HELIA, **23**, Nr. 33, p.p. 1-18, (2000) UDC 633.854.78:632.4

BIOCHEMICAL AND HISTOLOGICAL CHANGES ASSOCIATED WITH DOWNY MILDEW (*Plasmopara halstedii* (Farl.) Berl. and de Toni) INFECTION IN SUNFLOWER (*Helianthus annuus* L.)

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Received: December 22, 1999
Accepted: September 07, 2000

SUMMARY

Biochemical changes associated with downy mildew infection in sunflower revealed an increase in the total soluble proteins, 0-40% ammonium sulfate fractionated proteins and total soluble sugars. Molecular sieve chromatography of 0-40% ammonium sulphate fraction revealed for presence of a high molecular weight protein and polysaccharide in the downy mildew infected sunflower leaves. Antibodies raised against the high molecular weight protein and polysaccharide were used in the western blot/dot blot analysis. It has been shown that the downy mildew disease also induces PR proteins, which have antigenic homology with PR-S protein, a member of the PR-5 class of proteins. An increase in iPA (isopentenyl adenosine) content in the infected sample was observed. A positive correlation exists between iPA level, 0-40% protein and total sugar content. Histological studies revealed that the downy mildew fungal mycelium extensively proliferates, ramifies extensively the leaf tissue and forms a nutritional link with the host cell by producing the intracellular haustorium.

Key words: **sunflower, downy mildew, cytokinin, PR proteins, polysaccharides, histology**

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the major sources of vegetable oil and protein in India. The crop suffers from some major fungal diseases such as leaf

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spot, rust, downy mildew, collar rot and head rot, resulting in extensive yield losses. Downy mildew of sunflower caused by *Plasmopara halstedii* (Farl.) Berlese et de Toni, is of concern as it accounts for 15-20% of the yield loss. This disease is found in more temperate regions where emerging seedlings are exposed to low temperature and abundant rainfall. 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*., 1993). Downy mildew fungus is an obligate parasite and at least 7 races of this parasite are known (Gulya *et al*., 1991). The disease is characterized by poor seed set, damping-off, seed rot and systemic symptom like stunting of plants and total chlorosis of upper leaves. The affected plants bear abnormally thick, downward curled leaves that show prominent yellow and green epiphyllous mottling. A hypophyllous downy growth of the fungus, consisting of the conidiophores and conidia, develops and covers large areas that are concurrent with epiphyllous yellow spots (Kolte, 1990).

Plant pathogens bring about several changes at the cellular, molecular and hormonal levels, which needs to be thoroughly understood. Right from the dormant seed to maturity, plants are exposed to attack by a broad variety of potentially pathogenic microorganisms, predatory insects and other invertebrate pests (Shewry and Lucas, 1977). To counter these diverse threats, plants have evolved an active defense mechanism in which they express specific genes and synthesize large number of pathogenesis-related proteins (PR) that act directly on pathogens inhibiting their growth (Bradley *et al*., 1992).

PR proteins have been defined as plant proteins that are induced in response to both biotic stresses, like pathogens and pests, and abiotic stresses, like UV, wounding and osmotic stress (Van Loon, 1997). Based on serological properties, molecular mass and sequence data, PR proteins have been grouped into 11 major classes (Van Loon *et al*., 1994) which comprise four families of chitinases (PR-3, PR-4, PR-8 and PR-11), β-1,3-glucanases (PR-2), proteinase inhibitors (PR-6), peroxidase (PR-9), thaumatin like PR-5 family, and the birch allergen Betv1- related PR-10 family. PR-1 family has unknown biochemical properties. However, Alexander *et al*. (1993) predicted that PR-1a may exert direct fungicidal activity by slowing down pathogen establishment. PR proteins of class -2 having glucanase activity and PR proteins of classes -3, -4, -8 and -11 having chitinase activity are reported to have a role in degrading fungal cell wall (Schlumbaum *et al*., 1986; Mauch *et al*., 1988). PR-5 class of proteins showing high homology to thaumatin-sweet tasting protein from fruits of a monocot *Thaumatococcus daniellii*, exerts antifungal activity by permeabilizing fungal membranes (Anzlovar *et al*., 1998).

In this paper we describe the biochemical changes associated with downy mildew infection, the partial purification of the high molecular weight protein and polysaccharide, variations in the hormonal levels and histological changes associated with downy mildew infection of sunflower leaves.

MATERIALS AND METHODS

Sunflower seeds (*Helianthus annuus* L.), cv. Morden, were pre-germinated, sown in pots and allowed to grow under natural light for 30 days. As downy mildew fungus is an obligate parasite, the infection was inducted by rubbing an infected leaf over uninfected leaves. An equal number of healthy seedlings were also maintained in natural condition.

Extraction and ammonium sulphate fractionation of leaf proteins

Fresh leaves from 30-day-old downy mildew infected and healthy plants were cut into small pieces and homogenized in a blender with chilled acetone (1:10 W/V). The homogenate was filtered through Whatman 1 filter paper. The sediment was washed with chilled acetone, until most of the pigments were removed. The powder was air dried and stored at -20°C.

Isolation of PR proteins

Acetone powder was stirred with extraction buffer (0.05 M Tris HCl buffer, pH 7.2 containing 0.05 M EDTA, 0.01 M β-mercaptoethanol, 25 mM ascorbic acid and PVPP 100 mg/g of acetone powder) (1:20 W/V) at 4°C for 10 min. The slurry was centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was subjected to 0-40% and 40-60% ammonium sulphate precipitation. The precipitates were collected by centrifugation at 15,000 rpm for 20 min at 4°C and dissolved in the minimum quantity of extraction buffer, dialyzed against double distilled water using 12 kD cut off membrane, and lyophilized.

Sephadex G-200 molecular sieve chromatography

The 0-40% ammonium sulphate fraction from both healthy and infected sunflower leaves was subjected to gel filtration chromatography on Sephadex G-200 column (30 x 2 cm) equilibrated with 0.02 M Tris HCl pH 7.2 containing 150 mM NaCl, 5 mM β-mercaptoethanol, 0.02% NaN_3 and 0.1% Triton X-100. About 40 mg of the protein in 4 ml of 0.05 M Tris HCl buffer was loaded and 2 ml fractions were collected. The elution profile for protein, phenolic and sugar content was followed by measuring the absorbances at 280, 328 and 490 nm, respectively (Dubois *et al.*, 1956).

Determination of protein, phenolic, sugar and iPA content

The protein content in the samples was estimated by the dye binding method (Bradford, 1976) using BSA as standard. The phenolic content in the samples was determined by measuring the absorbance at 328 nm using chlorogenic acid as standard. The sugar/polysaccharide and reducing sugar contents were determined by the method described by Dubois *et al.* (1956) and A.O.A.C. (1980), respectively. The iPA level in leaves was determined by the indirect ELISA method using anti-

body raised against iPA-BSA conjugate as per the method described by Shashidhar *et al*. (1996).

SDS-PAGE analysis

SDS-PAGE was carried out according to the method described by Laemmli (1970). The protein samples were dissolved in Laemmli's buffer and incubated for 1 h at 42°C. About 300 μg of protein out of the clear supernatant were applied. SDS gel electrophoresis was carried out on 5% stacking gel and 12% separating gel, using Tris Glycine buffer pH 8.8 at 100 V and bromophenol blue as the tracking dye. The proteins were visualized using the silver staining (Sambrook *et al*., 1989), periodic acid-Schiff staining (Leach *et al*., 1980) and western blot analysis using IgY raised for the high molecular weight protein and polysaccharide and PR-S antibody (Pierette Geoffroy, France). The IgY for the high molecular weight protein and polysaccharide was raised and purified as per the protocol described by Song *et al*. (1985). The gels/blots were documented using the Herolab Easy Image Plus System.

Western blot and dot blot analysis

The proteins in the gel after SDS-PAGE were transferred to the nitrocellulose membrane as described by Towbin *et al*. (1979). The nitrocellulose membrane was blocked using 1% BSA in TBS (50 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$ pH 7.0) for 4 h at 4°C. The membrane was incubated with primary antibody (1:1,000 V/V of IgY for the high molecular weight protein and polysaccharide, and 1:2,000 V/V IgG for PR-S protein) in TBS containing 0.5% BSA for 4 h. The membrane was washed four times with TBS for 10 min each and incubated with secondary antibody (1:10,000 V/V for IgY and 1:500 V/V for gig), conjugated with alkaline phosphatase for 4 h and washed four times with TBS for 5 min each. Bands were developed using NBT/BCIP substrate solution in 0.1 mM Tris HCl buffer, pH 9.0 containing 5 mM $MgCl_2$ and 10 mM NaCl.

Histological studies

Leaf pieces measuring approximately 1 cm^2 were fixed using Carnoy's B fixative for 2 h and later stored in 70% alcohol. The leaf pieces were dehydrated using different ratios of alcohol-butanol series and infiltrated with paraffin wax. 10 μ thick sections were cut using Erma Rotary Microtome and fixed onto glass slides using an adhesive (gelatin). The sections were then deparaffinized using xylene/butanol and stained with periodic acid-Schiff's reagent and mercuric bromophenol blue. The sections were cleared using xylene/butanol series and mounted using DPX.

RESULTS

Soluble proteins, total sugar and reducing sugar in healthy and DM infected sunflower leaves

Increases in the total soluble proteins and protein content of 0-40% ammonium sulphate fraction were observed in the infected leaf samples when compared with the healthy ones, followed by a decrease in the protein content of 40-60% ammonium sulphate fraction in the infected leaf samples. The results indicated that the total sugar content in the downy mildew infected leaves was higher when compared with the healthy leaves, followed by a decrease in the reducing sugar content in the downy mildew infected leaves (Table 1).

Table 1: Soluble proteins, total soluble sugars, reducing sugars and iPA levels in healthy and downy mildew infected sunflower leaf samples*

	TSP	0-40%	40-60%	TSS	RS	iPA
Healthy	83.66 ± 2.96	20 ± 2.21	6.55 ± 0.138	4.53 ± 0.127	0.291 ± 4.4	1.02 ± 0.09
Infected	106.33 ± 3.52	30.1 ± 2.25	1.63 ± 0.295	5.02 ± 0.046	0.191 ± 4.4	2.71 ± 0.39
CD at 5%	NS	NS	0.805	0.565	12.44	0.898

* Data presented in the table are the mean of quadruplicate values ± standard error
TSP - total soluble proteins expressed as mg/g of acetone powder
0-40% ammonium sulphate fractionated proteins expressed as mg/g of acetone powder
40-60% ammonium sulphate fractionated proteins expressed as mg/g of acetone powder
iPa - iPa levels expressed as pico moles/g fresh weight of leaf sample

An increase in the polysaccharide content in the crude and 0-40% ammonium sulphate fraction of downy mildew infected leaves was observed (Figure 1). The total sugar content represented the polysaccharides as well as the glycoproteins present in the leaves as the low molecular weight sugars were removed from the samples by dialyzing extensively against water using 12 kD dialysis membrane.

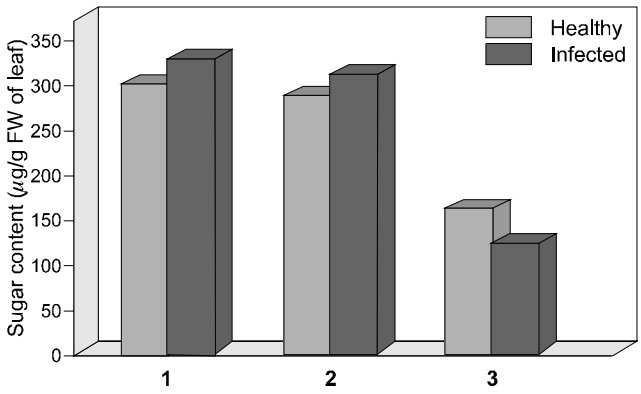


Figure 1: Total polysaccharide content in crude, 0-40% and 40-60% ammonium sulphate fractions. All fractions were dialyzed against water using 12 kD dialysis membrane.
1. Crude,
2. 0-40% ammonium sulphate fraction and
3. 40-60% ammonium sulphate fraction.

Hormonal levels

Isopentenyl adenosine (iPA) levels as estimated by ELISA showed significant increases in the downy mildew infected samples compared with the healthy ones (Table 1, Figure 2b). Correlations between iPA and protein fractions revealed that there existed a positive correlation between 0-40% protein fraction (r=0.773) and a negative correlation with 40-60% protein fraction (r=-0.897) with increase in iPA content (Table 1, Figures 2a and 2b). In the downy mildew infected leaf tissue an increase in total sugar content was observed, which showed positive correlation with iPA but not with reducing sugars (Table 2, Figure 2c).

Table 2: Variation in leaf thickness of sunflower leaves infected with *Plasmopara halstedii*

Tissue	Thickness (µm)	
	Healthy	Infected
Total leaf	127.50	157.25
Upper epidermis	12.75	12.75
Palisade tissue	51.00	51.00
Spongy tissue	63.75	93.50

However, the relative concentration of ABA and DHZR determined for these samples showed no significant variation (personal communication).

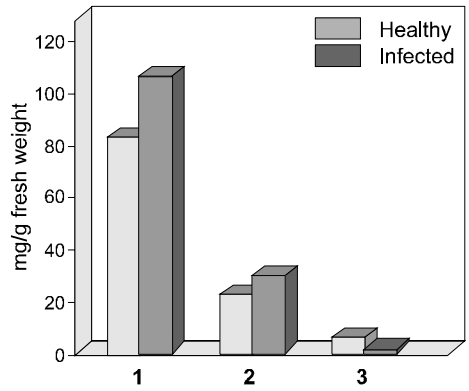


Figure 2a: Relative levels of total soluble proteins, 0-40% and 40-60% ammonium sulphate fractionated proteins in healthy and downy mildew infected sunflower leaves expressed as mg per g of fresh weight.
1. Total soluble proteins,
2. 0-40% soluble proteins,
3. 40-60% soluble protein.

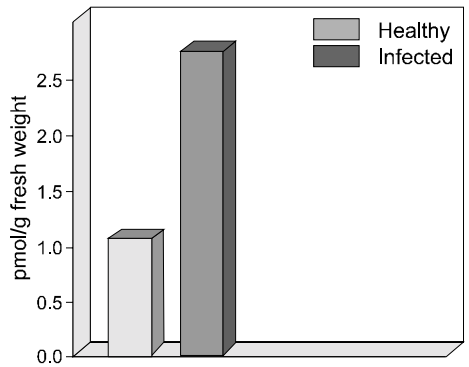


Figure 2b: Level of iPA (pmol/g FW of leaf) in healthy and downy mildew infected sunflower leaves.

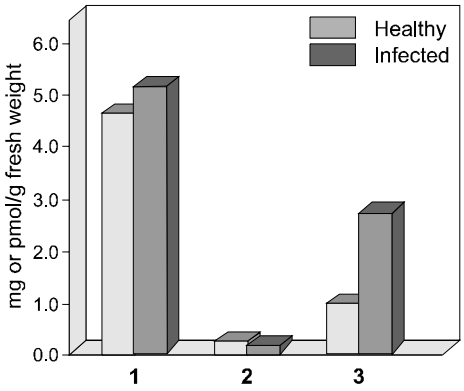


Figure 2c: Relative levels of total sugars, reducing sugars and iPA content in healthy and downy mildew infected sunflower leaves.
1. Total soluble sugars (mg/g of leaf sample),
2. Reducing sugars (mg/g of leaf sample),
3. iPA (pmol/g FW of leaf).

SDS-PAGE of crude, 0-40% and 40-60% protein samples

SDS-PAGE was carried out for the crude, 0-40% and 40-60% ammonium sulphate protein fractions from both infected and healthy sunflower leaves. The bands were less visible and less apparent in the infected sample lanes when compared with the healthy sample lanes (Figure 3).

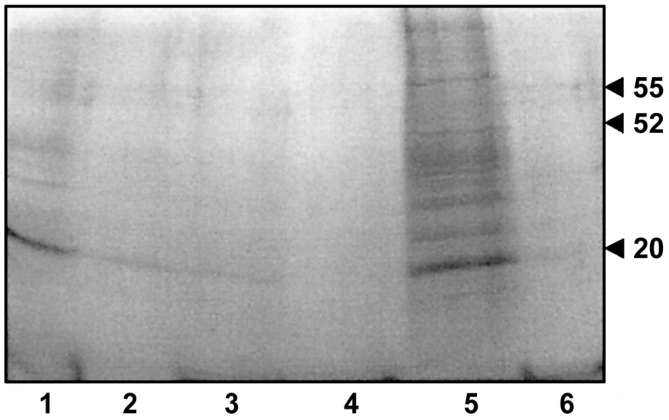


Figure 3: Total soluble proteins, 0-40% and 40-60% protein patterns of healthy and downy mildew infected samples of sunflower on a 12% sodium dodecyl sulphate polyacrylamide gel. 300 µg of protein loaded onto each well. Lanes 1, 3 and 5 - healthy, lanes 2, 4 and 6 - infected, lanes 1 and 2 - total soluble proteins, lanes 3 and 4 - 0-40% ammonium sulphate fractionated proteins, and lanes 5 and 6 - 40-60% ammonium sulphate fractionated proteins.

A prominent major band of approximate molecular weight 20 kD appeared in all the lanes. Two closely moving bands of approximate molecular weight 55 kD were also found in all the samples. The proteins in the infected samples stained positive to periodic acid-Schiff's stain. The banding pattern was observed to be a

smear in the high molecular weight region indicating the presence of glycoproteins. None of the proteins in the healthy sample stained positive for glycoproteins (data not shown).

Partial purification of 0-40% protein

The elution profile of 0-40% ammonium sulphate protein fraction from infected samples on Sephadex G-200 column showed the presence of high molecular weight protein (eluting between 11 and 18 fractions) and a polysaccharide (eluting between 20 and 40 fractions) but not in the healthy ones (Figures 4a and 4b).

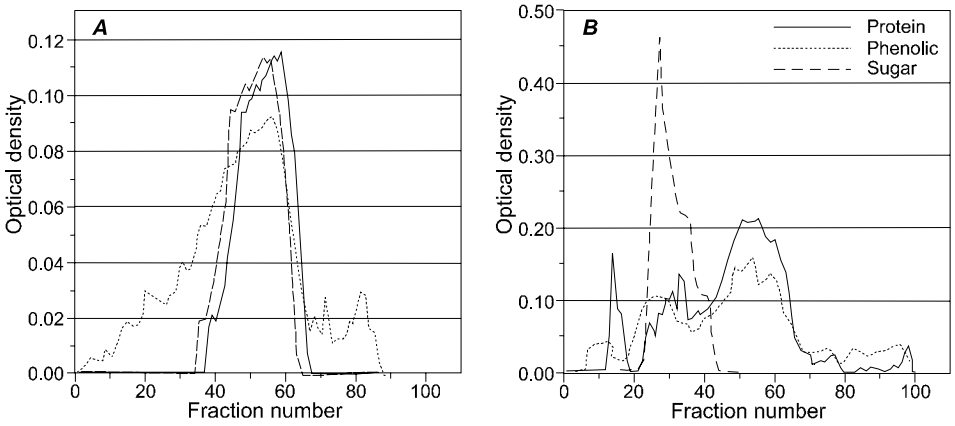


Figure 4: Elution profile of 0-40% ammonium sulphate protein fraction from healthy ***(A)*** *and downy mildew infected* ***(B)*** *sunflower leaf samples fractionated on Sephadex G-200 column (30 x 2 cm). 40 mg of protein sample loaded on column. The elution profile for proteins, phenolics and sugars were monitored by measuring the absorbance at 280 ηm, 328 ηm and 490 ηm, respectively.*

The low molecular weight protein fractions (eluting between fractions 35 and 65) were common for both healthy and infected samples. However, this fraction was more closely associated with the phenolic compounds in the infected samples (Figure 4b). The phenolic content was associated with protein in both healthy and infected samples. Further, high molecular weight polyphenolic compounds (eluting between fractions 14 and 34) which were not associated with proteins were observed in the healthy samples (Figure 4b). It is generally known that the presence of polysaccharides and glycoproteins causes poor resolution of proteins on SDS gels as well as poor protein staining with silver stain and Coomassie brilliant blue. Results from our studies, as shown in Figures 1, 4a and 4b, reveal the presence of high molecular weight polysaccharides. The poor separation as well as staining as observed in our SDS gels (Figure 3) may be due to the interference of high molecular weight polysaccharides and the polysaccharides and the polyphenolics associated with low molecular weight proteins.

Western blot and dot blot analysis

For immunizing the birds, the high molecular weight protein fraction and high molecular weight polysaccharide from infected samples were used and the IgYs were used in the western blot analysis. The IgY for polysaccharide showed smear stain in the high molecular weight region for the infected samples but not in the healthy ones (Figure 5a).

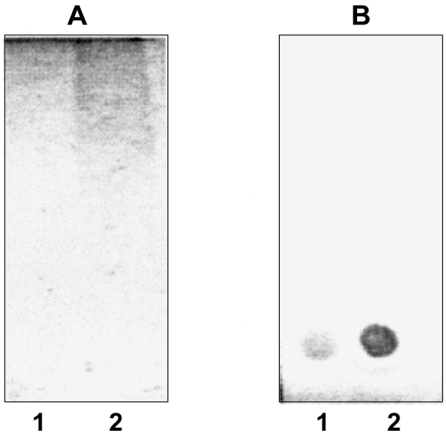


Figure 5:
a) *Western blot analysis of 0-40% ammonium sulphate fractionated proteins from healthy and downy mildew infected samples using IgY for high molecular weight polysaccharide.*
b) *Dot blot analysis of 0-40% ammonium sulphate fractionated healthy and downy mildew infected samples probed with IgY for high molecular weight polysaccharide.*
1. healthy and 2. infected.

Since the staining was poor, we carried out the dot blot analysis for the same samples. High color intensity in the infected sample on the blots was observed (Figure 5b). These results indicate the presence of the high molecular weight polysaccharide in the downy mildew infected but not in the healthy samples. The IgY for protein detected two closely moving bands of approximate molecular weight 50 kD and intensity of staining was found to be higher for the infected sample although no protein bands were found in the protein gel. However, another band of an approximate molecular weight around 45 kD (Figure 6a) which was detected on protein gels did not show any positive reaction with IgY raised against the high molecular weight protein (Figure 6b).

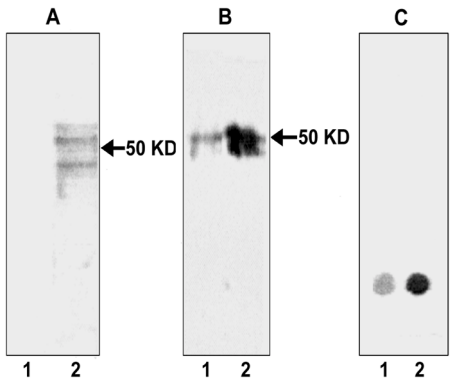


Figure 6:
a) *Gel photograph showing the high molecular weight proteins bands of 0-40% protein fraction eluted from Sephadex G-200 column. The protein bands were visualized by silver staining. 1. healthy and 2. downy mildew infected samples of sunflower.*
b) *Western blot analysis of the 0-40% healthy and infected samples using IgY for high molecular weight protein. Arrow indicates approximate molecular weight of 50 kD.*
c) *Dot blot analysis of 0-40% healthy and infected samples probed with IgY raised against the high molecular weight protein.*

A dot blot analysis for the same sample indicated high color intensity in the infected samples on the blot (Figure 6c). The western blot analysis using PR-S antibody revealed that two proteins of approximate molecular size 30 kD in infected samples cross-reacted with the antibody but not in the healthy samples (Figure 7). From this study it can be inferred that downy mildew infection in sunflower induces the *de novo* synthesis of polysaccharides, proteins which have the antigenic homology with PR-5 class of proteins, IgYs raised for high molecular weight polysaccharides and proteins from the downy mildew infected leaf samples.

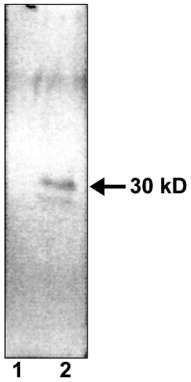


Figure 7: Immunoblot analysis of proteins contained in the 0-40% protein fractions from healthy and downy mildew infected samples. 100 µg of protein separated by SDS-PAGE, electrotransfered onto a nitrocellulose membrane and probed with antibody specific to the tobacco PR-S protein, a member of PR-5 class. Arrow indicates the approximate molecular weight of the protein to be around 30 kD.
1. healthy and
2. infected.

Histological studies

10 μm thick sections from healthy and downy mildew infected sunflower leaves were stained with PAS, TB and MBB and taken for micrometric observations. The observations revealed that the upper epidermis, palisade and spongy parenchyma of the healthy leaf were intact showing no distortions (Figure 8a). However, most of the sections of infected leaf showed no clear distinguishable epidermis (Figure 8b). The sections also revealed ramified mycelia in the palisade and spongy tissue with haustoria penetrating the cells resulting in the release of cellular contents (Figure 8c). The haustoria appearred to be double walled (Figure 8d).

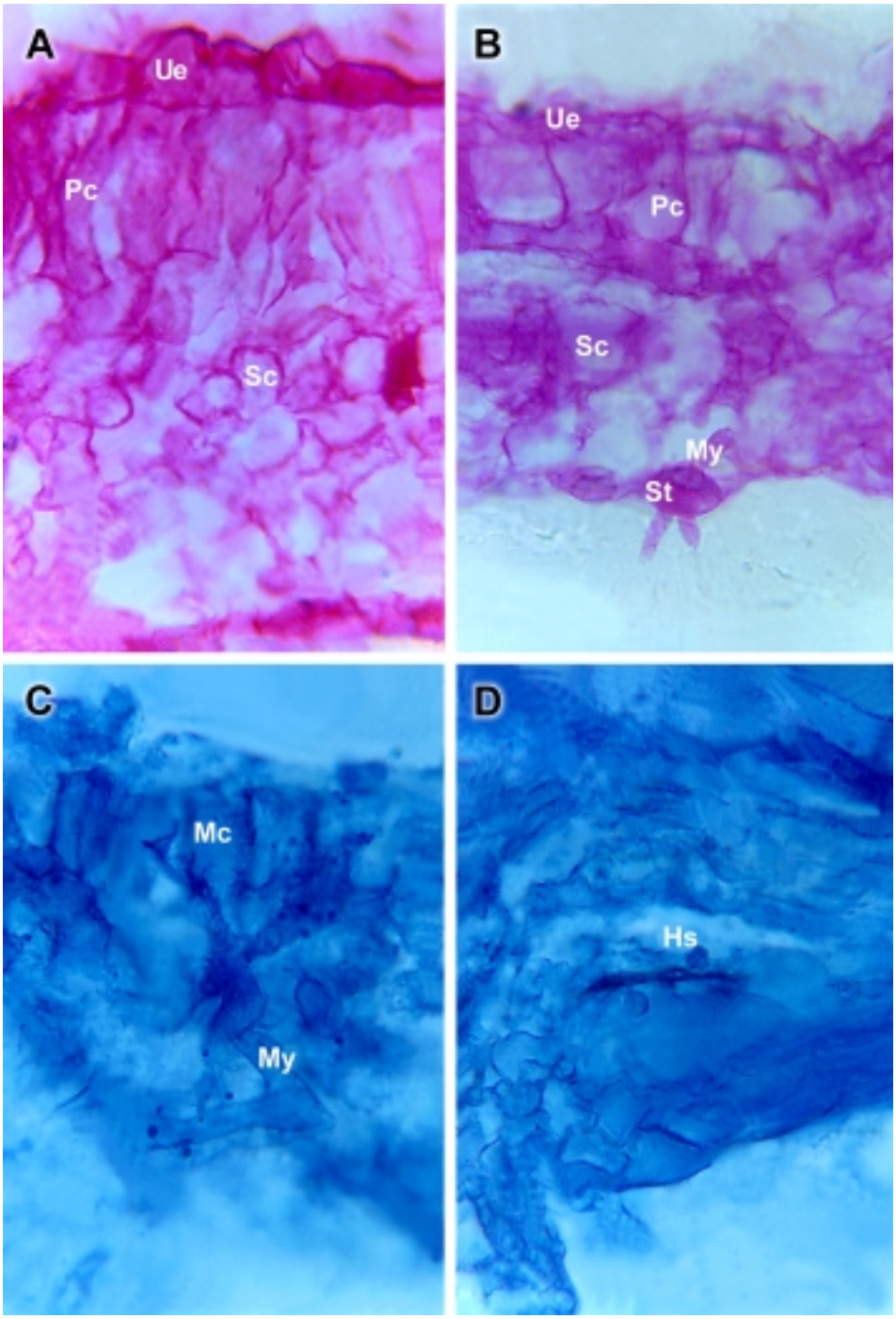
Micrometric studies revealed that there occurred an overall increase in total leaf thickness in the infected section (157.25 μm) as compared with the healthy one (127.5 μm). The thickness of the spongy tissue showed considerable increase in the infected tissue (93.5 μm) as compared with the healthy one (63.75 μm). The palisade zone did not show any significant difference between the infected and healthy leaves (Table 2).

DISCUSSION

In fungus-infected plants, the total soluble nitrogen and protein contents of the host-pathogen complex generally increased during the early stage of the disease. Increase in total soluble proteins may be a sequel to the infection process wherein

the plant tries to defend itself from the invading pathogen by triggering on the synthesis of a new set of proteins like toxin-binding proteins, chitinases, glucanases and polygalacturonases which are otherwise not present in the plants free from infection (Goodman *et al*., 1986). PR-1 protein was found to be induced to very high levels (10,000 fold) in TMV-infected tobacco tissue as compared with healthy tissue and it accumulated to 1-2% of total leaf protein suggesting an active role for this protein in the defense response (Alexander *et al*., 1993). Pinto and Ricardo (1995) described a group of three acidic proteins of molecular weight in the range of 16,500 that accumulated in leaves of *Lupinus albus* infected with *Colletotrichum gloeosporioides*, that show high sequence homology with PR-10 proteins. In this study, the total soluble proteins extracted in 0.05 M Tris HCl, pH 7.2 and 0-40% protein content were found to increase in the downy mildew infected sample as compared with the healthy sample contrary to the 40-60% protein content which showed a significant decrease. A positive correlation exists between the protein content in the 0-40% fraction and iPA levels and a negative correlation exists between protein content in the 40-60% fraction and iPA levels. The results of the present study revealed a significant increase in the total sugar content in the downy mildew infected leaves when compared with the healthy leaves. However, a decrease in the reducing sugar content was observed in the infected leaves. Accumulation of polysaccharides upon infection is a known phenomenon. Callose polysaccharide containing high proportion of β-1,3-linked glucose is deposited adjacent to plasma membrane in response to various stresses. Callose deposition forms one of the first lines of defense against pathogens (Bell, 1981). Rapid accumulation of callose layer upon infection has been reported in maize infected with *Phytophthora cinnamomi* (Hinch and Clarke, 1982) and soybean (Kohle *et al*., 1985). Molecular sieve chromatography analysis of the 0-40% soluble fractions using Sephadex G-200 column revealed the presence of a high molecular weight polysaccharide in downy mildew infected samples. This polysaccharide component was found to express predominantly in the infected sample and such polysaccharides might exist as glycoproteins.

An increase in the sugar content in the crude and 0-40% ammonium sulphate fraction of downy mildew infected leaves were observed as compared with the healthy leaves. This sugar content represented the polysaccharides as well as the glycoproteins present in these samples since the carbohydrate estimations were carried out for the samples extensively dialyzed against water using 12 kD dialysis membrane. These results indicate that downy mildew infection triggers the synthesis of polysaccharides/glycoproteins in sunflower leaves.


A
Ue
Pc
Sc
B
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Pc
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My
St
C
Mc
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Figure 8: Histological sections of healthy and downy mildew infected leaves stained with periodic acid-Schiff and mercuric bromophenol blue method.
***a)** Transverse section of healthy leaf stained with periodic acid-Schiff method showing intact upper epidermis (Ue), palisade cells (Pc) and spongy cells (Sc) (x 400).*
***b)** Transverse section of downy mildew infected leaf stained with periodic acid - Schiff method showing distorted upper epidermis (Ue), palisade cells (Pc), spongy cells (Sc) and ramified mycelium (My) (x 400).*
***c)** Transverse section of downy mildew infected leaf stained with mercuric bromophenol blue method showing completely distorted mesophyl cells (Mc) and ramified mycelium (My) penetrating through the stomata (x 400).*
***d)** Transverse section of downy mildew infected leaves stained with mercuric bromophenol blue method showing the double walled haustorium (Hs) (x 400).*

It is generally known that the presence of polysaccharides/glycoproteins as well as phenolics results in poor resolution of proteins on denaturing SDS gels with Coomassie brilliant blue. Results from our study, as shown in Figures 1, 4a and 4b, reveal the presence of high molecular weight polysaccharides in the downy mildew infected samples. The poor resolution and staining of the protein bands in SDS gels may be attributed to the interference of high molecular weight polysaccharides and polyphenolics associated with 0-40% protein fraction.

Cytokinins are a major group of plant growth regulators that modulate a number of physiological and biochemical processes such as cell division, flowering, fruit set, ripening, leaf senescence, seed germination stomatal function and disease resistance (Davies, 1987; Mok and Mok, 1994; Goodman *et al*., 1986). Reduced symptom development was observed in resistant cultivars of *Nicotiana tabacum* upon infection with TMV when compared with the susceptible cultivar. Cytokinin analysis of the resistant and susceptible tobacco cultivars revealed a strong correlation between resistance to TMV and cytokinin concentrations (Balazs and Kiraly, 1981).

In a similar report, Memelink *et al*. (1987) revealed that the constitutive expression of isopentenyl transferase (ipt) gene, encodes for isopentenyl transferase an enzyme involved in cytokinin biosynthesis, associated with the regulation of expression of PR-genes. Storti *et al*. (1994) introduced the ipt gene into tomato cultivar susceptible to *Fusarium oxysporum* and observed that the transgenic tomato plants carrying ipt gene were more resistant to this fungal pathogen.

Extracts of rust, *Uromyces phaseoli*-infected bean and broad bean leaves showed increased cytokinin activity (Goodman *et al*., 1986). Neoplastic tissues of curled peach leaves infected with *Taphrina deformans* exhibited increased cytokinin activity. Three chromatographically similar cytokinins were detected in both healthy and infected leaves but those in the infected leaves were more active. Chromatographic analysis of the cytokinins from the diseased tissues revealed the presence of an additional analogue of cytokinin which is different from those present in healthy leaves (Sziraki *et al*., 1975).

Comparison of the iPA levels in the healthy and downy mildew infected sunflower leaf samples revealed a significant increase in the iPA content in the infected samples compared with the healthy samples, whereas the variations in the levels of ABA and DHZR were not significant (data not shown). Earlier studies also indicated that wound-induced signals and ethylene induce the expression of genes for basic PRs at higher levels than that of genes for acidic PRs, whereas the levels of auxins induce the expression of genes encoding for acidic PRs (Ohashi and Ohshima, 1992). Studies of sunflower infected with *Puccinia helianthi* revealed that the distribution of 0-40% (acidic proteins) and 40-60% (basic proteins) ammonium sulphate fractionated proteins show inverse relationship with 0-40% protein fractions and age of the leaf. Conversely, the ABA contents showed positive correlation between age of the leaf and 40-60% protein fraction (Prasad and John, 1994). From our results it can be concluded that a positive correlation exists between iPA but not between DHZR and synthesis of acidic PR class of proteins (0-40% protein fraction). Morphological observations also revealed that the symptoms of downy mildew infection were more predominant in younger leaves than in the older leaves. From these results it appears that downy mildew infection results in enhanced synthesis of iPA, 0-40% protein fraction and polysaccharides.

PR proteins are used as marker proteins to identify systemic acquired resistance (SAR) (Ryals *et al.*, 1996). Considerable efforts have been made to identify biochemical markers for SAR that could be used to distinguish it from other inducible plant resistance responses (Ryals *et al.*, 1996). Extensive studies have been conducted on raising antibodies against PR proteins to use them in detection of diseases, for example the PR-5 protein in *Brassica compestris* flower buds, PR-p69, a proteinase from tomato infected with citrus exocortis virus, PR-P and PR-Q proteins from tobacco (Cheong *et al.*, 1997; Vera and Conejero, 1990; Legrand *et al.*, 1987). In this study, antibody was raised against the high molecular weight polysaccharide and protein (50 kD) in chicken and IgY was purified. The western blot/dot blot analysis using these IgYs indicate the presence of high molecular weight polysaccharide/ proteins in the downy mildew infected samples. The western blot analysis of 0-40% fractions using IgY raised for high molecular weight protein from the downy mildew infected samples showed very high positive cross reaction with two closely moving 50 kD bands. Further, the western blot analysis revealed that PR-S antibody cross-reacted with two proteins of approximate molecular size 30 kD in downy mildew infected samples alone. From these studies it can be inferred that downy mildew infection in sunflower induces the *de novo* synthesis of proteins and polysaccharides which can be detected by using IgYs raised against high molecular weight polysaccharides and proteins, reported in this work, and the antibody for PR-S, which belongs to PR-5 class pathogenesis related proteins.

Rao *et al.* (1998) have shown that initiation of the downy mildew infection in the leaf tissue seems to be through the spores that germinate and enter through the upper epidermis. Further, the mycelia ramify in the palisade and spongy tissues

and the haustoria penetrate the cells of these tissues resulting in the discharche of cell contents. The sporangiophores bearing sporangia emerge through the lower epidermis causing damage in the process. Duletić-Laušević and Mihaljčević (1997) have shown that the variability among genotypes in resistance to fungal pathogens 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.

Histopathological studies of the downy mildew infected sunflower seedlings have shown that the penetration of the roots and the lower part of the hypocotyls occurs for both compatible and incompatible combinations. After penetrating the susceptible genotypes, the parasite develops intercellular hyphae and intracellular haustoria, leading to systemic infection. In contrast, in resistant plants, as soon as the colonization develops, hypersensitive reaction occur in the parenchyma, with the appearance of necrotic zones surrounded by dividing cells. Growth of the parasite is strongly inhibited and most hyphae are blocked before they reach the cotyledonary node (Mouzeyar *et al.*, 1993). Wehtje and Zimmer (1978) working with sunflower infected by downy mildew have shown that a thin layer of host cytoplasm surrounds the haustorium; it is separated from the large host vacuole by the invaginated tonoplast.

Converging with these studies we have shown that the downy mildew pathogen ramifies extensively throughout the leaf tissue and forms a nutritional link with the host cell by producing the intracellular haustorium. We have also shown that the haustorium appears double walled which may be a part of the host cell wall (Wehtje and Zimmer, 1978). Further studies are required along this line to determine the role of this host cell layer around the haustorium.

CONCLUSIONS

Pathogens bring about changes in biochemical, molecular and cellular mechanism in the host plant. From this study it is evident that downy mildew of sunflower induces the production of high molecular weight proteins, polysaccharides and also results in the increased synthesis of the cytokinin, iPA. It has been also shown that downy mildew infection induces proteins which have antigenic homology with PR-S protein, a member of PR-5 class of proteins. Histological results show that the downy mildew fungus proliferates extensively throughout the plant tissue and establishes a nutritional link by producing the intracellular haustorium. Thus, it becomes necessary to understand the exact roles of these biomolecules in *Plasmopara halstedii* and sunflower interaction, in order to elucidate the molecular mechanism of downy mildew infection in sunflower.

ACKNOWLEDGMENTS

We wish to thank Dr. Samasundar Joshi for his help with photomicrography work and Pierette Geoffroy for having provided us with the PR-S antibody. This research work is partially supported by the Karnataka State Council for Science and Technology as a graduate student project (BRP).

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MODIFICACIONES BIOQUIMICAS E HISTOLOGICAS RELACIONADAS CON LA INFECCION DEL GIRASOL (*Helianthus annuus* L.) POR EL AGENTE DE MILDIU (*Plasmopara halstedii* (Farl.) Berl. y de Toni)

RESUMEN

El estudio de modificaciones bioquimicas ligadas con la aparicion de mildiu en el girasol ha mostrado que ocurrio el aumento del contenido en proteinas solubles totales, proteinas que se fraccionan por 0-40% de sulfato de amonio y azucares solubles totales. El analisis cromatografico del tamiz molecular en la fraccion soluble por 0-40% de sulfato de amonio mostro que en las hojas de plantas infectadas eran presentes las proteinas de alto peso molecular asi como polisacaridos. Los anticuerpos que aparecieron como reaccion a la presencia de proteinas de alto peso molecular, y polisacaridos fueron analizados por el metodo "western blot/dot blot". Fue mostrado que el mildiu inducia la proteina PR que posee la homologia antigena con la proteina PR-S, miembro de la clase de proteinas PR-S. Fue notado el aumento del contenido en isopentenil adenosina (iPA) en las muestras infectadas. Existia la correlacion positiva entre el nivel de iPA, 0-40% de proteina y el contenido en azucares totales. Los estudios histologicos mostraron que el micelio de mildiu reproducia intensamente, hacia la ramificacion del tejido de hoja, asi como creaba el contacto nutritivo con la celula huesped por lo que desarrolla el haustorium intercelular.

CHANGEMENTS BIOCHIMIQUES ET HISTOLOGIQUES ASSOCIÉS À L'INFECTION DU TOURNESOL (*Helianthus annuus* L.) PAR LE MILDIOU (*Plasmopara halstedii* (Farl.) Berl. et de Toni)

RÉSUMÉ

L'observation des changements biochimiques associés à l'infection du tournesol par le mildiou a révélé une augmentation du total de protéines solubles, 0-40% de protéines fractionnées par le sulfate d'ammonium et une augmentation du total des sucres solubles. La chromatographie du tamis moléculaire de 0-40% de la fraction par le sulfate d'ammonium a révélé la présence de protéines de poids moléculaire élevé et de polysaccharides dans les feuilles des plantes infectées par le mildiou. Les anticorps apparus en réaction à la présence de protéines à poids moléculaire élevé et de polysaccharides ont été analysés par la méthode "western blot/dot blot". Il a été démontré que la maladie du mildiou induit aussi des protéines PR qui ont une homologie antigène avec la protéine PR-S, membre de la classe de protéines PR-5. Une augmentation du contenu d'isopenténule adénosine (iPA) a été constatée dans les échantillons infectés. Il existe une corrélation positive entre le niveau iPA, 0-40% des protéines et le contenu total de sucre. Les études histologiques ont démontré que le mycélium fongique du mildiou se reproduit et ramifie le tissu des feuilles de manière intensive et forme un lien nutritionnel avec la cellule hôte en produisant un haustorium intracellulaire.