BIOCHEMICAL AND SEROLOGICAL CHANGES IN SOYBEAN IN RELATION TO INTERACTION BETWEEN *Bradyrhizobium japonicum* AND *Fusarium oxysporum*

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This is to certify that Mrs Banani Sarkar has carried out her research work under our joint supervision. Her thesis entitled **"Biochemical and serological changes in soybean in relation to interaction between *Bradyrhizobium japonicum* and *Fusarium oxysporum*"** is based on her original work and is being submitted for the award of Doctor of Philosophy (Science) degree in Botany in accordance with the rules and regulations of the University of North Bengal.

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Introduction

Soybean [*Glycine max* (L.) Merrill] is one of the most important legume crops of the world and is a primary source of vegetable oil and protein. Each year soybean products become more important in formulating new, low cost nutritionally balanced high protein foods and beverages for human consumption. As soybean acreage has expanded throughout the world, diseases have increased in number and severity. Root rot of soybean caused by *Fusarium oxysporum* Schlecht is considered a potentially destructive disease in the tropics and subtropics. In this disease, infection is generally confined to root and lower stem region and when the disease is severe seedling emergence is slow and poor and seedling growth is stunted and weak. The pathogen is usually confined to the cortex but vascular elements may be invaded in advanced stages of the disease. Although the pathogen is confined to the root, nearly mature pods may be invaded under prolonged humid and wet conditions. The pathogen may cause latent infection of the seeds which remain apparently healthy (Sinclair, 1991).

Rhizosphere of plants contain a large number of microorganisms and one of the most intriguing and exciting problems is the study of complex interactions occurring among soil microorganisms and their effects on disease reaction in plants. The legume rhizosphere contains a large population of rhizobia, most of which are highly specific to their host. It has been clearly established that sustained high levels of nodulation by contrasting *Bradyrhizobium japonicum* strain can alter the productivity of the *Bradyrhizobium*-soybean symbiosis under green house and field conditions. (Israil, 1981; Fuhrmann, 1990; Miller and May, 1991) Since nodulation effectively increases the growth and yield of soybean plants, there is a common agricultural practice of inoculating soybean seeds or soil with *Bradyrhizobium japonicum* prior to sowing (Plate-I). Other than increasing yeild and growth, there are several reports that different species of *Rhizobium / Bradyrhizobium* are capable of protecting their hosts against fungal pathogens (Tu, 1978 ; Purkayastha, 1981; Chakraborty and Purkayastha, 1984; Chakraborty and Chakraborty, 1989).

Introduction of biocontrol agents into the soil is also becoming a common practice in recent years, of which *Trichoderma sp, Pseudomonas sp, Bacillus sp. etc* are generally used (Dunne et al, 1996; Hessenmuller and Zeller, 1996 and Mandeel, 1996). In certain cases combined effect of one or more antagonist in reducing disease severity has also been reported (Novicova, 1995 ; Dubey, 1996 ; Craft and Nelson, 1996). The reduction in disease intensity of a particular plant due to the activity of one or more microorganisms in the soil may be attributed to biochemical changes induced in the host. Most important biochemical changes in plants following pathogen infection are production of phytoalexins, changes in enzymes and in pathogenesis related proteins (Ji and Kuc, 1996 ; Wibber et. al. 1996; Urbanek et. al 1996; Kristensen et. al. 1997).

However, biochemical changes in host resulting from combined inoculations of antagonist and pathogen have been relatively less investigated though increased phytoalexin production has been implicated in certain cases (Skipp and Deveral, 1973; Chakrabory & Chakraborty, 1989). Closely related to the biochemical changes in host pathogen interactions is the phenomenon of common antigen sharing between host and pathogen. Close serological similarity between host and pathogen is considered to be one of the prerequisites for the successful establishment of a pathogen within the host. Little information is available on the serological changes in host due to microbial interactions among biocontrol agents and pathogens (Troxler et al ; 1997).

Considering that soybean is a very important legume crop which is generally preinoculated with *Bradyrhizobium* and that information on the biochemical and serological changes in soybean due to *B. japonicum - F. oxysporum* interaction are lacking, the following study was undertaken, the main objectives which are as follows : (a) selection of soybean cultivars resistant or susceptible to *F. oxysporum,* (b) determination of the effect of preinoculation of soybean seeds by *B. japonicum* on disease development, (c) determination of the effect of soil infestation with *Trichoderma harzianum* on disease development (d) *invitro* interaction studies between *F. oxysporum, B. japonicum* and *T. harzianum,* (e) estimation of phenylalanine ammonia lyase and peroxidase, from soybean roots inoculated with *F. oxysporum* and *B. japonicum* and evaluation of the quantitative differences between them, (f) extraction and

estimation of glyceollin in roots of soybean cultivars challenged with *F. oxysporum* and subsequent changes following interaction with *B. japonicum;* (g) analysis of protein changes by SDS-PAGE after inoculation with *F. oxysporum* and *B. japonicum ;* (h) raising of antisera against antigen preparation from soybean roots and mycelium of *F. oxysporum* ;(i) detection of serological cross reactivity between *F. oxysporum* and soybean varieties following agar gel double diffusion, immunoelectrophoresis and enzyme linked immunosorbent assay, (j) detection of *F. oxysporum* in infected tissues and in roots preinoculated with *B. japonium,* (k) determination of the cellular location of cross reactive antigens in soybean root tissues as well as of mycelia, conidia and chlamydospores of *F. oxysporum.*

Before going into details of the present investigation, a brief review in confirmity with this study has been presented in the following pages.

Literature Review

Plants respond to infection by producing physical and chemical barriers which function as wall reinforcements, antibiotics or lytic enzymes (Nemestothy and Guest, 1990), It has been established in detailed comparative study of resistant and susceptible host responses that the differences between them are quantitative rather than qualitative. Although susceptible plants possess the machinery necessary for resistance it is not activated in sufficient magnitude or speed to restrict the infection (Kuc 1983). The success or failure of infection is also determined by dynamic competition and final outcome is determined by the sum of favourable and unfavourable conditions for both the pathogen and host cell. Antigenic similarity between host and pathogen as a prerequisite for compatable host pathogen interaction has also been established.

An attempt has been made in the following pages to review available literature on biochemical changes in plants following infection and serological relationship between host and pathogen.

Biochemical changes in plants following infection

Biochemical changes in plants following infection or attempted infection by pathogens has been a subject of research by a number of workers (Ji and Kuc, 1996; Wibber et al., 1996; Kristensen et al, 1997). Accumulation of phytoalexin as defence response has been demonstrated in more than hundred plant species till now. Several reviews have appeared on the subject (Ward 1986; Lamb et at. 1989 ; Keen, 1990 ; Chakraborty et al. 1995, Purkayastha, 1995; Oku and Shiraishi, Kuc,1995).

Metraux and Boller (1986) reported that in the first leaves of young cucumber plants infected with fungal, bacterial or viral pathogens, the activity of chitinase (E.C.3.2.1.14) increased up to 600 fold in the infected areas of the leaf. In uninfected areas of infected leaves, chitinase was induced about 10 times less strongly. Chitinase was induced up to 100 fold in the uninfected second leaves of the plants. The increase of chitinase activity in the second leaves correlated well with an increase in resistance against a challenge infection by *Colletotrichum lagenarium.*Chitinase could also be induced about 10 fold by treatment with 10nl ml^{-1} ethylene for 27h. These plants showed enhanced resistance to infection by C. *lagenarium.* Treatment of the first leaves

of cucumber plants with necrotizing salt solutions caused a 20 to 50 fold increase of chitinase activity in the first leaves, a three to seven fold increase in the untreated second leaves and a slightly enhanced resistance against infection by C. *lagenarium.*

The expression of resistance and susceptibility to inoculation with zoospores of *Phytophthora megasperma* f. sp. *glycinea* race I was determined in roots, hypocotyls, and cotyledons of etiolated and green seedlings and in leaves of soybean cvs. Harosoy (rps_1) and Harosoy 63 (Rpsl). Gene-specific resistance was demonstrated in all organs tested, except for cotyledons of etiolated seedlings. In each case higher concentrations of the glyceollins accumulated in resistant than in susceptible reactions; the difference being greatest in hypocotyls and smallest in roots. The relative proportions of glyceollin I, II and III varied with the organ, exposure of the seedlings to light, the interaction type and the incubation period. Glyceollin I was relatively the most abundant isomer in roots and to a lesser extent in hypocotyls. Glyceollin III was relatively the most abundant isomer in leaves. Major differences in accumulation rates were observed in time-course experiments and, after reaching a maximum, all three isomers decreased in leaves and glyceollin I decreased in hypocotyls. Bhattacharyya and Ward (1986) suggested that concentrations and hence isomeric proportions were finally controlled by rates of biosynthesis and metabolism.

The authors further determined phenylalanine -ammonia lyase (PAL) activity in soybean hypocotyls at 25 or 33°C following infection with *Phytophthora megasperma* f. sp. *glycinea* or treatment with the abiotic elicitor, $AgNO_3$. PAL activity was less at 33°C than at 25°C in each of six cultivars examined but was lowest in two cultivars previously shown to develop susecptibility at 33°C Glyceollin accumulation was determined in response to $Ag\ NO_3$ treatment and was higher at 33°C than at 25°C in four cultivars tested. The increase was marginal with two temperature sensitive cultivars but more than 50% in two cultivars that remained resistant at 33°C. There were significant differences among 18 races of the pathogen for growth rates *in vitro.* The effect of temperature (25 or 33°C) on growth, sensitivity to glyceollin I and the interaction of temperature and glyceollin I sensitivity. Growth of some races (eg-race 2) was strongly inhibited at 33°C, that of others was similar at both temperatures,

and that of one (race 19) was faster at 33°C than at 25°C. Minimal restriction of growth at 33°C and relative tolerance to glyceollin I in race 4 combined with a major suppression of PAL activity and little increase in glyceollin accumulation at 33°C in Cv. Altona was consistent with temperature induced susceptibility in this race-cultivar interaction. The possibility that combinations of physiological variables in host and pathogen, rather than or in addition to, putative recognition systems, may define reaction type has been proposed. (Bhattacharyya and Ward, 1987).

Bhattacharyya and Ward (1987a) also reported that in unwounded soybean hypocotyls, pulse labelled with [^{14}C] phenylalanine and inoculated with *Phytophthora megasperma* f. sp. *glycinea,* rates of [^{14}C] incorporation and glyceollin I accumulation were higher in resistant than in susceptible responses throughout the time course of the experiment. This distinction was maskd in hypocotyls that were wounded and inoculated. In such hypocotyls, high rates of [^{14}C]-incorporation developed that were similar for the first 11 h in resistant and susceptible responses, although much more glyceollin I accumulated in the former. High rates of [^{14}C]-incorporation also developed in uninoculated wounded hypocotyls but only small amounts of glyceollin I of high specific radioactivity were detected. Estimates of phenylalanine ammonialyase activity indicated that the metabolic flux through phenyl alanine was limited in wounded controls but potentially very high in resistant responses. Differences in rates of [^{14}C]-incorporation and in specific radioactivity of accumulated glyceollin I presumably indicate differences in the relative contributions of mobile internal pools and externally applied phenylalanine, in addition to rates of biosynthesis. Rapid decline in [^{14}C] glyceollin I was demonstrated in wounded controls in pulsechase experiments with phenylalamine as chase, but not in inoculated hypocotyls, due to continued [^{14}C]-incorporation during the chase period. Rapid metabolism was demonstrated in all interactions and in wounds when cinnamic acid was used as the chase, but there was no evidence that differences in glyceollin I accumulation were due to differential rates of metabolism. Additional evidence for metabolic activity was provided by pulse feeding with [^{14}C] glyceollin I.

A glucan elicitor from the cell walls of the fungus *Phytophthora*

megasperma f.sp. *glycinea* also caused increases in the activities of the phytoalexin biosynthetic enzymes, phenylalanine ammonia - lyase and chalcone synthase, and induced the production of the phytoalexin, glyceollin, in soybean *(Glycine max)* cell suspension cultures when tested in culture medium containing 1.2 mmol / liter Ca^{2+} (Stab and Ebel, 1987). Removal of extracellular Ca^{2+} by treatment with ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid followed by washing the cells with Ca^{2+} free culture medium abolished the elicitor - mediated phytoalexin response. This suppression was largely reversed on readdition of Ca^{2+} , Elicitor - mediated enhancement of biosynthetic enzyme activities and accumulation of glyceollin was strongly inhibited by La^{3+} ; effective concentrations for 50% inhibition were (μmol / liter)/ 40 for phenylalanine ammonia - lyase, 100 for chalcone synthase, and 30 for glyceollin. Verapamil caused similar effects only at concentrations higher than 0.1 mmol / liter, whereas trifluoperazine and 8-(diethyl amino) - octyl - 3,4,5- trimethoxybenzoate did not affect enzyme induction by the elicitor in the concentration range tested. Uptake of $\mathcal{L}$-amino isobutyric acid into soybean cells, which was rapidly inhibited in the presence of the glucan elicitor, was not affected by La^{3+} nor was uptake inhibition by the elicitor relieved by La^{3+} . The Ca^{2+} ionophore, A 23187, enhanced phytoalexin biosynthetic enzyme activities and glyceollin accumulation in a close - dependent manner, with 50% stimulation (relative to the elicitor) Sodium azide was found to be most effective of the six metabolic inhibitors tested in reducing charcoal rot disease of soybean (cv. soymax) caused by *Macrophomina phoseolina* (Chakraborty and Purkayastha, 1987) Glyceollin production also increased significantly after induction of resistance by sodium azide treatment occurring at about 5 μmol / liter.

In another study Esnault et al (1987) determined the production of mRNAs for phenylalanine ammonia lyase and chalcone synthese in the first 5h following infection of intact etiolated soybean hypocotyls with zoospores of *Phytophthora megasperma* f.sp. *glycinea* mRNA was extracted from tissue excised from inoculated sites and mRNAS for the two enzymes detected by dot hybridization using corresponding cDNA probes. A major increase in mRNAs for both enzymes was detected by 3h following inoculation in an incompatible interaction but not in a compatible interaction. The results are consistent with the development of early differences in glyceollin biosynthesis in the two types of interaction.

Phenylalanine ammonia-lyase activity was reported to increase rapidly beginning 2h after inculation with *Phytophthora megasperma* (Drechs.) f. sp. *glycinea* (Hildeb). Kuan & Erwin race 1 in unwounded hypocotyls of soybean cv. Harosoy 63 (resistant) but did not change significantly in cv. Harosoy (susceptible). Small increases in phenyl alanine ammonia lyase activitiy also were caused by wounding. Activity increased more slowly in hypocotyls (cv. Harosoy 63) wounded just before inoculation than in intact inoculated hypocotyls, but most activity developed in hypocotyls wounded 12h before inoculation. There were comparable effects of wounding on symptom development. Trifoliate leaves of 14 day - old cv. Harosoy 63 plants are resistant, but trifoliate leaves of 12 day old cv. Harosoy 63 plants and 14 day old cv. Harosoy plants are susceptible to race 1. Increase in phenylalanine ammonia lyase activity following inoculation were demonstrated only in 14-day old Harosoy 63 plants but not until 24-36 h after the inoculation. Significant accumulation of glyceolin occurred by 24h. Susceptible trifotiate leaves of 12 day old cv. Harosoy 63 plants produced only low levels of glyceollin following either infection or treatment with the abiotic elicitor Ag NO_3, whereas trifotiate leaves of 14 day old cv. Harosoy plants produced high levels of glyccollin in response to $AgNO_3$. The authors concluded that trifoliate leaves of 12 day old, as opposed to 14 day old, cv. Harosoy 63 plants have not developed mechanisms that trigger responses to either infection or the abiotic elicitor or they are defi cient in metabolic processes that support glyceollin biosynthesis or other defence related responses (Bhattacharyya and Ward, 1988).

A pathogenesis related (PR) protein was found in both the infected and the uninfected leaves of cucumber plants inoculated on the first true leaf with a fungal, a bacterial or a viral pathogen. This host coded protein was detected up to five leaves above the infected leaf. The protein was purified from the intercellular fluid by ion- exchange chromatography and by high performance liquid chromatography on ion- exchange and phenyl sepharose columns. The purified PR-protein was shown to be a chitinase with a molecular mass of 28000 as determined by SDS polyacrylamide gel electrophoresis and by gel filtration (Metraux *et al*; 1988). Changes in the soluble protein pattern of leaves of eight lines of barley, carrying different resistance genes to mildew, were analysed by SDS polyacrylamide gel electrophoresis and isoelectric focusing (Bryngelsson

et al; 1988). Apparently new host proteins were induced by *Erysiphe graminis* f.sp. *hordei* in the incompatible reaction which were not present in the immune or susceptible response. These proteins are of low molecular weight, 13,500-27,000 d, and have either very low or very high isoelectric points. Thus, they resemble the pathogenesis related proteins found in many dicotyledonous species. In a further study, one of the pathogenesis related proteins, HV-1, in barley was isolated and characterized. *Hordeum vulgare* cv. Alva was challenged with an incompatible race of mildew *(Erysiphe graminis* f. sp. *hordei)* and the HV-1 protein was isolated from necrotized tissue by a combination of ammonium sulphate precipitation, anion exchange chromotography, hydrophobic interaction chromatography and chromatofocusing. The protein has a molecular weight of approximately 19KD and an isoelectric point of 3.4. It is not a glycoprotein as judged from its lack of reaction with concanavalin A and peanut agglutinin. The HV-1 protein was partially sequenced and compared to known protein sequences. The sequence of 28 amino acids had a 48% homology with the N-terminus of osmotin and NP24, proteins which are synthesized in response to salinity stress in tobacco and tomato, respectively. The same level of homology was also present in a maize trypsin / $\mathcal{L}$- amylase inhibitor and in thaumatin, an intensely sweet tasting protein from the West African shrub *Thaumatococcus daniellii* Benth. Even higher homology (58%) was discovered with the terminus of the PR-5 protein of tobacco (Bryngelsson and Green, 1989).

The effects of foliar application of growth substances and mineral nutrition of the host on the development of charcoal rot disease of soybean caused by *Macrophomina phaseolina* was tested by Chakraborty et al. (1989). Among the eight growth substances examined, gibberellic acid was most successful in reducing the disease serverity, followed by 3-indoleacetic acid and 2,3,5-triiodobenzoic acid. Low concentrations of these compounds stimulated while high concentrations inhibited the mycelial growth of *M. phaseolina invitro.* Substrate supplementation with different doses of N, P, K and Ca had varying effects on disease development. Disease increased considerably by both excess and deficient N and also by deficient Ca, while excess Ca conferred partial resistance. Glyceollin contents of host roots before and after excess Ca and gibberellic acid (10mg / L) treatments were estimated. Both compounds

significantly increased glyceollin production in infected roots. However, gibberellic acid induced glyceollin synthesis even in uninoculated roots. Changes in the host reaction towards increased resistance was correlated with increased phytoalexin production.

The possibility that susceptibility of immature leaves of soybean was due to deficiencies in metabolic precursors required for the expression of resistance and glyceollin synthesis was tested by supplying a range of compounds to the leaves prior to inoculation (Ward, 1989). These included sugars, glyceollin precursors and growth factors. Only glucose, galactose and sucrose were clearly effective and they promoted resistance to *Phytophthora megasperma* f. sp. *glycinea* race 1 in both cv. Harosoy 63 and in the normally susecptible cv. Harosoy. In both cvs supplied with glucose, resistance was associated with the accumulation of significant levels of glyceollin. More immediate precursors of glyceollin were ineffective and the possibility that glucose functions as a source of energy required for the development of resistant responses or for leaf maturation rather than as a source of glyceollin precursors has been discussed.

The differential regulation of the activities and amount of mRNAs for two enzymes involved in isoflavonoid phytoalexin biosynthesis in soybean was studied by Habereder et al. (1989) during the early stages after inoculation of primary roots with zoospores from either race -1 (incompatible host resistant) and race -3 (compatible, host susecptibility) of *Phytophthora megaspermum* f.sp. *glycinea,* causal fungus of rot disease. In the compatible interaction cloned cDNAs were used to demonstrate that the amount of PAL and chalcone synthase mRNA increased rapidly at the time of penetration of fungal germ tube into epidermal cell layer (1-2h after inoculation) concomitant with the onset of phytoalexin accumulation highest levels were reached after 7 h in the compatible and incompatible interaction Saindrenan et al. (1989) reported that the treatment of detached cowpea leaves with phosphite, the active breakdown product in plant tissues of fosetyl AL, lead to the cessation of growth of *Phytophthora cryptogen* within 24 h of inoculation. Pretreatment of leaves with $\mathcal{L}$-amino oxyacetate (AOA) an inhibitor of phenylpropanoid pathway increases the size of lesion in phosphite treated leaves, and induce a complete susecptibility. By 24h. after inoculation, PAL activity is higher in phosphite treated leaves than in

untreated. The effect of AOA on PAL activity are parallel with the effect of the increase of the spread of infection. The effect of ethylene on the induction of PAL by cell wall elicitor of *Colletotrichum lindemuthianum* was investigated by Hughes and Dickenson (1989) in leaves of susceptible and resistant cultivar of *Phaseolus vulgaris.* Induction of PAL without elicitor was negligible.

Li *et.al.* (1989) reported that extract from Hamimelon plant immunized with *Colletotrichum lagenarium* could effectively inhibit the growth of *Phytophthora melonis.* Activities of three enzymes in phenyl propanoid metabolism i.e. phenyl alanine ammonia lyase (PAL), cinnamate - 4 hydroxylase (CA4H) and 4-coumarate : COA ligase (4CL) were stronger in immunized plants. The activity of peroxidase was also significantly higher in immunized plant. A new protein was identified from immunized plants by 10% polyacrylamide gel electrophoresis, which had an isoelectric point of p^H 5.0 and a molecular weight of about 15.5 KD. The protein did not directly inhibit the germination of P. *melonis* conidia. Studies on the cell suspension cultures of bean *(Phaseolus vulgaris)* cv. *Imuna* which accumulated isoflavonoid phytoalexin on exposure to elicitor from the phytopathogenic fungus *Colletotrichum lindemuthianum* was carried out by Ellis et al. (1989). Phytoalexin accumulation was preceded by rapid increase in the activities of phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS). The relative levels of transcript from individual member of the CHS multigene family differ significantly at 1.5 h compared to 22.5 h after elicitation. More strikingly, three PAL genes were expressed in cultivar Imuna in response to fungal elicitor, whereas two are expressed in elicitor treated cell cultures Canadian order.

The effect of the product of the PAL reaction, transcinnamic acid (CA), on the appearance of individual PAL transcript in suspension cultured bean cells was studied by Mavandad *et al* (1990). Concentration of CA in excess of 10^{-4} mole inhibited appearance of elicitor induced transcript encoding PAL1, PAL2 and PAL3 when added to the cell at the same time as fungal elicitor. Addition of CA 4 hours post elicitation caused a major reduction in levels of all three PAL transcript, but with different kinetics and subsequent rates of recovery. The inhibition of accumulation of PAL 1, PAL 2, or PAL 3 transcript measured 3 h. after exposure to elicitor, as a function of the time of addition of CA postelicitation reflected the different rates of appearance of the three PAL

transcripts in the presence of elicitor alone. The inhibitory effect of CA as seen on PAL transcript were not observed for the constitutively expressed transcript H1, or the elicitor inducible 1,3-β - D glucanase. Analysis of *in vitro* translated polypeptides showed that some elicitor induced mRNA activities were not down regulated by CA, and that a number of other mRNA activities were induced by CA, thus providing further evidence for specificity in the action of CA on bean cells. Treatment of elicited cells with L-$\mathcal{L}$aminoxy-β phenyl propionic acid, a potent specific inhibitor of PAL activity, resulted in maintenance of elevated PAL transcript level beyond 12 h post elicitation, this effect being greatest for PAL transcript 2 and 3. .

Nemestothy and Guest (1990) reported that No. 2326, a cultivar of tobacco resistant to race O of the blank shank pathogen *Phytophthora nicotianae* var. nicotianae responded to stem inoculation of tobacco by rapidly accumulating sesquiterpenoid phytoalexins and activating phenylalanine ammonia lyase activity at the infection front. In cv. Hicks, a near-isogenic susceptible cultivar, both responses were slower. Pretreatments of leaf discs with propylene oxide, which killed the cells, mevinolin, a specific inhibitor of sesquiterpenoid biosynthesis, or the non-specific amino-transferase inhibitor, aminoxyacetic acid (AOA), inhibited post infection phytoalexin accumulation in both cuttivars, and induced susecptibility in cv. NC 2326. Amino hydrazinophenyl propionic acid (AHPP), a specific inhibitor of phenylalanine ammonia lyase activity and aminoeth oxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, do not affect the susecptibility of either cultivar.

Plants of the cv. Hicks are protected from infection by the systemic phosphonate plant protectant, fosetyl-Al. Sesquiterpenoid phytoalexins, lignin and ethylene accumulated, and phenylalanine ammonia lyase activity increased more rapidly in fosetyl - Al-treated Hicks stems than in untreated stems. Propylene oxide, mevinolin and AOA inhibited sesquiterpenoid phytoalexin accumulation and the effectiveness of fosetyl - Al in cv. Hicks. Fosetyl - Al did not enhance sesquiterpenoid phytoalexin biosynthesis in cv. NC 2326, and only marginally reduced pathogen growth in the initial stage of infection, before resistance was expressed. Mevinolin and AOA did not induce total susceptibility in fosetyl-Al treated NC 2326, indicating that factors other than sesquiterpenoid phytoalexins are also involved in the mode of action of fosetyl - Al in this cultivar.

Stem injection of tobacco cultivar Ky 14 with *Peronospora tabacina* or leaf inoculation with tobacco mosaic virus induced systemic resistance to both pathogens. The treatment also elicited a systemic increase in peroxidase activity which was positively correlated with induced resistance. Increases were evident in cytosol, intercellular fluid, and cell wall fractions. Upon challenge with *P. tabacina,* peroxidase activity further increased in the induced plants and remained higher after challenge as compared to the control plants. The isozyme patterns of peroxidase on isoelectric focusing gels showed an increase of two anionic peroxidases. Both peroxidases were positively correlated with induced resistance (*Ye et al.* 1990). They further reported that inoculation of three leaves of tobacco cv. Ky 14 with tobacco mosaic virus and incubation at 23°C for 3-12 days caused localized necrosis on the inoculated leaves where as plants held at 28°C after inoculation developed systemic mosaic symptoms. Pathogenesis - related (PR) proteins and activities of peroxidase, β-1.3 glucanase and chitinase systemically increased in the inoculated plants at 23°C. When TMV inoculated leaves were removed 12 days after inoculation at 23°C and plants were challenged with TMV or *Peronospora tabacina* and held at 23°C, induced resistance to *P. tabacina* and TMV was apparent. However, resistance to blue mould but not TMV was apparent when the procedure was repeated, except that plants were transferred to 28°C one day after challenge with TMV or P. *tabacina.* PR proteins and activities of peroxidase, β-1,3 glucanase and chitinase were further increased after challenge with P. *tabacina* or TMV in induced plants at both temperatures. Tn 86, a cultivar systemically susecptible to TMV, was protected against blue mould but not against TMV by stem inoculation with P. *tabacina* PR proteins were induced and the activities of peroxidase, β-1,3 glucanase and chitinase were greatly increased in the induced plants (Ye et al; 1990).

It has also been observed/pointed out that light / sucrose treatment of sunflower (*Helianthus annuus*) hypocotyl originated cyclic changes of PAL activity with alternative periods of increase and decrease of activity and maxima at 4 and 28 h (Jorrin et al 1990). Actinomycin D, corelycepin and cycloheximide inhibited both induction and decay of PAL activity. These result suggested that the turnover mechanism of PAL implicates *denovo* synthesis of enzyme, better than activation of inactive precursors, in response to the inducer agent (light

and sucrose) and subsequent synthesis of a putative proteinaccous PAL inactivating system responsible for PAL activity decay. Expression of PAL gene during tracheary element differentation was studied in mesoplyll cell suspension cultures of *Zinnia elegans.* Dose response curves of benzyl adenine (BA) and $\mathcal{L}$-naphthalene acetic acid (NAA) were obtained for the cultures in order to achieve the highest percentage. Of BA and NAA, 0.1 mg. l^{-1} and 0.06 mg. l^{-1} respectively, normally stimulated about 40% differentiation by 96 h of culture. The effect of same ratio but different amount of BA and NAA on tracheary element formation have been tested and the result indicated that the absolute amount of BA and NAA rather than the ratio of them were important for tracheary element formation in the *Zinnia* cultures. The cells when cultured in the presence of 0.001mg $.l^{-1}$ of BA and 0.06 mg. $.l^{-1}$ of NAA expanded and divided but did not differentiate. The level of PAL activity, synthesis of PAL protein and level of PAL mRNA peaked during 72 to 96 h when lignin was actively deposited. This indicated that the PAL gene was temporally and preferentially expressed in association with the lignification during tracheary element differentiation and thus it can be regarded as a molecular marker for the process. (Lin and Northcote, 1990).

In a study with carrot *(Daucus carota* L.cv. *kurodagosun)* suspension culture cells, PAL was slowly induced during anthocyanin synthesis which occurred in a medium lacking 2,4 dichlorophenoxy acetic acid and was also induced rapidly and transiently by transferring and diluting cells to fresh medium. Analyses of nucleotide sequence derived from PAL cDNAs revealed that the PAL mRNAs induced by transfer were transcribed from different carrot PAL genes than the PAL mRNAs induced during anthocyanin synthesis. Northern blotting, using probes derived from 3' non-coding region for PAL cDNAs confirmed that different PAL genes were induced during anthocyanin synthesis and after transfer. Induction of different PAL genes occur in response to difference in induction trigger (Ozeki *et al*, 1990). The timing of changes in protein synthesis pattern of elicitor treated [^{35}S] methionine labelled parsley cell *(Petroselinum crispum)* was analysed by two dimensional gel electrophoresis Five groups were distinguished from a large number of elicitor responsive as well as unresponsive proteins. Two groups were synthesized *denovo* either early or late after elicitor application; two other groups were

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strongly reduced in their rates of synthesis either early or late after elicitor application; and one group was not appriciably affected at all. The elicitor induced changes altered the total protein composition considerably. A few selected, induced protein were functionally identified. These included two early induced enzymes, (PAL) and 4-coumarate : CoA ligase (4CL) and a late induced enzyme, a bergaptol - O methyl transferase (BHT) which is specifically involved in the biosynthesis of furanocoumarin phytoalexin. The biological significance of the observed differential timing of changes in protein synthesis rates was discussed by Bollmann et al (1990).

Alfalfa (*Medicago sativa L.)* cell suspension cultures accumulated high concentration of the pterocarpon phytoalexin medicarpin, reaching a maximum within 24 hours after exposure to an elicitor preparation from cell walls of the phytopathogenic fungus *Colletotrichum lindemuthianum* (Dalhim et al. 1990). This was preceded by increase in the extractable activities of the isoflavonoid biosynthesis enzyme L-phenyl alanine ammonia lyase, cinnamic acid 4-hydroxylase, 4-coumarate Co-A-ligase, chalcone synthase, chalcone isomerase and isoflavone O-methyl transferase. Pectic polysaccharides were weak elicitors of phenylalanine ammonia lyase activity but did not induce medicarpin accumulation, whereas reduced glutathione was totally inactive as an elicitor in this system. The fungal cell wall extract was a weak elicitor of the lignin biosynthetic enzymes caffeic acid O-methyl transferase and coniferyl alcohol dehydrogenase, but did not induce appreciable increase in the activity of the hydrolytic enzymes chitinase and 1.3 β-D-glucanase. Accumulation of polyphenoloxidase, peroxidase and phenylalanine ammonia lyase from cucumber *Cummis sativus* L. leaves were studied during the period of 22 days after inoculation with cucumber powdery mildew. Results indicated that early rapid increase of polyphenloxidase, peroxidase and phenylalanine ammonia lyase activities was of great significance in the disease resistance.

The 1- Amino - 2 phenylethyl phosphonic acid (phep) stimulated growth in *Allium cepa* L grown *in vitro* for 30 days, and increased activity of phenylalanine ammonia lyase both per mg fresh wt. and mg protein. After 60 day phep reduced PAL activity (Knypl and Janas, 1990). It was also shown by Janas and Knypl (1990) that 1-amino 2-phenyl ethyl phosphonic acid (phep) enhanced phenyl alanine ammonia lyase activity (PAL) upto 7-6 fold times,

increased the level of phenylalanine upto 8 times and reduced amaranthin content in *Amaranthus caudatus* L., the effect being dependent on age of seeding, illumination, concentration and optical enantiomer of phep. D(+)-phep was a stimulator of PAL *in vivo* whereas L (-) - phep at 0.01 and 0.1 mM reduced PAL activity by about 20%, and stimulated it at 1mM. The inhibitory effect of phep on amaranthin accumulation was strengthened by L (-) phenylalanine which at 0.01 and 0.1mM stimulated amaranthin accumulation, phep reduced PAL activity and potentiated the stimulatory effect of D(+) phep on PAL. PAL activity in the seedlings oscillated at 12 h intervals; the oscillation pattern was not modified by phep.

Akhtar and Garraway (1990) reported that the increase in peroxidase activity and electrolyte leakage induced in maize (*Zea mays* L.) leaves by sodium bisulfite were causally related to the sodium bisulfite induced increases in sporulation of the pathogen *Bipolaris maydis* race T on infected maize leaves. Pretreatment of detached leaves of maize inbred W64A with sodium bisulfite (500 μg/ml) for 24 h in the dark at 28^{0}C increased peroxidase activity in the Tms cytoplasm (susceptible) isoline compared with the N cytoplasm (resistant) isoline. No such differences in peroxidase activity between the two isolines were observed when detached leaves were pretreated with double distilled water. The sodium bisufite induced increase in peroxidase activity persisted even when leaves pretreated with sodium bisulfite were inoculated with *B. maydis* race T and subsequently incubated for 48 h in the dark at 28^{0}C. Similarly, pretreatment with sodium bisulfite caused a greater increase in electrolyte leakage as well as in sporulation on the leaves of the susceptible than on those of the resistant isoline when compared with leaves not treated with sodium bisulfite. Sodium bisulfite showed no effect on sporulation *in vitro.* Leachates from the susceptible isoline pretreated with sodium bisulfite also caused greater increase in sporulation than those from the resistant isoline pretreated with sodium bisulfite.

In another study, phenylalanine ammonia lyase (PAL) and peroxidase activities were shown to increase slightly during prehaustorial stages of development of the leaf rust fungus *(Puccinia recondita* f.sp. *tritici)* in each combination of rust strain and wheat line used. Activity of both enzymes greatly increased during the formation of the first haustoria by avirulent and virulent

strains in an Lr 28 bearing line but not in an Lr 20-bearing line. PAL increased markedly in activity at the time when an avirulent strain was known to cause hypersensitivity in the Lr 28 bearing line and when another avirulent strain was known to cause cellular changes that preceded the expression of resistance in the Lr 20 bearing line. PAL increased less markedly and not at all at corresponding times when virulent strains developed in the Lr 28 and Lr 20 lines respectively. Decrease in the peroxidase activity occurred after the increase in PAL activity and were greater during resistance expression than in leaves infected with virulent strain. Cytochemical tests revealed enhancement of peroxidase activity in epidermal and guard cells in response to avirulent and virulent strains only. The implication of the changes are that aromatic and phenylpropanoid synthesis and peroxidation may be enhanced in wheat by infection and particularly during resistance expression. The products of these processes may play roles in both Lr 28 and Lr 20-based expression of resistance (Southerton and Deverall, 1990).

Spraying the lower surface of the first true leaf (leaf 1) of cucumber plants with 50 mM K_2HPO_4 induced systemic resistance to anthracnose caused by *Colletotrichum lagenarium,* 2 to 7 days later (Irving and Kuc, 1990). Within 16 h of application, the activities of peroxidase and chitinase increased in the induced leaf, and they continued to increase over the next 7 days reaching levels at least ten fold higher than in leaf 1 of plants treated with water. During this period, the activities of both enzymes were usually two-fold or higher in leaf 2 of plants induced with K_2HPO_4 than in plants treated with water. If the induced plants were not further treated or mock challenged with water on leaf 2, the enzyme levels increased slightly and levelled out at between two or four fold higher than in the corresponding control plants. If plants were challenged with C. *lagenarium* on leaf 2, the enzyme activity at the site of challenge increased in the induced plants prior to rising in the control plants. Large increases in enzyme activity were only evident after the lesions became visible (3-4 days after challenge) in the control plants and this only occurred at the site of challenge. Seven days after challenge, the enzyme activities in the leaf tissue adjacent to the site of inoculation were similar in both the control and induced plants. Correlations were made between peroxidase and chitinase activities induced by several treatments of leaf 1 and the level of protection observed in leaf 2 after challenge with C. *lagenarium.*

Estabrook et al, (1991), have used conserved and non-conserved regions of cDNA clones for phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) isolated from a soybean nodule cDNA library to monitor the expression of members of the two gene families during the early stages of the soybean *Bradyrhizobium japonicum* symbiosis. Their results demonstrated that subsets of the PAL and CHS gene families are specially induced in soybean roots after infection with *B. japonicum*. Furthermore by analyzing a super nodulating mutant line of soybean that differs from the wild type parent in the number of successful infections, the induction of PAL and CHS was shown to be related to post infection events. Nodulated roots formed by a Nod^+ Fix^+ strain of *B. japonicum,* resembling a pathogenic association, displayed induction of another distrinct set of PAL and CHS genes. It was suggested by the authors that symbiosis specific PAL and CHS gene in soybean are not induced by stress or pathogen interaction. Phenylalanine ammonia lyase in activating factor (IF) prepared from chloroplast isolated from sunflower (*Helianthus annus*) leaves was utilized to study its inactivating effect of L-phenyl alanine ammonia lyase from *Rhodotorula glutinis, in vitro*. The effect of inactivation by inactivating factor were compared with those caused by chemicals such as sodium borohydride and nitromethane. The sunflower inactivator acted as an enzyme and enzymatic inactivation caused irreversible loss of PAL catalytic activity accompanied by shortening of the enzyme molecule. However, the capacity of IF inactivated the PAL to bind to L-phenylalanine, the enzyme substrate was maintained. These effect of inactivating factor from sunflower leaves are quite different from those inhibitor isolated from different sources by other marker (Gupta and Creasy, 1991).

Peroxidase and phenylalanine ammonia lyase activities (PAL) was determined in leaves of healthy and inoculated *Brassica napus* cultivars, showing differential disease reaction towards a virulent and a weakly virulent strain of *Leptosphaeria maculans,* the black leg pathogen by Chakraborty *et al* (1993). Both enzymes showed increased activities as a result of inoculation ; PAL activity increasing as early as 12 h after inoculation. The most significant increase in both peroxidase and PAL activity was observed when the moderately resistant cultivar, Cresor, was challanged with the weakly virulent strain. Highest activity of the two enzymes was detected 2d after inoculation. Very low peroxidase activity was detected in both strains of L. *maculans,* while no PAL

activity was detectable in either of the strains. Cytochemical tests revealed increased peroxidase activity following inoculation mainly in the epidermal and guard cell.

Subcellular localization of the pathogenesis - related PR-1 proteins of unknown function was studied in roots of resistant *Nicotiana tabacum* cv. *xanthi* nc uninfected or infected *invitro* by the black root rot fungus *Chalara elegans,* using polyclonal or monoclonal antibodies raised against PR-1a (or b_1) protein (Tahiri-Aloui *et al,* 1993). In healthy tobacco roots, the PR-1 proteins were found to be present in low amounts in intercellular space material, over cell walls and over secondary thickenings of xylem vessels. All these cell compartments were significantly enriched in the PR-1 proteins in infected tobacco root tissues. Cell wall outgrowths, typically induced in hypodermal cells by *Chalara elegans* infection and wall appositions formed in cortical parenchymal cells in response to infection, were also the sites of PR-1 protein accumulation. In contrast, very little occurred in electron - translucent intercellular spaces. PR-1 proteins were also detected in the wall of both inter and intracellular fungal hyphae invading root tissues, but not in axenically cultured *Chalara elegans* or hyphae developing outside roots.

Carver *et al* (1994) reported that seedling leaves of oat cvs Maldwyn and Selma have no known major resistance genes to powdery mildew caused by *Erysiphe graminis* f. sp. *avenae,* but their susceptibility to infection is quantitative. Thus only a portion of fungal germlings successfully overcome cell defences to penetrate host epidermis to form haustoria OH-PAS ([[(2-hydroxyphenyl) amino] sulphinyl] acetic acid, 1,1-dimethyl. ethyl ester) is a potent, specific suicide inhibitor of CAD (cinnamyl alcohol dehydrogenase), an enzyme specifically involved with synthesis of lignin precursors. OH-PAS was shown to inhibit CAD from oat in vitro. For *in vivo* assays of effects on epidermal cell defences, the cut ends of excised seedling leaves were immersed in OH-PAS solution for 24h to allow uptake before inoculation with *E.graminis* conidia. Inoculated leaves were allowed OH-PAS uptake during a further 36 h incubation period. Initial experiments established that OH-PAS at 10^{-3} M decreased the frequency and intensity of localized autofluorescent host epidermal cell responses associated with primary germ tubes (PGTs) and appressoria. Concurrently, OH-PAS treatment doubled the proportion of appresoria forming

haustoria, i.e. it increased quantitative susceptibility by suppressing host cell defences. Similar results were obtained with 10^{-3} M AOPP ($\mathcal{L}$-aminooxy β-phenyl propionic acid), a competitive inhibitor of PAL (phenylalanine ammonia lyase) which catalyzes the first committed step in phenylpropanoid metabolism. Both inhibitors doubled the proportion of appressoria penetrating epidermal cells and forming haustoria. Both inhibitors reduced the frequency and intensity of localized autofluorescent epidermal host cell responses to PGTS and appressoria, although the effect of AOPP was somewhat greater than that of OH-PAS. Neither OH-PAS nor AOPP had any deleterious effects on fungal development. Results support the idea that host autofluorogens accumulating at sites of fungal germ tube contact with epidermal cells are phenolic compounds. In addition the study provides experimental evidence pointing to involvement of products synthesized as part of the lignin biosynthetic pathway in oat epidermal cell defence against attempted penetration by appressoria of E. graminis f.sp. *avenae.*

Seeding leaves of two pairs of near-isogenic barley lines were inoculated with conidia of the powdery mildew fungus, *Erysiphe graminis* D. C. f. sp. *hordei* Marchal, racc 3. by Clark *et al.* (1994). One set of isolines (RISQ 5678 R and RISQ - 5678-S) differed at the ML-O locus where the recessive allele (ml-O) confers a high degree of race non-specific, penetration based and papilla - associated resistance to *E. graminis,* while the dominant allele (Ml-0) allows a proportion of attacking fungal germlings to succeed in infection. The second isoline set (Algerian R and Algerian-5) differed at the Ml-a locus where the dominant allele confers race-specific, epidermal cell death resistance visible only by light microscopy. The recessive allele (Ml-a) allows a proportion of attacking fungal germlings to succeed in infection. Leaf samples were taken at 0,2,4,6,8,10,12,15,18,21 and 24 h after inoculation to examine the timing of host epidermal cell cytoplasmic aggregate responses (visible by light microscopy) relative to phenylalanine ammonia lyase (PAL) mRNA transcript accumulation (determined by quantitative northern blots), and PAL enzyme activity (using radiolabelled phenylalanine). *Erysiphe graminis* produced primary germ tubes (PGTs) within 2h and appressorial germ tubes within 6-10 h of inoculation. In all isolines, host epidermal cell cytoplasmic aggregates fromed and subsequently dispersed beneath PGTs, between 2 and 10 h and beneath appressoria betwen 6 and 15 h. Concurrently, biphasic patterns of PAL transcript accumulation,

typed by plaks at 4 and 12 h occurred in all isolines. Temporal patterns of PAL enzyme activity were roughly similar to those of PAL transcript accumulation. Fungal germ tube contact initiated host epidermal cell cytological responses common to all isolines, induced PAL transcript accumulation, and increased PAL activity regardless of the Mendelian inheritance of "major gene-resistance factors" in the barley isoline sets. Thus there was PAL induction associated with a general defence to infection, but no unusually strong correlation between PAL induction and major gene resistance was found.

Phenylalanine ammonia lyase (PAL) activity in barley leaves was also measured at intervals after inoculation with the powdery mildew pathogen *Erysiphe graminis* f. sp. *hordei.* Measurements were made at times that included the periods of attempted penetration by the *E.* graminis primary germ tube and appressorium. The results demonstrated that extractable enzyme activity increased at 6 h and between 12-15h after inoculation, times consistent with attempted penetration by the primary germtube and the appressorium, respectively. Enzyme activity increased regardless of the resistance or susceptibility of the barley cultivar to the fungus suggesting that the response was non specific and was not a reflection of the resistance or susceptibility of the cultivar to the pathogen prior to the time of penetration. When barley was inoculated with the nonpathogen E. *pisi,* only a single period of elevation in PAL enzyme activity was detected. This was consistent with the fact that, unlike E. *graminis,* E. *pisi* does not produce a primary germ tube. The enzyme activity increased between 9-15h after inoculation, consistent with the time of the attempted penetration of the leaf by the E. *pisi* appressorium. Northern blot analyses to detect the time of appearence of PAL mRNA indicated that the level of the message began to increase at 0.5 h after inoculation with both fungi, and that the intensity of the increase was greatest in response to E *pisi.* The results have been discussed with respect to the presumed importance of host phenolic compound metabolism that occurs as a response to the fungal infection process. (Shiraishi et al, 1995).

In order to determine whether the level of phenylalanine ammonia-lyase (PAL) activity in sorghum mesocotyl tissues is elevated in response to inoculation. Orcyzk *et al* (1996) used sorghum cultivars that differed in their ability to synthesize anthocyanins in response to light. These cultivars provided a means

of distinguishing the light induced high background levels of PAL from increases in PAL that occur as a result of attempted infection. The induction of PAL as a response to both light and to attempted fungal infection was further confirmed by a time course study of RNA blot hybridizations with a barley PAL cDNA. Results showed that PAL transcripts in the cultivar that did not synthesize anthocyanins began to accumulate within 3 h of exposure of the tissue to light but that the level of the transcript decreased rapidly thereafter. However, when the same cultivar was inoculated with the nonpathogen *Bipolaris maydis* the intensity of the PAL transcript remained elevated throughout a period of 24h after inoculation. The results demonstrated that in this monocotyledonous host it is necessary to separate the naturally occuring high levels of PAL activity that are induced by light from the induction of PAL enzyme activity which occurs as a response to attempted fungal infection.

Four phytoalexins were identified from inoculated stems of cocoa genotypes resistant to *Verticillium dahliae.* Following purification by repeated flash chromatography and TLC, they were identified by NMR and GC-MS. The most abandant and polar compound was the triterpenoid arjunolic acid; two related phenolics were 3,4 dihydroxyacetophenone and 4. hydroxy acetophenone. The least polar was unambiguously identified as the most stable form of elemental sulphur, cyclooctasulphur S_8, by Gc- Ms and X-ray crystallography. Respective toxicities to V. *dahliae* conidia (ED 50 germination in $\mu g\ ml^{-1}$) were 12.8, 92.5, 7.2, 3.6. Sulphur and arjunolic acid first appeared after 10 and 3 days respectively, were present in the wood of stems at levels well above (7/ x13) those required for toxicity and they persisted for long (7/ 50 days) periods; they were found only after infection whereas the phenolics were detected in control stems and were enhanced to similar extents by infection or wounding . Sulphur accumulation was localized to xylem cells. In contrast, condensed tannins, although increasing approx. two. fold after infection, were performed, of low toxicity (ED_{50} 7/ 383 $\mu g\ ml^{-1}$) and concentrations in two resistant and one susceptible genotypes were not significantly different. This is the first report of phytoalexins in *T.* cacao and of sulphur accumulation in plants linked with active defence (Resende *et al*; 1996).

Groundnut leaves (cutlivar TMV2) infected with the fungal pathogens, *Cercospora arachidicola, Phaeoisariopsis personata* and *Puccinia arachidis*

accumulated 1830, 664 and 162 nml phytoalexins g^{-1} fresh wt., respectively, 4 weeks after inoculation, whereas leaves infested with *Frankliniella* sp. for the same period contained 1.25 nmol phytoalexins g^{-1} fresh wt. (Rao et al. 1996). Spraying abraded leaves with salicylic acid (0.01M) resulted in the accumulation of 1270 nmol phytoalexins g^{-1} fresh wt. 120 h after treatment and irradiation of abaxial leaf surfaces with U.V. light (254nm) for 48h and incubation in the dark for a further 96h caused the accumulation of 393 nmol phytoalexins g^{-1} fresh wt. Compounds with U.V. spectra corresponding to isoflavanones were almost exclusively synthesized in response to abiotic elicitors but in leaves infected with fungal pathogens formononetin, diadzen, and medicarpin were also present, though as minor components.

Reiss and Bryngelsson (1996) found that the perthotrophic fungus *Drechslera teres,* the causal agent of net blotch disease in barley, induces the accumulation of pathogenesis related (PR) proteins in barley leaves which was demonstrated by isoelectric focusing. The same protein pattern was also found in leaves treated with a toxin extract from the culture filtrate of *D. teres* as well as after infection with *Erysiphe graminis* f.sp. *hordei* or *Puccinia hordei.* Some of the proteins induced by infection with *D. teres* were charecterized as peroxidases, β-1,3- glucanases and chitinases by isoenzyme analysis. Immuno detection following western blots demonstrated that the induced proteins are the same as those that accumulate after inoculation of barley with *E. graminis;* basic PR-1a and b proteins, thoumatin like (TL) proteins, β-1,3 glucanases and chitinases. The accumulation of PR-1 type proteins, chitinases and TL- proteins was analysed quantitatively by ELISA.

Oligonucleotide primers made complementary to conserved sequences in phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) genes previously cloned from other species were used to amplify segments of the corresponding genes from sorghum Dycui et at (1996). Greater than 70% base sequence identity with homologous genes confirmed that a 535 bp clone (PAL 1-1) and a 620 bp clone (CHS2G) were derived from the coding regions of PAL and CHS respectively. When used to probe genomic digests, the two clones detected 3 and 5 Eco RI fragments, respectively. One fragment for each probe was polymorphic in the parents of a mapping population, permitting a locus for each gene to be located on a sorghum RFLP linkage map. RNA gel blot analyses

were performed on extracts from control seedlings and from seedlings challenged with either of two fungal sorghum pathogens or a pathogen of maize that elicits a hypersensitive response in sorghum. Although low levels of PAL and CHS mRNA transcripts were present constitutively in the controls, both rapidly accumulated to high levels following inoculation with the maize pathogen, *Bipolaris maydis,* and decreasing amounts of mRNA were apparent by 120h post inoculation. Since challenge with *Sporisorium reilianum* was by needle inoculation, an additional control of mock inoculations was included for this pathogen. However, no differences in the amount of mRNA for either gene were detected over the sampling period, whether samples from uninoculated, mock inoculated or spore inoculated seedlings were tested. Differences in the timing and level of mRNA accumulation were detected in seedlings after inoculation with *Peronosclerospora sorghi.* While seedlings of both resistant and susceptible cultivars accumulated higher levels of PAL and CHS mRNA than uninoculated controls, the accumulation of mRNA in resistant cutlivars was higher and longer rasting than that in susceptible cutlivars.

Three groups of *Orobanche cumana* seedings were distinguished according to the peroxidase content of the cells in the radicles : (1) those with neither extracellular nor intracellular peroxidase and whose radicles have a smoth apex (these were classified as non-infective); (2) those with a high peroxidase content of the nuclei and the cytoplasm layer adjacent to the cell wall, as well as excretion of peroxidase from the apex of the radicles : (3) those with a similarly high peroxidase activity in the parasite cells, but without extracellular excretion (Antonova and Terborg, 1996). The apices of the radicles of the last two groups are swollen. It has been suggested that these belong to *O.cumana* races C and D respectively. The extracellular peroxidase in *O. cumana* race C reacts with phenolic compounds, which are lignin precursors of the host, resulting in host resistance due to the formation of lignin layers in sunflowers possessing the Or 3 gene for resistance. The absence of extracellular peroxidase in *O.cumana* race D prevents lignin - formation and enables the parasite to attach to the host vascular system. Comparison on these data with the information on the earlier *O. cumana* races A and B, and older sunflower cultivars, points to a crucial role of peroxidase in the process of breeding new sunflower cultivars and the evolution of new *O. cumana* races.

Phytophthora megasperma f.sp. *glycinea* infection greatly induced the synthesis and accumulation of β-1,3 glucanases and chitinases in hypocotyls and leaves of soybean seedings. Native polyacrylamide gel electrophoresis and isoelectric focusing were used to identify and quantify the isoforms of the two hydrolases in soybean tissues. Some β-1,3 glucanase isoforms were constitutively experssed in soybean, but other isoforms only accumulated in soybean or only in a particular organ of soybean after inoculation with *P.m.* f. sp. *glycinea* infection or after treatment with mercuric chloride. The pI 8.4 isoform, Gb2 of β-1,3 glucanase constitutively present in soybean hypocotyls and leaves completely disappeared after mercuric chloride treatment. *P.m.* f. sp. *glycinea* infection and mercuric chloride treatment leads to the accumulation of distinctly different isoforms of chitinase in soybean tissues. The acidic chitinase isoform Ca 4 was induced and accumulated in hypocotyls, but not in leaves, after pathogen infection and mercuric chloride treatment. In general, β-1,3 glucanase and chitinase isoforms accumulated in the diseased soybean tissues during symptom development in both compatible and incompatible interactions. The results also suggested that pathogen infection and chemical stress may function in a different organ specific manner to induce isoforms of β-1, 3 glucanase and chitinase in soybeans (Seung and Byung, 1996).

Accumulation patterns of specific isozymes of pathogenesis related proteins in the resistant tomato genotypes 71B², NCEBR-1, NCEBR-2 and th susceptible cultivar Piedmont following inoculation with *Alternaria soloni* were described by Lawrence et al (1996). Western blot analysis demonstrated that four isozymes of chitinase (26,27, 30 and 32 KDa) were induced in all genotypes upon challenge with A. *solani,* but only resistant lines had significantly higher constitutive levels of the 30 KDa isozyme as well as total chitinase activity. In addition, the 30 kDa chitinase isozyme was found to accumulate to significantly higher levels in resistant lines during pathogenesis than the susceptible genotype. Two isozymes of β-1,3 glucanase (33 and 35 KDa) were detected in all genotypes, but a slightly higher constitutive level was detectable in all resistant lines when compared to the susceptible. Similar accumulation patterns of these isozymes were observed in all genotypes during the course of pathogenesis. Purified preparations of acidic and basic tomato chitinase and β-1,3 glucanase isozymes were tested for their antifungal activity against A. *solani* invitro. Results

presented in this study indicate that only basic isozymes of chitinase and β-1, 3 glucanase were inhibitory to A. *solani* whereas, no inhibitory activity was observed with the acidic isozymes. The results of this study suggest that a higher constitutive level of chitinase and β-1,3 glucanase and the induction pattern of a 30 KDa chitinase isozyme in early blight resistant breeding lines is related to genetically inherited resistance of tomato to A. *solani.*

It was also reported by Wibber et al (1996) that in the interaction between *Cladosporium fulvum* and tomato, resistance against the fungus correlates with early induction of transcription of genes encoding apoplastic chitinase and1,3 β-glucanase and the accumulation of these proteins in inoculated tomato leaves. For vacuolar, basic isoforms of chitinase and 1,3-β glucanase, however, early gene transcript accumulation was observed in both incompatible and compatible interactions. Only temporal differences in gene transcript accumulation were observed for each isoform studied. Expression of the acidic chitinase gene was observed primarily near leaf vascular tissue. Expression of the basic chitinase and the basic and acidic 1, 3-β glucanase genes was less confined to particular tissues. No preferential accumulation of gene transcripts in tissue near penetrating hyphae was observed in compatible or incompatible interaction.

Injection of purified race specific elicitors, AVR4 and AVR9, in tomato genotypes cf4 and cf9, respectively, induced primarily differential expression of acidic chitinase and acidic 1,3-β glucanase. The induction, observed most abundantly in resistant genotypes, correlated well with the difference in gene expression previously observed in time course experiments of compatible and incompatible C. *fulvum* tomato interactions. An acidic β-1,3- glucanase and three isoforms of chitinase (A,B,C) were isolated and purified from cucumber (*Cucumis sativus* L.) leaves inoculated with a necrogenic fungus, *Colletotrichum lagenarium.* Tests of antifungal activity to C. *lagenarium in vitro* were conducted with the purified β-1,3 glucanase, the three purified isoforms of chitinase and with intercellular wash fluids (ICF) from cucumber leaves above those inoculated with C. *lagenarium* or those treated with water. β-1,3 glucanase alone significantly inhibited spore germination at a concentration of 250 $\mu g\ kl^{-1}$ buffer solution and inhibited the growth of C. *lagenarium* at a concentration of about 7 μgml^{-1} agar medium. The latter concentration is equivalent to the concentration in infected cucumber leaves 7 days after inoculation with *C. lagenarium* and in

leaf 2 of induced plants 3 days after challenge with the fungus. Chitinase A and B did not inhibit spore germination or fungal growth at the concentrations tested, whereas chitinase C inhibited spore germination only at 1 mg ml^{-1} and inhibited fungal growth at 66 μg ml^{-1} agar medium, the highest concentration tested. β-1,3 glucanase in combination with chitinase C inhibited fungal growth synergistically. Lysis of hyphal tips and abnormal growth were observed in the presence of a mixture of β-1,3 glucanase and chitinase C. Intercellular wash fluid (ICF) and concentrated ICF (5x) from control and induced plants did not inhibit fungal growth. However, the crude ICF in the presence of purified β-1,3 glucanase and chitinase C had antifungal activity (Ji and kuc, 1996).

Generation of superoxide anion [0-2(0)] and peroxidase activity were significantly increased in bean leaves infected with incompatible and compatible pathogens : *Botrytis fabae* and *Botrytis cinerea,* respectively, but the induction was greater on direct inoculation with B. *fabale,* than with *B. cinerea* as determined by Urbanek et al (1996). A slightly higher 0-2(-.) level was also detected in the parts of leaves surrounding the inoculation side. Overproduction of 0-2(-.) was observed earlier than the increase in peroxidase activity. Pretreatment of the leaves with methyl jasmonate enhanced both 0-2(-.) production and peroxidase activity following inoculation with B. *cinerea.* Induction of superoxide dismutase activity after the infection was less pronounced than changes in 0-2 (-.) level. The differences in the rate of NADH oxidation in the extracts from control and inoculated leaves, correlated with the differences in the rate of 0-2 (-.) production. The results indicate that 0-2 (-.) level is one of the essential factors responsible for the difference in the interactions between bean plant and compatible and incompatible pathogens.

Tobacco plants (*Nicotiana benthamiana L.*) have been transformed with a T-DNA vector construct carrying the cDNA pBH6-301, encoding the major pathogen induced leaf peroxidase (Prx8) of barley, under control of an enhanced CaMV 356 promoter. Progeny from three independent transformants were analyzed genetically, phenotypically and biochemically. The T-DNA was steadily inherited through three generations. The barley peroxidase is expressed and stored to the intercellular space in the transgenic tobacco plants. The peroxidase can be extracted from the intercellular space in two molecular forms from both barley and transgenic tobacco. The tobacco expressed forms are

indistinguishable from the barley expressed forms as determined by analytical isoelectric focusing (PI 8.5) and western blotting. Staining for N-glycosylation showed that one form only was glycosylated. The N-terminus of purified Prx8 from transgenic tobacco was blacked by pyroglutamate, after the removal of which, N-terminal sequencing verified the transit signal peptide cleavage site deduced from the cDNA sequence. Phenotype comparisons show that the constitutive expression of Prx8 lead to growth retardation. However, an infection assay with the tobacco powdery mildew pathogen *Erysiphe cichoracearum* did not indicate that the transgenic plants had achieved enhanced resistance. (Kristensen *et al, 1997).*

Serological relationship between host and Pathogen

It is now well established that immune system functionally similar to that of animals exists in plants. The serological cross reactivity between host and pathogen has been a subject of considerable interest to a number of workers and a number of reviews pertaining to this area have been published previously (DeVay and Adler, 1976; Clark, 1981; Chakraborty, 1988, Purkayastha, 1994). Detection of plant pathogenic fungi within host tissues by serological means is a relatively recent development and a number of recent reviews have been published by workers along this line (Hansen and Wick, 1993; Werres & Steffens 1994).

Conidia of F. *oxysporum* f. sp. *vasinfectum* was reported to contain an antigen that cross reacted with antiserum to cotton root tissue antigens (Charudattan and Devay, 1981). In agar gel double diffusion tests, one precipitin band was formed when antiserum to cotton antigens was reacted with crude fungal antigens, or when antiserum to crude fungal antigens was reacted with cotton antigens. The cross reactive antigen from fungal conidia (CRA) was isolated, purified and partially characterized. The CRA migrated as a single band in polyacrylamide or agar gel electrophoresis, and sedimented as a single band during analytical ultracentrifugation. It was antigenic in rabbits and was a protein carbohydrate complex. The major cross-reactive antigenic substance (CRA), common to cotton (*Gossypium hirsutum)* and certain fungal parasites of cotton roots, was further purified to homogeneity from conidial cultures of F. *oxysporum* f. sp. *vasinfectum (Devay et al., 1981).* Agar gel double diffusion tests indicated

the presence of CRA not only in *F. oxysporum* f. sp. *vasinfectum* and in cotton roots and seed but also in *Thielaviopsis basicola*. Indirect staining of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross sections of roots, cut near or just below the root hair zone, the CRA was concentrated mainly around xylem elements, the endodermis and epidermis cells and was present throughout the cortex tissue. Protoplasts prepared from cross sections of young cotton roots also contained the CRA which was concentrated in the region of the plasmalemma. Treatment of conidia and mycelia of F. *oxysporum* f. sp. *vasinfectum.* with antiserum to cotton and using indirect staining with FITC indicated that the CRA was mainly present in hyphal tips and in patch like areas on conidia.

Johnson *et at.* (1982) used antiserum prepared to homogenates of washed *Epichloe typhina* mycelium grown in a liquid medium in an enzyme - linked immunosorbent assay (ELISA) to detect antigens of the fungus in tall fescue (*Festuca arundinacea)* tissue samples. Very low concentrations (100μg/ l) of freeze dried E. *typhina* mycelium could be detected and the pathogen could also be detected in individual seeds of tall fescue. Of 14 fungal genera tested, including *Acremonium, Claviecps, Helminthosporium, Pythium, Rhizoctonia* and *Sclerotium* all showed reactivities less than 0.1% that of *E. typhina*. Cross reactive antigen shared between soybean cultivars and *Macrophomina phaseolina* causing charcoal rot disease was detected. Rabbit antisera were raised against root antigens of soybean cultivars (Soymax and UPSM-19) and *M. phaseolina* isolate (M.P.1) and tested against homologous and heterologous antigens following immunodiffusion tests. When antiserum of M. *phaseolina* was reacted against its own antigens and antigens of susceptible soybean cultivars (Soymax, R-184) strong precipitation reactions were observed. In case of resistant cultivars (UPSM-19 andDS-73-16) no such reactions were observed. Reciprocal cross reactions between antiserum of the resistant cultivars and antigens of three isolates of *M.phaseolina* also failed to develop even weak precipitation bands. Four antigenic substances were found to be common between the susceptible soybean cultivars and isolates of M. *phaseolina* in immunoelectrophoretic tests, but no common antigens were

detected between resistant cultivars and the fungus. Purkayastha and Chakraborty (1983) further detected that in susceptible soybean plants (cvs. Soymax and R-184) a close relationship exists between lower production of glyceollin and presence of common antigens. The production of glyceollin was much higher in resistant soybean cultivars (cvs. UPSM-19 and DS-73-16) where common antigens were absent (Chakraborty and Purkayastha, 1983). Alba *et al.*, (1983) also detected common antigens in extracts of *Hemileia vastatrix* urediniospores and of *Coffea arabica* leaves and roots Antisera were made to both a whole cell and cell wall preparation of *Eutypa armeniacae.* Rhodamine isothiocyanate (RITC) conjugated antisera were tested for reactivity with various fungi on glass slides by Gendloff *et el* (1983). Both antisera showed low specificity, but specificity was improved by cross - adsorption of the RITC. conjugated cell wall antiserum with *Phomopsis viticola.* Woody cross sections from concord grapevines inoculated with E. *armeniacae* and also inhabited by various other fungi were stained directly with the conjugated anti - *Eutypa* rabbit serum. In an indirect staining procedure, sections were treated with anti-*Eutypa* rabbit gamma globulin. Both procedures specifically stained hyphae in wood sections. Hyphae stained indirectly in woody vine sections showed a much brighter fluorescence than analogous hyphae stained by the direct method. Fungi of some species that reacted strongly with the direct method on glass slides did not always react when stained indirectly in woody vine sections in which they were growing.

Indirect immunofluorescence performed by using antisera to culture filtrate molecules of *Phaseolus schweinitzii* has been used to demonstrate the presence of mycelium, and on occasions chlamydospores, in naturally and artificially infested soil samples. The authors could thus identify the kind of progagule most likely to be the source of field isolates of the organisms; this information, which could not be obtained by using selective media, strongly suggested that the pathogen could survive saprophytically in the soil. In contrast, isolated mycelial cell wall preparations did not prove to be a suitable source of immunogenic material for these studies (Dewey *et al, 1984).*

Agnelon and Dunez (1984) used double antibody sandwich ELISA and indirect ELISA technique for the detection of *Phoma exigua* in inoculated tubers and sprouts and in stems grown from these tubers. The fungus was detected in

these different tissues with var- *foveata* being more aggressive, demonstrating the applicability and sensitivity of the techniques. The antibodies produced to the two varieties of the fungus were not specific to their own varieties. They also reacted with *Phoma tracheiphila* but did not react with several other common potato pathogens.

Reddy and Ananthanarayanan (1984) investigated the presence of *Ganoderma lucidum* in betelnut by the fluorescent antibody technique. Presence of *Selerotinia sclerotiorum* in sunflower was also detected by Walcz *et al,* (1989) by enzyme - linked immuno sorbent assay (ELISA).

Immunodiffusion, immunoelectrophoretic and crossed immuno-electrophoretic analyses of rice antigens in relation to sheath rot disease revealed a serological relationship between susceptible rice cultivars and isolates of the causal organism of sheath rot, *Acrocylindrium oryzae*. One precipitin band was observed when the antigen preparation of A *oryzae* was cross-reacted with its own antiserum or against the antisera of four susceptible rice cultivars. No precipitin band was detected between the antiserum of the resistant cv. Mahsuri and antigen preparations from three isolates of A. *oryzae* or between the antigens of the resistant cultivars Mahsuri and Rupsail and the antiserum of *A. oryzae*. Crossed immunoelectrophoretic tests confirmed that there was a common antigen between Mahsuri and Jaya, and between Mahsuri and CR-126-42-1. The precipitin band between the antigen preparation of Jaya and A. *oryzae* was found to be similar (Purkayastha and Ghosal, 1985). The common antigenic relationship between soybean cultivars and *Colletotrichum dematium* var. *truncata* was also ascertained by Purkayastha and Banerjee (1986) following immunodiffusion, immunoelectrophoretic and crossed immunoelectrophoretic tests. At least one antigen was found to be common between host cultivar and the pathogen. No antigenic relationship was observed either between soybean cultivars and the non pathogen (*C. corchori*) or avirulent pathogen (*C. dematium*). Alba and Devay (1985) detected cross reactive antigens in crude preparations and in purified preparations from mycelia of *Phytophthora infestans* Race-4 and Race -1, 2, 3, 4, 7 with antisera for potatoes cv. King Edward and cv. Pentland Dell by using an indirect ELISA technique. It was suggested that the fungal mycelia do not easily release cross reactive antigens into synthetic media where they grow; that most of *P. infestans* cross

reactive antigens were thermolabile and that they could be concentrated by precipitation in the presence of 40% saturated ammonium sulphate (SAS). An antigenic disparity was observed when 40% SAS from P. *infestans* Race-4, mycelial preparation was assayed with antisera for King Edward and Pentland Dell.

Sections of leaves of *Nicotiana tabecum* infected with *Peronospora hyoscyami* f. sp. *tabacina* and of *Erythronium americanum* infected with *Ustilago heufleri* treated with an antiserum directed against the fimbriae of *U. violacea* Fuckel and other fungi were then treated with protein. A - gold complexes to detect the presence and location of fimbrial antigens following transmission or scanning electron microscopy. The infected leaf sections were heavily labelled with gold particles indicating the presence of fimbrial antigens, whereas the control preparations showed only a low background level of labelling. Gold particles were detected on the sections of hyphae, on haustoria and on the nearby plant cells. The intensity of labelling was much higher for P. *hyoscyami* f. sp. *tabacina* than for *U. heufleri* and was particularly high in the walls of the former species. Relatively high levels of labelling occurred over the cells of infected host tissues but little or no labelling occurred over the cells of uninfected host tissues or of the infected host tissues treated with a range of serological controls. This level of labelling was not associated with specific host structures in P. *hyascyami,* but was frequently associated with the chloroplast in U. *heufleri.* The antigens detected inside the host plant cells appeared to indicate that fungal fimbrial protein, either as polymerised fibrils or as isolated sub-units, could penetrate the host plasma membrane and therefore entered the host cytoplasm. (Day *et al., 1986).* Hyphae of *Verticillium dahliae* were detected in cotton root tissue with an ELISA by Gerik *et al.* (1987). A soluble protein extract of V.*dahliae* was used to prepare a specific rabbit antiserum. The reaction of this rabbit antibody to the hyphae of V. *dahliae* was detected with an alkaline phosphatase antirabbit IgG conjugate that hydrolyzed the substrate, napthol - As- phosphate, to a product that reacted with a diazonium salt, yielding a colored precipitate outlining the fungal hyphae. The hyphae were readily observed on and in the root cortex of the host using a dissecting microscope.

Purkayastha and Ghosal (1987) also compared the antigenic

preparations from two isolates of *Macrophomina phaseolina*, a pathogen of groundnut, four non-pathogens of groundnut (viz., *Corticium sasakii, Colletotrichum lindemuthianum* C. *corhori,* and *Botrytis allii)* and five cultivars of *Arachis hypogea* by immunoserological techniques. Common antigens were found among the susceptible cultivars of groundnut and two isolates of M.*phaseolina,* but not between nonpathogens and groundnut cultivars. No antigenic similarity was found between nonpathogens and *M. phaseolina* isolates. Cross immunoelectrophoretic tests confirmed that at least one antigen was common between cv. J-11 and cv. TMV-2, cv. Kadiri 71-1 and cv. TMV-2 and cv. Kadiri 71-1 and isolates of *M.phaseolina.*

Further, changes in antigenic patterns after chemical induction of resistance in susceptible soybean cultivar (soymax) to *Macrophomina phaseolina* was determined by Chakraborty and Purkayastha (1987). Sodium azide (100 μg / ml) altered antigenic patterns in susceptible cultivar (soymax) and reduced charcoal rot disease. Similar results were also obtained by Ghosal and Purkayastha (1987) in susceptible rice cultivar (Jaya) and *Sarocladium oryzae* after altering disease reaction (sheath rot) by the application of gibberellic acid and sodium azide (100 μg / ml). Gerik and Huisman (1988) observed that colony density of *V. dahliae* increased with distance from the root tip, with the maximal density occurring more than 1 cm from the root apex. Colony densities at distances more than 1cm from the tip were relatively constant. The mean colony length of *V. dahliae* was 7.3mm, and increased colony length was correlated with distance from root apices. Hyphae of V. *dahliae* were present through the entire depth of the cortex, and were greatest in the interior of the root cortex at the surface of the vascular cylinder. The colony appearence was consistent with growth of hyphae from the root surface toward the stele. Hyphae of *V. dahliae* also were found within numerous cortical cells. Colonies of *Fusarium oxysporum* similarly stained, were found to be mostly confined to the root surface and the outer cortex.

In enzyme linked immunosorbent assay, antiserum raised against pooled mycelial suspensions from five isolates (designated pf 1, pf2, pf3, pf 10 and pf 11) of *Phytophthora fragariae* detected homologous soluble antigens at protein concentrations as low as 2 μg/ ml (Mohan, 1988). Fungal antigens could also be detected in extracts of strawberry plants infected with P.

fragariae. Root extracts prepared from the alpine strawberry *Fragaria vesca* and *F. ananassa* cv. Cambridge Favourite infected with any of the five isolates studied produced strong reactions in ELISA. In F. *vesca,* ELISA positive material was detectable 6-8 days after inoculation before macroscopic symptoms appeared. The cultivar Red Gauntlet, resistant to pf1, 2 and 3 but susceptible to pf 10 and 11, reflected this differential response in ELISA : the absorbance produced by extracts of plants infected with virulent isolates was significantly higher than that obtained with the corresponding extracts of plants inoculated with avirulent isolates.

An agri-diagnostic *Phytophthora* multiwell ELISA kit, developed for detection of *Phytophthora* in plant tissue, also readily detected *Phytophthora* in soil where soybeans were damaged by P. *megasperma* f. sp. *glycinea* (Pmg) (Schmitthenner, 1988). Only low level of *Phytophthora* were detected in soil stored at 3°C. Following cold storage, high levels of *Phytophthora* could be detected directly from soil, after *Phytophthora* damping off of soybean seedlings was induced. But low *Phytophthora* detection was obtained from soybean leaf discs floated on water over infested soil for 24 hrs. Pmg was the only *Phytophthora* isolated from such leaf discs using selective media. It was concluded that *Phytophthora* was detected best with an ELISA test of soil with actively rotting roots or from leaf disc baits with actively growing mycelium.

Amouzon *et al.*, (1988) reported that the antiserum obtained by injecting robbits with mycelial protein extracts of one strain of *Phytophthora fragariae* had a dilution end point of 1:64 in double diffusion and 1/512,000 in indirect ELISA. This serum could detect 11 different strains of P. *fragariae* in pure culture and the pathogen in naturally infected or inoculated roots. Although the sensitivities of direct DAS and indirect ELISA were comparable, th direct DAS-ELISA was more specific for the detection of *P. fragariae* in strawberry roots. The antiserum failed to react with 18 fungal species isolated from underground parts of strawberry but reacted with some strains of *P cactorum,* which parasitized only rhizomes but not roots, and *Pythium middletonii,* which was isolated sometimes in association with *P. fragariae* from strawberry roots. In inoculated strawberry roots, *P. fragariae* was detected reliably by ELISA several days before oospores were found and before symptoms developed. Thus direct DAS.ELISA may be useful for early detection of infection and for the detection of latent infections of strawberry plants by P. *fragariae.*

Resting spores of *Plasmodiophora brassicae* from soil and root were detected by indirect fluorescent antibody technique. Infested soil and roots were stained the fluorescent antibody technique with the IgG and FITC conjugated antirabbit IgG-Sheep IgG. Resting spores were effectively detected and also clearly differentiated from small particles of soil and tissues of plant in the reflected light fluorescence microscope(Arie *et al.* 1988). An indirect ELISA for quantitative detection of *P. herpotrichoides* infections in wheat was developed by Unger and Wolf (1988). All tested isolates of the virulent varieties P. *herpotrichoides* var. *herpotrichoides,* P. *herpotrichoides* var. *acuformis* or the W - and R-type reacted on a high level in th test, while the less virulent *P. angnioides* was assessed only with 40% and the avirulent *P. aestiva* with 20% of the homologous reaction. No cross reactions occurred with extracts of 11 other species of *in vitro* cultivated fungi nor with plant material infected with other pathogens. The infection profile throughout the leaf sheaths was clearly reflected by ELISA. The examination of 24 stem base samples from the field showed that the values assessed by ELISA correlated well also with the disease indices of naturally infected plant material.

Antigens prepared from two resistant cultivars (UPSM-19 and DS 73-16) and two susceptible cultivars (DS-74-24-2 and PK-327) of soybean and three strains of *Myrothecium roridum* (M-1, ITCC-1143 and ITCC-1409), a causal organism of leaf spot disease were tested against antisera of pathogen. Immuno-diffusion tests revealed that common antigens were present only between the virulent strain and susceptible host cultivars. But no such cross reactive antigen was detected in case of resistant cultivars (UPSM-73-16). Immunoelectrophoretic analysis showed that one common antigen was shared by rocket immunoelectrophoresis (Ghosh and Purkayastha, 1990). Cross reactive antigens (CRA) were also detected between susceptible soybean cultivars and the virulent strain of *Colletotrichum dematium* var. *truncata* but CRA could not be detected between soybean cultivars and an avirulent strain of C. *dematium* or non-pathogen (*C. corchori*). Results of immuno diffusion and immunoelectrophresis showed absence of common antigen between resistant cv. UPSM-19 and the pathogen, while the results of indirect ELISA indicated the presence of common antigen between the two at a very low level. Alteration in antigenic patterns of soybean leaves after induction of resistance by the treatment of cloxacillin (100μg / ml) were further detected by Purkayastha and Banerjee (1990).

Watabe (1990) reported on the usefulness of immunofluorescent antibody technique for detection of *Phytophthora* in soil. Autofluorescence and the nonspecific staining of soil particles interfered with the detection of the fungi in soil. Pretreatment of the samples with gelatin rhodamine conjugate prevented the samples from the autofluorescence and the nonspecific staining and therefore permitted the immunofluorescent antibody staining in soil. Stained *Phytophthora* was easily detected on the yellow orange background.

A polyclonal antiserum, prepared in a rabbit immunized with a mycelium extract of *Phytophthora infestans* reacted in an enzyme linked immunosorbent assay (ELISA) with mycelial extracts of two *Phytophthora* species but not with those of 10 other microorganisms found on potato. P. *infestans* mycelium in leaf tissue was readily detected by ELISA using either the plate - trapped antigen or F (ab^1) antibody fragment techniques. The amount of mycelium in leaf extracts was estimated by comparing the values obtained in ELISA with those for known concentrations of P. *infestans* mycelium (Harrison *et al., 1990)* Ricker *et al. (1991)* detected water solube antigens produced by *Botrytis cinerea* in picked and naturaly infected grape juice by using an enzyme immunoassay with an indirect format of antibody HRPO conjugated bound to polyclonal rabbit antibodies directed against *B. cinerea* (anti BcIgG). Protein A purified γ globulin from an early bled antiserum (803.7), which reacted primarily with low molecular weight compounds present only in extracts of B. *cineria,* was used to specifically detect *B.cinerea* and quantify levels of infection in juice from infected grape berries. Late bled higher titre antiserum (803-19), which cross reacted with proteins and carbohydrates present in extracts from species of *Botrytis, Aspergillus, Penicillium* and *Uncinula,* was used ot quantify the levels of rot caused by presence of multiple fungi. Minimum detectable levels of infections, based on mixture of clean and infected juice, were.25-.5% with (803-7)/IgG, and 0.2% with 803-19 IgG. Cross reactivity of anti BeIgG with extracted antigens (water soluble) from sterile and reproductive strains of several fungi was negligible in early bled antiserum and increased in subsequent collections. The increase in cross reactivity in late bled antiserum corresponded with an increase in the overall serum titres for anti BeIgG to antigens from *B. cinerea.*

Polyclonal antisera prepared against purified mycelium proteins from *Verticillium dahliae,* the predominant fungus species in the potato early drying

complex was tested against crude mycelial preparations of *Verticillium* spp. using indirect ELISA (Sundaram, 1991). It reacted positively with 11 to 12 V. *dahliae* isolates from potato, cotton and soil but negatively with one isolate from tomato. The antisera did not react with mycelial proteins from *Fusarium* spp. from potato and cotton, with a *Colletotrichum* sp. from potato or with a isolate of *Rhizoctonia solani* from sugarbeat. Double antibody sandwich ELISA, using polyclonal antisera, detected V. *dahliae* and *V.allo-atrum* in infected roots and stems of potato.Benson (1991) compared two commercial serological assay kits to a culture plate method for detection of *Phytophthora cinnamomi* in root samples from infected azaleas. Both the multiwell E Kit and the rapid assay F kit detected. *P. cinnamomi* on azalea roots beginning 1 week after inoculation. Agreement between immunoassay kits and culture plate results for detection of *P. cinnamomi* was not consistent beginning 3-5 week after inoculation. Root symptoms, but not foliar symptoms, of *Phytophthora* root rot were evident during this period. There was a positive correlation between root rot severity in green house trials and root sample absorbance (multiwell) or meter reading (rapid assay) but not between symptom severity and immunoassay results. The multiwell kit detected *P. cinnamomi* in root samples containing as little as 1.0% infected root tissue. In a commercial nursery survey, 5, and 15% of the azalea root samples of two nurseries had positive ELISA values that were unconfirmed by culture plate. The rapid assay kit detected *P. cinnamomi*, was easy to use, and gave results in a short time.

Methods for sampling turf grass tissue were compared for their effectiveness in monitoring *Pythium* blight epidermis with enzyme linked immunosorbent assay (ELISA). Samples consisted of whole plants picked by hand and assayed as whole plants; whole plants sectioned into lower, middle and upper strata components; leaf clippings collected with a red mower set at a 1.2cm cutting height. ELISA readings for mowed samples generally matched those for whole plucked samples. Fluctuations in detectable *Pythium* antigens were most pronounced on the uppermost stratum compared with moderate to very little change in ELISA readings for the two lower strata. Several episodes of *Pythium* antigen increase were detected by ELISA assays of mowed samples, although signs and symptoms of *Pythium* blight were not evident. However, increase in ELISA readings for *Pythium* coincided with, but did not generally

precede, the onset of blight symptoms with a 2 to 3 day sampling interval. Shane (1991) concluded that antibody aided detections was useful for verification of diagnosis and determination of general *Pythium* population fluctuations, but was not satisfactory for advanced detection of blight epidemics.

Results of conventional isolation techniques for *Pythium violae* were compared with the assay of cavity spot lesions using polyclonal antibodies raised to *P. violae or P. sulcatum,* in competition ELISA (Lyons and White, 1992). Where lesions were artificially induced the test confirmed which pathogen was causal. With cavities developed on the field grown carrots *P. violae* again predominated and the ELISA confirmed this. In one sample *P. sulcatum* was also isolated from a small number of lesions and was not detected in ELISA. The competition ELISA did not indicate presence of either *Pythium* in a range of non-cavity spot lesions from which attempts at isolation were also negative. Linfield (1993) elicited polyclonal antiserum against a strain of *Fusarium oxysporum* f. sp. *narcissi* GCRI 80/26) and a specific and sensitive enzyme linked immunosorbent assay was developed. Antiserum raised to cell wall fractions gave better recognition than that to cytoplasmic fractions. Recognition was equally good in artificially and naturally infected bulbs. Little cross reactivity in bulb tissue was shown by three other bulb-rotting fungi. Nine isolates of *F. oxysporum* f. sp. *narcissi* from a wide geographic area gave similar results in an indirect ELISA of mycelial extracts, although some crossreactivity was observed with two other *Fusarium* spp. Four *Fusarium* spp and four other fungi showed little cross reactivity. Ten days after inoculation the pathogen was readily detected in the base plate area of three *Narcissus* cultivars and points remote from the inoculation site in the most susceptible cultivar. A direct correlation was observed between positive results in the enzyme linked immunosorbent assay and recovery of pathogen on selective medium.

Polyclonal antibodies produced against culture filtrate and mycelial extracts immunogen preparations from the soybean (*Glycine max*) fungal pathogen *Phomopsis longicolla* were purified to the immunoglobulin fraction and tested in indirect enzyme linked immunosorbent assay (ELISA) and in double antibody sandwich ELISA (DAS-ELISA) (Brill et al, 1994). The PABs raised to culture filtrate were more specific but less active in binding to members of the *Diaporthe. Phomopsis* complex than were those raised to the mycelial

extract immunogen preparation. DAS-ELISA was more specific and 100-fold more sensitive in detecting members of the complex than was indirect ELISA. Variability in specificity between different PABs was lower in DAS-ELISA compared with indirect ELISA. Immunization of one rabbit with culture filtrate over an extended time resulted in maximum anti *P longicolla* activity after three immunization, and the activity became constant against most members of the complex at the same time. Reactivity to some culture of *P. longicolla* was undetectable following the fourth and fifth immunization, where as reactivity to all of other cultures of the complex remained high. Polyclonal mouse ascite antibodies were raised against soluble protein extracts of chlamydospores and mycelium. The IgG fraction was purified and biotin labelled to device a fungal capture sandwich ELISA. ELISA detected both brown and grey cultural types of T. *basicola* and had negligible corss reactivity with other soil borne fungi. The minimum detection limit of ELISA was between 1 and 20 ng of T. *basicola* protein depending on the assay. T. *basicola* could be detected in cotton roots two days after inoculation. At this time, initial symptoms was apparent. The antibody also was used to observe T. *basicola* on cotton roots with immunofluoresecnce microscopy (Holtz *el al.*, 1994).

Purkayastha and Pradhan (1994) observed that three strains of *Sclerotium rolfsii* were serologically different and their pathogenecities also differed markedly with host cultivars. Virulent strains 266 and 23 showed common antigenic relationship with their respective susceptible host cultivars but not resistant cultivars. Antigenic change in a susceptible cv. AK-12-24 after treatment with a systemic fungicide kitazin was also evident. They suggested that resistance could be induced in susceptible plants if specific antigens were eliminated by suitable treatment. Antigens obtained from six Tocklai varieties of tea, four isolates of *Bipolaris carbonum* and non-pathogens of tea (*Bipolaris tetramera* and *Bipolaris setarae),* were compared by immunodiffusion, immunoelectrophoresis and indirect ELISA in order to detect cross reactive antigens (CRA) shared by the host and the parasite. CRA were found among the susceptible varieties (TV-9,17 and 18) and isolates of *B. carbonum* (BC 1, 2, 3 and 4). Such antigens were not detected between isolates of *B. carbonum* and resistant varieties (TV-16, 25 and 26), non-pathogens and tea varieties, as well as non pathogens and isolates of *B. carbonum.* Indirect staining of

antibodies using fluorescein isothiocyanate (FITC) indicated that in cross sections of leaves (TV-18), the CRA were concentrated mainly around epidermal cells. Treatment of mycelia and conidia of *B. carbonum* with antisera to leaves (TV-18) and indirect staining with FITC indicated the presence of CRA in the young growing hyphal tips and conidia, (Chakraborty and Saha, 1994).

A serological test was developed by Jamanx and Spire (1994) that allows the early detection of infection of young petals by *Sclerotinia sclerotiorum* an important pathogen of rapeseed. Soluble mycelial extracts of *S. sclerotiorum* were used to produce the first generation antiserum. This was not specific for *S. sclerotiorum* in double DAS ELISA and allowed the screening of cross reacting fungal species such as *Botrytis cinerea,* a pathogen commonly present on rapeseed petals Using a polyclonal anti *B. cinerea* serum enabled the adsorption by serial cycles, of *S. sclerotiorum* antigens commonly to B-*cinerea* Residual antigens were used as immunogens for the production of two second generation antisera (S1 and S2) which were then tested by DAS-ELISA. Cross reactions with *B.cinerea* decreased with purification cycles of the immunogen whereas cross-reactions with some unrelated fungi slightly increased. S. *sclerotiorum* and *B. cinerea* were distinguishable using antiserum S2.

Among the 12 varieties of tea tested against three isolates of *Pestalotiopsis theae* , causal agent of grey blight disease, Teen Ali - 17/1/54 and TV-23 were found to be highly susceptible while CP-1 and TV-26 were resistant under identical conditions. Leaf antigens were prepared from all the tea varieties, three isolates of P. theae and a non pathogen of tea (*Biplaris tetramera).* Polyclonal antisera were raised against mycelial suspensions of P. *theae* (isolate Pt.2) and leaf antigens of Teen Ali-17/1/54 and CP-1. These were compared by an immunodiffusion test and enzyme linked immunosorbent assay to detect cross reactive antigens (CRA) shared by the host and the parasite. CRA were found among the susceptible varieties and isolates of P. *theae* (Pt-2 and 3). Such antigens were not detected between isolates of P. *theae* and resistant varieties, *B. tetramera* and tea varieties or isolates of P. *theae.* Indirect staning of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross sections of tea leaves the CRA was concentrated in the epidermal cells and mesophyll tissues. CRA was present in the young hyphal tips of the mycelia and on the setulae and appendages of the conidia of *P. theae* (Chakraborty *et al., 1995).*

Wakeham and White (1996) raised polyclonal antisera against whole (coded : 16/2), and sonicated (coded :15/2) resting spores of *Plasmodiophora brassicae* as well as soluble components prepared by filfration and ultracentrifugation (coded : SF/2). Cross reactivity of all three antisera with a range of soil fungi, including *Spongospora subterranea* was low. Test formats including Western blotting, dip-stick, dot blot, indirect enzyme linked immunosorbent assay (ELISA) and indirect immunofluorescence were assessed for their potential to detect resting spores of *P. brassicae* in soil. Dot blot was least sensitive, with a limit of detection level of $1x10^7$ resting spores g^{-1} in soil. With Western blotting the lower limit of detection with antiserum 15/2 was $1x10^5$. This antiserum showed the greatest sensitivity in a dip-stick assay, indirect ELISA and indirect immunofluorescence, for all of which there was a limit of detection of $1x10^2$. The indirect ELISA was successful only after the substitution of alkaline phosphatase by protein. A conjugated horseradish peroxidase. Of the assays tested, indirect immunofluorescence appears to be the most rapid and amenable assay for the detection in soil of low levels of resting spores of *P. brassicae.*

Polyclonal antiserum was raised against mycelial extract of *Pestalotiopsis theae* and immunoglobulin fractions were purified by ammonium sulfate fractionation and chromatography on DEAE sephadex. In ELISA, antiserum diluted upto 1.16000 detected homologous antigen at 5 mg / L concentration and at 1:125 antiserum dilution fungal antigens could be detected at concentration as low as 25 μg / L. In 15 varieties of tea tested, from Darjeeling, UPASI and Tocklai Research stations, absorbance values of infected leaf extracts were significantly higher than those of healthy extracts at 40 mg/L concentration in indirect ELISA. ELISA positive material was detected in tea leaves as early as 12h after inoculation with P. *theae.* At antiserum dilution upto 1 : 125, pathogen could be detected in infected leaf extracts upto 2mg / L concentration. The results demonstrated that ELISA can be used for early detection of P. *theae* in leaf tissues even at very low level of infection (Chakraborty *et al., 1996).*

Two monoclonal antibodies (MAbs) were produced against soluble antigens from the 'Ascochyta complex' fungi by Bowen *et al (1996).* Specificity of MAbs was tested by ELISA using antigen coated wells. MAbs secreted by

the monoclonal hybridoma cell line JIM 44 recognized epitopes present in the antigen preparations from *Mycosphaerella pinodes* and *Phoma medicaginis* var. *pinodella*, but not those present in preparations from *Ascochyta pisi*. At high tissue culture supernatent concentration, MAbs produced by the monoclonal line JIM 45 recognized epitopes from all three fungi, however, on dilution of MAb the antigens from A. *pisi* were recognized preferentially to those from *M.* pinodes and P. *medicaginis* var. *pinodella*. On the basisof heat and periodate treatment of the antigens from the three fungi it can be concluded that the epitope recognized by JIM 44 is carbohydrate in nature, whereas that recognized by JIM 45 is proteinaceous in nature, carried on a glycoprotein antigen. Antigen preparations from other fungi, including other pea pathogens, non pathogens associated with pea and other fungi closely related to the 'Ascochyta complex.' were not detected with either of the two MAbs. Antigen preparations from peas could be used to differentiate healthy and infected seeds in a dot-blot assay, therefore indicating the potential of using the MAbs in the development of a diagnostic test for infection of *Pisum* seeds by the 'Ascochyta complex' fungi.

A polyclonal antiserum was raised against spore balls of *Spongospora subterranea* f.sp *s*ubterranea by Walsh *et al (1996)* to detect as little as 0.02 spore balls in an enzyme linked immunosorbent assay (ELISA). In spiked soil samples, the antiserum detected 100 spore balls per g soil. However, the different spore ball contamination levels were discriminated better in ELISA tests at concentrations above 2000 spore balls per g soil than at lower concentrations. In contrast, a bioassay test based on baiting soils with tomato seedlings gave good discrimination of spore ball contamination levels in spiked soils containing <1000 spore balls per g soil and poor discrimination of levels in spiked soils containing >2000 spore balls per g soil. Tests on a limited number of field soils suggested ELISA may be capable of predicting disease levels on tubers grown is such soils better than the bioassay. The antiserum did not react with 30 other microorganisms tested, including many that are saprophytes or pathogens on potatoes and resting spores of the taxonomically related *Plasmodiophora brassicae.* It detected spore balls of different cultivar origin equally well. It also detected spores from different geographical origins. An attemp to improve the sensitivity of the serological detection through

concentrating spore balls from field soils by sieving was unsuccessful. Cross absorption of the antiserum with uncontaminated field soil increased the sensitivity of detection of spore balls in spiked soil samples four fold. The ability of the antiserum to discriminate contaminated field soil from an uncontaminated soil was much improved by using the gamma globulin fraction or cross-absorbed serum. Western blot analysis revealed that the antiserum detected a number of different proteins the most distinct of which had a molecular weight of slightly less than 6.5 KDa. A technique was developed to suppress autofluorescence of spore balls, allowing immunofluorescence studies to be carried out. Using this technique in conjunction with indirect FITC immunofluorescence discrete bright fluorescent spots were visualized using the specific serum. With the non-specific serum, only a very dull background fluorescence was evident.

Materials and Methods

3.1. PLANT MATERIAL

3.1.1. Source of seeds

Seeds of different cultivars of soybean (*Glycine max* (L.)Merrill) were collected from the Pulses and Oil Seeds Research Station, Berhampore, West Bengal. Ten cultivars namely Soymax, JS.2, UPSM-19, Bragg, PK-327, Punjab1, PK-564, 17-PK-472, 18-PK-564, 19-PK-466, were used. Seeds were air dried and stored at room temperature (30±2°c) as well as at 20°c. Since the seeds lost their viability after one year, it was necessary to procure seeds every year.

3.1.2. Growth of plants

Soybean seeds were sown in sandy soil (soil : sand-1:1) contained in earthenware pots (10 plants / 25cm. dia pot). Prior to sowing, seeds were disinfected with 95% ethyl alcohol (Orellana *et al.,* 1976) for 3-5 minutes to remove superficial contaminants, followed by several washings with sterile distilled water. The plants were grown in the Nursery of the Department of Botany, University of North Bengal under natural conditions of day light and temperature (26-35°C). The pots were watered daily with ordinary tap water. The plants were grown during March to October.

3.2. FUNGAL CULTURES

3.2.1. Source of Cultures

A virulent strain of *Fusarium oxysporum* Schlecht (ITCC NO. 1803)obtained from Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi was used throughout this investigation. The culture was maintained on PDA medium by regular subculturings.

A culture of *Trichoderma harzianum Rifai* (ITCC No. 1894) obtained from Indian Agricultural Research Institute, New Delhi, India, was also used for experimental purposes.

3.2.2. Completion of Koch's Postulate

Surface sterilized soybean seeds were sown in pots which were

previously infested with conidia and mycelia of *F. oxysporum.* Reisolation of pathogen was done from infected roots. These were collected, washed, cut into small pieces, treated with 0.1% $HgCl_2$ for 3-5 minutes, rewashed with sterile distilled water and transferred to PDA slants. After 10 days, the isolated organism was examined, compared with the original stock culture of F. *oxysporum* and its identity was confirmed.

3.2.3. Stock Cultures

The cultures were maintained on PDA slants and stored under 3 different conditions [5°C, 20°C and at room temperature 30±2°C). The culture was examined at a regular interval to test its viability and pathogencity of the fungus.

3.2.4. Assessment of mycelial growth

3.2.4.1. Solid media

To assess mycelial growth of *F. oxysporum* in solid media, the fungus was first grown in petridishes, each containing 20ml of PDA and incubated for 7 days at 30°C. Agar block (4mm dia) containing the mycelia was cut with a sterile cork borer from the advancing zone of mycelial mat and transferred to each petridish containing 20 ml of sterilized media. Following solid media were used for assessment of grwoth.

Potato dextrose agar (PDA)

Peeled Potato	-	40.00 g
Dextrose	-	2.00 g
Agar	-	2.00 g
Distilled water	-	100 ml.

Richards Agar (RM)

KNO_3	-	1.00 g
KH_2PO_4	-	0.50 g
$MgSO_4, 7H_2O$	-	0.25 g
$FeCl_3$	-	0.002 g
Sucrose	-	3.00 g
Agar	-	2.00 g
Distilled water	-	100 ml.

Yeast extract mannitol agar (YEMA)

Yeast extract	-	0.040 g
Mannitol	-	1.00 g
$MgSO_4$, $7H_2O$	-	0.02 g
K_2HPO_4	-	0.05 g
NaCl	-	0.01 g
Agar	-	2.00 g
Distilled water	-	100 ml.

Carrot juice Agar (CJA)

Grated Carrot	-	20.00 g
Agar	-	2.00 g
Distilled water	-	100 ml.

Czapek - dox agar (CDA)

$NaNO_3$	-	0.20 g
K_2HPO_4	-	0.10 g
$MgSO_4$, $7H_2O$	-	0.05 g
KCl	-	0.05 g
$FeSO_4$, $7H_2O$	-	0.001 g
Sucrose	-	3.00 g
Agar	-	2.00 g
Distilled water	-	100 ml

All these petridishes were then incubated at 30°C for the desired period. Finally the mycelia were strained through muslin cloth, collected in aluminium fail of known weight, dried at 60°C for 96 h, cooled in a desicator and weighed.

3.2.4.2. Liquid media

To assess the mycelial growth of F. *oxysporum* in liquid media, the fungus, was first allowed to grow in petridishes containing 20ml of PDA and were incubated at 30°C for 7 days. From the advancing zone of the mycelial mat, agar block (4 mm-dia) was cut with a sterilized cork borer and transferred to each Ehrlenmeyer flask (250ml) containing 50ml of sterilized liquid medium for the desired period at 30°C. Finally, the mycelia were strained through muslin cloth, collected in aluminum foil cup of known weight, dried at 60°C for 96 h, cooled in a desiccator and weighed.

3.3. AGGLUTINATION RESPONSE OF CONIDIA

The agglutination response of condia was determined following the method of Lis and Sharon (1986) and Cristimzio *et al* (1988). Concanavalin A (Con A), *Helix pomatia* agglutinin (HPA), *Ulex europaeus* agglutin -I (UEA - I) and wheat germ agglutinin (WGA) of Sigma Chemical, USA, were diluted (1mg/ ml) with 50 mM phosphate buffered saline (PBS), pH 7.2 and were used for agglutination reactions. Con A solution contained 1mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$.

3.3.1. Preparation of conidial suspension

Agglutination tests were done with ungerminated spores. At first thefungus was allowed to grow on PDA slant for 6 days at 30°C. Conidial suspension was prepared by adding 3-5 ml of sterile distilled water. The resulting suspension was centrifuged at 3500g for 15 min at 4°C. The pellet was washed thrice with cold PBS and resuspended in the PBS to a concentration of approximately $6x10^6$/ml. The conidial suspensions were used immediately after preparation.

3.3.2. Agglutination tests

Ungerminated conidial suspension (10μl) was taken in a slide and incubated with diluted lectin solution (10μl) in a moist chamber at room temperature for various incubation times (upto 2h). During incubation, slides were gently swirled several times to ensure maximal cellular contact. Agglutination of conidia was observed under Leica Leitz Biomed microscope in bright field and arbitrarily scaled from '0' (no agglutination) to '4' (maximum agglutination).

3.4. BACTERIAL CULTURE

3.4.1. Source of culture

An isolate of *Bradyrhizobium japonicum* (SB119) was obtained from the Indian Agricultural Research Institute, New Delhi. The culture of *B. japonicum* was maintained in Yeast Extract Mannitol Agar (YEMA) medium during the investigation and sub-culturing were done at a regular interval.

3.4.2. Inoculation, re-isolation and identification of the bacterium

Seeds of soybean after disinfection with 95% ethanol were inoculated with *B. japonicum,* kept overnight and sown. Following nodulation, firm large nodules were selected for isolation of bacterium. They were first detached from the root, washed, disinfected with 95% ethanol and rewashed with sterile distilled water. Each nodule was placed in the centre of a sterile petridish, 1 or 2 drops of sterile distilled water was added to it and crushed with a sterile scalpel (Peltier *et al., 1961).* Sterilized YEMA medium was poured into petridishes and inoculated with bacteria (using the crushed nodule as inoculum) and incubated at 37°C. After 6 days raised, moist, and glistening colonies were observed in petridishes. Typical colonies were selected for Gram staining and some were transferred aseptically to YEMA slants. After 7 days of incubation, cultures were compared with the original stock culture of B. *japonicum* and its identity was confirmed.

3.4.3. Assessment of Bacterial growth

B. *japonicum* was grown on YEMA slants and bacterial suspension of known concentration ($1x10^6$ bacteria / ml) was prepared from 6-8 day old culture. YEMA medium was prepared, dispensed in Ehrlenmeyer flasks (50ml / 250 ml flask), sterilized at 15 lbs. p.s.i pressure for 15 minutes and inoculated with the bacterial suspension (0.5ml/flask). After a desired period of incubation, growth was measured by colorimetry (Eklund and Lankford, 1967). The concentration of bacterial suspension was determined (bacteria / ml of medium) by noting its OD value and compared with a standard value. The standard value was determined by cell counting method.

3.5. PRODUCTION OF INOCULA AND INOCULATION TECHNIQUE

3.5.1. *Fusarium oxysporum*

i) Pot screening - *F. oxysporum* was grown on sterilized sand - maize meal medium (sand maize meal -1:1) and incubate for 10 days at room temperature. After 10 days, fungus soil mixture (100 gm medium / 12" pot) was prepared by hand mixing, watered and kept for 3-4 days. Then surface sterilized seeds (20 seeds / pot) were sown in each pot at 2-3 cm depth.

3.5.2. *Trichoderma Sp.*

To study the effect of*Trichoderma* sp. on disease development first the inocula were prepared. *Trichoderma* sp. was grown on sterilized soil : wheat bran (1:1) medium. After 7 days incubation, the cultures of *Trichoderma* sp. was added to the soil where already F.*oxysporum* was infested before 3-4 days (Raghuchander *et. al. 1993).* Then seeds were sown.

3.5.3. *Bradyrhizobium japonicum*

6-8 days old cultures of *Bradyrhizobium japonicum* was selected for seed bacterization. At first bacterial suspension was prepared and concentration was determined by noting its OD (no. of bacteria / ml of medium) and compared with a standard value. Seeds were surface sterilized and then soaked in a bacterial suspension overnight before sowing.

3.6. ASSESSMENT OF DISEASE INTENSITY

Plants were examined after 7, 14, 21 and 28 days of inoculation. Disease intensity was assessed on the basis of percentage loss in dry weight of roots and colour intensity of the infected roots as described by Chakraborty and Shil (1989). After a desired period of incubation, plants were uprooted, washed, dried at 60°C for 96 h and weighed. For determination of root rot index roots were graded into 5 groups on the basis of percentage loss in dry weight of root in relation to control, and a value was assigned to each group (vix. 1-25% loss in weight =0.25, 26-50% = 0.50, 51-75% = 0.75, 76-100%=1). The root rot index in each case was the quotient of the total values of roots of replicate plants and the number of plants. Colour intensity of roots was expressed as light brown (+); dark brown (++) blackish brown (+++) and black (++++).

The number of nodules in each case was counted.

3.7. EXTRACTION OF ENZYMES FROM HEALTHY & INFECTED ROOTS

3.7.1. Phenylalanine ammonia lyase

For the extraction of phenylalanine ammonia lyase (PAL), the method of

Bhattacharya & Ward (1987) was followed. Seeds of two soybean cultivars (Soymax & JS-2) were surface sterilized, and half were inoculated with *B. japonicum.* Seeds were then sown in soil. Fifteen day old plants were uprooted and dipped in sterile distilled water as well as in mycelial and spore suspension of *F. oxysporum.* At a regular 4 h intervals, roots of each treatment (1g) were excised and crushed with a mortar and pestle with 0.1M sodium borate buffer. pH-8.8 containing 2 mM β-mercaptoethanol. The slurry was centrifuged in a microcentrifuge at 15,000 g for 20 minutes. The supernatant was collected and after recording its volume, was immediately stored at - 20°C until required.

3.7.2. Peroxidase

To extract peroxidase, the method of Chakraborty & Kapoor (1993) was followed with modifications. Both bacterized and non-bacterized fifteen day old plants were uprooted and dipped in sterile distilled water as well as in mycelial and spore suspension of *F. oxysporum.* At a regular 4 h interval, roots of each treatement (1g) were exiced and crushed with a mortar and pestle with 0.1 M sodium borate buffer, pH 8.8. The homogenate was centrifuged in a microcentrifuge at 15,000 g for 30 minutes and the resulting supernatants were assayed for peroxidase activity.

3.8. ASSAY OF ENZYME ACTIVITIES

3.8.1. Phenylalanine ammonia lyase

PAL activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenyl alanine spectrophotometrically. The reaction mixture contained 300 μM sodium borate, pH 8.8, 30 μM/L-phenylalanine and 0.5 ml of supernatant in a total volume of 3 ml. Following incubation for 1 h at 40°C the absorbance at 290 nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as μg cinnamic acid produced in one minute / gm fresh weight of tissue.

3.8.2. Peroxidase

For determination of peroxidase activity, 100 μl of crude extract was

added to the reaction mixture consisting of 1 ml of 0.2 M sodium phosphate buffer (pH-5.4), 100 µl of 4mM/L H_2O_2, 100 µl of 0- dianisidine (5 mg/ml) and 1.7 ml of distilled water. Peroxidase activity was assayed spectrophotometrically at 460nm by monitoring the oxidation of O-dianisidine in presence of H_20_2. Specific activity was expressed as the increase in absorbance of 460 nm/gm tissue / minute.

3.9. ESTABLISHMENT OF CALLUS

3.9.1. Culture media

For the callus induction, MS basal media (Murashige and Skoog, 1962) was used. Following stock solutions were prepared :

MS-1 (20x)

a)	KNO_3	-	38.0 g
	NH_4NO_3	-	33.0 g
	$MgSO_4,7H_2O$	-	7.4 g
	KH_2PO_4	-	3.4 g
	Double distilled water	-	1L
b)	$CaCl_2$, $2H_2O$	-	8.82 g
	Double distilled water	-	500 ml

Solution (a) and (b) were mixed and the volume was adjusted to 2L with double distilled water and stored at 4^0C.

MS-II (100x)

$MnSO_4$	-	2.23 g
$ZnSO_4$, $7H_2O$	-	860 mg.
H_3BO_3	-	620 mg
KI	-	83 mg
Na_2Mo_4, $2H_2O$	-	25 mg
$CuSO_4$, $5H_2O$	-	2.5 mg
$CaCl_2$, $6\ H_2O$	-	2.5 mg
Double distilled water	-	1L

MS -II (20x)

$Na_2EDTA, 2H_2O$	-	746 mg
Boiling double distilled water	-	80 ml
$Fe_2SO_4, 7H_2O$	-	556 g
Double distilled water	-	80 ml

$FeSO_4, 7H_2O$ solution was added to Na_2EDTA solution with vigorous stirring and volume was adjusted to 200 ml with double distilled water.

MS-IV (100x)

Myo - inositol	-	100 mg
Thiamine HCl	-	0.5 mg
Nicotinic acid	-	0.5 mg
Pyridoxin HCl	-	0.5 ng
Glycine	-	2 mg
Double distilled water		100ml.

MS - V

For 10 ml

Glycine	-	10 mg.
KI	-	8.3 mg in 20 ml

MS-VI

MSI, II, III, IV, Vand VI were mixed in the following ratio.

MS - I	-	100 ml
MS - II	-	50 ml
MS - III	-	5 ml
MS - IV	-	10 ml
MS - V	-	2 ml
MS - VI	-	2 ml

The medium was supplemented with 3% sucrose, 0.8% -1% agar, 1g/L 2,4.D (1mg / L) and .625mg/ L BAP (Kato 1989). Finally volume was made 1L by double distilled water and sterilized.

3.9.2. Callus induction

Seeds of different soybean cultivars were taken and surface sterilized with 90% ethanol for 5 min. and washed five times with sterile distilled water

for the removal of ethanol. After final washing, seeds were transfered into semi-soild media contained in flasks for germination. After 7-10 days, seedlings were cut in a sterile condition and finally one piece was transferred to each culture tube. They were incubated under 16 h photoperiod at 26°C and observed regularly.

3.10. EXTRACTION AND SEPARATION OF GLYCEOLLIN

3.10.1. Roots

To extract glyceollin, the method of Keen *et al.* (1971) was followed with modifications. Fifteen day. old plants were inoculated with F *oxysporum* following water culture method as stated earlier. After 24, 48 and 72 h. of inoculation roots were used for extraction of glyceollin. Infected as well as healthy roots (30g) were homogenised with 120 ml of 95% ethanol in an electric blender. The extracts were filtered through filter paper and the residues were re-extracted with an equal volume of 80% ethanol and filtered. The ethanol extracts were combined and reduced in volume in a rotary film evaporator at 45°C. The concentrate was extracted thrice with equal volume of ethyl acetate. The ethyl acetate fraction was dehydrated with sodium sulphate and dried at 45°C. The residue was dissolved in ethyl acetate (0.1 ml g^{-1} fresh wt. of roots) and was used in subsequent experiments.

For separation and chemical detection of glyceollin, thin layer chromatograms were prepared with silica gel G (0.2mm thickness) and activated for 1 h at 80°C. Aliquots of ethyl acetate extracts of roots were spotted and developed in a solvent system (Benzene : Methanol 95 : 5), dried and examined under UV-light or sprayed with Diazotized, P-nitroaniline (5ml of 0.5% P-nitroaniline solution in 2N HCl + 0.5 ml of 5% aqueous sodium nitrite solution + 15 ml of 20% aqueous sodium acetate solution).

RF value was determined in each case.

3.10.2. Callus

For extraction of glyceollin from callus, spore suspension of *F oxysporum* was placed on each callus and incubated for 24-72h. Extraction of glyceollin was done as described above

3.11. ULTRAVIOLET SPECTROPHOTOMETRY AND QUANTIFICATION OF GLYCEOLLIN

For UV-spectrophotometric studies, ethyl acetate extract (50μl) was spotted on TLC plates and developed in Benzene : methanol (95:5) solvent system and allowed to dry. The silica gel from unsprayed reacting zones was scraped off and eluted in spec methanol. The eluates were stored at 5^0C for overnight and centrifuged to remove the silica gel. These eluates were examined by UV-spectro. photometry (Sico, Model Digispec 200 GL) and the absorbance at 285 nm were determined.

Quantification : Quantity of glyceollin ($C_{20}H_{19}O_5$) was estimated from UV-spectrophotometric curve by assuming molar extinction co-efficient of 10800 at 286nm as described by Bhattacharyya and Ward (1985).

$$\text{Molar extinction co-efficient (E)} = \frac{\text{OD. of the tested solution}}{\text{Concentration (x)* of the tested solution (moles/litre)} \times \text{Path length of the cell (cm)}}$$

* x = moles / litre converted to g / litre by multiplying moles with molecular weightof glyceollin ($C_{20}H_{19}O_5$)

Results have been expressed in μg/g fresh weight of roots.

3.12. PETRIDISH BIOASSAY OF PHYTOALEXIN

In case of petridish bioassay, 0.2 ml of ethyl acetate extract of infected roots was taken in a sterilized petridish (9 cm. dia.) and allowed to dry. Then, 0.2 ml of ethanol was added to it, followed by 20ml of sterilized PDA (20 ml / petridish) and mixed well. Each petridish was inoculated with an agar block (4 mm. diam.) containing 4-day old mycelia of F. *oxysporum* and incubated at 30$\pm$ 1^0C. Diameter of mycelial mat was measured after 24, 48, and 72 h of inoculation and compared with the controls.

3.13. PREPARATION OF ANTIGEN

3.13.1. Root Antigen

Root antigens were extracted from healthy and *F. oxsporum* infected soybean roots following the method of Chakraborty & Saha (1994). Seeds of soybean cultivars were grown in earthen pots containing *Fusarium* infested soil as well as in sterilized washed soil separately. Healthy and infected plants were uprooted after two week intervals, washed with cold water and kept at -15^{0}C for 1 h. Finally, roots (20 g fresh weight) were crushed with sea-sand in mortar and pestle in cold (4^{0}C) and stored at -15^{0}C for 1 h and homogenized with 20 ml. of 0.05M sodium phosphate buffer supplemented with 10 mM Sodium metabisulphite and 0.5mM magnesium chloride. Homogenate was strained through cheese cloth and then centrifuged (12,000g) at 4^{0}C for 1 h and known quantity of ammonium sulphate was added to it for 100% precipitation (Green & Hughes, 1965), kept at 4^{0}C. Precipitate was dissolved in the same extractive buffer (pH-7.4) and dialysed against 0.005 M phosphate buffer for 24 h at 4^{0}C. During this period 10 changes were given. The dialysate (i.e., soluble protein) was used for antisera production and for gel electrophoretic study.

3.13.2. Mycelial Antigen

Mycelial antigen was prepared following the method of Chakraborty & Saha (1994). Initially fungal mycelia (4mm disc) were transferred to 250 ml Ehrlenmeyer flasks each containing 50ml of sterilized liquid Richards medium (g/1 distilled water, sucrose, 30; KNO_3, 10; KH_2PO_4, 5; $MgSC_4$, $7H_2O$, 2.5 and $FeCl_3$, 0.02) and incubated for 10 days at 30 $\pm$ 1^{0}C. For extraction of soluble antigens, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (30 g fresh wt.) were homogenized with 0.05 M sodium phosphate buffer (pH-7.2) supplemented with 10mM sodium metabisulphite and 0.5 mM magnesium chloride and 0.85% NaCl in mortar and pestle in presence of sea sand. Cell homogenates were kept overnight at 4^{0}c and then centrifuged (15000g) for 30 min at 4^{0}C. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at 4^{0}C. After this period the mixture was centrifuged

(15000 g) for 30 min at 4^{0}C, the precipitate was dissolved in 10ml 0.05M sodium phosphate buffer (pH-7.2). The preparation was dialysed for 72 h through cellulose tubing (Sigma Chemical Co. USA) against 1 L of 0.005 M sodium phosphate pH -7.2) with 10 changes. Then the dialysed matarial was stored at -20^{0}C and used as antigen for the preparation of antiserum and other experiments.

3.13.3. Protein Estimation

Soluble proteins were estimated following the method as described by Lowry *et. al.* (1951). To 1 ml of protein sample 5 ml of alkaline reagent (0.5 ml of 1% $CuSO_4$ and 0.5 ml of 2% potassium sodium tartarate, dissolved in 50 ml of 2% Na_2CO_3 in 0.1N NaoH) was added. This was incubated for 15 min at room temperature and then 0.5 ml of Folin Ciocalteaus reagent (diluted 1:1 with distilled water) was added and again incubated for 15 min for colour development following which optical density (OD) was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumim (BSA).

3.14. SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEIN

3.14.1. Preparation of Slab Gel

Stock solutions

For the preparation of gel, the following stock solutions were initially prepared as described by Laemmli (1970) & Sambrook *et. al.* (1989)

(A) Acrylamide and N, N'-methylenebisacrylamide :

Acrylamide	-	29 g
N, N' - methylenebisacrylamide	-	1 g
Distilled water	-	100 ml

Solution was filtered, and pH adjusted to 7.

(B) Sodium dodecyl sulphate

SDS	- 10 g
Distilled water	- 100ml

(Stored at room temperature)

(C) Lower gel buffer (1.5 M Tris)

Tris	-	18.18 g
Distilled water	-	100 ml

pH was adjusted to 8.8

(D) Upper gel buffer (0.5 M Tris)

Tris	-	6.06 g
Distilled water	-	100 ml

pH was adjusted to 6.8

(E) Ammonium peroxidisulphate (APS)

Ammonium peroxidipsulphate	-	0.1 g
Distilled water	-	1.0 ml

(freshly prepared each time)

(F) Tris - glycine electrophoresis buffer

(25mM Tris Base ; 250 mM glycine)

5x stock can be made;

Tris Base	-	15.1 g
Glycine	-	94 g

in 900 ml of dH_2O, pH was adjusted to 8.3

Then 50 ml of 10% SDS was added and volume made upto 1000 ml.

(G) 1xSDS gel loading buffer :

50 mM Tris Cl (pH - 6.8)

100 mM dithiothreitol.

2% SDS

0.1% bromophenol blue

10% glycerol

Slab gel Preparation

For slab gel preparation, two glass plates (17cmx19cm) were washed with dehydrayted alcohol and dried. Then 1 mm thick spacers were placed between the glass plates at the two edges and the 2 sides of glass plates were

sealed with gel sealing tape and kept in the gel casting unit. Resolving gel solution was prepared as follows :

H_2O	-	11.9 ml
30% Acrylamide mix	-	10.0 ml
1.5 M Tris (P^H 8.8)	-	7.5 ml
10% SDS	-	0.3 ml
10% APS	-	0.3 ml
TEMED	-	0.012 ml

The gel solution was cast very slowly and carefully up to a height of 12 cm by a syringe. The gel was overlayered with water and kept for 2-3 hrs for polymerization. Then stacking gel solution was prepared as follows :

H_2O	-	6.8 ml
30% acrylamide mix	-	1.7 ml
1 M Tris (p^H-6.8)	-	1.25 ml
10% SDS	-	0.1 ml
10% APS	-	0.1 ml
TEMED	-	0.01 ml

After polymerization of resolving gel, water overlay was decanted off and a 13 well 1 mm thick comb was placed. Stacking gel solution was poured carefully up to a height of 4 cm over the resolving gel and overlayered with water. Finally the gel kept for 30 min for polymerization.

3.14.2 Sample preparation

Sample was prepared by mixing the sample protein with 1x SDS gel loading buffer (final volume 80μl). All the samples were floated in boiling water bath for 3 min. After cooling upto 80 μl of each sample was loaded in a predetermined order into the bottom of the wells with a microliter syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging in molecular weight from 30 to 200 KD (Carbonic anhydrase - 29,000, Albumin(egg) - 45,000, Albumin (bovine) - 66,000, Phosphorylase b - 97,400,

β-galactosidase - 116,000 and Myosin - 205,000) was treated as the other samples and loaded in a separate well.

3.14.3. Electrophoresis

Electrophoresis was performed at 25 mA for a period of 3 h until the dye front reached the bottom of the gel.

3.14.4. Fixing & Staining

For fixing the fixer solution was prepared as follows -

Glacial Acetic Acid	-	10 ml
Methanol	-	20 ml
Distilled water	-	70 ml

The entire gel was removed from the glass plates and then the stacking portion was cut off from the resolving gel. After that gel was soaked for 20 h in the fixer for fixing.

The staining solution was prepared as follows :

Coomassie Brilliant Blue R 250	- 0 .25 g
Methanol	- 45 ml
Distilled water	- 45 ml
Acetic Acid	- 10 ml

At first, gels were stained by staining solution for 2-3 h and finally soaked with destaining solution (methanol : dH_2O : Acetic acid - 4.5 : 4.5:1) until the background became clear.

3.15. ANTISERA PRODUCTION

3.15.1. Rabbits and their maintenance

For the production of antisera against different fungal and root antigens, New Zealand white, male rabbits were used. Before immunization, the body weights of rabbits were recorded and were observed for at least one week inside the cages. They were regularly fed with 500 g green grass each time in the morning and evening. Every alternate day they were also given 50-75 g of

green seeds soaked in water. Besides this, they were given saline water after each bleeding for three consecutive days. Cages were cleaned everyday in the morning for better hygeinic conditions.

3.15.2. Immunization

Antisera were raised in separate rabbits against antigen preparations of mycelia of *F. oxysporum* as well as healthy root antigen of UPSM-19. Before immunization normal sera were collected from each rabbit. In each case 1ml of antigen emulsified with an equal volume of Freund's complete adjuvant (Difco) were injected intramascularly, repeating the doses at 7 days intervals with Freund's incomplete adjuvant (Difco) for 7 consecutive weeks.

3.15.3. Bleeding

Blood was collected from the marginal ear vein puncture 3 days after sixth week of first immunization and subsequently seven times more every fortnight. During bleeding, rabbits were placed on their backs on a wooden board after taking them out from the cage. The board was fixed at a 60^0 angle. The neck of the rabbit was held tight in the triangular gap at the edge of the board, and the body was fixed in such a way that the rabbits could not move during the bleeding. The hairs were removed from the upper side of the ear with the help of a razor and disinfected with rectified spirit. Then the ear vein was irritated by xylene and an incision was made with the help of a sterile blade and blood samples (2 ml) were collected in a sterile graduated glass tube. After collection, all precautionary measures were taken to stop the flow of the blood from the puncture. For clotting, the blood samples were kept at 30^0C for 1 h and then the clot was loosened with a sterile needle and the antiserum was clarified by centrifugation at 2000 g for 10 min. Finally, blood samples were distributed in 1 ml vials and stored at - 20^0C until required.

3.16. PURIFICATION OF Ig G

3.16.1. Precipitation

IgG was purified following the method of Clausen (1988). The antiseurm

(5ml) was diluted with two volumes of distilled water and an equal volume of 4.0 M ammonium sulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16 h at 22^{0}C. Then it was centrifuged at 10,000g for 1 h at 22^{0}C and the precipitate was dissolved in 5 ml of 0.02 M sodium phosphate buffer, pH 8.0.

3.16.2. Column preparation

Initially DEAE sephadex (Sigma Co.USA) was suspended in distilled water overnight after which the water was decanted off and the gel was suspended in 0.005 M phosphate buffer, pH 8.0. The buffer washing was repeated 5 times. The gel was next suspended in 0.02 M phosphate buffer, pH 8.0 and was applied to a column (2.6 cm in dia, 30 cm high) and allowed to settle for 2 h.After that 25 ml of 0.02 M phosphate buffer (pH 8.0) was applied to the gel material.

3.16.3. Fraction collection

At the top of the column, 2 ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.3 M. The initial elution buffer (1) was 0.02 M sodium phosphate buffer p^H 8.0 (diluted from a 0.10 M sodium phosphate buffer pH 8.0 containing 16.86 g Na_2HPO_4, $2H_2O$ + 0.731 g NaH_2PO_4, H_2O/L.). The final elution buffer (2) was 0.30 M sodium phosphate buffer pH 8.0.

The buffer (1) was applied in a flask in which one rubber connection from its bottom was supplying the column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). During the draining of buffer (1) to column, buffer (2) was sucked into buffer (1) thereby producing a continuous rise in molarity. Finally, 40x5 ml fractions were collected and the optical density (OD) values were recorded by UV-spectrophotometer at 280 nm.

3.17. IMMUNODIFFUSION TEST

3.17.1. Preparation of agar slides

The glass slides (5cmx5cm) were degreased successively in 90%(v/v) ethanol : di-ethyl ether (1:1v/v) and ether, then dried in hot air oven and sterilized inside the petridish each containing one slide. A conical flask containing Tris-barbiturate buffer (pH 8.6) was placed in boiling water bath; when the buffer was hot, 0.9% agarose was mixed to it and boiled for the next 15 min. The flask was repeatedly shaken in order to prepare absolutely clear molten agarose which was mixed with 0.1% (w/v) sodium azide (a bacteriostatic agent). The molten agarose was poured on the glass slides (6 ml / slide) and allowed to solidify. After that 3-7 wells were cut on the agar plate with a sterilized cork borer (4 mm dia.) at a distance of 5mm. from the central well.

3.17.2. Diffusion

Agar gel double diffusion test was performed following the method of Ouchterlony (1967). The antigens and undiluted antisera (50 μl/well) were pipetted directly into the appropriate wells and diffusion was allowed to contiune in a moist chamber for 48-72 h at 25^{0}C.

3.17.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9% NaCl and 0.1% NaN_2) for 72h with 6 hourly changes to remove unreacted antigen and antibody widely dispersed in the agarose. Then slides were staine with 0.5% coomassie blue (0.5 g coomassie blue, 5 g $HgCl_2$, 5ml glacial acetic acid, 95 ml distilled water) for 10 min. at room temperature. After staining slides were washed thrice in destaining solution [2% (v/v) acetic acid) for 5 h to remove excess stain. Finally, all slides were washed with distilled water and dried in hot air oven for 3 h at 50°C

3.18. IMMUNOELECTROPHORESIS

3.18.1 Preparation of agarose slides

The slides (7.5x2.5cm) were degreased, dried and sterilized as described earlier. Thin and uniform layer (2mm thick) of fluid agarose medium (0.9% agarose, 0.1% NaN_3 dissolved in 0.05 M barbitol buffer (pH - 8.6) was poured on each slide taking care that no air bubble was present in the agarose medium. This was necessary in order to avoid any irregularity which may cause asymmetrical migration and diffusion during electrophoretic separation or the immunodiffusion. The slides were kept in petridishes and stored at 4°C until use.

3.18.2. Electrophoresis

Two central wells (3 mm dia) were cut out from the agarose plate of each slide following the conventional method (Ouchterlony, 1967). Slides were placed in the middle compartment of the electrophoretic box. The anode and cathode chambers were filled with barbitol buffer (0.05 M pH 8.6). Antigens (40μl) were introduced into the wells. Filter paper strips (Whatman) were soaked in buffer and laid on both ends of the slides which connected the buffer solution in the anode and cathode compartments with the agarose surfaces. An electric current (2.5 mA slide ; 10v / cm) was passed through the slides for 3 h at 4°C. After electrophoresis the current was discontinued.

3.18.3. Diffusion.

A longitudinal trough parallel to the long edge of the slide was cut in the agarose plates in between two wells and the undiluted antiserum (400μl) was pipetted into the trough. Diffusion was allowed to continue in a moist chamber for 48-72h at 25°C.

3.18.4. Washing, drying and staining of slides

After immunodiffusion, slides were washed, stained and destained as mentioned earlier. Then all slides were dried in hot air oven for 3 h at 50°C.

3.19. ENZYME LINKED IMMUNOSORBENT ASSAY

3.19.1. Indirect ELISA

Following buffers were prepared for Indirect ELISA following the method as described by Chakraborty *et al.* (1995).

1. Antigen coating buffer (Carbonate - bicarbonate buffer 0.05 M, pH -9.6)

Stocks			
A	Sodium Carbonate	=	5.2995 g
	Distilled water	=	1000 ml
B	Sodium bicarbonate	=	4.2 g
	Distrilled water	=	1000 ml

160 ml of stock solution 'A' was mixed with 360 ml of stock solution 'B' pH was adjusted to 9.6.

2. Phosphate Buffer saline (0.15 M PBS, pH-7.2)

Stock			
A	Sodium dihydrogen phosphate	=	23.40 g
	Distilled water	=	1000 ml
B	Disodium hydrogen phosphate	=	21.2940g
	Distilled water	=	1000 ml

With 280 ml of stock solution 'A', 720 ml of stock solution 'B' was mixed and the pH of the mixed solution was adjusted to 7.2. Then 0.8% NaCl and 0.02% KCl was added to the solution.

3 0.15 M Phosphate buffer saline - Tween (0.15M PBS - Tween, pH-7.2).

To 0.15 M phosphate buffer saline, 0.05% Tween 20 was added and the pH was adjusted to 7.2.

4. Blocking reagent (Tris buffer saline, pH-8.0)

(0.05 M Tris, 0.135 M NaCl, 0.0027M KCl).

Tris = 0.657 g

NaCl = 0.81 g

KCl = 0.223 g

Distilled water was added to make up the volume upto 100 ml. Then pH was adjusted to 8.0 and 0.05% Tween 20 and 1% bovine serum albumin (BSA) were added.

5. Antisera dilution buffer (0.15 M PBS-Tween, pH 7.2)

In 0.15 M PBS Tween, pH 7.2, 0.2% BSA, 0.02% Polyvinyl polypyrrolidone, 10,000 (PVPP, 10,000) and 0.03% sodium azide (NaN_2) was added.

6. Substrate

Sigma Fast PNPP substrate tablet sets were used. Each tablet set yields the following when dissolved in 20 ml of distilled water.

PNPP = 1.0 mg/ml

Tris buffer = 0.2 M

7. Stop Solution

0.3 M NaOH solution was used to stop the reaction.

ELISA was performed following the method as described by Chakraborty *et al.* (1995). Plant and fungal antigens were serially diluted with coating buffer and the diluted antigens were loaded (200 µl/ well) in a Nunc 96 well ELISA plate. After loading, plate was inculated at 25°C for 4 h. The plate was then washed four times under running tap water and once with PBS - Tween and each time, plate was shaken dry. Subsequently, 200 µl of blocking agent was added to each well for blocking the unbound sites and the plate was incubated at 25°C for 1h. After incubation, plate was washed as mentioned earlier. Purified antiserum (IgG) was diluted in antisera dilution buffer and loaded (200 µl / well) to each well and incubated at 4°C overnight. After a further washing 200 µl of antirabbit IgG goat antiserum labelled with alkaline phosphatase (Sigma

Chemicals, USA) was added and incubated at 37°C for 2 h. Plate was washed, dried and loaded with 200 µl of Pnitrophenyl phosphate substrate in each well and incubated in dark at room temparature for 30-45 min. Colour development was stopped by adding 50 µl / well of 0.3M NaOH solution and absorbance was determined in an ELISA reader (Cambridge Tech. Inc. USA) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.19.2. Dot -blot ELISA

For Dot ELISA following buffers were prepared as described by Hammond and Jordon (1990) with modifications.

1. Antisera dilution buffer (Carbonate - bicarbonate buffer 0.05M, pH9.6)

Stocks			
A	Sodium carbonate	=	5.2995 g
	Distilled water	=	1000 ml
B	Sodium bicarbonate	=	4.2 g
	Distilled water	=	1000 ml.

160 ml of stock solution 'A' was mixed 360 ml of stock solution 'B' pH was adjusted to 9.6.

2. Tris HCl Buffer (10 mM Tris, pH - 7.4)

Tris	-	1.2114 g
Distilled water	-	1000 ml

pH was adjusted to 7.4, then 0.9% NaCl and 0.05% Tween 80 was added. (TBS - T80)

3. Blocking Reagent

(10mM TBS - T80, pH -7.4)

In 100 ml TBS - T80, 3 gm BSA was added.

4. Antigen dilution buffer

(10mM TBS - T80, pH - 7.4)

In 10 mM TBS-T80, pH -7.4, 0.01 M Sodium diethyldithiocarbamate, 2%

pyrollidone (PVP), 1%, BSA and EDTA was added.

5. Substrate

Sigma Fast Red TR salt and Naphthol AS-Mx phosphate were used. Each Sigma Fast Red TR/Naphthol AS-MX tablet set contains the following when dissolved in 10 ml d H_2O.

Fast Red TR salt	-	1 mg / ml
Naphthol AS-MX	-	0.4 mg / ml
Levamisol	-	0.15 mg / ml
Tris buffer	-	0.1 M

For dot-blot ELISA, nitrocellulose membrane (NCM) filters (IMMOBILON-NC, HAHY 0.45 um, Sigma USA) first floated in distilled water for 5-10 second. Antiserum was diluted with carbonate bicarbonate buffer (pH - 9.6) and then NCM filters were dipped in this diluted antiserum. After 4 h incubation, NCM filters were rinsed 5 times with 10 mM TBS-T80 (pH - 7.4). After that NCM was blocked by putting it in blocking solution for 10-15 min (3% BSA in TBS-T80) following which it was rinsed as stated before. Antigen was diluted 1 : 1 with antigen dilution buffer and spotted (5-10μl) on to the dry antiserum coated NCM and incubated at 4^{0}C overnight. After washing, alkaline phosphatase (Sigma chemicals, USA) conjugated with IgG was added to each plastic dish and NCM was floated in the solution and incubated for 1-2 h at 27^{0}C. NCM was washed, dried and finally floated in the substrate developing solution. After 30-45 minutes colour dots were developed. NCM was rinsed with deionized water to stop the reaction.

3.20. FLUORESCENCE ANTIBODY STAINING AND MICROSCOPY

Indirect fluorescence staining of cross sections of soybean roots, fungal spores and mycelia were done using FITC labelled goat antirabbit IgG following the method of Chakraborty & Saha (1994).

3.21.1. Cross section of soybean roots

Initially, fresh cross sections of soybean roots were cut and immersed in

phosphate. buffer saline (PBS) pH 7.2 containing 0.8% NaCl and 0.02% KCl. Following this, sections were treated with normal serum or antiserum diluted (1:125) with PBS (pH 7.2) and incubated for 30 min at 27°C . After incubation, sections were washed thrice with PBS Tween (pH 7.2) for 15 min and transferred to 100 µl of diluted (1:40) goat antiserum specific to rabbit globulins and conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30 min at 27°C. All prepations with FITC labelled antibodies were carried out in darkness or very low light. After that, sections were washed thrice with PBS Tween as mentioned above and then mounted on a grease free slide with 10% glycerol. A coverslip was placed on the section and sealed. Fluorescence of the root sections were observed using Leica Leitz Biomed microscope with fluorescence optics equipped with ultra violet (UV) fifter set I3. Tissue section were photographed under both phase contrast and UV fluorescent conditions for comparison of treatment.

3.21.2. Mycelia

Fungal mycelia were grown in PDB. After 4 days of inoculation young mycelia were taken out from the flask and kept in grooved slide. After washing with PBS (pH -7.2) mycelia were treated with normal sera or antisera diluted (1:125) with PBS, and incubated for 30 min at 27°C. Then mycelia were washed thrice with PBS Tween as mentioned above and treated with goat antirabbit IgG (whole molecule) conjugated with fluorescein isothiocyanate (Sigma) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 30 min at 27°C. After incubation, mycelia were washed thrice in PBS and mounted in 10% glycerol. A cover glass was placed on mycelia and sealed. The slides were then observed as before.

3.21.3. Conidia and Chlamydospore

Fungal conidia and chlamydospores were collected from 15 day-old culture and a suspension of this was prepared with PBS (pH 7.2). Conidial suspensions were taken in micro centrifuge tube and centrifuged at 3000 g for 10 min and the PBS supernatant was discarded. Then 200 µl of diluted antiserum (1:125) was added into the microcentrifuge tube and incubated for 2h at 27°C.

After incubation, tubes were centrifuged at 3000 g for 10 min and the supernatant was discarded. Then the spores were rewashed 3 times with PBS-Tween by centrifugation as before and 200 μl of goat antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and the tubes were incubated in dark at 26^{0}C for 1h. After the dark incubation excess FITC antisera was removed by repeated washing with PBS - Tween and the spores were mounted on glycerol jelly and observed under Leica microscope, equipped with I3 UV fluorescence filter and photographs were taken.

Experimental

4.1 Pathogenicity test of *F. oxysporum* on different cultivars of soybean

Pathogenicity of *F. oxysporum* was tested on ten cultivars of soybean (Soymax, Bragg, Punjab1, 19-PK 466, PK-327, JS-2, UPSM-19, PK-564, 17-PK 472 and 18-PK-564). Inoculation technique and disease assessment have been described in detail under materials and methods. Results are presented in Table 1, Fig. 1 and Plate II (Figs. A&B). Disease assessment was carried out after 14 and 28 days of inoculation with *F. oxysporum.* Assessment was on the basis of percentage loss in dry weight of inoculated roots in relation to respective control as well as on the colour intensity of infected roots. Results revealed that among all the cultivars tested UPSM-19 with a percentage loss of 75.2% was the most susceptible, followed closely by JS-2 with 73.4% loss in dry weight. Soymax was the most resistant followed by Bragg with percentage losses in dry weight of roots being 27.2 and 31.4 respectively. On the basis of significant test of the different cultivars in relation to the most susceptible and the most resistant, the cultivars could be broadly divided into resistant and susceptible types. Soymax, Bragg, 19-PK-466, PK-327 and Punjab-1 were the resistant group where the percentage loss in dry weight varied from 27.2% to 38.9% 28 days after inoculation. Colour intensity of infected roots of these groups did not show much variation, the maximum intensity being deep brown.The other cultivars i.e. JS-2, UPSM-19, PK-564, 17-PK 472 and 18-PK-564 were the susceptible ones and percentage loss in dry weight of roots varied from 62.2% (PK-564) to 75.2% (UPSM-19) ofter 28 days of inoculation. Colour intensity of the infected roots also varied from deep brown to black.

4.2. Cultural characteristics of microorganisms

Three microorganisms have been mainly used in this study. These are *Fusarium oxysporum, Bradyrhizobium japonicum* and *Trichoderma harzianum.* The cultural characteristics of these have been studied and are described below.

Table 1 : Pathogenicity test of *Fusarium oxysporum* on different cultivars of Soybean.

Cultivars	% loss in dry mass of roots (mg)*		Colour intensity of infected roots**		Root rot index plant ***	
	14 days	28 days	14 days	28 days	14 days	28 days
Soymax	14.1 ± 1.1	27.2[a] ± 2.1	-	+	0.25	0.50
Bragg	22.5 ± 1.4	31.4[a] ± 3.1	+	++	0.25	0.50
Punjab 1	25.2 ± 2.1	38.1[a] ± 1.2	-	+	0.25	0.50
19-PK-466	27.3 ± 1.7	38.9[a] ± 3.8	+	++	0.50	0.50
PK-327	28.5 ± 2.3	37.2[a] ± 2.5	-	+	0.50	0.50
JS-2	43.5 ± 3.4	73.4[b] ± 3.8	++	++++	0.50	0.75
UPSM-19	39.2 ± 2.2	75.2[b] ± 2.9	++	+++	0.50	0.75
PK-564	33.4 ± 2.8	62.2[b] ± 2.5	+	+++	0.50	0.75
17-PK-472	37.0 ± 0.98	63.5[b] ± 1.9	+	++	0.50	0.75
18-PK-564	34.8 ± 3.0	65.6[b] ± 3.5	+	++	0.50	0.75

* In relation to control on the basis of 3 separate trials of 10 plants each. Values with same superscript not significant at P = 0.01.

** + = light brown; ++ = deep brown, +++ = blackish brown ; ++++ = black.

*** Root rot index computed on the basis of % loss in dry weight of roots : 1-25% loss = 0.25 ; 26-50 % = 0.50 ; 5 -75% = 0.75 and 76 -100% = 1.

Plate II (figs. A & B) : A - Healthy and *F.oxysporum* infected plants of soybean (cv.JS-2); **B** - Portion of infected roots

Pathogenicity test of *F.oxysporum* on soybean cultivars

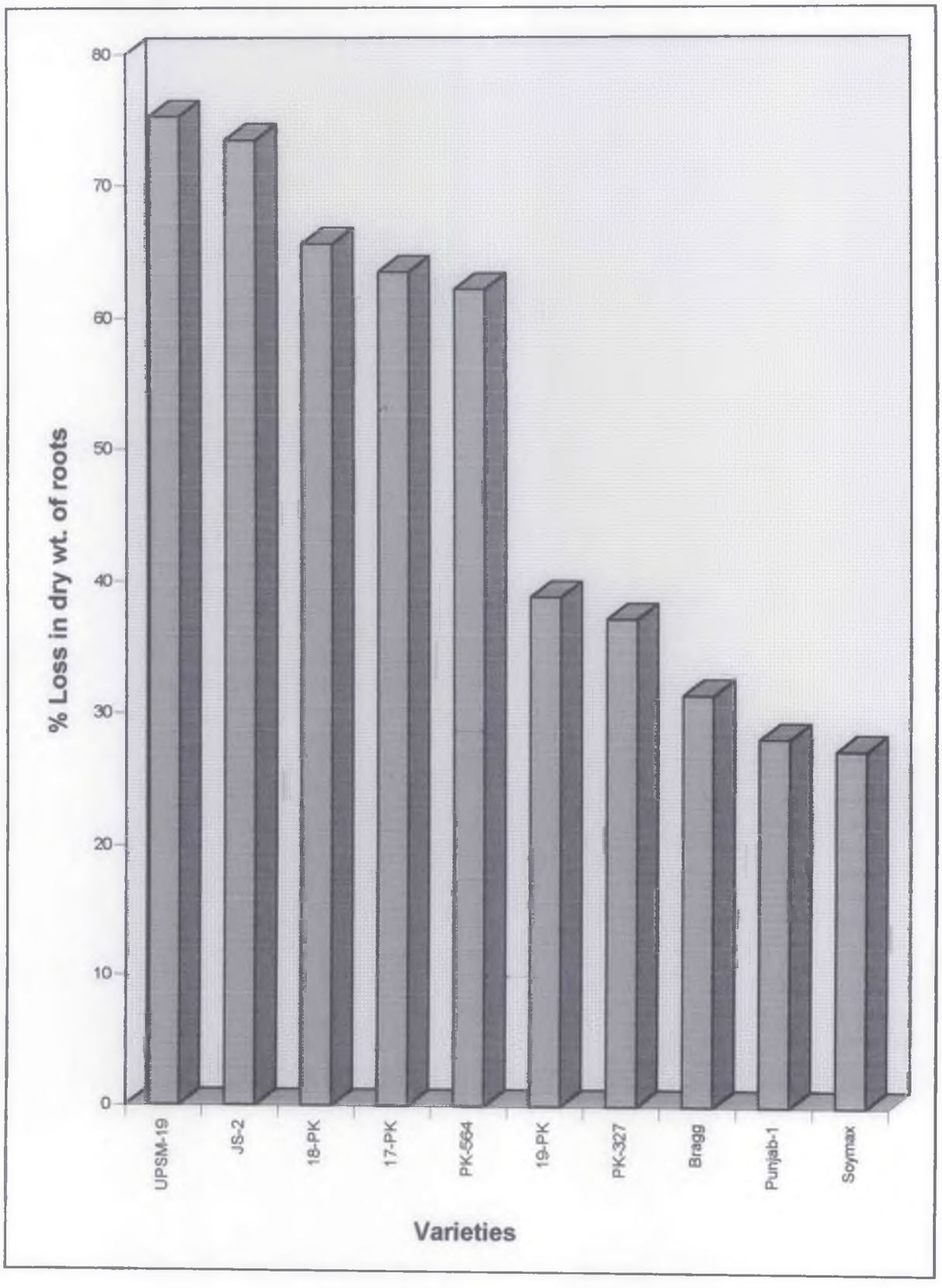


Fig.1

4.2.1. *F. oxysporum*

This is a fast growing fungus reaching 4-6 cm diameter within 4 days. Aerial mycelium sparse to abundant and floccose, becoming felted usually with a purple or violet tinge, more intense at the stromatic agar surface. Microconidia generally abundant, borne on sparsely branched conidiophores, never forming chains; not septate, ellipsoidal to cylindrical, 5-12x2-4 µm. Macroconidia fusiform, moderately curved, pointed at both ends, basal cells pedicillate, 3-4 septate, 20-60 x 3-5µm (Plate III, fig. A). Chlamydospores terminal or intercalary in hyphae.

Besides the general microscopic observations regarding morphology of the conidia, attempts were made to charecterize conidial wall by studying the agglutination effect of four lectins (Con A, HPA,UEA-1, WGA) on conidia. Agglutination procedure has been described in materials and methods. Agglutinations were examined under the light microscope and arbitrarily scaled from 0-4 (0= no agglutination; I=1-25% ; 2=26-50% ; 3-51-75% : and 4=76-100% agglutination.Microscopical examination revealed that conidia were strongly agglutinated by con A and HPA and to lesser degrees by WGA and UEA-1(Table 2&Plate III, figs. B-D). Strong agglutination with Con A suggest that surface components are glycoconjugates containing L -D glucopyranoside and / or L -D mannopyranoside residues, while agglutination with HPA suggest the presence of also glycoconjugates containing N-acetyl L -D galactosaminyl residues. Agglutination with WGA and UEA - 1 lectins which have affinity for N-acetyl L -D glucosamine and L- fucose suggested the presence of these sugar residues, though to a lesser extent on the conidial wall surface.

4.2.2. *B. japonicum*

This is a slow growing bacterium with slow and scanty growth, streak slightly raised, glistening, opaque white and grows well in medium containing mannitol. Gram negative rods varying in size from 3-5 µm in length and 1.5-3µm in breadth. Bacteroids present, sometimes branched forming T or V shaped structure.

Table 2 : Agglutination response of conidia of *F. oxysporum* to different lectins.

Name of lectins	Agglutination of conidia of *F. oxysporum* *
Concanavalin A (Con A)	4
Helix pomatia (HPA)	4
Ulex europaeus (UEA -1)	3
Wheat germ agglutinin (WGA)	2

* 0 = No agglutination; 0-25 = 1; 26-50 = 2 ; 51-75 = 3 ; 76.100 = 4.

4.2.3. *T. harzianum*

Fast growing hyaline colonies bearing repeatedly branched conidiophores in tufts with divergent, often irregularly bent, flask shaped phialides. These grow best in day light and in the dark they quickly loose the capacity to sporulate. Colonies reaching over 9 cm diameter in 5 days at 20°C . Conidia subglobose to short oval, measuring approximately 2.8-3.2 x 2.5x2.8 µm (Plate VI, fig. A)

4.3. Factors affecting growth

The effect of various cultural conditions on the growth of *F. oxysporum* and *B. japonicum* were tested, since the growth of microorganisms both *invitro* and in natural conditions is greatly influenced by different factors like temparature, pH of the sorrounding medium and available nutrients.

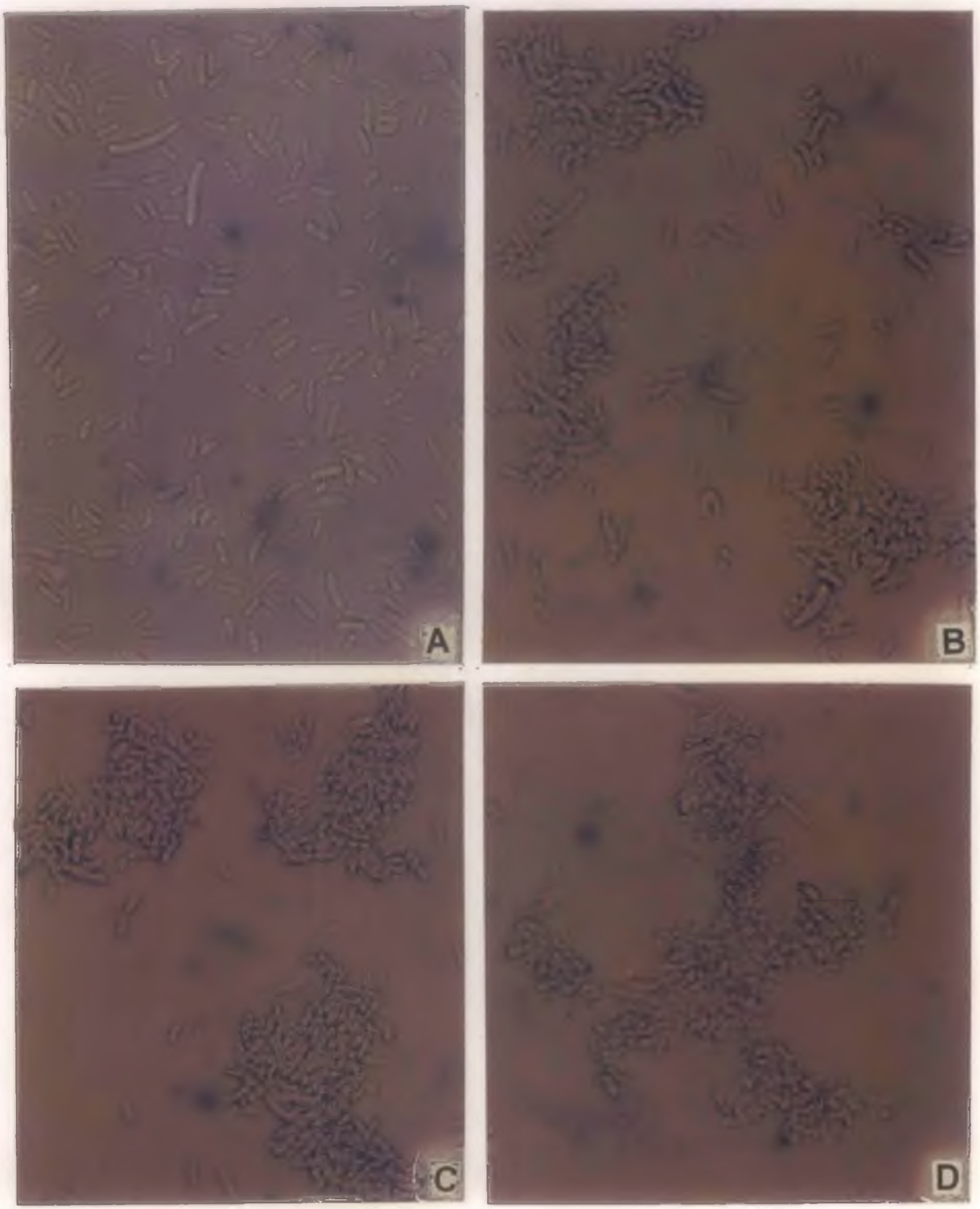


Plate III (figs. A-D) : Agglutination response of conidia of *F.oxysporum.* Control **(A)** ; After treatment with UEA-1 **(B)**, ConA **(C)** & HPA **(D)**

4.3.1. *F. oxysporum*

The growth and sporulation of *F. oxysporum* varies depending upon the available cultural conditions. The cultural conditions affecting the growth of *F. oxysporum* which were tested in this study are different media, incubation period, pH, different nitrogen and carbon sources.

4.3.1.1. Media

The growth of *F. oxysporum* was tested on a number of media, both solid and liquid and results were recorded.

4.3.1.1.1. Solid

F. oxysporum grew well in all the solid media tested (Potato dextrose agar, Richards agar, Yeast extract mannitol agar, Joffe's agar, Czapek dox agar and Carrot juice agar). Results (Table-3) revealed that two days after inoculation maximum growth was observed in PDA (25.3mm) and the least in Richards agar (17.3mm). This increased to 69.6 and 61.6 mm respectively after 6 days of growth while, after 8 days the mycelial growth in most of the media covered the petridish area (90mm). In Joffes agar medium the growth was submerged while in all other media, growth on the upper sufface of the petridish was white and fluffy. On the reverse side of the petridish a purplish colouration was observed after 8 days of growth in YEMA while black colour was observed in Czapek-dox agar.

4.3.1.1.2. Liquid

Mycelial growth was assessed in five different liquid media (Potato dextrose broth, Richards, Yeast extract mannitol, Joffes, and Czapek dox). Mycelial dry weights were determined after 6,9 and 12 days of growth. Maximum growth was obtained in Richards medium after 9 days of growth (581.3 mg dry weight) and minimum in Joffe's medium (Table-4). Czapek dox and potato dextrose broth also supported good growth while in yeast extract mannitol medium growth was rather suppressed. (Fig. 2).

Table 3 : Effect of different media on mycelial growth of *F. oxysporum*

Medium	Diameter of mycelia (mm)[a]			
	2 *	4 *	6 *	8 *
Potato dextrose agar	25.3 ± 0.72	51.6 ± 0.72	69.6 ± 0.98	90.0 ± 0.57
Richards agar	17.3 ± 0.72	43.6 ± 0.91	61.6 ± 1.26	90.0 ± 1.15
Yeast extract mannitol agar	23.6 ± 0.72	50.6 ± 1.18	68.0 ± 1.15	89.3 ± 0.27
Joff's agar	22.6 ± 0.54	46.3 ± 0.50	65.3 ± 0.98	90.0 ± 1.73
Czapek dox agar	21.3 ± 0.88	38.0 ± 2.31	58.3 ± 1.18	81.6 ± 0.72
Carrot juice agar	23.3 ± 0.54	49.6 ± 1.18	66.6 ± 0.54	86.3 ± 1.09

a Average of 3 replicates

* Days after inoculation

± Standard error.

4.3.1.2. Incubation period

F. oxysporum was grown in Richards medium for a period of 18 days. Mycelial growth was recorded after 3,6,9,12,15 and 18 days. Maximum Mycelial growth (553.0 mg dry weight) was recorded after 9 days of growth (Table 5, Fig. 3). After 3 days of growth mycelial dry weight was only approximately 52.0 mg which sharply increased to 413.5 mg after a further 3 days of growth. The reduction in growth after 9 days was gradual and after 18 days the growth was 276.5 mg dry weight. No sporulation was observed after 3 days growth while abundant sporulation appeared after 6 days.

Effect of different media on mycelial growth of *F.oxysporum*

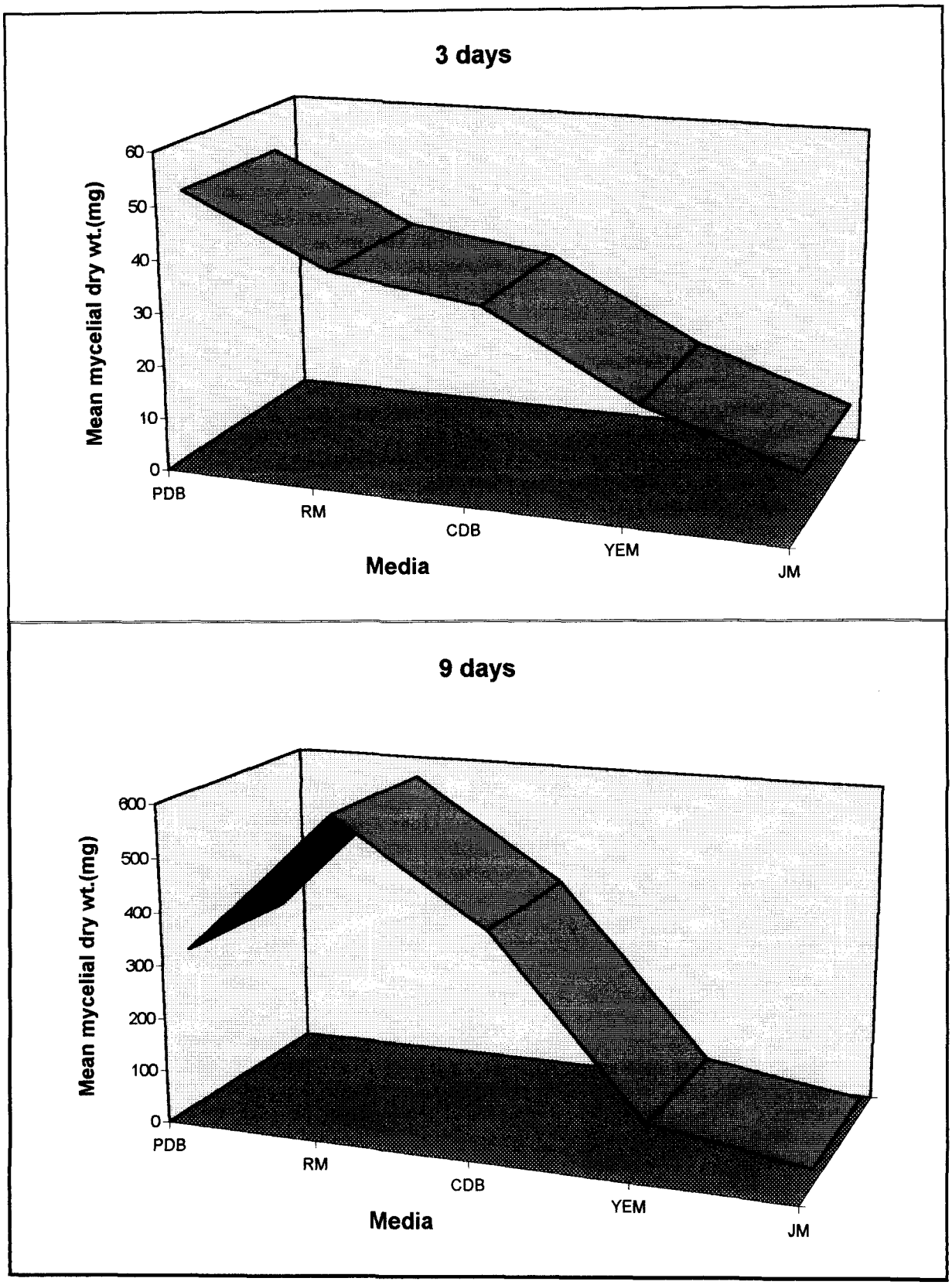


Fig.2

Table 4 : Effect of different liquid media on mycelial growth of *F. oxysporum*

Medium	Mean mycelial dry wt. (mg)[a]		
	6 *	9 *	12 *
Potato dextrose	253.6 ± 1.65	306.0 ± 2.16	260.0 ± 1.24
Richards	417.6 ± 1.18	581.3 ± 1.65	376.5 ± 2.60
Yeast extract Mannitol	60.3 ± 1.36	73.6 ± 2.22	52.5 ± 1.44
Joffe's	27.3 ± 0.78	30.3 ± 1.44	17.0 ± 1.82
Czapek dox	299.5 ± 2.60	396.3 ± 1.96	370.3 ± 0.78

a = Average of 3 replicates.

* = Days after inoculation.

± = Standard error

4.3.1.3. pH

The growth of all microorganisms are affected to a great degree by the pH of the medium in which they grow. To determine the effect of pH, buffer systems have to be used to stabilize the pH. In the present investigation buffer solutions with pH values ranging from 3 to 10 (3,4,5,6,7,8,9,10) were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of M/30. The pH was finally adjusted using N/10 HCl or N/10 NaOH in each case. Double strength medium and the buffer was sterilized separately by autoclaving for 15 minutes at 15 lbs p.s.i. Equal parts of the buffer solution and the medium (RM) were mixed before use. Each flask containing 50 ml of the medium was then inoculated with mycelial block of *F. oxysporum* and incubated for 9 days at 28 ± 1°C. Results (Table-6) revealed that *F. oxysporum* grew to a lesser or greater extent

Effect of incubation period on growth of microorganisms

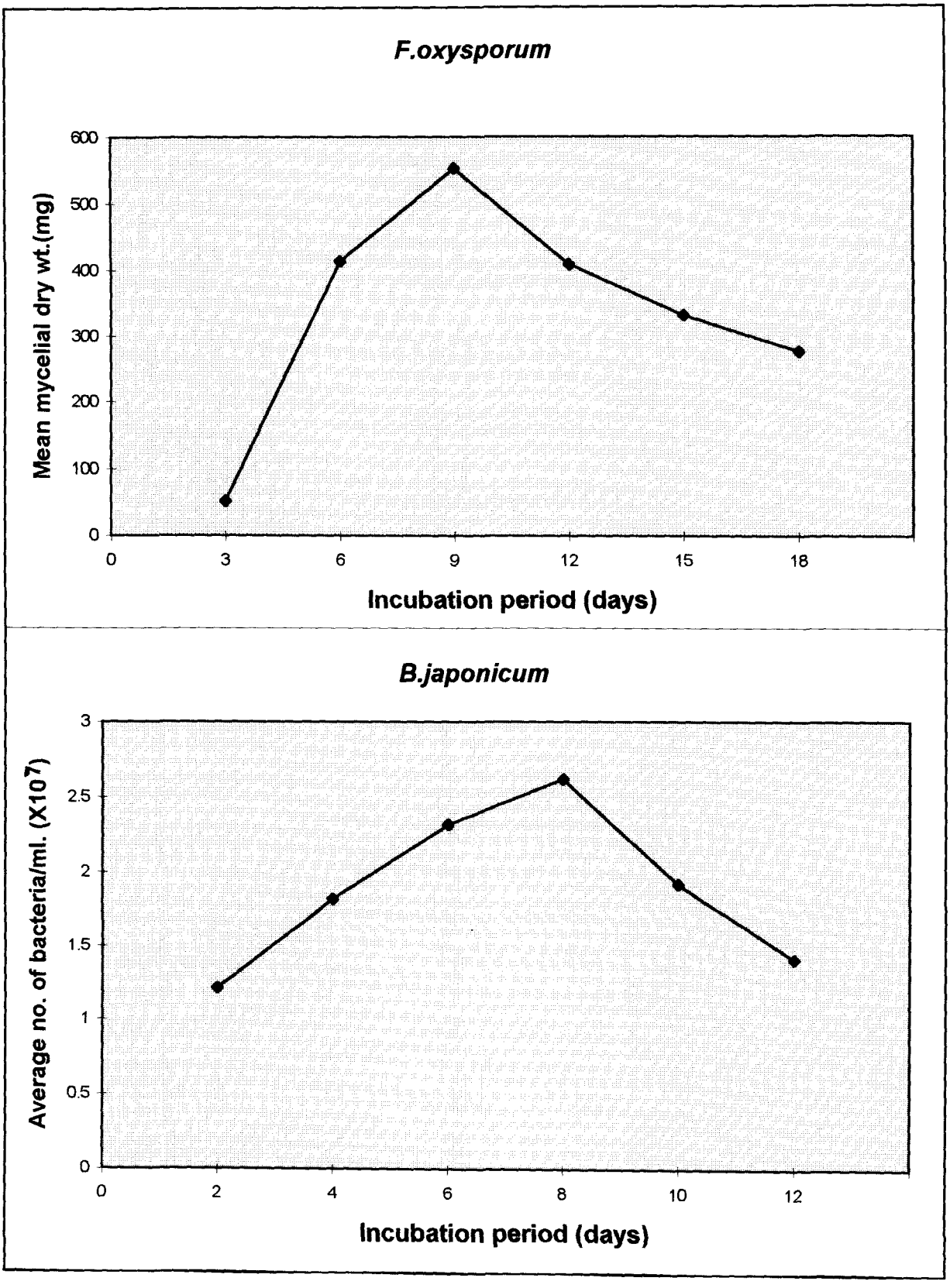


Fig.3

Table 5 : Effect of incubation period on Ithe growth of *F. oxysporum.*

Incubation time (Days)	Average dry wt. of mycelia (mg)*
3	51.6 ± 1.73
6	413.5 ± 1.15
9	553.0 ± 2.02
12	409.3 ± 1.96
15	331.6 ± 2.62
18	276.5 ± 1.51

* = Average of 3 replicates.

± = Standard error.

in all the pH tested, though maximum growth was recorded at pH 5 (642.5 mg dry weight).Minimum growth was recorded in pH3 followed by pH10.

4.3.1.4 Nitrogen Sources

The growth of any organism is dependent on the available nutrition and nitrogen is undoubtedly the single most important nutrient. The availability of nitrogen depends to a great degree on the form in which it is supplied. Thus the most suitable nitrogen source for any particular microorganism can only be determined by testing a number of sources including both inorganic and organic. In this study the effect of different nitrogen sources (both inorganic and organic) on the mycelial growth of *F. oxysporum* was tested. Basal Richards medium without any nitrogen source was considered as control. Mycelial dry wt was determined after 6,9 and 12 days of inoculation. Results (Table -7) revealed that among all the sources tested, both inorganic and organic, yeast extract was the best source followed by peptone. Organic sources were in general better source than inorganic ones. Minimum growth was evident when ammonium sulphate was used as nitrogen source. In the basal medium without nitrogen insignificant growth was recorded.

Table 6 : Effect of different pH on mycelial growth of *F. oxysporum.*

pH of medium[a]	Mean mycelial dry wt (mg)*
3	287.5 ± 2.60
4	530.0 ± 4.62
5	642.5 ± 3.75
6	606.0 ± 1.15
7	557.3 ± 4.06
8	525.3 ± 3.31
9	428.5 ± 2.08
10	391.0 ± 2.31

[a] Richards medium

* Average of 3 replicates

± Standard error.

4.3.1.5. Carbon sources

The ability of fungi to grow in different media depends on their capacity to utilise the available nutients of which carbohydrates are the major ones. All carbohydrates are not utilized by the fungus in the same rate and so the growth rate varies with different carbon sources. Eight different carbon sources were used in the present investigation, keeping Richard's medium as the basal. Data were recorded after 6, 9 and 12 days of inoculation. Among the different sources, dextrose was the optimum source while lactose supported minimum growth (Table - 8). All other sources supported moderately good growth. Growth without any carbohydrate was minimum.

Table 7 : Effect of different nitrogen sources on mycelial growth of *F. oxysporum*

Nitrogen sources	Mycelial dry wt. (mg)*		
	6 days	9 days	12 days
Inorganic			
Potassium nitrate	439.5 ± 2.02	604.5 ± 1.44	417.0 ± 1.71
Calcium nitrate	250.0 ± 4.92	536.6 ± 4.40	481.3 ± 1.97
Sodium nitrate	276.0 ± 6.34	349.0 ± 6,02	307.0 ± 5.20
Ammonium sulphate	71.0 ± 1.70	108.0 ± 4.04	82.3 ± 1.91
Organic			
Yeast extract	658.0 ± 2.62	1.009 ± 2.56	681.0 ± 2.87
Peptone	279.0 ± 5.40	951.6 ± 4.84	771.3 ± 6.44
Beef extract	260.3 ± 3.60	862.3 ± 1.79	623.0 ± 1.97
Casein acid hydrolysate	433.3 ± 1.52	836.6 ± 3.23	749.0 ± 3.46
Control (without nitrogen)	10.8 ± 0.98	50.6 ± 0.72	41.2 ± 1.02

[a] Basal medium - Richards.

* Average of 3 replicates.

± Standard error.

4.3.2. *B. japonicum*

Effect of various factors on the growth of *B japonicum invitro* were also determined in order to ascertain the optimum growth conditions. The various factors tested in this investigation include incubation period, pH as well as nitrogen and carbon sources in the medium. Growth of bacteria was assessed on the basis of OD values as described in materials and methods.

Table 8 : Effect of different carbon sources on mycelial growth of *F. oxysporum.*

Carbon sources[a]	Mycelial dry wt (mg)*		
	6 days	9 days	12 days
Sucrose	426.5 ± 0.86	547.0 ± 3.46	413.5 ± 3.17
Dextrose	688.0 ± 1.73	904.0 ± 3.46	560.5 ± 2.02
Fructose	556.5 ± 3.75	723.0 ± 2.89	650.0 ± 0.57
Lactose	55.3 ± 0.98	155.0 ± 3.46	88.0 ± 2.62
Sorbose	208.0 ± 1.73	596.5 ± 1.44	464.5 ± 2.60
Starch	297.0 ± 0.57	468.0 ± 2.94	409.6 ± 1.51
Mannitol	184.5 ± 0.86	702.3 ± 1.96	547.6 ± 3.31
Sorbitol	179.5 ± 3.17	617.0 ± 1.15	505.0 ± 2.89
Control (without sugar)	-	-	-

[a] Basal medium - Richards

* Average of 3 replicates

± Standard error.

4.3.2.1. Incubation Period

The bacterium was allowed to grow in yeast extract mannitol broth and growth measurements were done at every two day intervals, since this was a slow growing bacterium. Results presented in Table 9 and Fig. 3 revealed that growth increased for a period of upto 8 days after which it declined. The average number of bacteria /ml after 8 days of growth was more than double that of two days growth.

4.3.2.2. pH

It is well known that the pH of the medium usually plays on important role in the growth of microorganisms. The utilization of nutrients depends partially upon the pH of the culture medium. Hence in the present investigation the

Table 9 : Effect of different incubation period on the growth of *Bradyrhizobium japonicum.*

Incubation time (Days)	Average number of bacteria / ml.
2	$1.21x10^7$
4	$1.82x10^7$
6	$2.32x10^7$
8	$2.62x10^7$
10	$1.92x10^7$
12	$1.41x10^7$

effect of different pH was also studied on the growth of *B. japonicum.* The pH range varied from 3 to 10. It was observed that *B. Japonicum* grew best at pH 7 and also in pH 6 while the growth in pH either above or below this range was lesser. There was a sharp decline in growth at pH 10 while at pH 3 also the bacterium grew to a moderate extent. (Table 10)

4.3.2.3. Nitrogen Sources

The growth of *B. japonicum* in media with different nitrogen sources was tested, using yeast extract mannitol broth as the basal medium. Maximum growth was obtained with either yeast extract or casein acid hydrolysate as the nitrogen source. Among the inorganic sources calcium nitrate supported maximum growth followed by ammonium sulphate (Table 11)

Table 10 : Effect of different pH on the growth of *B. japonicum.*

pH of medium[a]	Average number of bacteria / ml
3	$2.32x10^7$
4	$2.48x10^7$
5	$2.62x10^7$
6	$2.69x10^7$
7	$2.79x10^7$
8	$1.73x10^7$
9	$1.37x10^7$
10	$8.09x10^7$

a Yeast Extract Mannitol

Incubation period - 8 days.

4.3.2.4. Carbon Sources

Eight different carbohydrates were tested to study their effect on the growth of *B. japonicum.* Among the tested carbohydrates mannitol supported maximum growth followed by sorbitol while minimum growth took place in fructose and sorbose. (Table -12). The results seen to indicate that the most easily utilizable sugars for this bacterium are the sugar alcohols (mannitol and sorbitol).

4.4. Effect of pre-inoculation on disease development.

The rhizosphere of a plant does not contain any single organism but rather, a large number of micro-organisms interact in the rhizosphere which finally determines how the particular host responds to a pathogen. In the present investigation it was considered worthwhite to study how two microorganisms i.e. *B. japonicum* and *T. harzianum,* both beneficial to the plant affect the development of root root disease caused by *F. oxysporum.* The effects were studied both singly as well as jointly.

Table 11 : Effect of different nitrogen sources on the growth of *B. japonicum*

Nitrogen sources[a]	Average number of bacteria / ml.		
	6 days	9days	12 days
Inorganic			
Potassium nitrate	3.03×10^6	8.09×10^6	4.04×10^6
Calcium nitrate	9.10×10^6	2.02×10^7	8.09×10^6
Sodium nitrate	3.03×10^6	5.05×10^6	4.04×10^6
Ammonium sulphate	7.07×10^6	1.11×10^7	8.09×10^6
Organic			
Yeast extract	2.42×10^7	2.73×10^7	1.82×10^7
Peptone	1.11×10^7	2.32×10^7	1.31×10^7
Beef extract	1.31×10^7	2.12×10^7	1.51×10^7
Casein acid hydrolysate	2.32×10^7	2.73×10^7	1.71×10^7
Control (without nitrogen)	1.01×10^6	1.51×10^6	5.05×10^5

[a] Basal medium - Yeast extract mannitol

4.4.1. *B. japonicum*

In order to study the effect of preinoculation with *B. japonicum* on root rot disease, four sets of plants were grown in earthenware pots containing soil : sand mixture as described in materials and methods. These four sets included - (i) uninoculated seed sown in noninfested soil; (ii) uninoculated seeds grown in soil infested with *F. oxysporum* : (iii) seeds inoculated with *B. japonicum* and sown in noninfested soil and (iv) seeds inoculated with *B. japonicum* and sown in soil infested with *F. oxysporum*. All the sets were watered regularly and uprooted after 14 and 28 days following which disease assessment was made as described in materials and methods. Results are presented in Table 13 and 14, Fig. 4 and Plate IV (figs. A & D). Bacterization of seeds reduced disease intensity in all tested cultivars (Soymax, Bragg, Punjab 1, JS-2, UPSM-19 and

Table 12 : Effect of different carbon sources on the growth of *B. japonicum.*

Carbon sources[a]	Average number of bacteria / ml.		
	6 days	9 days	12 days
Sucrose	1.61×10^7	1.92×10^7	1.31×10^7
Dextrose	2.12×10^7	2.22×10^7	1.71×10^7
Fructose	4.04×10^6	5.05×10^6	1.01×10^6
Lactose	1.71×10^6	1.92×10^7	1.71×10^7
Sorbose	3.03×10^6	4.04×10^6	2.02×10^6
Starch	5.05×10^6	9.10×10^6	7.07×10^6
Mannitol	2.52×10^7	2.83×10^7	1.92×10^7
Sorbitol	1.82×10^7	2.32×10^7	1.71×10^7
Control (without carbon)	-	-	-

[a] Basal medium - Yeast extract mannitol

PK-564) both after 14 and 28 days of inoculation. The difference in all cultivars was significant at both 5% and 1% level after 28 days of inoculation.

Production of nodules in the bacterized seeds was affected to a certain degree when grown in presence of *F. oxysporum* as the number of nodules per plant registered a reduction in this case.

4.4.2. *T. harzianum*

T. harzianum is a commonly used biocontrol agent and in this study experiments were conducted to determine the effect of *T. harzianum* on root rot disease development. Both *T. harzianum* and *F. oxysporum* were inoculated in the soil as described in detail in materials and methods. Plants were uprooted and disease assessment was done as before. Results (Table 15 and Fig.5)

Effect of pre-inoculation with *B.japonicum* on soybean root rot

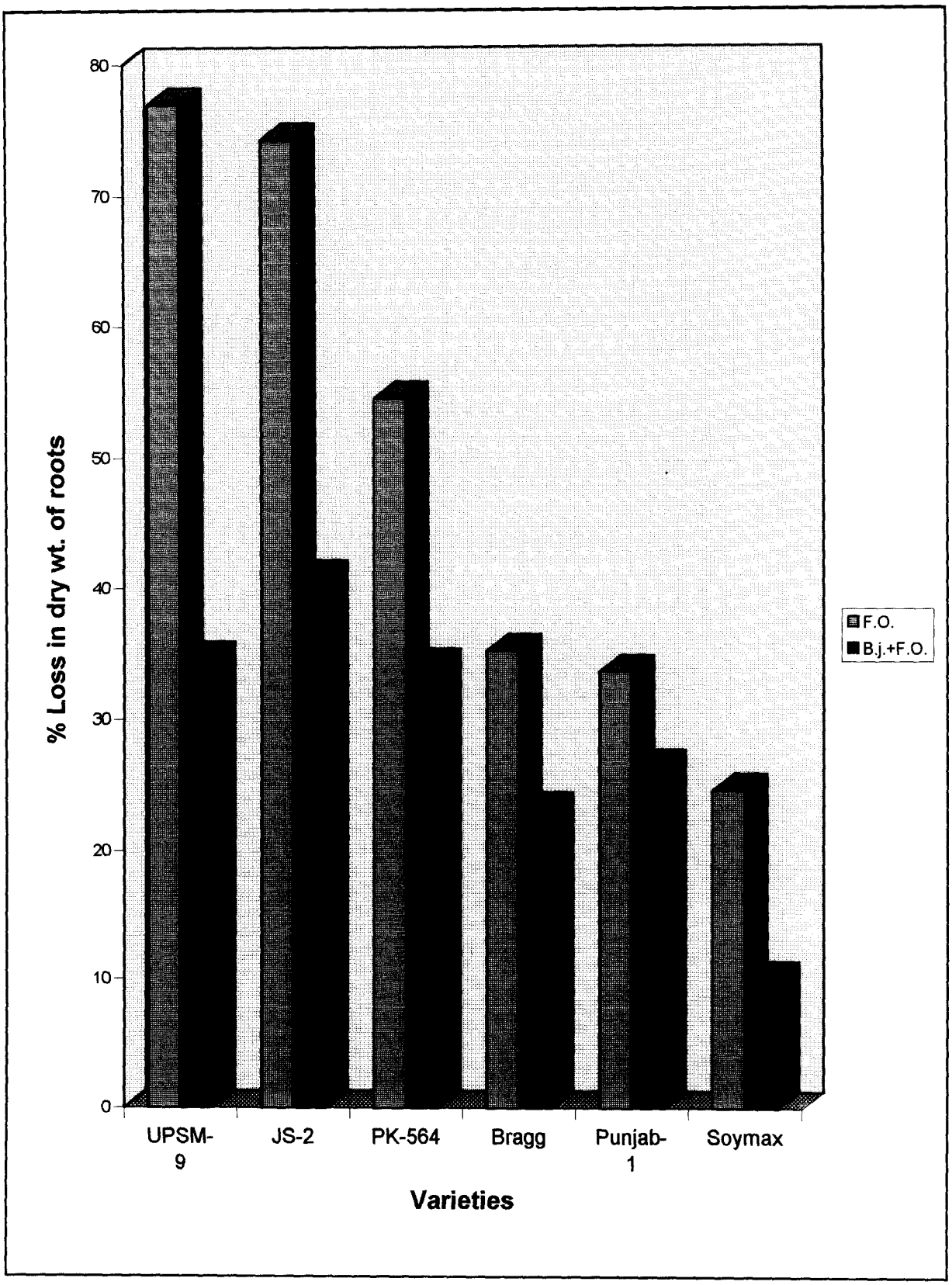


Fig.4

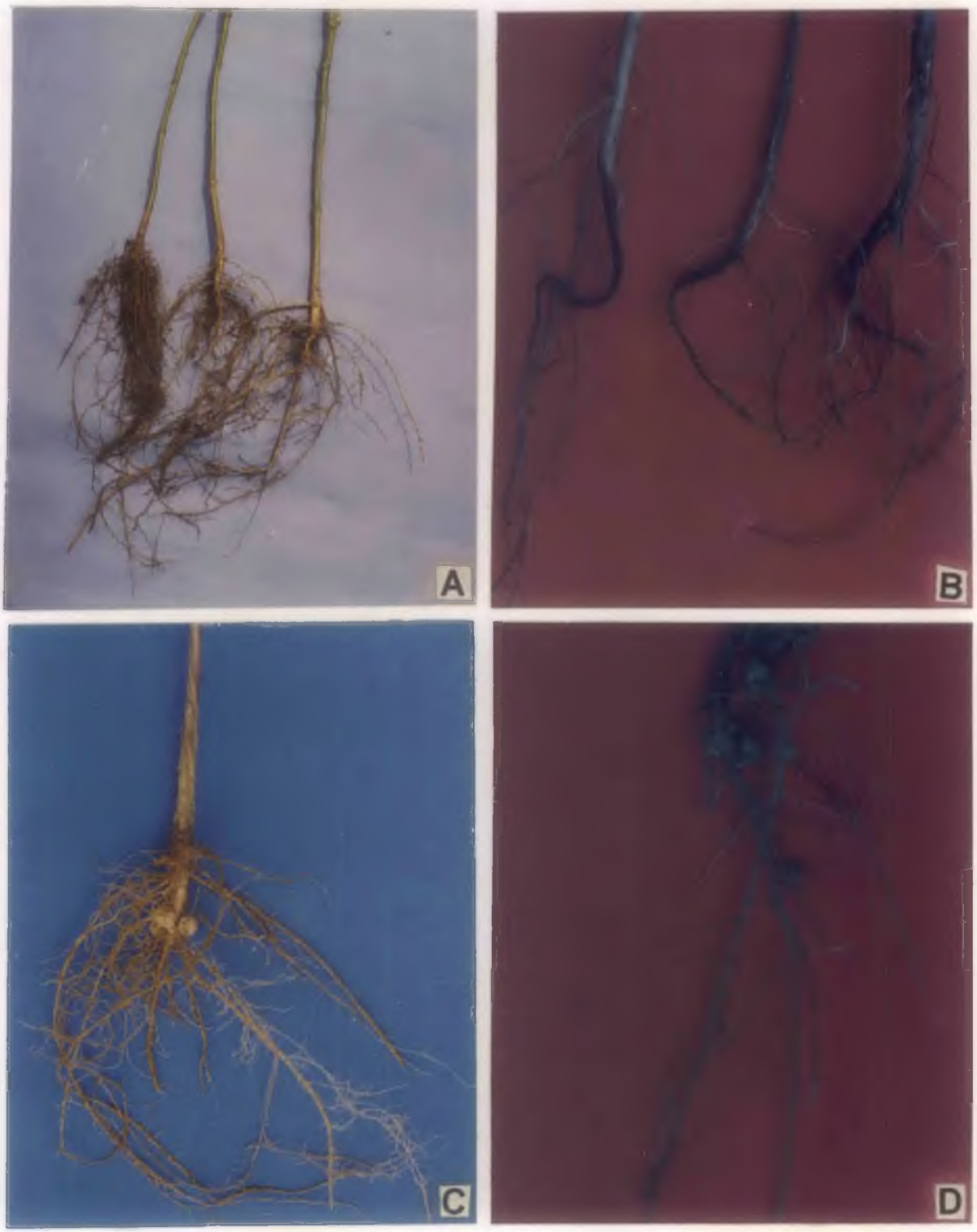


Plate IV (figs. A - D) : Portion of soybean roots . Healthy **(A)** ; Inoculated with *F. oxysporum* **(B)** , *B.japonicum* (**C**) & *B.japonicum* + *F.oxysporum* **(D)**

Table 13 : Effect of pre inoculation of soybean seeds with *Bradyrhizobium japonicum* on root rot disease development (14 days after inoculation)

Cultivar	Dry wt. of roots/plant*		% loss in dry wt.	Colour intensity*	Dry wt. of roots/plant*		%loss in drywt.	Colour intensity
	Healthy	Inoculated			*B.j*	*B.j +F.o*		
Soymax	130.3 ± 1.22	110.6 ± 0.72	15.12[a] ± 1.02	-	158.0 ± 1.24	140.3 ± 0.98	11.39[a] ± 0.82	-
Bragg	121.6 ± 1.65	89.3 ± 1.18	26.56[a] ± 1.18	+	137.0 ± 0.98	107.3 ± 1.90	21.68[a] ± 1.04	-
Punjab 1	114.0 ± 1.18	81.3 ± 1.44	28.69 ± 1.14	+	156.6 ± 1.15	119.3 ± 1.63	23.82 ± 1.81	-
JS-2	112.6 ± 1.65	66.6 ± 0.98	40.32[a,b] ± 1.89	++	128.0 ± 1.44	93.3 ± 1.96	27.11[a,b] ± 1.21	+
UPSM-19	129.3 ± 0.98	71.6 ± 0.98	44.49[a,b] ± 1.36	++	126.3 ± 1.65	96.3 ± 2.37	23.75[a,b] ± 1.45	+
PK-564	119.6 ± 1.44	78.3 ± 2.12	34.53 ± 1.89	+	146.3 ± 2.05	100.6 ± 2.12	31.24 ± 1.14	-

* In relation to control on the basis of 3 separate trials of 10 plants each.

** + - Light brown ; + + - Deep brown ; +++ - Blackish brown ++++- Black

a Difference between % loss in dry wt. due to *F. oxysporum* inoculation and due to *B. japonicum* inoculation significant at 5% level, and a, b = difference significant at 5% and 1% level in 't' test.

Table 14 : Effect of pre-inoculation of soybean seeds with *Bradyrhizobium japonicum* on root disease development (28 days after inoculation)

Cultivar	Dry wt. of roots/plant*		% loss in dry wt.	Colour* intensity	Dry wt. of roots/plant*		%loss in dry wt.	Colour inensity
	Healthy	Inoculated			*B.j*	*B.j +F.o*		
Soymax	213.0 ± 1.96	160.6 ± 2.59	24.60 ± 1.02	+	234.0 ± 2.16	210.3 ± 2.37	10.13 ± 1.04	-
Bragg	171.3 ± 1.18	111.6 ± 3.03	35.20 ± 1.25	++	204.3 ± 2.23	157.0 ± 2.83	23.15 ± 1.62	+
Punjab 1	169.0 ± 2.02	112.3 ± 1,65	33.55 ± 1.25	+	199.3 ± 1.96	146.6 ± 2.16	26.44 ± 1.14	-
JS-2	160.6 ± 3.42	41.3 ± 3.44	74.20 ± 2.51	++++	173.6 ± 2.37	102.6 ± 2.65	40.90 ± 2.05	++
UPSM-19	184.3 ± 2.59	42.6 ± 3.03	76.89 ± 2.14	+++	189.6 ± 2.67	124.0 ± 2.83	34.60 ± 2.18	+
PK-564	165.3 ± 2.32	75.3 ± 2.94	54.45 ± 1.89	+++	184.0 ± 1.90	121.3 ± 1.18	34.08 ± 2.24	+

* In relation to control on the basis of 3 separate trials of 10 plants each

** + - light brown; ++ Deep brown ; +++ Blackish brown ; ++++ - Black.

Difference in % loss in dry wt. due to inoculation with *F. oxysporum* and with *B. japonicum* + *F. oxysporum* significant at 1% level in 't' test in all cases.

revealed that disease intensity in both the cultivars tested (Soymax and JS-2) was reduced markedly by *T harzianum. T harzianum* also reduced nodulation since the number of nodules was lesser when plants were grown in presence of *T harzianum.*

Table 15 : Effect of *T. harzianum* on disease development by *F. oxysporum* in soybean (28 days after inoculation)

Cultivar	Treatment	Dry wt. of roots/plant (mg)*	Colour intensity **
	Healthy	265.0 ± 1.81	+
	T. harzianum	262.8 ± 2.04	+
Soymax	*F. oxysporum*	190.2 ± 1.30	++
	F. oxysporum + *T. harzianum*	216.3 ± 1.40	
	Healthy	271.3 ± 2.06	+
	T. harzianum	269.4 ± 2.10	+
JS-2	*F. oxysporum*	94.6 ± 1.08	+++ +++
	F. oxysporum + *T. harzianum*	203.8 ± 2.06	+

* Based on 3 separate trials of 10 plants each.

**+ - light brown; ++ - Dark brown : +++ - Blackish brwon ;

++++ - Black.

Effect of pre-inoculation with *T.harzianum* on soybean root rot

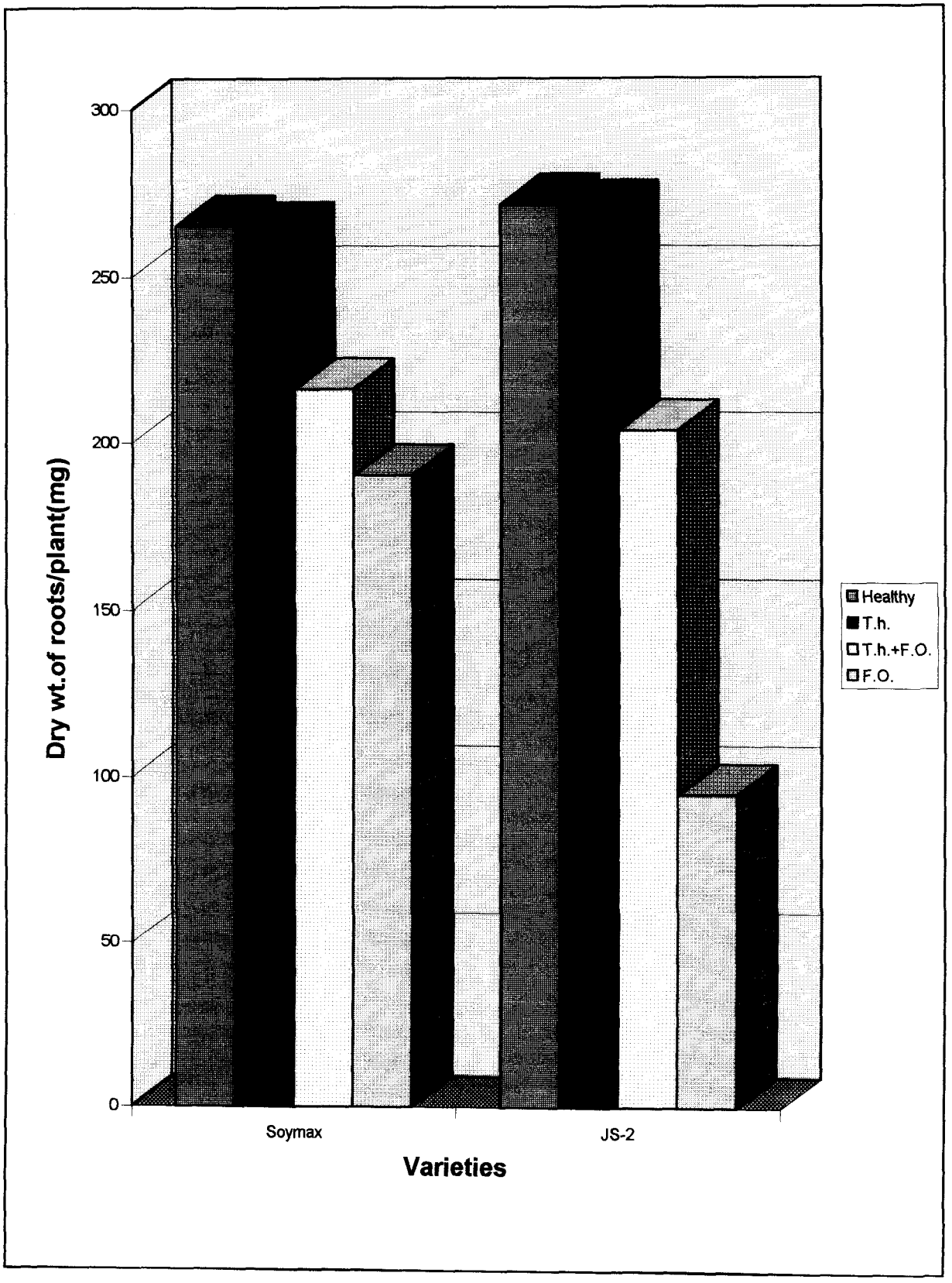


Fig.5

4.4.3. *B. japonicum* + *T. harzianum*

Since both *B. japonicum* and *T. harzianum* reduced disease intensity when treated separately, it was next decided to determine how joint inoculation with both of these microorganisms affect the development of root rot disease. For this, eight sets of plants were grown as follows, (i) uninoculated control, inoculated with (ii) *B. japonicum, (iii) T. harzianum ; (iv) B. japonicum + T. harzianum ; (v) F. oxysporum ; (vi) B. japonicum + F. oxysporum ; (vii) T. harzianum+ F. oxysporum (viii) B. japonicum + F. oxysporum + T. harzianum.* Assessment of disease revealed that though both *B. japonicum* and *T. harzianum* reduced disease intensity when treated separately, the effect of joint inoculation was hightly significant. Only about 4% loss in dry weight of roots was observed in joint inoculation as against 70% loss in dry weight when. *Fusarium* alone was inoculated (Table 16, Fig. 6). Inoculation with *B. japonicum* alone reduced percentage loss to a approximately 35% while *T. harzianum* reduced it to about 20%.

4.5. *Invitro* interaction studies

Results of the previous experiments indicated that both *B. japonicum* and *T. harzianum* reduced root rot disease intensity caused by *F. oxysporum.* This reduction in disease could be caused either due to inhibition of growth of the pathogen by the biocontrol agent or due to modification of biochemical changes in the host due to preinoculation with the respective organisms. Therefore in order to determine the mechamism by which disease reduction is brought about by *B. japonicum* and *T. harizianum,* a series of experiments were conducted. Initially *invitro* studies were carried out to determine whether any of the two organisms had antagonistic property.

4.5.1. *B. japonicum* and *F. oxysporum*

To determine the effect of *B. japonicum* on the growth of *F. oxysporum* and vice-versa, experiments were conducted in both solid and liquid medium.

Table 16 : Effect of *B. japonicum* and *T. harzianum* on disease development by *F. oxysporum* (cv. JS-2)

Treatment		Dry wt. of roots / plant (mg)*	Colour intensity**
Uninoculated	Control	252.6	-
Treated	*B. japonicum*	278.4	-
	T. harzianum	256.4	-
	B. japonicum + *T. harzianum*	259.1	-
Inoculated	*F. oxysporum*	80.0 (68.33)	++++
Treated	*B. japonicum* + *F. oxysporum*	182.9 (34.30)	++
	T. harzianum + *F. oxysporum*	204.2 (20.36)	++
	B. japonicum + *F. oxysporum* + *T. harzianum*	249.2 (3.82)	+

* Based on 3 separate trials of 10 plants each after 28 days of inoculation with *F. oxysporum*

** + - Light brown ; ++ - Dark brown ; +++ Blackish brown : ++++ Black

Figures in parenthesis indicate percentage loss in dry wt. of roots in relation to respective control.

Effect of *B.japonicum* and *T.harzianum* on soybean root rot

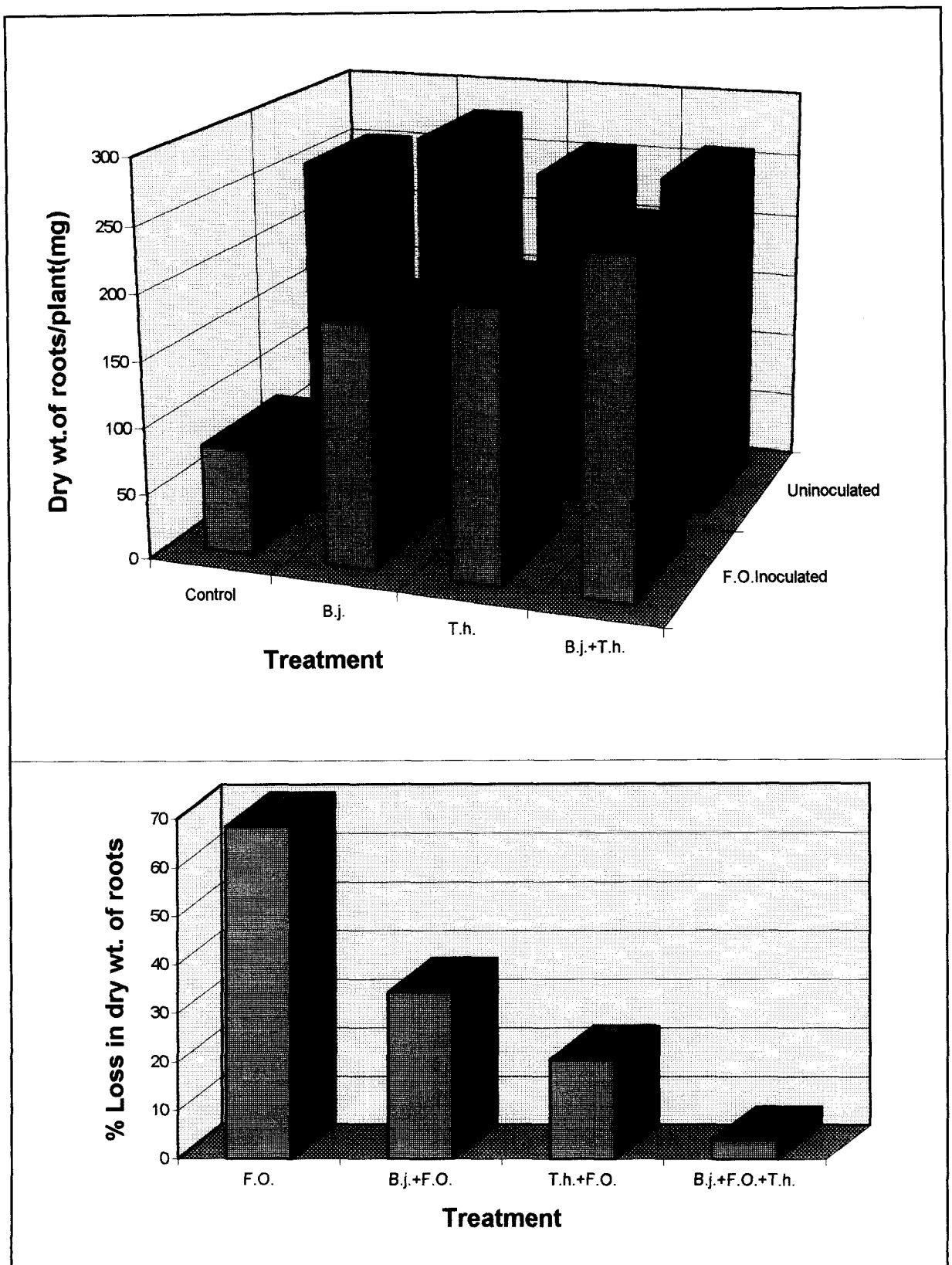


Fig.6

4.5.1.1. Solid medium

Pairing experiments were carried out with *B. japonicum* and *F. oxysporum* on YEMA. In the petridish (9 cm dia) a block of *F. oxysporum* was placed near one edge, while a streak of *B. japonicum* was made on the opposite edge. A block of *F. oxysporum* alone served as control. The petridishes were incubated for a period of 7 days after which observations were made. It was observed that *F. oxysporum* overgrew the bacterium and covered the petridish (Plate V, figs. A&B). This indicated that *B. japonicum* was not antagonistic to *F. oxysporum.*

4.5.1.2. Liquid medium

Interaction between *B. japonicum* and *F. oxysporum* was also studied in liquid culture. For this 50 ml YEM were taken in 250 ml Ehrlenmeyer flasks and inoculated either with a 4mm block of *F. oxysporum,* 0.5 ml of bacterial suspension ($1X10^7$ bacteria / ml) or both together. At 3 day intervals mycelial dry weight and bacterial growth were assessed as described in materials and methods. Results (Table 17) revealed that the growth of both organisms was inhibited to a slight degree in mixed culture. Growth of *F. oxysporum* was only reduced insignificantly whereas there was a greater reduction in growth of *B. japonicum.*

Invitro studies between *B. japonicum* and *F. oxysporum* therefore revealed that *B. japonicum* was not antagonistic to *F. oxysporum* and possibly reduced disease by changes in the host metabolism.

4.5.2. *T. harzianum* and *F. oxysporum*

Since T. hazianum significantly reduced root rot disease caused by *F. oxysporum invivo,* the effect of *T. harzianum* on the growth of the pathogen was determined *invitro.* Invitro interactions were studied on solid medium following which the effect of the culture filtrate of *T. harzianum* on the mycelial growth of *F. oxysporum* was also determined.

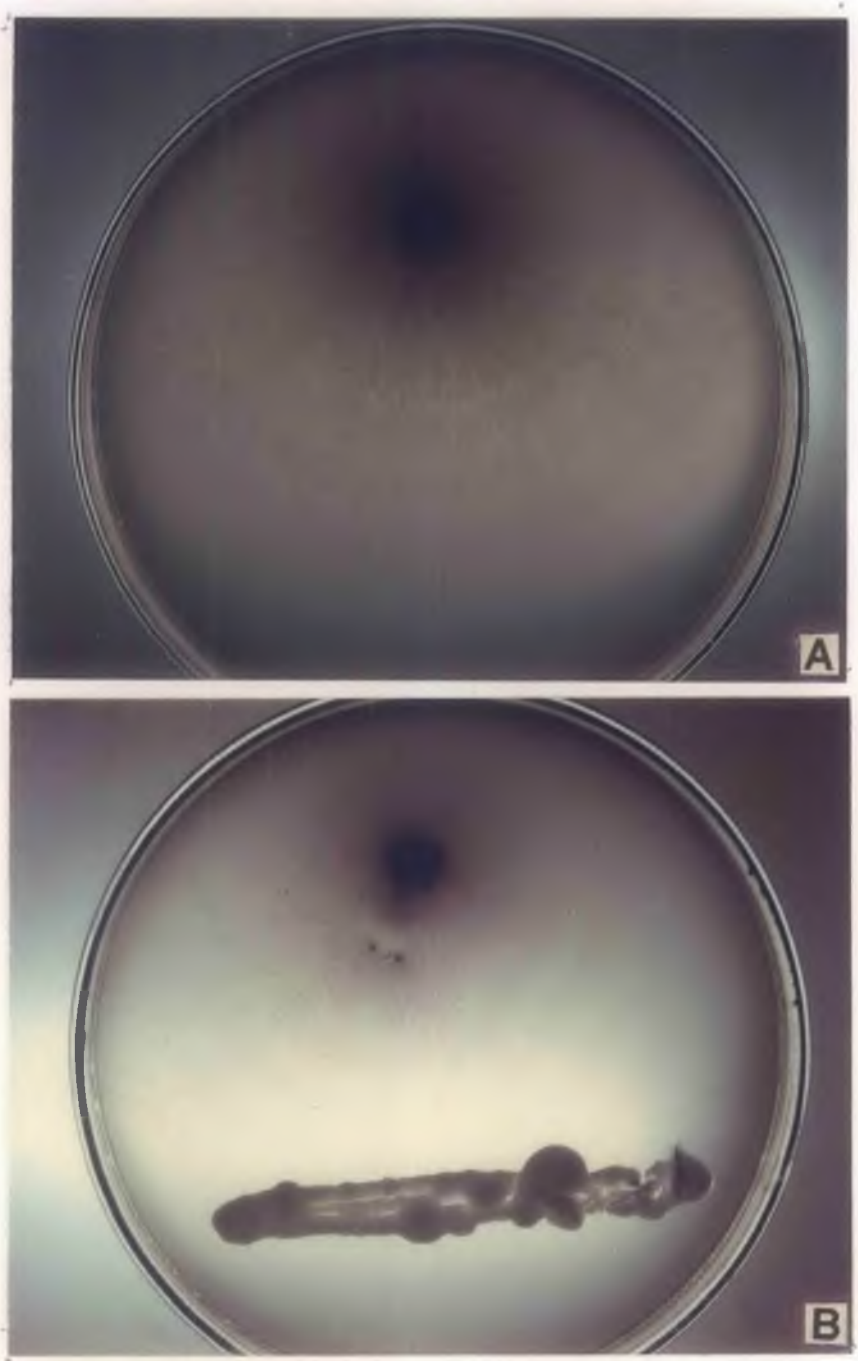


Plate V (figs. A & B) : Growth of *F.oxysporum* on YEMA.
A - Control ; **B** - Paired with *B. japonicum*

Table 17 : Interaction between *F. oxysporum* and *B. japonicum* in liquid culture.

Organism	Average dry wt. of mycelia (mg)**			Average no. of bacteria /ml		
	6*	9*	12*	6*	9*	12*
F. oxysporum	56.6 ± 0.98	71.6 ± 1.18	47.0 ± 1.24	-	-	-
B. japonicum	-	-	-	1.61×10^7	2.93×10^7	1.41×10^7
F. oxysporum + *B. japonicum*	49.6 ± 0.54	68.3 ± 1.18	43.3 ± 0.72	8.09×10^6	1.21×10^7	1.01×10^7

* Days after inoculation

** Mean mycelial dry wt. of 3 replicates

Growth medium - Yeast extract mannitol

± Standard error.

4.5.2.1. Solid medium

For this experiment, mycelial blocks of both *T. harzianum* and *F. oxysporum* were placed on PDA medium in petriplates. The two fungi were allowed to grow separately (control) as well as jointly. Observations were made after a period of 8 days and it was seen that *T. harzianum* inhibited the growth of *F. oxysporum* significantly (Plate VI, figs. B&C). *T. harzianum* overgrew *F. oxysporum*, thus inhibiting its growth. The growth and sporulation of *T. harzianum* was not affected by *F. oxysporum*.

4.5.2.2. Culture filtrate

As the growth of *F. oxysporum* was inhibited by *T. harzianum* it was decided to test whether *T. harzianum* secretes any antifungal compounds in culture. For this initially *T. harzianum* was grown in PDB for 6 days after which the culture filtrates were collected by harvesting and centrifugation. Half of the

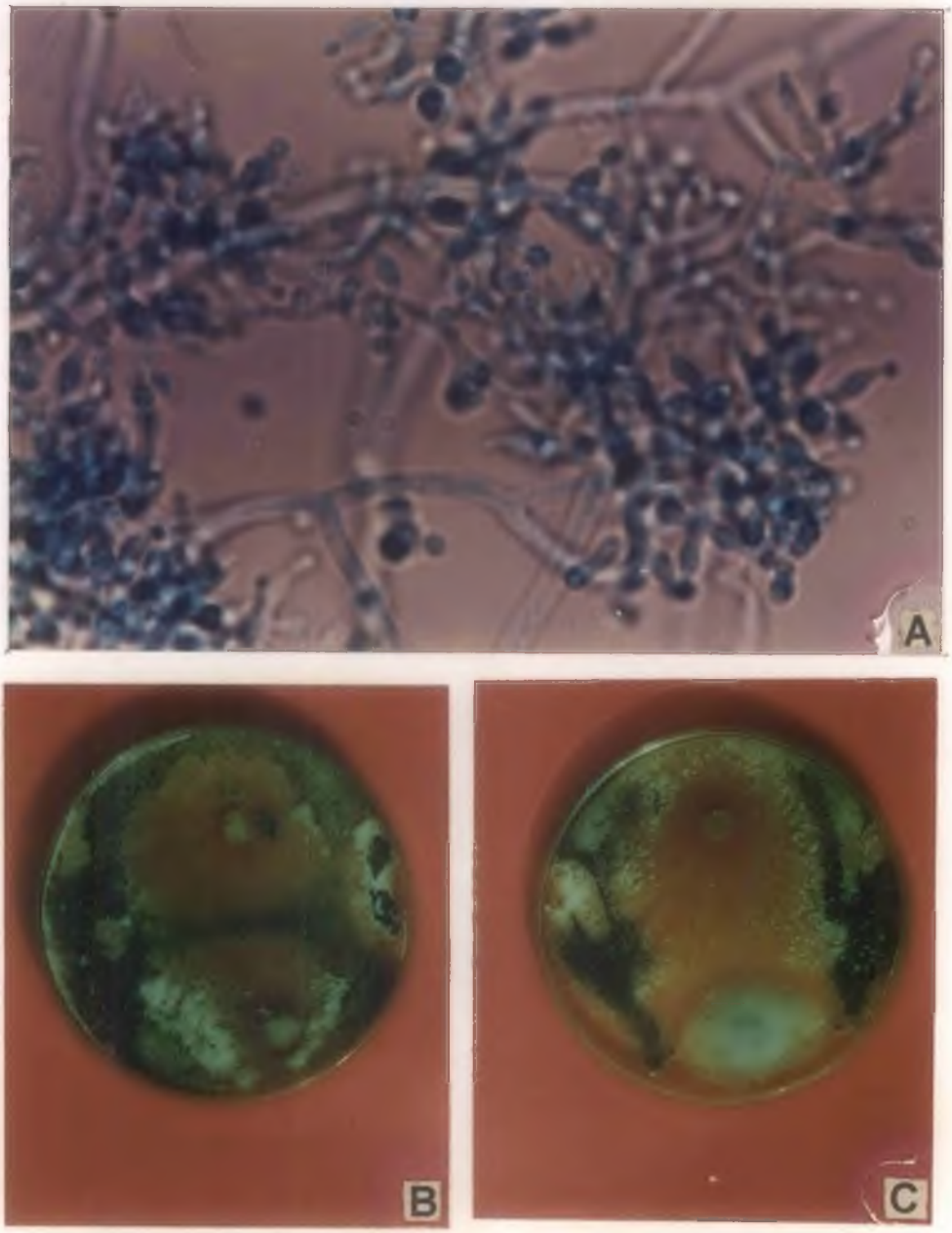


Plate VI (figs. A - C) : A - Mycelia and conidia of *T.harzianum*;
B & C - Pairing of *T.harzianum* and *F.oxysporum* on PDA;
B - *T.harzianum* alone; **C** - *T.harzianum* and *F.oxysporum*

culture filtrate was sterilized by autoclaving at 15 lbs p.s.i pressure for 15 minutes and the other half was sterilized by passing through a G5 bactofilter. Mycelial blocks of *F. oxysporum* was put into both outoclaved and non autoclaved culture filtrates and allowed to grow for a period of 9 days. *F. oxysporum* was also allowed to grow in fresh PDB which served as control. Results (Table 18) revealed that the culture filtrate of *T. harzianum* inhibited and growth of *F. oxysporum* to about 50%. The inhibition was evident in both autoclaved and cold sterilized culture filtrate. The antifungal principle secreted into culture by *T. harzianum,* therefore seems to be heat stable.

Results of *invitro* interaction studies between *F. oxysporum* and *T. harzianum* therefore revealed that *T. harzianum* was antagonistic to *F. oxysporum.* It probably reduced disease intensity by inhibiting the growth of the pathogen and not by bringing about any change in the biochemical mechanisms of the host.

Table 18 : Effect of culture filtrate of *T. harzianum* on the growth of *F. oxysporum.*

Treatment *	Average dry wt. of mycelia (mg)[a,b]
Control	539.6 ± 2.37
Cell free culture filtrate	
Autoclaved	377.6 ± 3.73
Unheated	369.0 ± 3.21

* Basal medium - Potato dextrose broth.

a Incubation period - 9 days

b Average of 3 replicates

± Standard error.

4.5.3. *B. japonicum* and *T. harzianum*

In the next phase of *invitro* interaction studies the interaction between *B.japonicum* and *T. harzianum* were also studied, since it was previously observed that joint inoculation with both *B. japonicum* and *T. harzianum* reduced disease significantly. Hence *invitro* studies were carried out both on solid as well as in liquid medium.

4.5.3.1. Solid medium

In paired experiments involving *T.harzianum* and *B. japonicum* both of these were allowed to grow on YEMA in petridishes as described previously. It was observed that within a period of 8 days *T. harzianum* overgrow the bacterial streak and covered the petriplates. (Plate VII, figs. A&B).

4.5.3.2. Liquid medium

T. harzianum and *B. japonicum* were inoculated into YEM jointly as well as separately as already described. Mycelial dry weights and bacterial growth were determined after 6,9 and 12 days of growth. Results (Table 19) showed that the growth of *T. harzianum* was only affected slightly by *B. japonicum* but the growth of *B. japonicum* was reduced to a greater extent by *T. harzianum.*

Thus, results of all *invitro* interaction studies between the three microorganisms clearly brings out the fact that while *T. harzianum* was antagonistic to *F. oxysporum, B. japonicum* was not antagonistic to either *F. oxysporum* or *T. harzianum.* The observed disease reduction by *T. harzianum* seems to be by its antagonistic effect on the pathogen while that of *B. japonicum* seems to be by other means, probably changes in the host metabolism. Hence further biochemical studies to determine the mechanism by which disease reduction occurs was carried out only with *B. japonicum* and no further tests were done with *T. harzianum.*

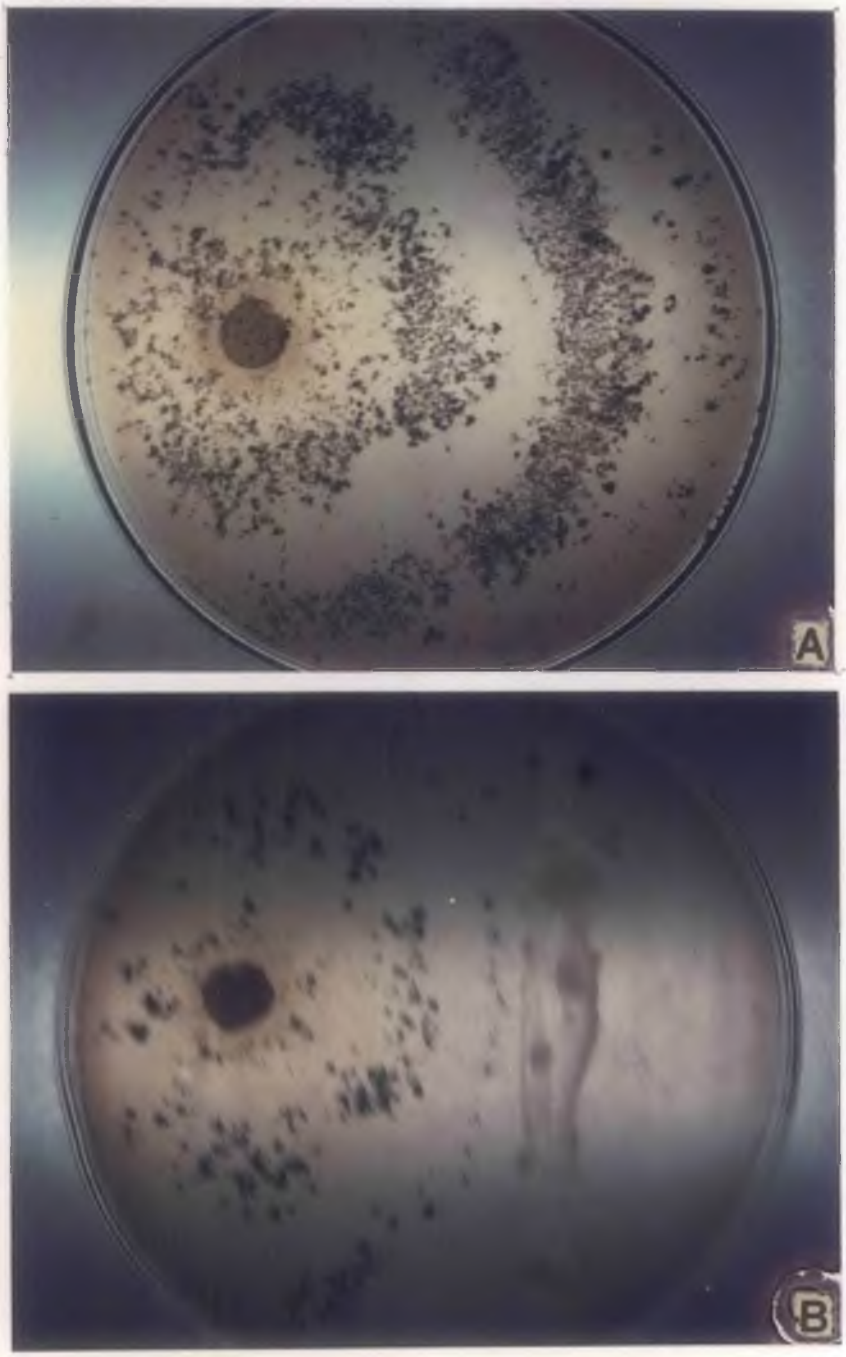


Plate VII (figs. A & B) : Growth of *T.harzianum* on YEMA;
A - Control ; **B** - Paired with *B.japonicum*

Table 19 : Interaction between *T. harzianum* and *B. japonicum* In liquid culture.

Organism	Average dry wt. of mycelia (mg)**			Average no. of bacteria /ml		
	6*	9*	12*	6*	9*	12*
T. harzianum	32.6 ± 1.18	47.3 ± 0.72	42.6 ± 0.27	-	-	-
B. japonicum	-	-	-	1.71×10^7	2.83×10^7	1.51×10^7
T. harzianum + *B. japonicum*	18.6 ± 0.54	38.3 ± 0.27	32.3 ± 0.98	6.06×10^6	1.11×10^7	9.10×10^6

* Days after inoculation

** Mean mycelial dry wt. of 3 replicates

Growth medium - Yeast extract mannitol

± Standard error.

4.6. Assay of phenylalanine ammonia lyase activity in soybean roots

Phenylalanine ammonia lyase was extracted from soybean roots at 4h intervals after inoculation with *F. oxysporum* and activity was assayed as described under materials and methods. For PAL assay two cultivars were selected, one resistant (Soymax) and other susceptible (JS-2) to *F. oxysporum*. Four sets of treatments were prepared i.e. healhy, inoculated with *F. oxysporum*, *B. japonium* and *B. japonicum* +*F. oxysporum*. Results are expressed as µg cinnamic acids produced by one gm root tissue /minute. Results are presented in Tables 20 and 21 and Figs. 7 & 8. It was observed that in general Soymax had higher PAL activity than JS-2 and following inoculation with *F. oxysporum* PAL activity increased from 4 h onwards till 16 hours of inoculation after which

it started declining. Inoculation with *B. japonicum* also increased PAL activity to some extent in relation to control but not as much as inoculation with *F. oxysporum.* In both cultivars, maximum increase in PAL activity was obtained following inoculation with *B. japonicum* + *F. oxysporum.*

Table 20 : Phenylalanine ammonia lyase (PAL) activity in soybean roots (cv. Soymax)

Time after inoculation with *F.o. (h)*	µg cinnamic acid produced / g / min*.			
	Healthy	Inoculated with *F.o.*	Inoculated with *B.j.*	Inoculated with *B.j* + *F.o*
4	2.97± 0.02	5.98 ± 0.01	3.90 ± 0.05	8.05 ± 0.05
8	3.08 ± 0.02	6.95 ± 0.18	3.43 ± 0.07	8.73 ± 0.07
12	3.20 ± 0.01	7.19 ± 0.52	4.21 ± 0.03	9.23 ± 0.02
16	2.98 ± 0.02	7.93 ± 0.09	4.18 ± 0.01	9.93 ± 0.23
20	3.01 ± 0.06	6.93 ± 0.02	4.21 ± 0.03	8.66 ± 0.23
24	2.98 ± 0.03	6.86 ± 0.07	4.16 ± 0.01	8.57 ± 0.05

* On the basis of 3 experimental sets.

± Standard error.

4.7. Assay of peroxidase activity in soybean roots.

Peroxidase is also one the enzymes generally involved in defence mechanism of a host. In this study therefore other than PAL, peroxidase activity was also assayed in soybean roots following inoculation with different microorganisms as already mentioned. Detailed procedure of extraction and

PAL activity in healthy and inoculated soybean roots (cv.Soymax)

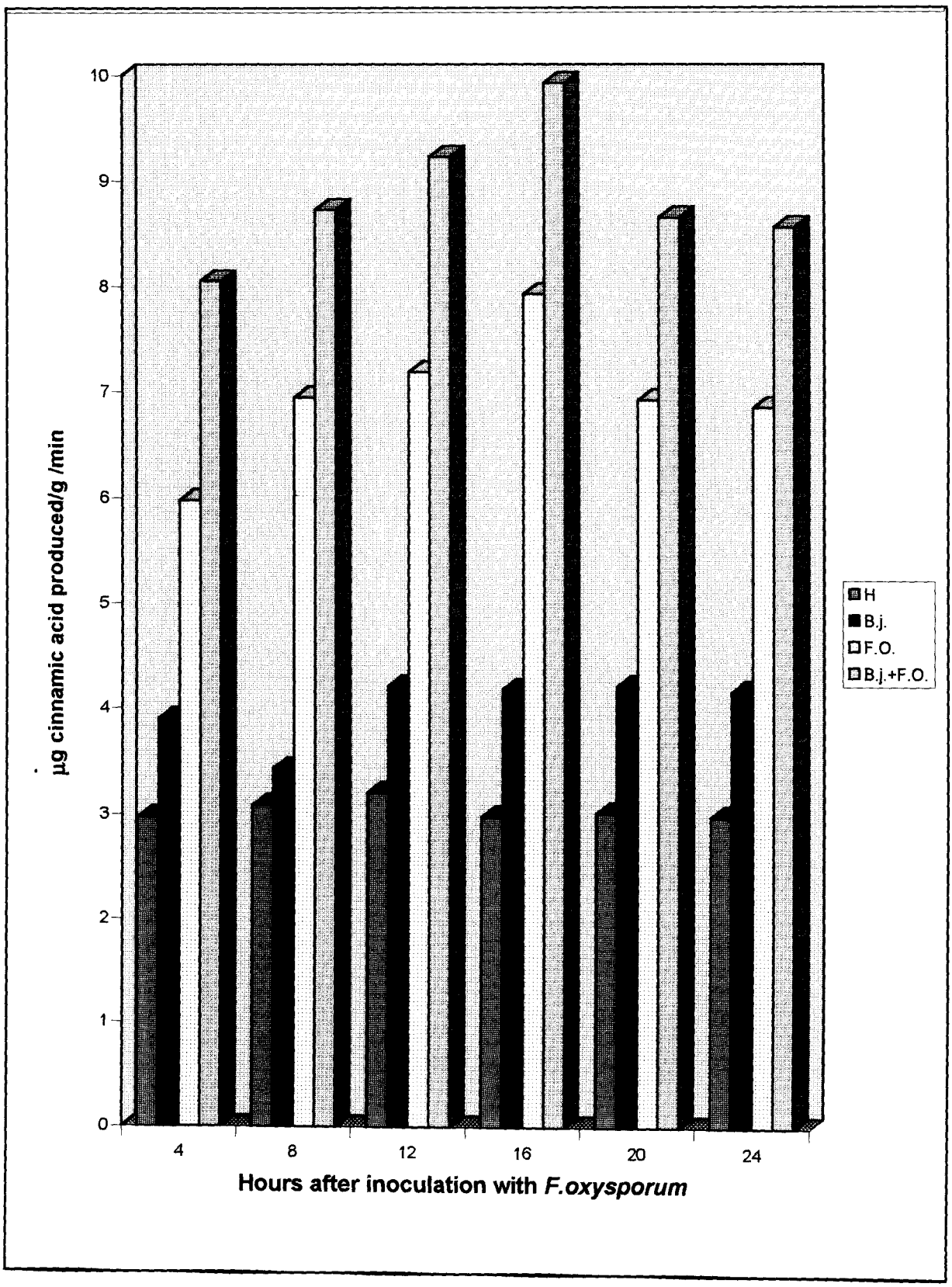


Fig.7

PAL activity in healthy and inoculated soybean roots (cv.JS-2)

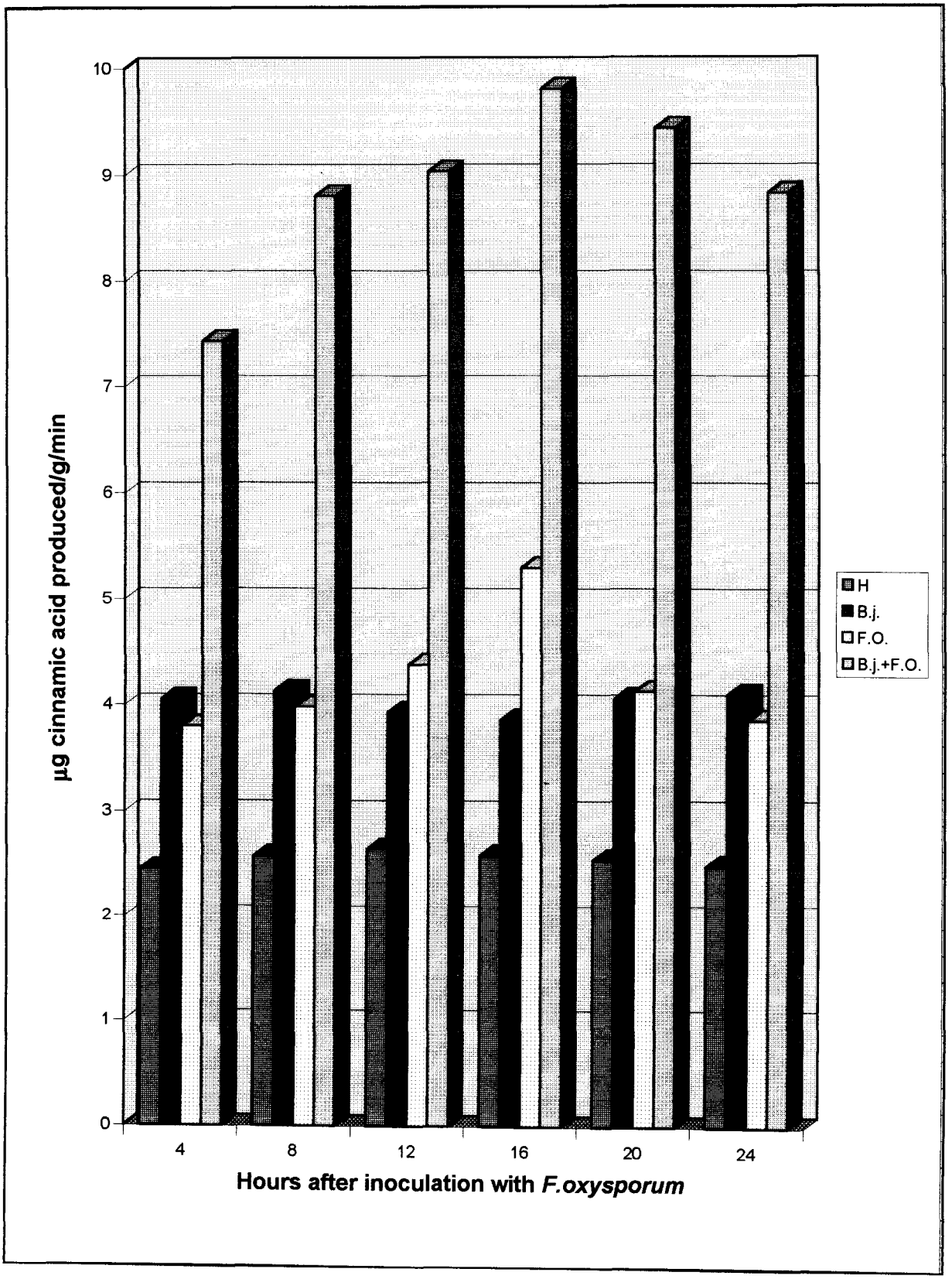


Fig.8

Table 21 : Phenylalanine ammonia lyase (PAL) activity in soybean roots (cv. JS-2)

Time after inoculation with *F.o. (h)*	µg cinnamic acid produced / g / min*			
	Healthy	Inoculated with *F.o.*	Inoculated with *B.j.*	Inoculated with *B.j + F.o*
4	2.45 ± 0.02	3.80 ± 0.05	4.05 ± 0.04	7.43 ± 0.07
8	2.57 ± 0.02	3.98 ± 0.03	4.13 ± 0.05	8.80 ± 0.05
12	3.64 ± 0.04	4.38 ± 0.03	3.93 ± 0.02	9.03 ± 0.10
16	2.58 ± 0.02	5.30 ± 0.05	3.86 ± 0.05	9.80 ± 0.16
20	3.54 ± 0.01	4.13 ± 0.02	4.06 ± 0.02	9.43 ± 0.07
24	2.48 ± 0.04	3.86 ± 0.02	4.10 ± 0.05	8.83 ± 0.09

* On the basis of 3 experimental sets.

± Standard error.

assay have been given in materials and methods. Peroxidase activity has been expressed as change in absorbance at 460 nm brought about by enzyme extract from one gram root tissue / minute. Results as presented in Tables 22 and 23 and Figs. 9 and 10 showed that maximum activity was obtained after 12h of inoculation with *F. oxysporum* in both the cultivars. Activities in both cultivars were more or less similar and inoculation with *B. japonicum* did not greatly affect the activity. Neither was the activity increased following inoculation with both *F. oxysporum* + *B. japonicum*.

Table 22 : Peroxidase activity In healthy and inoculated roots of soybean (cv. Soymax)

Time after noculation with *F.o. (h)*	Δ A 460 nm / g tissue / min			
	Healthy	Inoculated with *F. o*	Inoculated with *B. j*	Inoculated with *B.j + F.o*
4	6.92	6.03	6.84	5.75
8	6.21	6.09	6.17	6.10
12	6.72	11.63	6.92	7.96
16	7.15	8.15	7.00	8.92
20	6.35	6.70	6.40	7.14
24	5.66	5.62	5.42	4.92

Table 23 : Peroxidase activity in healthy and inoculated roots of soybean (cv.JS-2)

Time after inoculation with *F.o. (h)*	Δ O.D at 460 nm / g tissue / min			
	Healthy	Inoculated with *F. o*	Inoculated with *B. j*	Inoculated with *B.j + F.o*
4	7.30	7.20	7.50	7.45
8	6.93	6.85	7.08	6.84
12	6.75	12.80	7.02	9.64
16	7.42	9.25	7.36	8.54
20	7.22	7.68	7.46	7.02
24	6.86	6.58	6.90	6.74

Peroxidase activity in healthy and inoculated soybean roots (cv.Soymax)

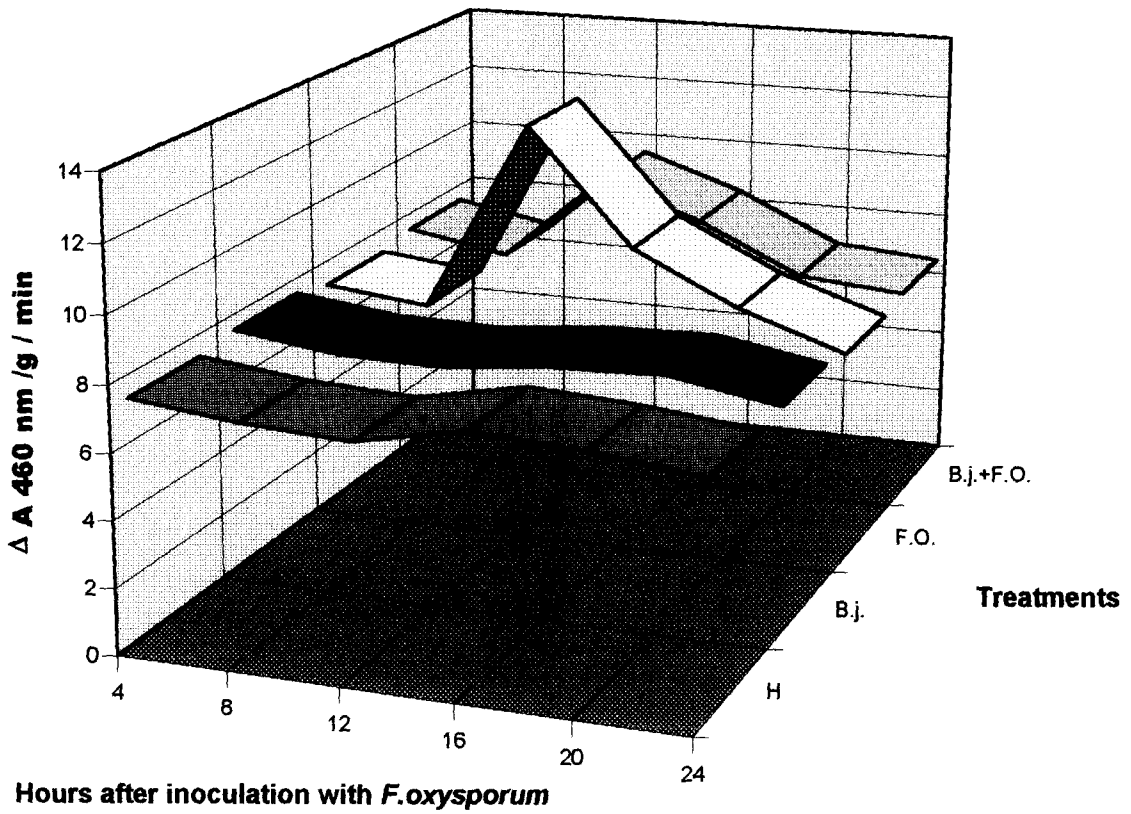


Fig.9

Peroxidase activity In healthy and inoculated soybean roots (cv.JS-2)

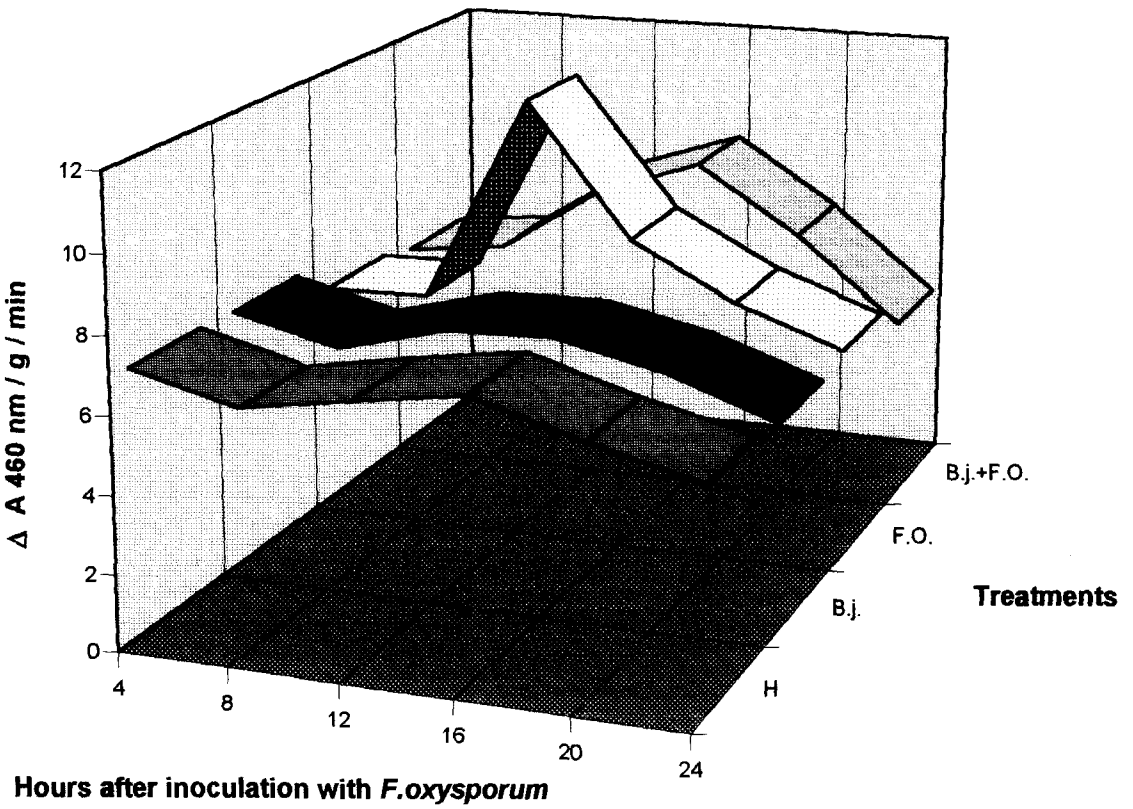


Fig.10

4.8. Glyceollin content

Among the biochemical changes in plants following inoculations with pathogen, accumulatin of phytoalexin is perhaps the most well documented (Bhattacharyya and Ward, 1986; Paxton, 1995; Avazkhodjaev et. at, 1995). Increased production of glyceollin (phytoalexin) has been reported in soybean following inoculation with a number of agents where resistant responses have been elicited. In this study therefore it was considered worthwhile to determine changes in glyceollin accumulation in a resistant and susceptible cultivar following inoculations with different microorganisms. Glyceollin extraction has been done from roots as well as from callus induced from cotyledons.

4.8.1. Roots

Glyceollin was extracted from roots of the two cultivars Soymax (resistant) and JS-2 (susceptible) after 24h of inoculation with *F. oxysporum.* Extraction procedure and quantification have already been described under materials and methods. Four treatments were considered in this case also. Glyceollin was initially detected on thin layer chromatograms on the basis of the appearance of yellow spots after spraying with p-nitroaniline. Glyceollin could only be detected in treatments with *F. oxysporum* and *B. japonicum* + *F. oxysporum*. Presence of glyceollin in the crude extracts of *F. oxysporum* as well as *B. japonicum* + *F. oxysporum* inoculated roots was further confirmed by petridish bioassay of th extracts. In petridishes where the medium was mixed with the above extracts inhibition in mycelial growth of *F. oxysporum* was obtained to about 75 %..

These extracts were further partially purified by thin layer chromatography and quantified from the absorbance values obtained at 286 nm. Results revealed that glyceollin content was much higher in the resistant roots following inoculation with *F. oxysporum* than in the susceptible ones (Table 24, Fig. 11). Further a marked increae in glyceoliin content was obtained following inoculation with both *B.japonicum* and *F. oxysporum.*

Table 24 : Glyceollin content of soybean roots after different treatments.

Treatment	Glyceollin content (µg / g fresh wt. of roots)	
	cv. Soymax	cv. JS-2
F. oxysporum	462 ± 5.8	250 ± 2.0
B. japonicum + *F. oxysporum*	541 ± 7.3	428 ± 5.5

Mean of 3 separate experiments
No glyceollin was detected in roots of healthy or *B. japonicum* inoculated plants.

4.8.2. Callus

Since the pathogen of root rot disease of soybean, *F. oxysporum* has also been reported as a latent infection in seeds (Sinclair, 1991) this created an interest to determine whether glyceollin accumulated in seed tissues following inoculation with *F. oxysporum* at rates comparable to those in roots. For this purpose initially calli were induced from cotyledons of cv. Soymax (Plate - VIII, figs. A&B) as described in materials and methods. About 2 mounth - old calli were inoculated with spore suspension of *F. oxysporum* and glyceollin was extracted from this tissue as already mentioned. The extracts were tested for the presence of glyceollin on both TLC and by petridish bioassay. Both of these confirmed the presence of glyceollin in the extracts after inoculation. Quantification of glyceollin content indicated that the cells of the callus accumulated glyceollin following inoculation at rates comparable to those in infected roots (450 µg / g tissue), while no glyceollin could be detected in healthy cells.

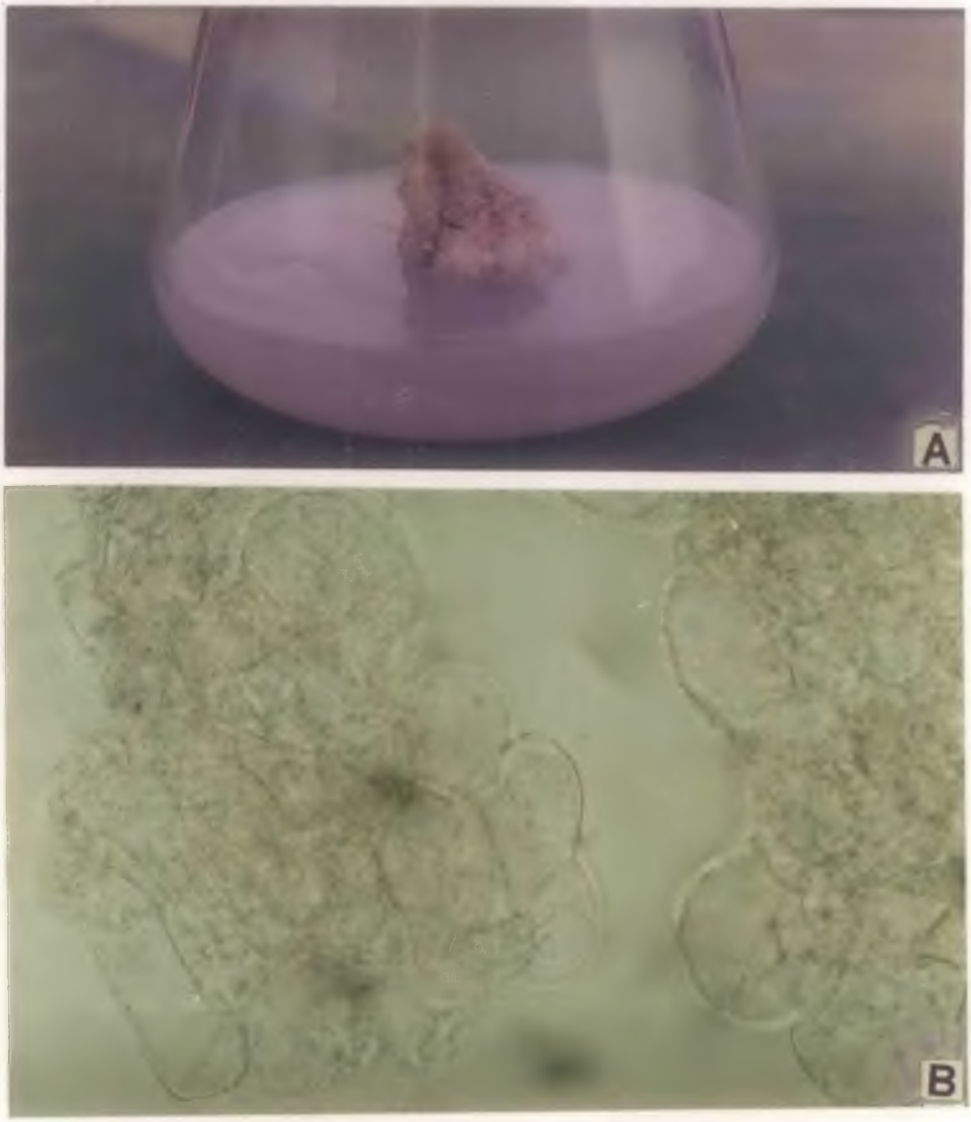


Plate VIII (figs. A & B) : A - Cotyledonary callus of soybean;
B - Cells from the callus

Glyceollin content of healthy and inoculated soybean roots

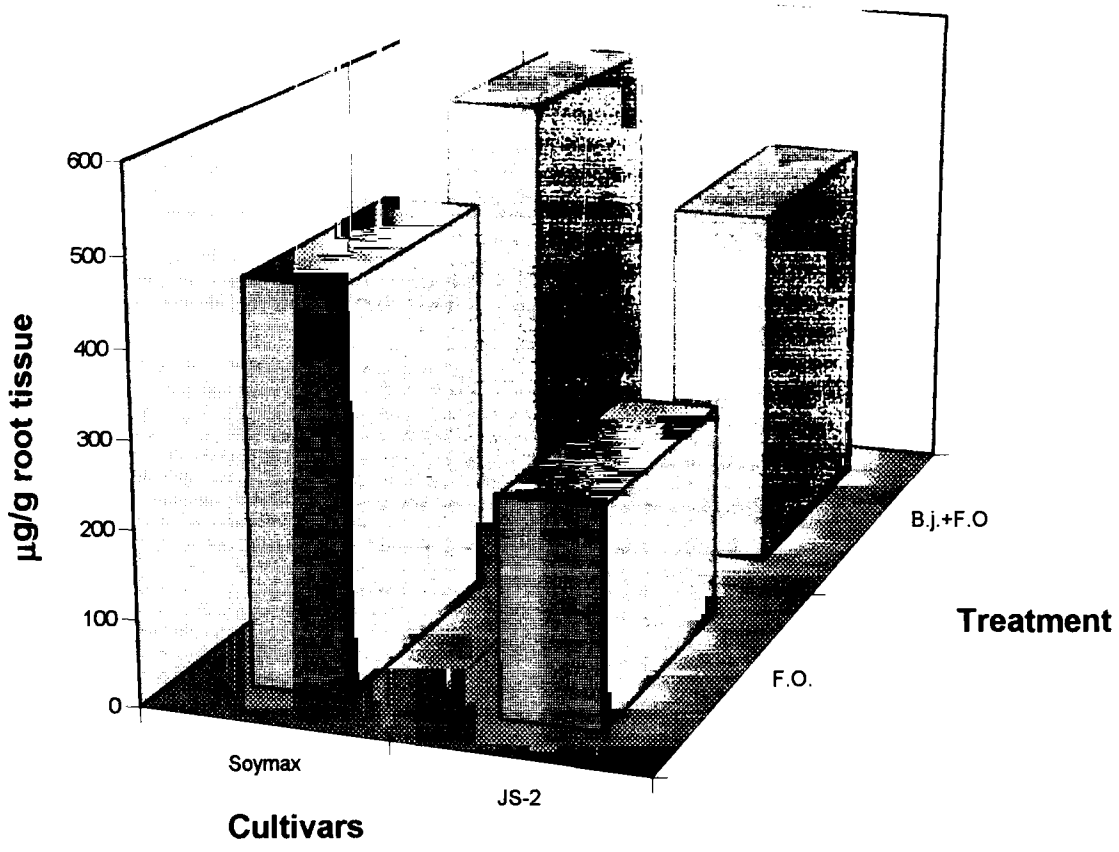


Fig.11

4.9. Analysis of proteins

Proteins are major biochemical components in all plants, Proteins are generally known to either increase or decrease due to infection by pathogen and more importantly their patterns may also change. Hence for a thorough investigation into the biochemical changes following inoculation, it was considered imperative to determine changes in the protein contents as well as protein patterns in soybean tissues. For this purpose a number of experiments were designed.

4.9.1. Protein content

Protein was extracted from the mycelia of *F. oxysporum,* soybean roots and soybean seeds and contents were estimated. Detailed procedures for extraction and estimation have been presented under materials and methods.

4.9.1.1. *F. oxysporum*

Estimation of mycelial protein content revealed that *F. oxysporum* had protein content of 2.37 mg / g/ fresh weight tissue.

4.9.1.2. Soybean roots

Proteins were extracted from roots of both Soymax (resistant) and UPSM-19 (susceptible) and estimated. Susceptible cultivar had higher protein content then the resistant cultivar while in both cultivars inoculation with either *B. japonicum*, *F. oxysporum* or *B. japonicum* + *F. oxysporum* increased protein content (Table 25, Fig. 12). This increase is greatest with *F. oxysporum* inoculation.

Table 25 : Protein contents of healthy and infected soybean roots

Cultivars	Protein content (mg / g root tissue)*			
	Healthy	Inoculated with *B. j*	Inoculated with *F. o*	Inoculated with *B.J + F.o*
Soymax	1.16	2.75	2.26	2.50
UPSM-19	1.42	3.65	1.80	3.25

* 28 days after inoculation with *F. oxysporum*.

4.9.1.3. Soybean seeds

Seeds were considered for studies in proteins since in nature latent infection of *F. oxysporum* is also to be found in the seeds. Surface sterilized seeds of two cultivars were placed on PDA medium in petridishes containing actively growing mycelia of *F. oxysporum*. For control, seeds were placed on media without any fungal growth, After a period of 6 days the seeds were taken out, external mycelia were washed off after which proteins were extracted and contents estimated. Results (Table 26) revealed that soybean seeds of both cultivars had much higher protein content than the roots. In this case also, following inoculation protein content increased in relation to control (Fig. 13).

Table 26 : Protein contents of healthy and *F. oxysporum* inoculated soybean seeds

Cultivars	Protein content (mg/ g tissue) *	
	Healthy	Inoculated with *F.o.*
Soymax	57.12	67.5
UPSM - 19	55.24	68.0

* 6 days after inoculation.

Protein contents of healthy and inoculated soybean roots

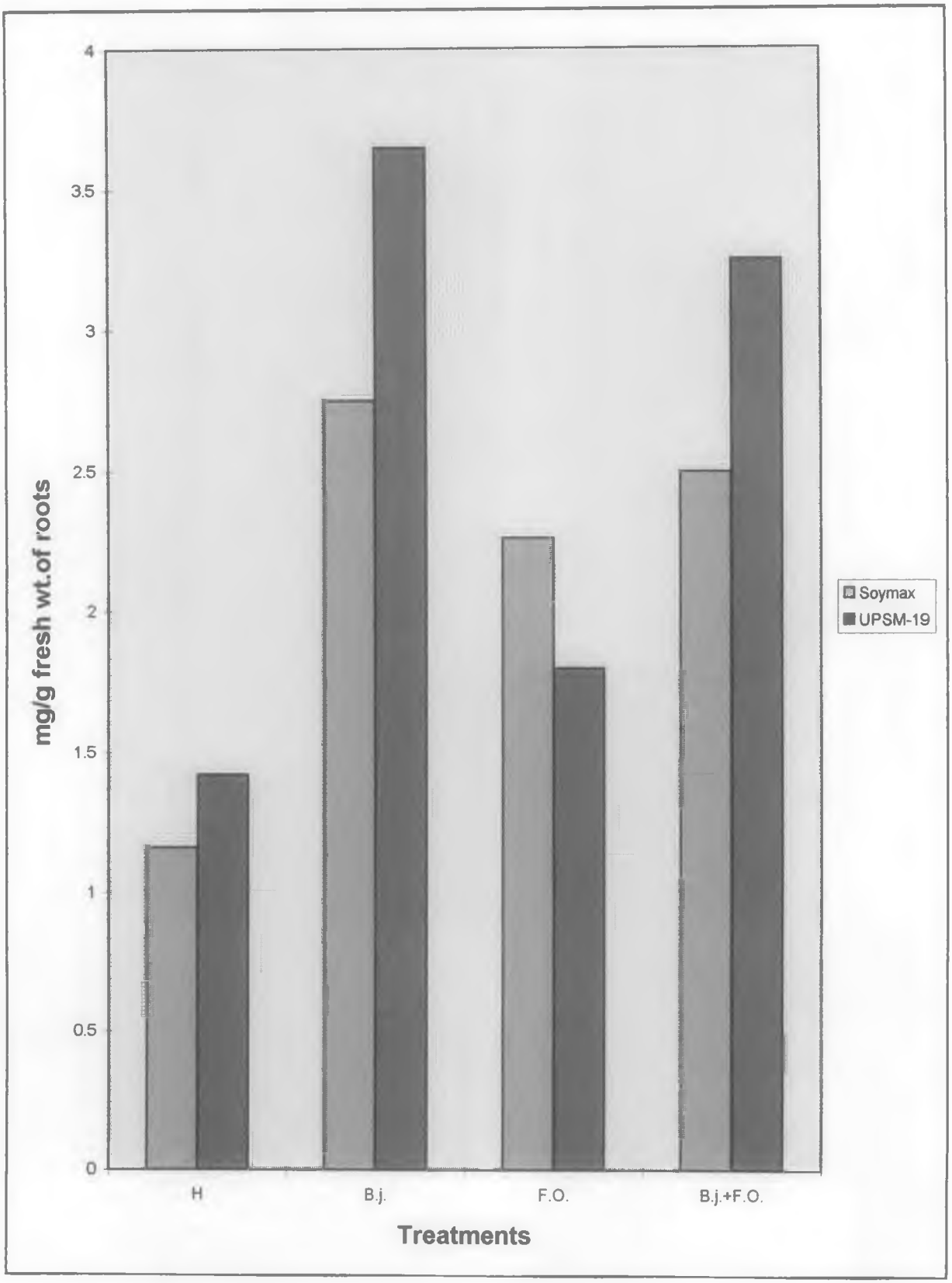


Fig.12

Protein content of healthy and inoculated soybean seeds

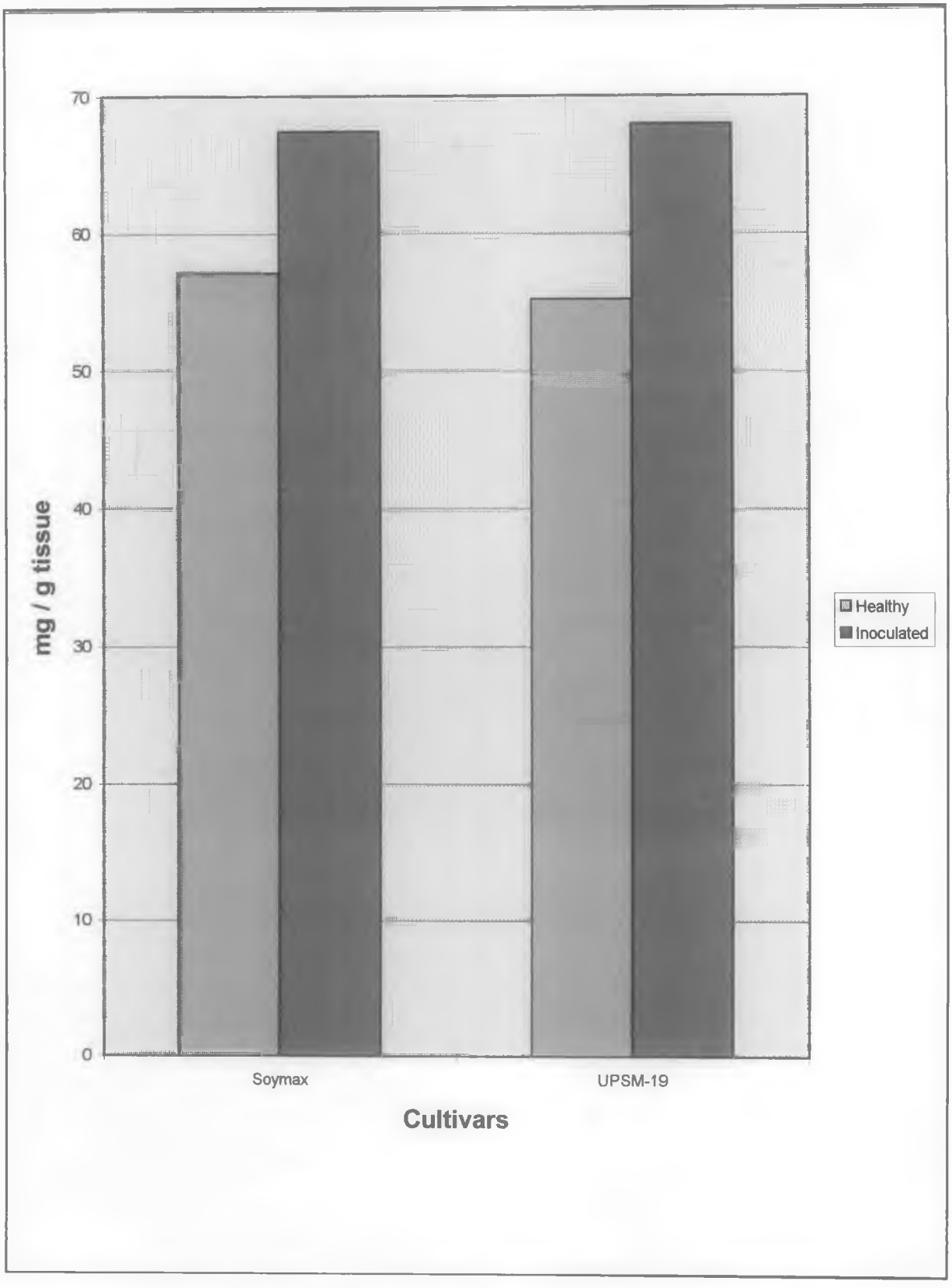


Fig.13

4.9.2. SDS - PAGE

Proteins extracted from the different sources were further analysed by SDS - PAGE following the method described under materials and methods. A number of bands were visualized in all cases following staining with coomassie blue. Molecular weights were determined from the known molecular weights of a mixture of 6 proteins as mentioned in materials and methods.

4.9.2.1. *F. oxysporum*

Mycelial protein exhibited about 35 bands ranging in molecular weight from 200 KDa to 14 KDa. The bands were of varying intensity and the lower molecular weight proteins were more prominent (Plate IX, fig. A).

4.9.2.2. Soybean roots

SDS - PAGE analysis of the different proteins revealed that the protein patterns in all four treatments were more or less similar. Roots after inoculation with *B. japonicum* had some extra bands specially in the lower molecular weight region. Two extra bands of high molecular weight were noticed following inoculation with any of the organisms (Plate IX, fig. B).

4.9.2.3. Soybean seeds

Extracts of healthy seeds of soybean exhibited some very prominent bands of approximate molecular weights 95, 70, 65, 50 KDa and very prominent one at a very low molecular weight. Following infection in the susceptible cultivar all the prominent bands disappeared excepting the one at 50 KDa. In the resistant cultivar most of the bands were still present but the intensity were much reduced. In this case two new bands at low molecular weight were evident. (Plate IX, fig. B).

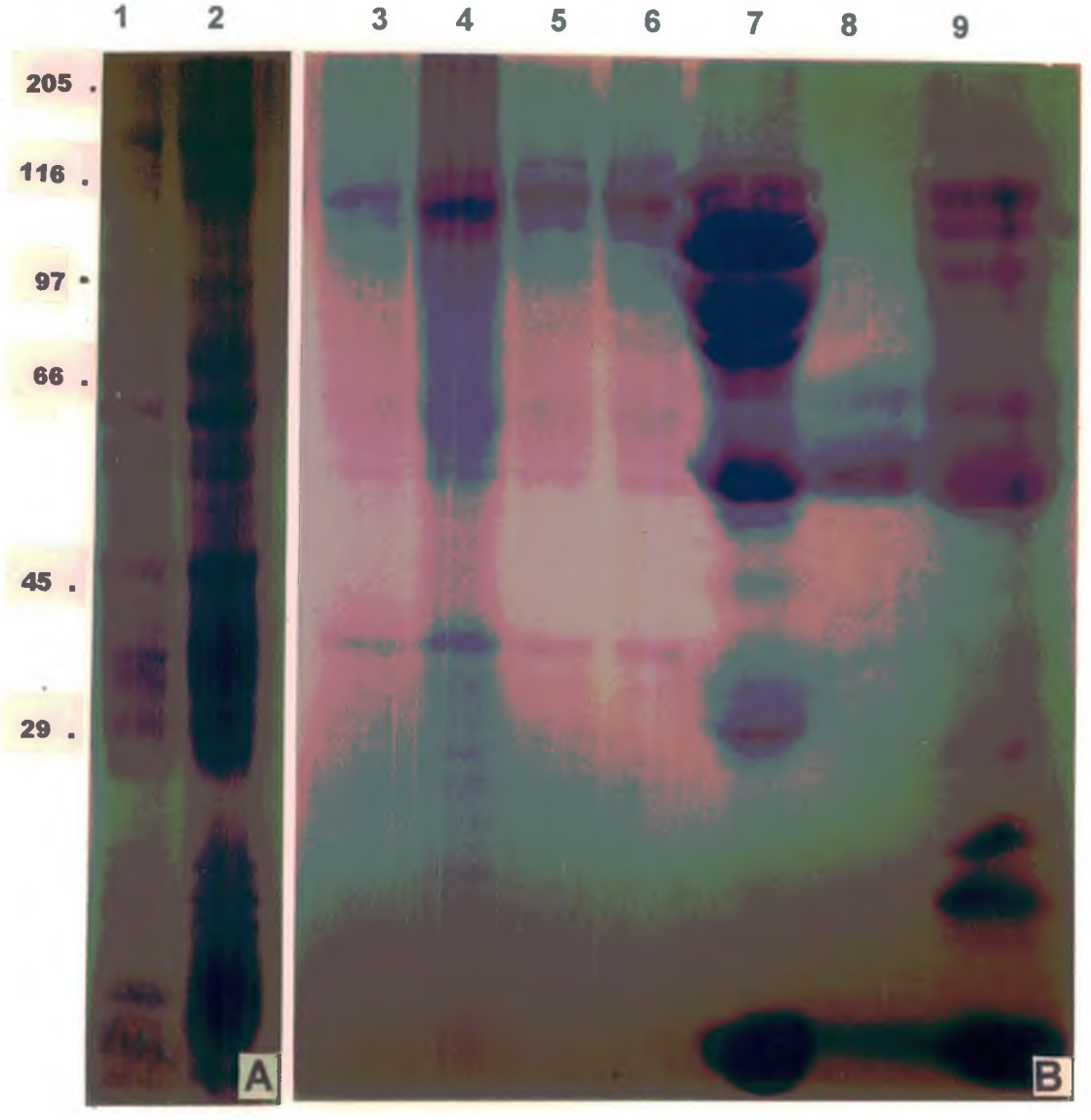


Plate IX (figs. A & B) : SDS-PAGE analysis of proteins . **A** - Mycelial proteins of *F. oxysporum* (**lanes 1 & 2**) ; **B** - Soybean root and seed proteins (**lanes 3 - 9**) ; **Lanes 3 - 6** : Root proteins of cv. Soymax (**3** - Healthy ; **4** - *B. japonicum* inoculated ; **5** - *F.oxysporum* inoculated & **6** - *B.japonicum* + *F.oxysporum* inoculated) ; **Lanes 7 - 9** : Seed proteins ; **7** - Healthy ; **8 & 9**- *F.oxysporum* inoculated ; **8** - cv. UPSM-19 & **9** - cv .Soymax

4.10. Detection of cross reactive antigens (CRA) between *F. oxysporum* and soybean cultivars

Existing studies on plant or animal host and their pathogens suggests that whenever an intimate and continuing association of cells of host and parasites occurs partners of this association have a unique resemblance to one another involving one or more antigenic determinants. Resistance or susceptibility of a plant to infection and disease development may be dependent also on the antigenic relationship of host and pathogen. The greater the antigenic similarity the greater the possibility of immunological tolerance and likelihood that the pathogen will become established in the host and cause disease. Conversely greater antigenic disparity may trigger off certain responses which lead to biochemical changes resulting in resistant responses.

Considering the above, in the present investigation following studies on several biochemical changes in soybean after inoculation with *F. oxysporum* and / or *B. japonicum,* attempts have been made to determine the serological relationship between soybean and *F. oxysporum* as well as to determine changes due to inoculation with *B. japonicum.* For this purpose immunodiffusion tests, immunoelectrophoresis, ELISA and immunofluorescence have been used.

4.10.1. Immunodiffusion

For immunodiffusion tests, initially antisera were raised against *F. oxysporum* as well as a susceptible soybean cultivar (UPSM-19) as described in materials and methods. The effectiveness of antigen preparation from soybean cultivar (UPSM-19) and *F. oxysporum* in raising antibodies was checked initially by homologous cross reaction following agar gel double diffusion tests. Control sets involving normal sera and antigens of both the host and pathogen were all negative. Antiserum of *F. oxysporum* was tested with homologous antigens as well as antigens of 10 soybean cultivars and also a non-host (*Camellia sinesis*) and a non-pathogen (*Glomerella cingulata*). Strong precipitin reactions occurred when antiserum of *F. oxysporum* was exposed to its own antigen or antigens of susceptible cultivars (JS-2, UPSM-19, PK-564, 17-PK-472,18-PK564). With resistant cultivars (Bragg, Punjab 1, Pk-327, 19.PK-466), weak precipitin reaction was observed while in case of the most resistant cultivar (Soymax) no precipitin band could be discerned (Table 27, Plate X). No reaction was observed

with antigen of non-host or non-pathogen.

In reciprocal test using antiserum raised against cv. UPSM-19 precipitin reactions were observed when this antiserum was exposed either to antigen of *F. oxysporum* or antigens of the different soybean cultivars. In this case also no precipitin reaction was observed when reacted with antigen of *C. sinensis* or *G. cingulata.*

Table 27 : Detection of cross reactive antigens among soybean cultivars and *F. oxysporum* using agar gel double diffusion[a]

Antigens	Antisera	
	F. oxysporum	**Soybean (cv. UPSM-19)**
Host		
Soybean cultivars		
Soymax	-	+
Bragg	±	+
Punjab - 1	±	+
19-PK-466	±	+
PK-327	±	+
JS-2	+	+
UPSM-19	+	+
PK-564	+	+
17-PK-472	+	+
18-PK-564	+	+
Nonhost		
Camellia sinensis	-	-
Pathogen		
Fusarium oxysporum	+	+
Non - pathogen		
Glomerella cingulata	-	-

[a] Common precipitin band :

+ Strong precipitin band

± Weak precipitin band.

- Precipitin band absent.

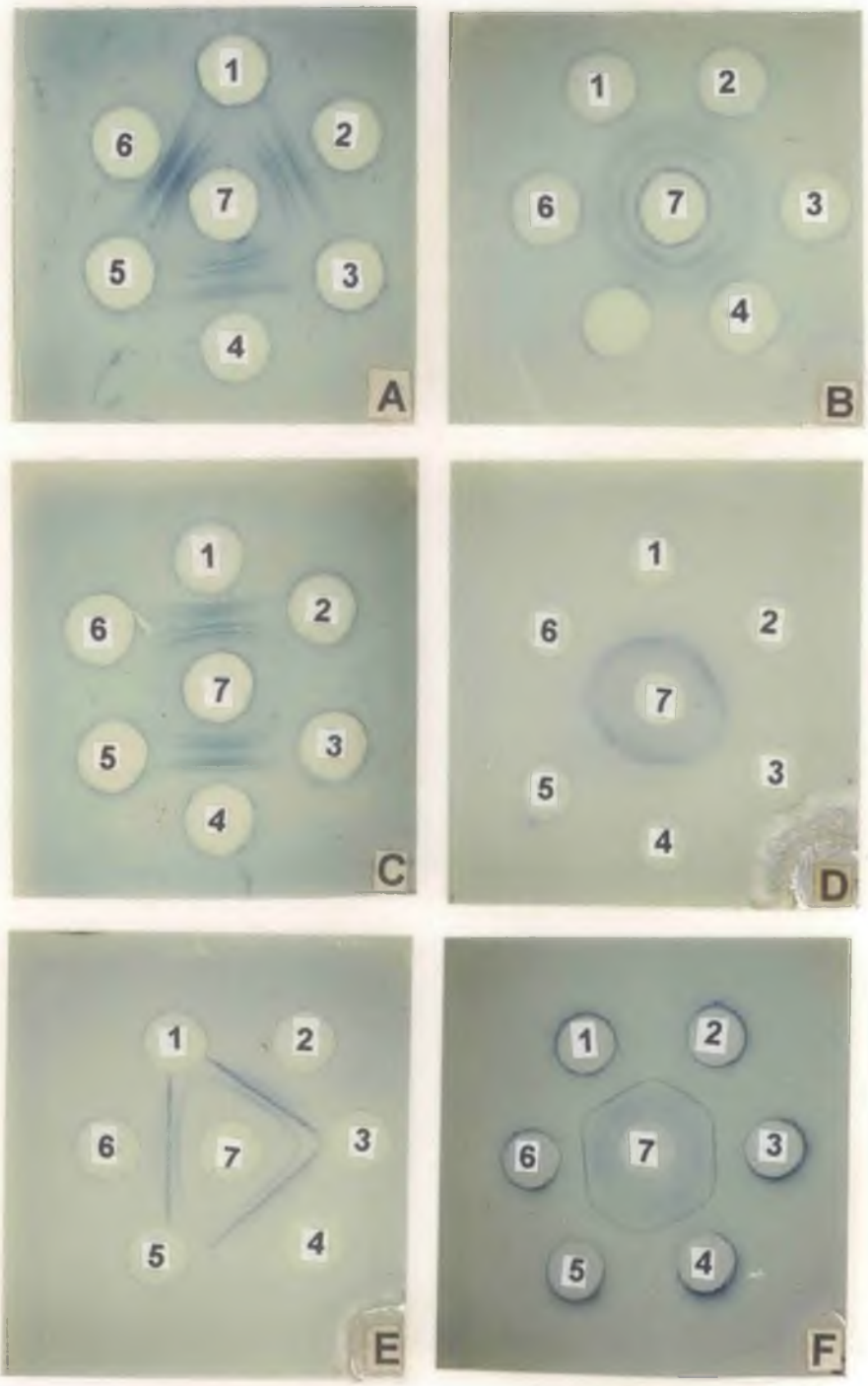


Plate X (figs. A- F) : Agar gel double diffusion tests. Central wells (7) contain antisera of *F.oxysporum* **(A,C,&E)** and soybean (cv.UPSM-19) root **(B,D & F).** Peripheral wells (1-6) contain antigens. **A** - *F.oxysporum* (2,4,6), *G.cingulata* (1,3,5) ; **B** - cv.UPSM-19 (1 ,2, 3 , 4, 5, 6) ; **C** - *F.oxysporum* - (1,4), cv.Soymax (2,3) *C.sinensis* (5,6); **D** - *F.oxysporum* - (1,2,3,4,5,6) ; **E** - cv.UPSM-19 (2,4,6) ; cv.Soymax (1,3,5) ; **F** - cv.Soymax (1,2,3,4,5,6)

4.10.2. Immunoelectrophoresis

Results of immunodiffusion tests were confirmed by immunoelectrophoresis using similar antiserum antigen combinations. Results of immunodiffusion tests revealed the presence or absence of cross reactive antigens (CRA) between *F. oxysporum* and soybean cultivars. Many of the cross reactions in the immunodiffusion gave diffused precipitin bands which could not be clearly distinguised. It was also not clear whether precipitin reactions in all cases were due to single or several antigenic substances. Therefore, further resolution was attempted by subjecting the antigens to electrophoresis before exposing them to antisera. Details of immunoelectrophoretic technique have been described under materials and methods.

Following the conventional immunoelectrophoretic set up, antigenic comparison of the ten cultivars of soybean, *F. oxysporum,* one non-host species (*Camellia sinensis*) and a non-pathogen of soybean (*Glomerella cingulata*) using antisera of pathogen (*F.oxysporum*) and host (UPSM-19) was done and results are presented in Table 28.

In homologous cross reactions, *F. oxysporum* and UPSM-19 antigens exhibited 6 and 5 precipitin arcs, each. In reactions of *F. oxysporum* antiserum with antigens of soybean cultivars; 4 precipitin lines were observed with susceptible cultivars and the number with other cultivars varied from 3-1. With Soymax (resistant cultlvar), no precipitin line was discerned. UPSM-19 also exhibited antigenic similarity with other cultivars. No lines were observed in reactions with either non-host or non-pathogens.

4.10.3. Enzyme linked immunosorbent assay

ELISA is an important technique in recent serological investigations which can specifically detect the presence of cross reactive antigens between host and pathogen or also detect the presence of pathogen infection in the host at very early stages. Several formats of ELISA have been developed in recent years of which direct antigen coated (DAC) ELISA is the most commonly used one in case of fungal pathogens. Besides DAC ELISA, Dot Blot ELISA

Table 28 : Antigenic comparison of soybean cultivars, pathogen, non-pathogen and non-host by immunoelectrophoresis.

Antigens	Total no of precipitin lines	
	Antisera	
	F. oxysporum	UPSM-19
Host		
Soybean cultivars		
Soymax	0	3
Bragg	1	3
Punjab-1	3	3
19-PK-466	3	4
PK-327	2	3
JS-2	4	4
UPSM-19	4	5
PK-564	3	3
17-PK-472	3	3
18-PK-564	3	3
Pathogen		
Fusarium oxysporum	6	4
Non-host		
Camellia sinensis	0	0
Non-pathogen		
Glomerella cingulata	0	0

can also be used for rapid detections. In the present investigation most of the tests have been performed using DAC ELISA while one emperiment was carried out using dot blot ELISA.

For ELISA, initially a series of experiments were carried out to determine the optimum conditions of the different variables i,e. enzyme, antiserum and antigen.

4.10.3.1. Optimization of ELISA

For optimization, 3 major variables were considered i,e. enzyme, antiserum and antigen dilutions. Optimization was carried out in homologous reactions.

4.10.3.1.1. Enzyme dilution

Various dilutions of the enzyme (alkaline phosphatase) was tested in ELISA using *F. oxysporum* antiserum (1 : 125 dilution) and homologous antitgen (5 µg/ml). Dilutions ranged from 1 : 10,000 to 1 : 50,000, Results (Table - 29, Fig - 14) revealed that absorbance values at 405nm decreased with increasing enzyme dilution. Absorbance values at 1 : 10,000 dilution was 1.7 which decreased to 0.4 to for 1 : 50,000 dilution. Enzyme dilution of 1 : 10,000 was used for all further experiments.

Table 29 : ELISA reaction of anti *F. oxysporum* antiserum and homologous antigen with various dilution of alkaline phosphatase.

Enzyme dilution[a]	Absorbance at 405 nm			
	Exp-1	Expt-2	Expt-3	Mean
1:10,000	1.687	1.724	1.692	1.701 ± 0.001
1:20,000	1.438	1.329	1.355	1.374 ± 0.028
1:30,000	1.201	1.226	1.192	1.206 ± 0.001
1:40,000	0.842	0.838	0.856	0.845 ± 0.005
1:50,000	0.421	0.422	0.428	0.424 ± 0.002

Mycelial antigen concentration 5 µg / ml

Anti *F. oxysporum* antiserum (1:125 dilution)

[a] Alkaline phosphatase.

± Standard error.

Effect of enzyme dilution on ELISA reaction

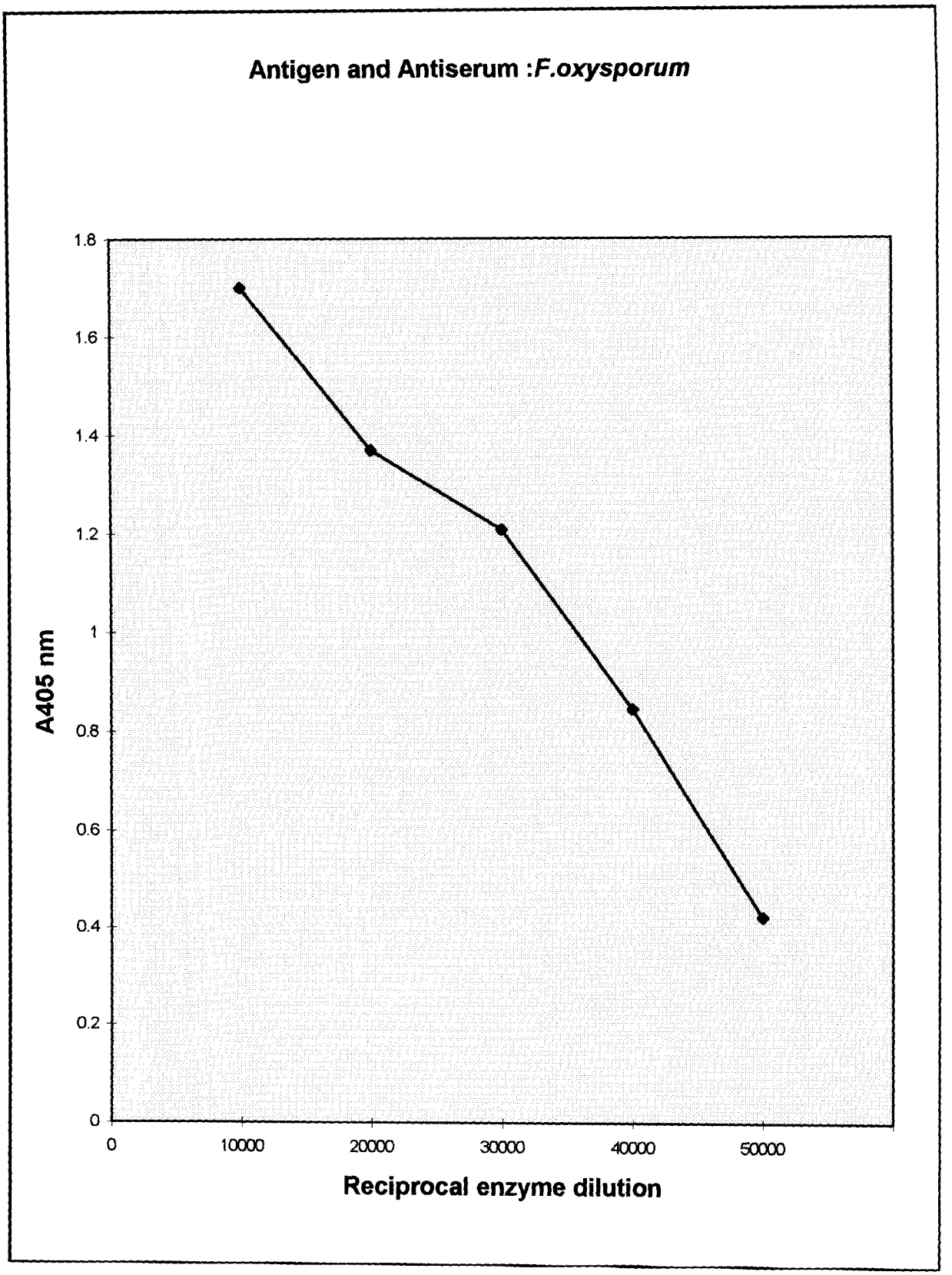


Fig.14

4.10.3.1.2. Antiserum dilution

Antiserum raised against *F. oxysporum* was used at doubling dilutions from 1 : 125 to 1 : 4000 against homologous antigen at a concentration of 5 µg/ ml in DAC ELISA. ELISA responses as indicated by A 405 nm values revealed that ELISA reactivity decreased with increasing dilutions. At 1 : 125 dilution absorbance value was 1.851 which decreased to 0.46 incase of 1.4000 dilution (Table 30, Fig. 15). Hence 1 : 125 dilution was used in further experiments.

Table 30 : ELISA reaction with various dilution of anti *F.oxysporum* antiserum and homologous antigen.

Antiserum dilution	Absorbance at 405 nm			
	Exp-1	Expt-2	Expt-3	Mean
1:125	1.863	1.844	1.836	1.847 ± 0.006
1:250	1.525	1.531	1.525	1.527 ± 0.001
1:500	1.015	0.997	1.008	1.006 ± 0.004
1:1000	0.863	0.903	0.876	0.880 ± 0.009
1:2000	0.578	0.582	0.575	0.578 ± 0.002
1.4000	0.467	0.462	0.465	0.464 ± 0.001

Mycelial antigen concentration - 5 µg / ml
Enzyme dilution -1 : 10,000
± Standard error.

4.10.3.1.3. Antigen dilution

Antigen dilution used for this experiment ranged from 3200 ng (3.2 µg) / ml to 25 ng / ml, ELISA reactivities were tested using two antiserum dilutions i,e. 1 : 125 and 1 : 250. In both cases antigen concentrations as low as 25 ng/ ml was still detectable by ELISA. ELISA reactivities decreased with increasing dilutions (Table 31, Fig. 16).

Effect of antiserum dilution on ELISA reaction

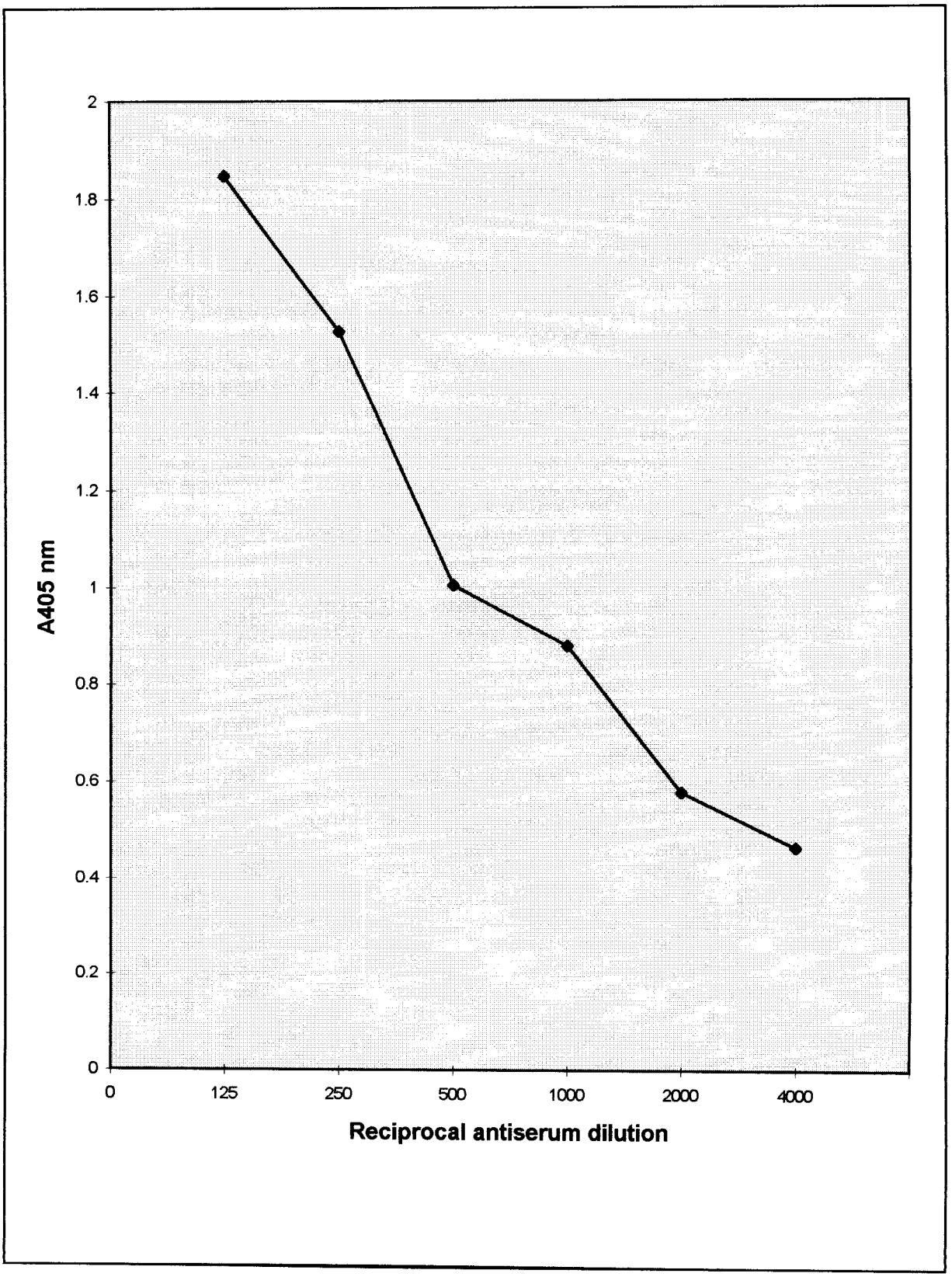


Fig.15

Table 31 : ELISA reaction with various concentration of mycelial antigen of *F. oxysporum* and homologous antiserum.

Antigen conc. (ng / ml)	Absorbance at 405 nm			
	Expt.-1	Expt.-2	Expt.-3	Mean
25	0.591	0.573	0.594	0.586 ± 0.005
50	0.676	0.674	0.693	0.681 ± 0.004
100	0.746	0.741	0.701	0.729 ± 0.011
200	0.803	0.804	0.816	0.807 ± 0.003
400	0.979	0.936	0.932	0.949 ± 0.012
800	1.019	0.997	1.003	1.006 ± 0.005
1600	1.213	1.294	1.210	1.239 ± 0.022
3200	1.531	1.551	1.528	1.530 ± 0.007

* Anti *F. oxysporum* antiserum dilution - 1:125

Enzyme dilution 1 : 10,000

± Standard error.

4.10.3.2. Comparison of ELISA reactivity among antigens of different soybean varieties against antiserum of *F. oxysporum*.

For detection of CRAs between soybean and *F. oxysporum* ELISA reactions were carried out with *F. oxysporum* antiserum and antigens of different soybean cultivars. Absorbance values in ELISA reavealed that higher absorbance values were obtained for the susceptible cultivars (JS - 2, UPSM - 19, PK - 564) than the resistant ones (Soymax, 19 - PK 466, PK - 327). In ELISA involving antigens of non-host very low values were recorded (Table 32, Fig 17). In reciprocal tests when antiserum raised against the susceptible soybean cultivar (UPSM - 19) was reacted with antigens of other varieties A 405 values did not differ significantly except in homologous combinations (Table 33, Fig. - 18) : The values varied among the different cultivars but no correlation could be drawn in this case.

Effect of antigen dilution on ELISA reaction

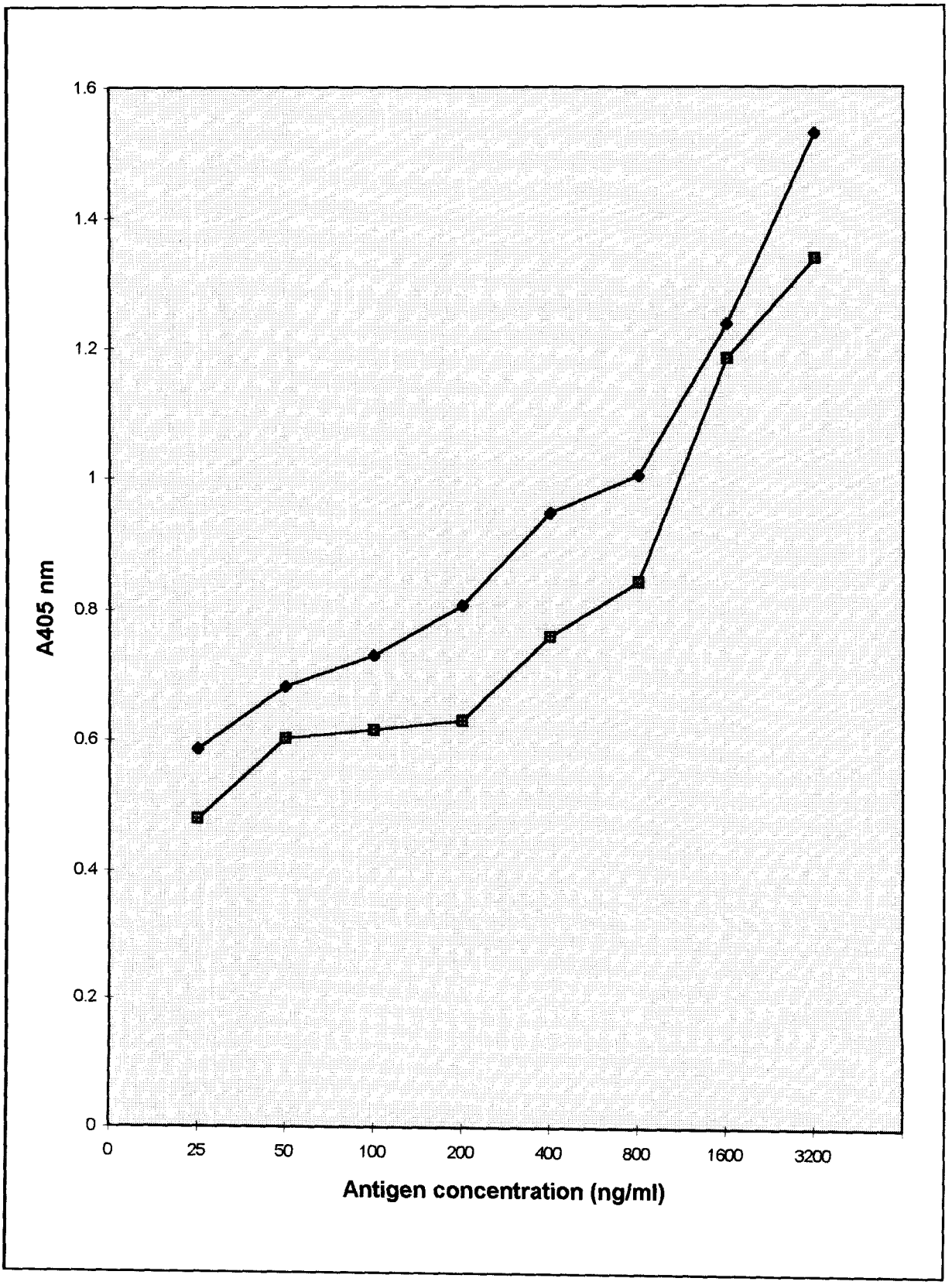


Fig.16

Table 32 : Indirect ELISA values (A 405) of combinations of antigens of host varities, non-host, pathogen and anti-serum of *F. oxysporum*.

Antigens	Absorbance at A 405nm			
	Expt - 1	Expt - 2	Expt. - 3	Mean.
Host				
Soymax	0.882	0.862	0.851	0.865 ± 0.010
JS - 2	1.538	1.506	1.529	1.524 ± 0.007
UPSM - 19	1.542	1.563	1.522	1.542 ± 0.014
Bragg	1.021	0.936	0.981	0.979 ± 0.020
Punjab - 1	1.038	1.121	0.989	1.049 ± 0.037
PK - 564	1.428	1.391	1.402	1.407 ± 0.038
17 - PK-412	1.218	1.114	1.238	1.190 ± 0.014
19 - PK-466	0.918	0.904	0.893	0.905 ± 0.014
PK - 327	0.929	0.939	0.962	0.943 ± ,0.016
18 - PK-564	1.318	1.298	1.307	1.306 ± 0.001
Non-host				
Camellia sinensis	0.320	0.285	0.369	0.325 ± 0.001
Pathogen				
Fusarum oxysporum	1.826	1.842	1.856	1.841 ± 0.001

Anti *F. osysporum* antiserum -1 : 125 dilution
Antigen concentration 40 μg/ml.
± Standard error.

DAC-ELISA response of *F.oxysporum* antiserum

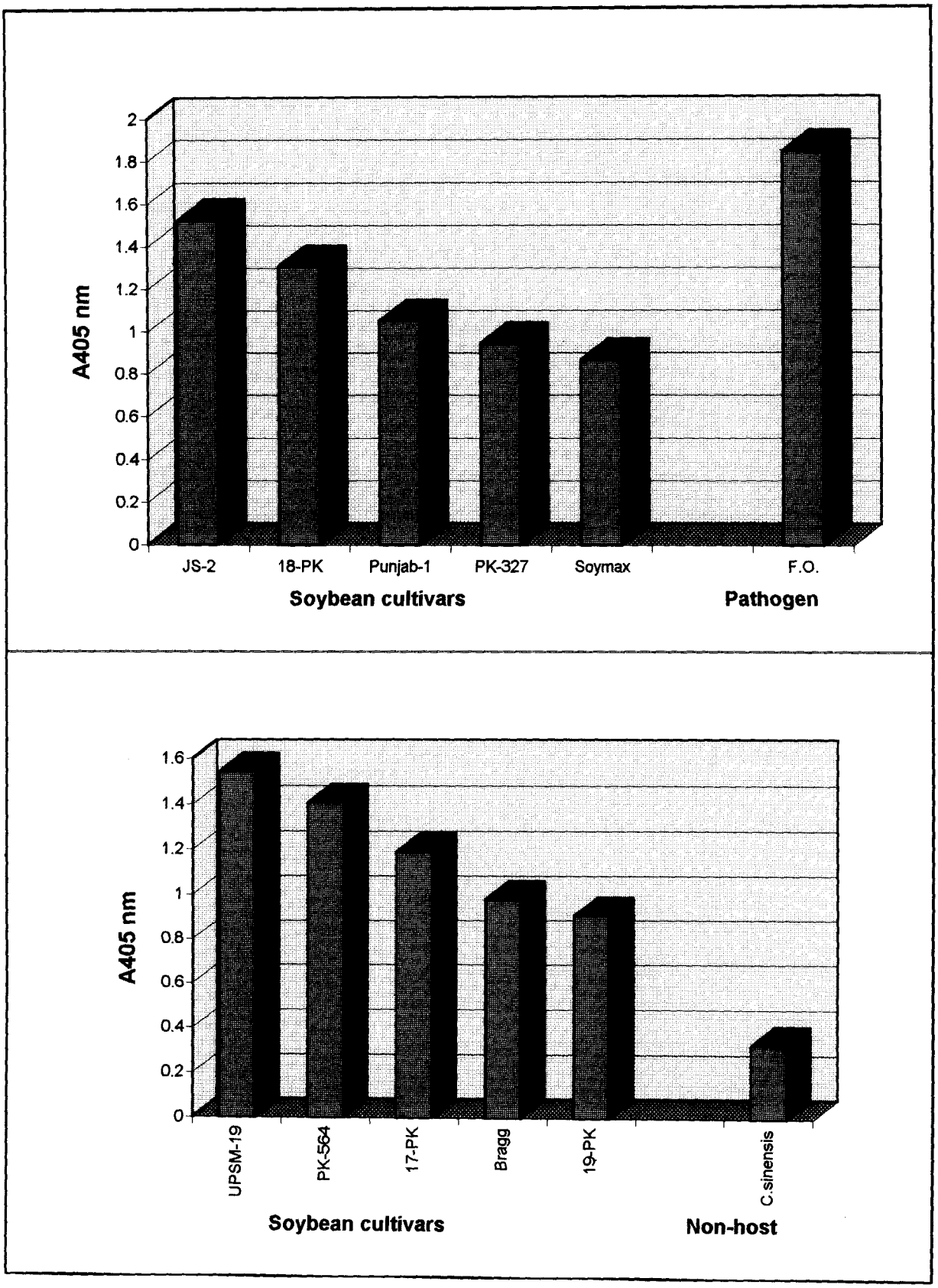


Fig.17

Table 33 : Indirect ELISA values of combinations of antigens of host varities, non-host, pathogen and antiserum of soybean (UPSM-19)

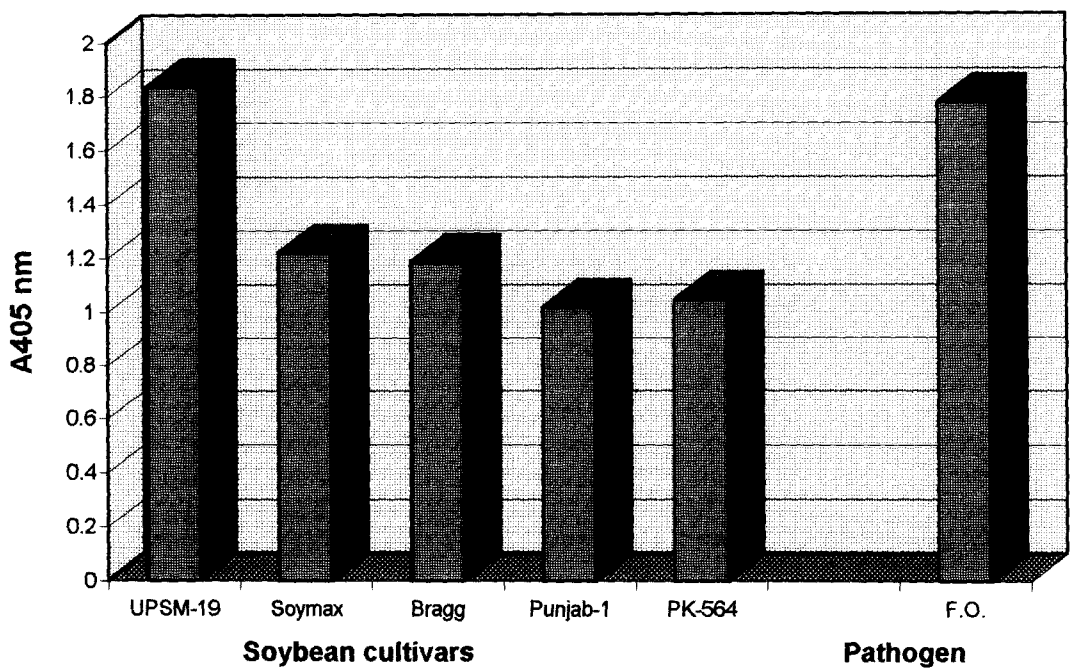
Antigens	Absorbance at 405 nm			
	Expt - 1	Expt.- 2	Expt. - 3	Mean
Host				
Soymax	1.218	1.208	1.232	1.219 ± 0.003
JS - 2	1.508	1.512	1.518	1.513 ± 0.002
UPSM - 19	1.828	1.834	1.842	1.835 ± 0.003
Bragg	1.226	1.228	1.102	1.185 ± 0.034
Punjab 1	0.982	1.062	1.004	1.016 ± 0.040
PK - 564	1.013	1.148	0.965	1.042 ± 0.045
17 - PK-472	1.124	0.998	1.062	1.061 ± 0.030
19 - PK-466	1.058	0.988	1.092	1.046 ± 0.025
PK - 327	0.842	0.839	0.892	0.858 ± 0.014
18 -PK. 564	1.046	1.128	1.039	1.071 ± 0.023
Pathogen				
F.oxysporum	1.742	1.763	1.819	1.775 ± 0.020
Non pathogen				
G. cingulata	0.245	0.249	0.250	0.248 ± 0.001

Antiserum dilution 1 : 125

Antigen concentration 40 μg/ml.

± Standard error.

DAC-ELISA response of soybean (cv.UPSM-19) antiserum



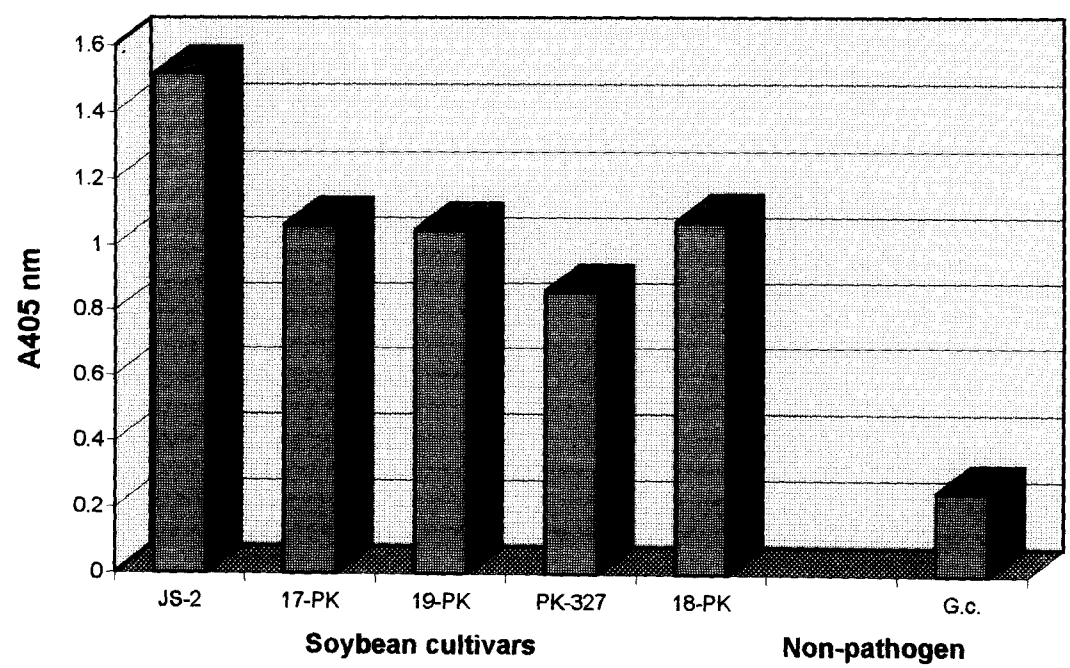


Fig.18

4.11. Detection of pathogen in infected tissues

4.11.1. *F. oxysporum* inoculated

Since a serological relationship exists between *F. oxysporum* and soybean as evidenced by previous experiments, the possibility of detection of *F. oxysporum* in soybean tissues by serological means was considered. Pathogen detection in host tissues by the use of ELISA with the antiserum raised against the pathogen is an effective method. Difference in ELISA readings between healthy and infectecd antigens indicates the measure and extent of infection. In the following experiments attempts have been made to detect *F. oxysporum* in root and seed tissues.

4.11.1.1. Root

In this experiment antigens were prepared from healthy and *F. oxysporum* infected roots of soybean of two cultivars - UPSM-19 and Soymax. These antigens at a concentration of 40 µg / ml were tested against anti *F. oxysporum* antiserum at 1:125 dilution using two ELISA formats.

4.11.1.1.1. DAC ELISA

This is the commonly used ELISA format for quantitative detection of fungal pathogen in host tissues. DAC ELIS A tests were performed as mentioned above and results are presented in Table 34 and Fig.19. Absorbance values for infected root antigen preparations of both the cultivars were significantly higher than their respective healthy extracts at P=0.01

4.11.1.1.2. Dot Blot ELISA

The efficacy of antiserum raised against *F. oxysporum* in detection of infection was further confirmed by dot blot ELISA. Dot blot ELISHA has been judged in many cases as the most sensitive and simple method for pathogen charecterization and detection (Lazerovits, 1990) In the presnt investigation observation of the membrane after the assay revealed deep coloured spots in homologous reactions. In healthy extracts faint coloured spots appeard whereas in case of infected extracts the colour intensity of the spots was much higher (Table 35)

Table 34 : ELISA values showing reaction of antiserum of *F. oxysporm* with antigens of healthy and and inoculated roots of soybean.

Cultivars	Treatment	Absorbance at 405 nm			
		Expt 1	Expt 2	Expt 3	Mean
UPSM-19	Healthy	1.428	1.432	1.446	1.435 ± 0.004
	Inoculated[a]	1.828	1.841	1.838	1.836 ± 0.003
Soymax	Healthy	0.850	0.864	0.861	0.858 ± 0.005
	Inoculated[a]	1.262	1.258	1.236	1.252 ± 0.001

Antigen concentration - 40 µg / ml

Anti *F. oxysporum* antiserum used at 1: 125 dilution

[a] 28 days after inoculation with *F. oxysprum*

± Standard error

Difference between healthy and inoculated values significant at P = 0.01.

Table 35 : Dot blot ELISA values of healthy and *F. oxysporum* inoculated soybean tissues.

Antigen preparation	Antiserum of *F. oxysporum*	
	Intensity of Spots	
	Healthy	Inoculated
cv. UPSM-19		
Root	+	++
Seed	+	++
cv. Soymax		
Root	+	++
Seed	+	++
F. oxysporum		+++

Colour intensity : Low(+); Medium(++); High (+++).

ELISA values of soybean root antigens with pathogen antiserum

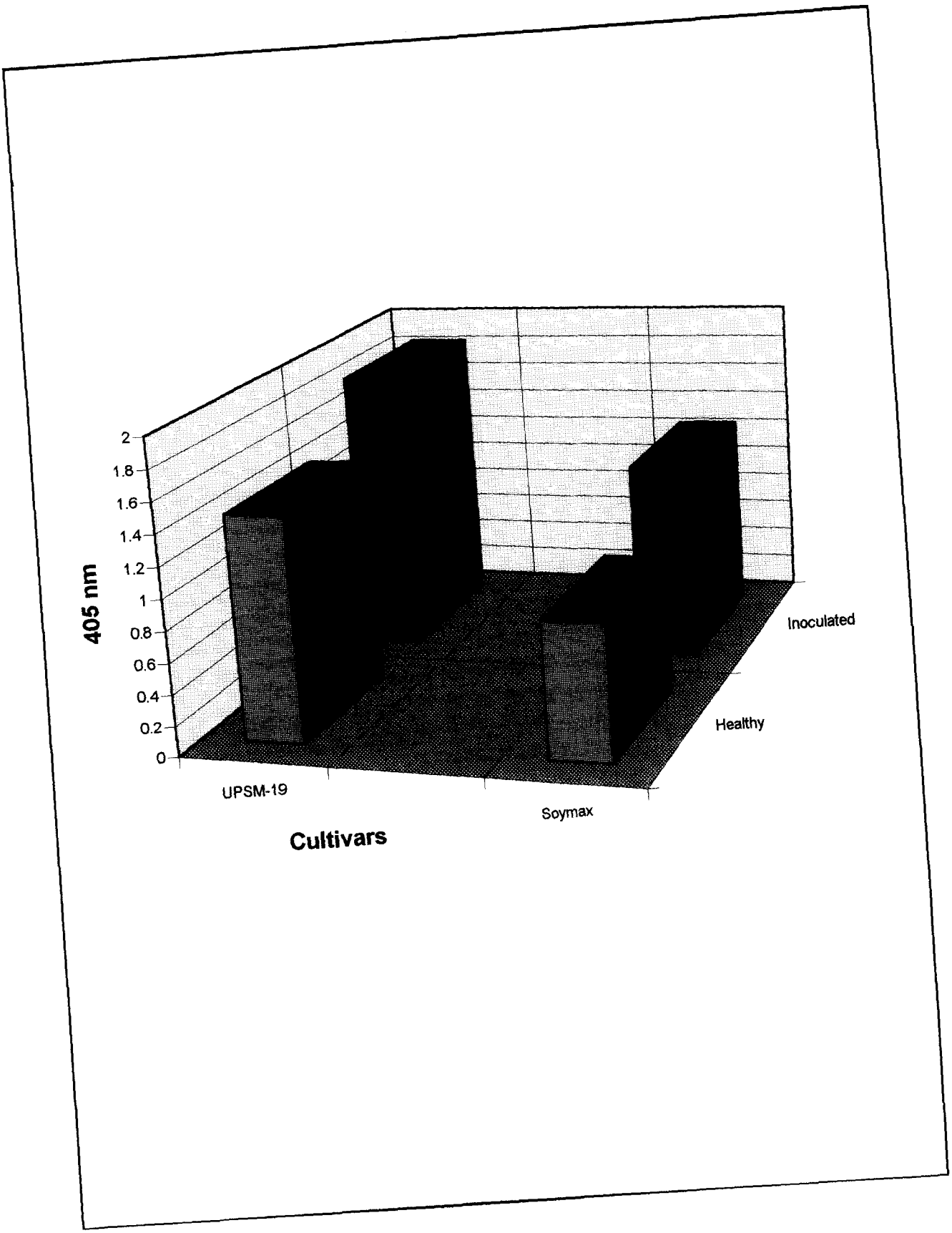


Fig.19

4.11.1.2. Seed

Latent infection of seeds by various pathogens is of quite common occurrence and *F. oxysporum* has also been previously reported to cause latent infection. In such cases serological techniques become all the more important because these can detect even very small amounts which by other techniques may be undetected. Hence ELISA was carried out with antigens of healthy and inoculated seed sample and antiserum raised against *F. oxysporum.*

4.11.1.2.1. DAC ELISA

Results of DAC ELISA performed as mentioned above revealed that absorbance values of infected seed extracts were significantly higher than those of the healthy extracts at P=0.01 (Table 36, Fig-20) Infection could be detected in both the cultivars tested.

4.11.1.2.2. Dot blot ELISA

Infected seed extracts exhibited deep coloured spots in dot blot ELISA as compared to less intense spots in healthy extracts (Table - 35). Thus infection in seeds could also be detected by visual estimation of the intensity of spots. It was graded for partial quantification.

4.11.2. Inoculated with *B. japonicum* and *F. oxysporum*

Results of previous experiments had established that preinoculation with *B. japonicum* reduced root rot discase caused by *F. oxysporum* and these were attributed to biochemical changes occurring in the soybean tissues. It has also been established that ELISA values of infected extracts was greater than healthy ones. Hence in the next experiment it was decided to investigate the changes in ELISA responses due to preinoculation with *B. japonicum.*

The antigens were prepared from healthy, *B. japojnicum* inoculated, *F-oxysporum* inoculated and *B. japonicum* + *F. oxysporum* inoculated roots of the two cultivars (UPSM-19 and Soymax) as described previously. These antigens were then tested against anti *F. oxysporum* antiserum in DAC ELISA. Results (Table 37, Fig. 21) revealed no significant differences between healthy

Table 36 : ELISA values showing reaction of antiserum of *F. oxysporum* with antigens of healthy and inoculated seeds of soybean.

Cultivars	Treatment	Absorbance at 405 nm			
		Expt 1	Expt 2	Expt 3	Mean
UPSM-19	Healthy	1.350	1.344	1.361	1.351 ± 0.004
	Inoculated[a]	1.840	1.828	1.836	1.834 ± 0.002
Soymax	Healthy	0.876	0.875	0.880	0.877 ± 0.001
	Inoculated[a]	1.256	1.390	1.306	1.317 ± 0.031

Antigen concentration - 40 µg / ml
Anti *F. oxysporum* antiserum used at 1: 125 dilution
[a] 6 days after inoculation with *F. oxysporum*
± Standard error.
Difference between healthy and inoculated values significant at P = 0.01.

and bacterized root antigens. Inoculation with *F. oxysporum significanlly* increased the ELISA values in both cultivars. Values for *B. japonicum* + *F. oxysporum* inoculated extracts were higher than those of either healthy or *B. japonicum* inoculated roots but lesser than those of *F. oxysporum* inoculated ones.

4.12. Cellular location of major cross reactive antigen by immunofluorescence.

In the present study the presence of CRA shared by soybean and *F. oxysporum* has been detected by immunodiffusion, immunoelectrophoresis and ELISA. Changes in ELISA responses due to infection with *F. oxysporum* as well as preinoculation with *B. japonicum* has also been recorded. It was next

ELISA values of soybean seed antigens with pathogen antiserum

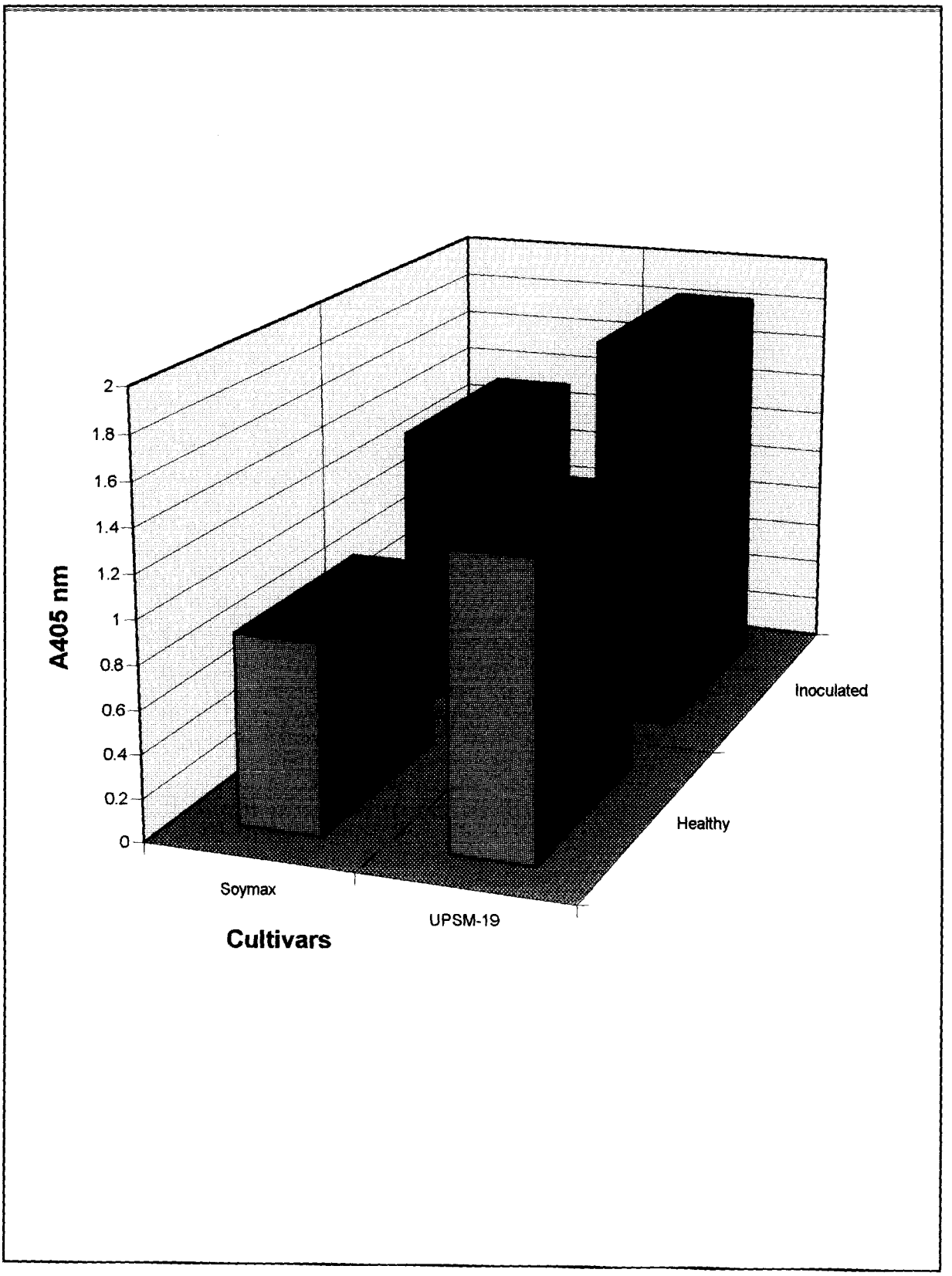


Fig.20

Table 37 : ELISA values of healthy and treated root antigens of two soybean cultivars reacted with pathogen antiserum.

Cultivars	Treatment*	Absorbance at 405 nm			
		Expt 1	Expt 2	Expt 3	Mean
UPSM-19	Healthy	1.439	1.448	1.526	1.471 ± 0.023
	Inoculated *(F.o)*	1.836	1.872	1.849	1.852[a] ± 0.009
	Inoculated *(B.j)*	1.462	1.386	1.475	1.441 ± 0.022
	Inoculated *B.j ± F.o.*	1.642	1.569	1.602	1.604[a] ± 0.017
Soymax	Healthy	0.850	0.863	0.861	0.858 ± 0.017
	Inoculated *(F.o)*	1.238	1.214	1.302	1.251[a] ± 0.021
	Inoculated *(B.j)*	0.836	0.840	0.844	0.841 ± 0.003
	Inoculated *B. j + F.o*	1.062	1.092	0.992	1.049[a] ± 0.024

Antigen concentration 40 µg / ml

Anti *F. oxysporum* antiserum used at 1:125 dilution.

* 28 days after inoculation with *F. oxysporum*

± Standard error.

[a] Difference with respective healthy extracts significant at P = 0.01.

ELISA response of healthy and inoculated soybean root antigens

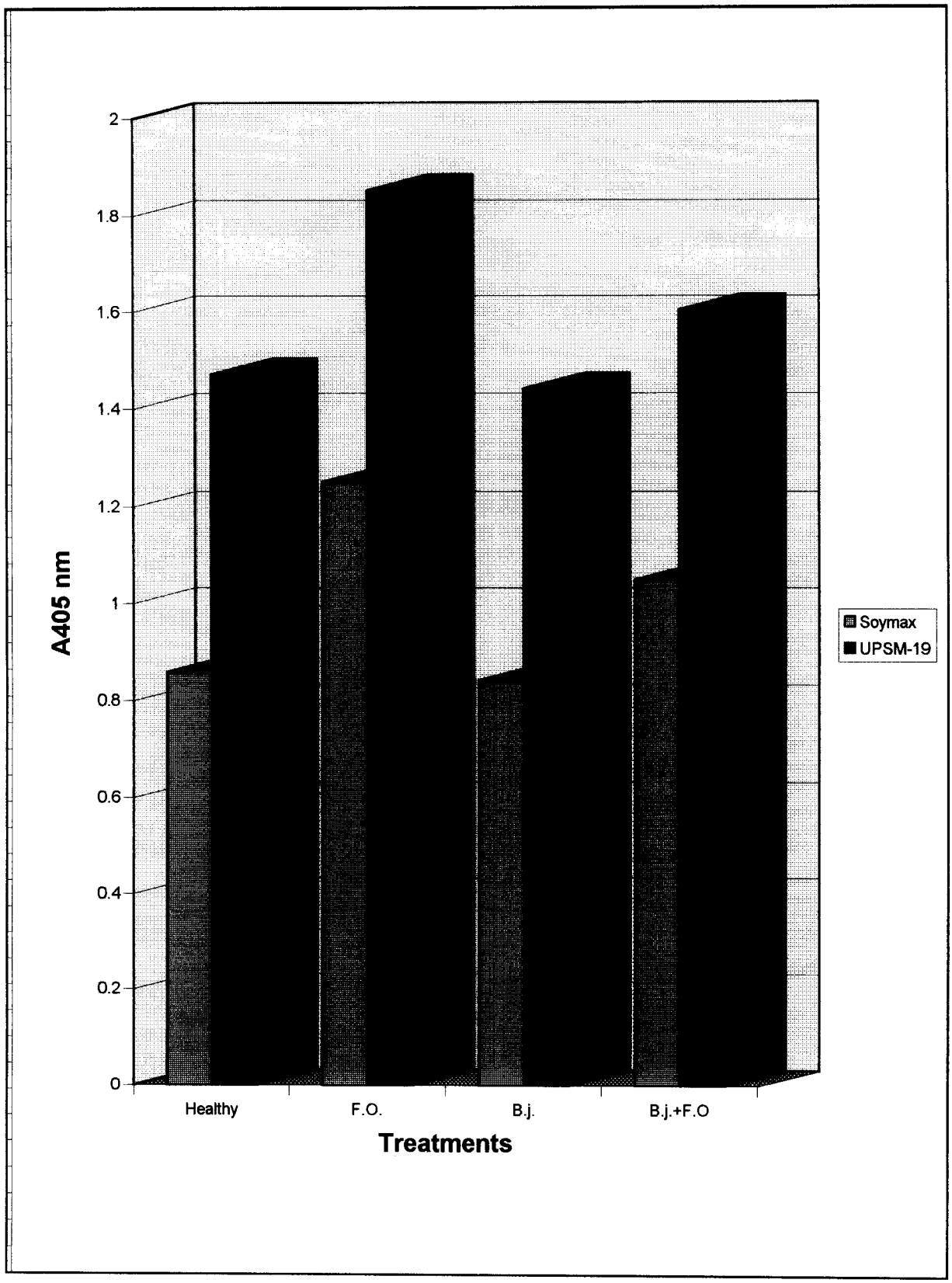


Fig.21

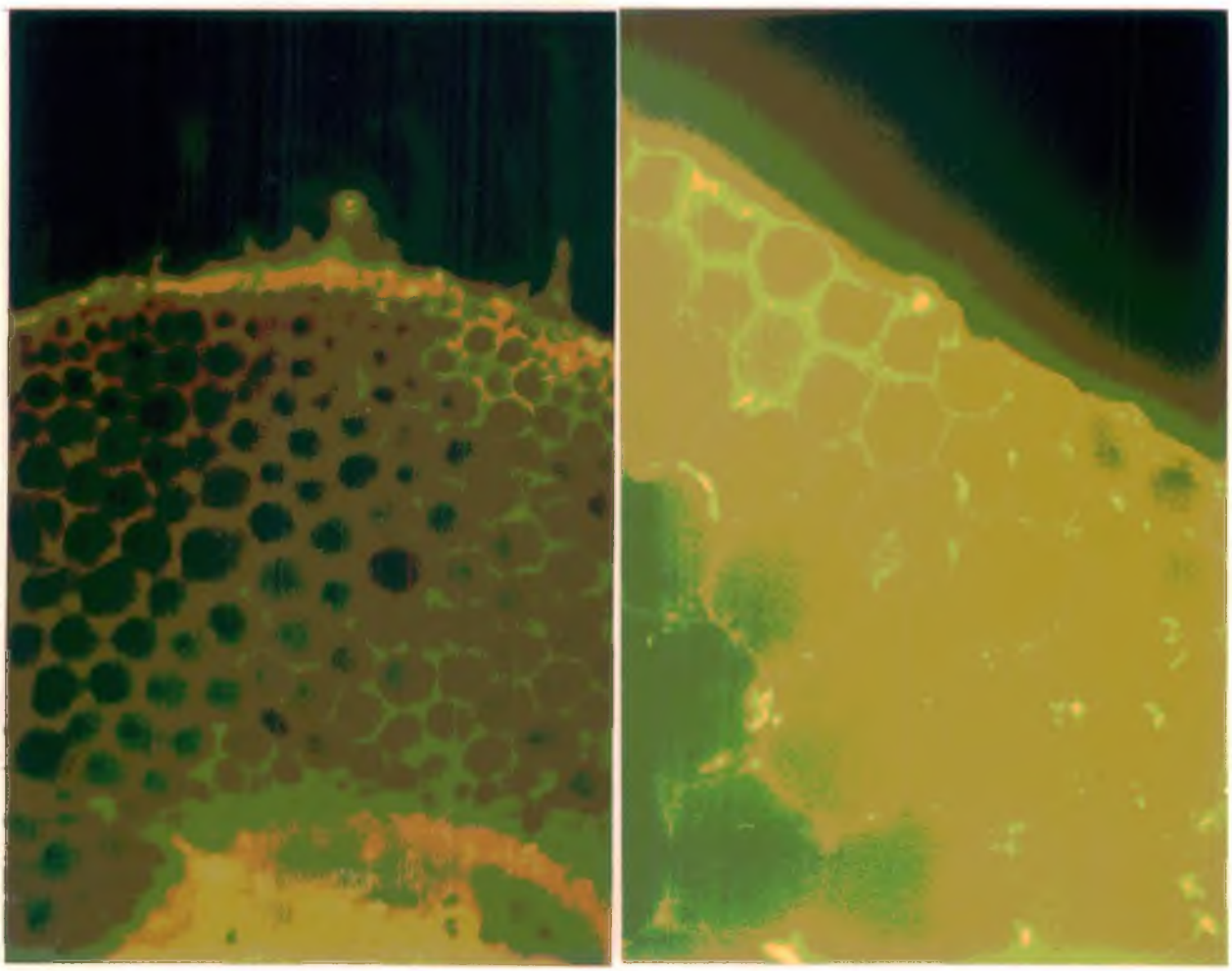


Plate XI (figs. A & B) : Fluorescent antibody staining of soybean root tissues (cv.UPSM -19) for cross reactive antigens shared with *F.oxysporum* . Root sections treated with antiserum to *F.oxysporum* and FITC - antibodies of goat specific for rabbit globulins ; **A** - Portion of root section ; **B** - magnified view of epidermal and cortical region

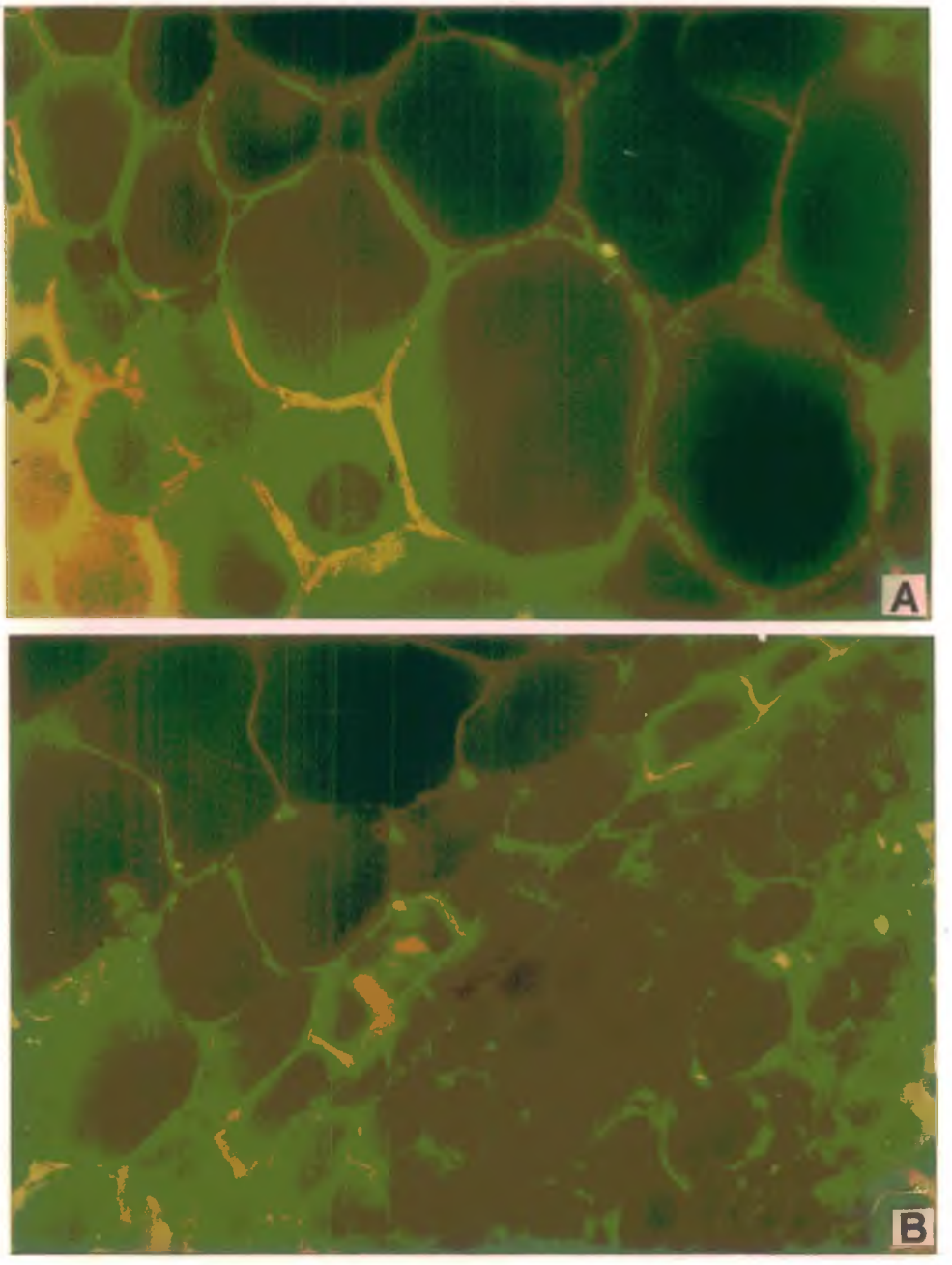


Plate XII (figs. A & B) : Fluorescent antibody staining of soybean root tissues (cv. UPSM -19) for cross reactive antigens shared with *F.oxysporum* . Root sections treated with antiserum to *F.oxysporum* and FITC - antibodies of goat specific for rabbit globulins. Enlarged view of : **A** - cortical region ; **B** - cortical,endodermal and stelar region

decided to determine the tissue and cellular location of CRA in root sections as well as in mycelia, conidia and chlamydospores of *F. oxysporum* by flourescent antibody labelling with fluorescein isothiocynate (FITC). Detailed methods of antibody staining have already been described in materials and methods.

4.12.1. Root Sections

Fresh cross sections of roots of two cultivars (Soymax and UPSM-19) were made and tested either with antiserum of UPSM-19 or *F. oxysporum* after indirectly labelling with FITC. When the sections were treated with antiserum of F. oxysporum indirectly labelled with FITC it was observed that in the sections of susceptible cultivar (UPSM-19), CRA was concentrated around xylem elements, endodermis, cortical tissues and epidermal cells (Plate XI, figs. A&B & XII, figs. A&B) while in the resistant cultivar (Soymax) fluorescence was concentrated mainly in the epidermal cells and partly in the cortical cells (Plate XIII, figs. A&B).

4.12.2. Mycelia, Conidia and Chiamydospore

Treatment of *F. oxysporum,* mycelia, microconidia, macroconidia as well as chlamydospores with its own antiserum as well as antiserum of UPSM-19 followed by FITC labelling resulted in general fluorescence in all cases. (Plate XIV, figs. A-C). None of these were autofluorescent nor did they fluoresce when treated with normal serum followed by FITC.

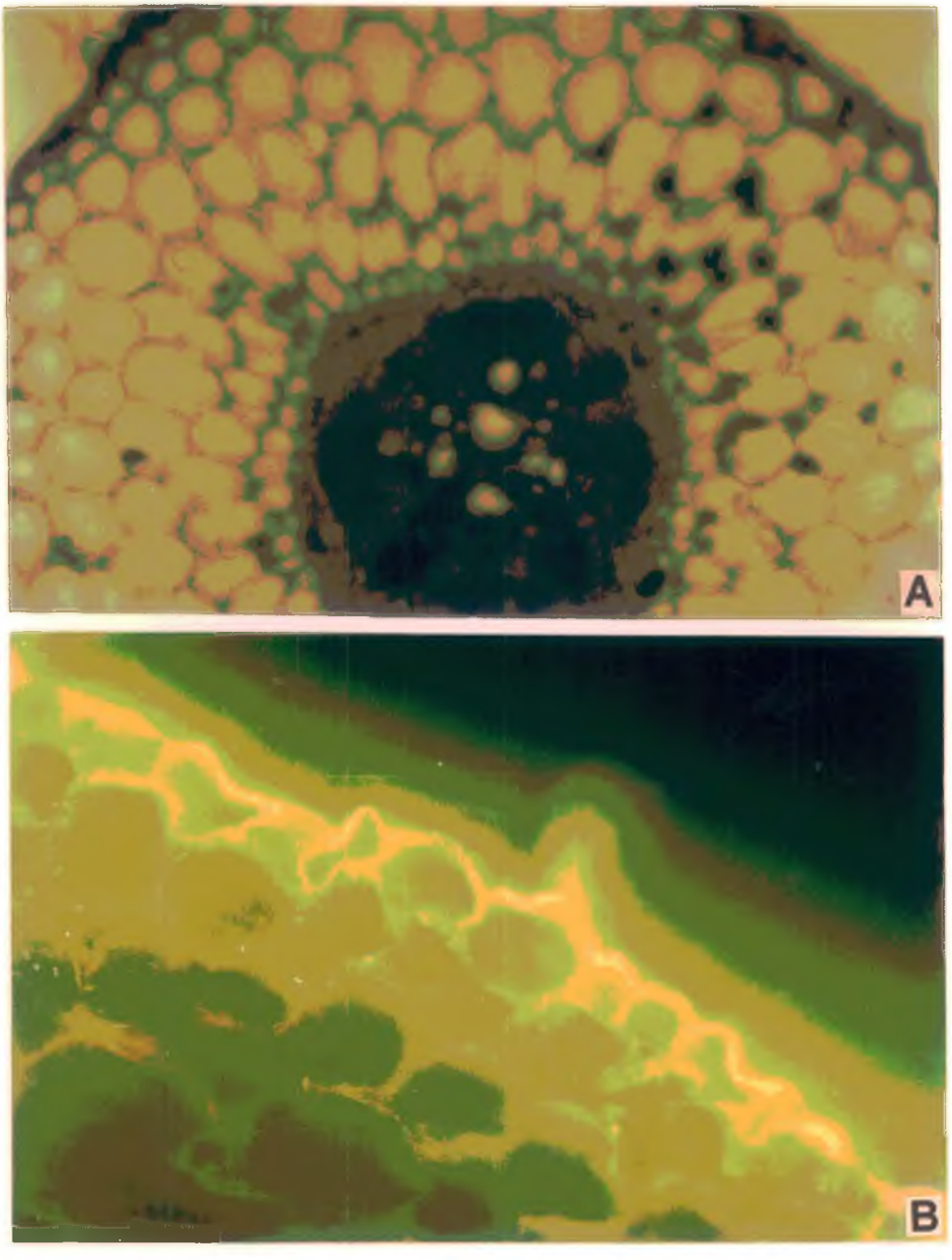


Plate XIII (figs. A & B) : A - Unstained root section of soybean (cv.UPSM -19) ; **B** - Portion of root section of cv. Soymax treated with antiserum to *F.oxysporum* and FITC-antibodies of goat specific for rabbit globulin

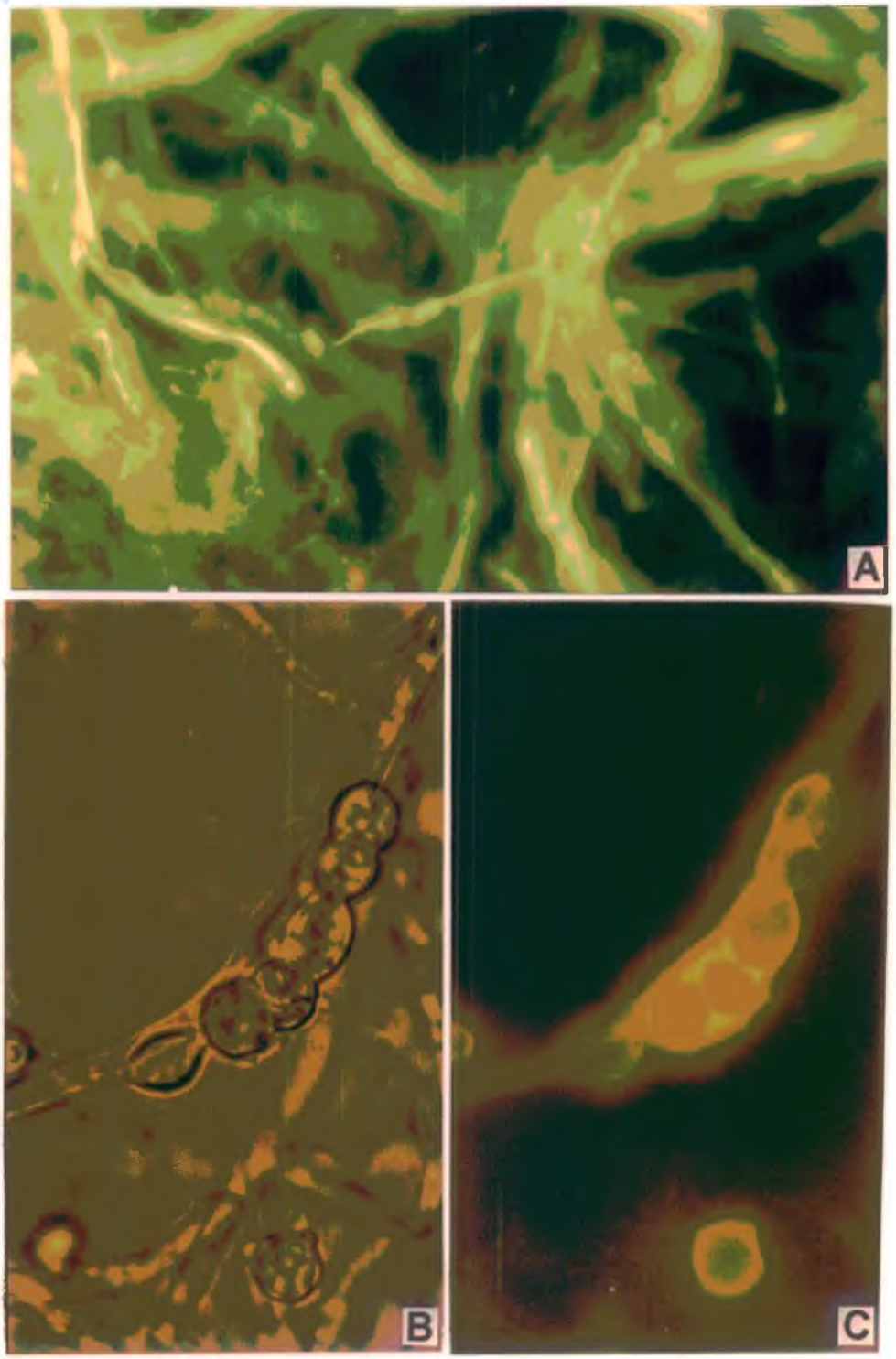


Plate XIV (figs. A - C) :Mycelia (**A**) and chlamydospores (**B&C**) of *F.oxysporum*. **A & C** - Treated with antiserum to *F.oxysporum* and FITC - antibodies of goat specific for rabbit globulin; **B** - Under light microscope

Discussion

In nature, plants generally have a versatile multi-component defense adequately equipped to provide them protection against most of their potential pathogens; only a few of them can overcome this defense and cause disease (Sinha, 1995). Varieties within a host species are resistant when they possess one or more resistant gene(s) and susceptible when they lack any such gene. The spatial and temporal deployment of plant defense responses involves the complex interplay of signal events often resulting in superimposition of signaling processes (Graham and Graham, 1996). In a host-pathogen interaction, a potential pathogen may recognize features of a plant which signal the suitability of that plant for parasitism. On the otherhand, the potential host may be able to detect or recognize the potential fungal pathogen or non-self and use the initial act of recognition to trigger a range of induced resistance mechanisms (Callow, 1982 & 1983; Purkayastha, 1994). Every plant possesses a diverse array of resistance mechanisms operating at different levels and successful infection is dependent on the ability of pathogen to avoid or overcome each in turn.

In the present study, at the onset, pathogenicity test of *Fusarium oxysporum* was carried out on ten cultivars of soybean. Of the ten cultivars tested, Soymax was the most resistant followed by Bragg while JS-2 and UPSM-19 were most susceptible. Differences in pathogenicity of *Macrophomina phaseolina* to different cultivars of soybean have been reported by previous workers (Agarwal et al 1973; Gangopadhay, *et al.* 1973. Oswald and Wyllie 1973; Purkayastha *et al.* 1981). It has been reported that most fungal and viral pathogens of soybean seedlings, plants, pods and seeds have an asymptomatic or latent period after infection or colonization (Sinclair, 1991). Of the reported diseases of soybean atleast 10-15, including *F. oxysporum* are known to cause latent infection. It has been shown by Rizvi and Yang (1996) that soybean seedlings contain number of fungal flora, some of which are pathogenic and others are non-pathogenic. Percentages of major fungal taxa isolated from soybean seedlings were- *Rhizoctonia solani* 27.5%, *Fusarium* sp 11.9%, *Pythium* sp and *Phytophthora sojae* cumulatively 60.5%. Repeated pathogenicity tests confirmed that *Pythium* sp, *Phytophthora sojae* and R. *solani* were the major causal fungi associated wit the seedling disease complex of tested soybean.

Deposition of pathogen inoculum on the host surface is followed by spore germination, penetration and growth of mycelia within the host tissue. This depends on a number of factors which may be environmental or of host origin. In view of this, the effect of some of the important factors on mycelial growth of *F. oxysporum* and bacterial growth of *B. japonicum* have been investigated *invitro. Maximum* mycelial growth of *F. oxysporum* occurred at an incubation period of 9 days, p^H 5 with yeast extract as nitrogen and dextrose as carbon source. Since the nature of the fungal spore wall is important in the initial recognition and subsequent infection processes, in the present study the nature of conidial wall component of *F. oxysporum* was determined by agglutination test. Responses of conidia to different lectins revealed that the surface mainly contained L-D glucopyranoside and N-acetyl L-D galactosaminyl residues. Cristinzio et al (1988) reported that the surface of conidial wall of *F. solani* and *F. oxysporum* contained L-D mannosyl and L-D galactosaminyl residues while these were not found to occur in the outermost layer of the conidial wall of *F. culmorum, F. sambucinum, F. graminearum, F. aveneceum, F. moniliformae* or *F. xylariodes.* On the basis of studies with FITC-conjugated lectins Mercure *et al.* (1995) reported that the material released from conidia of *Colletotrichum graminicola* was a glycoprotein and contained glucose and or mannose. In case of *B. japonicum* optimum incubation period was 8 days, pH was 7 and maximum growth occurred with yeast extract and mannitol as nitrogen and carbon sources, respectively.

As *B. japonicum* is a usual component of soybean rhizosphere, experiments were conducted to determine the effect of seed bacterization with *B. japonicum* followed by growth of plants in *F. oxysporum* infested soil on disease development. Results clearly indicated that preinoculation with *B. japonicum* reduced disease intensity significantly. Such results have also been obtained by several previous workers. Tu (1978) observed that the development of root rot disease of soybean caused by *Phytophthora megasperma* decreased with increasing rhizobial population in the soil. Tu demonstrated that rhizobia could colonize hyphae particularly at their tips. Savada (1982) reported that as a result of interaction of *Rhizobium melilotii* and *Fusarium oxysporum* in the rhizosphere of alfa-alfa both root rot and nodulation was decreased. Similar findings were also reported by Chakraborty & Chakraborty

(1989) in case of *R. leguminosarum* and *F. solani, sp. pisi* on pea. Fully sporulated culture of *Bacillus cereus* and sterilized filtrates of this culture were effective in protecting alfa-alfa seedlings against damping off (Handelsman et. al 1990). A cell free culture filtrate of *B. subtilis* was also reported to significantly reduce disease intensity on alfa alfa seedlings from 56% to 16%, although treatment of seedlings with washed cell suspension had no influence on disease (Douville and Boland, 1992). Suspensions from washed or non-washed *Saccharomyces cerevisiae* cells and filtrates of the suspension reduced the development of *Colletotrichum graminicola* as well as the expression of anthracnose on maize leaf when they were previously or concomitantly treated with these preparations (Dasilva and Pascholati 1992). The authors attributed the reduction of the development of *C. graminicola* and disease expression on the leaves by filtrates of cell suspension of *S. cerevisiae* to a thermolabile substance or complex of substances released from the cells into the filtrates. Crop improvement and disease suppression by a *Bacillus* sp. from pea rhizosphere was also reported by Kumar (1996). Rupe *et al.* (1996) isolated fungi from the rhizosphere and rhizoplane of healthy or mildly diseased plants collected in areas of fields severely affected by sudden death syndrome of soybean (SDS) caused by *Fusarium solani.* Of the 151 fungi evaluated for control of this disease in greenhouse test, 46 demonstrated control activity. None of these isolates had *invitro* antibiosis activity at either high or low nutrient levels. Predominent fungi demonstrating biological control activity were strains of *F. solani* and *F. oxysporum.* In the present study, nodulation was also reduced to some extent by *F. oxysporum.* Treatment of the soil with *T. harzianum* prior to infestation with *F. oxysporum* also gave significant reduction in disease. Further, joint inoculation with *T. harzianum* and *B. japonicum* (in the soil and seed respectively) reduced disease intensity to an even greater degree indicating that neither *T. harzianum* nor *B. japonicum* inhibited the activity of the other. Role of *Bradyrhizobium japonicum* and *Trichoderma* sp. in the control of root rot of soybean has also been studied by Ehteshamul-Haque and Ghaffar (1995). *T. harzianum, T. viride, T. hamatum, T. koningi* and *T. pseudokoningi* significantly controlled the infection of 30 day old seedlings by *Macrophomina phaseolina, Rhizoctonia solani* and *Fusarium* sp. In 60 day old plants *Trichoderma* sp. and *B. japonicum* inhibited the growth of *R. solani* and *Fusarium* sp. whereas the use of *B. japonicum* with other species of

Trichoderma controlled the infection by *M. phaseolina*. Novikova et al (1995) also reported that application of mixture of alirin B and alirin S (biopreparations) reduced severity of cucumber root rot and also stimulated plant developmt and resulted in yield increase. The application of biopreparation shifted the ratio between antagonistic and phytopathogenic microbes in soil and led to the increase in antagonistic species.

Reduction of disease intensity by a particular biocontrol agent may be due to antagonistic effect of the biocontrol agent which may directly inhibit the growth of the pathogen *invivo*. On the other hand the biocontrol agent may induce some biochemical changes in the host which trigger off defense responses. In the present study therefore, attempts were made to determine the mechanism of action of *B. japonicum* and *T. harzianum* which led to reduction in disease severity. At first, interaction studies were conducted *invitro* involving the three microorganisms. Results clearly indicated tht *T. harzianum* was antagonistic to *F. oxysporum* and inhibited its growth significantly while *B. japonicum* in no way inhibited the growth of *F. oxysporum*. The results obtained are in confirmity with those of several previous workers. Cubeta et al (1985) reported that when a *Bacillus subtilis* isolate from soybean was tested for antagonism against 26 fungi commonly associated with soybean it showed fungicidal activity against *Penicillin* sp and fungistatic to all others. Autoclaved filtrates of *B. subtilis* culture inhibited growth and stroma formation of *Phomopsis* sp. El-Abyad *et. al* (1993) reported that 80% concentration of culture filtrate of *Streptomyces pulcher* or *S. canescens* significantly inhibited spore germination, mycelial growth and sporulation of *Fusarium oxysporum* f. sp *lycopercisii, Verticillium albo-atrum* and *Alternaria solani*. In the present study also both autoclaved and nonautoclaved culturate filtrate of *T. harzianum* reduced the growth and sporulation of *F. oxysporum*. Hessenmuller and Zeller (1996) reported that 50 antagonistic bacterial isolates out of the genera *Agrobacterium, Bacillus, Enterobactor* and *Pseudomonas* inhibited the mycelial growth of *Phytophthora* sp. in dual cultures. The most inhibitory activity with a reduction of 68% was shown by an isolate from *B. licheniforms against* P. *cactorum*. 2-4 diacetyl phloroglucinol a secondary metabolite produced by *Pseudomonas fluorescens* - F113 was capable of protecting sugarbeet against the causal agent of damping off, *Pythium ultimum*. *Stenotrophomonas*

maltophilia strain W81(P) produced chitinase and protease enzymes and was capable of conferring plant protection against the disease causing activity of *Pythium ultimum invitro.* On the otherhand reports are also available of biocontrol activity where no inhibition in growth was observed (Chakraborty & Chakraborty, 1989; Rupe et al, 1996).Bertagnolli(1996.) reported that cell free culture filtrates of the fungal plant pathogen *Rhizoctonia solani* isolate 2B12 inhibited the growth of the biocontrol agent *Bacillus megatarium* and *Trichoderma harzianum by secretion* of endoprotease, exochitinase, glucanase and phospholipases. In the present study, *F. oxysporum* and *T. harzianum* both inhibited the growth of *B. japonicum* to a certain extent *invitro.*

It was established from the results of the present investigation that disease reduction by two tested microorganisms - *B. japonicum* and *T. harzianum,* were achieved probably by two different mechanisms. *T. harzianum* inhibited the growth of *F. oxysporum* and the observed disease reduction was by its direct antagonistic effect on *F. oxysporum. T. harzianum* is known to have antagonistic effect on plant pathogenic fungi and results of all the previous and present study taken together pointed to antagonism as the most probable mechanism. Hence no further tests were carried on with *T. harzianum. B. japonicum* on the other hand did not have any antagonistic effect against *F. oxysporum* and several further experiments were designed and carried out to determine the mechanism by which *B. japonicum* reduced disease intensity.

Phytoalexin production is one of the most extensively studied inducible defense responses. These constitute a chemically heterogenous group of substances belonging to various classes of natural products. Among these are isoflavonoids, sesquiterpenoids, polyacetylenes as stilbenoids (Ebel and Grisebach,1988). The major phytoalexin accumulating in soybean root infection site is the substituted pterocarpen glyceollin and some of its other isomers glyceollin II and III. Phenylalanine ammonia lyase is one of the early key enzymes in the biosynthesis of glyceollin. It has been shown that application of R-(1 amino 2 phenylethyl) phosphonic acid which is an inhibitor of phenylalanine ammonia lyase to soybean root infection system gave rise to complete loss of resistance of a soybean cultivar to a normally incompatible *Phytophthora megarperma* Race 1 (Waldmueller and Grisebach,1987). Considering the above, in the present investigation, changes in the activity of

phenyl alanine ammonia lyase (PAL) and accumulation of glyceollin after disease reduction by *B. japonicum* was determined. Peroxidase is another enzyme related to induced resistance in several cases (Parent and Asselin 1984; Kuc, 1985). Peroxidase is reported to have an important function in secondary cell wall biosynthesis by polymerizing hydroxy and methoxy cinnamic alcohols into lignin and forming rigid cross links between cellulose, pectin, hydroxyproline rich glycoproteins (HRGP) and lignin (Grisebach,1981). Lignification, systemic accumulation of HRGP and cell wall apposition are associated with induced resistance to blue mold in tobacco (Ye *et al.* 1990) Hence, in the present investigation peroxidase was also selected as one of the enzymes which could be involved in induction of resistance.

Activity of both PAL and peroxidase were assayed in four sets of plants consisting of healthy,*B japonicum* inoculated, *F. oxysporum* inoculated and *B. japoniucm* + *F. oxysporum* inoculated of a resistant (Soymax) and a susceptible (JS-2) cultivar. PAL activity was higher in the resistant cultivar after inoculation with *F. oxysporum,* but reached a peak in both cultivars after 16 h. of inoculation with F. oxysporum. A significant observation here was that inoculation with *B. japonicum* and *F. oxysporum* increased PAL activity greatly in both the cultivars. Thus the activity of this enzyme seemed to be related to the observed disease reduction by *B. japonicum.* On the other hand the activity of peroxidase did not increae in treatments involving joint inoculation with *B. japonicum* and *F. oxysporum.* In relation to healthy control however, the activity of peroxidase increased significantly when inoculated with *F. oxysporum* in both the cultivars; maximum activity was obtained after 12h of inoculation with *F. oxysporum.* Results of the present investigation therefore clearly bring out certain interesting facts. Firstly PAL seems to be involved in disease resistance mechanism, since its activity was higher in case of incompatible reaction as also when disease resistance was induced by preinoculation with *B. japonicum.* Secondly peroxidase did not seem to be involved in specific compatible / incompatible reaction but rather it seemed to accumulate as a general result of infection.

Works of several previous authors have also demonstrated a definite role of PAL in host resistance. (Bhattacharyya & Ward, 1986; Bonhoff *et al.*, 1986; Southerton and Deverall, 1990; Chakraborty *et al.* 1993; Shiraishi *et al.* 1995; Edens *et al* 1995). Bhattacharyya and Ward (1987) reported that PAL

activity of soybean was enhanced in the first few hours following inoculation in the resistant but not in the susceptible response of soybean hypocotyls to *P. megasperma* f. sp glycinea. Considering that PAL is an important enzyme in the biosynthesis not only of glyceollins but also of lignins, phenolic compounds in general and melanins, all of which have been associated with resistant responses in various host plants the authors suggested that activity of PAL could be a useful indicator of the activation of defence related responses. They also demonstrated that induction of susceptibility to the pathogen by changes in temperature conditions was associated with the suppression of PAL activity. Esnault et al. (1987) suggested that production of mRNAS for enzymes leading to phenyl propanoid biosynthesis in soybean hypocotyls was an early response to infection with an incompatible but not with a compatible race of *P. megasperma* f. sp. glycinea. They postulated that the biosynthetic steps controlled by PAL and CHS (chalcone synthase) are relatively remote from those involed in the final elaboration of the glyceollins. Cuypers *et. al.* (1988) also demonstrated that the timing of PAL mRNA accumulation in potato differed markedly between two types of interaction, compatible and incompatible races of *Phytophthora infestans.* A marked increase in the accumulation of PAL mRNA was observed 3 hour after inoculation of the incompatible race at the infection sites where as it was 6 hour after inoculation of the compatible race. It was also reported by Yamada et al. (1989) that treatment of etiolated pea epicotyl tissue with elicitor activated the accumulation of PAL and CHS mRNAS within 1 hour, followed by an increase in PAL activity and pisatin biosynthesis. Concomitant presence of suppressor with elicitor resulted in delay of this host defence reaction.

Southerton & Deverall (1990) have shown that in the wheat - rust (*Puccinia recondita f. sp. tritici*) interaction increase in PAL levels that could be associated with resistance expression occur between 16 and 20 h after inoculation when effects upon the fungus were first seen and when resistant host cell began to collapse. They suggested that PAL may play a more regulatory role in wheat line as in a number of other plants. Similar results have also been reported by Chakraborty *et al* (1993) in *Brassica napus* inoculated with different strains of *Leptospharia maculans.* They demonstrated that a weakly virulent strain elicited more PAL activity as early as 12 h after

inoculation in the resistant cultivar. While PAL activity decreased after 2 days in the susceptible cultivars, in the moderately resistant cuitivar it attained a plateau and did not show any decrease even 3 days following infection. On the basis of this the authors suggested that the maintenance of PAL activity at a high level for a considerable period may be one of the factors responsible for the incompatibility reaction. Shirashi et al. (1995) on the other hand demonstrated increased PAL activity in the barley leaves after inoculation with the powdery mildew pathogen *Erysiphe graminis f.* sp. *hordei* in both resistant and susceptible cultivars. They suggested that the response was not specific and was not a reflection of the resistantce or susceptibility of the cultivar to the pathogen. Elevation of PAL levels by the host was considered to be the direct response to attempted penetration by the fungi.

In the present study, peroxidase increased as a result of infection but not during induced resistance. Activity of peroxidase was greater in the susceptible cultivar than in the resistant one. Akhtar and Garraway (1990) observed an increased peroxidase activity in the susceptible isoline compared with the resistant one when both were treated with sodium bisulphite prior to inoculation with the pathogen *B. maydis*. The sodium bisulfite-induced increase in peroxidase activity persisted even when leaves pretreated with sodium bisulfite were inoculated with *B. maydis* race T and subsequently incubated for 48 h in the dark at 28°C. Concomitant with increased peroxidase activity due to sodium bisulfite, increased sporulaion of *B. maydis* on maize leaves and increased electrolyte leakage was also observed. These results therefore support the finding that increased peroxidase activity can be correlated with increased infection. On the otherhand there are also reports of increased peroxidase activity due to induction of disease resistance. Ye et al (1990) reported that stem infection of tobacco cultivars with *Peronospora tabacina* or leaf inoculations with tobacco mosaic virus induced systemic resistance to both pathogens with a simultaneous increase in peroxidase activity. They further observed enhanced PR proteins as well as peroxidase, β-1,3 glucanase and chitinase activities in induced plants (Ye et al, 1990a). Irving & Kuc (1990) also obtained increased activities of peroxidase and chitinase in plants sprayed with K_2HPO_4 prior to inoculation with *Colletotrichum lagenarium*. This treatment also induced systemic resistance as well as increased chitinase activity. They

suggested that systemic induced resistance resembled passive resistance as it relied upon increased levels of enzymes associated with plant defense in a manner similar to physical barriers.

Subsequent to studies on enzyme responses in soybean, accumulation of glyceollin was determined in plants following different treatments already described. Significant differences in glyceollin level were obtained between susceptible and resistant cultivars inoculated with the pathogen. Increased glyceollin levels were also obtained when resistance was induced due to preinoculation with *B. japonicum*. A number of previous workers have presented conclusive elidence that resistant cultivars of different host species produce more phytoalexins in response to fungal infection than the susceptible ones. (Ingham *et al* 1981; Tegtmeier and Van Etten 1982, Kumar and Sridhar 1984, Rouxel et al 1989; Abazkhodjaev *et al* 1995; Zeringeu 1995, Essenberg and Pierce 1995.

Of the different host pathogen interactions studied till date soybean-*Phytophthora megasperma* has perhaps been the most widely studied (Keen *et al* 1971; Bhattacharyya and Ward 1986, 1987 and 1988; Ebel and Grisebach 1988, Ward, 1989; Yoshikawa 1995). Similar results have also been obtained in soybean with other pathogens (Kaplan *et al* 1980; Purkayastha *et al*. 1981; Purkayastha & Chakraborty 1983; Long *et at*. 1985 ; Chakraborty & Purkayastha 1987). In spite of extensive work on the role of phytoalexins in host resistance relatively little work has been done on their role in induced resistance. Purkayastha et at. (1981) reported that induced resistance of soybean due to *B. japonicum* could not be correlated with increased glyceollin accumulation. Further, they attributed the observed disease reduction to rhizobitoxin produced by *R. japonicum* (Chakraborty & Purkayastha, 1984). Contrary to this observation Chakraborty & Chakraborty (1989) obtained increased phytoalexin (4-hydroxy 2.3.9 trimethoxy pterocarpan) in pea where resistance was induced by *R. leguminosearum* against *Fusarium solani* f. sp *pisi*. These results are in agreement with the present findings. Mithoefer *et al*. (1996) reported that fungal β.glucan induced glyceollin synthesis in soybean was suppressed by cyclic 1,3-1, 6-β glucans from *B. japonicum*. They have demonstrated that both the fungal and the bacterial β-glucans are ligands of β glucan binding site which are putative receptors for the elicitor signal compounds in soybean roots.

Whereas the fungal β glucans stimulated phytoalexin synthesis even at low concentrations, the bacterial glucans appear to be inactive even at relatively high concentration. Competition studies indicated that increasing concentrations of the bacterial glucans progressively inhibited stimulation of phytoalexin synthesis.

In the present investigation after having established the role of PAL, peroxidase and glyceollin in induced resistance, further experiments were conducted on changes in proteins and serological relationships between soybean and *F. oxysporum*. Two cultivars, one resistant (Soymax) and the other susceptible (UPSM-19) were selected and studies were conducted not only in the roots but also seeds as *F. oxysporum* in many cases is present as a latent infection in soybean seeds. Results revealed that protein contents did not differ significantly in the two cultivars but the seeds had very high protein content as compared to the roots. Inoculation with the pathogen led to increase in the protein content in both roots and seeds. In roots, inoculation with *B. japonicum* increased protein content but this could not be correlated to induced resistance. Analysis of root protein by SDS-PAGE revealed the presence of some extra bands in *B. japonicum* inoculated roots but no major difference were obtained after induction of resistance. In case of seed proteins infection led to absence of many of the common bands. In this case therefore even though protein content increased after inoculation the number of protein bands decreased. Zhang and Smith (1996) reported that inoculation of soybean with genistein preincubated *B. japonicum* increased soybean protein content and dry matter yeild under short season conditions. Velicheti et al (1992) observed increased quantity of lipoxygenases and soybean seed lectin in the seed coat of soybean infected with *Phomopsis longicolla*. Higher levels of lectins were observed in cultivars of soybean resistant to *Phytophthora sojae*.

Involvement of antigens in disease reaction of plants has also been reported where antigenic similarity in susceptible / compatible host parasite interactions have been obtained. Purkayastha (1995) suggested that phytoalexins and plant antigens together could be involved in disease reaction and that production of phytoalexins depends on the interactionof host parasite proteins or specific antigens. Hence in the present study investigations on the serological relationship between *F. oxysporum* and soybean cultivars were

next determined. Results of immunodiffusion, immunoelectrophoresis and ELISA clearly revealed more antigenic similarity between susceptible cultivars and the pathogen or the presence of cross reactive antigens in the susceptible host-pathogen interaction. Several earlier studies have also implicated the importance of common antigens in host pahtogen compatibility. The occurrence of CRA and their involvement in various host parasite combinations have been demonstrated by a number of previous authors (Alba *et al* 1983; Chakraborty & Purkayastha 1983; Alba & Devay 1985; Purkayastha & Banerjee 1986; Ghosh and Purkayastha 1990; Purkayastha & Pradhan 1994; Chakraborty & Saha 1994 and Chakraborty *et al.* 1995). Among all available serological techniques for detection of CRA, enzyme linked immunoserbent assay is probably the most sensitive. In the present study antisera were raised against antigenic preparation of *F. oxysporum* and soybean roots which were initially purified and IgG fraction was used in all further tests. This was necessary to minimise non specific binding which may interfere with the actual antigen-antibody reaction. At the onset the sensitivity of the assay was optimized. ELISA readily detected CRA in antigen preparations of *F. oxysporum* at a concentration of 5 μg / ml with 1:125 antiserum dilution. Among the ten soybean cultivars tested high absorbance values were obtained in reaction of anti *F.oxysporum* antiserum with antigenic preparations from susceptible cultivars. In order to confirm that the observed cross reactivity between *F. oxysporum* and susceptible cultivar was specific, antigen preparations from non host (*C. sinensis*) and non-pathogen (*G. cingulata)* were also assayed with antisera of *F. oxysporum* and susceptible soybean cultivar (UPSM-19).None of the above reactions showed any reactivity to ELISA.

Differential response of the different cultivars of soybean to *F. oxysporum* has been established by the pathogenicity test and cross reactivity test. Following this, the ability of the antiserum of *F. oxysporum* to detect the pathogen in infected root was tested in ELISA. ELISA could detect infection in both susceptible and resistant cultivars. Pathogen detection in the host tissue by ELISA has also been reported by a number of previous workers (Mohan, 1988 ; McDonald, 1990; Lyons and White, 1992; Linfield, 1993 : Chakraborty *et al.* 1996). Since significant differences were obtained in ELISA between healthy and infected extracts, in the next phase ELISA were performed

with root antigens after inoculation with *F. oxysporum, B. japonicum* or *F. oxysporum* + *B. japonicum.* Significant differences were obtained between healthy and *F. oxysporum* or *B. japonicum* + *F. oxysporum* inoculated root antigens but not beteen healthy and *B. japonicum* inoculated extracts. ELISA values in *B. japonicum* + *F. oxysporum* antigens were lower than those with *F. oxysporum* alone. Thus reduction in disease due to *B. japonicum* inoculations reduced the ELISA *reactivity.* Antiserum raised against *F. oxysporum* could not detect *B. japonicum.* Results were similar in both the cultivars.

It is also important in studies on host parasite relationship to determine the cellular location of cross reactive antigens which are involved in host sesceptibility / compatibilty with the pathogen. For this, in the present investigation fluorescence tests were conducted with sections of soybean roots as well as mycelia, conidia and chlamydospore of *F. oxysporum.* Cross sections of soybean roots were treated either with anti-*F. oxysporum* or anti UPSM-19 antiserum followed by staining with FITC conjugated anti rabbit globulin specific goat antiserum. In sections of suceptible cultivar (UPSM-19), bright fluorescence developed which was concentrated mainly around epidermal cells, the endodermis and xylem elements and was distributed throughout the cortial tissues. In roots of cv Soymax, fluorescence was noticed only in the epidermal cells and cortical tissues. Treatment of mycelia, conidia and chlamydospores with homologous antiserum and FITC showed a general fluorescence. DeVay *et al.* (1981) determined the tissue and cellular location of major CRA shared by cotton and *F. oxysporum f. sp. vasinfectum.* On the basis of strong fluorescence obtained at the epidermis, cortex, endodermis and xylem tissues, they suggested that the CRA detrminants in roots have a general distribution. The cellular location of CRA between tea and *Bipolaris carbonum* and tea and *P. theae* have also been established (Chakraborty and Saha, 1994; Chakraborty *et al.*,1995).

Detection of pathogen in host tissues using antibody based immunofluorescent technique also been reported by several previous authors (Dewey *et al,* 1984; Watabe, 1990; Wakeham and White, 1996). Troxler *et al.* (1997) used immunofluorescence microscopy to investigate the colonization of tobacco roots by the biocontrol agent *Pseudomonas fluorescens* CHAO and its physical relationship with the black root rot fungus *Thielaviopsis basicola.*

The pseudomonad delayed colonization of the interior of tobacco roots by *T. basicola.*

It can be generalized from the aforesaid considerations that host-pathogen interaction is a complicated process, and cannot be viewed in isolation, since in nature, disease is never the outcome of a single host-pathogen interaction but rather the cumulative effects of a number of interactions. Detailed studies on the interaction between the rhizosphere microorganisms of soybean - *B. japonicum, T. harzianum* and *F. oxysporum,* the former two beneficial and the latter, a pathogen have thrown some light on the complicated mechanisim. It has been established that for effective induced resistance to occur, several non-specific host defense responses may be activated. As with other host pathogen systems, resistance has been correlated with increased PAL activity and glyceollin content, but no role for peroxidase in induced resistance has been established. It is also evident that biochemical changes in soybean are closely related to changes in antigenic relationships between host and pathogen. No direct evidence for correlation of biochemical defense responses and serological changes have yet been forthcoming, but it has been suggested that if cross reactive antigens have a functional role other than in recognation phenomena, it probably will be found in the infection process and be subject to the over riding effects of substances such as phytoalexins or other inhibitory substance already present in host tissues or induced by parasitic microorganisms (DeVay *et al.* 1981). Thus it can be conclusively stated that the final resistance / susceptibility of host to a pathogen cannot be attributed to a single factor, but the outcome of a number of responses occurring at different cellular levels.

Summary

(i) A review of literature pertaining to this investigation has been presented which deals with biochemical changes in plants following infection and serological relationship between host and pathogen.

(ii) Materials used in this investigation and experimental procedures have been described in detail.

(iii) Pathogenicity of *Fusarium oxysporum* was tested on 10 cultivars of soybean (Soymax, Bragg, Punjab-1, 19-PK-466, PK-327, JS-2, UPSM-19, PK-564, 17-PK-472 and 18 PK-564). Soymax and Bragg were found to be most resistant, while JS-2 and UPSM-19 were susceptible.

(iv) Agglutination test of conidia of *F. oxysporum* with different lectins revealed strong agglutination with ConA and HPA. The presence of glycoconjugates containing glucose and or mannose residues as well as N-acetyl galactosaminyl residues on the outer surface of the conidial wall was confirmed.

(v) Maximum growth of *F. oxysporum* occurred after 9 days of incubationand at a pH of 5. Yeast extract was the most effective nitrogen source and dextrose the most effective carbon source for optimum growth of *F. oxysporum*

(vi) Maximum growth of *B. japonicum* occurred at an incubation period of 8 days and at pH 7. Optimum growth of *B. japonicum* occurred using yeast extract as nitrogen source and mannitol as carbon source.

(vii) Intensity of root rot disease decreased significantly in all tested cultivars when soybean seeds were bacterized with *B. japonicum. Trichoderma harzianum* decreased intensity of root rot caused by *F. oxysporum.* Joint pre inoculation with *B. japonicum* and *T. harzianum* reduced disease intensity to the greatest extent.

(viii) *B. japonicum* did not exhibit any antagonistic effect against *F. oxysporum.* In paired cultures, however, *T. harzianum* inhibited the growth of *F. oxysporum.* Culture filtrate of *T. harzianum* also reduced mycelial growth of *F. oxysporum.*

(ix) Phenylalanine ammonia lyase (PAL) activity in *F. oxysporum* inoculated roots was higher in cv. Soymax (resistant) than in

cv. UPSM-19. Maximum PAL activity was obtained 16 h after inoculation with *F. oxysporum*. Among all treatments, maximum PAL activity was exhibited *B. japonicum* + *F. oxysporum* inoculated roots.

(x) Peroxidase activity increased in both cultivars after inoculation with *F. oxysporum*. After 12 h of inoculation with *F. oxysporum* peroxidase activity attained a peak. Pre-inoculation with *B. japonicum* did not increase such activity.

(xi) Glyceollin contents of *F. oxysporum* inoculated roots of both Soymax (resistant) and JS-2 (susceptible) cultivars and cotyledonary callus were estimated. Resistant cv. contained relatively more glyceollin than the susceptible one. Bacterization of seeds with *B. japonicum* caused significant increase in glyceollin content in both cultivars.

(xii) Mycelial protein of *F.oxysporum* was estimated and analysed by SDS-PAGE. More than 35 bands of different molecular weights were obtained.

(xiii) Protein contents of resistant and susceptible cultivar did not differ significantly. Protein content of seeds was much higher than that of roots. As a result of inoculation with *B. japonicum* and *F. oxysporum* protein content increased.

(xiv) SDS-PAGE analysis of proteins from roots after different treatments revealed no significant differences in protein pattern though *B. japonicum* inoculated roots had a few extra bands. In seeds, some major protein bands disappeared after inoculation with *F. oxysporum*.

(xv) Polyclonal antisera were raised against antigen preparations from mycelia of *F.* oxysporum and roots of soybean (cv. UPSM-19).

(xvi) In agar gel double diffusion tests antiserum of *F. oxysporum* and UPSM-19 were cross reacted with antigens of the ten soybean cultivars, a non-host (*Camellia sinensis)* and a non pathogen of soybean (*Glomerella cingulata)* separately strong precipitin reactions occurred in homologous reactions and in reactions involving susceptible cultivars. No precipitation occurred with the resistant cultivar, non host or non-pathogen.

(xvii) Immunoelectrophorectic tests revealed cross reactivity of anti *F.oxysporum* antiserum and susceptible soybean cultivars.

(xviii) Antisera were purified by ammonium sulphate precipitation and DEAE - Sephadex chromatography. IgG obtained after purification was used for ELISA tests.

(xix) Optimum conditions for ELISA reactions were determined. An antiserum dilution of 1:125 and an enzyme (alkaline phosphatase) dilution of 1:10,000 were optimum. Antigens up to a concentration of 25 ng/ml were detected by ELISA in homologous reactions.

(xx) Comparison of ELISA reactivity of all the 10 cultivars of soybean with antiserum of *F. oxysporum* revealed highest absorbance in the susceptible cultivars (JS-2 and UPSM-19) and lowest in the resistant one (Soymax).

(xxi) *F. oxysporum* in infected root and seed tissues were detected using DAC-ELISA formats.

(xxii) Detection of infection was also done by dot-blot ELISA.

(xxiii) Absorbance values of root antigens from *B. japonicum* + F. oxysporum inoculated roots were lower than those of *F. oxysporum* inoculated roots. Antigens of *B. japonicum* inoculated roots exhibited similar ELISA reactivity as healthy roots when reacted with *F. oxysporum* antiserum.

(xxiv) Cross sections of soybean roots (cvs. Soymax and UPSM-19) when treated with antiserum of *F. oxysporum* and then with FITC developeda bright fluorescence. In cv. UPSM-19 CRA was spread throughout the section, whereas in resistant cv. it was concentrated mainly in the epidermal cells and partly in the cortical cells.

(xxv) Mycelia, conidia, or chlamydospores of *F. oxysporum* when treated with homologous antiserum or anti-UPSM-19 antiserum and then with FITC revealed bright fluorescence mainly on young hyphae and throughout the surface of conidia and chlamydospores.

(xxvi) Implications of the results have been discussed.

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