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Changes in Anatomical Features and Protein Pattern of Sunflower Partially Resistant and Susceptible Lines During Infection By Virulence Factors of *Sclerotinia Sclerotiorum*

Maryam Monazzah[1], Sattar Tahmasebi Enferadi[1,*], Zohre Rabiei[1] and Alessandro Mattiello[2]

[1]Department of Plant Molecular Biotechnology, Institute of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, PO Box 14925/16, Iran.

[2]Department of Agri-Food, Animal and Environmental Sciences, University of Udine, Udine, Italy.

[*]Corresponding Author: Sattar Tahmasebi Enferadi. Email: tahmasebi@nigeb.ac.ir.

Abstract: *Helianthus annuus* L. as an oil seed crop is widely grown throughout the world. One of the most destructive diseases of sunflower is stem rot caused by *Sclerotinia sclerotiorum.* Oxalic acid is the major virulence factor of this necrotrophic pathogen. It is important to further investigate plant responses to this non-specific toxin. Therefore, in the present study, we compared the patterns of total soluble proteins and xylem morphology of partially resistant and susceptible sunflower lines after treatment with Sclerotinia culture filtrate. The basal stems of both lines were treated with 40 mM oxalic acid (pH 3.7) of fungus culture filtrate and samples were collected at 24, 48 and 72 hours post treatment. In SDS-PAGE protein pattern new protein bands appeared in both lines after treatment. These observations suggest induction of stress-related proteins upon culture filtrate treatment. The identities of these new proteins need to be more clarify in future investigations. The changes in xylem morphology and degree of lignification of both lines was studied by light microscopy and microtome sectioning techniques after treatment with *S. sclerotiorum* culture filtrate. Anatomical investigations revealed changes in xylem diameter and xylem lignification of treated lines at various time points. More lignin deposition in xylem vessels of partially resistant line has been observed after treatment. In addition, the size of xylem vessels in partially resistant line has been sharply decreased upon pathogen filtrate treatment. The results of this study will help us gain a more complete understanding of resistance mechanisms to this cosmopolitan and devastating pathogen.

Keywords: Microtome; Xylem; *Helianthus annuus*; *Sclerotinia sclerotiorum;* Stem rot

1 Introduction

The sunflower, *Helianthus annuus* L. is an economically important crop through the world. *Sclerotinia sclerotiorum* is one of the most destructive plant pathogens that infect over 400 plant species worldwide including sunflower and canola [1].

Oxalic acid (OA; $H_2C_2O_4$) is a nonspecific toxin that has been known as the main pathogenicity determinant of *S. sclerotiorum* [2]. OA have several roles during infection process including direct plant toxicity [3], enhancing the activities of cell wall degrading enzymes [4], suppression and induction of programmed cell death (PCD) in plant cells [5,6].

S. sclerotiorum infection induced changes in transcriptome and proteome levels in plant hosts [7-12]. The proteome-level study can convey us to identify the real molecules involved in plant defense responses [13]. Understanding the molecular mechanisms underlying plant-pathogen interactions contribute to develop sunflower lines with higher levels of resistance to Sclerotinia.

Noyes and Hancock [14] reported that there is 15 times more oxalic acid in the wilted leaves of sunflower plants than in the control plants. The pH of xylem sap measured at a point distant from the lesion areas was remarkably lower than that of the uninoculated tissues. Severe damage to vascular fibers and xylems was only observed in susceptible species of Brassica under *S. sclerotiorum* infection [15]. In addition, OA by several mechanisms causes wilting in infected hosts. It forms oxalate crystal in the plant xylem and causes embolisms that consequently leads to water loss in plant tissues. It was demonstrated that water stress has an effect on vessel morphology [16].

Therefore, in this study, we aimed to investigate morphological changes of xylem vessels in two partially resistant and susceptible lines of sunflower upon *S. sclerotiorum* virulence factors treatment. The relationship between vessel morphology and plant pathogen infection is less clear and requires more explanation. In order to study differential responses of partially resistant and susceptible sunflower lines to Sclerotinia culture filtrate, we compared the electrophoretic patterns of total protein of both lines after treatment. Our study revealed differential accumulation of proteins during plant defense responses in both lines.

2 Material and Methods

2.1 Preparation of Pathogen Culture Filtrate

Sclerotia of *S. sclerotiorum* were placed onto potato dextrose agar (PDA) medium in a petri plate and incubated at 28°C. Hard black sclerotia were directly gathered from naturally infected sunflower field. Mycelial plug of approximately 1 cm in diameter were cut by a sterile blade and placed onto the liquid Czapek-Dox medium. It was incubated at 28°C for 30 days to have all metabolites of the pathogen in the culture filtrate. Then, the liquid medium was separated from developed mycelium and filtered through the 0.22 μm sterile filters [17]. The OA concentration in broth medium was estimated by high performance liquid chromatography (HPLC). HPLC analysis was performed using Agilent Eclipse XDB-C18 column (4.6 × 250 mm) with ultraviolet detector set at 240 nm. Standard curve was created with known concentrations of OA, in order to calculate OA concentration in the liquid medium.

2.2 Plant Material and Treatment Procedure

Partially resistant (AC 4122) and susceptible HA 89 (Reg. no. GS-39, PI 642062) sunflower lines were provided by University of Udine (Udine, Italy) [17]. Seeds of both lines were germinated on ½ MS medium containing vitamins and agar [17]. The stems of 14-day old sunflower seedlings were slightly scratched by a sterilized pipette tip [8] and then treated with 10 ml culture filtrate. The stems were sampled at 24, 48 and 72 time points after treatment and stored at -70°C until use. The control samples were treated by Czapek-Dox medium.

2.3 Total Protein Extraction and Quantitation Assay

One gram of sunflower stems was ground to a fine powder using liquid nitrogen. The protein extraction has been done by cold 10% TCA in acetone containing 0.07% DTT (w/v) and 1mM PMSF. The samples was incubated at -20°C overnight to enhance protein precipitation. Then, the microtubes were centrifuged at 16000 g for 20 minutes (4°C). The supernatant was discard and the pellet was resuspended four times with 1 ml cold acetone with 0.07% (w/v) DTT. The protein pellet was completely dried and solubilized in 20 mM Tris HCL buffer (pH = 7.5) containing 8 M urea, 2 M thiourea, 50mM DTT, 4% CHAPS. Then, the microtubes were centrifuged as described above. The supernatant was stored at -70°C until use [8]. The Bradford protein assay was used to measure protein concentration in samples [18].

2.4 SDS-PAGE

Protein samples were separated on 5% stacking gel and 12% separating gel in a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, CA, USA). SDS-PAGE was run at constant voltage at 80 V for 2 hour.

Finally, the gels were stained with Coomassie Brilliant Blue R250 (CBB-R250) for 20 min and then destained with 40% methanol and 10% acetic acid until bands were clearly visible.

2.5 Sample Preparation for Light Microscopy

Transverse sections of sunflower stems at all sampling time points were cut by hand using a sharp razor blade. The sections were dyed with freshly prepared phloroglucinol-HCl solution and examined for the presence of lignin [19].

2.6 Sample Preparation for Microtome Sectioning

The segments of the sunflower stems were fixed in FAA solution containing 37% formaldehyde, 96% ethanol and glacial acetic acid for 24 h at room temperature. The material was washed with running tap water for 6 h to remove extra fixative and then dehydrated in a graded ethanol solutions ranging from 30% to 100%. The tissues were embedded in paraffin. They were sectioned with Ultra Cut microtome and stained with hematoxylin and eosin (H&E). The average area of vessels in the stem section of both lines was determined by analyzing images using ImageJ software (version 1.52 g).

2.7 Statistical Analysis

The experiment was done in a completely random plan with three replicates for each treatment. Data from the light microscopic observation and protein concentration were analyzed with ANOVA method followed by Fisher's LSD ($p \leq 0.05$) using the SPSS software (version 16).

3 Results

3.1 Total Soluble Protein

Soluble protein content in stems of the partially resistant line increased significantly at 48 and 72 sampling times. While in the susceptible line it was only increased significantly at 48 h post treatment (Fig. 1). The highest total protein level was observed at 48 h post treatment in both lines (about 1.5-fold).

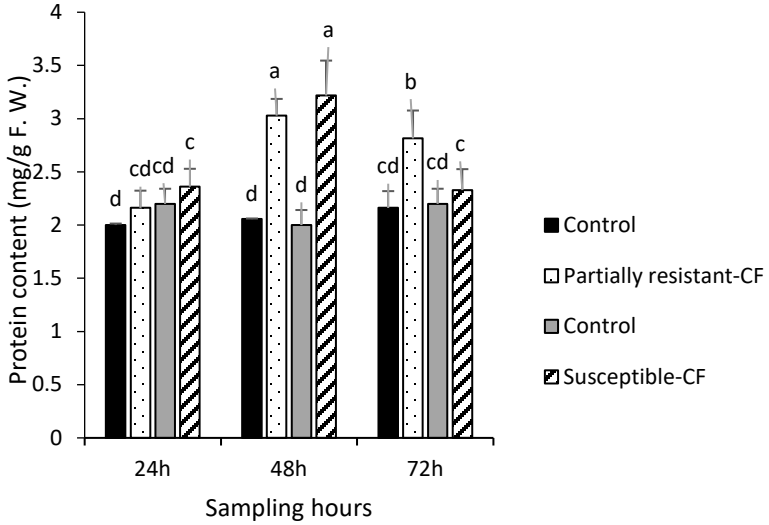


Figure 1: Protein concentration in the stem of partially resistant and susceptible sunflower lines in non-infected plants and samples treated by pathogen culture filtrate (CF). Columns represent mean ± SD with

three replicates. Different letters indicate a significant difference at $p \leq 0.05$ based on ANOVA and Fisher's LSD test

3.2 SDS-PAGE Patterns of Total Soluble Protein

In SDS-PAGE protein pattern, new protein bands appeared in both lines after treatment (Fig. 2). New bands at high molecular weight (> 200 kDa) and lower molecular weight (below 20 kDa) accumulated in the stem of both lines at 24 h, 48 h and 72 h after treatment. New protein bands at molecular weight between 25-30 kDa and 45 kDa have been detected in partially resistant line, as well.

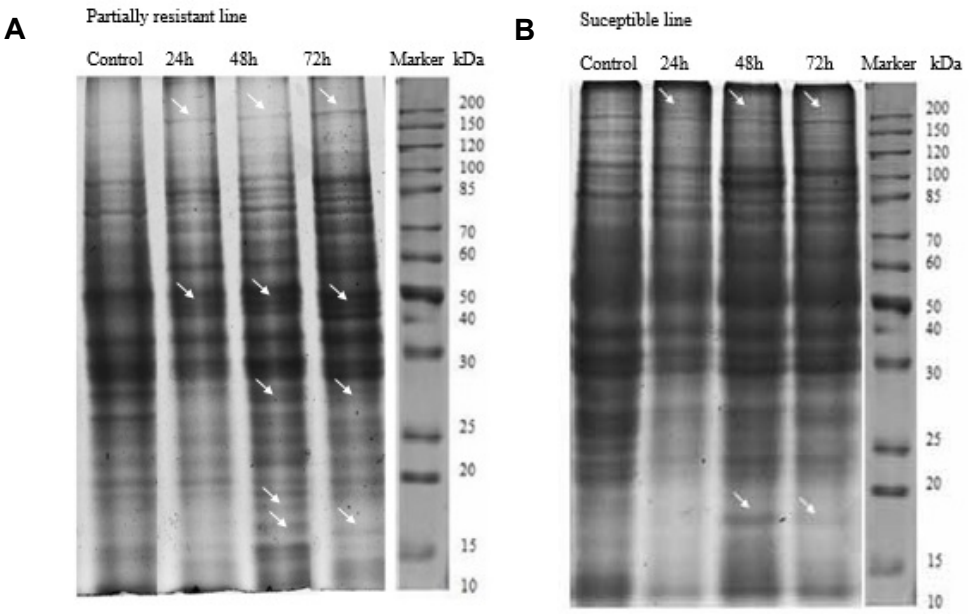


Figure 2: Protein patterns of partially resistant (A) and susceptible (B) sunflower lines at 24 h, 48 h and 72 h post treatment with pathogen filtrate. The arrows indicate bands which were either appeared newly or disappeared after treatment

3.3 Light Microscopic Observations

Phloroglucinol-HCl staining was used to estimate lignification in the stem of susceptible and partial resistant sunflower line in various time points (Fig. 3). This solution reacts with coniferaldehyde units in lignin and the red color intensity approximately reflects the total lignin content [19]. It seems that the stems of partially resistant plants at 72 h after treatment were much more intensely stained compared to the control sample (Fig. 3(H)).

In H&E staining, the data showed that there is a relationship between vessel size and treatment. The size of the xylem vessel was reduced significantly in partially resistant plants after pathogen filtrate treatment at all sampling time points (Figs. 4 and 5). It was reduced about 1.5-times at 24 h and 48 h and 3.5 times at 72 h post treatment.

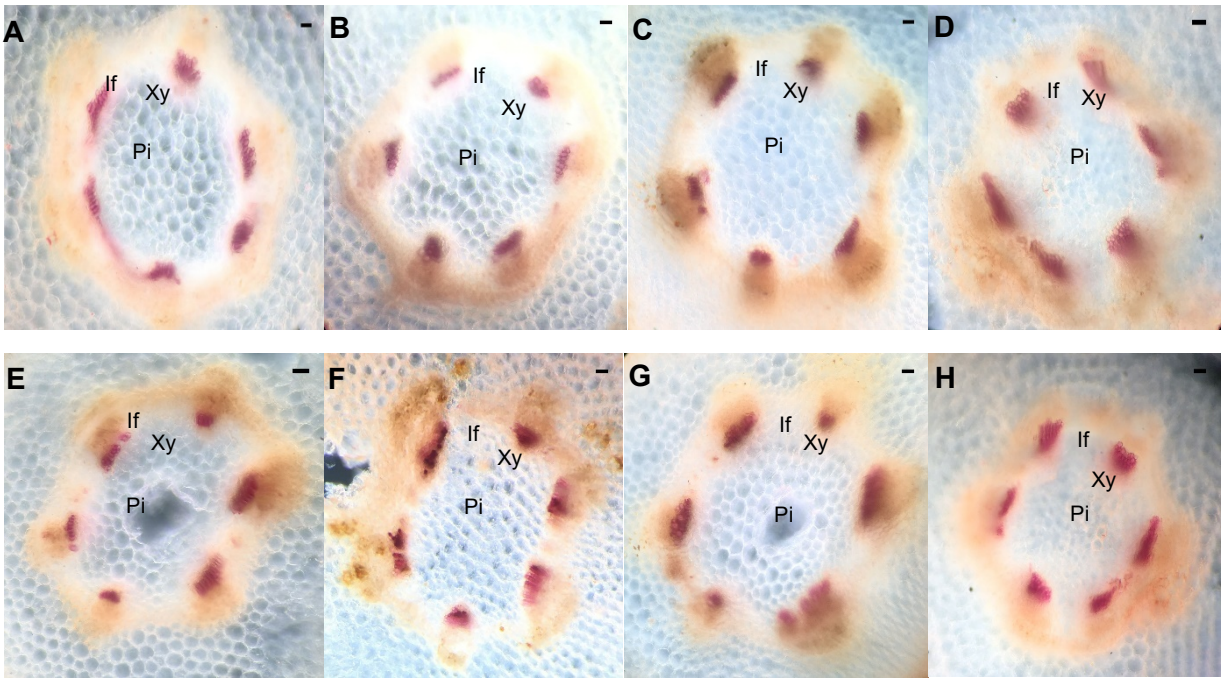
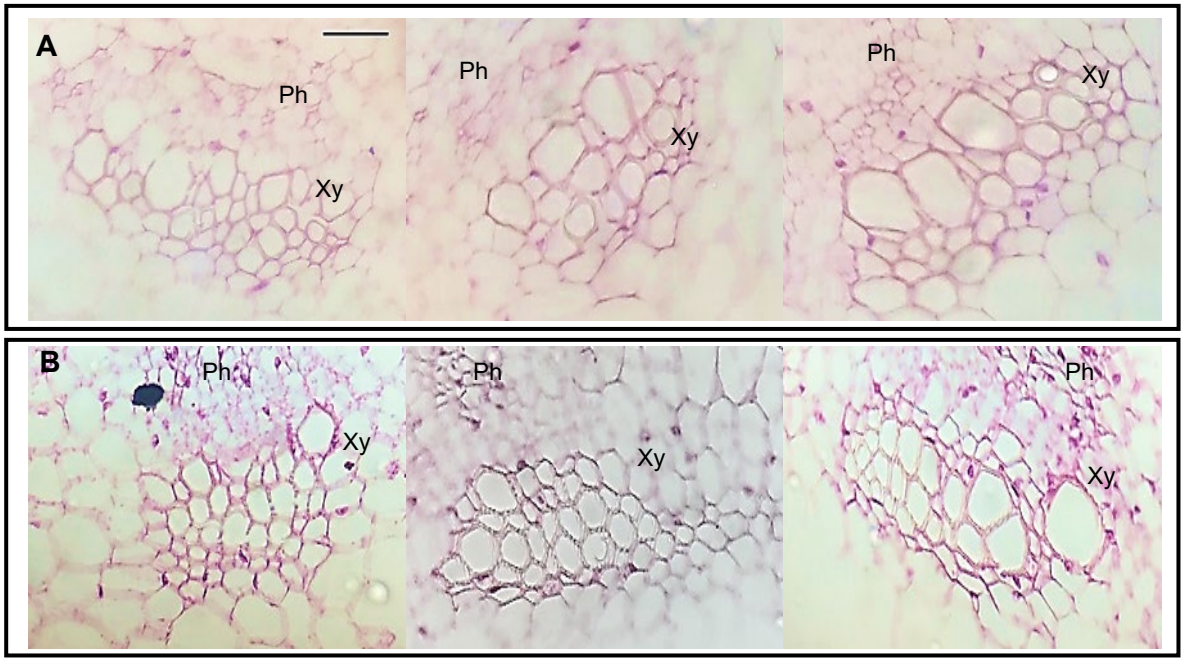
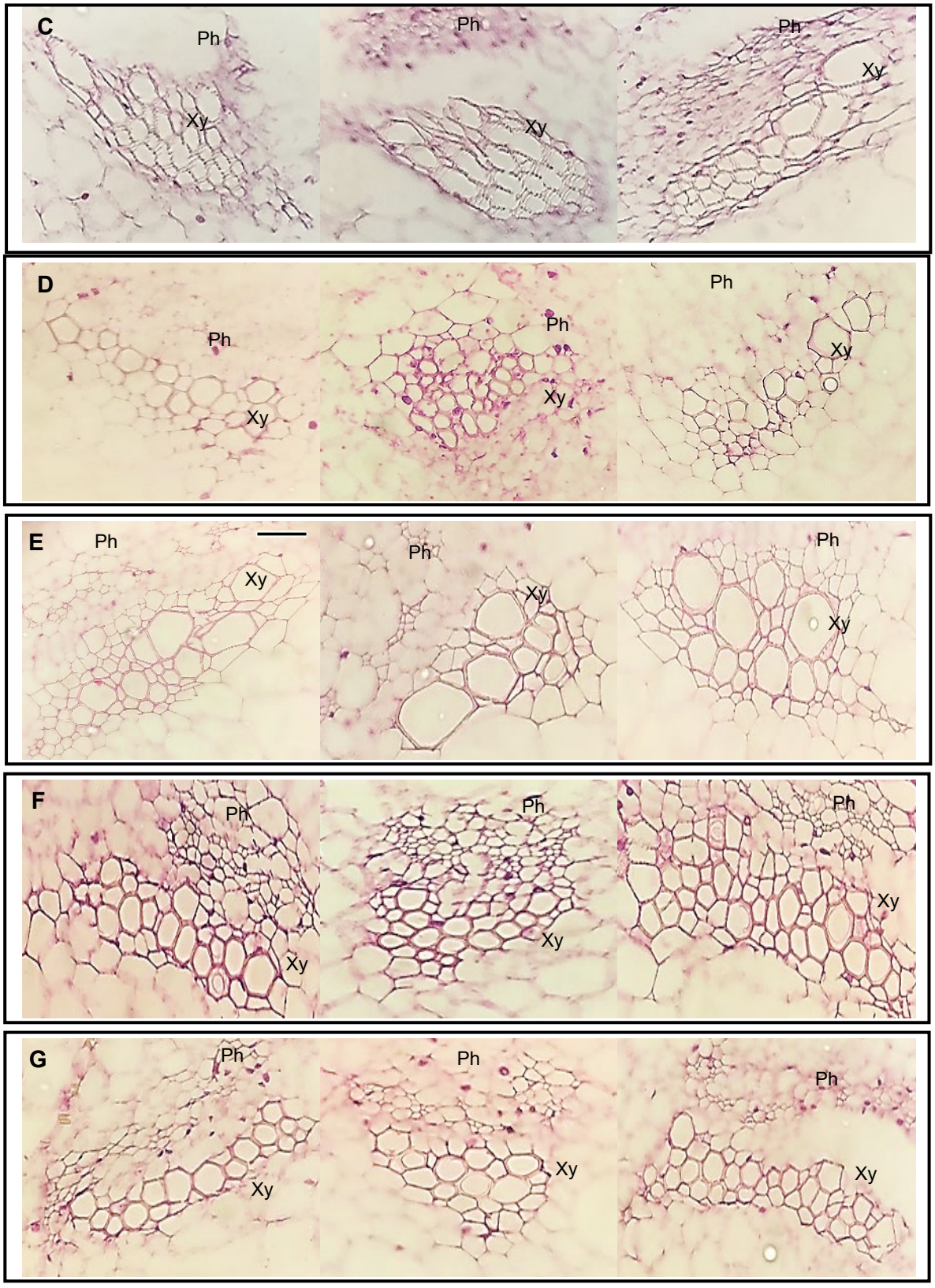


Figure 3: Hand-cut sections of untreated susceptible sunflower line (A), susceptible line treated with pathogen filtrate at 24 h (B), 48 h (C) and 72 h (D); untreated partially resistant sunflower line (E), partially resistant line treated with pathogen filtrate at 24 h (F), 48 h (G) and 72 h (H) for lignin staining. Staining was done using phloroglucinol-HCl. Lignin staining is red. Xy, Xylem; If, Interfascicular fiber; Pi, Pith. Bar = 0.06 mm for (A) to (H), 10x magnification

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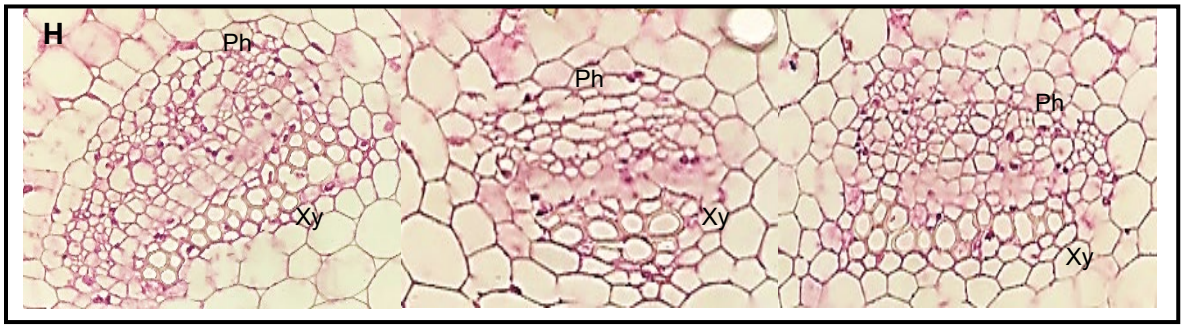

Figure 4: Sunflower stem sections were made by microtome. Morphological features of the xylem of susceptible and partially resistant lines at various time points; untreated susceptible sunflower line (A), susceptible line treated with pathogen filtrate at 24 h (B), 48 h (C) and 72 h (D); untreated partially resistant sunflower line (E), partially resistant line treated with pathogen filtrate at 24 h (F), 48 h (G) and 72 h (H). Staining was done using eosin and hematoxylin. Xy, Xylem; Ph, Phloem. Bar = 0.05 mm for (A) to (H), 40x magnification

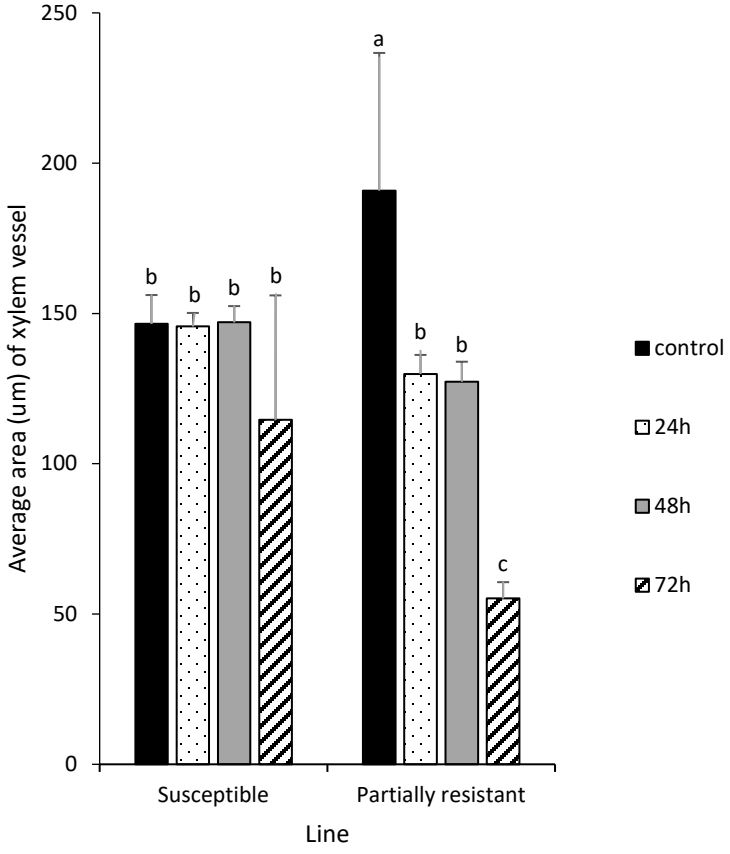

Figure 5: Average area (µm) of vessels of stems after pathogen filtrate treatment for susceptible and partially resistant lines. Columns represent mean ± SD with three replicates. Different letters indicate a significant difference at $p \leq 0.05$ based on ANOVA and Fisher's LSD test

4 Discussion

4.1 Protein Patterns of Total Proteins in Susceptible and Partially Resistant Lines of Sunflower

The plant immune system consists of effector-triggered immunity (ETI) and pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) pathways [20]. PTI is a quantitative host resistance which activates against different types of pathogens including necrotrophic pathogens. Recognition of PAMPs/DAMPs raises warning alarm and activation of set of downstream defense responses [21]. These defense responses can be constitutive or inducible in plants. The result of our study suggest the induction of stress-related proteins upon culture filtrate treatment. We observed the induction of protein bands at low (below 20 kDa) and high molecular weight (200 kDa) in both lines. However, there was obvious difference of protein band patterns between two lines at molecular weight ranging from 25 to 50 kDa. In partially resistant line, new protein bands also accumulated at molecular weight between 25-50 kDa. Most pathogenesis-related proteins have low molecular weight ranging from 8 kDa to 50 kDa [22]. In our previous study, we observed that the transcript abundance of two pathogenesis-related genes, *PDF 1.2* and *PR5-1*, were higher in the partially resistant line compared to those in the susceptible line [23,24]. However, the identities of these new proteins need to be more clarify in future investigations.

4.2 Changes in Xylem Vessels of Sunflower Lines After Culture Filtrate Treatment

The Phloroglucinol-HCl staining also showed that more lignin deposition in the cell walls of xylem of partially resistant line after pathogen filtrate treatment. In our previous studies [23,24], we found that the activity of phenylalanine ammonia lyase enzyme (PAL) as well as shikimate dehydrogenase enzyme were much more pronounced in partially resistant line than susceptible line. In addition, some proteins such as peroxidase and laccase were only up-regulated in partially resistant line that might point out the critical role of the phenylpropanoid pathway in sunflower defense mechanism against *S. sclerotiorum* infection.

The relationship between vessel morphology and plant pathogen infection is less clear and need further explanation. In the present study, analysis of stem sections showed the reduced size of xylem in partially resistant line after treatment with pathogen filtrate. There are several reports that Sclerotinia enters the xylem vessels during plant hosts colonization [25,26]. Oxalic acid causes wilting symptoms in plant hosts. Oxalate crystal formation that lead to xylem occlusion and embolisms [27,28] or stomatal opening induced by OA [16] are known to cause water stress in plant. Several reports have shown that water availability have an impact on vessel size and hydraulic conductivity [29-31]. Anatomical investigation showed that vessel diameter was reduced under drought stress [32-34]. A reduced size of xylem vessels subjected to moderate water deficiency that may be associated with control of water flow and a reduction in vulnerability to drought-induced xylem embolism [35]. The more total pit area in the large vessel and stomatal dysfunction in vascular wilt diseases appear to cause increased rates of water deficiency that lead to xylem embolism [36]. The role of xylem diameter in the resistance to vascular wilt diseases was reported in previous studies [37,38]. Grape cultivars with wide xylem vessel were more susceptible to *P. chlamydospora* [38]. Several advantages of small size vessels has been addressed in literature; a) more efficient pathogen compartmentalization, b) prevent the spread of phytotoxins and conidia in a quicker and more efficient way, c) more efficient respond to disease and conductivity of xylem sap due to less vulnerability to drought-induced embolism [29,37,38]. Several studies reported modifications in vascular anatomy of plant hosts under pathogen attack [39,40]. Increase in vessel number and increase in vessel diameter have been observed in resistant and susceptible genotypes of soybean following infection with *Phialophora gregata*, respectively [40]. Similarly, reduced vessel number, diameter, and vascular cambium were observed in tomato under *Fusarium oxysporum* f. sp. *lycopersici* infection compared to the healthy plants [39]. Change in xylem diameter observed in the present study may be related to an adaptation to pathogen filtrate treatment and loss of water caused by OA. These observations also suggest different mechanisms of resistance to virulence factors of Sclerotinia in sunflower.

In conclusion, electrophoretic patterns of total soluble proteins showed a rapid induction of newly synthesized proteins in both lines after treatment with pathogen filtrate. However, in partially resistant line, new protein bands were also observed at molecular mass between 25-50 kDa. These newly induced protein bands might be involved in sunflower resistance pathways and induced in response to *S. sclerotiorum* invasion. The biological importance of these responsive proteins need to be investigated more in future studies. Our study also showed the role of xylem morphology and its impact on xylem susceptibility to *S. sclerotiorum* infection. The findings in this study help us gain a more complete understanding of resistance mechanisms to this cosmopolitan and devastating pathogen which could provide some useful information for breeding programs.

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