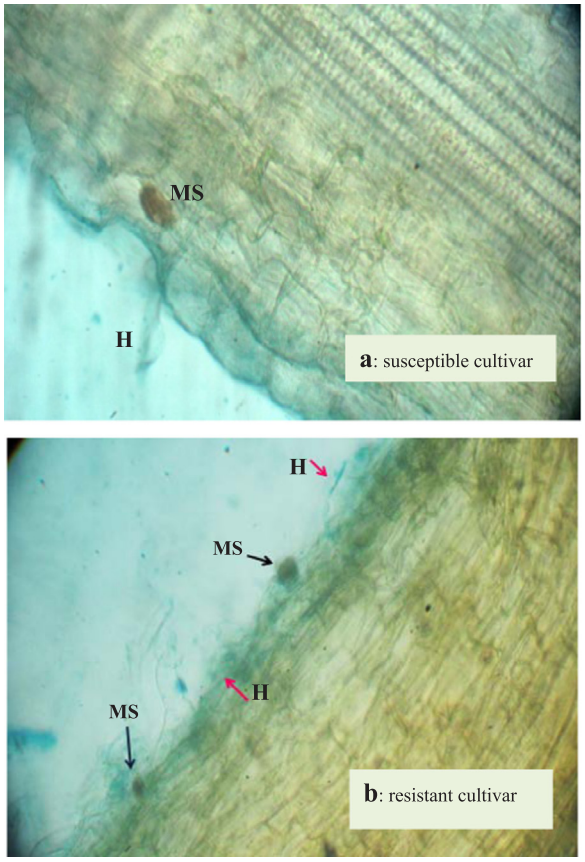

Fig. 2. : Comparison between susceptible and resistant soybean cultivars in terms of *M. phaseolina* microsclerotia germination and hyphae development. **a**: susceptible cultivar, **b**: resistant cultivar, **MS**: Microsclerotia attached to the roots surfaces, **H**: The generated hyphae on the roots surface. Magnification 60×.

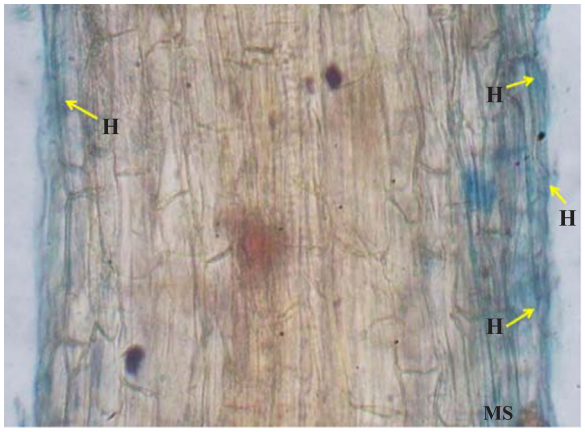

Fig. 3. Hyphal penetration into the epidermal cells and cortex of susceptible cultivar 4 days after inoculation with *M. phaseolina* that was observed only in the susceptible cultivar. **MS**: Microsclerotia attached to the roots surfaces, **H**: The generated hyphae on the roots surface or penetrated hyphae into the epidermal cells and cortex. Magnification 60×.

development on the soybean roots took place similarly in both cultivars (Figs. 2a and 2b), hyphae penetration was observed (4 days after inoculation) only in the susceptible cultivar (Fig. 3). The penetration into the lateral roots was similar to that of the main roots. According to the histopathologic observations, hyphal penetration into the roots of susceptible cultivar occurred shortly after microsclerotia germination, whereas in the resistant cultivar no penetration was observed during

the same period of time (Fig. 2b). The hyphae penetrated directly into the epidermal cells and cortex. In general, these findings suggest that microsclerotia germination-related events are not connected to the cultivar, however, the time it takes to penetrate into the roots tissue and start the process of infection, differs from cultivar to cultivar. To infect the host plants, the pathogen must be attached to the root's surface and then penetrate into the inner tissues after passing through the protective layers such as cell wall. Therefore, the cell wall is the first protective barrier against the pathogen (Baird et al., 2003). At this stage, the thickness of the cuticle layer and cellulose cell wall of the epidermal cells play a key role in preventing pathogen penetration into the tissues (Agrios, 2005). With regard to the above, the thickness of the cuticle layer or cell wall may cause the later pathogen penetration into the root of resistant cultivars. However further studies are needed to confirm these findings.

3.3. Pathogen colonization into infected inner tissues

Histopathological findings, observed on 5th, 6th, 7th, 8th and 9th days after inoculation, confirmed that the pathogen colonized roots intercellular spaces in the susceptible cultivar. During early stages of colonization, in the susceptible cultivars, the pathogen continued growing and spreading into adjacent cells so that more hyphae were observed in intercellular spaces of epidermal cells rather than intracellular spaces (Fig. 4a). By contrast, in the resistant cultivar, the development of hyphae was more limited to intracellular spaces, in other words, the development of hyphae in intercellular spaces and their expansion into the adjacent cells was much slower and less in comparison to susceptible cultivar (Fig. 4b). The necrotic symptoms on the surface of the root cells in both cultivars were observed 7 days after inoculation, however, these symptoms were considerably more severe in the susceptible cultivar compared with resistant cultivar (Figs. 5a and 5b). At the same time, leaf chlorosis was observed in the susceptible cultivar but no visible change appeared on the leaves of the resistant cultivar. In the susceptible cultivar, the hyphae reached the vascular tissues (stele) within 9 days after inoculation, whereas the hyphae development in the resistant cultivar was only limited to several layers of epidermal cells (Fig. 6a, b and c).

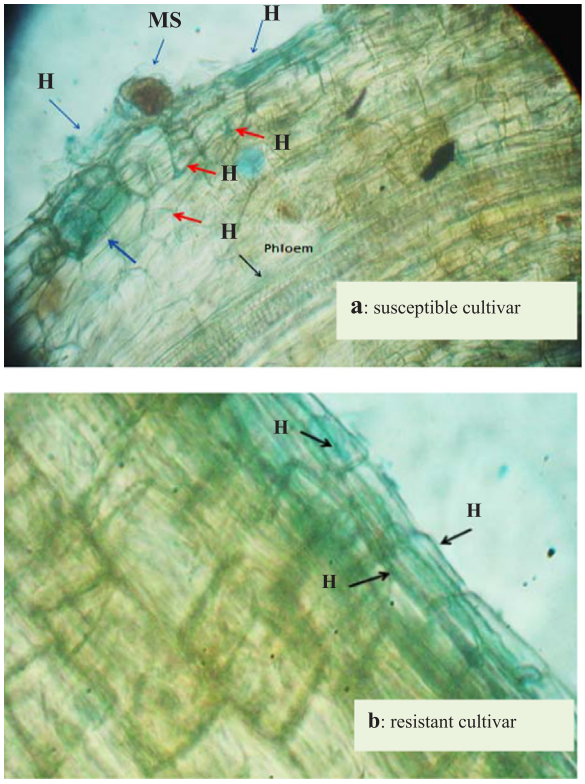

Fig. 4. : Six days after inoculation with *M. phaseolina*, the pathogen colonized roots intercellular spaces in the susceptible cultivar, whereas the hyphae development in the resistant cultivar was only limited to several layers of epidermal cells. **a**: susceptible cultivar, **b**: resistant cultivar, **MS**: Microsclerotia attached to the roots surfaces, **H**: The generated hyphae on the roots surface or penetrated hyphae into the epidermal cells and cortex. Magnification 60×.

3.4. Hyphae development and adventitious roots formation

On the 9th day of inoculation, the density of hyphae in the sensitive cultivar significantly increased in some parts of the roots, especially near the stele (Fig. 7a). By contrast, no considerable change in hyphae development and density was observed in the resistant cultivar and hyphae colonization was only limited to several layers of epidermal cells (Fig. 7b). Irrespective of the lack of successful colonization at the microscopic level, the most important and interesting macroscopic observation in the resistant cultivar was the formation of adventitious roots on the crown of soybean plants, a phenomenon that was not observed in susceptible cultivar (Fig. 8). In the susceptible cultivar, wounds appeared on the crown.

3.5. Microsclerotia formation

In the susceptible cultivar, the brown colored hyphae that formed around the stele as a visual indicator of microsclerotia formation, appeared 12 and14 days after inoculation (Figs. 9a, 9b, 9c and 10) whereas, in the resistant cultivar no change was observed.

4. Discussion

In the current study, the selected resistant cultivar showed higher resistance to *M. phaseolina*. Considering the penetration of the pathogen into the roots tissue of the resistant cultivar and the spreading of the hyphae into the host tissues (Figs. 2b and 4b), it can be concluded that there is no complete resistance to *M. phaseolina* in soybean cultivars. Given that resistance is the ability of a host to protect itself from a pathogen or reduce pathogen establishment but tolerance is the ability of a host to deal with a given established pathogen, so “tolerance” is a better term to describe cultivars that survive pathogen exposure.

The comparative study of infection events in susceptible and tolerant soybean cultivars revealed differences in pathogen's behavior in two cultivars. According to the observations, the infection process (from microsclerotia germination on the roots surface to the appearance of new microsclerotia in stele region) in the susceptible cultivar completed 14 days after inoculation. During this period, disease symptoms such as leaf chlorosis, wilting and necrosis on the crown and the upper region of taproot were observed in the susceptible cultivar, whereas in the resistant cultivar, the pathogen could only penetrate into the roots epidermal cells and cortex.

After colonization of epidermal and cortical cells, the pathogen colonized deeper tissues such as mesophyll tissues in the susceptible cultivars. This developmental pattern has been observed in chickpea (Singh et al., 1990). Histopathology studies have shown that hyphae penetrate directly into the epidermal cells and then further move toward the mesophyll tissues intercellularly. Direct penetration has also been reported in fungi like *Phoma medicaginis* in alfalfa leaf (Castell-Miller et al., 2007), *P. clematidina* on clematis (*Clematis* spp.) leaf surface (Van de Graaf et al. 2002) and *Stagonospora nodorum* in wheat leaf (Solomon et al. 2006). Generally, microsclerotia form appressoria over host epidermal cells, developing hyphae which enter between epidermal cells. The hyphae grow inter and intracellularly, and attack cells by mechanical or enzymatic action. Intracellular colonization occurs after lamella and cell wall disintegration (Ammon et al., 1974; Ammon et al., 1975). Following epidermal and cortex invasion, *M. phaseolina*

Fig. 5. : Necrosis symptoms on the roots surface of susceptible (**a**) and resistant (**b**) cultivars 7 days after inoculation. Necrosis lesions (shown by arrows) caused by disease progression were more noticeable in the susceptible cultivar and were associated with leaves yellowing. Aboveground symptoms in the resistant cultivar were not distinctive and there were limited lesions on the roots surface.

colonizes the vascular system developing microsclerotia on xylem vessels which may lead to their blockage. Xylem vessel blocking causes wilt symptoms (Ilyas and Sinclair, 1974).

In the resistant cultivar, unlike the susceptible cultivar, the hyphae penetrated into intracellular spaces (Fig. 4a and b). Therefore, we can postulate that intracellular development of hyphae in the resistant cultivar, as a barrier, hinders rapid colonization of the pathogen through intercellular space of the plant's roots. However, further complementary investigations are necessary to fully understand the role of non-specific barriers (e.g. thicker cellulose cell wall or pectin layer) as

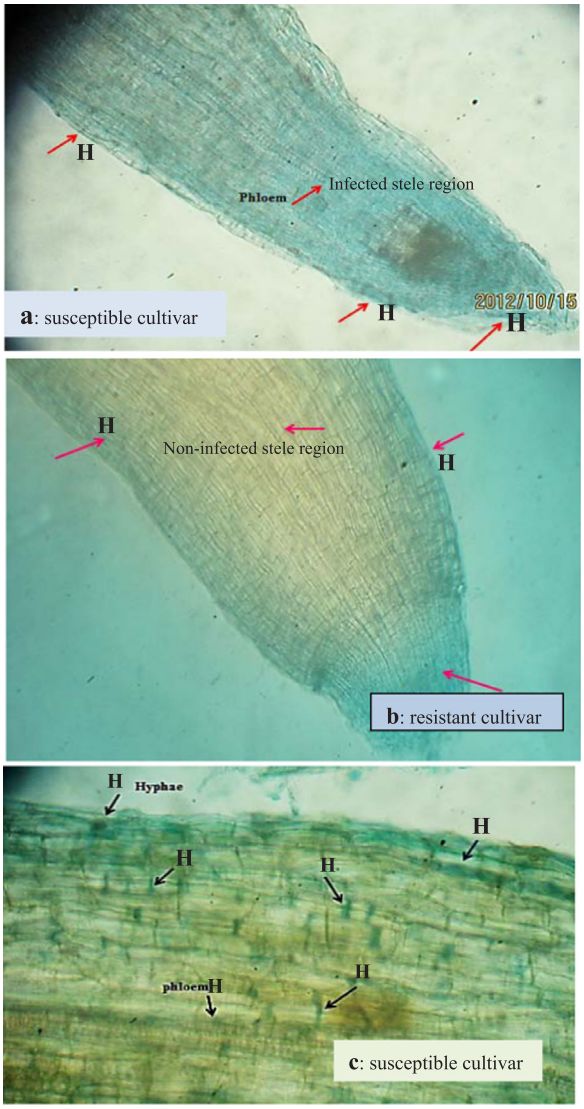

Fig. 6. : Comparison between susceptive (a) and resistant (b) cultivars in terms of root colonization by *M. phaseolina*. **c**: nine days after inoculation with *M. phaseolina* and development of hyphae in intercellular spaces in susceptible cultivar. In the susceptible cultivar, the hyphae reached the stele region within 9 days after inoculation. Magnification 60×. **H**: The generated hyphae.

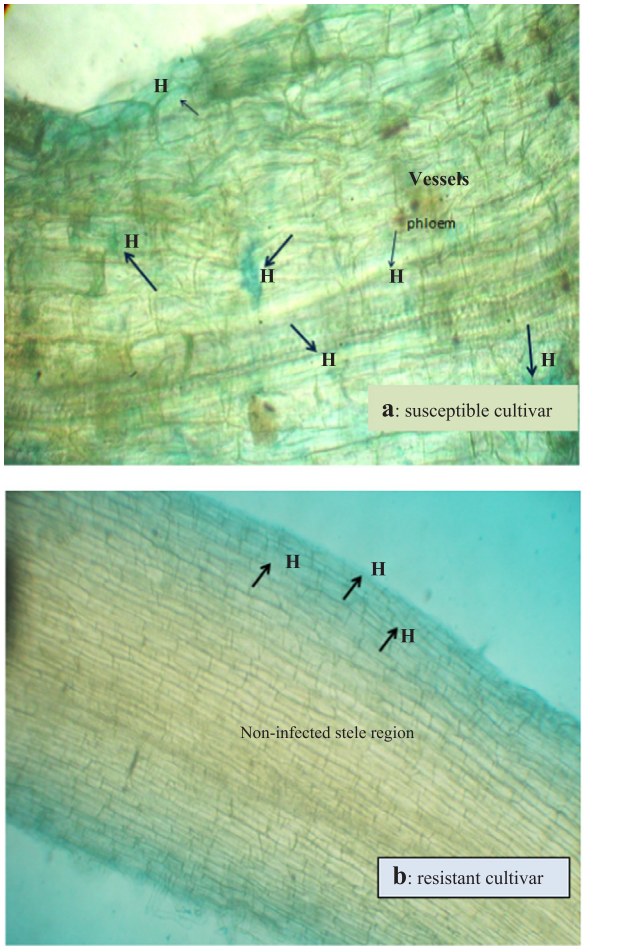

Fig. 7. : Nine days after inoculation with *M. phaseolina*. The hyphae are denser in the susceptible cultivars, whereas there is no considerable change in hyphae density. **a**: susceptible cultivar, **b**: resistant cultivar, **H**: The generated hyphae on the roots surface or penetrated hyphae into the epidermal cells and cortex. Magnification 60×.

Fig. 8. : Nine days after inoculation with *M. phaseolina*, adventitious roots (shown by arrows) appeared on the crown of resistant cultivar (up), whereas wound progression was observed in the susceptible cultivar (down).

an important factor in *M. phaseolina* resistance in soybean cultivars. No cell wall collapse was observed by the ninth day of inoculation in any of the cultivars, but in the susceptible cultivar from ninth day onwards, cell wall collapse was observed after accumulating the hyphae near the stele (Fig. 9a, b and c). Middle lamella of parenchyma cells may have been either degraded or dissolved due to activation of cell decomposing enzymes like cyanide hydratase as reported by Sexton and Howlett (2001). Given the slow progression of the pathogen in the resistant cultivar and limited hyphal penetration into several layers of epidermal cells, although necrosis symptoms were observed on the roots surface, no signs of intercellular walls collapse was observed (Fig. 7b).

In a susceptible cultivar, the required time to complete pathogen's life cycle is much less than that in a resistant cultivar, therefore, delay in completion of pathogen's life cycle (microsclerotia production) can be considered as one of the resistance strategies in resistant cultivars. Failure to produce microsclerotia in a resistant cultivar will result in the production of healthy and pathogen-free seeds and thus eliminate the risk of disease outbreak in upcoming years.

Charcoal rot causal agent, *M. phaseolina*, is known as a necrotrophic pathogen due to its necrotic symptoms and devastating effects on host plants (Bellaloui et al., 2012; Acharya et al., 2013). The current observations indicated that in the susceptible cultivar, the pathogen spread throughout the tissue intercellularly, whereas in the resistant cultivar it remained confined to the focus of infection, so there was no further propagation. Investigations between the 9th and 12th days

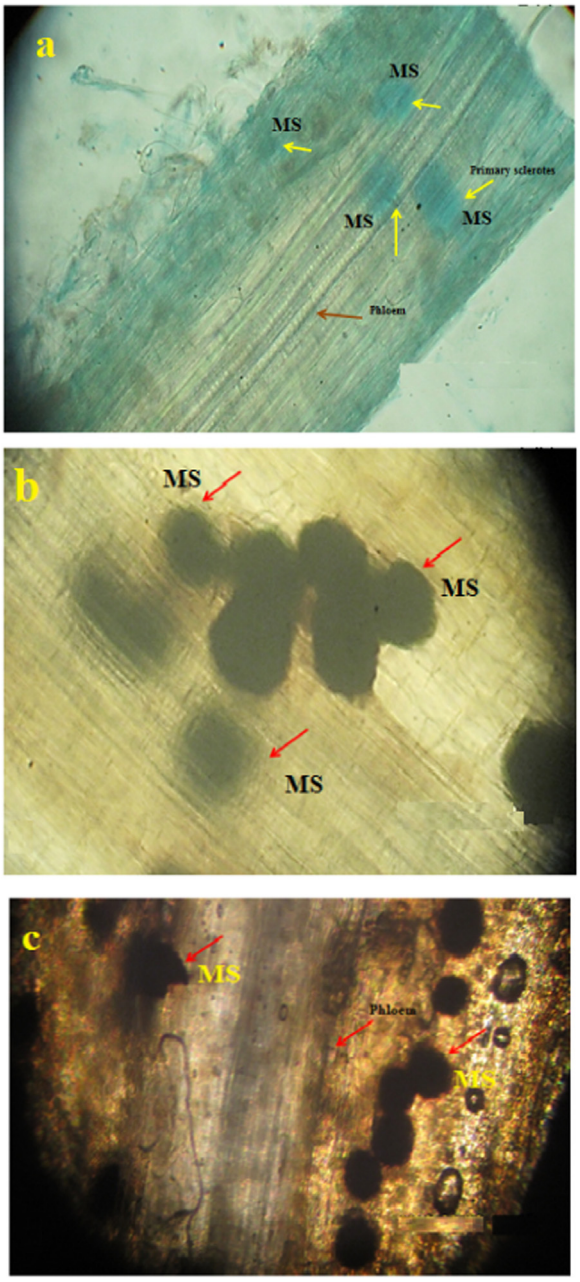

Fig. 9. : Microsclerotia formation in the susceptible cultivar. **a**: condensed hyphae near the vascular system of the roots, **b**: Melanin secretion around condensed hyphae (12 days after inoculation), **c**: microsclerotia formation into the roots, **MS**: Microsclerotia. Magnification 60×.

revealed that cell death and tissue collapse occurred by entering the pathogen into the necrotrophic phase. If the cell death occurs, the pathogen would need a nine-day biotrophic phase to settle inside the plant so that it could colonize the stele tissue. Accordingly, it can be concluded that *M. phaseolina* takes advantages of hemibiotrophic pathogens' strategy in which plant cells do not die immediately after entering the pathogen. Instead, the pathogen enters into a biotrophic phase to supply required energy for microsclerotia production. Possibly, the pathogen remains in the biotrophic phase until the production of microsclerotia and upon reaching the vascular region, at the end of the growth period, enters into the necrotrophic phase.

The cell-to-cell progression of the pathogen in a susceptible cultivar causes systemic infection in plants, but in a resistant cultivar the infection is local and involves only the first few layers of the cells. Despite

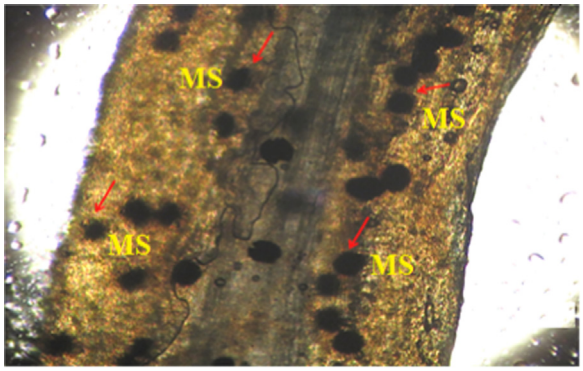

Fig. 10. 14 days after inoculation with *M. phaseolina*, the microsclerotia formation has completed in the susceptible cultivar. The roots have completely destroyed and filled with microsclerotia. **MS**: Microsclerotia.

the spreading of the hyphae in all intercellular spaces, in the susceptible cultivar, no microsclerotia were produced before reaching the hyphae to the stele region. The cell walls collapse happened on the 9th day of inoculation followed by hyphae started to produce microsclerotia throughout the colonized regions. Mechanical stresses, enzymatic digestion, natural pores and ulcers are among the most important factors helping the pathogen to penetrate into the cells of a plant (Horbach et al., 2011) (Fig. 10).

In the current study, in addition to above mentioned factors, the ulcers caused by growing the roots, were found to be a passage for pathogen penetration into the plant. Furthermore, the infection of the new secondary roots can happen by infected adjacent cells in the early stages of their formation (Fig. 11 a, b and c). According to these findings, in a systemic infection, roots get infected at early stages of differentiation and even before contacting the pathogen in the soil. This process increases primary inoculum production for the following years, as every single of these roots is able to produce a large number of microsclerotia. The formation of adventitious roots around the crown in the resistant cultivar is a response to pathogen that renders plants more resistant to pathogen's damages. This is the first report of formation of adventitious roots around the crown in soybean challenged with *M. phaseolina*. However, similar results were reported by Agrios (2005) due to Fusarium infection.

5. Conclusion

The results obtained in this study show that the main events of infection of soybean cultivars with *M. phaseolina* are: (i) attachment of microsclerotia to the root surface, hyphae development and penetration through epidermis and colonization of the cortex cells, 4 days after inoculation, (ii) hyphal penetration through endodermis layer and invasion of vascular tissues, 9 days after inoculation; (iii) colonization of all plant root tissues by the pathogen, including complete hyphae spreading, appearance of condensed hyphae near the vascular system, microslorotia formation and melanin secresion around condensed hyphae, 12 days after inoculation and (iv) completion of microsclerotia formation and roots tissue destruction,14 days after inoculation. In addition, our findings demonstrate that microsclerotia germination and hyphae development, as pathogen's pre-penetration steps, are not linked to resistance as these events took place similarly and at the same time span in both cultivars. Moreover, the observation confirmed that the infection of secondary roots through adjacent infected cells can happen at cell differentiation stage just before their formation. The obtained results will help to better understand the infection process and resistance or tolerance mechanisms in soybean, which may help breeders to release new disease resistant soybean cultivars.

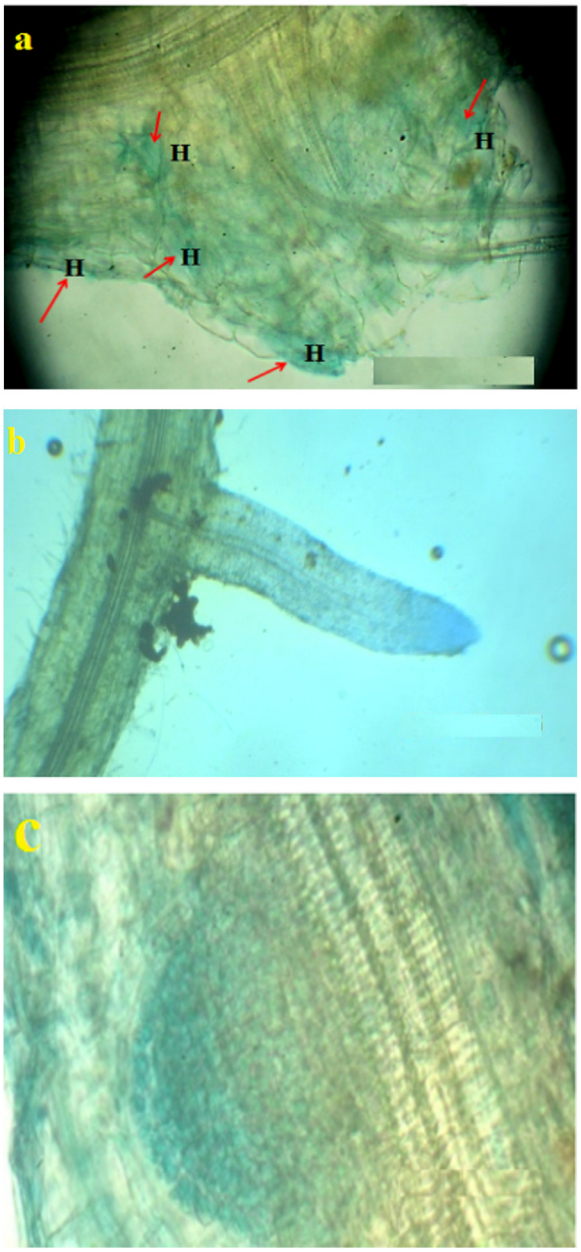

Fig. 11. a: Hyphae penetration site and secondary roots growth, **b**: infected secondary root in the susceptible cultivar, **c**: secondary roots differentiation and infection through adjacent cells in the early stages of their formation. Magnification 60×. **H**: The generated hyphae.

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Corrigendum

Corrigendum to "Histopathology of charcoal rot disease (Macrophomina phaseolina) in resistant and susceptible cultivars of soybean" [Rhizosphere 7 (2018) 27–34]

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The authors would like to inform the readers that Figure 1 is incorrectly referenced as being from Wrather et al., 2007. The correct citation is:

David R.J. Cruz, M.S., 2011, Kansas State University, MSc thesis http://krex.k-state.edu/dspace/handle/2097/10747. On page 14, Figure 1-1, is the original figure that includes citations used to construct the disease cycle, namely: Pedersen, 2003; Wrather et al., 2007.

Pedersen, P. 2003. Soybean growth stages (Ames, Iowa State University Extension).

Wrather, J. A., Shannon, J. G., and Mengistu, A. 2007. Impact of soybean planting date on soil population density of *Macrophomina phaseolina*. Plant Health Progress doi:10.1094/PHP-2007-0917-03-RS.

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