

The Infection Processes of *Sclerotinia sclerotiorum* in Basal Stem Tissue of a Susceptible Genotype of *Helianthus annuus* L.

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Abstract

Sunflower, *Helianthus annuus* L., is a major oil seed crop widely cultivated throughout the globe. White mold, caused by necrotrophic pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary, is a common and widespread pathogen of sunflower. The infection process of *S. sclerotiorum* was studied in the stem base of infected host tissues by light microscopy and Hemi-thin sectioning techniques. Host-pathogen interactions were examined at the plant surface and cellular level of a susceptible genotype (C146), 12, 24 and 48 h of post inoculation. The results showed that the appressoria were formed and the hyphal strands branched upon contact of pathogen with the host surface. Moreover a direct penetration of fungal hyphae was observed through the cuticle within 12h of inoculation. Microscopic observation of inoculated tissues after 24 hours revealed that fungal hyphae have developed both inter- and intra-cellular layer. Moreover, the fungal hyphae growth was incremental among and inside the host cells. The host cells were completely colonized by fungal mycelium 48 hour after inoculation, leading to a tissue collapse. The hyphae invaded the dead host tissues and emerged from the host tissue after extensive colonization. The present study has detailed the infection processes and pathogen development both at plant surface and inside the host tissues. The results of this study will be useful for engineering of disease-resistant genotypes and development of markers for screening pathogen resistance individuals.

Keywords: basal stem rot, microscopy, plant-pathogen interaction, sunflower, white mold

Introduction

White mold, caused by the necrotrophic fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most devastating and cosmopolitan soil borne plant pathogen that infects over 500 species of plants worldwide including important field and fruit crops, ornamentals, trees, shrubs and numerous weeds (Saharan and Mehta, 2008; Garg *et al.*, 2010). The majority of these hosts are dicotyledonous plants, although a number of agriculturally significant monocotyledonous plants such as onions and tulips are also host of this pathogen (Boland and Hall, 1994). Among the various hosts, sunflower (*Helianthus annuus* L.) is an important plant species that is susceptible to *S. sclerotiorum* infection, almost during its entire life cycle. Sunflower cotyledons, apical buds, base of stems, leaves and heads are usually susceptible to infection (Gulya *et al.*, 1997). *S. sclerotiorum* causes stem rot in sunflower which is one of the most important plant diseases in many areas of the world, mainly due to lack of effective control-

ling systems. Rapid drying of the leaves and development of lesions on the tap roots and basal portion of the stem provoke plants to die within a few days after the onset of wilting (Dorrell and Huang, 1978). Yield losses can reach 100% when the climatic conditions are suitable for the fungus (Sackston, 1992). In Iran, infection of sunflower by basal stem disease is considered as a potential threat that seriously affects crop production. The pathogen (fungus) is recognized by the fluffy white mycelium and black sclerotia that develops on the surface of lesions (Bolton *et al.*, 2006).

To date, a number of sunflower genotypes with different levels of resistance to stem rot have been identified, but no full-resistant genotype has been reported yet (Hahn, 2002). Therefore, development of resistant varieties through breeding programmes is an important aim. Management of plant diseases through chemical and cultural practices are usually ineffective and the level of host resistance to the pathogen is inadequate (Bolton *et al.*, 2006; Li *et al.*, 2008). Studies of host-pathogen interactions at

the cellular level will contribute to the development of more effective disease-control measures (Tariq and Jeffries, 1986).

The compatible interaction of *S. sclerotiorum* with several different host species has been studied since the pioneering work of de Bary (1886, 1887) who demonstrated the formation of appressoria from germinating ascospores. Subsequent researches were undertaken in bean (Abawi et al., 1975; Lumsden and Dow, 1973; Lumsden and Wergin, 1980; Tariq and Jeffries, 1986), soybean (Sutton and Deverall, 1983), lettuce (Purdy, 1958; Tariq and Jeffries, 1984), tomato (Purdy and Bardin, 1953; Purdy, 1958), potato (Jones, 1976), pea (Huang and Kokko, 1992), oil-seed rape (Garg et al., 2010; Huang et al., 2008) as well as in sunflower (Rodriguez et al., 2004; Sedun and Brown, 1987). All above investigations reported the infection processes of *S. sclerotiorum* in compatible interactions. These studies demonstrated that fungal pathogen may enter into plant cells through direct penetration, enzyme and toxin effects, mechanical pressure, or indirectly through wounds or natural openings such as lenticels or stomata (Isacc, 1992). Different tissues of the host plant (stem, root, cotyledon petiole, leaf petiole, etc.) can be penetrated differently by the same fungus. Each species of fungal pathogen may display different ways to penetrate host under different conditions (Isacc, 1992). In most cases, *Sclerotinia* penetrates directly the cuticle by appressoria and not through stomata (Boyle, 1921). The enzymatic digestion of the cuticle also plays a role in the penetration process (Tariq and Jeffries, 1986).

Pathogenic fungi usually facilitate the host colonization process by production of a wide range of cell wall degrading enzymes (CWDEs), including pectinases, β -1, 3 glucanases, glycosidases, cellulases, xylanases and cutinases (Annis and Goodwin, 1997). It was also shown that *S. sclerotiorum* is capable for synthesizing extracellular CWDEs and oxalic acid (Bolton et al., 2006; Donaldson et al., 2001; Godoy et al., 1990; Lumsden, 1976; Marciano et al., 1982, 1983; Maxwell and Lumsden, 1970) which is accumulated in the water-soaked lesions (Riou et al., 1991). During the host-pathogen interaction, *S. sclerotiorum* secretes CWDEs that can facilitate penetration, tissues maceration and degradation of plant cell wall components (Riou et al., 1991).

The ability of *Sclerotinia* spp. to invade the host and the mode of penetration into host tissues depend on the type

of inoculum, the nutrient status of the fungus, properties of the host and effects of the surrounding environment. Two types of inoculums can initiate infection such as, germinated ascospores and mycelia (Abawi et al., 1975). In stem rot disease of sunflower, myceliogenic germination of soil-borne sclerotia results in mycelia that can directly attack plant tissues (Holley and Nelson, 1986).

However, no sufficient structural studies have been conducted yet on mode of penetration and interactions of sunflower basal stem and *S. sclerotiorum* during the infection process.

The aim of present work is to provide cellular and histological information during the infection process and to describe the infection processes of *S. sclerotiorum* on sunflower basal stem.

Materials and methods

Plant materials

C146 is a F₂ recombinant inbred line (RIL) coming from a cross between PAC2 and RHA266 (Poormohammad Kiani et al., 2007). C146 is susceptible to *Sclerotinia* basal stem rot (Davar et al., 2010). Seeds of C146 line were sown in 10×12 cm pots filled with sterilized soil after surface sterilization with a 6% sodium hypochlorite solution and three washes in sterile distilled water. Tab. 1 summarizes the properties of the soil used in the experiments. The soil was silty clay with a pH of 7.6 and an EC of 0.6 dSm⁻¹. Plants were grown in a controlled environment with a 12 h day with a day-light intensity of 200 mEm⁻²s⁻¹, 65% relative humidity and a day/night temperature of 24/18 (±1°C) for 4 weeks, until they reached to growth stage V6-V8 (Schneider and Miller, 1981).

Fungal isolate and inoculums production

The fungal isolate SSU107 was previously isolated from an infected sunflower and reported to be moderately pathogenic on sunflower hybrid cv. 'Iroflor' (Davar et al., 2010). The isolate were grown on PDA [(Potato Dextrose Agar (39 g l⁻¹, pH 6, Merck)] medium and were incubated in the dark at room temperature (25°C) for 3 days.

Artificial inoculation

At V6-V8 growth stage, mycelial plugs of the isolate (3 mm diameter) were cut from the growing edge of the colony (3-day-old on PDA) and were placed against the basal

Tab. 1. Some physical and chemical properties of the soil used in this study (average values)

Soil parameters	pH	Ec×10 ³ (dS m ⁻¹) ^a	P (mg kg ⁻¹)	K (mg kg ⁻¹)	N (Total) (%)	Mg (meq l ⁻¹)	Ca (meq l ⁻¹)	
\bar{X}	7.60	0.60	39.3	565	0.11	1.87	1.89	
Soil parameters	Cl (meq l ⁻¹)	OC ^b (%)	SP ^c (%)	CaCO ₃ (%)	HCO ₃ (%)	Sand (%)	Silt (%)	Clay (%)
\bar{X}	0.80	0.73	49.9	11.84	3.57	16	44	40

^a Ec×10³, electrical conductivity; ^b OC, organic carbon; ^c SP, saturation percentage

stem of the sunflower plants. The stem and mycelial plug were wrapped with Parafilm for 48 h to preserve humidity, following the method of Price and Colhoun (1975). Completely randomized design with three replications was used in this experiment. Observation of treatments were carried out at different time post inoculation intervals of 12, 24 and 48 h.

Sample preparation for light microscopy

Inoculated stems were sampled for anatomical studies at 12, 24 and 48 h after inoculation. Samples were first fixed in an acetic acid: ethanol: formaldehyde (Glacial Acetic Acid 5 ml: Ethanol (70%) 90ml: Formaldehyde (38%) 5ml) solution for 15 h and then were washed with distilled water for 8 h. Cross sections were stained with 1% cotton blue in lactophenol (Li et al., 2004) and rinsed in lactophenol for 10 min. The sections were examined and photographed using a Zeiss Axioplan 2 microscope with a Nikon digital photograph system.

Sample processing for Hemi-thin sections

The infected segments of the sunflower stems were fixed in 2.5% glutaraldehyde in 0.05M phosphate buffer, pH 7.2, for 48 h at room temperature. Material was post-fixed with 2% (w/v) osmium tetroxide buffer for 2h at 20°C before being dehydrated in a graded ethanol solutions ranging from 50% to 100% (dry ethanol). Preparations were embedded in Spurr epoxy resin (Spurr, 1969). Hemi-thin sections (500nm thick) were cut on an Ultra Cut microtome and were mounted on glass slides and stained with Toluidine blue. Sections were examined with a Zeiss Light microscope.

Results

The light microscopic observations of the fungus in the sunflower basal stem are presented in Fig. 1, 2 and 3. There was a slight increase in the length of aerial mycelia on the basal stem and surface of the host tissue, 12 h after inoculation, which intensely stained by cotton blue (Fig. 1A). After contact with the host, appressorium was formed and the hyphal strands were branched. The hyphal apices on the surface of stem tissue showed dichotomous branching that gave rise to simple appresoria (Fig. 1B). Penetration proceeded directly through the cuticle (Fig. 1B).

After penetration of the host cuticle, hyphae were developed between the cuticle and the epidermal cell layer and in the cortex (Fig. 1C). The hyphae that moved into the cortex were developed inter- and intra-cellular levels (Fig. 1D, E and F). After the initial infection period (12-24 h), the hyphae were branched and developed rapidly from the basal stem tissues. There was inter- and intra-cellular colonization of parenchyma (Fig. 2G), in which fungal hyphae was branched profusely. The hyphae continued growing in inter and intracellular spaces and extensively invaded the dead host tissues, 24-48 h after pathogen invasion. (Fig.

2H, I and J). The ramifying hyphae also invaded the vascular tissues of sunflower stem and entered the vessels and interfascicular regions (Fig. 2J). As the infection progressed, the pathogen reached the pith of the stem (Fig. 2H and I). A 48 h after inoculation, host cells were disorganized and eventually collapsed (Fig. 2H, I and J). Hyphae were observed on the surfaces of stems after 48 h.

The hemi-thin sectioning method was performed for better visualization of fungus penetration inside the plant tissues. The hemi-thin sections of inoculated stems were observed by light microscopy and the micrographs corresponded to the development of the fungus inside the host tissues. Toluidine blue stain and observations under bright light, was in agreement with our previous cotton blue staining results, indicating that hyphae of *S. sclerotiorum* is growing in inter and intra-cellular space of cortex tissue (Fig. 3A and B).

Discussion

Although the symptoms of *Sclerotinia* white rot disease have been described by various authors (Abawi et al., 1975; Garg et al., 2010; Rodriguez et al., 2004; Sedun and Brown, 1987), but the histological aspects of the infection of sunflower basal stem by *S. sclerotiorum* have not been documented. As first report, this study describes the infection process of *S. sclerotiorum* on the basal stem of a susceptible sunflower genotype.

The structural investigation of the artificial inoculation with *S. sclerotiorum* hyphae showed the occurrence of direct penetration through the cuticle in sunflower. The present study also reports the presence of subcuticular hyphae in basal stem of sunflower infected by *S. sclerotiorum*. Some other studies have shown that infection by *S. sclerotiorum* hyphae is usually penetrate directly through the cuticle (Abawi et al., 1975; Lumsden and Dow, 1973; Purdy, 1958), which is in agreement with the present results. Direct penetration has also been reported for other fungi such as *Phoma macdonaldii* in sunflower (Roustae et al., 2000). Freeze and bruising injuries are important factors associated with infection of cabbage by *S. sclerotiorum* (Hudyncia et al., 2000). Tariq and Jeffries (1984) showed that infection of healthy tissue by myceliogenic infection depends on the formation of infection cushions on the host surface. The infection process is characterized by the formation of an appressorium, mechanical penetration of the cuticle and formation of inflated infection hyphae that develop inter and intracellular colonization of the host tissue (Lumsden and Wergin, 1980).

Infection cushions and/or appresoria were observed on the surface of stem base of studied genotype (Fig. 1B). It has been reported that appresoria were formed unless penetration occurs directly via stomata (Jones, 1976; Prior and Owen, 1964). Formation of these structures requires contact stimulus (Abawi et al., 1975; Garg et al., 2010; Purdy 1958).

After contact with the host surface, the hyphal branch strands dichotomously formed dome-shaped infection cushions (Fig. 1B and C). Similarly, previous researches on *S. sclerotiorum* and other related pathogens such as *S. trifoliorum* and *S. rolfsii* also showed that the infection cushion assists with breaching the cuticle barrier of the host epidermis by applying mechanical pressure for fungal entry (Boyle, 1921; Garg *et al.*, 2010; Lumsden and Dow, 1973; Lumsden and Wergin, 1980; Purdy, 1958) and/or by enzymatic disintegration of the host surface (Prior and Owen, 1964; Tariq and Jeffries, 1986). However, dissolution of the cuticle and epidermal cells, which results from enzyme function, is occurred when the infection cushions appear.

Microscopic observation of tissues 24 h post inoculation revealed that fungal hyphae have extensively devel-

oped at both inter and intracellular levels and growing between and through cells (Fig. 1D, E, F and 3A, B). These observations clearly resembled the previous reports, in which penetration of host tissue by hyphae have been described at inter and intracellular levels (Abawi *et al.*, 1975; Purdy, 1958).

Undoubtedly, the infection hyphae are responsible for breaching the host's defenses and initiate colonization of host tissue. The infection hyphae are associated with the advancing margins of visible lesions on hosts (Boyle, 1921; Lumsden and Dow, 1973). These hyphae probably are responsible for changes in infected host tissue which include histological alterations in pectic materials in cell walls (Lumsden and Dow, 1973), death of cells (Boyle, 1921; Hancock, 1972), copious accumulation of fluids

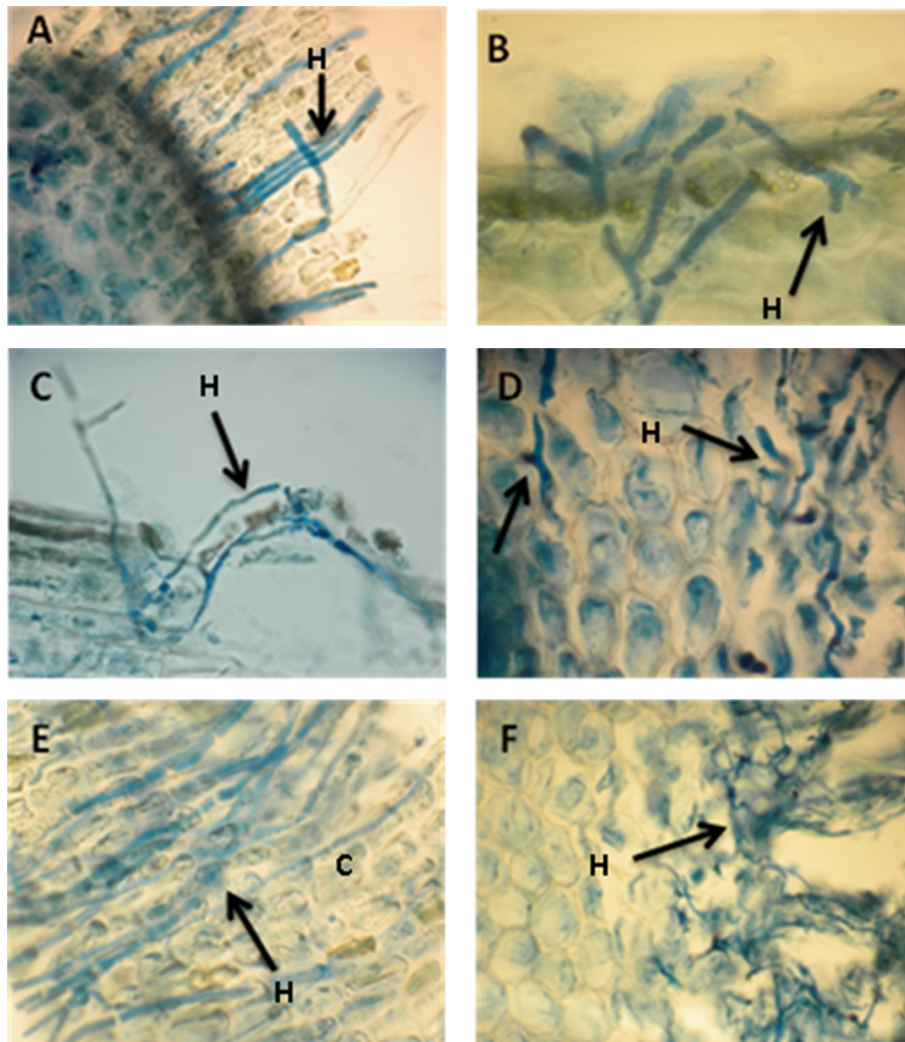


Fig. 1. Light micrographs following inoculation of *S. sclerotiorum* isolate SSU107 onto basal stem of susceptible 'C146' sunflower (A-F). The samples were fixed in an acetic acid: ethanol: formaldehyde solution, stained with 1% Cotton Blue in lactophenol, and photographed using a Zeiss Axioplan 2 microscope photograph system. Arrows indicate fungal hyphae. Micrographs showing increase in the aerial hyphae on basal stem (A), appressorium formation, hyphal branching (B), development of hyphae and colonization of the fungus (C, D) and 24 h (E, F) after inoculation. In inoculated tissues after 24 hours (E and F) revealed that fungal hyphae growth was incremental among and inside the host cells. H = hyphae, C = Cortex

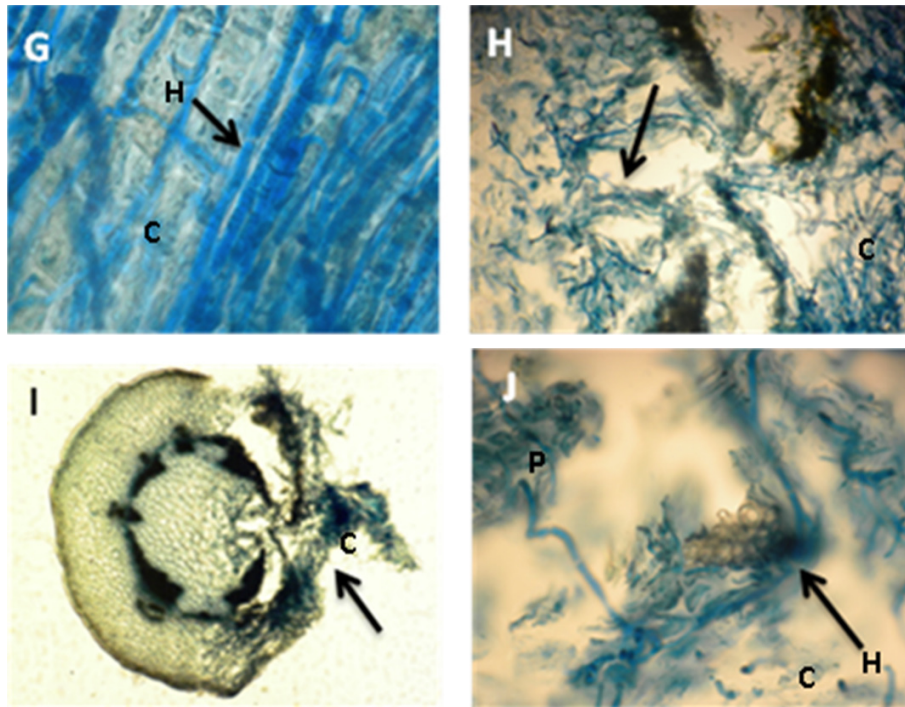


Fig. 2. Light micrographs on infection and extension of *S. sclerotiorum* in host tissues. Cross sections showing the penetration of *S. sclerotiorum* isolate SSU107 into basal stem of sunflower 48 h after inoculation. Samples were fixed in an acetic acid: ethanol: formaldehyde solution, stained with 1% Cotton Blue in lactophenol. G-J Micrographs showing development of the hyphae and colonization of the fungus 48 h after inoculation. Micrographs showing dense colonization of stem tissue by hyphae of *S. sclerotiorum* (G). The hyphae had entered into the host tissues (H). Host cortical cell disintegrate after sclerotinia invasion (H, I and J). Arrows indicate fungal hyphae. C = Cortex, H = Hyphae, P = Pith

and water-soaking in advancing margins (Hancock, 1972; Lumsden and Dow, 1973).

Lumsden and Dow (1973) demonstrated that after colonization of host tissue by *Sclerotinia* infection hyphae (12-24 h after penetration), branches are developed and extensively ready to invade the dead host tissue by penetrating cell walls at both inter and intracellular levels. This was evidently observed in the histological examination of the study that extensive colonization of cells

caused the death of stem tissues cells (Fig. 1F, 2H, I and J). Rodriguez *et al.* (2004) reported inter and intracellular colonization and cell collapse in flower tissues of sunflower. This might be related to changes occurring in tissues in response to the pathogen, such as cell collapse and changes in cell wall composition. Cosson *et al.* (1993) and Pratts-Pérez *et al.* (2000) studied wild and cultivated genotypes of sunflower and identified that necrotized tissues synthesize compounds which appear to be phytoalexins.

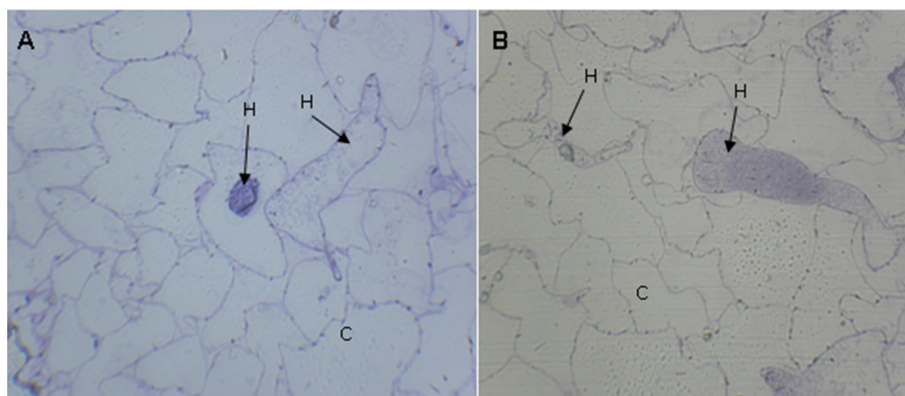


Fig. 3. Hemi-thin cross section showing colonized hyphae which spread inter and intracellularly in the infected stem tissue. (A and B) Observations made using light microscopy. Sections showing that fungal hyphae appear stained with toluidine blue within the stem tissues. The hyphae were developed among and inside of cortex cells. Arrows indicate fungal hyphae. H = hyphae, C = Cortex

Pathogenesis is a complex process involving the pathogen's inherent capabilities and multiple factors that govern penetration and infection of a host plant. The host plant has various defense mechanisms that must be inactivated or nullified before a disease can be developed. This interaction between host and pathogen also depends on the environmental factors. The pathogen's invasion mechanisms include producing the cell wall and middle lamella dissolving enzymes, toxins, enzymes to degrade host tissue and defense substances, and rapidity in host plant infection. *S. sclerotiorum* facilitates penetration and colonization of host plant tissues by secreting multiple pectinolytic enzymes.

The present study has detailed the infection processes and pathogen development both on the plant surface and inside the host tissues. These results can be used for strategic engineering of disease-resistant genotypes and developing markers for screening of resistance individuals.

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