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DOWNY MILDEW DISEASE CAUSED BY *Plasmopara halstedii* (Farl.) Berl. AND de Toni. IN SUNFLOWER (*Helianthus annuus L*): IDENTIFICATION OF PATHOGENESIS RELATED PROTEINS AND HISTOLOGICAL CHANGES

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SUMMARY

Downy mildew disease caused by *Plasmopara halstedii* is emerging as one of the major diseases of sunflower in the Indian sub-continent. The yield loss due to this pathogenic fungus is accounting for more than 50% in the susceptible sunflower genotypes. The results of the present study clearly indicated that downy mildew causes extensive cellular changes, leading to hypertrophism and leakage of the cellular contents. A reduction in the total soluble protein was recorded in the infected leaves with no reduction in the percent of heat-stable proteins. Further, characterization of the PR proteins in the downy mildew infected leaves resulted in the isolation of two 100 kD proteins which appear to be specific to downy mildew infection.

Key words: Sunflower PR proteins, *Plasmopara halstedii*, histology

INTRODUCTION

Sunflower, one of the potential sources of vegetable oil and protein, is one of the major oilseed crops in India. With the increase in the area and the production of sunflower, there is a concomitant increase in the severity and the incidences of diseases especially, *Alternaria helianthi*, *Puccinia helianthi* and *Plasmopara halstedii*, which ultimately account for the yield loss anywhere between 30-40% (Kolte, 1990). Downy mildew disease caused by the fungus *Plasmopara halstedii* (Farl.) Berl. and de Toni. is a fairly new disease and its occurrence is recorded in Indian

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sub-continent in recent years. Sunflower genotypes with genetic resistance to downy mildew have been identified and incorporation of such resistance through breeding programs is being done in many parts of the world (Sackston, 1992; Mouzeyar *et al.*, 1994).

Plant pathogens usually bring about innumerable changes both at the cellular and molecular levels in the host so that they efficiently colonize it and propagate there. Such changes include cell membrane damage, alterations in the cell permeability and turgor pressure inside the cell followed by excessive ion leakage, accumulation of phytoalexins, depositions of phenolic compounds and cell wall proteins, synthesis of pathogenesis-related proteins and localized cell death (van Loon, 1997; Schaffer, 1994). A comprehensive knowledge of the biochemical basis for interactive and response phenomenon between host and pathogen at the cellular and molecular levels need to be probed to understand the genetic mechanism of resistance to diseases and other stress responses in plant systems.

Morden, a short-duration variety with high yield potential, susceptible to downy mildew and other diseases has been selected for use in this study. The objectives of the present study were (1) to evaluate the histological changes in sunflower leaves caused by downy mildew infection and (2) to examine whether the pathogen induces the synthesis of pathogen related-proteins (PRs) in sunflower as related to infection.

MATERIAL AND METHODS

Histological studies

Leaf pieces measuring one square centimeter from healthy and infected leaves (including a part of uninfected tissue) of sunflower were fixed in Carnoy's B fixative for two hours and later stored in 70% alcohol. The leaf pieces were dehydrated using alcohol-butanol series and infiltrated with paraffin wax. Section of 10 μ thick were cut using Erma rotary microtome and stored in a dust free chamber. The sections were deparaffinized using xylene-butanol series and stained with periodic acid-Schiff's reagent. After the staining the sections were cleared in xylene-butanol series and mounted using the DPX mountant.

Sample preparation for protein analysis

Acetone powder of 30-day old downy mildew - infected and healthy leaves from the genotype Morden were made by homogenizing the leaves with chilled acetone and subsequently washing with another aliquot of chilled acetone for removing the chromogenic substances. The acetone powder was air dried and stored at -20°C till further use.

Isolation of PRs

The acetone powder was homogenized with 50 mM Tris-HCl buffer, pH 7.2, containing 50 mM EDTA and 10 mM 2-mercaptoethanol (1:5 W/V) at 5°C, and centrifuged at 15,000 rpm at 5°C for 20 min. The clear supernatant collected after centrifugation was subjected to ammonium sulphate fractionation. The 40-60% ammonium fractionated proteins were dissolved in $1/10^{th}$ of the original volume and dialyzed extensively against water and lyophilized before subjecting it for purification on DEAE Sephadex-G25.

Thermal stability of the soluble proteins

Known amounts of the total soluble proteins were subjected to thermal denaturation by incubating the samples at 80°C for 30 min. The samples were cooled in an ice bath and centrifuged at 15,000 rpm for 20 min at 5°C. The supernatant was removed and the sediment dissolved in 20 mM Tris-HCl buffer, pH 7.2, containing 2% sodium lauryl sulphate (SDS) and 5% 2-mercaptoethanol. The protein content in the samples was estimated by measuring the absorbance at 280 nm.

DEAE Sephadex G-25 ion-exchange chromatography

DEAE Sephadex G-25 was equilibrated with 50 mM Tris-HCl buffer, pH 7.2, containing 50 mM EDTA and 10 mM 2-mercaptoethanol and 0.002% sodium azide. Twenty mg of the protein sample were applied onto the column (1.0 cm x 10.0 cm) and the unbound proteins were washed off using the same buffer until the OD at 280 nm almost attained the baseline. The bound proteins were eluted using 0.0 – 2.0 M sodium chloride gradient in the same buffer. Measuring the absorbance at 280 and 328 nm for proteins and phenolics, respectively, monitored the elution profile of the proteins. The suitable fractions were pooled and dialyzed extensively against distilled water overnight with two changes. The dialyzed proteins were lyophilized and stored at –20°C until further use.

SDS-PAGE analysis

The protein samples were dissolved in Laemmli's buffer (20 mM Tris-HCl buffer pH 6.8 containing 2% SDS amd 5% 2-mercaptoethanol) and incubated for one hour at 37°C (Laemmli, 1970). Before applying onto the gel, the samples were spun for 15 min at 15,000 rpm at room temperature. About 200 µg protein out of the clear supernatant was applied. SDS gel electrophoresis was carried out on 5% stacking gel and 12% separating gel, using Tris-Borate buffer at pH 8.8 at 100V, and bromo phenol blue as the tracking dye. The proteins were visualized using the silver staining method (Merril *et al.*, 1981) and the gels were documented using the Herolab Easy image plus system.

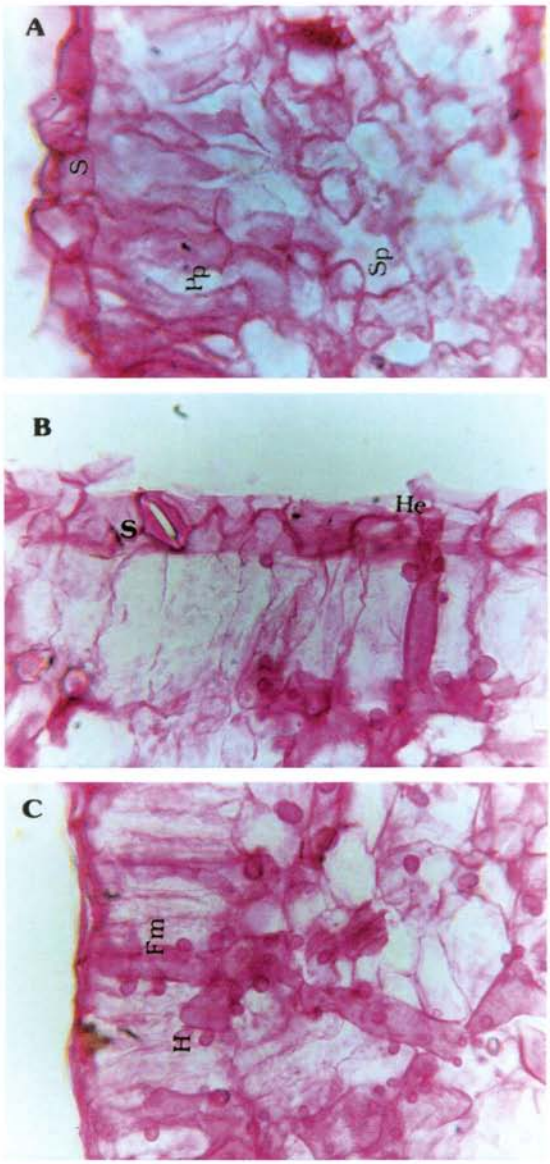

Figure 1: Histological sections of control and downy mildew-infected leaves.
A. Transverse section of the leaf showing intact upper epidermis and the mesophyll tissue (x 400).
B. Transverse section of downy mildew-infected leaf showing the entry of the fungal mycelium into the upper epidermal layer of the cell (x 400).
C. Transverse section of the downy mildew-infected leaf showing the proliferated fungal mycelium with haustoria projecting into the mesophyll cells (x 400).
Pp : Palisade parenchyma; Sp: Spongy parenchyma; Fm: Fungal mycelium; H: Haustoria; S: Stomata; He: Hyphal entry through epidermis

RESULTS AND DISCUSSION

Systemic infection by the downy mildew pathogen, *Plasmopara halstedii*, causes a variety of changes in plant metabolism and development like reduction in growth, dwarfing of the plant, abnormally thick and downward-curled leaves that show prominent yellowish and pale green mottling, malformation in the development of head, plants remaining sterile or with poor seed setting, white downy cover underneath the leaf associated with chlorotic appearance on the upper side of the leaf. Earlier reports also revealed that the stems and leaves become brittle, and show loss of phototrophic and negative geotrophic responses. In case of the old leaves, the symptom expression is delayed until flowering without any chlorotic symptoms on the leaves (Viranyi, 1978; Kolte, 1990; Sackston, 1992; Mouzeyar *et al.*, 1994; Torresan *et al.*, 1998).

The present studies have shown that the initiation of infection by the downy mildew fungus in the leaf tissue seems to be through the spores that germinate and enter through the upper epidermis (Figure 1B). Further the mycelia ramify in the palisade and spongy tissue and the haustoria penetrate the cells of these tissues resulting in discharge of cell contents (Figure 1C). The sporangiophores bearing sporangia emerge out through the lower epidermis leading to its damage (Figure 1C and Table 1).

Table 1: Variation in leaf thickness of sunflower leaves infected with *Plasmopara halstedii*

	Thickness (µm)	
Tissue	Healthy	Infected
Total leaf	177.1	219.5
Upper epidermis	15.4	7.7
Palisade tissue	69.3	107.8
Spongy tissue	77.0	103.9
Lower epidermis	15.4	distorted or broken

Further, it has been shown that the variability in the resistance among the genotypes is not a function of the anatomical variation but it is based on either the pathogen's capability or incapability to penetrate the host tissue (Duletić-Laušević and Mihaljčević, 1997). Studies have shown that the downy mildew fungus has significant effect on the leaf tissues, which is reflected by hypertrophied cells in both palisade and spongy tissue. Further, this has an impact on the tissue thickness (Table 1). The individual palisade cells show greater variation in size and shape (Table 2). The plastids in the infected cells show variation in number as compared with healthy cells (Figures 1A and 1C). There is an increase in the number of plastids and their irregular distribution in the infected cells unlike the row of plastids along the cell margins as seen in the healthy cells. The size of the plastids remains almost uniform (1.02µm) throughout the tissue in the healthy leaf whereas, in the infected tissue, variation in the plastids size ranges from 1.02 to 3.85µm.

Table 2: Variation in cell size and shape in the downy mildew-infected sunflower leaves

		Cell size (μm)	
		Healthy	Infected
		Palisade layer	
Cell size	- length	57.7	84.7
	- breadth	11.6	11.5
Cell shape		Cylindrical	Irregular
		Spongy layer	
Cell size		7.7 to 15.4	distorted or broken
Cell shape		Globular to irregular	distorted or broken

Estimation of the phenolics and soluble protein in the healthy and the downy mildew-infected leaf samples clearly indicated significant variation in their contents (Table 3). However, the soluble content in both healthy and uninfected leaves from the infected plants seems to show little variation. The thermal denaturation studies indicate that the sunflower leaf samples contain higher levels of thermotolerant proteins, no matter if infected with downy mildew or not, as compared with the thermosensitive proteins (Table 3).

Table 3: Total phenolics and soluble protein content in the sunflower leaves infected by *Plasmopara halstedii*

	(mg per g of sample)		(% of soluble proteins)	
	Soluble proteins	Phenolics	HS proteins	HU proteins
Healthy	152.0	0.599	80.8	19.2
DM infected	69.0	0.184	77.3	22.6
DM uninfected[a]	130.0	0.422	79.5	20.4

[a] Uninfected (visually) leaf sample from the infected plants respectively
HS proteins: heat stable proteins; HU proteins: heat unstable proteins

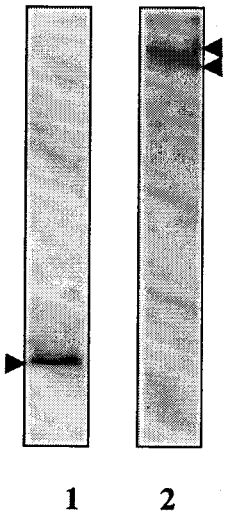

Figure 2: SDS-PAGE pattern of the DEAE cellulose purified proteins from sunflower leaves. Lane 1: 20 kD protein from the healthy leaf & Lane 2: two closely moving 100 kD proteins from downy mildew-infected leaf

The results of earlier studies indicate that plants after subjecting to any of biotic or abiotic stresses synthesize stress proteins like HSPs (temparature), osmotin (salinity) and PRs (bacteria, virus or fungi) as a measure of response to overcome the deleterious effects (Bell, 1981; Bowels, 1990; Keen, 1992: van Loon, 1997). Pathogenesis related proteins (PRs) are unique host-specific proteins, whose synthesis is triggered during biotic stress. They are found too highly soluble in extremes of pH, thermostable and resistant to proteolytic enzymes (van Loon, 1997). The thermostable proteins in the downy mildew-infected and healthy sunflower leaves showed no significant variation in their content. But the ammonium sulphate fractionation followed by ion-exchange chromatography on DEAE-sephadex G-25 revealed the presence of single 280nm absorption peak eluting at 80 mM sodium chloride concentration in case of the downy mildew-infected samples as compared with the peak eluted at 50 mM sodium chloride concentration in the healthy sample. The SDS-PAGE analysis of these peaks revealed the presence of a 20 kD protein in the control (data not shown) and a unique set of two closely moving protein bands of molecular size 100 kD in the downy mildew infected sample (Figure 2). Since these proteins were treated with β-mercaptoethanol during the processing of proteins during electrophoresis, the SDS-PAGE analysis clearly indicated that these proteins were monomers in nature. Attempts are underway for developing a screening kit for downy mildew in sunflower using the polyclonal antisera for these proteins.

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MILDIU DE GIRASOL (*Helianthus annuus*) CAUSADO POR *Plasmopara halstedii* (Farl.) Berl Y De Toni: IDENTIFICACIÓN DE PATOGÉNESIS RELACIONADA CON CAMBIOS HISTOLÓGICOS Y CON PROTEINAS

RESUMEN

El Mildiu, enfermedad de girasol, causada por *Plasmopara halstedii* está emergiendo como una de las más importantes en el subcontinente de India. Las pérdidas de rendimiento debidas a este hongo patógeno representan más del 50 % en los genotipos susceptibles. Los resultados del presente estudio indicaron claramente que el mildiu causa cambios celulares extensivos, que dan lugar a hipertrofismo y pérdidas de los contenidos celulares. Una reducción en las proteínas solubles totales fue observada en las hojas infestadas con no reducción en el porcentaje de proteínas estables al calor. Además la caracterización de las proteínas PR en las hojas infestadas por mildiu resultó en el aislamiento de dos proteínas 100 KD que son específicas a la infección por mildiu.

LE MILDIOU DU TOURNESOL, *Plasmopara halstedii* (Farl.) Berl ET De Toni: IDENTIFICATION DES PROTÉINES ET DES MODIFICATIONS HISTOLOGIQUES LIÉES À LA PATHOGÉNÈSE

RÉSUMÉ

Il ressort que l'agent du mildiou, *Plasmopara halstedii*, est l'une des principales maladies du tournesol aux Indes. Les pertes de rendement occasionnées par ce champignon pathogène atteignent plus de 50 %, chez les génotypes sensibles. Les résultats de cette étude indiquent clairement que le mildiou provoque des modifications cellulaires importantes, conduisant à l'hypertrophie et à la perte des constituants cellulaires. On observe une réduction des protéines totales solubles dans les feuilles infectées et un maintien de la teneur des protéines thermostables. La caractérisation détaillée des protéines PR dans les feuilles infectées par le mildiou a conduit à l'isolement de deux protéines de 100 kD, spécifiques de l'infection par le mildiou.