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Infection of Jute Seedlings by the Phytopathogenic Fungus *Macrophomina phaseolina* Mediated by Endogenous Lectin*

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Abstract: The phytopathogenic fungus *Macrophomina phaseolina* infects many plants, e.g., jute (*Corchorus capsularis*), soybean (*Glycine max*) and peanut (*Arachis hypogaea*). Fungal adhesion to the host surface mediated by initial binding and recognition is an important event which subsequently leads to infection and pathogenesis to host plants. Lectins bind well to the carbohydrates present on plant cell surfaces. Thus, lectins in phytopathogenic fungi may play a role together with cell-wall degrading enzymes in the infection process. The degree of infection of jute seedlings by a 60-day-old mycelial suspension of *M. phaseolina* increased to the same level as that of a young (7-day-old) mycelial suspension with the addition of *M. phaseolina* lectin ($35 \mu\text{g mL}^{-1}$) to the incubation medium. The intensity of infection of the jute seedlings by the 60-day-old mycelia was unchanged even after the addition of L-asparagine ($35 \mu\text{g mL}^{-1}$) to the medium suggesting that increased fungal infectivity was not a result of the use of *M. phaseolina* lectin as a nutrient source. Agglutinin production in *M. phaseolina* infected jute seedlings was 87 ng per seedling on the fifth day of infection as quantitated by ELISA. The amount of *M. phaseolina* lectin accumulated in the culture media increased if host (jute seedlings) cell wall or its components, e.g., such as pectin, cellulose, xylan was added to the mycelium. So, this lectin is suggested to play a role in the recognition and infection process of host plants.

Key words: *Macrophomina phaseolina*, lectin, jute seedlings, pathogenesis, enzyme-linked immunosorbent assay

Introduction

Pathogenesis is a process of development of disease by microorganisms including fungi. Fungal invasion occurs chiefly by germ tubes produced by of spore germination or by growth of existing structures, viz., mycelial hyphae or appressoria (Hinch and Clarke, 1980) that has been stimulated by host plant exudates (Flentze *et al.*, 1963). The infecting hyphae may penetrate the cell wall directly, or enter through wounds made by degradation of host tissue by cutinolytic, pectinolytic (Brookhouser and Weinhold, 1979) and cellulolytic enzymes.

Lectins, which are abundantly present in animals, plants, fungi and microorganisms, can act as mediators of cellular and molecular recognition in a wide range of biological systems (Sharon and Lis, 1989) since they bind specifically to carbohydrates. Attachment of fungal pathogens to a host cell's surface is a prerequisite for parasitic infection and the infection process

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may involve binding between complementary molecules on both host and fungal cell surfaces (Hohl and Balsiger, 1988). One hypothesis is that carbohydrates and lectins in the cell wall may play an important role in specific adhesion and recognition in host-pathogen interactions (Longman and Callow, 1987). Lectin-carbohydrate interaction has been proposed to mediate adhesion in several fungal-host systems. Examples of such interactions include plant pathogens (Hinch and Clarke, 1980), fungal-fungal interactions (mycoparasitism) (Nordbring-Hertz and Chet, 1986), fungal-algal interactions (lichen symbiosis) (Petit, 1982; Petit *et al.*, 1983), fungal-insect interactions (Chabasse *et al.*, 1988; Ishikawa *et al.*, 1981) and nematode-trapping fungi (Nordbring-Hertz and Mattiasson, 1979; Rosenzweig and Ackroyd, 1983). Thus, fungal-host interactions mediated by lectins of fungal origin may be important in pathogenesis, together with cell-wall degrading enzymes. The detection of lectins in phytopathogenic fungi is interesting which can give possible mechanism of host-pathogen interactions. For this reason the present investigation has been planned to study the effect of endogenous lectin, *M. phaseolina* lectin (MPL) on the infection of jute seedlings by the phytopathogenic fungus *M. phaseolina*.

Materials and Methods

Materials

Jute seeds (JRO 524) were obtained from the Central Research Institute for Jute and Allied Fibers (Barrackpore, West Bengal, India). Freund's complete and incomplete adjuvant, Tween-20, sodium dodecyl sulfate, *O*-phenylenediamine, Tris, citrate-phosphate buffer tablets, Bradford's reagent, L-asparagine, pronase P from *Streptomyces griseus* type VII and horseradish peroxidase type VI were purchased from Sigma, (St. Louis, Mo). Pectin, cellulose, xylan from Birchwood were obtained from Dr. S. Sengupta, Indian Institute of Chemical Biology, Kolkata, India.

Microorganism

The phytopathogenic fungus *M. phaseolina* (MTCC 166) was obtained from Institute of Microbial Technology, Chandigarh, India and maintained in potato dextrose agar medium as previously described (Bhowal *et al.*, 1999).

Purification of Extracellular Lectin

M. phaseolina was grown in Richard's medium for seven days and mycelia were separated from the culture filtrate by vacuum filtration. The culture filtrate was concentrated by lyophilization and stored at -20°C until use. Hemagglutinating activity and protein content of the culture filtrate were determined as previously described (Bhowal *et al.*, 1999). Mycelia, including sclerotia, were homogenized and suspended in water (1%).

M. phaseolina lectin (MPA) was purified from the culture filtrate of the above phytopathogen which revealed that it is a glycoprotein consisting of a single polypeptide chain of M_r 34 kD (Bhowal *et al.*, 2005).

Preparation of Immunoglobulin G (IgG)

Antisera against MPL was raised and the IgG fraction was separated from the whole rabbit antisera by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Campbell *et al.*, 1970; Majumder and Chatterjee, 1996).

Preparation of Enzyme-labeled Antibody

Horseradish peroxidase (6.7 mg, ~2000 unit) was activated overnight with 0.2 mL of 1.25% glutaraldehyde in 10 mM PBS, pH 7.4 at 20°C and conjugated with rabbit anti-MPL IgG (5 mg mL⁻¹) (Engvall and Perlmann, 1972; Lind, 1986).

Role of MPL on the Infection of Jute Seedlings by M. phaseolina

Jute seeds were surface sterilized with 0.1% HgCl₂ solution and after thorough washing were spread on sterile moistened filter paper in petri dishes containing either young (7-day-old) or old (60-day-old) mycelial suspension. To some of the petri dishes containing the old mycelial suspension different concentrations of purified MPL (5, 15, 25 and 35 µg mL⁻¹) or 35 µg mL⁻¹ L-asparagine were added and incubated for twelve days. MPL (25 µg mL⁻¹) was incubated separately with glucuronic acid (12.5 mM) for 2 h at 37°C and the conjugate (MPL-GUA) was added to the petri dishes containing the old mycelial suspension. The same set of experiments without mycelial suspension was treated as a control. Three petri dishes were taken for each supplementation. All petri dishes were kept at 30°C in the dark. The degree of infection of seedlings was recorded by visual observation of the symptoms, i.e., brown coloration of the seedlings.

Quantitation of M. phaseolina Lectin in M. phaseolina Infected Jute Seedlings

Surface sterilized jute seeds were germinated for 3 days in petri dishes containing 2% nutrient agar medium and the seedlings were incubated with the 7-day-old mycelial suspension for up to twelve days. Each day 150 seedlings were taken and roots and shoots were extracted with TBS, pH 7.2 and centrifuged at 1200×g for 5 min. The amount of MPL in supernatants was measured by ELISA (Voller, 1982) in a 96-well-U bottomed Titertek immunoassay plate (Flow Laboratories, UK). Each well was coated with 100 µL anti-MPL IgG (1:1000 dilution in BBS, pH 8.4) for 2 h at 37°C. The plate was washed thrice with PBS, pH 7.4 containing 0.05% (v/v) Tween-20 (PBST). Thereafter, 100 µL of BBS containing 1% (w/v) BSA was added to each well and incubated for 1 h at 37°C. The plate was successively washed with PBST and PBS and incubated with the extracts of *M. phaseolina*-infected jute seedlings for 2 h at 37°C. To quantify MPL produced in the infected seedlings, a standard curve was prepared with purified MPL (0.05-20 µg/100 µL). After washing the plate was incubated with 100 µL of IgG-HRP (1:500) for 2 h at 37°C, washed as usual, followed by addition of 100 µL of 0.04% *O*-phenylenediamine in 0.1M citrate phosphate buffer (pH 5.4) containing 0.01% H₂O₂ to each well. The plate was kept for 25 min in the dark at 25°C and the absorbance of each well was measured at 492 nm in a Titertek Multiskan MK Plus II automatic ELISA reader. Non-infected seedlings served as control.

Isolation of Plant Cell Wall

Cell walls were isolated following the method of Kellens (Kellens). Surface-sterilized jute seeds were germinated on moistened filter paper in petri dishes at 30°C in the dark. Sprouts of 5-days-old jute seedlings were frozen in liquid nitrogen and pulverized in a mortar. The powder was extracted with PBS and centrifuged at 6000×g for 10 min; the process was repeated twice. The residue was boiled with ethanol (95%) for 2 h and filtered. The cell walls were washed consecutively with ethanol (15% v/v), chloroform-methanol mixture (1:1 v/v) and acetone, dried and kept at -20°C for further use.

Effect of Plant Cell Wall and Different Cell Wall Constituents on the Production of M. phaseolina Lectin

Four sets of culture media (30 mL) each containing twenty-four flasks were incubated with *M. phaseolina* in the presence of cell wall and its constituents. In one set, isolated cell wall (2.5 mg) was added to each of the twenty-four flasks and in other three sets of flasks either pectin, cellulose, or xylan (1.5 mg each) was. Flasks without cell wall or any cell walls constituent were used as controls. Three flasks from each set were taken at regular intervals of time up to 21 days. Mycelia were separated from the culture filtrate through vacuum filtration and weights of mycelia were determined after drying at 105°C for 24 h. Culture filtrates were concentrated by lyophilization and MPL content were determined by ELISA.

Results

The young (7-day-old) mycelial suspension of *M. phaseolina* in water was more virulent and produced greater levels of infection in jute seedlings than the old (60-day-old) (Table 1, Fig. 1A and 1B). However, the old fungal suspension in water supplemented with MPL infected the jute seedlings to a greater extent. The degree of infection increased gradually with an increase in MPL concentrations (5-25 $\mu\text{g mL}^{-1}$). The old fungal suspension containing 25 $\mu\text{g mL}^{-1}$ of MPL was the most effective and infected the jute seedlings as like as young fungal suspension (Fig. 1C). The germination of seeds occurred but further growth was completely inhibited (Fig. 1D) when suspension was supplemented with MPL (35 $\mu\text{g mL}^{-1}$). The old fungal suspension containing L-asparagine produced the same degree of infection on the germinated jute seeds as caused by the old suspension alone. This observation indicated that the virulence of the pathogen was not due to the nutritional value of MPL. Preincubation of MPL with glucuronic acid reduced the infection of jute seedlings (Fig. 1E). In the absence of fungal culture, the MPL alone in the incubation medium had no effect on the infection of jute seeds (Fig. 1F).

MPL content in the jute seedlings upon infection continued to increase gradually and reached maximum, 87 ng per seedling on fifth day of inoculation (Fig. 2). Thereafter, MPL content declined gradually and was 8 ng per seedling at the twelfth day.

Table 1: Infection of jute seedlings by *M. phaseolina*

Treatments	Amount added ($\mu\text{g mL}^{-1}$)	Degree of infection
Jute seeds incubated with		
I Young (7-day-old) mycelial suspension	MPL 0	++++
	35	germination occurred but seedlings appearance completely prevented
II Old (60-day-old) mycelial suspension	MPL 0	+
	5	++
	15	+++
	25	++++
	35	no seedling appearance
	L-Asn 35	+
III Old (60-day-old) mycelial suspension	MPL-GUA	++
IV Without mycelial suspension	MPL 0	-
	35	-

(-) no infection, (+) little infection, (++) light infection, brown colored roots, (+++) brown colored roots and moderate infection on stem bases, (++++) heavy infection, brown colored roots and stems

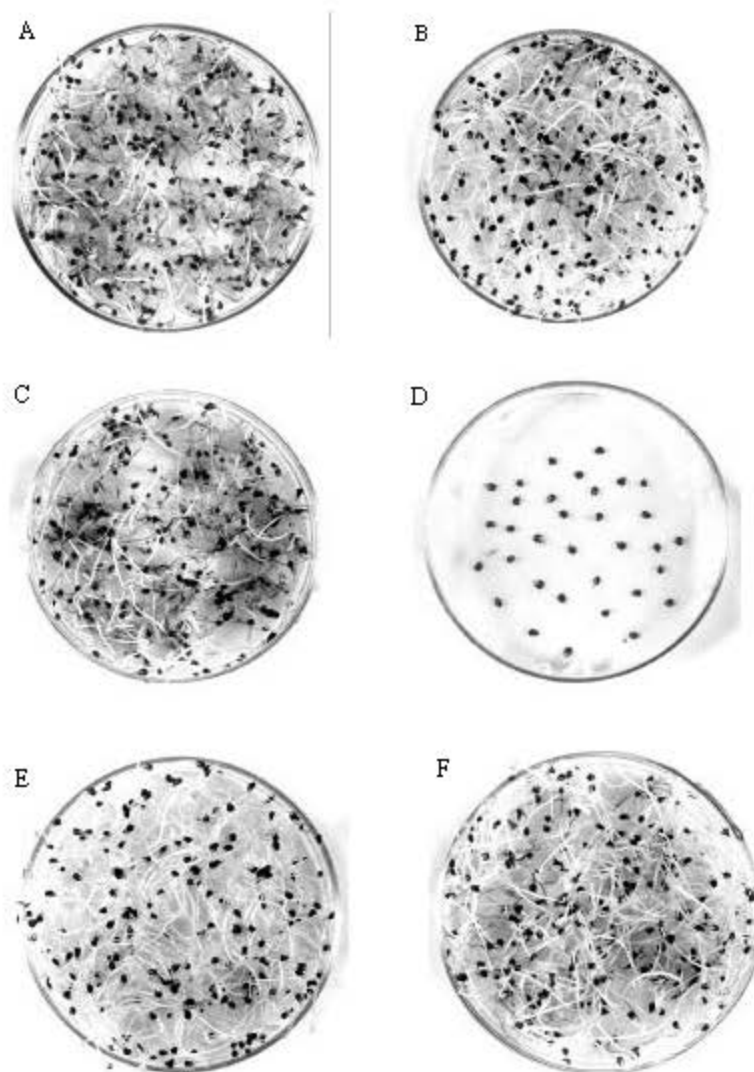


Fig. 1A: Infection of jute seeds in the presence of young (7-day-old) mycelial suspension of *M. phaseolina*
B: Infection of jute seeds in the presence of old (60-day-old) mycelial suspension of *M. phaseolina*
Fig. C: Infection of jute seeds in the presence of old (60-day-old) mycelial suspension of *M. phaseolina* containing $25 \mu\text{g mL}^{-1}$ of MPL
Fig. D: Infection of jute seeds in the presence of old (60-day-old) mycelial suspension of *M. phaseolina* containing $35 \mu\text{g mL}^{-1}$ of MPL
Fig. E: Infection of jute seeds in the presence of old (60-day-old) mycelial suspension of *M. phaseolina* containing $35 \mu\text{g mL}^{-1}$ of MPL and 12.5 mM GUA
Fig. F: Infection of jute seeds in the absence of mycelial suspension of *M. phaseolina* containing $35 \mu\text{g mL}^{-1}$ of MPL

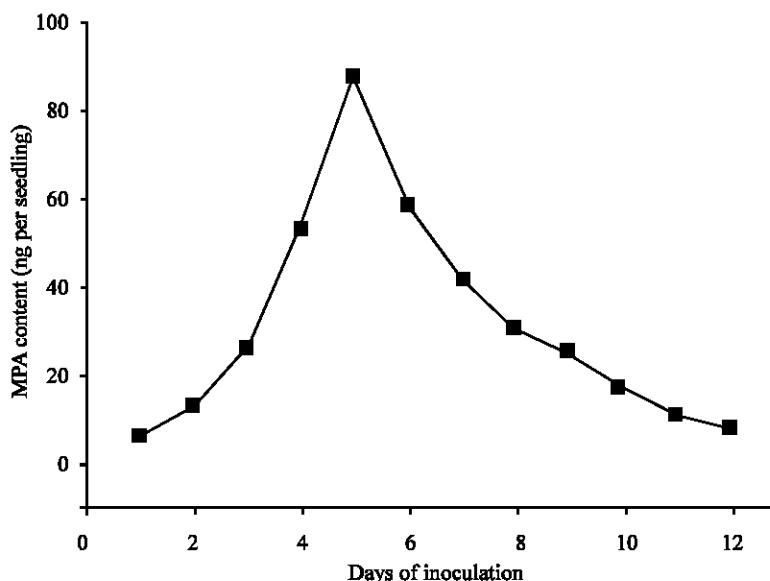


Fig. 2: MPL content in *M. phaseolina* infected jute seedlings

The dry weight of mycelia grown in the presence of cell wall did not differ from the control which was devoid of cell wall (Fig. 3A). Similar results were obtained when the culture media contained different cell wall constituents such as pectin, cellulose or xylan (Fig. 3A). There are however, marked differences in the agglutinin concentration in the culture filtrates. After seven days MPL concentrations in the culture filtrates supplemented with or without the addition of cell wall, pectin, cellulose or xylan were found to have increased with respect to the control and highest increase in this respect was noted at the twelfth day (Fig. 3B). Maximum agglutinin concentrations in the cell wall, pectin, cellulose or xylan treated culture media were 12, 12, 11 and 10 mg/100 mL, whereas it was only 5 mg/100 mL in the control. This increase in agglutinin concentration in the culture filtrate was not due to increase in mycelial growth as evidenced from Fig. 3A.

Discussion

Recognition is prelude to the attachment of fungal pathogens to their host plants by formation of appressoria (Carver and Ingerson, 1987; Staples and Macko, 1980) and has been suggested to be mediated by lectins. *M. phaseolina* lectin besides sialic acids binds to glucuronic acid also and gum arabic, which contains glucuronic acid as one of its sugar constituents (Bhowal, 2002).

Infection of jute seeds by *M. phaseolina* mycelial suspension increased following the addition of MPL to the suspension. The old mycelial suspension together with MPL became as effective as fresh suspension in infecting the jute seedlings. This was not due to the additional quantity of nutrients, since addition of L-asparagine to the medium did not alter the virulence of old mycelial suspension. Preincubation of MPL with glucuronic acid (12.5 mM) solution reduced the infection. From all these findings it could be suggested that MPL plays a role in the recognition process of host plant. These initial binding and recognition are preliminary steps for firm adhesion and subsequent infection to the roots. Similarly, fungal infection by the specific binding of zoospores of *Phytophthora*

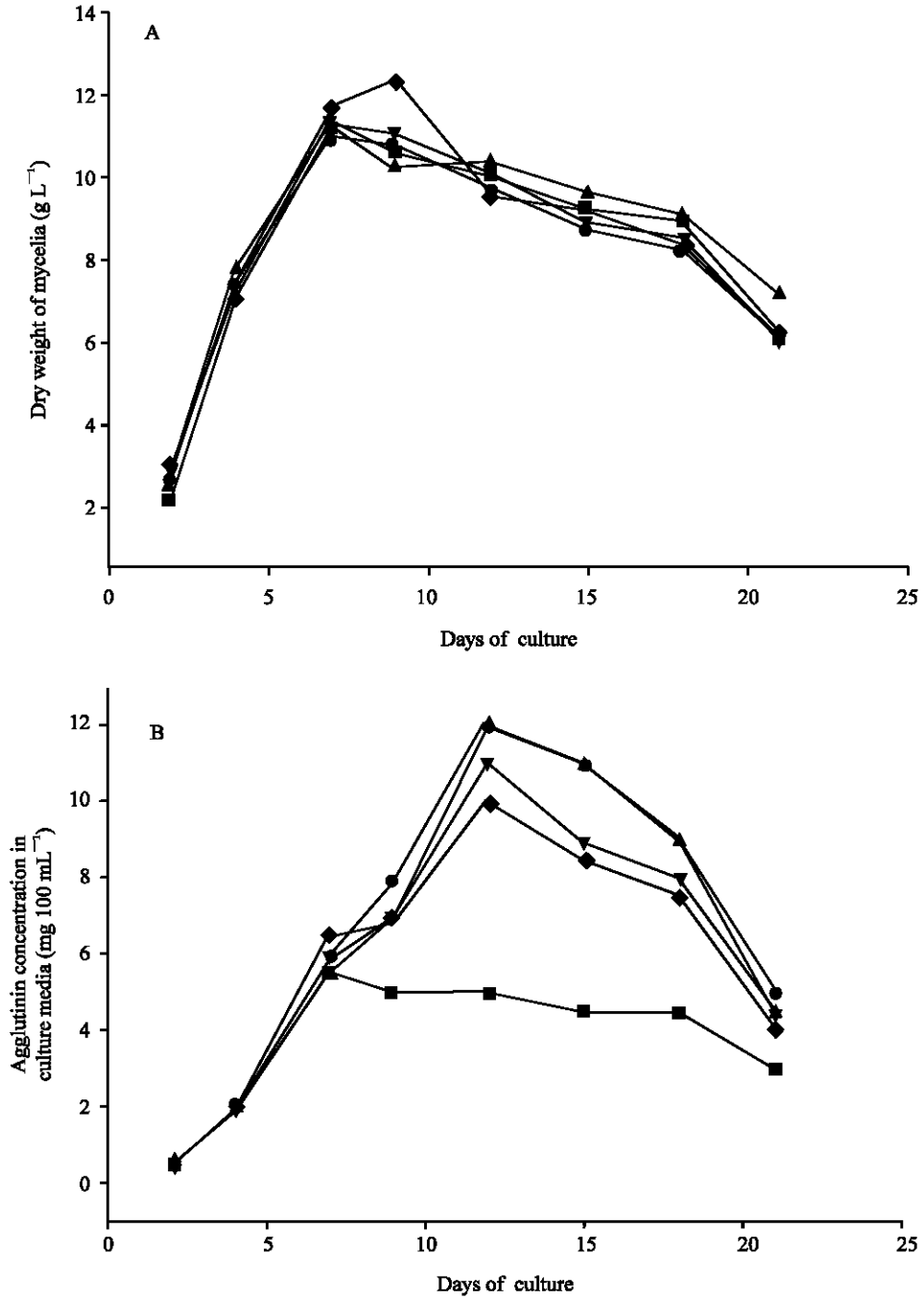


Fig. 3A: Effect of isolated cell wall and its different components on the dry weight of mycelia, control (■), cell wall (●), pectin (▲), cellulose (▼), xylan (◆)

Fig. B: Effect of isolated cell wall and its different components on the MPL accumulation in culture media (Symbols are as in Fig. 3A)

cimamomi to *Zea mays* roots was mediated by lectin which bound to fucosyl residues of the secreted root surface slime (Hinch and Clarke, 1980). Subsequent infection of host plant root tissue occurred through germ tube and ultimately spoiled the host plant. Zoospore adhesion was appreciably reduced by treatment of the roots with *Ulex europaeus* agglutinin (UEA I) whereas preincubation with L-fucose completely abolished the effect of the lectin. Another example of attachment and recognition of specific sugar residues on the host surface was the interactions of zoospores of *Pythium aphanidermatum* to the root surfaces of cress *Lepidium sativum*. Zoospores-root adhesion could be abolished by trypsinization of zoospores or incubation of zoospores with exogenous root polysaccharide. From the above results it was suggested that lectin-like receptors on the zoospore surface interact with sugar residues in root surface mucilage initiating zoospore attachment and subsequent infection (Longman and Callow, 1987).

It is well understood that fungus during its development and growth on host plant surface liberates enzymes which degrade cell wall releasing several of its components, which play an important role in the initial stages of infection. These fragments in some cases take part in defense mechanism of host (Hahn *et al.*, 1981) and in other cases trigger certain reactions in the pathogenesis resulting in a successful infection. The present study has demonstrated the augmentation of MPL production in the culture medium by the addition of cell wall and its different constituents. The cell wall and its components act as elicitors for fungal growth as well as lectin synthesis. Similar results were reported in the infection of wheat grains by another phytopathogenic fungus *Rhizoctonia solani* (Kellens).

From the above findings it may be hypothesized that *M. phaseolina* lectin which is synthesized and secreted in response to cell wall and its constituents interacts with host plasma membrane causing suppression of host defense mechanism which in turn leads to successful infection. Similar mechanism has been described by lectin binding to wheat protoplasts (Kogel *et al.*, 1985). However concrete evidence for this hypothesis is not obtained.

Lectins show also enzymatic activity (Finkelstein *et al.*, 1983; Shannon and Hankins, 1981) and it has been suggested that many enzymes such as proteases, phosphatases and peroxidases/catalases are involved in the infection of nematodes by *Arthospora oligospora* (Tunlid *et al.*, 1992; Veenhuis *et al.*, 1985). However, the purified lectins affect the enzymes activity through formation of co-functional complexes with the enzyme and substrate for the function of microbial lectins as proposed by Gilboa-Garber and (Gilboa-Garber and Garber, 1989).

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