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Sclerotinia-Induced Accumulation of Protein in the Basal Stem of Resistant and Susceptible Lines of Sunflower

Robab DAVAR[1], Reza DARVISHZADEH[2,3], Ahmad MAJD[4]

[1]*Payame Noor University, Faculty of Science, Department of Biology, Po Box 19395-3697 Tehran, Iran; Davar.Robab@gmail.com*

[2]*Urmia University, Department of Agronomy and Plant Breeding, Urmia, Iran*

[3]*Urmia University, Institute of Biotechnology, Urmia, Iran*

[4]*Tarbiat Moallem University, Faculty of Science, Department of Biology, Tehran, Iran*

Abstract

Sunflower, *Helianthus annuus* L. is a major oilseed crop widely cultivated across the globe. White mold, caused by the necrotrophic pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary, is a common and widespread pathogen of sunflower. Changes in a partially resistant and a susceptible sunflower line infected with *Sclerotinia sclerotiorum* were studied 12, 24 and 48 h after inoculation. Electrophoretic patterns and quantitative changes in soluble proteins were determined in the basal stem region. Soluble proteins were accumulated post infection in the partially resistant line. A rapid accumulation of stress-related, low molecular weight proteins was induced in both lines by different pathways. By 12 and 24 h post inoculation, stress proteins with molecular masses of 27 kDa had accumulated in infected stems of the partially resistant line. SDS-PAGE results showed the accumulation of proteins with a molecular mass of 55 kDa in the susceptible line and the absence of this band in the resistant line. This relatively faster response to *Sclerotinia sclerotiorum* invasion could be partially responsible for the resistance or susceptibility to this pathogen. The differences between lines may also indicate further avenues worth exploring in host-pathogen relations which could ultimately lead to selection and production of new lines with higher levels of resistance to *Sclerotinia sclerotiorum*.

Keywords: *Helianthus annuus* L., low-molecular-weight proteins, *Sclerotinia sclerotiorum,* SDS-PAGE, soluble proteins

Introduction

Sunflower, *Helianthus annuus* L., is one of the major plant oil crops and is widely cultivated around the world. White mold, caused by the necrotrophic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most devastating and cosmopolitan soilborne plant pathogens, and infects over 500 species of plants worldwide including important field crops, fruit crops, ornamentals, trees, shrubs and numerous weeds (Saharan and Mehta, 2008). White rot caused by *Sclerotinia sclerotiorum* is a major yield-limiting disease of sunflower in the temperate regions of the world. The fungus can attack several plant parts and cause stalk rot/wilt or head rot (Gulya *et al.*, 1997). Rapid drying of the leaves and development of lesions on the tap roots and basal portions of the stem cause plants to die within a few days after the onset of wilting (Dorrell and Huang, 1978). Yield losses can reach up to 100% when weather conditions are favorable for the fungus (Sackston, 1992). In Iran, infections of the sunflower basal stem are considered a potential threat to the entire crop.

To date, sunflower genotypes with different levels of resistance to stem rot have been identified, but no fully resistant genotypes are available (Davar *et al.*, 2010), and so breeding resistant varieties is an important objective. Different features, related to the resistance mechanisms of the plant could be applied for screening the host genotypes (Bazzalo *et al.*, 1991).

Plants possess both pre-formed and inducible mechanisms for resisting pathogen invasion. Extant morphological barriers, secondary metabolites (phytoanticipins), and antimicrobial proteins must be avoided or overcome if pathogens are to successfully invade a plant (Slusarenko *et al.*, 2000). Resistance, according to Agrios (1988) is the ability of an organism to exclude or overcome, completely or in some degree, the effect of a pathogen or other damaging factor. Once contact has been established, elicitors produced and released by the pathogen induce further defenses, comprising the reinforcement of cell walls, the production of phytoalexins, and the synthesis of defense-related proteins (Slusarenko *et al.*, 2000). Defense related genes encode a variety of proteins including enzymes controlling secondary metabolism, pathogenesis related (PR) proteins and regulatory proteins that control the expression of other defense related genes (Dixon *et al.*, 1994).

The induced resistance response of plants to diseases correlates intimately with the accumulation of PR proteins. Pathogenesis-related proteins degrade the fungal cell wall and cause lysis of the fungal cell. Chitin and glucan oligomers released during degradation of fungal cell walls

act as elicitors that elicit various defense mechanisms in plants (Frindlender *et al*., 1993). PR proteins are localized in extracellular regions. When plant pathogenic microorganisms invade plants, they initially multiply in the intercellular spaces of the plant cells. Therefore, the accumulation of antimicrobial proteins in the intercellular space is important for their antimicrobial ability to inhibit the invading pathogens (Lee *et al*., 2008).

PR proteins represent major quantitative changes in soluble protein during the defense response and usually are monomers with a low molecular mass (8-50 kDa) (Stintzi *et al*., 1993).

Induction and accumulation of soluble defense proteins make the plant resistant to pathogen invasion (Van Loon, 1997), and have been correlated with defense against pathogen invasion in many plants, such as cucumber (Rasmussen, 1991), green gram (Ramanathan *et al*., 2000), tobacco (Beaudoin-Eagan and Thorpe, 1985) and tomato (Bashan *et al*., 1985).

Zhao *et al*. (2007) showed the enhanced accumulation of PR proteins in response to *Sclerotinia sclerotiorum* in *Brassica napus*. Low molecular pathogen-related protein was also isolated from *Helianthus annuus* flowers by its ability to inhibit the germination of fungal spores (Marcela *et al*., 2000).

In this study, is been examined whether a susceptible and a partially resistant line of sunflower responded differently to *Sclerotinia sclerotiorum* infection through differential protein accumulation and electrophoretic patterns of the total soluble proteins in the basal stem of infected and noninfected plants. Also is been studied the relationship between the accumulation of stress soluble proteins and the development of induced resistance in susceptible and partially resistant lines.

Materials and methods

Plant material, fungal isolate and Experimental design

Two sunflower lines, C71 and C146, identified as partially resistant and susceptible respectively to isolate SSU107 of *Sclerotinia sclerotiorum* in previous experiments (Davar *et al*., 2010), were used to study any change in electrophoretic patterns of the total soluble proteins of sunflower during the infection process. Seeds were surface sterilized in 2% NaOCl for 3 min, washed three times in sterile distilled water, and sown in 10×12 cm pots filled with sterilized soil collected from the research farm of Urmia University, Iran. The soil was a silty clay with a pH of 7.6 and an EC of 0.6 dSm^{-1}. Tab. 1 summarizes the properties of the soil used in the experiments. Plants were grown for 4 weeks in a controlled environment at temperatures of 24±1°C, 65% relative humidity, and a 12 h photoperiod with light intensity of 200 $\mu Em^{-2}s^{-1}$, until plants were at growth stage V6 to V8 (at least six to eight leaves on a plant) (Schneiter and Miller, 1981).

Isolate SSU107 was cultured on PDA (Potato Dextrose Agar (39 $g\,l^{-1}$, pH 6) medium and grown in the dark at room temperature (25°C). At the V6 to V8 growth stage, mycelial plugs (3 mm diameter) of isolate SSU107 were cut from the growing edge of 3 day old colonies and were placed against the basal stem of the sunflower plants. The stem and mycelial plug were wrapped with Parafilm for 48 h to maintain humidity, following the method of Price and Colhoun (1975). The experimental design was completely randomized with three replications. Fresh sunflower basal stem samples of inoculated and control plants were harvested 12, 24 and 48 h post inoculation. Samples were frozen in liquid nitrogen and then stored at -80°C until biochemical assays could be performed.

Protein extraction

The frozen stem samples were ground to a fine powder in liquid nitrogen and total protein extracted with ice-cold 0.1 M Tris HCl buffer (pH 7.5) containing 5% (w/v) PVP (4:1 buffer volume:fresh weight). The homogenate was centrifuged at 13000 g for 15 min at 4°C, and the supernatant containing the soluble stem proteins was used for determining protein concentration and SDS-PAGE analysis (Abedi *et al*., 2011).

Quantitation of protein using the Bradford assay

The protein concentration was spectrophotometrically determined at 595 nm using the Protein Assay Dye Reagent Concentrate (BioRad) according to Bradford's method (1976). Bovine serum albumin was used as standard reference in this investigation (Stoscheck, 1992).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Flat bed SDS-PAGE was performed with a vertical BioRad System. Was prepared a 100×70×0.5 mm dimension acrylamide gel. Protein samples were separated on 12% SDS- PAGE gels. Prior to electrophoresis, samples were mixed with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% [w/v] glycerol, 5% [v/v] β-mercaptoethanol, 0.001% [w/v] bromophenol blue) and boiled for 5 min. The running buffer was prepared according to Garfin (1990). The buffer system in the strips

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

pH	Ec×10[3a] ($dS\,m^{-1}$)	P ($mg\,kg^{-1}$)	K ($mg\,kg^{-1}$)	N (Total) (%)	Mg ($meql^{-1}$)	Ca ($meql^{-1}$)	Cl ($meql^{-1}$)	OC[b] (%)	SP[c] (%)	CaCO3 (%)	HCO3 (%)	Sand (%)	Silt (%)	Clay (%)
7.60	0.60	39.3	565	0.11	1.87	1.89	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

formed a discontinuous buffer system together with the gel buffer. A high molecular weight ladder of 250 KD (Fermentas) was used for detection of protein bonds on the gel. Electrophoresis was performed at constant current of 50 mA in the BioRad electrophoretic apparatus set at 150 V for 1 hour.

Gels were silver-stained following the silver staining method described by Blum *et al*. (1987). The gels (fixed in TCA) were incubated for 20 min three times in 5% (v/v) methanol, soaked for 1 min in 40 mg/l of Na_2S_2O4, washed two times in water, and incubated for 20 min in a 0.2% (w/v) $AgNO_3$ solution containing 0.035% (v/v) formamide. For protein coloration, the gels were washed two more times and then soaked in a solution of 3% (w/v) Na_2CO_3, 0.035% (v/v) formamide, and 40 mg/l of $Na_2S_2O_4$.

Statistical analysis

Spectrophotometric protein concentration data are presented as means of three readings from three independent experiments. Significant differences were assessed by Student's *t* test. Differences were considered to be significant at the $P \leq 0.05$ level.

Result and discussion

Changes in total soluble proteins

There were significant differences in soluble stem protein content between the two lines when infected by *Sclerotinia sclerotiorum*. Soluble protein content in stems of the partially resistant line C71 increased 12 h and 24 h post inoculation by *Sclerotinia sclerotiorum* (to a maximum of 1.3 times). In stems of the susceptible line C146 there was no significant induction in soluble protein content (Fig. 1).

Malencic *et al*. (2010) also reported that soluble protein content increased in soybean genotypes 12 h and 24 h post inoculation by *Sclerotinia sclerotiorum*. Naglaa and Heba (2011) showed that total soluble protein content in leaves of resistant flax lines increased significantly after powdery mildew invasion. Inoculation with the fungus *Sclerotinia sclerotiorum* induced a rapid accumulation of stress proteins in tomato plants (Gorovits *et al*., 2007).

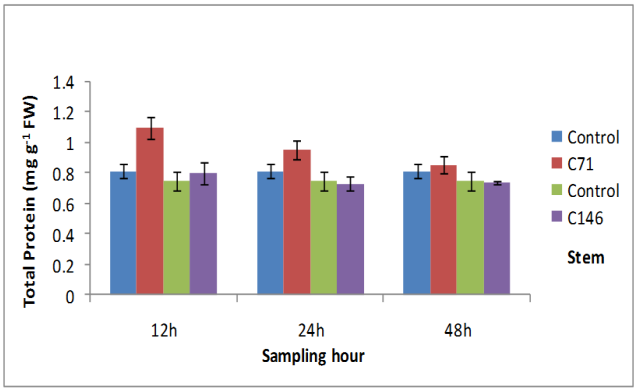

Fig. 1. Levels of soluble proteins in non-infected and *Sclerotinia sclerotiorum* infected stems of two sunflower lines. Data are means ± SD of triplicate samples from three independent experiments. Differences were considered to be significant at the $P \leq 0.05$ level

Bacterial and fungal plant pathogens elicit the synthesis of host proteins which restrict the spread of the pathogens to healthy tissue (Datta *et al*., 1999). Pathogenesis-related proteins are considered stress-related proteins produced in response to infections by viruses, viroids, bacteria and fungi, and are thought to function in the acquired resistance against further infection (Van Loon, 1989). Gnieszka and Iwona (2003) reported that the presence of pathogenesis-related proteins, such as chitinase and thaumatin, in the tissue infected by pathogens, positively correlates with plant resistance to micro-organisms. However, in contrast to most other types of stress proteins, they accumulate in plant tissues to levels that are easily detectable on gels by general protein stains (Van Loon, 1989). Induction of pathogenesis-related proteins has since been found to be invariably linked to necrotizing infections giving rise to systemic acquired resistance (SAR), and has been taken to be a marker of the induced state (Kessmann *et al*., 1994).

Changes in electrophoretic patterns of soluble stem proteins in resistant and susceptible sunflower

To check if there were any changes in polypeptide composition between lines, total soluble stem proteins were size-fractionated on SDS-PAGE. Fig. 2 and 3 show the electrophoretic patterns of soluble stem proteins of partially resistant (C71) and susceptible (C146) lines at 12, 24 and 48 h after inoculation with the fungus *Sclerotinia sclerotiorum*. A number of proteins accumulated in both susceptible and partially resistant lines after pathogen invasion (Fig. 2 and 3). A rapid accumulation of stress-related, low-molecular-weight proteins was induced in both lines in different pathways. Lanes loaded with soluble proteins from the partially resistant line showed a clear difference between control and infected plants. At 12 and 24 h post inoculation, soluble proteins with a molecular mass of 27 kDa accumulated in stem of C71 infected plants. This response was more pronounced at 12h post inoculation and protein band density was high. This polypeptide pattern was detected only in the partially resistant line.

Disease resistance in plants is manifested by limited symptoms, reflecting the inability of the pathogen to grow and spread, and often takes the form of a hypersensitive reaction (HR). Following a HR reaction, systemic acquired resistance leads to the induction of numerous plant genes encoding defense proteins (Ryals *et al*., 1994). These proteins include structural proteins that are incorporated into the extracellular matrix and participate in the confinement of the pathogen, as well as enzymes of secondary metabolism; for instance, those involved in the biosynthesis of plant antibiotics and pathogenesis-related proteins, which represent major quantitative changes in

soluble protein during a defense response. All have been identified in polyacrylamide gels run either in the absence or in the presence of SDS and stained with general protein stains. Pathogenesis-related proteins usually have low molecular mass (8-50 kDa) (Stintzi *et al*., 1993). Marcela *et al*. (2000) isolated a 16 kDa protein from *Helianthus annuus* flowers that inhibits the germination of *Sclerotinia sclerotiorum* spores. Raju *et al*. (2008) reported higher levels of pathogenesis-related proteins in roots and shoots of a resistant cultivar of chickpea than in a susceptible cultivar upon inoculation with the fungus *Fusarium oxysporum*. Black spot disease in rose also induced accumulation of soluble proteins, particularly 27 and 36 kDa pathogenesis-related proteins (Suo and Leung, 2002).

Two polypeptide bands, with molecular mass ranging from 55 to 60 kDa, were only observed in the control plants and disappeared in the *Sclerotinia*-inoculated stems of the partially resistant line (Fig. 2). This may indicate that this particular polypeptide was initially sensitive to *Sclerotinia sclerotiorum* invasion and, hence, disappeared at *Sclerotinia* treatment over the 12, 24 and 48 h post inoculation. Previous studies have clearly shown that oxidative stress may be triggered when plants encounter an aggressive pathogen (Nanda *et al.,* 2010). Oxidative stress can disrupt normal metabolism by oxidizing DNA, RNA, lipids, and proteins affecting the integrity of cell membranes and inactivating key cellular functions (Singh *et al.*, 2009). So, breakdown of proteins and decrease of pathogenesis related (PR) proteins under oxidative stress are expected. Decline of the PR proteins level was detected in viral infected tobacco during programmed cell death, known as the hypersensitive response (HR) in plants (Mittler *et al.,* 1998). Malencic *et al*. (2010) also reported that proteins content influenced in soybean genotypes inoculated by *Sclerotinia sclerotiorum*. Lee *et al*. (2006) monitored changes in the rice proteome responding to fungal pathogen *Rhizoctonia solani* and observed decreased PR proteins associated with abiotic stress.

At 12, 24 and 48 h post inoculation, a new protein band with a molecular mass of 55 kDa appeared in soluble stem proteins of the susceptible line (Fig. 3). The results showed the accumulation of new proteins in the susceptible line and the absence of this band in the partially resistant line.

Certain plant genes are required for susceptibility to plant pathogens. Vogel *et al*. (2002) analyzed the PMR6 mutant of *Arabidopsis* and showed that it exhibits enhanced resistance to powdery mildew. Resistance is associated with a recessive, loss-of-function mutation in PMR6. These points suggest that PMR6 might function as a plant disease susceptibility factor rather than as a component of host defense responses. Resistance and susceptibility are opposite sides of the same coin, and the study of disease resistance cannot be conducted without reference to susceptibility.

In conclusion, by using SDS-PAGE the current study revealed that inoculation with the fungus *Sclerotinia sclerotiorum* induced a rapid accumulation of stress-related proteins in a partially resistant sunflower line. This more rapid response to *Sclerotinia sclerotiorum* invasion could be partially responsible for the resistance or susceptibility to this pathogen. The findings in this paper provide benefits in the better understanding of *Sclerotinia* biotic stress and the understanding of stress related physiology in sunflower. These differences between lines, also may point to further pathways in exploring host-pathogen relations which could ultimately lead to selection and production

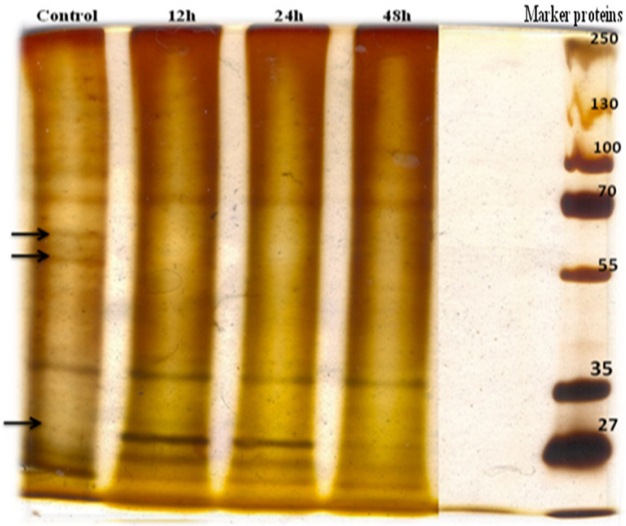

Fig. 2. SDS-PAGE patterns of total leaf soluble protein fractions from a resistant C71 sunflower genotype at 12, 24 and 48 h post inoculation. The arrows indicate bands which were influenced after *Sclerotinia sclerotiorum* invasion

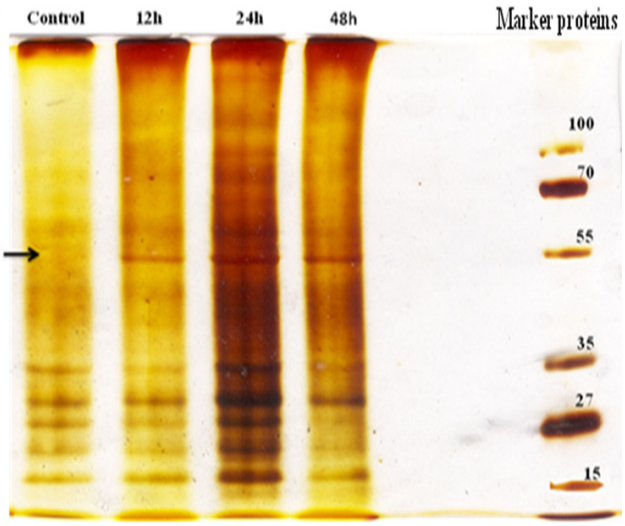

Fig. 3. SDS-PAGE patterns of total leaf soluble protein fractions from a susceptible C146 sunflower genotype at 12, 24 and 48 h post inoculation. The arrows indicate bands which were induced after *Sclerotinia sclerotiorum* invasion

of new genotypes with higher levels of resistance to *Sclerotinia sclerotiorum* and also to some other plant pathogens.

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