Chemical induction of resistance in soybean plants to *Sclerotium rolfsii* Sacc.

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This is to certify that Mr. Pankaj Mitra has carried out his research work at Department of Plant Pathology, Uttar Banga Krishi Viswavidyalaya, Cooch Behar and at Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal under the supervisions of Dr. A.K.Chowdhury and Professor B.N.Chakraborty. His thesis entitled **"Chemical induction of resistance in soybean plants to *Sclerotium rolfsii* Sacc."** is based on his 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

Active immunization has achieved remarkable success as a method for disease control in men and animals, but little use has been made of this method in the management of plant diseases. Because of the basic similarities in the biochemical processes that support life, one is tempted to think that primary defence mechanism may have evolved along similar lines in animals and plants and that active immunization may also occur in plants. Major strategies for disease control are based on the assumption that susceptibility is due to a lack of resistance gene and the disease in a susceptible variety can be controlled either by incorporating resistance gene in it or by treating it with chemicals, that inhibit the development of pathogen. Since suitable resistance varieties are not always available, we have become over-dependent on the use of toxic plant protectant chemicals for plant disease control. The ever increasing use of such toxic chemicals and often their injudicious applications has its attendent hazards and often poses environmental problems, as many of them may disturb the fine ecological balance that exists in nature. We have now become acutely conscious of the pollution hazards and in that context, search for safer alternative approaches to plant disease control has become very urgent.

Disease resistance and susceptibility in plants do not represent any absolute values. Even a susceptible host variety shows resistance to its pathogen at certain growth stages and under certain cropping conditions or stress situations and rarely perishes. This would suggest that even a susceptible variety has a potentially by effective defence mechamism and that by manupulating cropping conditions or by creating stress, it may be possible to elicit the expression of such latent defence potential during host parasite interactions. This constitutes the very basis of induced resistance in plants as a possible disease control measure. Since the first report of induction of resistance in plants against their fungal pathogens by prior inoculation with their less virulent forms, the emphasis had been mostly on biological induction of resistance. Only lately, any serious attention has been focused on the possibility of chemical induction of resistance in plant host. Considerable evidence has now accumulated to show that prior inoculation of susceptible plant host with an avirulent form of pathogen, cultivar of non–pathogenic races of pathogen, other pathogens of both homologous and heterologous nature or non–pathogen or even prior application of the heat killed or sonicated suspension of the pathogen or the metabolic or

constitutive components of both pathogen and non-pathogen, can provide it significant levels of protection from the subsequent attack of the virulent form of pathogen (Goodman, 1967, 1980 ; Kuc, 1982, 1984 ; Matta – 1971, 1980 ; Purkayastha, 1994 ; Mukhopaddhya , 1997 ; Sequeira, 1983 ; Sinha, 1990). Plants so protected develop only limited number of lesions and often show limitations of lesion size. In some cases, such induced or acquired resistance is systemic in nature and persists effectively over fairly long periods. Even though effective field level protection has been achieved by this method against many diseases of tobacco, bean, cucumber and melons, various logistical problems relating to the large scale production of the inducer biotic agent and its application to the crop under field conditions make this approach both cumbersome and uneconomic and heavily limit its utility as a measure for plant disease control particularly in the Indian agricultural perspective. In that context, the possibility of disease control through chemical induction of resistance assures much greater significance.

Information is now available that treatment with diverse groups of nonconventional chemicals , mostly phytoalexin inducers with little or no direct toxicity can reduce symptom expression in many crop diseases significantly and restrict resultant damage within tolarable limits (van Andel, 1966 ; Wain and Carter, 1972; Chakraborty and Purkayastha, 1987; Lazarovits, 1988 ; Sinha, 1989; Purkayastha, 1994 ; Mohr and Cahill, 2001). It has been demonstrated that prior treatment with many chemicals in the nature of metal salts, amino acids, plant growth regulators and miscellaneous organic compounds are effective in inducing high level of resistance in susceptible rice plant against brown spot (Sinha and Hait, 1982) and blast diseases (Sinha and Sengupta, 1986), in soybean against charcoal rot (Purkayastha and Chakraborty, 1989), in chick pea against fusarium wilt (Chowdhury and Sinha, 1996), in peanut against *Sclerotium* infection (Chowdhury and Sinha, 1997) and in rice against *Rhizoctonia solani* (Bhattacharyya and Roy, 2000) . These and other studies established that many such compounds can provide crop plants with effective systemic protection from their pathogens and that such effect often persists over fairly long periods. In most of these cases no direct toxic action of these chemicals on the pathogens can be envisaged, rather these seem to act as "Sensitizer" and activate host defense through a conditioning of its tissue.

Soybean [*Glycine max* (L) Merrill] is one of the major pulse crops (Plate-1) under Indian conditions, particularly important as a source of protein for both human and animal consumption and also a rich source of cooking oil with heavy use in vanaspati industry. It is also used in the manufacture of paints, varnishes, water proof goods, glycerine, rubber substitues, fire works and explosive (Kale, 1985). Soybean is also recognised by medical science for its high content of lecithin which is essential for building up the nervous system .

Sclerotium blight , southern blight or southern stem rot of soybean incited by *Sclerotium rolfsii* Sacc. (=*Corticium rolfsii* Curzi.) , a soil borne pathogen of very aggressive nature causes considerable damage to soybean (Punja,1985). During the first month of plant development or during late reproductive stages symptom usually appears as white , cottony , fan like mycelium which grows over the stem surface on seedlings and mature plant . Infected seedlings or older plants often are killed . The fungus produces an abundance of globular , tan to reddish brown or dark brown sclerotia about the size of mustard seeds . The sclerotia are associated with the mycelium on the surface of the plant. This disease is very difficult to control by conventional chemicals and no soybean variety resistant to *Sclerotium rolfsii* is yet available. Considering the undoubted importance of soybean in Indian agriculture , it was felt worthwhile to explore the possibilities of control of the above disease by the use of non conventional phytoalexin inducer chemicals.

Basic objectives of the present investigation were (a) to achieve effective control of *Sclerotium* infection by developing high level and durable resistance in susceptible soybean plants by wet seed treatment with a select group of non-conventional chemicals , at their optimum concentration and for optimum duration, (b) to investigate the nature of biochemical changes that may be associated with induction of resistance in soybean plants, (c) to detect cross-reactive antigens shared between *S.rolfsii* and soybean varieties and their cellular location and (d) to determine the serological changes after induction of disease resistance.

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

Plate 1: Soybean [*Glycine max* (L.) Merrill] plants (variety – Macs58) grown in the Experimental field (inset picture- soybean pods)

Literature Review

In general, plants respond in two different ways to pathogens. There is either no obvious interaction, or an interaction occurs and is, in the extreme cases, either incompatible or compatible. The biochemical events occurring in interactions between host or non-host plants with potential pathogens are basically similar, but their timing of appearance and both the intensities and patterns depend on their genomic as well as on environmental conditions. It has been established that different plant species, even varieties within a species may have fine differences in biochemical make-up particularly in respect of phenolics, lignins, proteins and various other metabolic as well as constitutive components as also in their enzyme components and they also differ in their responses to infection in most of these respects. Such biochemical differences in the host responses to inoculation with their potential pathogens have been studied for many host pathogen combinations, and the sum of all available data suggests that most, or all of them, are part of a typical resistance responses of plants, one often determining over the other as the major defence mechanisms. Variations may occur more at the level of timing of induction, location and relative amounts, than in terms of an all-or-none response. Immune systems in plants play a significant role in the host response to the pathogen, and though different from that of animals, is functionally similar. Plants, in general, have their own natural immunity against several diseases; this could also be induced by physical, chemical or biological agencies (Purkayastha, 1994).

A short and comprehensive review on the biochemical changes following induced resistance in plants and serological relationship between host and parasite have been presented below.

Biochemical changes following induced resistance in plants

Well-documented evidences are now available to indicate that effective resistance can be induced in plant host by prior restricted inoculation with the same pathogen or inoculation with its mildly virulent or avirulent races or other homologous or heterologous pathogen or even prior treatment with their metabolites or constitutive compounds. Many chemicals with little or no toxicity *in-vitro* against the pathogen have also been tested occasionally for plant disease control. These include metal salts, amino acids, plant growth regulators and various other

compounds with varied chemical and biological action. Initial information in this respect was reviewed by van Andel (1966) and Wain and Carter (1972). There has been increased activity based on this approach and the resultant information has been reviewed by number of workers (Sequeira, 1979; Matta, 1980; Hamilton, 1980; Goodman, 1980; Langcake, 1981, Kuc, 1987; Lazarovits, 1988; Sinha, 1989; Kessmann *et al.*, 1994; Sticher *et al.*, 1997; Hammerschmidt and Becker, 1997) and more recently by Hammerschmidt (1999).

Significant levels of success has been achieved by using chemicals of widely diverse nature without any direct toxic action against many plant diseases (Sinha, 1984). Apart from chemicals, physical agents such as X-ray (Purkayastha and Ghosh, 1983), UV (Bridge and Klarman, 1973) and biological agents (Sinha and Das, 1972, Chakraborty and Chakraborty, 1989) are also known to alter disease reaction. Numerous molecules have been implicated in mediating disease resistance. There is evidence that some products either of biotic or abiotic origin are capable of activating the host's defense reaction by accumulating secondary metabolites or "Stress" metabolites such as phytoalexin, in treated (physically or chemically) plants (Darvill and Albersheim, 1984; Purkayastha, 1986). Several elicitors of phytoalexin synthesis also induce the expression of other host plant defense responses (e.g.,proteinase inhibitor synthesis and accumulation of hydroxyproline-rich glycoproteins). In some of the recent reviews the mechanisms of induced resistances in plants have been well documented (Ouchi, 1983; Sequeira, 1983; Halverson and Stacey, 1986; Madamanchi and Kuc, 1991).

Oku (1960) presented evidence indicating that resistance of rice plants to *Cochliobolus miyabeanus* could be broken down by treatment with reducing agents such as ascorbate or glutathione. The resistance of rice plants against hyphal penetration by *C. miyabeanus* could partially be attributed to fungal oxidation product, perhaps quinones, derived from host cells or membranes.

The influence of gibberellic acid on the seedling blight of corn was noted by Wilcoxon and Sudia (1960). They observed that treatment of maize hybrid seed with 5, 10 and 20 ppm gibberellic acid enhanced the severity of seedling blight. Use of nickel chloride as foliar spray to tea plants (*Camellia sinensis*) for the control of blister blight caused by *Exobasidium vexans* was demonstrated by Venkataram

(1961). Percentage shoot infection was lower in nickel chloride than in the cuprous oxide treatment. Hale *et. al.*, (1962) reported that growth regulators (viz. Indole-3-acetic acid, napthalene acetic acid, 2,4-dichlorophenoxy acetic acid and maleic hydrazide) caused an increase in size and number of leaf spot/ plant on the susceptible inbred corn like K-44 and the resistant line K-41 when the plants were inoculated with *Helminthosporium carbonum.*

Severity of lesion development on the hypocotyls of red kidney bean increased by foliar applications of gibberellic acid when plants were grown in soil infested with *Rhizoctonia solani,* isolate Rh-5. However, when the plants were treated with gibberellic acid and grown in soil infested with two other pathogenic isolates of *R. solani*, severity of the disease was not affected. The increased virulence of Rh-5 was probably caused by root excretions resulting from the gibberellic acid treatments (Peterson *et al.*, 1963). It was speculated by Daly and Deverall (1963) that hormonal concentration in a leaf could be important in controlling the development of a pathogen. The initial establishment of the disease could be due to hormonal changes brought about by entry of pathogen. Foliar application of different concentration of IAA and GA to detached bean leaves had little effect on lesion production by *Botrytis fabae* and *Botrytis cinerea* and were ineffective in the spread of lesion by *B. cinerea* (Purkayastha and Daverall, 1965).

The effect of maleic hydrazide (MH) on wheat and barley rust were studied by Joshi (1965). The solutions of maleic hydrazide (0.02 percent) were administered to wheat roots and barley seedlings at the time of emergence. The doses, however, varied between 50-120 ml/plot (10 cm. diam). Barley plants (varieties Bolivia and Oderbrucker) treated with 0.2% MH solution (110ml/plot) showed reduction in growth and higher susceptibility of plants to *Puccinia hordeii.* The response was very poor in case of Agra local variety.

Foliar or soil application of CCC [(2-chloroethyl) trimethyl ammonium chloride] reduced the infections of bean seedlings by *Sclerotium rolfsii* (Tahori *et al.*, 1965). But Crosier and Yountburg (1967) reported that CCC (2-4 pounds of CCC/acre) was ineffective against *Tilletia foetida* on winter wheat when used alone as foliar spray. Sinha and Wood (1967) have shown that IAA reduced wilt disease of tomato caused by *Verticillium alboatrum.* On the other hand, maleic hydrazide

(300 ppm), greatly retarded growth of the plant and made them susceptible. Cycocel and napthalene acetamide gave good control of disease over a range of concentrations when applied to the soil in which the plants were growing. Of the other growth regulating substances tested, 2, 4, 5-trichlorophenoxyacetic acid increased disease at some concentrations and reduced it at others.

Chalutz and Stahmann (1969) induced pisatin formation in carrot tissue by ethylene. However, production of pisatin in pea tissues in response to ethylene treatment was less than that induced by fungi. It is possible that ethylene could induce some of the enzymes (phenyl alanine deaminase) involved in the biosynthesis of the pisatin. Carrot roots treated with IAA, 2, 4-D and 2,4,5-T also elicited coumarin accumulation. In all cases production of isocumarin was related to the amount of ethylene produced by root tissue. Foliar spray with either GA_3 or CCC (both 1 and 100 ppm) increased susceptibility of jute seedlings growing in *Macrophomina* infested soil. Maximum susceptibility was observed when treated with GA_3 but minimum in case of CCC treated plants (Purkayastha *et al.*, 1972). Under the influence of IAA and GA_3 some aspects of host parasite relationships were studied by Valken (1972). He reported that IAA increased the *Fusarium* wilt of tomato while the reverse result was obtained with GA_3.

Furrer and Staulfer (1972) demonstrated that by the application of cycocel in combination with nitrogen, yields of spring wheat was augmented; lodging and eye spot caused by *Cercosporella herpotrichoides* was reduced. Bojarezuk and Ruszkowski (1972) also noted that application of cycocel (3-4kg/ha) at the end of tillering reduced eye spot infection in wheat and rye and increased yield in both cases. The effects were more pronounced in varieties susceptible to lodging with high nitrogen fertilizer. Efficacy of cycocel against grey rot of grapevine caused by *Botrytis cinerea* was tested by Natalina and Svetvov in 1972. About 25-50% reduction in the incidence of disease was recorded after spraying vine with cycocel. But cycocel treatment increased the infection of jute caused by *Septoria nodorum.*

Artificial application of natural and synthetic chemicals could also induce disease resistance in plants. Sharma (1973) reported that application of DL-tryptophan, IAA and HCN induced resistance in some sorghum varieties to *Colletrotrichum graminicola.* Particularly, 0.12% and 0.062% of KCN (in place of

HCN) proved to be effective in inducing the resistance.The most encouraging results were obtained with 25+5, 25+10 and 50+5 ppm concentrations of DL-tryptophan and zinc respectively (zinc is known to take part in conversion of tryptophan to IAA). By the application of IAA (50 ppm) resistance to *C. graminicola* was noticed more than DL-tryptophan (25 or 50 ppm).

Sad and Rashid (1973) recorded that 16 ppm CEPHA (2-chloro ethane phosphonic acid) controlled the chocolate spot disease of potato and induced the production of small size tubers and tuber crackie (4%). Similarly, 25 ppm GA also controlled the disease but induced knobiness (12%) and sprouting (15%) of tubers in the field. On the other hand application of IAA (80-50 ppm) did not influence disease but induced the production of large size tubers. It is interesting to note that a mixture of CEPHA and IAA when sprayed 2 weeks after flowering decreased disease incidence considerably and undesirable side effects produced by CEPHA alone were not observed.

The gibberellins and tri-iodobenzoic acid decreased severity of charcoal rot disease of soybean under all experimental conditions (Oswald and Wyllie, 1973). The effects of indole acetic acid and kinetin on the development of *Verticillium* wilt of cotton was explained by Abrarov *et al.*, (1973). These compounds inhibited the spread of necrosis of leaf blades and stimulated formation of leaves and generative organs.

The role of auxins in leaf spot incidence in ragi was discussed by Vidyasekaran (1976). Young leaves of ragi (*Eleusine coracana*) was resistant to blight disease caused by *Helminthosporium tetramera* while the older leaves were highly susceptible. Young leaves contained more auxin than the older leaves. The IAA treatment inhibited spore germination and growth of the pathogen only at high concentrations.

The effect of foliar application of plant hormones on the development of anthracnose disease caused by *Colletotrichum corchorum* in two cultivars of jute (*Corchorus capsularis*) were studied by Purkayastha and Ray (1977) under identical conditions. These hormones were also tested on the growth of the pathogen *in-vitro,* Gibberellic acid (10 and 100 ppm) and indole acetic acid (10 ppm) increased disease susceptibility in both resistant and susceptible cultivars. These compounds stimulated mycelial growth of *C. corchorum* at a low (0.1 ppm) concentrations. Apparently there

was no correlation between mycelial growth and pathogenecity of fungus. Inflorescence of grapevine sprayed with 10 ppm gibberellic acid significantly reduced *Botrytis* infection (Rivera and Mavrich, 1978). Mercuric acetate caused accumulation of rishitin and lubimin in potato tuber discs. Accumulations of these terpenoids was not directly correlated to the necrotic reaction. When two cultivars of *P. vulgaris* showing different degrees of susceptibility were treated with $HgCl_2$, the yield of phytoalexin was similar in both the cultivars. However, the accumulation pattern differed when inoculated separately with 3 isolates of *Botrytis cinerea* differing in virulence (Cheema and Haard, 1978).

In the glass house, application of 2,4-D (40% butyl ester) and atrazine (72%) increased susceptibility of soybean to blight disease caused by *Sclerotium rolfsii.* Incidence was higher in plants with low or high sugar content, but lowest on those with normal sugar content. It was also noted that monosodium phosphate, zinc sulphate, 2,4-D and atrazine were mildly phytotoxic (Carlos, 1979). Some growth retardants mitigated *Verticillium* wilt and increased yield of cotton. Particularly the application of chloromegnat [(2-chloroethyl) trimethyl ammonium chloride], Pix (N,N-dimethyl piperidinium chloride) and chemagro-8728 (tributyl) slightly migrated the severity of symptoms of *V. dahliae* on cotton and reduced internal populations of the pathogen in the petioles, cotton yield was increased (10.29%) by these treatments (Erwin *et al.*, 1979).

A fungicide known as 2,2-dichloro-3,3-dimethyl cyclopropane carboxylic acid (WL 28325) has been found to activate the natural resistance of rice plants against blast disease caused by *Pyricularia oryzae.* The activity of WL-28325 is unique in that it does not itself stimulate phytoalexin production but rather increase the capacity of rice plants to synthesize more momilactones (rice phytoalexins) in response to fungal infection. The antifungal activity of rice phytoalexin may be the basis for its disease reduction properties (Cartwright *et al.*, 1980). The effect of mercuric chloride on glyceollin synthesis or degradation of glyceollin was tested by Moesta and Grisebach (1980). They observed that $HgCl_2$ produced only a slight effect on the biosynthetic activity but strongly inhibited glyceollin degradation.

The effect of foliar spray of bacitracin, chloramphenicol and GA on rhizosphere microflora of pea seedlings (*P. sativum* L.) infected with *V. dahliae* was

studied. The antibiotics increased fungus and actinomycetes counts and reduced the bacterial populations in the rhizosphere. The GA reduced all three groups of micro-organisms while 100 ppm increased actinomycetes slightly. Foliar spray also affected the percentage occurrence of particular genera of fungi in the rhizosphere, for examples, *Trichoderma* spp. were stimulated by all treatments, the maximum being with 10 ppm GA. Foliar spray however, markedly reduced disease severity (Ramarao and Isacc, 1980).

The effect of three growth substances 6-Furfuryl aminopurine (Kinetin), 6-Benzyl aminopurine (BAP) and gibberellic acid (GA_3) on the development of charcoal rot disease of soybean caused by *Macrophomina phaseolina* was studied by Chakraborty and Purkayastha (1981). Two foliar sprays with 1 or 10 ppm GA_3 at an interval of 3 days before inoculation of plants reduced the disease significantly. But the application of 10 ppm kinetin or BAP markedly augmented the disease.

Eight chemicals reported to induce phytoalexin in plants were used for wet seed treatment in an attempt to develop resistance in susceptible rice seedlings to *Drechslera oryzae*, the brown spot pathogen. While all produced appreciable effects, cysteine, thioglycollic acid, cycloheximide, sodium selenite, p-chloromercuribenzoate and lithium sulphate caused marked reduction in symptoms in rice seedlings when challenge inoculation was done at the age of 3-4 weeks. With sodium selenite and thioglycollic acid the induced effect persisted upto 8 weeks after sowing. A second treatment in the form of foliar spray with these chemicals caused sharp increases on protection, but these disappeared 2 weeks after treatment. Leaf diffusates from 2 week old seedlings in different treatments showed considerable fungitoxicity, which declined with seedling age and become practically non-existent by the end of fourth week. Inoculation with treated plants at this age resulted in moderate to marked toxicity in their diffusates. Seed treatment was found to be more effective than foliar spray treatment (Sinha and Hait, 1982). Accumulation of phytoalexin in excised cotyledons of *P. vulgaris* was detected when treated with 10^{-4}M abscissic acid or benzylaminopurine (BAP). In case of former, cotyledons were incubated both in light and in dark but in case of latter, they were kept under light only (Stoessel and Magnalato, 1983).

Furocoumarin (Phytoalexin) was induced in celery by copper sulphate (Bier and Oertelli, 1983). Capsidiol (pepper phytoalexin) production has also been induced in fruits of *Capsicum annum* by 0.1M copper sulphate, sodium nitrate and chloramphenicol (Watson and Brooks, 1984). Gibberellic acid (GA_3) induced momilactone synthesis in treated inoculated (with *Acrocylindrium oryzae*) leaf sheaths and coleoptiles. Since GA_3 is a degraded diterpene it may act as a precursor of gibberellin mediated enzyme (associated with momilactone biosynthesis) which may account for the elicitation of momilactone synthesis in rice plants (Ghosal and Purkayastha, 1984). Seed treatment of wheat with dilute concentration of nickel chloride and barium sulphate significantly induced resistance to *Drechslera sorokiniana* (Chakraborty and Sinha, 1984).

Twenty out of twenty four chemicals known to induce phytoalexin production in other plants when used as seed treatment provided effective protection to 3-week-old susceptible wheat seedlings against inoculation with *Helminthosporium sativum.* The number of lesions was very significantly reduced by most of the treatments and there was evidence for inhibition of lesion expansion in a few. Studies with twelve of the more effective chemicals showed that the protection effect persisted at significant levels even in 5 week old plants and that at this stage this inhibiting effect on lesion expression was more pronounced in most of the treatments. Different treatments led to the development of a moderate to high level of fungitoxicity in young wheat seedlings which markedly declined with age of the plant and disappeared in 5 week old plants. When inoculated at the age of 3 or 5 weeks, plants receiving most of the treatments developed appreciably higher fungitoxicity than the untreated plants (Hait and Sinha, 1986).

Chakraborty and Purkayastha, (1987) studied the effect of six metabolic inhibitors (viz. sodium iodoacetate, 2,4-dinitrophenol, sodium fluoride, sodium malonate, sodium azide and sodium molybdate) on the development of charcol rot disease of soybean (cv. Soymax). The effect of sodium azide (100 μg/ml) was found to the most significant among the metabolic inhibitors tested, in reducing the disease symptom. The reduction in disease was evidenced by minimum loss in weight of roots and minimum root rot index. The glyceollin content of soybean roots before and after disease reactions by sodium azide treatment was estimated and compared. The

production of glyceollin was maximum when plants were treated with sodium azide followed by inoculation with M.phaseolina. Sodium azide induced glyceollin synthesis even in uninoculated soybean plants.

The effect of foliar application of growth substances 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 (3-indole-acetic acid, 2,4-dichlorophenoxyacetic acid 2,3,5-tri-iodobenzoic acid, 2-napthoxyacetic acid, L-naphthalene acetic acid, gibberellic acid, 6-furfuryl amino purine and 6-benzyl aminopurine) examined, gibberellic acid was most successful in reducing the disease severity, followed by 3-indole acetic acid and 2,3,5-tri iodobenzoic acid. Low concentrations of these compounds stimulated while high concentrations inhibited the mycelial growth of *M. phaseolina in vitro*. Glyceollin contents of host roots before and after treatments with gibberellic acid (10mg/l.) were estimated; this compound significantly increased glyceollin production in infected roots.

Spray with $AgNO_3$ and $CuCl_2$ solution on the leaves of *Brassica juncea* and *B. napus* also caused accumulation of phytoalexin and the effect of cycloheximide suggested that its accumulation was associated with induced plant metabolism. Phytoalexin was detected in *B. juncea* and *B. napus*, 6h and 18h after challenge with $CuCl_2$ a non-specific elicitor. *B. juncea* always accumulated 4 to 10 times more phytoalexin than did *B. napus* (Rouxel *et al.*, 1989).

Purkayastha and Banerjee (1990) used six antibiotics (penicillin, cloxacillin, tetracyclin, chloramphenicol, cephaloridine and kanamycin) as foliar spray on a susceptible soybean cultivar (soymax) to induce resistance against anthracnose. Among the six antibiotics tested cloxacillin and penicillin induced maximum resistance against anthracnose. Spraying the lower surface of the first true levels of cucumber plants with 50M K_2HPO_4 induced systemic resistance to anthracnose caused by *Colletotrichum lagenarium*. Correlations were made between peroxidase and chitinase activities induced by several treatments on first leaf and the level of protection observed in leaf 2 after challenge with *C. lagenarium* (Irvan and Kuc, 1990). Reduction in barley stripe disease induced by *Helminthosporium gramineum* was observed by Mathur and Bhatnagar (1991) when seeds were immersed in ferrous sulphate solution or moistened with sulphates of manganese and cobalt before sowing.

Wet seed treatment with phytoalexin inducer chemicals and related compounds protected rice plants from the attack of both brown spot and blast diseases. Such compounds were effective at dilute concentrations, mostly non-hazardous and with little or no fungitoxic effect at the concentration employed. Many of the chemicals have equally strong effective action against both diseases; some are more effective against one than against the other. Sarkar and Sinha (1991) concluded that such chemicals may be mostly acting through an induction of general host resistance and also provide in the process a broad spectrum action effective simultaneously against a group of pathogens.

Effectiveness of 19 non-conventional (mostly non-toxic) chemicals in wet seed treatment (10^{-4} to 10^{-2}M) in controlling wilt of tomato (cv. Patharkuchi) caused by *Fusarium oxysporum* f. sp. *lycopersici* was demonstrated by Mandal and Sinha (1992). While most of the compounds could reduce wilt symptoms appreciably, cupric chloride, ferric chloride, zinc chloride, manganese sulphate, mercuric sulphate, L-cysteine, IAA and DL-methionine showed very strong protective effect. These reduced leaf symptoms by 52 to 71%, prevented mortality completely and also limited vascular colonization by the pathogen. Most of the test compounds showed little or no *in vitro* fungitoxicity at their effective concentrations and stronger protection was often achieved at lower than higher concentration. These non-conventional chemicals act in plant disease control not by toxic action but by inducing resistance in susceptible tomato plants, mediated through host tissue conditioning.

A varied group of non-conventional, non-toxic compounds such as metal salts, growth regulators, amino acids, metabolic inhibitors, and biological compounds used as seed treatment (at 10^{-6} to 10^{-8} M) were tested in controlling *Sclerotium* rot of soybean, *Fusarium* wilt of chickpea and *Sclerotium* rot and early leaf spot of peanut. The significant levels of protection were achieved with cupric chloride and ferric chloride in soybean; IAA, chitosan, cycloheximide in chickpea; IAA, chitosan in peanut against *S.rolfsii* and IAA, DL-phenylalanine, cycloheximide, barium sulfate and lithium sulfate against *Cercospora* infection (Chowdhury and Mitra, 1999; Chowdhury, 2000).

Serological relationship between host and parasite :

It is generally accepted that the cells recognize one another through pairs of complementary structures on their surfaces: a structure on one cell carries encoded biological information that the structure on the other cell can decipher. This idea represents an extension of the lock and key hypothesis. Each and every living plant has its own immune system functionally similar to that of animals. Conclusive evidences are now available to confirm the existence of phytoimmunity but unlike humoral immunity or specific target of antibodies which commonly operates in animals. A number of reviews pertaining to serological relationships between host and parasite have already been published (DeVay and Adler, 1976; Chakraborty, 1988; Hansen and Wick, 1993; Werres and Steffens, 1994).

Three species of the genus *Phytophthora* were studied serologically by gel diffusion and immunofluorescence. The type and concentration of nitrogen source, the amount of inoculum, and the age of mycelium greatly affected the antigenic efficacy of the preparations. The antigens were found to be localized in the young growing tips of the hyphae. Species specific sera were obtained and proved efficient for the identification of *P. cactorum,P. cinnamomi* and *P. erythroseptica* (Burrell *et al.*, 1966). Serodiagnostic methods for the differentiation between susceptible and resistant Egyptian cotton varieties with *Fusarium oxysporum* and *Citrus* sp. with *Phytophthora citrophthora* have been described by Abd-El-Rehim and Hashen (1970) and Abd-El-Rehim *et al.*, (1971a). Serological and immunoelectrophoretical studies on resistance and susceptible watermelon varieties to *Fusarium semitectum* also revealed that the cultivars could be differentiated by the titre or the time after which reaction occurred between antisera specific to the pathogens and seed globulins. In this case only $\alpha_2\beta$ globulin was present in the resistant varieties (Abd-El-Rehim *et al.*, 1971b).

Wimalajeewa and DeVay (1971) detected common antigenic relationship between *Zea mays* and *Ustilago maydis.* A pair of compatible haploid lines and two diploid solopathogenic lines of *U. maydis* were used in immunological studies. *Hordeum vulgare* var. "California Mariout" and *Avena sativa* var. "Victory" were taken as resistant hosts. Certain antigens were found common between corn and *U. maydis.* A strong antigenic relationship existed between the solopathogenic lines 132

and 3 day-old oat seedlings. Barley did not have any antigen in common with any of the *U. maydis* lines tested. Antigenic comparison of the four lines of *U. maydis* did not indicate any quantitative significant serological differences among them. Charudattan and DeVay (1972) compared common antigenic relationship among four cotton varieties and isolates of *Fusarium* and *Verticillium* species. One antigenic substance was common among the varieties of cotton and isolates of *Fusarium oxysporum* f. sp. *vasinfectum, F. solani* f. sp. *phaseoli, Verticillium albo-atrum* and *V. nigrescens*. Cotton varieties which were resistant or susceptible to *Fusarium* wilt as well as pathogenic and nonpathogenic isolates of *F. oxysporum* f.sp. *vasinfectum* shared the common antigens. The common antigens were not shared between cotton and nonpathogen (*F. moniliformae*). The common antigenic determinant shared by cotton and the fungal isolates does not appear to be related to the severity of wilt symptoms, but it may affect host pathogen compatibility during the process of root infection.

The presence of *Aspergillus* and *Penicillium* spp. which were predominantly isolated from stored barley grains, were confirmed by immunofluorescence, but the amounts of mycelium involved were small. *Alternaria* spp. though not isolated from 3 of the samples, mycelium were detected in grains of all the samples in small amounts (Warnock, 1973). Fluorescent antibody staining of *Fusarium culmorum* using antiserum raised against the mature hyphae of the fungus was reported by Hornok and Jagicza (1973). Both the direct and indirect methods of the fluorescent antibody technique resulted in a specific yellow-green immunofluorescence, however, indirect staining was always more intense. There was a significant difference in the intensity of the fluorescence between the mycelia of the homologous *F. culmorum* strain and those of the heterologous *F. graminearum* and *F. culmorum* strains. All the other *Fusarium* species tested showed no detectable fluorescence. The heterologous strains of *F. culmorum* and *F. graminearum* could not be distinguished. No significant difference was found between the 'culmorum' strains from different host plants. The serum specific for *F. culmorum* and *F. graminearum* is suitable to separate these species from other fungi.

Common antigen was shared by both avirulent and virulent isolates of *F. oxysporum* f.sp. *vasinfectum* with disease resistant and susceptible lines of cotton. In

all cases, the fungal isolates invaded and parasitized cortical tissues of cotton roots, but only those fungal isolates that caused disease became established in the vascular system (Kalyanasundaram *et al.*, 1978).

Rabbit antiserum rose against soluble extracts of *Phytophthora infestans* (Race-4) and tubers of "Aran Banner" and "Golden Wonder" potato cultivars showing field susceptibility and resistance respectively to late blight were used to test for the presence of common antigens between extracts of the fungus and various host and non-host plants (Palmerley and Callow, 1978). Cross reactive antigen was detected between *P. infestans* (Race-4) and potato tubers of both the field susceptible and field resistant cultivars and also between the fungus and leaves of tomato and tobacco. Common antigens were not detected between *P. infestans* (Race-4) and leaves of non-host (mungbean, pea, radish, cucumber and maize), or between potatoes and the alternative pathogen, (*Fusarium solani* var. *caeruleum*) and two non pathogens (*Ustilago maydis* and *Phytophthora cinnamoni*).

Fluorescent antibodies were used to study the survival of zoospore cysts of *Phytophthora megasperma* and *P. cinnamoni* in soil (Mac Donald and Duniway, 1979). Germinated zoospore cysts of both the species were stained by an immunofluorescent detection technique and counted. Under laboratory conditions, zoospores cysts of *P. megasperma* survived no longer than three weeks at water potentials ranging from 0-15 bars, and under field conditions they survived no longer than two weeks. Zoospore cysts of *P. cinnamoni* survived no more than 3 weeks in soil at water potential of 0 to –1 bar under either field or laboratory conditions. However, at water potentials of –5 or –15 bars, zoospore cyst of *P. cinnamoni* survived upto 6 weeks in the laboratory.

An antiserum, prepared to homogenates of washed *Epichloe typhina* mycelium grown in a liquid medium, was used in an ELISA to detect antigens of the fungus in tall fescue (*Festuca arundinacea*) tissue samples by Johnson *et al.*, (1981). ELISA could detect as low as 100 ng of freeze dried *E. typhina* mycelium/ml and could detect *E. typhina* in individual seeds of tall fescue. Of 14 fungal genera tested, including *Acremonium, Claviceps, Helminthosporium, Pythium, Rhizoctonia* and *Sclerotium* all showed reactivities less than 0.1% that of *E. typhina.*

Major cross reactive antigenic substances (CRA) common to cotton (*Gossypium hirsutum*) and certain fungal parasites of cotton roots, was isolated and purified to homogeneities from conidial cultures of *Fusarium oxysporum* f.sp. *vasinfectum.* Agar gel double diffusion tests indicated the presence of crossreactive antigen not only in *F. oxysporum* f.sp. *vasinfectum* and cotton roots and seeds but also in *Theielaviopsis basicola* (Devay *et al.*, 1981a). Indirect staining of antibodies and labelling with FITC indicated that in cross sections of roots, cross reactive antigen (CRA) was concentrated mainly around xylem elements, the endodermis and epidermis cells and was present throughout the cortex tissue. Protoplasts prepared from cross section of cotton roots also contained the CRA which was concentrated in the region of the plasmalemma. Treatment of mycelia and conidia of the pathogen with antiserum of cotton and using indirect staining with FITC indicated that the CRA was mainly present in hyphal tips and in patch like areas on conidia (DeVay *et. al.*, 1981b). Four formae speciales of *F. oxysporum* (*dianthi, mclonis, pisi, lycopersici*) and three physiological races (1,2,3) of *F. oxysporum* f.sp. *melonis* have been differentiated by Iannelli *et al.*, (1982) using agargel double diffusion technique. Three isolates of *F. oxysporum* f.sp. *dianthi* race 2 and 10 of f.sp. *lycopersici* race 1 tested against the appropriate antisera reacted the same within each races.

Gendloff *et al.*, (1983) produced antisera against 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. Specificity of the sera was improved by cross adsorption of the RITC-conjugated cell wall antiserum with *Phomopsis viticola.* Wood 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 serum and then stained with RITC labeled goat anti-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.

The presence of *Ganoderma lucidum* in roots of betel nut was detected by the induction of fluorescent antibodies (Reddy and Ananthanarayanan, 1984). Indirect immunofluorescence microscopy was used by Dewey *et al.*, (1984) to detect the binding of species-specific antisera against *Phaseolus schweinitzii* antigens to extracellular macromolecules secreted by the fungus, to cell surface of basiodiospores, and to the cell surface and cross walls of mycelia. Common antigenic determinants were present in extra cellular culture filtrate material and walls of mycelia, chlymodospores and basidiospores. Indirect immunofluorescence, performed by using antisera to culture filtrate molecules has been used to demonstrate the presence of mycelium, and on occasions chlamydospores, in naturally and artificially infested soil samples. Identification of the kind of propagule most likely to be the source of field isolated of the organisms was possible by immunofluorescence and 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.

Chard *et.al.*, (1985) carried out immunofluorescence tests, involving 34 species of fungi on an anti-*Mycena galopus* serum raised against a partially purified antigenic fraction. Cross-reacting fluorescence was produced primarily by *Mycenia*, deuteromycetes and ascomycetes species. Non-*Mycena* basidiomycetes generally showed less fluorescence. Absorption of the antiserum with mycelium from cross-reacting fungi resulted in a reduction in fluorescence of cross-reacting species, mostly to an acceptable control level.

Rabbit antisera were raised against antigens of *Macrophomina phaseolina* (isolate MP_1) and roots of soybean cultivars Soymax and UPSM-19 which were susceptible and resistant respectively to charcoal rot disease. These antisera were used in agar gel double diffusion tests for the presence of common antigens between isolates of *M. phaseolina* and soybean cultivars. Immunoelectrophoretic tests revealed that four antigenic substances were common between the susceptible soybean cultivars and the fungus (Chakraborty and Purkayastha, 1983). A close relationship between lower production of glyceollin by soybean cultivars and presence of common antigen following immunodiffusion and immunoelectrophoretic tests was also described by Purkayastha and Chakraborty (1988).

Immunological comparisons of teliospore surfaces using polyclonal antisera and monoclonal antibodies indicated that *Tilletia controversa* and *Tilletia caries* were very similar (Banowetz *et al.*, 1984). Although two polysaccharide antigens were present in teliospore extracts, these components appeared to be immunologically identical in both species and no protein antigens were demonstrated by either electrophoretic or immunologic means. None of seven fluorescein-lebelled lecitins bound these teliospores, even after the spores were treated with 8M Urea to enhance exposure of potential lecitin binding sites. An antibody "double sandwich" enzyme immunoassay demonstrated quantitative differences in the numbers of certain monoclonal antibody binding sites of the two fungi, although these differences did not provide a basis for the unambiguous detection of either bunt species in contaminated wheat shipments. Specific antibodies to *Phoma exigua* var. *foveata* and var. *exigua* were isolated and immunoenzymatic techniques (double antibody sandwich ELISA and indirect ELISA) were used by Aguelon and Dunez (1984) to test for the fungus 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. They also reacted with 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. The ELISA technique also showed good specificity and sensitivity in detecting "Sclerotinia antigen" at a concentration as low as 10 ng/ml, from both artificially and naturally infected host plants (Walcz *et al.*, 1985).

Cross reactive antigens were detected in crude preparations and in purified preparations from mycelia of *Phytophthora infestans* Race-4 and Race 1,2,3,4,7 with antisera of potatoes cv. King Edward and cv. Pentland Dell by using an indirect ELISA technique, (Alba and DeVay, 1985). Results suggests that the fungal mycelia did not easily release cross reactive antigens into synthetic media where they grew; that most of the *P. infestans* cross reactive antigens were thermo liable and that they could be concentrated by precipitation in the presence of 40% saturated ammonium sulphate (SAS). The results also revealed an antigenic disparity when 40% SAS from *P. infestans* Race-4 mycelial preparation was assayed with antisera for King Edward and Plentland Dell.

Immunodiffusion, immunoelectrophoretic and crossed immunoelectrophoretic analysis of rice in relation to sheath rot disease revealed a serological relationship between susceptible rice cultivars and isolates of *Acrocylindrium oryzae* (Purkayastha and Ghosal, 1985). One precipitin band was observed when the antigen preparation of *A. oryzae* was cross reacted with its own antiserum or with the antisera of four susceptible rice cultivars. No precipitin band was detected between the antiserum of resistant cv. Mahsuri and antigen preparations from isolates of *A. oryzae* or between the antigens of the resistant cultivars Mashuri 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 Banerjee (1986) determined common antigenic relationship between soybean cultivars and *Colletotrichum dematium* var. *truncata* 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*). Alterations in disease (charcoal rot) reaction as well as alteration in antigenic pattern were evident in soybean roots by the application of sodium azide (Chakraborty and Purkayastha, 1987).

Antigen from two isolates of *Macrophomina phaseolina* a pathogen of groundnut, four non pathogens of groundnut (viz. *Corticium sasakii, Colletotrichum lindemuthianum, C. corchori* and *Botrytis allii*), and five cultivars of *Arachis hypogea* were compared by immunodiffusion, immunoeolectrophresis and crossed immunoelectrophoretic studies for the presence of cross reactive antigens (Purkayastha and Ghosal, 1987). Common antigens were found among the susceptible cultivars of groundnut and two isolates of *M. phaseolina,* but not between non pathogens and groundnut cultivars. No antigenic similarity was found between non-pathogens and *M. phaseolina* isolates. Cross immunoeletrophoretic tests confirmed that at least one antigen was common between cv. J-11 and cv. TMV-2 and cv. Kidiri-71-1 and isolates of *M. phaseolina.*

Gerik *et al.*, (1987) detected hyphae of *Verticillium dahliae* in cotton root tissue with an indirect enzyme linked immounoassay. A soluble protein extract 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. *Phytophthora fragariae* was detected by ELISA in roots of strawberry cv. 'Teniva'. Because of high sensitivity of ELISA, presence of fungal antigen was demonstrated before symptoms could be detected in microscopical observations (Werres and Casper, 1987).

Gleason *et al.*, (1987) detected seed borne infection of soybean by *Phomopsis longicola* using indirect ELISA and immunoblot assay. Antigen of *P. longicola* was detected by indirect ELISA in as little as 250ng of dried mycelium/ml of extract. The antiserum reacted with mycelium preparation of *Diaporthe phaseolarum* var. *sojae* showed comparatively little or no reaction when tested against 7 other seed borne fungi. Extracts of whole seeds and of seed coats produced a non specific background reaction. *P. longicola* could be detected in individual symptomless infected seeds. A single infected seed coat not be readily detected by indirect ELISA. Immunoblot assay was developed to overcome problems with nonspecific interference in indirect ELISA. Indirect ELISA absorbance values for bulked samples of seed coat halves from the same 10 seed lots correlated weakly with agar plate bioassay but strongly ($P<0.01$) with incidence of symptomatic seeds because SIBA detected only viable *P. longicola* and ELISA did not discriminate between live and dead fungus, the author concluded that SIBA should be a better indicator of pathogen activity on seeds after planting. The two types of serological assay apparently measure different aspects of the disease, however, and both may be useful for evaluating soybean seed lot quality.

Schmitthener (1988) reported the development of an agridiagnostic *Phytophthora* multiwell ELISA kit for detection of *Phytophthora* in plant tissue, which also readily detected *Phytophthora* in soil where soybeans were damaged by *P. megasperma* f.sp. *glycinea* (Pmg). Only low levels of *Phytophthora* were detected in soil stored at 3^0C. Following cold storage high levels of *Phytophthora* could be detected directly from soil, after *Phytophthora* damping off of soybean seedlings was

induced. But *Phytophthora* detection was obtained from soybean leaf discs floated on water over infested soil for 24 hours. Pmg was the only *Phytophthora* that could be detected best with an ELISA test of soil with actively rotting or from leaf disc baits with actively growing mycelium.

An indirect ELISA for quantative detection of *P. herpotrichoides* infections in wheat was presented 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 the test, while the less virulent *P. anguioides* 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 sheath 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.

Antiserum raised against pooled mycelial suspension from five isolates (designated Pf 1, Pf 2, Pf 3, Pf 10 and Pf 11) representing five physiologic races of *Phytophthora fragariae,* used in an enzyme linked immunosorbent assay (ELISA) detected homologous soluble antigens at protein concentrations as low as 2ng/ml. 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, which was resistant to Pf 1,2 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 with avirulent isolates (Mohan, 1988).

Amouzou-Alladaye *et al.,* (1988) reported that the antiserum obtained by injecting rabbits 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 direct ELISA were comparable, the direct DAS ELISA was more specific for the detection of *P. fragariae* in strawberry roots. The antiserum failed to react with some strains of *P. cactorum* and *Pythium middletonii.* In inoculated strawberry roots, *P. fragariae* was detected reliably by ELISA several days before oospores were found and before symptoms developed.

Indirect fluorescent antibody technique was also applied for diagnosis of the clubroot of Brassicaceae caused by *Plasmodiophora brassicae.* Immunoglobulin (γ - globulin; 0.91mg/ml) was purified from antiserum against resting spores of *P. brassicae* prepared from a rabbit. Resting spores and root were stained by fluorescent antibody technique with the IgG and FITC conjugated anti rabbit 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).

The antisera raised against *Penicillium verrucosum* var. *verrucosum* were characterized by immunofluorescence and by ELISA for their reactivity with 44 strains of moulds (Fuhrmann, *et al.*, 1989). Antigenically, *P. verrucosum* var. *verrucosum* appeared to be similar to strains belonging to subgenus *furcatum*, but strongly different from *Penicillium frequentans.* Specific absorption of antibodies to antigens confirmed the existence of similar biochemical structures on *Penicillium frequentans, Aspergillus versicolor* and *A. fumigatus* .

Competitive types of two novel enzyme linked immunosorbent assay (ELISA) for *Fusarium* species were developed by Kitagawa *et al.*,(1989). Antiserum against a strain (F504) of *Fusarium* species was elicited in rabbits, and a highly specific, sensitive and accurate ELISA for the homologous strain was developed by using the antiserum with β-D-galactosidase-labelled antirabbit IgG as the secondary antibody and cell fragments of the strain attached to Amino Dylark balls as a solid phase antigen in a heterologous competitive ELISA. The modified system was a general assay for 10 strains for four *Fusarium* species with a high sensitivity. The specificity of the heterologous competitive ELISA was shown to be limited to *Fusarium* species. The potential of polyclonal antisera and monoclonal antibodies to differentiate the EAN and NAN aggressive subgroups of *Ophiostoma ulmi* was explored by Dewey *et al.*, (1989). Polyclonal antisera, when tested by ELISA, cross reacted widely with

unrelated species and failed to distinguish between the two aggressive subgroups but small quantative differences were found, particularly between antigen secreted overnight by EAN and NAN germlings. Out of 33 cell lines that secreted monoclonal antibodies positive for *O. ulmi* approximately one third were non-specific; 11 were specific either to species or subspecies. Two-cell line differentiated mycelial antigens of the aggressive isolates of *O. ulmi* from those of the non-aggressive subgroup, but non-antigens from surface washings. Only quantative differences were detected between the EAN and NAN aggressive subgroups. Almost all the monoclonal antibodies and antiserum recognized antigen present in surface washings of cultures or solid medium in cell free culture fluids, and in substances secreted overnight by germinating spores. Most of the monoclonal antibodies appeared to have potential diagnostic value; they gave readings two-fold to ten fold higher with extracts from diseased than from healthy tissue. However, one cell line that secreted antibodies specific to *O. ulmi* cross reacted strongly with extracts of healthy tissue.

Antiserum (anti PfM) rose against mycelial suspensions of *Phytophthora fragariae* isolates reacted strongly with antigens from several *Phytophthora* species. Some cross reactions with antigens from *Pythium* sp. were decreased by fractioning on an affinity column of Sepharose 4B bound to extracts of *Fragaria vesca* roots infected with *P. fragarie.* The affinity purified anti PfM retained its high cross reactivity with the various *Phytophthora* species tested. It also detected infection of rasberry and strawberry roots by some *Phytophthora* sp. (Mohan, 1989).

Phelps *et al.*, (1989) reported the development of an enzyme linked immunosorbent assay (ELISA) for isomarticin, a napthazarin toxin produced by *Fusarium solani.* A carbodrimide procedure was used to couple the heptan isomarticin to BSA for the immunogen and to alkaline phosphatase for enzyme linked assays. The resulting assay had a detection limit of 2ng/ml for isomerticin, other napthazarin toxin were detectable at less than 10ng per well in ELISA plates. The assay was specific for napthazarins. The cross reactivity with a number of phenolic compounds including the closely related napthoquinones was 3 order of magnitude less sensitive.

Glycoconjugates on the surface of zoospores and cysts of the pathogenic fungus *Phytophthora cinnamoni* have been studied by Hardhan and Suzaki (1989) using fluorescin FITC lebelled lectins for fluorescence microscopy and flow

cytometry, and ferritin and gold labelled lectins for ultrastructural analysis. Of the 3 lecitins used, only Con-A binds to the surface of the zoospores, including the flagella and water expulsion vacuole. This suggests that of accessible sachharides, glucosyl or mannosyl residues predominate on the outer surface of the zoospore plasma membrane. Labelling of zoospores in which intracellular sites are accessible indicates that the soybean agglutinin binding material is stored in vescicles that lie beneath the plasma membrane. Quantitation of soybean agglutinin labelling showed that maximum binding occurs 2-3 min. after the induction of encystment.

Watabe (1990) reported immunofluorescent antibody technique as a useful method for detecting *Phytophthora* in soil. But the autofluorescence and the nonspecific staining of soil particles interfered with the detection of the fungi in soil. However, pretreatment of the samples with gelatin-rhodammine conjugate prevented the samples from autofluorescence and nonspecific staining and therefore permitted the immunofluorescent antibody staining in soil. Stained *Phytophthora* was easily detected on the yellow-orange background.

Antisera were raised against antigens of three strains of *Myrothecium roridum,* two susceptible and one resistant cultivar of soybean for analysis or cross reactive antigens. Results of immunodiffusion revealed that common antigens were present only between the virulent strain and susceptible host cultivars, but no CRA was detected in case of resistant cultivars (UPSM-19, DS-73-16). Immunoelectrophoretic analysis showed that one common antigen was shared by susceptible host and the virulent strain (M-1). This was further confirmed by both crossed and rocket immunoelectrophoresis (Ghosh and Purkayastha, 1990). Alteration in antigenic pattern in soybean leaves were also observed after induction of resistance against *Colletotrichum dematium* var. *truncata* following treatment with cloxacillin (Purkayastha and Banerjee, 1990).

Antibodies of three isolates each of *Armillaria mellea,A .ostoyae,A .tabescena* and *Lentinula edodes* were isolated from eggs of immunized laying hens. The reactivity of each antibody preparation with all isolates was examined using an enzyme linked immunosorbent assay (ELISA). The cross reactivity of the antibody preparations to a given *Armillaria* species varied considerably when tested against isolates of other *Armillaria* species. Several antibody preparations were capable of

distinguishing isolates of the homologous species from isolates of the heterologous species. The specificity of the antibodies present in egg was dependent on time elapsed since immunization. Eggs laid 3 week after immunization with *Armillaria* species isolate possessed antibodies that were most specific for isolates of that species. The intergeneric cross-reactivity was found to be smallest with antibodies from eggs laid 5 weeks or more after immunization (Burdsall *et al.*, 1990).

A polyclonal antiserum prepared in 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 micro-organisms found on potato. *P. infestans* mycelium in leaf tissue was readily detected by ELISA using either the plate trapped antigen or $F(ab)_2$ antibody fragment techniques. The amount of mycelium in the 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 soluble antigens produced by *Botrytis cinerea* in picked and naturally infected grape juice by using enzyme immunoassay with an indirect format of antibody HRPO conjugates bound to polyclonal rabbit antibodies directed against *B. cinerea* (Anti Bc IgG). Protein-A purified Gama globulin from an early bled antiserum (803-7), which reacted primarily with low molecular weight compounds present only in extracts of *B. cinerea*, was used to specifically detect *B. cinerea* and quantify levels of infection in juice from infected grape berries. Late bled higher titer antiserum (803-19), which cross reacted with proteins and carbohydrates present in extracts from species of *Botrytis*, *Aspergillus*, *Penicillium* and *Uncinula* was used to 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 0.25-5%, with (803-7)IgG, and 0.2% with (803-19)IgG. Cross reactivity of all anti Bc IgG collections were low with infected grape juice. In contrast, cross reactivity of anti Bc IgG 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 antisera corresponded with an increase in the overall serum titers for anti Bc IgG to antigens from *B. cinerea*. Non specific binding of 803-19 IgG was high with extracts

from *A. niger* and an unidentified sp. of *Penicillium,* which suggested numerous epitopes common to antigens from these fungi.

In ELISA, antisera raised against *Phialophora mutabilis* reacted strongly with its homologous antigen and cross reacted strongly to moderately with six other *Phialophora* soft rot spp. (Daniel and Nilsson, 1991). With the exception of *Ceratocystis* sp. the serum reacted weakly or not at all with 11 other mold, blue and rots fungi. Extracts from *Fusarium oxysporum* and *F. oxysporum* var. *redolens* isolates were compared by means of electrophoresis and cross immunoelectrophoresis. Both fungi appeared almost identical serologically. Relative amounts of their corresponding proteins differed but quantative patterns of the proteins were nearly the same with the anti- *F. oxysporum* var. *redolens* serum; however, only one specific antigen was detected in the extract from this isolate. Ratej-Guranowska and Wolko (1991) concluded that although the obtained result indicated a strong similarity between *F. oxysporum* and *F. oxysporum* var. *redolens,* they were not sufficient for an unequivocal statement that the fungi belonged to the same species.

Sundaram *et al.,* (1991) reported that when antisera prepared against *Verticillium dahliae* were tested with crude mycelial preparations of *Verticillium* spp. using indirect ELISA, they reacted positively with 11 of 12 *V. dahliae* isolates from potatoes, cotton, soil, but negatively with one isolate from tomato. The antisera did not react with mycelial proteins of other fungi tested. Double antibody sandwich ELISA, using polyclonal antisera also detected *V. dahliae* and *V. albo-atrum* in infected roots and stems of potato.

Two commercial serological assay kits were compared by Benson (1991) to a culture plate method for detection of *Phytophthora cinnamomi* in root samples from inoculated azaleas. Both the multiwell E kit and F kit detected to *P. cinnamomi* on azalea roots beginning one week after inoculation. Agreement between immunoassay kits and culture plate results for detection of *P. cinnamomi* was most consistent beginning 3-5 weeks after inoculation. Root symptoms, but not foliar symptoms, of *Phytophthora* root rot were evident during this period. There was positive correlation between root severity in green house trails 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 10% infected root tissue. In a commercial nursery survey 5 and 15% of the azalea root samples at 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 also compared for their effectiveness in monitoring *Pythium* blight epidemics with ELISA. Samples consisted either of whole plants picked and assayed as whole plants, whole plants sectioned into lower, middle and upper starta components or leaf clipping collected with a mower. ELISA reading for mowed samples generally matched those for whole plucked samples (values 0.457 to 0.601). Fluctuations in detectable *Pythium* antigens were most pronounced on the upper most stratum compared with moderate to very little change in ELISA reading for 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, increases in ELISA readings for *Pythium* concided with, but not generally precede the onset of blight with 2-3 days sampling interval. It was concluded by the author (Shane, 1991) that antibody aided detection is useful for verification of diagnosis and detection of general *Pythium* population fluctuations, but the current method was not satisfactory for advance detection of blight epidemics.

The sensitivity of a *Phytophthora* specific immunoassay kit was tested on 17 species of *Phytophthora* collected throughout the world, including 18 isolates each of *P. cinnamomi* and *P. cactorum* by Pscheidt *et al.*, (1992). Kits were also used in a diagnosis of plant specimen with symptom characteristic of *Phytophthora infestans.* All isolates tested produced a positive result with the immunoassay kits. The lower absorbances relative to other species were obtained from *P. cinnamomi* and *P. megasperma.* Variation in absorbance was high among isolates of *P. cinnamomi* but low among *P. cactorum.* Clinic samples produced positive results with the immunoassay as did pure cultures of *Phytophthora* sp. isolated from those samples. Cross reactions occurred with several *Pythium* sp. isolated from those with several specimens infected with *Perinospora* sp. Other samples without typical *Phytophthora* symptoms but associated with other pathogens did not produce a positive reaction

with the immunoassay. Cross reactivity with some *Pythium* species made interpretation difficult, but when kit results were combined with field histories and symptomology, the immunoassay proved to be a useful tool for diagnosis.

Isolation of *Pythium* sp. from different soils in U.K. by conventional methods revealed *P. violae* to be most common, while *P. sulcatum* was isolated less frequently. Competition ELISA using polyclonal antibodies against *P. violae* and *P. sulcatum* confirmed the results of the conventional techniques. With cavities developed on the field grown carrots, ELISA confirmed the predominance of *P. violae.* 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 negative (Lyons and White, 1992).

A rapid serological test for detecting *Fusarium oxysporum* f.sp. *narcissi* in *Narcissus* was reported by Linfield (1993). Antiserum raised to cell wall fractions of *F. oxysporum* f. sp. *narcissi* 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 cross-reactivity was observed with two other *Fusarium* spp. and four other fungi showed little cross reactivity. Ten days after inoculation, the pathogen was detected in 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 enzyme linked immunosorbent assay and recovery of the pathogen on selective medium.

A double antibody sandwich ELISA test has been developed by Priestley and DeVay (1993), for the detection of *Pseudocercosperella herpotrichoides* using a high specific monoclonal antibody pH-10 as the capture antibody and genus specific polyclonal rabbit antisera as test antibody. The assay recognized extracts from plants both artificially and naturally infected with *P. herpotrichoides* giving at least 3 fold higher absorbance values with extracts of *P. herpotrichoides* infected tissue than with extracts from healthy tissue. The assay tested positively with all isolates of *P. herpotrichoides* including W type and R type isolator. The immunogen used was a

mycelial extracts from which high molecular weight proteins and glycoproteins were removed by SAS. The high mol. wt. fraction was shown to contain cross reactive antigens; it induced antiserum in mice that cross reacted with the other step base fungi even at high dilution.

Enzyme linked immunosorbent assay (ELISA) has also been used in the determination of fungal biomass during the early stages of infection of tuber disc of *Solanum tuberosum* by *Phytophthora infestans.* By optimizing the dilution of sample extracts and the dilution of primary anti-*P. infestans* antiserum, quantification of the biomass of *P. infestans* in zoospore inoculated tuber discs could be achieved by 8-18 h after inoculation (Beckman *et al.*, 1994). Differences in growth between avirulent and virulent isolates of *P. infestans* on the resistant potato cv. Kennebec were quantified by 32-48h after inoculation. On the resistant host, the growth of the avirulent isolate as detected by ELISA was essentially arrested by 16 h after inoculation, whereas that of the virulent isolate continued throughout the time course. On the susceptible host, however, the avirulent isolate appeared more aggressive than the virulent isolate. Two monoclonal antibodies and three polyclonal antisera were raised by White *et al.*, (1994) to cell wall/membrane fractions of *Pythium violae* and *Pythium sulcatum.* When screened with a collection of 40 isolates of genus *Pythium* including 20 species and the H-S group there was extensive cross-reaction. However, when the binding of the antibodies was assessed in an enzyme linked immunosorbent assay using cytoplasmic fraction antigens, the combined recognition pattern produced profiles unique to each species. In multivariety analysis, isolates of the same species tended to group together, while the affinity for fungi other than members of the genus *Phytophthora* was low.

Brill *et al.*, (1994) analyses two ELISA formats and antigen preparations against *Phomopsis longicolla.* The PAbs were purified to IgG fraction and tested in indirect ELISA, and in DAS-ELISA. 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 specifity 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 anti *P. longicolla* activity after 3 immunizations, and the activity become constant against most members of complex at the same time. Reactivity to some cultures of *P. longicolla* was in detectable following the 4th and 5th immunization, where as reactivity of all other cultures of the complex remained high.

Soluble protein extracts of chlamydospores and mycelium of *Thielaviopsis basicola* infecting *Gossypium hirsutum* were used to raise polyclonal mouse ascites antibodies. In a fungal capture ELISA, using the purified and biotin labelled IgG fraction both brown and grey cultural types of *T. basicola* were detected, while negligible cross reactivity was observed with other common 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 the time, initial symptoms were apparent. The antibody also was used to observe *T. basicola* on cotton roots with immunofluorescence microscopy. (Holtz *et al.*, 1994).

Jamaux and Spire (1994), developed a serological test that allows the early detection of infection of young petals of *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 DAS-ELISA and allowed the screening of cross reacting fungal species such as *Botrytis cinerea*, a pathogen commonly present on rapseed petals. Use of polyclonal anti-*B. cinerea* serum enabled the absorption by serial cycles, of *S. sclerotiorum* antigens common to *B. cinerea*. Residual antigens were used as immunogens for the production of the second generation antisera (S1 and S2) which were then tested by DAS-ELISA. Cross reactions with some unrelated fungi slightly increased. *S. sclerotiorum* and *B. cinerea* were distinguishable using antiserum S2.

Antigens obtained from tea varieties, isolates of *B. carbonum* and non pathogens of tea (*Bipolaris tetramera* and *Bipolaris setarae*), were compared by immunodiffusion and immunoelectrophoresis, in order to detect cross-reactive antigens (CRA) shared by the host and the parasite (Chakraborty and Saha, 1994). 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 the 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.* When three antisera preparations (T 18A, T 26A and BC 1A) at 1:125 dilution, were tested against homologous and heterologous antigens (5 and 25µg/protein/ml), indirect ELISA could readily detect cross reactive antigens in semipurified mycelial preparations at concentrations ranging from 5-25µg/ml with antiserum BCTA at 1:125 dilution. Antigenic preparations from *B. carbonum* (isolates BCI) exhibited higher absorbance value when cross reacted with antiserum of susceptible variety (TV-18) than the resistant one (TV-26). Antigen preparations from pathogen *B. carbonum* showed greater absorbance value than from the non-pathogen, *B. tetramera* in its cross reaction with antiserum of TV-18. Indirect staining of the antibodies raised against *B. carbonum* using fluorescein isothiocyanate (FITC) indicated that in cross sections of tea leaves (TV-18), the CRA concentrated mainly around epidermal cells. Treatment of mycelia and conidia of *B. carbonum* with antisera of leaves (TV-18) and indirect staining with FITC indicated the presence of CRA in the young growing hyphal tips and conidia.

Leaf and fungal antigens from 12 varieties of tea and from mycelia of three isolates of *Pestalotiopsis theae,* causal agent of grey blight disease were prepared. These antigens were compared with antisera raised against mycelial suspension of *P. theae* (Pt-2) and leaf antigens of Teenali – 17/1/54 and CP-1 by immunodiffusion tests (Chakraborty *et al.,* 1995) to detect crossreactive antigens (CRA) shared by the host and the parasite. CRA were found among the susceptible varieties and the pathogen (Pt-1,2 and 3). Such antigens were not detected between isolates of *P. theae* and resistant varieties. Antiserum preparation from leaf antigens of two varieties (Teenali 17/1/54 and CP-1) and mycelial antigen of *P. theae* each at a diluation of 1:125 were tested against homologous and heterologous antigens (25µg protein/ml). Results showed that indirect ELISA could readily detect cross reactive antigens both in crude and semi purified mycelial antigen obtained from *P. theae.* Higher absorbance values were obtained in reaction with Teenali 17/1/54 antiserum than when reacted with antiserum of CP-1 thus showing an antigenic disparity. When mycelial extracts of non-pathogen *Bipolaris tetramera* was tested in ELISA using antisera of CP-1, Teenali-17/1/54 and *P. theae,* no such cross reactivity was detected.

Using fluorescein isothiocyanate (FITC) and antibodies raised against *P. theae*, Chakraborty *et al.*, (1995) reported that CRA was present in the young hyphal tips of the mycelia, setulae and appendages of conidia of *P. theae* and in the epidermal and mesophyll tissue of cross sections of tea leaves.

An immunoassay for the detection of *Phomopsis* was used to detect levels of the mycotoxin in the epidermal peels from resistant and susceptible lupin cultivars asymptomatically infected with *Diaporthe toxica.* Quantifiable levels (>6.25μg/kg) of *Phomopsis* were detected in susceptible lupin cultivars but not in very resistant breeding lines or in the controls. These differences reflect the difference in resistance observed in the microscopical assay and mature plants in the field (Williamson *et al.*, 1995).

Polyclonal antiserum was raised against mycelial extract of *Pestalotiopsis theae* and immunoglobulin fractions were purified by ammonium sulfate fraction and chromatography of DEAE sephadex. In ELISA antiserum dilution upto 1:16000 detected homologous antigen at 5 mg/L concentrations 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 serum 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).

Wakeham and White (1996) raised polyclonal antisera to whole (coded-16/2), and sonicated (coded-15/2) resting spores of *Plasmodiophora brassicae* and two soluble components prepared by filtration and ultracentrifugation (coded-SF/2). Cross reactivity of all three antisera with a range of soil fungi, including *Spongospora subteranea* was low. Test formats including Western blotting, dipstick, dot blot, indirect ELISA and indirect immunofluorescence was 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 1 x 10^7 resting spores/g in soil. With Western blotting the lower limit of detection with antiserum 15/2 was 1:10^5. This antiserum showed the greatest

sensitivity in the dipstick assay in the indirect ELISA and indirect immunofluorescence, for all which, these was a limit of detection of $1:10^2$. The indirect ELISA was successful only after the substitution of alkaline phosphatase by protein-A conjugated Horse radish peroxidase. Of the assay 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 antisera were raised against *Fusarium oxysporum* and soybean roots (cv.UPSM-19). Cross-reactive antigens (CRA) shared by *F.oxysporum* and *Glycine max* were detected by indirect ELISA and their cellular location in root tissues were determined by immunofluorescence test. CRA were concentrated mainly around epidermal cells and distributed in the cortical tissues. CRA were also present in microconidia, macroconidia and chlamydospores of the fungus (Chakraborty *et.al.*, 1997). Immunological formats for detection of *Sphaerostilbe repens,* causal agent of violet root rot disease of tea, as well as biocontrol agents such as *Trichoderma harzianum* and *Trichoderma viride* from soil has been developed by Chakraborty *et.al.* (2000).

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 National Research Centre for Soybean , Indore , Madhya Pradesh , India . Six cultivars namely Macs-58 , J-80 , NRC-7 , Bragg , PK-262 , Pusa-16 were used. Seeds were air dried and stored at room temperature (28± 2^0C) as well as 20^0C . 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 / 25 cm diameter pot) . Prior to sowing , seeds were treated with 0.1% $HgCl_2$ for 5 minutes to remove superficial contaminants , followed by several washing with sterile distilled water .The plants were grown in two research station viz., Phytopathological Experimental Garden , Department of Botany, University of North Bengal and Bidhan Chandra Krishi Viswavidyalaya, North Bengal Campus , Cooch Behar under natural conditions of day light and temperature (26-35^{0}C) . The pots were watered daily with ordinary tap water. The plants were grown during March to October .

3.2 Fungal culture

3.2.1. Source of culture

A virulent strain of *Sclerotium rolfsii* Sacc . obtained from culture collection centre , Department of Plant Pathology, Bidhan Chandra Krishi Viswavidyalaya, North Bengal Campus, Cooch Behar. The culture was maintained on Potato Dextrose Agar medium by regular subculturings .

3.2.2. Completion of Koch's Postulate

Soybean seeds were surface sterilized with 0.1% $HgCl_2$ solution for 5 minutes, washed with sterile distilled water and sown in earthenware pots containing sandy soil. Seedlings (14 days old) were inoculated with *S. rolfsii.* Infected roots 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 7 days, the isolated organism was examined, compared with the original stock culture of *S. rolfsii* and its identity was confirmed.

3.2.3. Maintenance of Stock Cultures

The fungi listed in (Table –1) were grown on PDA slants and stored under 3 different conditions [5^0C, 20^0C and at room temperature 28^0C±2^0C]. Apart from weekly transfer for experimental work at a regular interval culture of *S. rolfsii* was examined in order to test its pathogenicity.

3.3.Test Chemicals

The following chemicals of diverse nature were used for seed treatment in different experiments. Most of the chemicals used were Sigma products and all were of analytical grade.

3.3.1. Metal Salts

a) Cupric chloride
b) Lithium sulphate
c) Ferric chloriole
d) Magnesium sulphate
e) Sodium molybdate
f) Manganese sulphate
g) Zinc chloride
h) Barium sulphate

3.3.2.Growth regulators

a) 2,4, - Dichlorophenoxy acetic acid (2,4,-D)
b) 2,4,5 – Trichlorophenoxy acetic acid (2,4,5-T)
c) Indole – 3 – acetic acid (IAA)
d) Cycocel [(2 chloroethyl) trimethyl ammonium chloride].

3.3.3.Other Compound

Chitosan (a polymer of β – 1,4 linked glucosamine) : The deacetylated derivative of natural biopolymar,chitin. It is the product of Bentech Laboratories Inc. USA.

Initially three concentrations ranging between 10^{-3}M to 10^{-5}M or 10^{-5}M to 10^{-7}M for metal salts and growth regulators while six concentrations ranging from 0.01% to 1.0% for chitosan were selected for *in-vitro* and *in-vivo* test and finally effective concentrations were chosen for further experiments.

3.4. Process of chemical treatment

Most of the chemicals were dissolved in distilled water. Chitosan was dissolved in 1% glacial acetic acid solution in distilled water and pH of the solution was then adjusted to 5.9 by adding 1 N NaOH. Indole-3-acetic acid, 2,4 – dichlorophenoxy acetic acid and 2,4,5 – trichlorophenoxy acetic acid were dissolved initially in a few drops of ethanol and then distilled water was added to make the require volume.

Seeds (100g) were surface sterilized by dipping them in 0.1% $HgCl_2$ solution for two minutes, thoroughly washed with distilled water and soaked separately in experimental chemical solution (200ml) for 24 hours. For chitosan treatment, surface sterilized seeds were treated with chitosan solution (2ml / 100 g of seed) followed by thorough shaking to spread the solution to seed surface as a thin film and then drying them before sowing in pots or field plots.

3.5 Inoculation technique

3.5.1. Sand maize meal culture

S. rolfsii was initially grown on sterilized sand maize meal medium (Sand:Maize meal-3:1) for six days at 28^0C. Finally the inoculum was mixed with sterile soil at the ratio 1:8. Fungus-soil mixture (50gm) were mixed with top soil of each pot containing 14 day- old soybean seedlings (10 plants/pot) and kept in glass house for observation of disease reaction.

3.5.2. Water culture

Fifteen surface sterilized (with 0.1% $HgCl_2$ solution for 5minutes) soybean seeds were sown in each pot containing autoclaved sandy soil. The seedlings were grown in the glass house conditions. *S. rolfsii* was grown in potato dextrose broth (100 ml broth/250 ml flask) at 30^0C for 10 days. Mycelial suspension containing sclerotia were prepared by homogenizer. Subsequently 15 day-old seedlings were uprooted from the experimental pots, the root system was washed thoroughly in running tap water, then rinsed twice in sterile distilled water and finally 10 seedlings were placed in each Erlenmeyer flasks (250 ml) containing 200 ml mycelial suspension and plugged with cotton. Control plants were kept in sterile distilled water.

3.6 Disease assessment

The external symptoms were assessed thrice (4,7 and 14 days) after inoculation with *S. rolfsii* . The plants were carefully uprooted, washed in tapwater and lesions at the colar region were intially examined and graded as follows : 0 = Plant is healthy ;1 = Incipient lesions at the collar region, 2 – 7 mm in length ; 2 = Large lesion, 8–12 mm in length, loss of turgor at the top i.e. drooping of tips; 3 = Extensive rotting at the collar region, wilting and drying of many leaves, drooping of the stem; 4 = Plant completely wilted, dead and dry.

Total number of plants per treatment ranges from 60-70 and mean disease index for a plant and percent disease index (PDI) were calculated as follows:

$$\text{Mean disease index} = \frac{\Sigma \text{ Disease index}}{\text{No. plants observed}}$$

$$\text{Percent Disease Index} = \frac{\Sigma \text{ Disease index} \times 100}{\text{No. plants observed} \times \text{maximum rating}}$$

In addition, percentage mortality of the plants were also recorded at the last date of sampling .

3.7. Fungitoxicity assay of chemicals

The fungitoxicity of test chemicals were assayed as follows. Filter papers were soaked in test chemical solutions, 25 sclerotia of *S. rolfsii* were placed on each filter paper kept in sterile petridishes (90mm diam). There were four replicates for each treatment. The petridishes were incubated at 28^0C for 48 hours. Then the percentage of germination of sclerotia were determined under a binocular microscope.

3.8. Extraction of enzymes

Four enzymes viz., Pectolytic enzyme, Polyphenol oxidase, Peroxidase and Phenylalanine ammonia lyase were extracted from healthy and *S. rolfsii* infected soybean plants. For *S. rolfsii* infected soybean plant, 2-3 cm collar region were collected and while collecting tissue samples from inoculated plants, every attempt has been made to keep the unaffected green portion to a minimum.

3.8.1. Pectolytic enzyme

Five grams of infected plant tissue was crushed with 5ml of 0.05M citrate phosphate buffer at pH 5.0 and centrifuged at 5000 r.p.m. for 20 min and the supernatant was taken .Four ml of 0.3% polygalacturonic acid in 0.05M citrate phosphate buffer (pH 5.0) was taken with 1ml of tissue extract and this reaction mixture was kept at 30^0C for 1 hour and then placed in ice to stop the enzyme activity and measured the amount of galacturonic acid using this mixture as enzyme source .

3.8.2 Polyphenoloxidase

For the extraction of polyphenoloxidase the method of Jennings *et al.* (1969) was followed. Two grams of fresh tissue was taken in a pre-cooled glass mortar with 0.05M Tris HCl buffer pH 7.4 (5 ml/g) and ground with pestle at 0^0C with a pinch of neutral sand to facilitate good grinding . The homogenate was then centrifuged at 15,000 r.p.m. for 30 minutes at 0^0C . The supernatant liquid was then used as the source of enzyme.

3.8.3 Peroxidase :

Two grams of fresh tissue was taken in a pre-cooled glass mortar with phosphate buffer pH 6.0 (5ml/g) and a pinch of neutral sand and ground with pestle at 0^0C. The homogenate was then centrifuged at 15,000 r.p.m. for 30 minutes at 0^0C and the supernatant was used as the source of enzyme.

3.8.4 Phenylalanine ammonia lyase

One gram tissue was ground with a mortar and pestle with 5 ml/g sodium borate buffer in 2 mM mercaptoethanol [0.1 (M), pH – 8.8] . Slurry centrifuged at 15000g for 4 minutes and supernatant was collected and enzyme activity was assayed by measuring the production of cinnamic acid from L–phenyl alanine spectrophotometrically (Bhattacharya and Ward, 1987).

3.9 Assay of enzyme activity

3.9.1 Pectolytic enzyme

Pectolytic enzyme activity was measured following the method as described by Miller (1972). Initially the following reagents were prepared.

A. Sodium potassium tartarate (300g of this salt was mixed in 500ml of distilled water).

B. 3,5- Dinitrosalicylic acid (10g of this reagent was dissolved in 200ml of 2N sodium hydroxide).

C. DNS reagent : This reagent was prepared freshly by mixing solution (A) and (B) and making the volume upto 1 liter by addition of water.

One ml aliquot of the reaction mixture was taken in a test tube and 3ml of DNS reagent was added to it and the mixture was kept in a boiling water bath for 5 minutes, cooled and the volume was made upto 25ml with distilled water. The optical density was measured at 540 nm in Bausch and Lomb Spectronic-20 colorimeter. D-galacturonic acid was used as the standard (1 mg/ml) for the purpose of estimation.

3.9.2 Polyphenoloxidase

Polyphenoloxidase activities were assayed following the method of Jennings *et al* (1969). The reaction mixture consisted of 0.05 ml of crude extract, 3.0 ml of 0.02M citrate phosphate buffer (pH 6.0), 1.0 ml of proline (5.0 mg/ml) and 1.0 ml of catechol (2.0 mg/ml). The mixture was aerated, using glass capillary for two minutes, before addition of catechol which initiated the reaction and absorbance was measured at 420nm in a Bausch and Lomb Spectronic–20 colorimeter. Enzyme activity was expressed as the change in absorbance/0.05ml of extract (10mg of extracted tissue) per minute but final change of optical density after 30 minutes at 420nm was taken into consideration. Enzyme extract autoclaved for 20 minutes at 121^0C was used as the time control in all cases.

3.9.3 Peroxidase

Peroxidase activity was measured following the method of Addy and Goodman (1972).To 3ml of 0.05 M pyrogallol reagent in a colorimeter tube, 0.05ml of homogenate was added and thoroughly mixed. The tube was then inserted into Bauch and Lomb Spectronic –20 colorimeter at 420nm. After the colorimeter galvanometer had been adjusted to '0' optical density, 0.5ml of 1 percent H_2O_2 was quickly added to the tube which was then inverted once and immediately reinserted into the colorimeter. The change in optical density between 40 and 160 sec at 420nm was used to plot peroxidase activity. A change in the absorption by 0.01 per minute was accepted as a unit of activity. Results were expressed as unit of activity g/ tissue.

3.9.4 Phenylalanine ammonia lyase

The reaction mixture was prepared with 0.3 ml of 300 µm sodium borate buffer (pH – 8.8), 0.3ml of 30µm L- phenylalanine, 0.5ml of supernatant and 1.9 ml distilled water was added to make the total volume of 3.0ml. The reaction mixture was incubated for 1h at 40^0C and finally the absorbance at 290nm was noted in UV-spectrophotometer. The enzyme activity was expressed as µg cinnamic acid produced in one minute/g fresh weight of tissue.

3.10. Total phenol

3.10.1 Extraction

Tissues freshly collected from plants, washed with distilled water and then cut into 1-2 cm pieces. Such pieces were used to extract phenol following the procedure of Biehn *et al.* (1968) with minor modifications.

Five grams of fresh tissue were put into boiling 80% ethanol (5ml/g tissue) in a water bath and kept for 10 minutes. The extract was cooled in a pan of cold water and then the tissue was crushed in a mortar with pestle for 5-10 minutes and the extract passed through two layers of cheese cloth .The ground tissue was re-extracted for three minutes in boiling 80% ethanol (using 3ml of alcohol for every g of tissue). The two ethanol fraction were pooled together and the mixture then evaporated in vacuum (at 40^0C) and the residue was suspended in glass distilled water, acidified to pH 4.5 with (N) HCl, and extracted three times with equal volume of ethyl acetate. The ethyl acetate fractions were then taken together and evaporated to dryness in vaccum at 40^0C. The dry material was then taken in 10 ml of ethanol.

3.10.2 Estimation

The total phenol content was estimated using Folin – ciocalteau reagent following the method of Mahadevan and Sridhar (1982).

One ml of extract was pipetted into a graduated test tube, to which 1ml of Folin – ciocalteu reagent was added followed by 2ml of Na_2CO_3 (20%) solution. The tube was shaken and heated on a boiling water bath for 1min. and then cooled under running tap water. The resulting blue solution was diluted to 25 ml with distilled

water and its absorbance was measured at 650 nm in a Bausch and Lomb Spectronic – 20 colorimeter. For comparison a blank containing ethanol and reagents was used. Total phenol was determined as catechol equivalent after comparing with the standard curue prepared from distilled catechol, obtained by using the same reagents. Total phenol was expressed as mg/g fresh weight of tissue.

3.11. Ortho – dihydroxy phenol

3.11.1 Extraction

Plant tissues (2g) were cut into pieces and immediately immersed in 20ml of boiling alcohol. After 15 minutes of boiling it was cooled and then crushed in a mortar with neutral sand using ethanol. The slurry was centrifuged at 3000 r.p.m. for 20 minutes and the supernatant was taken for O-dihydroxyphenol estimation using Arnow's reagent.

3.11.2 Estimation

The O-dihydroxyphenol was estimated following the method of Mahadevan and Sridhar (1982). One ml of alcoholic tissue extract was taken in a test tube to which 2 ml of 0.5N HCl, 1 ml of Arnow's reagent ($NaNO_2$-10g ; Na_2MoO_4-10g, Distilled water – 100 ml.), 2ml of 1 N NaOH were added and mixed thoroughly in room temperature following which the volume of the reaction mixture was raised to 10ml. Optical density was recorded in a Baush and Lomb Spectronic-20 colorimeter at 515 nm. A blank was prepared for comparison by adding 1ml of alcohol instead of tissue extract with other reagents. Standard curve was prepared with different concentrations of catechol. Results were expressed as mg/g fresh weight of tissue.

3.12 Soluble protein

3.12.1 Extraction

Soluble protein were extracted from soybean root tissue (collar region) following the method of Chakraborty *et al.* (1995). Root tissue (1g) were homogenized with 0.05M Sodium phosphate buffer (pH 7.2) containing 10mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$, 2mM polymethyl sulphonyl fluoride (PMSF) in mortar with pestle at 4^0C with sea sand. The homogenate was centrifuged at 4^0C for 20 minutes at 10,000 r.p.m. and the supernatant was used as crude protein and immediately stored at -20^0C for further use.

3.12.2 Estimation

Soluble proteins were estimated following the method as described by Lowry *et al.* (1951). To 1ml of protein sample 5ml of alkaline reagent (0.5ml of 1% $CuSO_4$ and 0.5 ml of 2% potassium sodium tartarate, dissolved in 50ml of 2% Na_2C0_3 in 0.1N NaOH) was added. This was incubated for 15min at room temperature and then 0.5ml of Folin ciocalteau reagent (diluted 1 : 1 with distilled water) was added and again incubated for 15 min for colour development following which optical density (O.D.) was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.13 Calcium and magnesium

3.13.1 Extraction

Plant samples were first air dried for 2 days and then oven dried at 40^0C for 24 hr. Then 0.5g of such dried plant samples were digested with 10ml of Tri–acid mixture (Conc. $HN0_3$. Conc H_2S0_4 and Conc. $HClO_4$ at a ratio of 10 : 1 : 4). After digestion the volume was made up to 50ml with distilled water and filtered through Whatman 42 filter paper.

3.13.2 Estimation

The calcium and magnesium content in plant tissue was determined following the method of Black (1965).

Five ml of above filtrate was taken in a 100ml conical flask for estimation of ca^{++} and Mg^{++} and 2.5 ml of NH_4Cl- NH_4OH buffer was added to it. A few drop of NaOH solution was added to raise the pH to 11.0. Then few drops of Eriochrome black T indicator was added and titrated against standard EDTA solution (0.01M), and the colour changed from wine-red to pink.

For estimation of calcium, 5ml of aliquot and 2.5ml of NH_4Cl – NH_4OH buffer was taken in 100ml conical flask to which a few drops of NaOH solution was added to raise the pH to 12.0 and then it was titrated with standard EDTA solution (0.01M) using calcon indicator, the colour changed from pink to blue.

3.14 Lignin

3.14.1 Extraction

Stem tissue (1g) was cut into 0.5 cm pieces and ground in 5ml of cold 0.01M phosphate buffer, pH 7.0. The solids were collected by centrifugation (1000 g) for 10 min. and washed with 10ml cold buffer thrice and cold water twice. The residue was further extracted with 10ml hot ethanol thrice and then successively with ethanol, acetone and diethyl ether. After air drying, lignin was extracted with 5ml 0.5M NaOH at 70^0C for 16 h. The residue was removed by centrifugation (1000g) for 5min and the supernatant was adjusted to pH 8.0 with 2.5 M HCl and the extract was dialyzed against water for 24h.

3.14.2 Estimation

Lignin was estimated following the method of Stafford (1960) as modified by Ride (1975). The spectrum of absorbance was recorded from 230 to 380nm in a Backman UV – spectrophotometer by diluting 0.5 ml of extract with 2.5ml of 0.06 M phosphate buffer, reading the sample at pH 12.0 directly against at pH 7.0.

3.15 Extraction and separation of glyceollin

To extract glyceollin, the method of Keen *et.al* (1971) was followed with modifications. Fifteen day old plants were inoculated with *S.rolfsii* following water culture method. In this case, *S. rolfsii* was grown in potato dextrose broth at 30^0C for 10 days. Mycelial suspension containing sclerotia were prepared by homogenization. Subsequently plants uprooted from experimental plot, the root system washed thoroughly in running tap water following sterile distilled water and transferred in 500ml flasks. After 24, 48 and 72h. of inoculation, roots were used for extraction of glyceollin. Infected as well as healthy roots (30g) were homogenised with 120ml of 95% ethanol in an electrical 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 45^0C.The residue was dissolved in ethyl acetate (0.1ml/g 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 (0.2 mm thickness) and activated for 1h at 80^0C. 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 + 0.5ml of 5% aqueous sodium nitrite solution + 15ml. of 20% aqueous sodium acetate solution). Rf value was determined in each case.

3.16 Ultraviolet spectrophotometry and quantifcation 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 scrapped off and eluted in spec methanol. The elutes were stored at 5^0C for overnight and centrifuged to remove the silica gel. These elutes were examined by UV- spectrophotometry (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 286 nm as described by Bhattacharya and Ward (1985).

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

X* = moles / liter converted to g/liter by multiplying with molecular weight of glyceollin ($C_{20}H_{19}O_5$). Results have been expressed in µg/g fresh weight of roots.

3.17 Petridish bioassay of phytoalexin :

In case of petridish bioassay, 0.2 ml of ethyl acetate extracts of infected roots were taken in 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 (20ml / petridish) and mixed well. Each petridish was inoculated with an agar block (4 mm.dia.) containing 4 day old mycelia of *Sclerotium rolfsii* and incubated at 30 ± 1^0 C. Diameter of mycelial mat was measured after 24,48 and 72 hours of inoculation and compared with the controls.

3.18 Preparation of antigen

3.18.1. Root antigen

Root antigens were extracted from healthy and *Sclerotium rolfsii* infected soybean roots following the method of Chakraborty and Saha (1994) . Seeds of soybean cultivars were grown in earthen pots containing sterilized soil. When the seedlings were at the age of 14 days they were inoculated with fungal inocula . Healthy and infected plants were uprooted after two-week intervals, washed with cold water and kept at 15^0 C for 1 hour. Finally, roots (20 gm fresh weight) were crushed with sea sand in mortar and pestle in cold (4^0C) and stored at -15^0C for 1 hour and homogenized with 20ml of 0.05M sodium phosphate buffer supplemented with 10 mM sodium metabisulphite and 0.5 mM magnesium chloride . Homogenate was strained through cheese cloth and then centrifuged (12,000g) at 4^0C for 1 hour and known quantity of ammonium sulphate was added to it for 100% precipitation (Green and Hughes, 1995), kept at 4^0C. Precipitate was dissolved in the same extractive buffer (pH-7.4) and dialysed against 0.005M phosphate buffer for 24 hour at 4^0C. During this period 10 changes were given. The dialysate (i.e. soluble protein) was used for antisera production and for gel electrophoretic study.

3.18.2. Mycelial antigen.

Mycelial antigen was prepared following the method of Chakraborty and Saha (1994). Initially fungal mycelia (4mm disc) were transferred to 250ml Erlenmeyer flasks each containing 50ml of sterilized liquid Richards medium (g / 1 distilled water, sucrose, 30; KNO_3, 10; KH_2PO_4, 5; $MgSO_4$, $7H_2O$, 2.5 and $FeCl_3$, 0.02) and incubated for 10 days at 30 ± 1^0C. For extraction of soluble antigens, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (30g fresh wt.) were homogenized with 0.05M sodium phosphate buffer (pH-7.2) supplemented with 10mM sodium metabisulphite and 0.5mM magnesium chloride and 0.85% NaCl in mortar and pestle in presence of sea sand. Cell homogenates were kept overnight at 4^0C and then centrifuged (15000g) for 30 min at 4^0C. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at 4^0C. After this period the mixture was

centrifuged (15000g) for 30min at 4^0C, the precipitate was dissolved in 10ml 0.05M sodium phosphate buffer (pH-7.2). The preparation was dialysed for 72h 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 material was stored at -20^0C and used as antigen for the preparation of antiserum and other equipments.

3.19 SDS-polyacralamyde gel electrophoresis of total soluble protein

3.19.1 Preparation of Slab Gel

3.19.1.1 Stock solutions

For the preparation of gel, the following stock solutions were initially prepared as described by Laemmli (1970).

(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	100 ml.

(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 peroxidisulphate 0.1 g

Distilled water 1.0 ml

(freshly prepared each time)

(F) Tris-glycine electrophoresis buffer

(25 mM Tris Base ; 250 mM glycine)

5x Stock can be made;

Tris Base 15.1 g

Glycine 94 g

In 900ml of d. H_2O, pH was adjusted to 8.3. Then 50 ml of 10% SDS was added and volume made upto 1000 ml.

(G) 1x SDS gel loading buffer :

50 mM Tris Cl (pH-6.8)

10mM β-Mercaptoethanol

2% SDS

0.1% bromophenol blue

10% glycerol.

3.19.1.2 Slab gel preparation:

For slab gel preparation, two glass plates (17cm X 19cm) were washed with dehydrated alcohol and dried. Then 1 mm thick spacers were placed between the glass plates and the two edges and the 2 sides of glass plates were sealed with grease and 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 (pH 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 over layered 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 (pH-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 upto a height of 4 cm over the resolving gel and overlayed with water. Finally the gel kept for 30 min for polymerization.

3.19.2 Sample preparation

Sample was prepared by mixing the sample protein with 1 x 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 micro liter 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.

3.19.3 Electrophoresis

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

3.19.4 Fixing and 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 20h in the fixer for fixing.

The staining solution was prepared as follows –

Coomassie Brilliant Blue R250	- 0.25 g
Methanol	- 45 ml
Distilled water	- 45 ml
Acetic Acid	- 40 ml

At first, gel were stained by staining solution for 2-3h and finally soaked with destaining solution (methanol : d H_2O : Acetic acid - 4.5 : 4.5 : 1) until the background become clear.

3.20 Antisera production

3.20.1 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. They were regularly fed with 500g green grass. Besides, every alternate day they were also given 50-75g of chickpea seeds soaked in water. Beside this, they were given saline water after each bleeding for two consecutive days. Cages were cleaned with Phytofresh every day in the morning for better hygienic conditions.

3.20.2 Immunization

Polyclonal antibody against mycelial antigens of *S. rolfsii* was prepared by immunizing rabbits. Before immunization normal sera were collected from each rabbit. Each time antigen emulsified with an equal volume of Freund's complete/incomplete adjuvant (Difco) and was injected intramuscularly. Doses were repeated at 7 days intervals and continued for 9 consecutive weeks.

3.20.3 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 (2ml) 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 1h and then the clot was loosened with a sterile needle and the antiserum was clarified by centrifugation at 2000g for 10 min. Finally, blood samples were distributed in 1ml vials and stored at -20^0C until required.

3.21 Purification of IgG

3.21.1 Precipitation

IgG was purified following the method of Clausen (1988). The antiserum (5ml) was diluted with two volumes of distilled water and an equal volume of 4.0 M ammoniumsulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16h at 22^0C. Then it was centrifuged at 10,000g for 1h at 22^0C and the precipitate was dissolved in 5ml of 0.02M Sodium phosphate buffer, pH 8.0.

3.21.2 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 0.02M phosphate buffer, pH 8.0 and was applied to a column (2.6cm in dia, 30 cm high) and allowed to settle for 2h. After that 25ml of 0.02M phosphate buffer (pH 8.0) was applied to the gel material.

3.21.3 Fraction collection

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02M to 0.03M . The initial elution buffer (1) was 0.02M sodium phosphate buffer pH 8.0 (diluted from a 0.10M sodium phosphate buffer pH 8.0 containing 16.86g Na_2HPO_4 , $2H_2O$ + 0.731g NaH_2PO_4 , H_2O/L.).The final elution buffer (2) was 0.30M 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, 40 x 5 ml fraction were collected and the optical density (OD) values were recorded by UV- spectrophotometer at 280 nm.

3.22 Immunodiffusion test

3.22.1 Preparation of agar slides

The agar slides (5cm x 5cm) were degreased successively in 90% (v/v) ethanol: di-ethyl ether (1:1 v/v) and ether, then dried in hot air oven and sterilized inside the Petri dish each containing one slide. A conical flask containing Trisbarbiturate 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 repetedly 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 a glass slides (6 ml/slide) and allowed to solidify. After 3-7 wells were cut on the agar plate with a sterilized cork borer (4 mm dia.) at a distance of 5 mm from the central well .

3.22.2 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 continue in a moist chamber for 48-72h at 25^{O}C.

3.22.3 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 stain with 0.5% coomassie blue (0.5g coomassie blue, 5g $HgCl_2$, 5ml glacial acetic acid, and 95ml distilled water) for 10min. at room temperature. After staining slides were washed thrice in destaining solution [2% (v/v) acetic acid] for 5h to remove excess stain. Finally, all slides were washed with distilled water and dried in hot air oven for 3h at 50^0C.

3.23 Immunoelectrophoresis

3.23.1 Preparation of agarose slides.

The slides (7.5 X 2.5 cm) were degreased, dried and sterilized as described earlier. Thin and uniform layer (2mm thick) of fluid agarose medium (0.97% agarose, 0.1% NaN_3 dissolved in 0.05 barbitol buffer (pH-8.6) was poured on each slide taking care that no air buble 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 4^0C until use.

3.23.2 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 (0.05M 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 3h at 4^0C. After electrophoresis the current was discontinued.

3.23.3 Diffusion

A longitudinal through 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 25^0C.

3.23.4 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 3h at 50^0C.

3.24 Enzyme linked immunosorbent assay

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

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

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

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

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

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

With 280 ml of stock solution 'A', 720ml 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).

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

Tris	0.657 g
NaCl	0.81 g
KCl	0.223 g

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

5. Antisera dilution buffer (0.15M 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:

The substrate P-nitrophenyl phosphate at 1.0 mg/ml in substrate buffer (1.0% [W/V] diethanolamine , 3mM NaN_3, pH 9.8) was added at 100µl per well.

7. Stop solution

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 (100µl / well) in Coster EIA/RIA 8 well flat bottom strip ELISA plate. After loading, plate was incubated at 25^0C for 4h. The plate was then washed three times under running tap water and once with PBS- Tween and each time, plate was shaken dry. Subsequently, 100µl of blocking agent was added to each well for blocking the unbound sites and the plate was incubated as 25^0C for 1h. After incubation, plate was washed as mentioned earlier. Purified antiserum (IgG) was diluted in antisera dilution buffer and loaded (100µl / well) to each well and incubated at 4^0C overnight. After a further washing 100µl of antirabbit IgG goat antiserum labelled with alkaline phosphatase (Sigma Chemicals, USA) was added and incubated at 37^0C for 2h. Plate was washed, dried and loaded with 100µl of Pnitrophenyl phosphate substrate in each well and incubated in dark at room temperature for 60 min. Colour development was stopped by adding 50µl / well of 3 M NaOH solution and absorbance was determined in an ELISA reader (Trans Asia Lisa-5 ELISA machine) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.25 Immunofluorescence

Indirect fluorescence staining of fungal mycelia were done using FITC labelled goat antirabbit IgG following the method of Chakraborty and Saha (1994).Fungal mycelia were grown in Richards solution. 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 3 min at 27^0C. 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 27^0C. 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 using Leica Leitz Biomed microscope with fluorescence optics equipped with ultra violet (UV) filter set 13. Mycelia were photographed under both phase contrast and UV fluorescent conditions for comparison of treatment.

Experimental

4.1. Pathogenicity test of *Sclerotium rolfsii* on different soybean varieties

Sclerotium blight of soybean [*Glycine max* (L) Merrill] incited by *Sclerotium rolfsii* Sacc.(*Corticum rolfsii* Curzi), a soil borne rotting pathogen of very aggresssive nature cause a considerable damage to one of our major legume crops with hundreds of food,feed and industrial uses. In the first experiment six soybean varieties viz. PK-262, Bragg , NRC-7 , Pusa – 16 , J-80 and Macs–58 were screened in respect of their disease reaction to infection with *Sclerotium rolfsii* . The plants were inoculated at the age of 14 days by adding sand-maize meal grown inoculum to the soil. First symptom of this disease was the sudden wilting of a branch of a plant which was in complete or partial contact with the soil. Leaves turned brown, wilted and remain attached to the plant (Plates 2 & 3) . A whitish growth of the fungal mycelium was seen at the junction of the branch with the stem closed to the soil level, which was the most favoured point of attack. With time, the disease progresses and a white mycelial web spreads over the soil and the basal canopy of the plant followed by the appearance of the Sclerotia of mustard seed size on the infected areas. The entire plant was killed but sometimes only 2-3 branches get affected. In its advance stage infection was very much prominent in the root system also (Plate- 4).

Symptoms were assessed at regular intervals, disease index were computed . Results recorded 14 days after inoculation when the symptoms appeared to be fearly advanced are presented in Table-1, Fig.1. It is evident from Table-1 that all six varieties are susceptible to *Sclerotium rolfsii* pathogen as evident from the data on disease index , percent disease index and mortality percentage , recording 2.8 to 3.2 , 70 to 80% and 71 to 88% respectively. Among the six soybean varieties tested, Macs-58 showed highly susceptible to *S.rolfsii* and all other varieties have more or less similar disease reaction. Hence Macs-58 was selected for further experiments.

S.rolfsii is a facultative parasite that occurs in diverse soils and has a very wide host range .The colonies of *S.rolfsii* were very fast growing on potato dextrose agar media, reaching 9 cm. diameter in 3 days at 28^0C, with white, cottony fan like mycelium and produces an abundance of globular, tan to reddish brown or dark brown sclerotia about the sizes of mustard seeds after 6-7 days (Plates – 5 & 6).

Plate 2 (A & B): Soybean plants (Macs-58) artificially inoculated with *Sclerotium rolfsii* [12 days after inoculation]
(A)Healthy ; (B) Infected

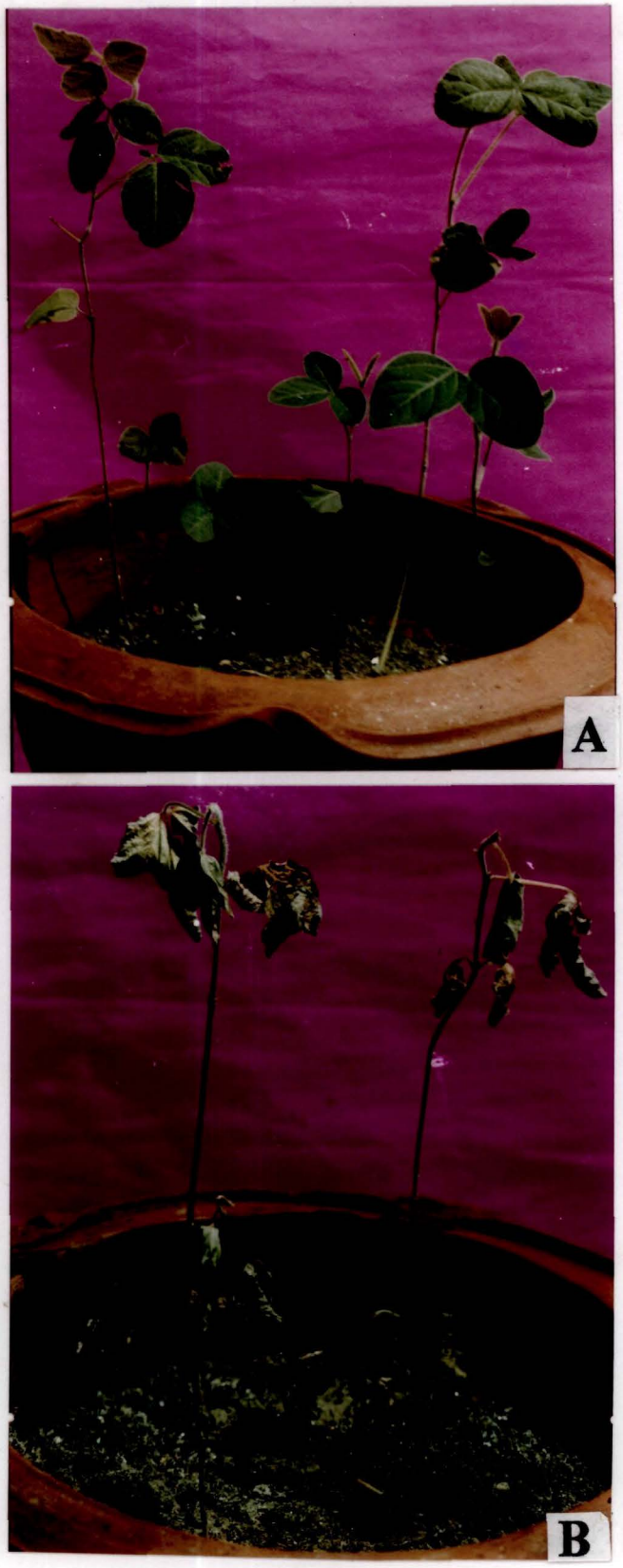


Plate 3 (A & B): Soybean plants (J-80) artificially inoculated with *Sclerotium rolfsii* [14 days after inoculation]
(A) Healthy ; (B) Infected

Plate 4 (A & B): Uprooted soybean plants (PK-262) artificially inoculated with *Sclerotium rolfsii*
(A) Healthy; (B) Infected

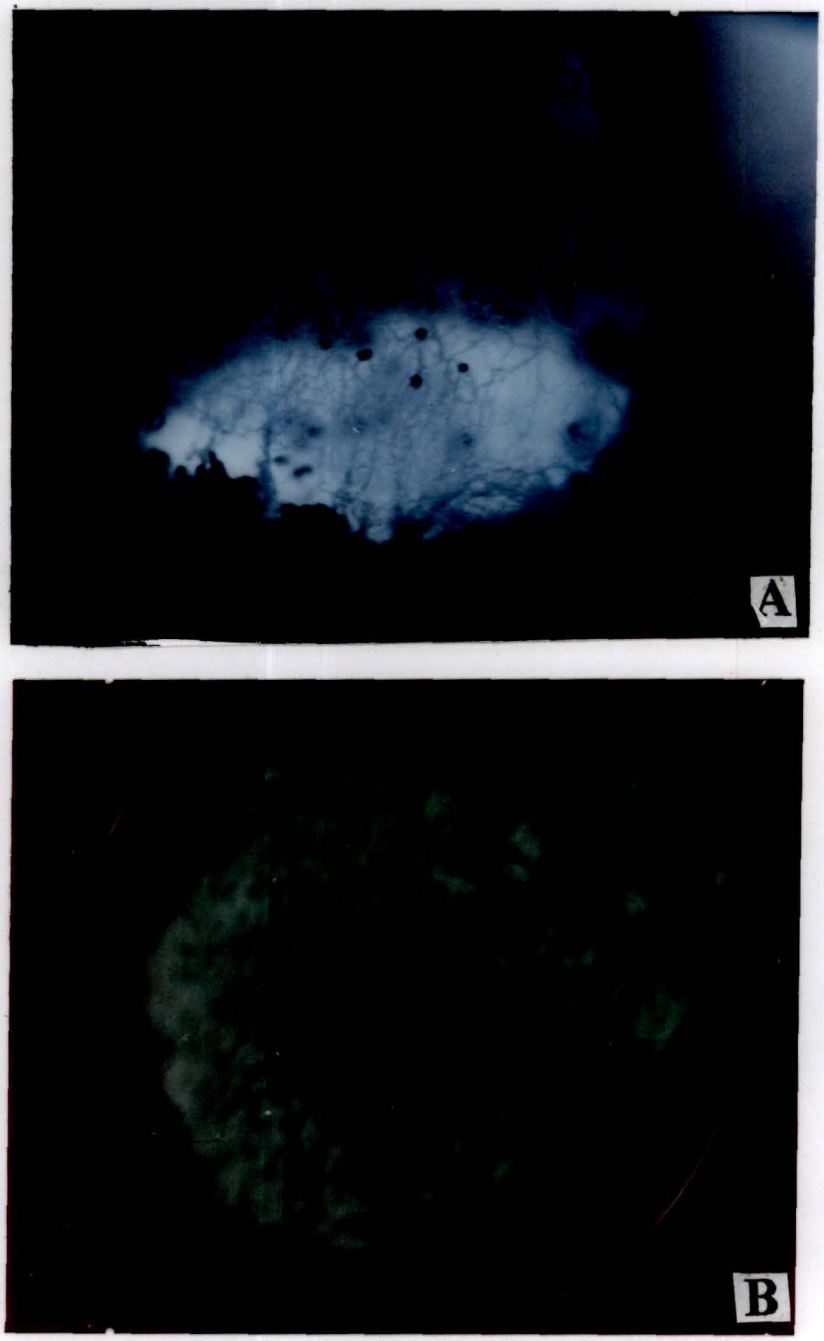


Plate 5 (A & B): *Sclerotium rolfsii* grown in Richard's medium
(A) Hyphal growth and sclerotia formation
(B) Close up view of sclerotia on mycelial mat

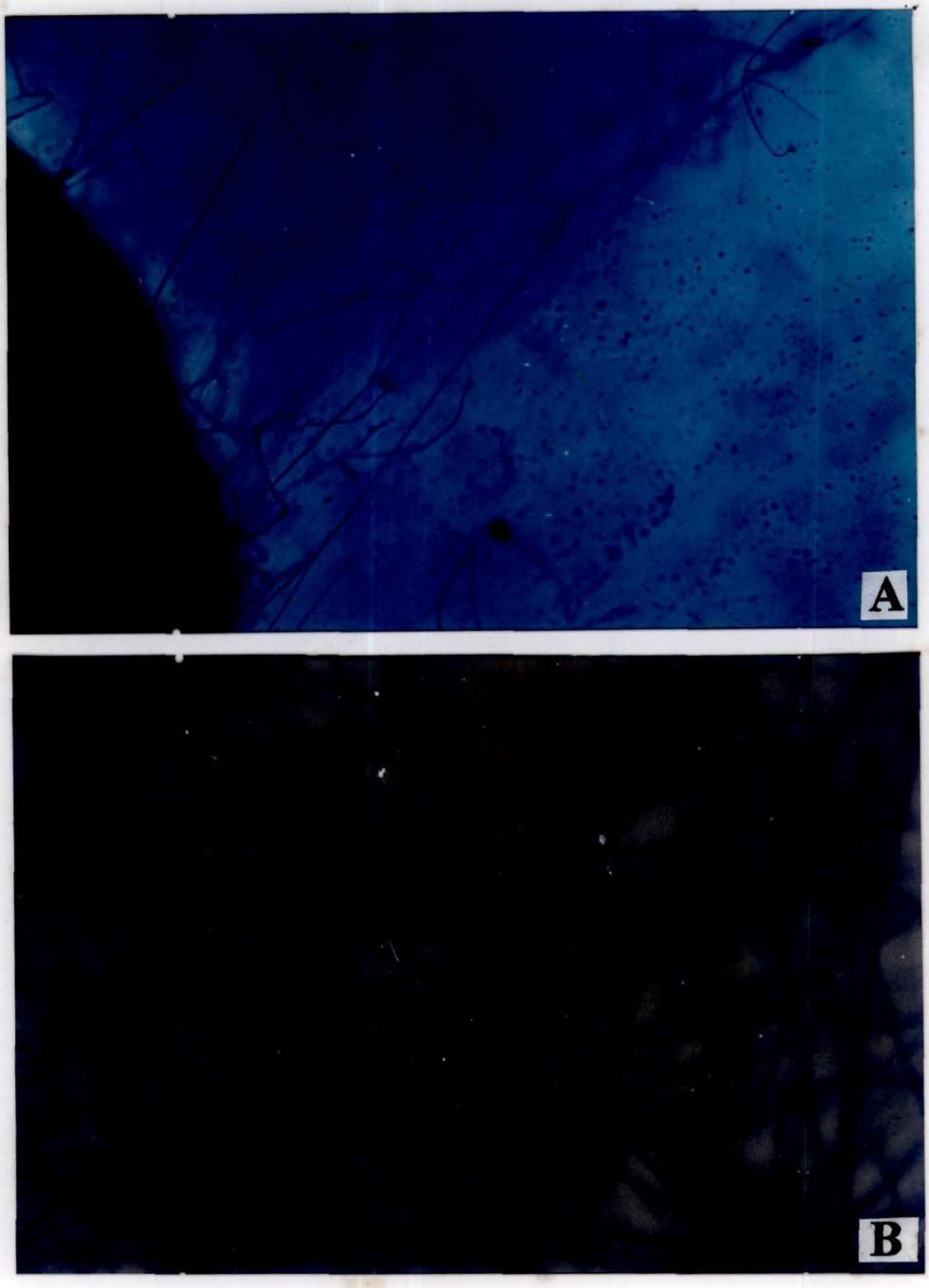


Plate 6 (A & B): Germinated sclerotium of *S.rolfsii* on glass slide
(A) Germination after 24h of incubation
(B) Close up view of mycelia

ıthogenecity test of *Sclerotium rolfsii* on different soybean varieties

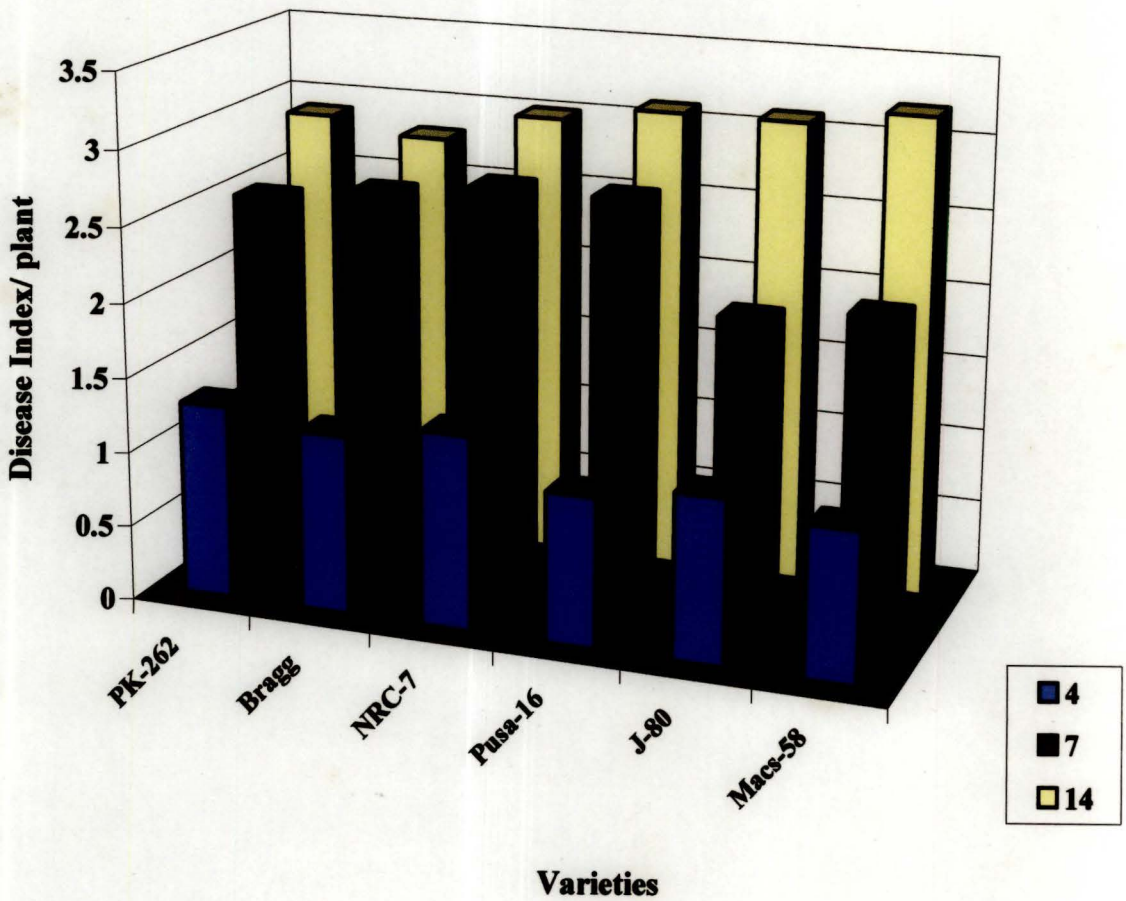


Fig. 1

Table-1 : Varietal reaction of soybean plants against *Sclerotium rolfsii*

Variety	Mean disease index/ plants.	Percent Disease	Plant mortality (%)
PK - 262	2.9	72.5	71.0
Bragg	2.8	70.0	73.0
NRC-7	3.0	75.0	76.0
Pusa-16	3.0	74.5	80.0
J-80	3.1	77.5	79.0
Macs-58	3.9	80.0	90.0

Results represent the averages of 60-70 plants per treatment, recorded at 14 days after inoculation

4.2. Comparison of glyceollin contents of soybean varieties after inoculation with *S.rolfsii*

It is evident from the results in Table-1and Fig.1 that among six soybean varieties (PK-262, Bragg, NRC-7, Pusa-16, J-80 and Macs-58) tested against *S.rolfsii* , Macs-58 was found to be highly susceptible. The differential response of soybean varieties towards *S.rolfsii* may be attributed to differences in their abilities to produce glyceollin (phytoalexin). Disease resistance of several crop plants has been correlated with the rate of production of phytoalexin by a number of previous research workers (Keen, 1981; Bhattacharyya and Ward, 1986; Purkayastha, 1995; Hammerschmidt,1999). In the present study, it was considered worthwhile to compare the accumulation of glyceollin in these varieties after challenge against *S. rolfsii* . Glyceollin was extracted and seperated from the infected roots of soybean. Healthy roots were also used for extraction which was considered as control. In this case, initially the healthy soybean plants were grown in sterilized soil under control

uprooted, washed thoroughly with sterile distilled water and dipped in mycelial and sclerotial suspension of *S. rolfsii* following the "water culture" inoculation technique as described under materials and methods. Yellowing of lower leaves and browning of root system was evident after 96h of inoculation in susceptible soybean varieties Macs-58. Sampling were done after 24 and 48h after inoculation for extraction of glyceollin. In order to detect glyceollin from infected soybean roots as well as to select the best solvent system (for better seperation), initially ethyl acetate fraction of root extracts loaded on TLC plates, developed in three different solvent systems viz. hexane:ethyl acetate: acetic acid (80:20:4); benzene: methanol (95:5) and chloroform: acetone: acetic acid (90:10:0.5) and then sprayed with p-nitroaniline. The Rf value of glyceollin obtained from infected roots (Table 2) were compared after co-chromatography with authentic sample. Finally, the solvent system hexane:ethyl acetate:acetic acid (80:20:4) was chosen.

Table 2 :Comparison of Rf values of authentic glyceollin with the glyceollin extracted from soybean roots inoculated with *S. rolfsii*.

Solvent system [a]	Rf values of glyceollin	
	A	B
Benzene : Methanol (95 : 5)	0.25	0.24
Hexane: Ethyl acetate: Acetic acid (80:20:4)	0.60	0.62
Chloroform : Acetone : Acetic acid (90:10:0.5)	0.48	0.45

A = Authentic glyceollin.

B = Glyceollin obtained from soybean roots inoculated with *S. rolfsii.*

[a] Spray reagent- Diazotised p-nitroaniline.

Methanolic solution of glyceollin when examined in a UV-spectrophotometer, maximum absorption was observed at 286nm. No such absorption was noted for healthy root extraction. After detection of glyceollin, its antifungal activity was tested following TLC plate bioassay and radial mycelial growth assay methods. The TLC plate bioassay using *Curvularia lunata* as a test organism, 10mm diam inhibition zone at Rf 0.60 was observed on the chromatogram after 96h of incubation at 25^{0}C. Silica gel corresponding to the inhibition zone (Rf 0.60) were removed from freshly prepared TLC plates and eluted by spec methanol. Purified eluants obtained after re-chromatography were tested for antifungal activity following sclerotial germination method. The relative antifungal activity of glyceollin was compared against *Sclerotium rolfsii, Fusarium oxysporium, Rhizoctonia solani* and *Macrophomina phaseolina* following petridish bioassay method. Inhibition of radial growth of the fungal species are presented in Table 3.

Table 3 : Inhibition of radial growth of fungi by glyceollin.

Fungi	**Percent inhibition of mycelial growth * (25 µg/ml glyceollin)**
Sclerotium rolfsii	94
Fusarium oxysporum	90
Rhizoctonia solani	92
Macrophomina phaseolina	86

*[Fungi were bioassayed in 2ml of medium in petriplates (35 mm diam.). Measurement were taken when net radial growth in the controls was 30±2 mm. Growth was calculated by measuring two diameters for each of three replicate colonies and substracting the diameter of the mycelial plug (2 mm) used to inoculate the plates.]

Thus, antifungal nature of glyceollin was confirmed. Accumulation of glyceollin from six different soybean varieties were detected after 24 and 48h of inoculation with *S. rolfsii.* It appears from the result that PK-262 and Bragg contained more glyceollin than Macs-58. This experiment was repeated twice and same trend in glyceollin accumulation was noticed in those six varieties. Average glyceollin content of six varieties after 24 and 48h of inoculation with *S. rolfsii* are presented in Table 4. Highest accumulation of glyceollin at 48h interval was noticed. Hence in further experiment this time period was considered for detection of glyceollin accumulation.

Table 4: Comparison ofglyceollin content of soybean roots infected with *S. rolfsii*

Varieties	Glyceollin content [a] (µg/g fresh wt. of tissue)	
	24h.	48h.
PK-262	286.7	392 .0
Bragg	236.8	377.2
NRC-7	168.0	329.4
Pusa-16	155.8	285.5
J-80	137.1	212.0
Macs-58	98.5	189.2

[a] Values shown are the mean from two determinations

4.3 Fungitoxicity assay of various chemicals on *S.rolfsii* and their effects on alteration of disease reaction

4.3.1. Fungitoxicity assay of test chemicals

Chemicals of diverse nature of which eight metal salts viz. cupric chloride , lithium sulphate , ferric chloride , manganese sulphate , sodium molybdate , manganese sulphate , zinc chloride , barium sulphate and three growth regulators viz. indole – 3- acetic acid ,

2,4- dichlorophenoxy acetic acid and 2,4,5 – trichlorophenoxy acetic acid and one biological compound , chitosan were tested against *Sclerotium rolfsii*. All these compounds were screened at a range of 3 concentrations each for their possible fungitoxic effect, if any, on sclerotial germination of pathogen. Both the percentage and nature of sclerotial germination make it clear that most of the test chemicals exhibited no toxic effect on the sclerotial germination at the concentrations screened. Only chitosan at 1.0% and 0.5% completely inhibited *Sclerotium* germination. The same compound at 0.3% and 0.1%, cupric chloride at 10^{-3}M, and ferric chloride at 10^{-3}M caused only mild inhibition (Table-5).

4.3.2. Seed treatment with chemicals and their effect on disease development

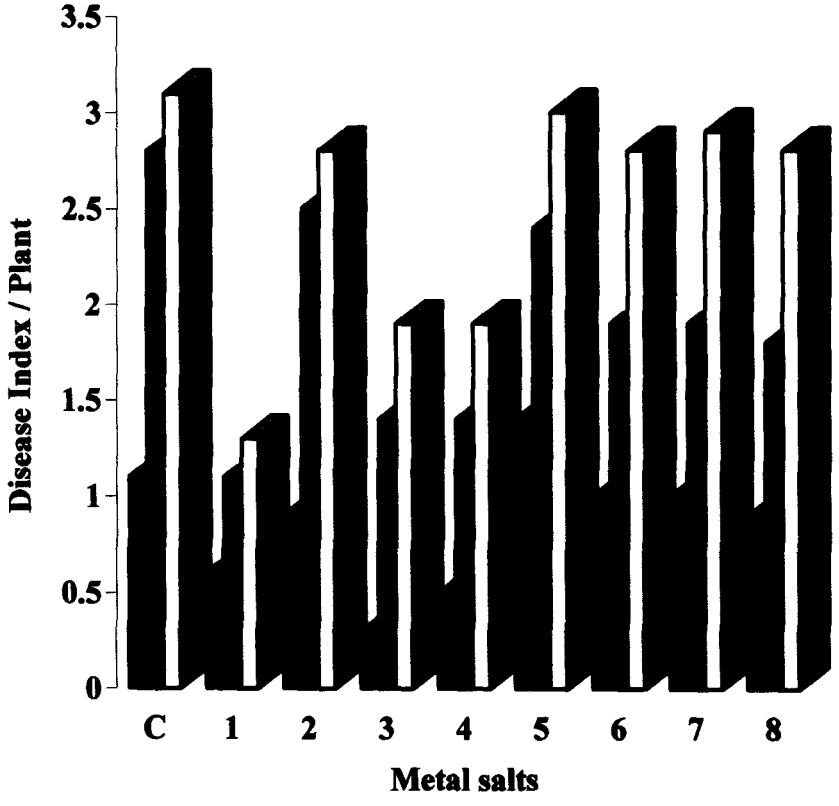
Non-conventional chemicals of diverse nature which includes eight metal salts viz. cupric chloride , lithium sulphate , ferric chloride , manganese sulphate , sodium molybdate, manganese sulphate , zinc chloride , barium sulphate and three growth regulators viz. indole – 3- acetic acid , 2,4- dichlorophenoxy acetic acid and 2,4,5 – trichlorophenoxy acetic acid and one biological compound , chitosan were used as seed treatment and their effect on disease development were studied on pot grown soybean plants. For this purpose, soybean seeds of susceptible variety (Macs-58) were soaked in above chemical solutions of desired concentration for overnight separately. Symptoms were assessed at regular intervals after inoculation of plants. All plants showing even incipent sign of rotting was taken as infected . It appears from the results that plants treated with cupric chloride , ferric chloride and manganese sulphate, provided soybean plants with high levels of protection against the rotting pathogen, the reduction in disease index in these treatments varying between 38% and 58%, being always highly significant (P=0.05). These treatments also checked plant infection by bringing down plant mortality from 73% to 42-53%. Best results were achieved with cupric chloride at 10^{-3} M. Plants treated with IAA, 2,4 -D, and chitosan also showed protective effects against the pathogen. These treatments reduced the disease symptoms by 25% to 57% as compared to the untreated plants at 14 days after inoculation and also brought down plant mortality recording from 70% to 39 in relation to untreated control plants.

Table 5 : Effect of test chemicals on sclerotial germination of *Sclerotium rolfsii in vitro*

Chemical / compound	Concentration	Germination (%)
Water (control)		95
Cupric chloride	10^{-3}M	55
	10^{-4}M	65
	10^{-5}M	76
Ferric chloride	10^{-3}M	60
	10^{-4}M	85
	10^{-5}M	90
Lithium sulphate	10^{-3}M	93
	10^{-4}M	95
	10^{-5}M	95
Sodium molybdate	10^{-3}M	75
	10^{-4}M	95
	10^{-5}M	95
Manganese sulphate	10^{-3}M	85
	10^{-4}M	90
	10^{-5}M	95
Zinc chloride	10^{-3}M	72
	10^{-4}M	90
	10^{-5}M	95
Magnesium sulphate	10^{-3}M	90
	10^{-4}M	95
	10^{-5}M	95
Indole 3-acetic acid	10^{-3}M	100
	10^{-4}M	
	10^{-5}M	95
2,4-dichlorophenoxy-acetic acid	10^{-5}M	95
	10^{-6}M	90
	10^{-7}M	95
	10^{-5}M	96
2,4,5-trichlorophenoxy acetic acid	10^{-6}M	96
	10^{-7}M	Nil
	1.0%	Nil
	0.5%	58
Chitosan	0.3%	60
	0.1%	75
	0.05%	90
	0.01%	

As a follow up experiment all the eight metal salts along with three plant growth regulators and one biological compound were further tested using mostly 3 concentrations ($10^{-3}$and 10^{-5}M) as seed treatment in order to determine the optimum concentration for induction of disease resistance in susceptible variety Macs-58. The results presented in Tables 6-9, confirm the earlier observation. The plants treated with 10^{-3}M cupric chloride and 10^{-3}M ferric chloride significantly (P=0.05) reduced symptoms as compared to the untreated plants as early as 7 days after inoculation. Subsequently, symptom development was distinctly slower in the treated plant and after 14 days these showed very significant differences in symptoms compared to untreated inoculated (control) plants. Regarding the concentration effect the higher concentration caused greater reduction of disease incidence. The plants treated with lithium sulphate had minor effect on the disease reduction. Cupric chloride and ferric chloride at their effective concentration i.e. at 10^{-3} M reduced the symptoms by 40% and 56% as compared to those in control plants, percent disease index from 75% in control plants to 45% and 32.5% while mortality percentage from 67% to 25% and 32% respectively (Table 8, Figs.2 & 3). The plants treated with manganese sulphate only showed strong protective effects at 10^{-4} M concentration against *S.rolfsii* which is evident as 53.3% less disease symptom in comparison to untreated inoculated (control) plants. This treatment also reduced the plant mortality substantially. The other chemicals i.e. sodium molybdate and magnesium sulphate had no effect on the reduction of disease incidence . It appears from Table 6, Fig.4 ; that susceptible plants (Macs58) treated with IAA at 10^{-3} M and 10^{-4} M concentration and 2,4-D at 10^{-6} M concentration significantly (P=0.05) reduced the disease index as compared to the control plants when final sampling was done. The plants treated separately with 3 concentrations (10^{-5}M – 10^{-7}M) of 2,4,5–T had no significant effect on reduction of disease incidence. Six different concetrations (0.01% to 1.0%) of chitosan were used for seed treatment and tested for their induced protective effect in soybean plants against *S.rolfsii.* Result presented in Table 7, Fig.5 indicate a graded concentration effect of chitosan on the reduction of disease index except with 1% chitosan. Plant mortality also gradually decreased with increasing concentration.

Effect of metal salts on disease development



C - Control
1 - Ferric chloride
2 - Cupric chloride
3 - Lithium sulphate
4 - Zinc chloride
5 - Barium sulphate
6 - Manganese sulphate
7 - Sodium molybdate
8 - Magnesium sulphate

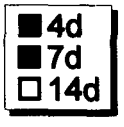


Fig. 2

Table 6 : Effect of wet seed treatment with chemicals at three concentration on disease development in soybean plants artificially inoculated with *Sclerotium rolfsii,* recorded at intervals of 4,7and 14 days

Treatment	Concentration (M)	Mean disease index/ plants.			Percent Disease index [a].	Plant mortality [a] (%)
		4 days	7 days	14 days		
Water (control)		0.9	2.1	2.9	72.5	80.0
I.A.A	10^{-3}	0.5	1.0	1.8(-37.9)[b]	45.0	51.0
	10^{-4}	0.3	0.7	1.0(-65.5)	25.0	35.0
	10^{-5}	0.6	1.2	1.9(-34.5)	47.5	42.0
2,4-D	10^{-5}	0.6	1.5	2.0(-31)	53.0	55.0
	10^{-6}	0.4	1.0	1.8(-37.9)	45.0	49.0
	10^{-7}	0.7	1.6	2.3(-20.6)	57.5	72.0
2,4,5-T	10^{-5}	0.6	1.7	2.4(-17.2)	60.0	77.0
	10^{-6}	0.6	1.4	2.1(-27.6)	52.5	65.0
	10^{-7}	0.5	1.0	2.0(-31)	50.0	53.0
C.D at 5%		0.16	0.73	0.51	15.13	17.25

a Plant Disease Index (PDI) and plant mortality percentage were computed at the last date of sampling.

b Values in the parenthesis indicate percentage reductions in terms of control

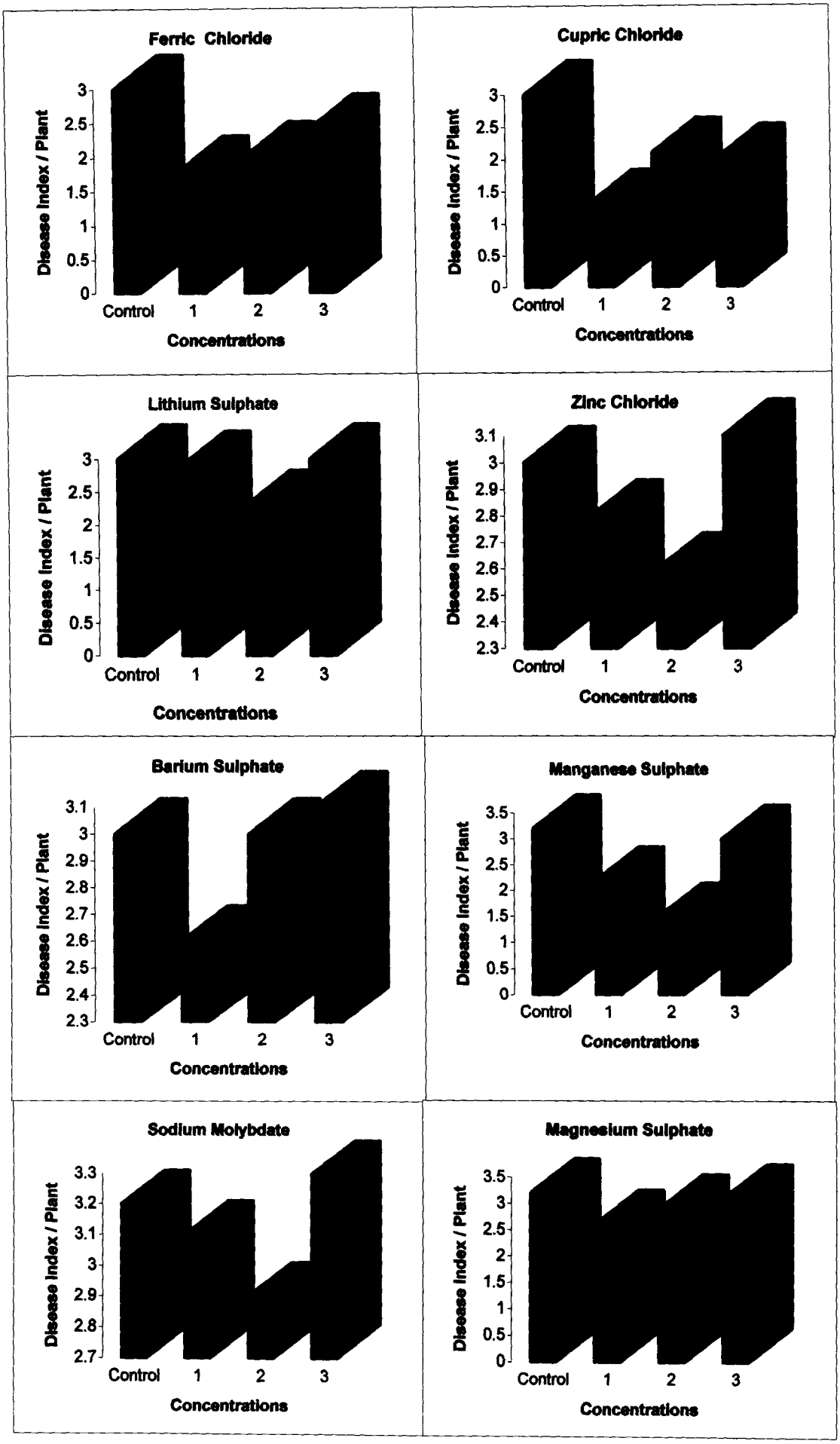
Table 7 : Effect of seed treatment with chitosan at different concentrations on disease development in soybean plants artificially inoculated with *Sclerotium rolfsii* ,recorded at 4,7and 14 days

Treatment	Concentration (%)	Mean disease index/ plants.			Percent Disease index [a].	Plant mortality [a] (%)
		4 days	7 days	14 days		
Water (control)		1.0	2.1	3.2	80.0	88.0
Chitosan	0.01	0.4	1.6	2.8(-12.5)[b]	70.0	78.0
	0.05	0.6	1.7	2.7(-15.6)	67.5	72.0
	0.1	0.5	1.0	1.9(-40.6)	47.5	46.0
	0.3	0.5	1.1	1.8(-43.7)	45.0	40.0
	0.5	0.3	0.9	1.3(-59.4)	32.5	28.0
	1.0	0.4	1.0	1.5(-53.1)	37.5	35.0
C.D at 5%		0.42	0.63	0.95	15.30	16.15

a. PDI and plant mortality percentage were recorded at 14 days after inoculation.

b. Values in the parenthesis indicate percentage disease reduction in terms of control

Effect of metal salts on disease development



(1) 10^{-3}M, (2) 10^{-4}M, (3) 10^{-5}M

Table 8: Effect of seed treatment with metal salts on disease development in soybean plants inoculated with *Sclerotium rolfsii*,[a].

Treatment	Concentration (M)	Mean disease index/ plants.	PercentDisease index [b].	Plant mortality [b] (%)
Water (control)		3.0	75.0	69.0
Cupric chloride	10^{-3}	1.7(-43.3)[c]	42.5	43.0
Ferric chloride	10^{-3}	1.2(-60)	30.0	31.0
Lithium sulphate	10^{-4}	2.4(-20)	60.0	61.0
Manganese sulphate	10^{-4}	1.7(-43.3)	42.5	42.0
Sodium molybdate	10^{-4}	2.8(-6.7)	70.0	67.0
C.D at 5%		0.71	18.2	15.6

a. Data recorded 14 days after inoculation.

b. PDI and plant mortality percentage were computed at the last date of sampling.

c. Values in the parenthesis indicate percentage reductions in terms of control.

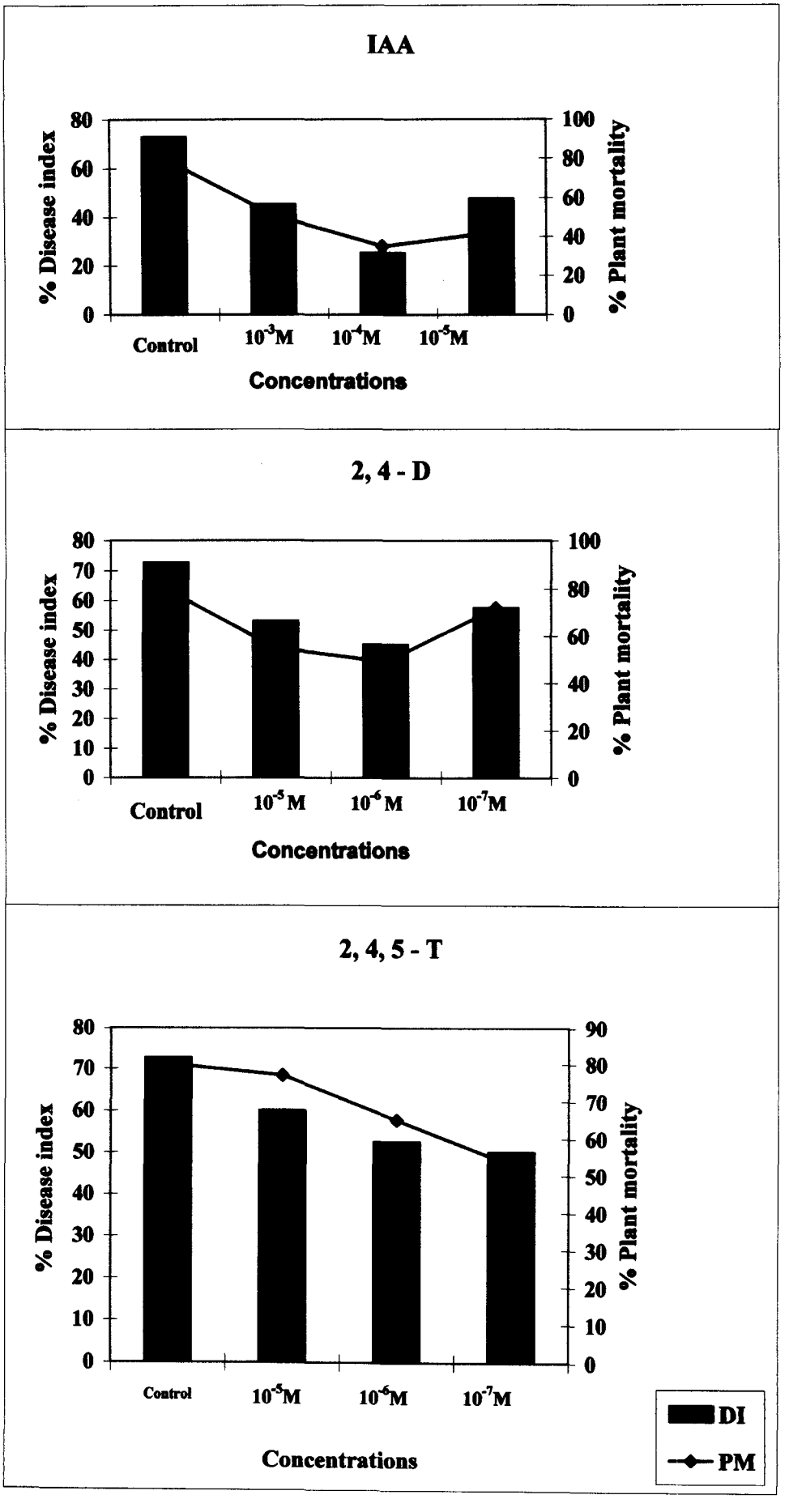

IAA
% Disease index
% Plant mortality
Control
10-3M
10-4M
10-5M
Concentrations
2, 4 - D
% Disease index
% Plant mortality
Control
10-5M
10-6M
10-7M
Concentrations
2, 4, 5 - T
% Disease index
% Plant mortality
Control
10-5M
10-6M
10-7M
Concentrations
DI
PM

Fig. 4

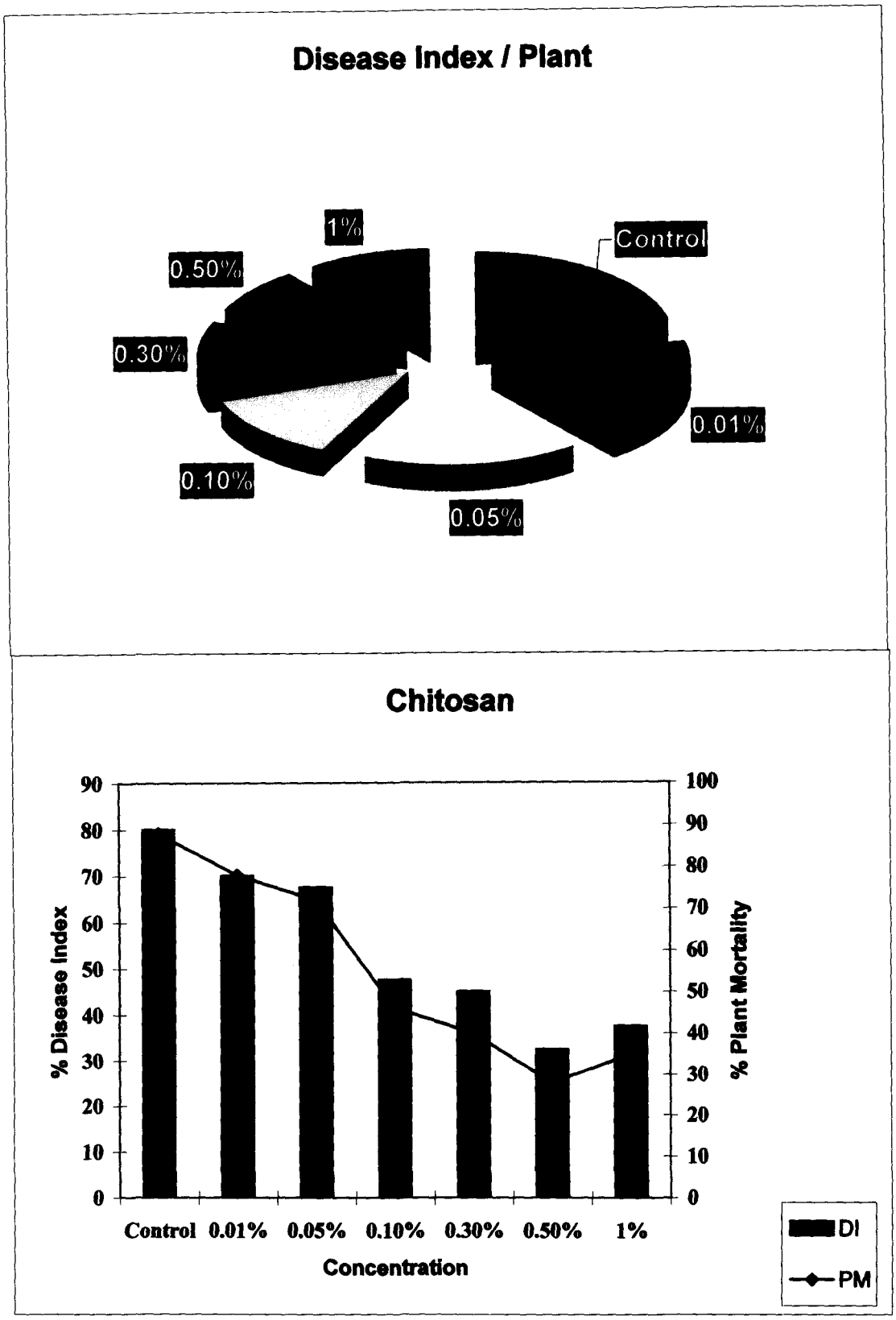

Disease Index / Plant
1%
Control
0.50%
0.30%
0.01%
0.10%
0.05%
Chitosan
% Disease Index
% Plant Mortality
90
80
70
60
50
40
30
20
10
0
100
90
80
70
60
50
40
30
20
10
0
Control
0.01%
0.05%
0.10%
0.30%
0.50%
1%
Concentration
DI
PM

Fig. 5

Table 9: Effect of seed treatment with chemicals on disease devolpment in soybean plants (cv. Macs-58) inoculated with *Sclerotium rolfsii.* [a]

Treatment	Conc.	Mean disease Index / plant.	Percent Disease index.	Plant mortality (%)
Water (control)		3.1	84.4	81.0
1.A.A.	10^{-4}M	0.9(-70.9)b	19.5	25.0
2.4 – D	10^{-6}M	2.5(-19.4)	65.0	60.0
2,4,5 – T	10^{-7}M	2.7(-12.9)	68.3	62.0
Chitosan	0.5%	1.2(-61.3)	30.0	32.0
C.D. at 5%		0.97	17.54	23.63

[s] Data recorded 14 days after inoculation.
[b] Values in parenthesis indicate percentage reductions in terns of contol.

4.4 Biochemical changes associated with induction of resistance in soybean plants

Previous results make it clear that wet seed treatment with many of the test compounds has an inhibitory effect on *Sclerotium* infection of soybean plants. This not only inhibit the initial successful establishment of infection and its subsequent spread but also substantially prevents mortality. Since no direct toxic effect on the pathogen can be envisaged for most of the test compounds, it is felt that these act indirectly through host responses that either (a) inactivate the pathogen, (b) restrict its spread or (c) inactivate or suppressed its mechanism of pathogenesis. To explore these possibilities, studies on changes in biochemical responses associated with chemically induced resistance in susceptible soybean plants were undertaken seperately with two groups of effective

chemicals. Areas investigated includes: total phenol, orthodihydroxyphenol, oxalic acid, calcium, magnesium and lignin contents; polyphenol oxidase, peroxidase, pectolytic enzyme and phenylalanine ammonialyase activities in healthy and infected tissue of untreated and treated plants. Five metal salts such as cupric chloride(10^{-3} M), ferric chloride (10^{-3} M),lithium sulphate(10^{-4} M), manganese sulphate (10^{-4} M) and sodium molybdate(10^{-4} M); three growth regulators such as IAA (10^{-4} M), 2,4 – D (10^{-6} M) , 2,4,5 –T(10^{-7} M) and chitosan (0.5%) were used for seed treatments. Both untreated and treated plants were artificially inoculated at the age of two weeks and the progress of symptom were recorded at intervals of 4,7 and 14 days after inoculation. On each sampling date plant materials were collected for biochemical studies and analysis were made following methods described earlier. Observations on symptom development in plants receiving treatment with the chemicals from which tissue samples were taken for biochemical studies noted for comparison. The plants treated with cupric chloride, ferric chloride and manganese sulphate suppressed *Sclerotium* infection from the earliest stage onwards and 43% to 60% reduction with respect to the control plants were recorded at the final stage of sampling . The chemicals like lithium sulphate and sodium molybdate had no effect on disease reduction. In the effective treatments , progress of disease was strikingly slowed down and as results mortality was brought down from 69%(control plants) to 31% (ferric chloride treated plants) and 43% (cupric chloride treated plants) . Plants treated with IAA and chitosan significantly (P=0.05) reduced the symptoms in soybean plants as compared to the untreated plants . In these treatments the plants had 61% to 70% less symptoms than in the control plants which showed severe symptoms and mortality was also markedly checked and was brought down from 81% in the untreated plants to 25% to 32% . The chemicals like 2,4-D and 2,4,5-T had less pronounced effect on all aspects of disease . IAA at 10^{-4} M concentration had the most marked protective effect in all respects. These results fully confirm the initial observations made with these compounds.

4.4.1. Total phenol

It appears from Tables 10 and 11; Fig.6 that different treatments differently stimulated phenol biosynthesis in soybean plants . Seed treatment caused marginal

increases in phenol level at all stages of sampling. Inoculation resulted in a moderate increase (20%) in phenol level in untreated plants at the early stages of infection , assessed 4 days after inoculation . But this effect rapidly declined with the age , i.e. at 7 and 14 days after inoculation . In the effective treated plants i.e. $CuCl_2$, $FeCl_3$ and $MnSO_4$, however , inoculation resulted in marked(78 - 87 %) and very significant (P = 0.05) increases 4 days after inoculation and the phenol level reaches peak , recording 7 days after inoculation , 83% to 101% higher phenol level but after 14 days , i.e. at the late stage of infection , this effect some what declined but 70% to 77% higher levels could still be noted . The final post- infection phenol levels in different treatments were 48% to 59% higher after 4 days , 68% to 85% higher after 7 days and 73% to 78% higher after 14 days of inoculation as compared to the control treatment . It is evident from the results that plants in different treatment showed no significant (P = 0.05) increases in phenol level over the untreated plant at different stages of sampling , between 4 and 14 days after inoculation . Infection mostly led to an increase in phenol level . Following infection the untreated plants recorded a moderate (25%) increase over healthy plants at the early stage of sampling but later this effect was less pronounced and recorded only marginally higher 3% to 13% levels . On the other hand the treated plants showed very significant (86%) increases quite early following inoculation and the stimulatory effects slightly less pronounced to record 70% higher levels at 7 days and 53% to 56% higher levels at 14 days after inoculation . The plants treated with IAA , the most effective treatment also showed highest stimulation in phenol level among the test compounds . Results clearly showed that susceptible plants in different treatment which displayed effective resistance to *Sclerotium* infection also developed much higher phenol levels when infected . Though any significant difference rarely existed among the responces of plants in different effective treatments , still the relation between their ability to induce resistance in host plants and to stimulate post infection increase in phenol level appears to be good . Maximum increase in phenol level was recorded for cupric chloride , the most effective compound , and minimum increase for sodium molybdate , the compound with least effect in disease suppression .

Table 10 : Effect of seed treatment with metal salts and / or inoculation with *S.rolfsii* on total phenol content in soybean plants, recorded at intervals of 4,7 and 14 days after inoculation .

Treatment	Total phenol (mg /g tissue) [a]					
	4days		7days		14 days	
	H	I	H	I	H	I
Water (Control)	1.74	2.10	1.75	1.91	1.74	1.75
Cupric chloride	1.78	3.34	1.76	3.54	1.76	3.12
Ferric chloride	1.75	3.12	1.75	3.21	1.74	3.03
Lithium sulphate	1.76	2.58	1.75	2.61	1.75	2.12
Manganese sulphate	1.78	3.29	1.79	3.48	1.78	3.03
Sodium molybdate	1.75	2.32	1.76	2.31	1.75	2.02

C.D. at 5%

Days X Treatment = 0.058

Days X Inoculation = 0.037

Treatment X Inoculation = 0.19

[a] =Mean of three replications

H= Healthy; I = Inoculated.

Table 11 : Effect of seed treatment with growth regulators and chitosan and / or inoculation with *S.rolfsii* on total phenol content in soyben plants recorded at intervals of 4,7 and 14 days after inoculation .

Treatment	Total phenol (mg/g tissue)[a]					
	4 days		7 days		14 days	
	H	I	H	I	H	I
Water (control)	1.72	2.15	1.74	1.98	1.74	1.80
1.A.A.	1.76	3.29	1.75	2.99	1.76	2.75
2,4 – D	1.75	2.75	1.76	2.45	1.75	2.25
2,4,5 – T	1.72	2.90	1.74	2.31	1.73	2.01
Chitosan	1.75	3.26	1.77	3.0	1.77	2.72

C.D. at 5%

Days x Treatment = 0.057

Days x Inoculation = 0.074

Treatment x Inoculation = 0.27

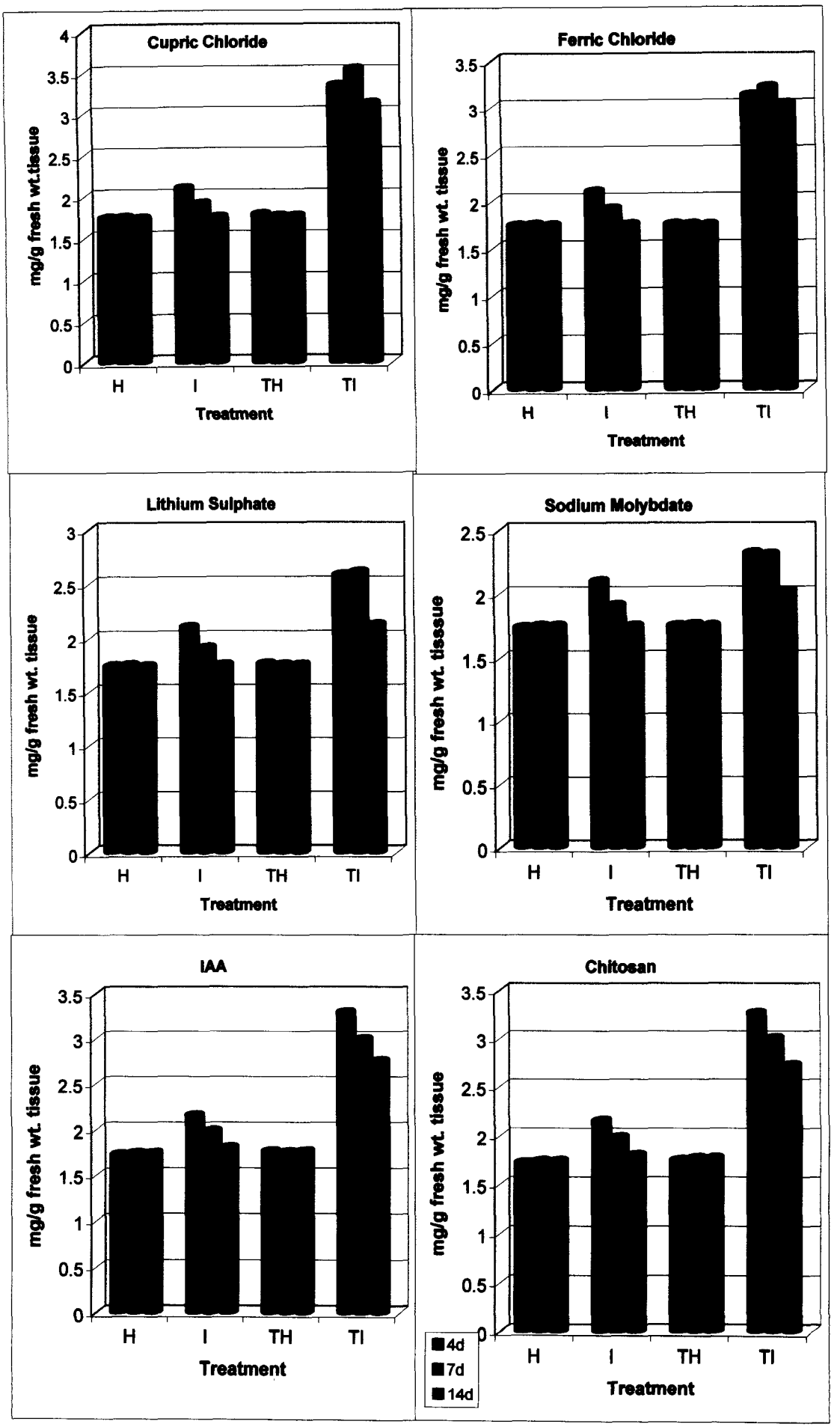
[a] = Mean of three replicarions

H = Healthy

I = Inoculated

NS = Not significant

Total Phenol content in soybean roots after induction of resistance



H- Healthy; I- Inoculated; TH- Treated healthy; TI- Treated inoculated

Fig. 6

4.4.2. Orthodihydroxy phenol

Table12 shows that treatments induced in susceptible plants vary small or no increases in O.D. phenol level over that in the untreated plants at different stages of sampling. Following inoculation, untreated plants recorded marked (70% increase) in O.D. phenol content 4 days after inoculation but this effect was less pronounced within next 3 days and during the next 7 days its content came down to only 8% increase. In three highly effective treatments, very significant increases were noticed after 4 days (81 – 90%) and seven day (84 – 108%) but during the next seven days the stimulatory effect declined so that 46% to 53% higher level was recorded. The plants in the least effective treatment with sodium molybdate recorded lower increases at all stages of sampling. The final post infection level of O.D. phenol in treated plants was significantly higher than that in the untreated plants, particularly in the three highly effective treatments, recording 11% to 17% after 4 days, 84% to 108% after 7 days and 46% to 53% after 14 days of inoculation (Fig. 7)

It is evident from Table 13 that treatment induced in susceptible plants had no significant increases in orthodihydroxyphenol level over that in the untreated plants at different stages of sampling . Following 4 days after inoculation, untreated plants recorded marked increases (100%) in orhtodihydroxy phenol content but these effects was almost disappeared at later stages of infection. In two highly effective treatments, very significant increases were noticed after 4 days (155 – 166 %) and 7 days (81 – 110 %) but during the next 7 days the stimulatory effect was further decline so that 72% higher level was recorded . The plants in the least effective treatment with 2,4,5 – T recorded lower increases at all stages of sampling. The final post infection level of orthodihydroxy phenol in higher effective treatments was significantly higher than that in the untreated plants.

Table 12 : Effect of seed treatment with metal salts and / or inoculation with *S.rolfsii* on ortho di -hydroxy phenol content in soybean plants, recorded at intervals. (at 4,7 and 14 days)

Treatment	Orthodihydroxy phenol content (mg/g tissue) [a]					
	4 days		7days		14 days	
	H	I	H	I	H	I
Water (Control)	0.10	0.17	0.12	0.13	0.12	0.13
Cupric chloride	0.11	0.20	0.12	0.25	0.13	0.19
Ferric chloride	0.10	0.19	0.13	0.24	0.13	0.20
Lithium sulphate	0.11	0.18	0.12	0.19	0.12	0.18
Manganese sulphate	0.10	0.19	0.12	0.24	0.12	0.20
Sodium molybdate	0.10	0.18	0.13	0.18	0.12	0.16

C.D. at 5%

Days X Treatment = NS

Days X Inoculation = 0.032

Treatment X Inoculation = 0.06

[a] =Mean of three replications

H= Healthy ; I= Inoculated.

NS = Not significant

Table 13 : Effect of seed treatment with growth regulators and chitosan and / or inoculation with *S.rolfsii* on orthodihydroxy phenol content in soybean plants, recorded at the intervals of 4,7and14daysafter inoculation

Treatment	Orthodihydroxyphenol (mg /g tissue)[a]					
	4 days		7 days		14 days	
	H	I	H	I	H	I
Water (control)	0.09	0.18	0.10	0.15	0.11	0.12
1.A.A.	0.09	0.24	0.11	0.20	0.11	0.19
2,4 – D	0.08	0.21	0.10	0.19	0.10	0.14
2,4,5 – T	0.10	0.20	0.10	0.16	0.11	0.13
Chitosan	0.09	0.23	0.10	0.21	0.11	0.19

C.D. at 5%

Days x Treatment = N S

Days x Inoculation = 0.03

Treatment x Inoculation = 0.05

[a] = Mean of three replications

H = Healthy

I = Inoculated

NS= Not significant

Ortho-dihydroxyphenol content in soybean roots after induction of resistance with metal salts

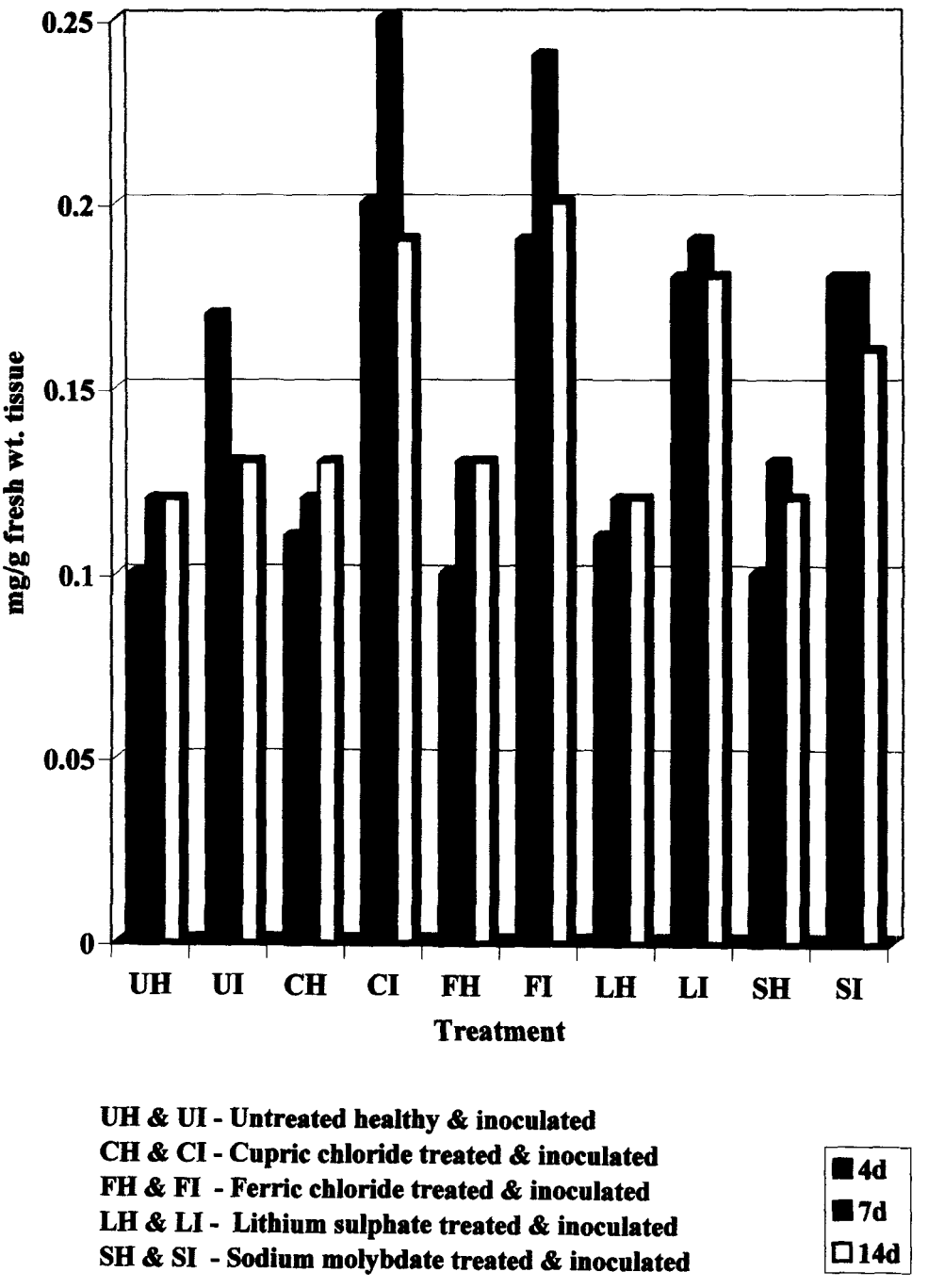


Fig. 7

4.4.3. Polyphenol oxidase

Treated susceptible plants invariably recorded mild to moderate (14 –28%) increase in the level of polyphenol oxidase activity than the untreated plants at different stages of sampling (Table 14). Following inoculation, polyphenol oxidase activity appreciably increased (28%) in susceptible plants after 4 days and further increased to attained 57% higher levels during the next 3 days. However, this effect sharply declined during the last 7 days, to much lower values, only 14 % higher than that in the untreated non-infected plants. At every stage of sampling, susceptible plants in 3 effective treatments responded to inoculation with greater increases of enzyme activity, as much as 112% to 157% after 4 days, 125% to 171% after 7 days and 66% to 100% after 14 days. Their post infection levels were also much higher than in the untreated plants, the differences varying between and 100% after 4, 63 and 72% after 7 and 87 and 112% after 14 days of inoculation. Barring the treatments with sodium molybadate and lithium sulphate which had the lesser protective effect and elicited much reduced responses, the other treatments with substantial protective effect elicited strong responses in all respect. The plants treated with cupric chloride, the most effective compound, elicited the maximum increases in enzyme activity following inoculation (Fig.8)

Treatment with compounds in susceptible soybean plants had no significant increase in the level of polyphenol activity than the untreated plant at different stages of sampling (Table 15). Following inoculation polyphenol oxidase activity appreciably increased (80%) in susceptible plants and further increased to attain 100% in higher levels during the next three days .With time the response become weaker so that after 14 days moderately higher (50 %) levels were recorded . Plants in two highly effective treatments also responded to inoculation with greater than normal increases and this effect persisted till the end with slightly reduced responces , their final post infection levels of polyphenol oxidase activity being always significantly higher than that in untreated plants .

Table 14 : Effect of seed treatment with metal salts and / or inoculation with *Sclerotium rolfsii* in soybean plants (cv. Macs – 58) on polyphenol oxidase activity, recorded at 4,7 and 14 days interval after inoculation.

Treatment	Δ O.D. / 10 mg tissue/min [a]					
	4 days		7days		14 days	
	H	I	H	I	H	I
Water (Control)	0.07	0.09	0.07	0.11	0.07	0.08
Cupric chloride	0.07	0.18	0.08	0.19	0.08	0.16
Ferric chloride	0.08	0.17	0.08	0.18	0.09	0.15
Lithium sulphate	0.08	0.13	0.08	0.13	0.08	0.14
Manganese sulphate	0.07	0.17	0.07	0.19	0.09	0.17
Sodium molybdate	0.08	0.10	0.08	0.12	0.07	0.09

C.D. at 5%

Days X Treatment = N.S.

Days X Inoculation = 0.030

Treatment X Inoculation = 0.04

[a] =Mean of three replications, expressed as the change in optical density / 0.05ml of extract after 30minutes.

H = Healthy

Ino = Inoculated.

NS = Not significant.

Table 15 : Effect of seed treatment with growth regulators and chitosan and / or inoculation with *S.rolfsii* in soybean plants (cv. Macs–58) on polyphenoloxidase activity, recorded at 4,7 and 14 days after inoculation

Treatment	Δ O.D. / 10 mg tissue/min [a]					
	4 days		7 days		14 days	
	H	I	H	I	H	I
Water (control)	0.05	0.09	0.06	0.12	0.06	0.09
1.A.A.	0.07	0.17	0.06	0.21	0.07	0.13
2,4 – D	0.05	0.15	0.07	0.16	0.07	0.10
2,4,5 – T	0.04	0.09	0.06	0.15	0.07	0.09
Chitosan	0.06	0.20	0.07	0.20	0.06	0.14

C.D. at 5%

Days x Treatment = N S

Days x Inoculation = 0.028

Treatment x Inoculation = 0.05

[a] = Mean of three replications and expressed as the changes in optical density / 0.05ml of extract after 30 minutes.

H = Healthy ; I= Inoculated ; NS= Not significant

Polyphenol oxidase activity in soybean roots after induction of resistance

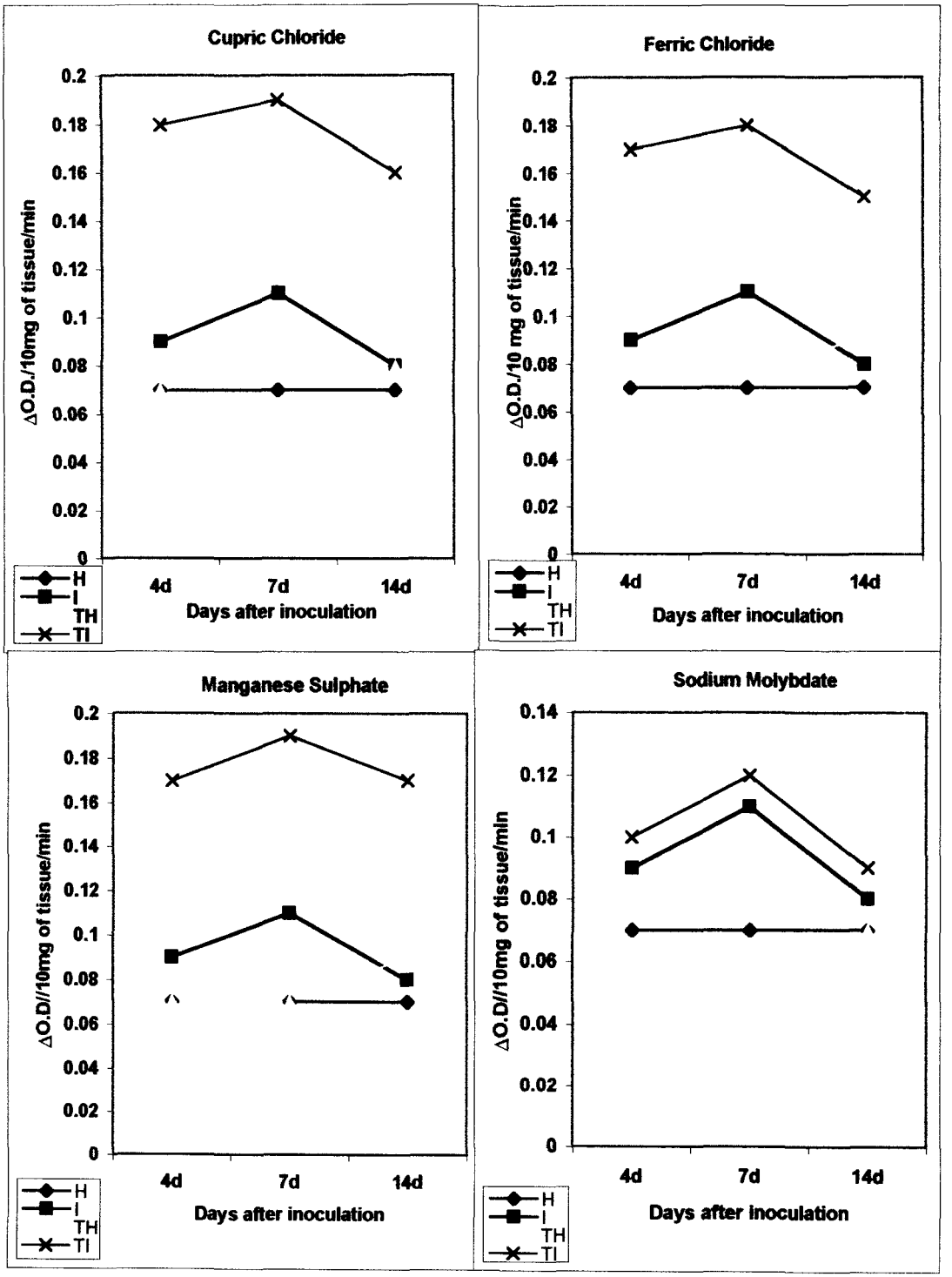


Fig.8

4.4.4. Peroxidase

It is evident from Table 16, Fig 9 that treated plants always had higher (3 – 12%) levels of peroxidase activity than the untreated plants at different stages of sampling. The susceptible plants responded to inoculation with considerable increases (66–80%) in enzyme activity between 4 and 7 days after inoculation, but during the next 7 days the stimulated activity sharply declined to only 32% increases. The plants in three effective treatments responded to inoculation with pronounced increases, 114% to 163% after 4 days and 167% to 207% after 7 days. Though this treatment induced effect declined with time still after 14 days, i.e. at the late stage of infection 81% to 110% higher levels could be noticed. Though all of the treatments recorded higher post infection levels of peroxidase activity, the three more effective treatments recorded much higher levels than the lesser effective treatments like lithium sulphate and sodium molybdate. Though maximum effect of treatments mostly occurred between 4 and 7 days of inoculation, generally the peak period of pathogenic activity, still significant effects persisted even at the late stage of infection.

Treated susceptible plants in two effective treatments viz. IAA and chitosan always had higher (2–15 %) levels of peroxidase activity than the untreated plants at different stages of sampling . The minimum increase always recorded with the least effective 2,4,5 – T treatment. The susceptible plants responded to inoculation with considerable increases (74 – 111 %) in enzyme activity between 4 and 7 days after inoculation , but during the next 7 day the stimulated activity sharply declined recording only 35% higher level of enzyme activity . The plants treated with IAA and chitosan, responded to inoculation with pronounced increases, 187% to 195% after 4 days and 205% to 218% after 7 days and 119% to 186% at the late stage of infection. Though maximum effects of treatments mostly occur between 4 and 7 days of inoculation, generally the pick period for pathogenic activity, still significant effects persist at the late stage of infection (Table 17).

Table 16 : Effect of seed treatment with metal salts and / or inoculation with *S.rolfsii* in soybean plants (cv. Macs – 58) on peroxidase activity recorded 4,7 and 14 days after inoculation

Treatment	Δ O.D. / g tissue/min X 100 [a]					
	4 days		7days		14 days	
	H	I	H	I	H	I
Water (Control)	12.8	21.3	13.0	23.5	13.5	17.9
Cupric chloride	13.5	35.6	13.5	41.5	13.0	27.3
Ferric chloride	14.0	30.0	14.3	38.3	14.6	26.5
Lithium sulphate	13.3	29.8	13.3	28.5	13.0	22.2
Manganese sulphate	14.3	38.8	14.6	41.5	14.6	30.5
Sodium molybdate	13.5	23.8	14.0	25.0	14.0	18.5

C.D. at 5%

Days X Treatment = 1.02

Days X Inoculation = 1.47

Treatment X Inoculation = 2.14

[a] = Mean of three replications; expressed as a change in the absorption by 0.01 per minute as a unit of activity.

H = Healthy, I = Inoculated

Table 17: Effect of seed treatment with growth regulators and chitosan and / or inoculation with *S.rolfsii* in soybean plants (cv. Macs – 58) on peroxidase activity, recorded 4,7 and 14 days after inoculation

Treatment	Δ O.D. / g tissue/min X 100 [a]					
	4 days		7 days		14 days	
	H	I	H	I	H	I
Water (control)	11.5	24.3	12.8	22.3	13.5	18.3
1.A.A.	12.8	37.8	13.5	43.0	13.8	39.5
2,4 – D	11.8	30.0	13.0	31.0	13.0	22.0
2,4,5 – T	11.8	29.8	13.0	24.8	13.5	20.3
Chitosan	13.3	38.3	13.5	41.3	13.8	30.3

C.D. at 5%

Days x Treatment = 1.04

Days x Inoculation = 1.85

Treatment x Inoculation = 3.54

[a] = Mean of three replicarions, expressed as change in the absorption by 0.01 per minute as a unit of activity

H = Healthy

I = Inoculated

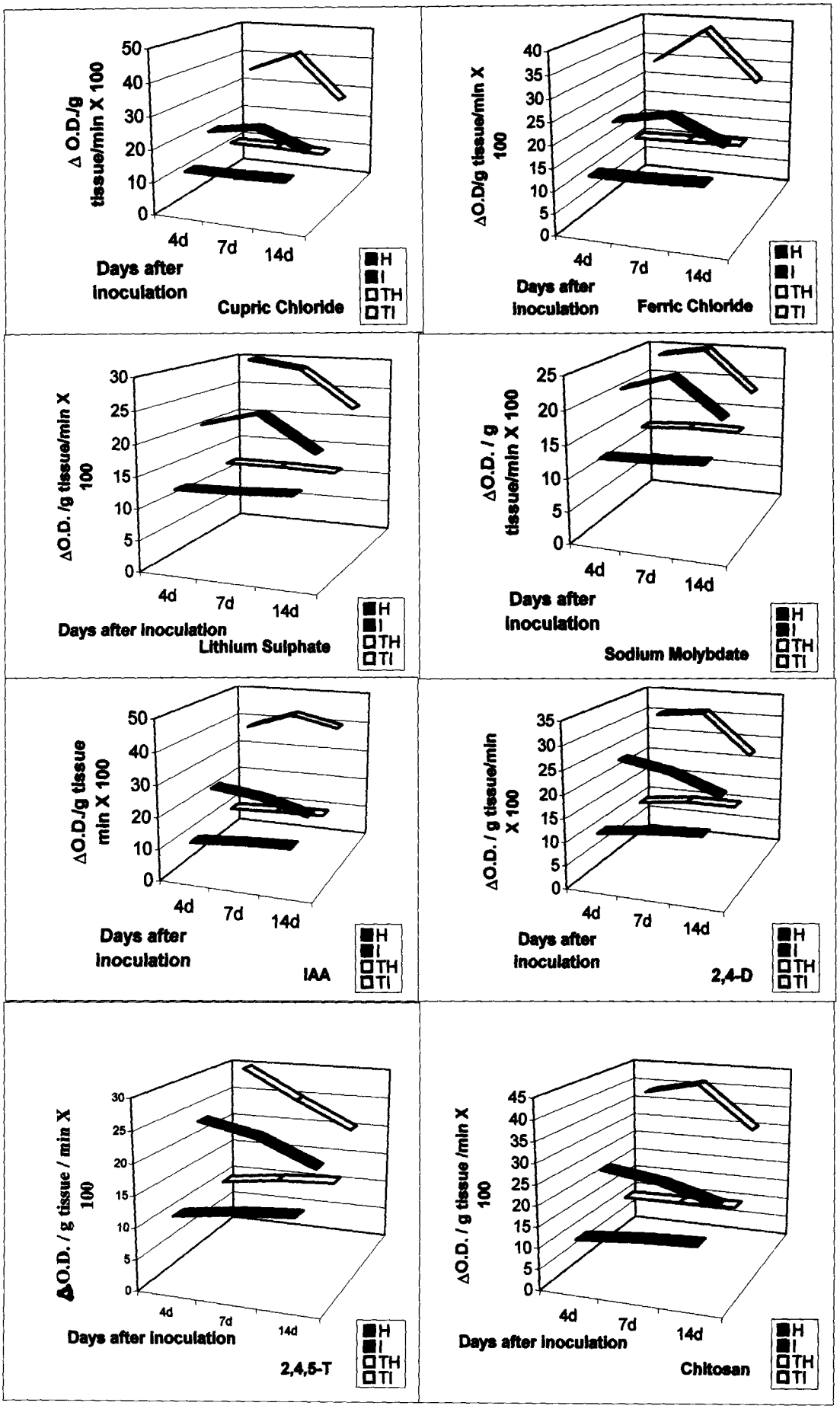

Δ O.D./g tissue/min X 100
Days after inoculation
4d
7d
14d
Cupric Chloride
H
I
TH
TI
ΔO.D/g tissue/min X 100
Ferric Chloride
ΔO.D. /g tissue/min X 100
Lithium Sulphate
ΔO.D. / g tissue/min X 100
Sodium Molybdate
ΔO.D./g tissue min X 100
IAA
ΔO.D. / g tissue/min X 100
2,4-D
ΔO.D. / g tissue / min X 100
2,4,5-T
ΔO.D. / g tissue /min X 100
Chitosan

Fig. 9

4.4.5. Pectolytic enzyme

Since rotting of stem tissue in *S. rolfsii* infected plants is known to result from pectolytic enzyme activity of fungal origin, if there had been any significant change in such enzyme activity following various treatments was investigated . Both polygalacturonase and pectin methyl galacturonase activity in the infected tissue were estimated on the basis of D – galacturonic acid released from their respective pectic substrates. Trends of changes in pectolytic enzyme activity in the infected plants in different teatments appeared to be nearly similar. Among the five different treatments, strong reduction (26 – 40%) in PG and PMG activities were recorded with cupric chloride, ferric chloride and manganese sulphate as compared to that in control plants at 7 days after inoculation, when the first sampling was done (Table 18). Later, the enzyme activities were declined with time both in control and treated plants but still the final levels in effective treatments were lower (21 – 30%) as compared to that in the untreated plants . The lesser effective lithium sulphate and sodium molybdate could reduce pectolytic enzyme activities to a small extent only. A good correlation was found between the supression of pectolytic enzyme activity and lowering of disease index in different treatments.

There was high pectolytic enzyme activity in *Sclerotium* infected tissue of untreated soybean plants 7days after inoculation. At this stage, infected tissue from effective treatments like IAA and chitosan show much reduced (25-36%) enzyme activity (Table 19). During the next seven days, the enzyme activity becomes less pronounced in the control plants as well as in other treatments. However, at this stage, the more effective treatments had 18-23% less activity than the control. Chitosan at 0.5% and 2,4,5-T at 10^{-4}M, the treatments exercising the most and least protective effect against *Sclerotium* infection, as well as suppression of pectolytic enzyme activity respectively (Fig.10)

4.4.6. Oxalic acid

As it is known that oxalic acid content may play a contributory factor in induced resistance by lowering the pH of the cell wall which is favourable for the cell wall degrading enzymes (mainly polygalacturonase type) to hydrolyze the pectates (Faboya *et.al*, 1983), its contents were estimated both in the treated and untreated infected plants. There is no significant difference in oxalic acid content of untreated

and treated healthy plants. The infection resulted significantly higher increase in the oxalic acid content in untreated than the treated plants and the final levels in the effective treatments viz., $CuCl_2$, $FeCl_3$ and $MnSO_4$ had (39 – 60 %) less after 7 days and 47% to 57% less after 14 days of inoculation as compared to that of the untreated infected plants (Table 20) Soybean plants treated with IAA or chitosan has no significant (P = 0.05) effect on changes in oxalic acid content after inoculation with *Sclerotium rolfsii* (Table 21)

Table 18: Effect of seed treatment on pectolytic enzyme activity in disease affected soybean stem tissue in various treatments, 7 and 14 days after inoculation.

Treatment	Amount of D-galacturonic acid released (mg /g fresh weight of tissue/hr)[a]			
	7days		14 days	
	Pectin	Sodium polypectate	Pectin	Sodium polypectate
Water (Control)	1.42	1.47	0.75	0.76
Cupric chloride	0.85	0.89	0.52	0.55
Ferric chloride	0.95	0.93	0.58	0.55
Lithium sulphate	1.25	1.35	0.67	0.69
Manganese sulphate	1.05	0.92	0.59	0.58
Sodium molybdate	1.40	1.43	0.75	0.73
C.D. at 5%	0.21	0.25	0.18	0.17

[a] Mean of three replictions and expressed as D- gulacturonic acid equivalent.

Table 19 : Effect of seed treatment on pectolytic enzyme activity in disease affected soybean stem tissue in various treatments , 7 and 14 days after inoculation .

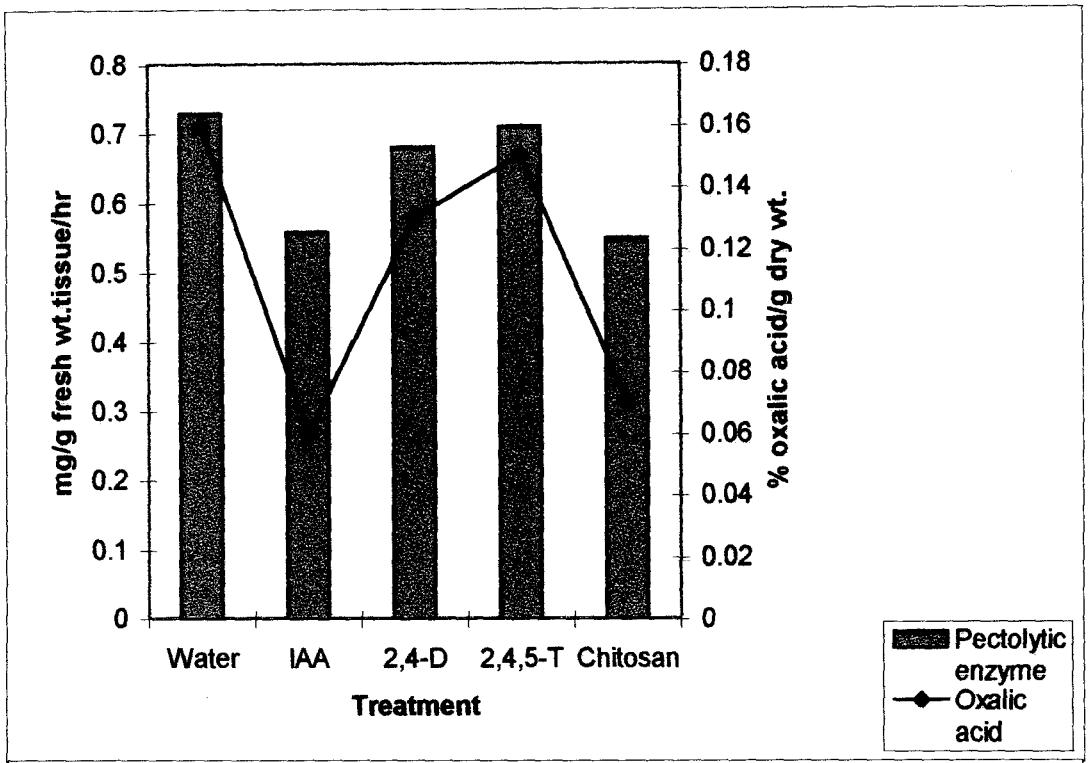
Treatment	Amount of D-galacturonic acid released (mg/ g fresh weight of tissue / hr)[a]			
	7 days		14 days	
	Pectin	Sodium polypectate	Pectin	Sodium polypectate
Water (control)	1.37	1.43	0.73	0.71
I.A.A.	0.89	0.91	0.56	0.58
2,4 – D	1.28	1.35	0.68	0.67
2,4,5 – T	1.30	1.40	0.71	0.68
Chitosan	1.02	0.92	0.55	0.55
C.D. at 5%	0.18	0.23	0.08	0.06

[a] Mean of three replications and expressed as D – galacturonic acid equivalent.

4.4.7. Calcium and magnesium content

It has been demonstrated that the pectolytic enzyme activity was significantly less in the infected stem tissue of the treated plants than in the control plants, and this was correlated with reduced rotting symptoms in the former. Both limited tissue rotting and reduced enzyme activity, have often been linked with greater accumulation of Ca^{++} and Mg^{++} ions at and around the infection side and conversion of pectic compounds to calcium and magnesium pectates that are less amenable to enzyme degredation (Reddy *et al.*, 1988) keeping this possibility in mind the amount of Ca^{++} and Mg^{++} ions in the stem tissue of soybean around the infection site was estimated following the methods describe earlier.

Pectolytic enzyme activity and oxalic acid concentration in soybean roots after induction of resistance



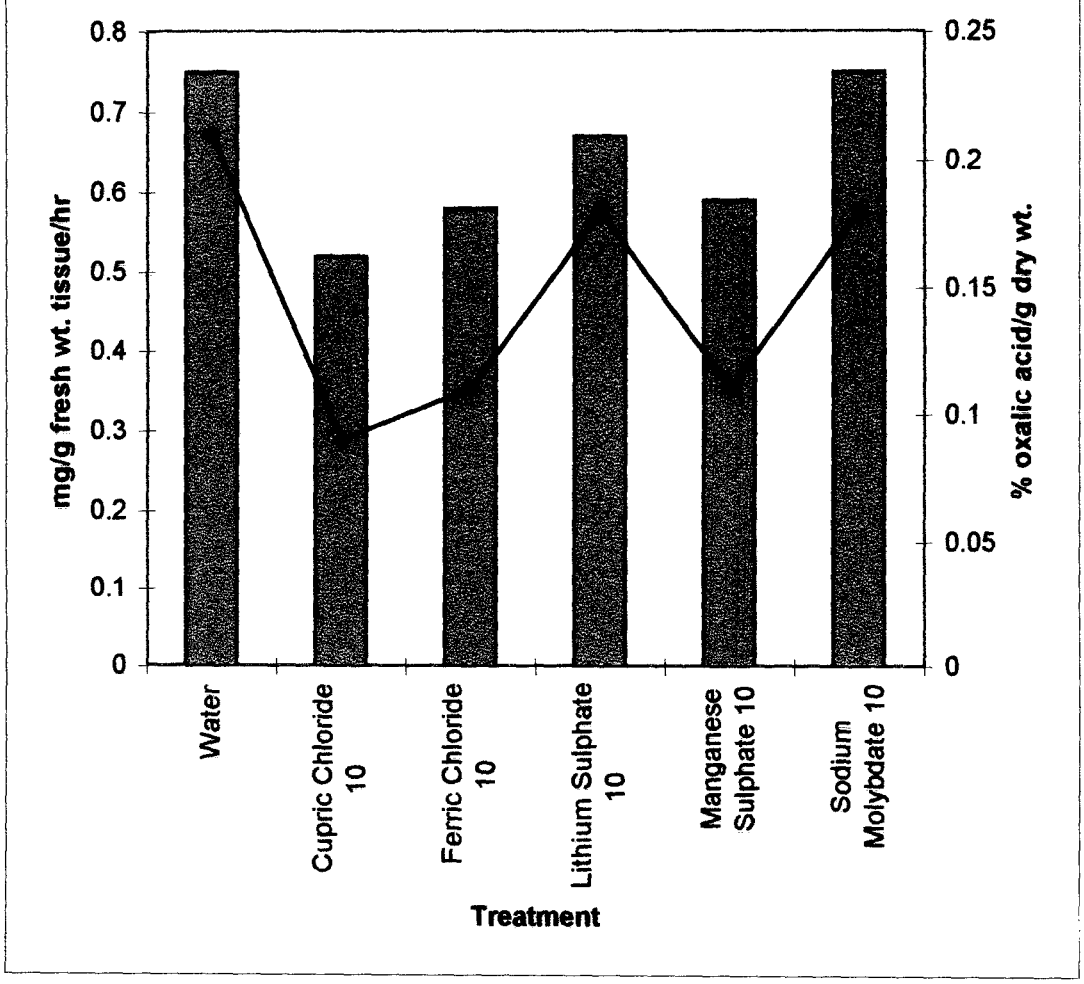


Fig.10

Table 20: Effect of seed treatment with metalic salts on oxalic acid concentration in soybean plants inoculated with *S.rolfsii*, 7 and 14 days after inoculation.

Treatment	% oxalic acid /g dry weight basis			
	7days		14 days	
	H	I	H	I
Water (Control)	0.03	0.28	0.03	0.21
Cupric chloride	0.03	0.11	0.04	0.09
Ferric chloride	0.04	0.17	0.04	0.11
Lithium sulphate	0.04	0.21	0.03	0.18
Manganese sulphate	0.03	0.12	0.04	0.11
Sodium molybdate	0.03	0.27	0.03	0.18
C.D. at 5%	NS	0.07	NS	0.05

[a]=Mean of three replictions .
H=Healthy ; I= Inoculated
NS = Not significant .

It appears from Table 22 that various treatments as such had little or no effect on the calcium content of the stem tissue. Infection resulted in small (4%) increase in the untreated plants but those in different effective like $CuCl_2$, $FeCl_3$ and $MnSo_4$ treatments responded with greater (14 –18 %) increases in calcium content. The final post–infection calcium content in those treated plants were also appreaciably higher (7 – 12%) than that in untreated plants. The metal salts like Li_2 So_4 and Na_2 MoO_4 had no or less effect on calcium content. The trend was almost similar in respect of magnesium content also. Following inoculation the magnesium level increased by 6% over that in noninfected untreated plants but the treated plants in effective treatments recorded 8% to 14% increase in magnesium level in response to inoculation. The final post infectional level of magnesium in these treated plants were also marginally (3 –7 %) higher. In respect of both calcium and magnesium accumulation, the maximum effect was achieved with ferric chloride.

Table 21 : Effect of seed treatment with growth regulators and chitosan on oxalic acid concentration in soybean plants inoculated with *S.rolfsii* 7 and 14 days after inoculation .

Treatment	% oxalic acid/ g dry weight basis [a]			
	7 days		14 days	
	H	I	H	I
Water (control)	0.04	0.22	0.04	0.16
I.A.A.	0.04	0.09	0.03	0.06
2.4 – D	0.03	0.16	0.04	0.13
2,4,5 – T	0.04	0.18	0.03	0.15
Chitosan	0.04	0.09	0.04	0.07
C.D. at 5%	NS	0.06	NS	0.04

[a] = Mean of three replicarions

H = Healthy

Ino = Inoculated

NS = Not significant

The concentration of calcium and magnesium in soybean plants as influenced by seed treatments with IAA, 2,4-D, 2,4,5-T and chitosan are presented in Table 23. Following inoculation, the untreated plants showed 4% increase in calcium content, where as the two effective treatments (IAA and chitosan) caused greater (23 – 25 %) increase. The final post infectional level of calcium in such treated plants were also appreciably (22 – 21 %) higher than that in the untreated infected plants. The trend was almost similar in respect of magnesium content also. In the untreated plants following inoculation, resulted in marginal increase (7%) but plants in two effective treatments showed appreciably higher (15 – 20 %) increase in magnesium content of stem tissue. Both in respect of calcium and magnesium content, the maximum increase was recorded with IAA, the most effective compound in protecting symptom expressing.

Table 22 : Effect of seed treatment with metal salts on calcium and magnesium content in soybean plants inoculated with *S.rolfsii*, recorded 14 days after inoculation.

Treatment	Concentration of calcium and magnesium (on g^{-1} dry weight basis) [a]			
	% of Ca		% of Mg	
	H	I	H	I
Water (Control)	1.21	1.27	0.49	0.52
Cupric chloride	1.22	1.42	0.48	0.55
Ferric chloride	1.21	1.43	0.49	0.56
Lithium sulphate	1.20	1.29	0.50	0.51
Manganese sulphate	1.20	1.37	0.49	0.54
Sodium molybdate	1.22	1.28	0.51	0.52
C.D. at 5%	NS	0.66	NS	0.02

[a]= Mean of three replications ; H= Healthy ; I = Inoculated

4.4.8. Phenylalanine ammonia lyase activity:

It has been clearly established that in the early experiments that wet seed treatment with some metal salts can induce strong protective effects in soybean plants against *Sclerotium rolfsii* infection. Such induced effects are mostly correlated with an increased biosynthesis of phenolics and stimulated oxidase activity at and around the site of host – pathogen interaction. It is well that phenylalanine ammonia lyase (PAL) is the first enzyme of the phenyl propanoid pathway and considered as the key enzyme in the regulation of the flux of the phenyl propanoid compounds such as lignin and their derivatives (Camm and Towers , 1973) and also appeared to be associated with hypersensitive reaction (Novacky and Acedo , 1970) . The PAL activity of soybean plants in different treatments and /or inoculation with *S. rolfsii* was estimated following the method desccribed earlier and the results are presented in Table 24 – 25, and Fig.11

Table 23 :Effect of seed treatment with growth regulators and chitosan on calcium and magnesium content in soybean plants inoculated with *S.rolfsii*, recorded 14 days after inoculation

Treatment	Concentration of calcium and magnesium (on g^{-1} dry weight basis)[a]			
	% of Ca		% of Mg	
	H	I	H	I
Water (control)	1.18	1.23	0.53	0.59
I.A.A.	1.19	1.49	0.53	0.64
2,4 – D	1.19	1.25	0.52	0.59
2,4,5 – T	1.18	1.24	0.53	0.58
Chitosan	1.20	1.48	0.53	0.61
C.D. at 5%	NS	0.45	NS	0.55

[a] = Mean of three replications ; H = Healthy ; I = Inoculated ; NS = Not significant

Treated susceptible plants recorded only very marginal increases in PAL activity over that in the untreated plants at different stages of sampling. The PAL activity in untreated plants following infection had mild increase (16%) after 7 days and the stimulatory effect weakened in them with time and become marginal during the later stage of infection, recording only 6.0% higher level after 14 days of inoculation .The plants in effective treatments responded to inoculation with greater increases in enzyme activity, as much as 73% to 86% after 7 days and 34% to 46% after 14 days. Their post infection were also much higher than in the untreated plants, the differences varying between 49 and 64% after 7 days and 31 and 44% after 14 days. The plants treated with cupric chloride, the most effective treatment, elicited the maximum increases in enzyme activity following inoculation and also led to the highest post-infection level.

Soybean plants treated with growth regulators and chitosan as such had little or no effect on the phenylalanine ammonia lyase activity at different stages of sampling, higher levels were always recorded with IAA and chitosan , the two most effective compounds (Table 25). Following infection the untreated plants had very marginal increases (7–14 %) in enzyme activity but susceptible plants in highly effective treatments recorded moderately higher (95%) increases but the quantum of increase became less pronounced thereafter and varied between 39% to 40% at the late stage . The final post infection levels in these treatments were always much higher than the untreated plants .

Table 24 : Effect of seed treatment with metal salts on phenyalalanine ammonialyase activity in soybean plants (cv. Macs-58) , inoculated with *S.rolfsii.*

Treatment	Phenylalanine ammonia-lyase activity (µg cinnamic acid released / g tissue / min .)[a]			
	7days		14 days	
	H	I	H	I
Water (Control)	92.5	108.0	90.0	95.5
Cupric chloride	95.5	178.0	94.0	138.0
Ferric chloride	93.0	161.0	93.5	125.5
Lithium sulphate	92.0	111.5	91.0	98.5
Manganese sulphate	94.0	169.0	93.5	137.0
Sodium molybdate	93.0	112.0	92.5	98.0

C.D. at 5%

Days X Treatment = N.S.

Days X Inoculation = 15.35

Treatment X Inoculation = 12.85

[a]=Mean of three replications ; H= Healthy, I = Inoculated; NS= Not significant .

PAL activity in soybean roots after induction of resistance

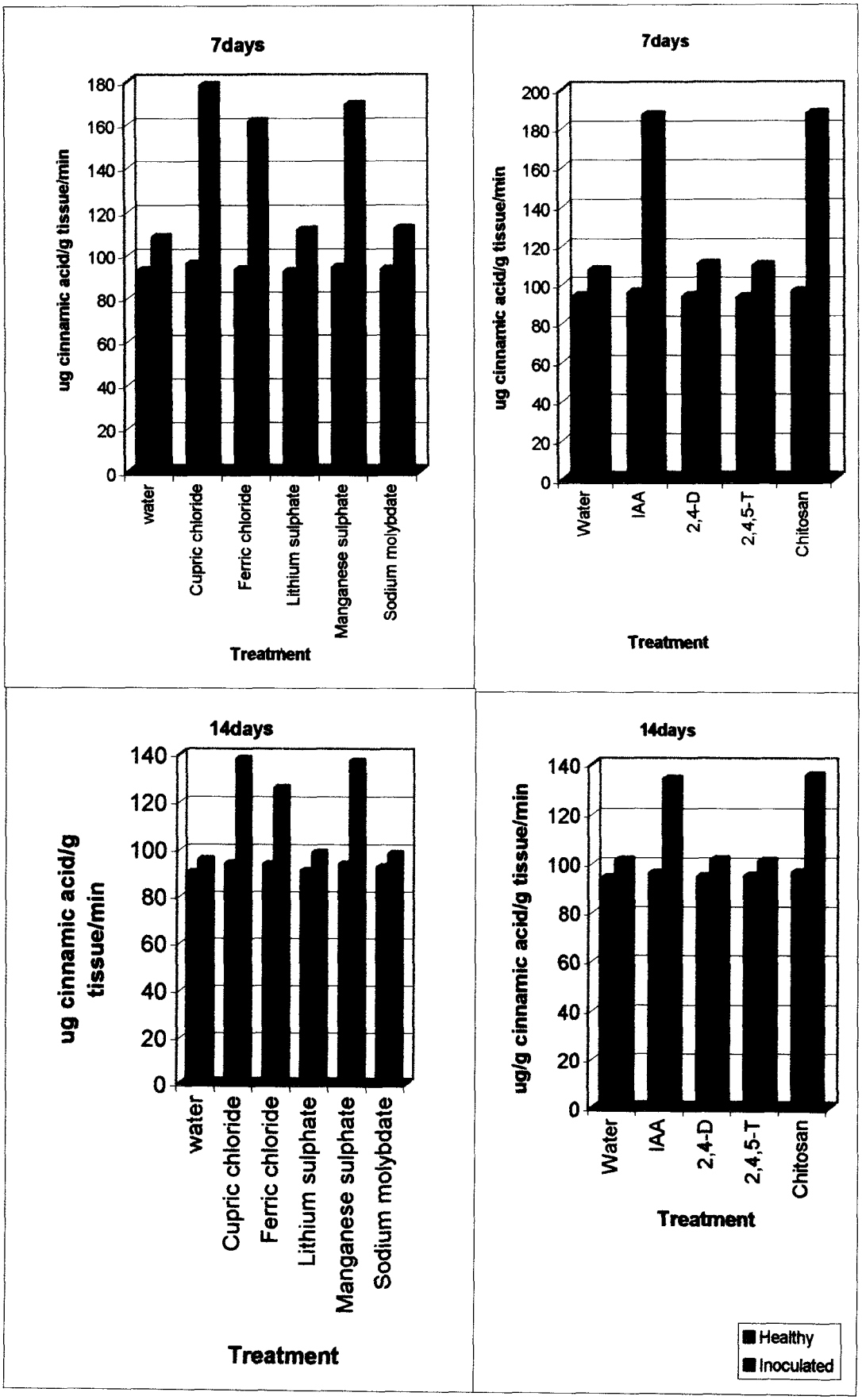


Fig.11

Table 25: Effect of seed treatment with growth regulators and chitosan and / or inoculation with *S.rolfsii* in soyben plants (cv. Macs – 58) on phenylalanine ammonia lyase activity, recorded 4,7 and 14 days after inoculation .

Treatment	Phenylalanine ammonia lyase activity (µg cinnamic acid released / g tissue/ min)[a]			
	7 days		14 days	
	H	I	H	I
Water (control)	93.5	107.0	94.0	101.3
I.A.A.	95.5	186.5	96.0	134.0
2,4 – D	93.5	110.3	94.5	101.5
2,4,5 – T	93.0	109.5	95.0	101.0
Chitosan	96.0	187.3	96.5	135.5

C.D. at 5% Days x Treatment = 1.16
Days x Inoculation = 12.56
Treatment x Inoculation = 15.42

[a] = Mean of three replications ; H = Healthy , I = Inoculated

4.5. Effect of seed treatment on lignification in soybean plants

Results described earlier indicated an association between resistance induced in soybean plants against *S. rolfsii* by chemical treatment and increased biosynthesis of phenolics and greater activity of peroxidase. It is well known that peroxidase is the terminal enzyme in phenyl-propanoid pathway and increased peroxidase activity is presumed to be associated with enhanced lignification, an important mechanism for limiting the spread of the pathogen. This possibility was explored in soybean plants using three highly effective treatments like cupric chloride(10^{-3} M), ferric chloride (10^{-3} M) and chitosan(0.5%). To quantitate lignin, the ionization difference spectra was examined from 230 to 380nm in a Beckman UV-spectrophotometer following the method as described earlier. The extracts of soybean

stem gave two major peaks at approximately 247nm due to simple phenolic groups and at 351nm, presumably due to phenols with conjugated side chains such as lignin. The absorbance difference at 351nm and 247nm of dialysed extracts of soybean stem at 4 days and 7 days after inoculation with *S.rolfsii* are presented in Table 26. The trend was nearly the same for the simple phenolics recorded at 247nm. Following inoculation, the untreated plants showed an increase in the absorbance (43-68%) but the final post-infection levels were higher in the treated plants than in the untreated plants, 73% to 78% after 4 days and 66% to 77% after 7 days, though the differences among the treatments were not significant. Here, plants treated with cupric chloride showed maximum increases in the absorbance.

More conclusive evidence for lignification was provided by estimating phenolic aldehydes resulting from oxidation with alkaline nitrobenzene (Heartley, 1973). Stem material was pre-extracted with organic solvents and cold alkali to remove free and esterified low molecular weight phenols and the residue containing the lignin 'core' was analysed. A significant (P=0.05) increase in all three characteristic aldehydes (vanillin, syringaldehyde and p-hydroxybenzaldehyde) was observed 7 days after inoculation with cupric chloride, ferric chloride and chitosan, but not after inoculating alone (Table 27). The results also indicated that the three treatments induced essentialy the same type of polymer, since lignin induced by the three compounds yielded similar relative proportions of aldehydes.

Table 26: Induced lignification in dialysed extracts of soybean plants in different treatments .

Treatment	ΔE at 351 nm		ΔE at 247 nm	
	4 days	7days	4 days	7days
Healthy, Uninoculated	0.23	0.24	0.16	0.16
Control [a]	0.29	0.35	0.23	0.27
Cupric chloride: Inoculated	0.47	0.58	0.41	0.48
Ferric chloride : Inoculated	0.44	0.56	0.40	0.46
Chitosan : Inoculated	0.48	0.55	0.41	0.45
C.D. (P = 0.05)	0.04	0.06	0.03	0.07

[a] = Inoculated and treated with water

Table 27: Yield of alkaline nitrobenzene oxidation products from soybean plants inoculated with *S.rolfsii*, harvested 7 days after inoculation.

Treatment	Yield [a]		
	Vanillin	Syringaldehyde	p-Hydroxy benzaldelyde
Healthy, Uninoculated	4.64	0.44	0.61
Contral [b]	5.29	0.56	1.03
Cupric chloride: Inoculated	9.58	3.26	6.05
Ferric chloride : Inoculated	8.28	2.65	5.77
Chitosan : Inoculated	9.25	3.20	5.80
C.D. (P = 0.05)	1.10	0.87	2.17

[a] =μ mol g^{-1} alkali extracted, mean of three samples.

[b] = Inoculated and treated with water

4.6 Accumulation of glyceollin in soybean plants before and after alteration of disease reaction by selected chemicals

In order to alter disease reactions in one of the highly susceptible soybean variety (Macs-58), a series of experiments were performed and finally on the basis of the results obtained with special reference to biochemical chages associated with induction of resistance , two chemicals viz. cupric chloride and ferric chloride were selected for further study. It was decided to investigate whether chemically induced resistance in Macs-58 was related to higher production of glyceollin. Since cupric chloride and ferric chloride markedly reduced disease in susceptible soybean variety, accmulation of glyceollin in this variety before and after alteration of disease reaction by the said chemicals were determined. To study the effect of cupric chloride and ferric chloride on the production of glyceollin, soybean seeds (variety- Macs58) were treated with $CuCl_2$ and $FeCl_2$ (10^{-3}M) as described under Materials and Methods, sown in the earthen pots and susequently the plants (15-old-plants) were treated with

said chemical(s) as foliar spray, uprooted and inoculated with *S.rolfsii* following water culture methods. The glyceollin content in soybean roots (cv. Macs-58) – untreated healthy, untreated and inoculated with *S.rolfsii* , treated with $CuCl_2$ and/or $FeCl_2$ as well as treated and inoculated with *S.rolfsii* were estimated after 24, 48 and 72h of inoculation. Results (Table-28) reveals that glyceollin reached a maximal concentration after 48h of inoculation with *S.rolfsii*. It is significant that $CuCl_2$ and $FeCl_2$ induced glyceollin synthesis in uninoculated soybean plants also (Table 28 & 29). Glyceollin accumulation reached maximum following 72h of treatment alone. In this case cupric chloride and ferric chloride act as abiotic elicitors of glyceoolin in soybean roots.

Table 28: Glyceollin accumulation in soybean roots (cv. Macs-58) at verious times after inoculation with *S.rolfsii* or treatment with non-conventional chemicals.

Time after inoculation or treatment (h)	Glyceollin content (µg/g fresh weight of roots)		
		Treatment	
	Inoculated with *S. rolfsii*	$CuCl_2$ (10^{-3}M)	$FeCl_2$ (10^{-3}M)
24	98 ± 2.2	87 ± 1.8	95 ±2.6
48	189 ± 3.6	12 1 ± 4.6	109 ± 3.3
72	125 ± 4.0	166 ± 3.9	135 ±4.5

Values represent the means ± SE from three experiments with three different preparations.

The glyceoolin content of soybean roots (Macs-58), before and after alteration of disease reactions by cupric chloride and ferric chloride treatments, were estimated and cmparisons were made 48h after inoculation as well as 48h after foliar treatment followed by inoculation. The production of glyceollin was maximum when plants were treated with these abiotic elicitors followed by inoculation with *S.rolfsii* (Table 29). The experiments were repeated three times and the results clearly indicate that the accumulation of glyceollin induced by $CuCl_2$ increases in soybean plants following inoculation with *S.rolfsii* than the $FeCl_2$ treatment.

Table 29: Effect of non-conventional chemicals on the accumulation of glyceollin in soybean roots (cv. Macs-58).

Plant treated with	Glyceollin content (µg/g fresh weight of roots)	
	Uninoculated	**Inoculated with *S. rolfsii***
Water (control)	0	190 ± 3.9
Cupric chloride (10^{-3}M)	144 ± 4.8	485 ± 4.2
Ferric chloride (10^{-3}M)	117 ± 5.2	332 ± 3.8

Glyceollin was extracted from soybean roots 48h after inoculation. Values represent the average of three separate experiments ± SE.

4.7. Analysis of host-parasite protein by SDS-PAGE

Plant immunity towards fungal pathogens may also depend on the speed and extent of protein synthesis induced in the host by the pathogen. In case of compatible combination, however, changes in protein configuration in the host may induce host accessibility to the pathogen, which is related to the induced susceptibility. There is also evidence that alteration in the protein sysnthesis in the plants can lead to the development of local resistance for immune layer around infection sites.

In view of these findings, attempts have been made to analyse the protein patterns of root , leaf as well as collar regions of soybean plants following infection alongwith the protein pattern of the fungal pathogen. Total soluble proteins were extracted from (a) roots of healthy and artificially inoculated soybean plants (PK-262, Bragg, NRC-7, PUSA-16, J-80 and Macs-58); (b) different parts – leaf, root as collar regions of healthy and artificially inoculated soybean plants (Macs-58)- 14 days after inoculation; (c) roots of untreated healthy control plants (Macs-58), inoculated with *S.rofsii*, treated with Chitosan (0.3%), IAA (10^{-4}M), $CuCl_2$ ((10^{-3}M), $FeCl_2$ ((10^{-3}M), as well as mycelial proteins of *S.rolfsii* (5-day-old and 12-day-old mycelia) and estimated . Experimental procedure has been described in detail under Materials and Methods. Results are presented in Table 30 and Plate 7 (A& B).

Table 30 : Protein content of soybean roots (Macs-58) treated with chemicals and inoculated with *S.rolfsii*

Treatment	Protein content (mg/g tissue)	
	Uninoculated	Inoculated with *S.rolfsii*
Water control	4.2 ± 0.23	5.4± 0.77
Chitosan (0.3%)	5.3± 0.44	4.0± 0.54
IAA (10^{-4}M)	4.4± 1.12	4.8± 0.50
$CuCl_2$ (10^{-3}M)	5.2± 0.93	4.6± 0.44
$FeCl_3$(10^{-3}M)	4.8± 0.80	4.1± 0.69

Protein content increased in untreated inoculated roots. Protein content in roots of treated plants also increased , however, in treated and inoculated roots protein content decreased. When protein samples of infected root, stem (collar regions) and leaf were compared with healthy control in SDS-PAGE , infected collar region yielded more protein. Soluble mycelial protein from 12-day-old culture of *S.rofsii* yielded maximum number of protein bands than 5-day-old culture (Plate 7 A).

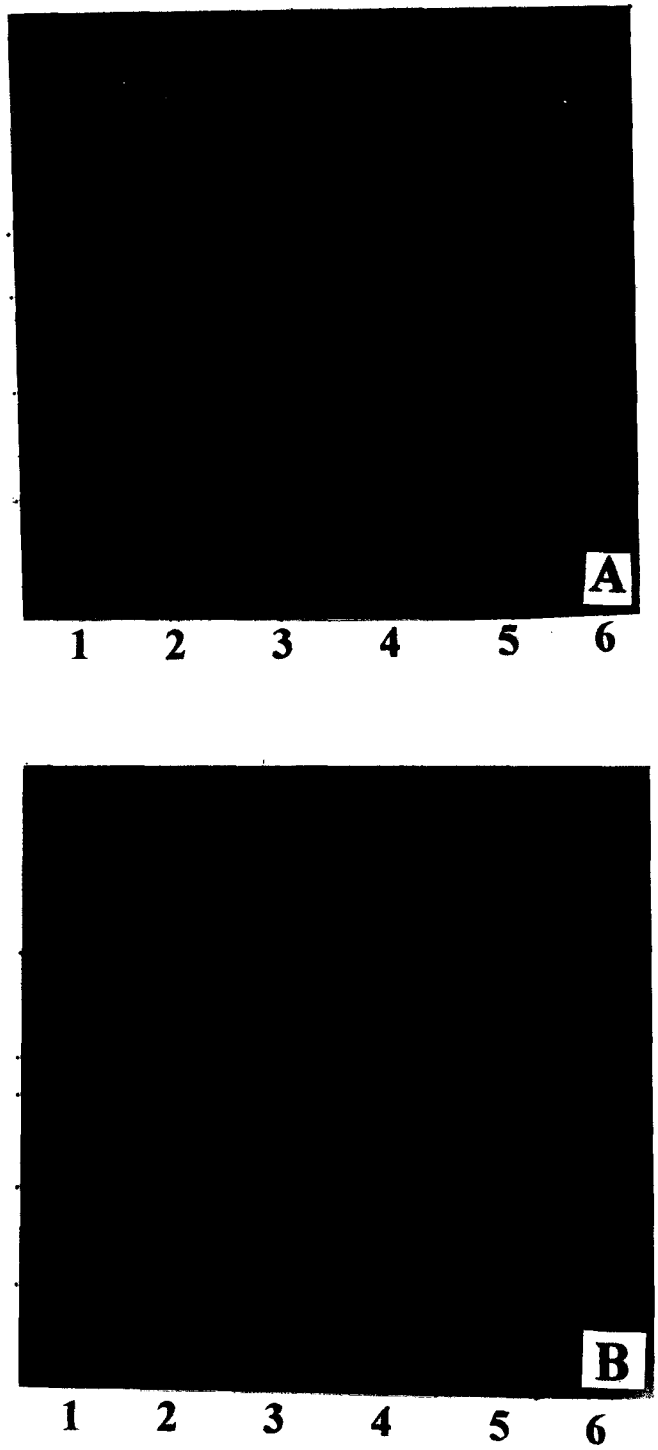


Plate 7 (A & B): SDS-PAGE analysis of soluble proteins
(A) Mycelial proteins of *S.rolfsii*; Lane 1-3: 5-day old;
4-6: 12-day old; (B) Soybean plants Lane 1&2: leaf;
3&4: collar region; 5&6: root
Healthy – 1,3 & 5; Infected – 2,4 & 6

4.8. Detection of cross reactive antigens between *S.rolfsii* and soybean varieties

The presence of cross reactive antigens (CRA)among host and pathogenic organisms is a well documented phenomenon. Existing studies on plant hosts and pathogens suggests that whenever, an intimate and continuing association of host and pathogen occurs, partners of this association have a unique serological resemblance to one or more antigenic determinants. Various methods have been generally used to detect the presence of CRA between host and parasite. In the present investigation, CRA between *S.rolfsii* and soybean varieties have been detected using immunodiffusion, immunoelectrophoresis and enzyme linked immunosorbent assay. Series of experiments performed and results obtained have been presented below.

4.8.1. Immunodiffusion

The effectiveness of antigen preparations from *S.rolfsii* and soybean roots (Macs-58) were checked by homologous cross reactions following agar gel double diffusion tests. Control sets involving normal sera and antigens of soybean roots and pathogen were all negative. Strong precipitin reaction occurred when antiserum raised against mycelia of *S.rolfsii* was reacted with its own antigen (soybean isolate) and the antigens of five more isolates of *S.rolfsii* from cowpea, pea , marigold, tea . (Plate 8 A). When anti *S.rolfsii* antiserum was cross reacted with root antigens prepared from four soybean varieties such as Macs-58, J-80, PK-262 and Bragg , precipitin bands were observed in immunodiffusion tests (Plate 8 C). No such precipitin reactions were observed in case of cross reactions between anti *S.rolfsii* antiserum and antigens preapred from two non pathgens of soybean plants (*Fomes lamaoensis* and *Ustulina zonata*). Reciprocal cross reaction between antiserum raised against soybean root antigen (Macs-58) and antigens of all six isolates of the pathogen (*S.rolfsii*) in agar gel double diffusion test develop one strong precipitin band (Plate 8 B) confirming common antigenic relationships among isolates of the pathogen (*S.rolfsii*) and highly susceptible soybean variety (Macs-58).

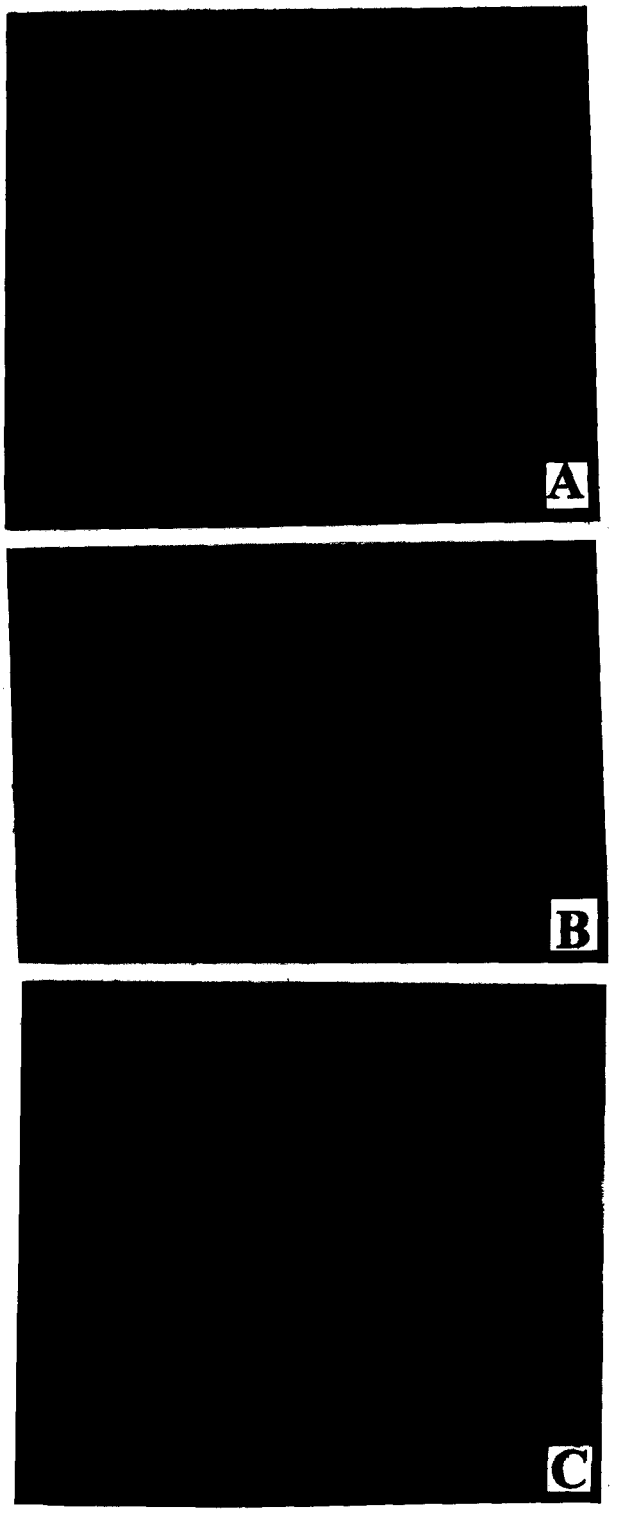


Plate 8 (A-C) : Agar gel double diffusion tests.
Central wells (7) contain antisera of *S.rolfsii* (**A&C**)
and soybean (Macs-58) root (**B**). Peripheral wells (1-6)
contain antigens.**A & B**–*S.rolfsii* isolates (1-6);**C**–*S.rolfsii*(1);
Macs-58 (2,3); J- 80 (4); PK-262 (5); Bragg (6)

4.8.2. Immunoelectrophoresis

The presence or absence of cross reactive antigens between isolates of *S.rolfsii* and soybean varieties were established by immunodiffusion tests. Many of the cross reactions in immunodiffusion tests gave diffused preciptin bands which could not be clearly distinguished. It was not clear, whether the preciptation reaction is due to single or several antigenic substances. In this experiment, antigenic comaprison among six soybean varieties and six isolates of the pathogen (*S.rolfsii*) and two non pathogens of soybean (*Fomes lamaoenis* and *Ustuliza zonata*) using antisera of pathogen (*S.rolfsii*) and host (Macs-58) were done following conventional set up. Results are shown in Plate 9 (A-C) and Table 31.

Table31: Antigenic comparison among soybean varieties, pathogen (*S.rolfsii*) and non pathogens

Antigens of host and parasite	**Total no. of precipitin lines**	
	Antisera of host and parasite	
	Macs-58	***S.rolfsii***
Soybean varieties		
Macs-58	3	2
PK-262	2	1
Bragg	3	1
NRC-7	3	1
J-80	2	2
Pusa 16	2	1
Pathogen (*S. rolfsii*)		
Isolate –1	2	4
Isolate-2	1	2
Isolate-3	2	3
Isolate-4	1	2
Isolate-5	2	2
Isolate-6	1	2
Non-pathogens		
Fomes lamaoensis	0	0
Ustulina zonata	0	0

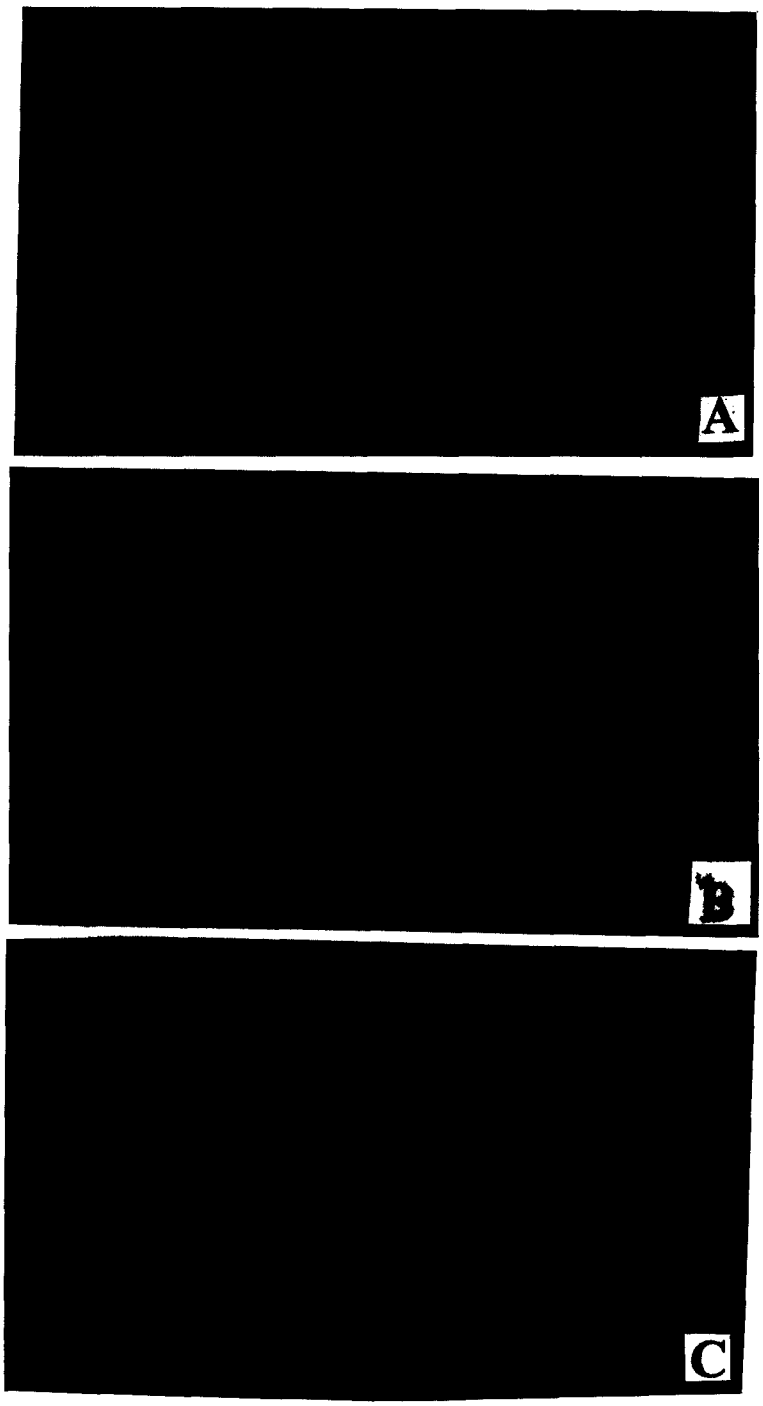


Plate 9 (A-C): Immunoelectrophoretic patterns of the antigens of soybean roots and mycelia of *S.rolfsii*. Central rectangular troughs charged with antiserum of S.rolfsii (**A&B**), soybean root–Macs58 (**C**) and the surrounding wells with antigens of *S.rolfsii* (1 & 2), Macs-58 (3 & 6); *Fomes lamaoensis* (4&5); Macs-58 (6),

4.8.3. Direct antigen coated enzyme linked immunosorbent assay (DAC-ELISA)

The enzyme linked immunsorbent assay is one of the most sensitive serological techniques for detection of CRA between host and pathogen as well as for detection of pathogens in diseased tissue. In indirect or DAC-ELISA, antigens are bound to the microtitre plates after which the antibody is allowed to bind to the antigen. To this antigen antibody complex, the conjugate (an antibdy conjugated to an enzyme) is added. Finally, a non coloured substrate is added which is converted to a coloured end product, which is generally detected by a reader. In the present investigation, DAC ELISA has been used in most of the experiments. Since ELISA depends on a number of factors and these varies from system to system, it was considered essential to optimize the conditions in this particular host-pathogen system.

4.8.3.1. Optimization of ELISA

Optimization of ELISA was done using IgG fraction of antisera raised against mycelial antigens of *S.rolfsii*. Three variables such as dilution of enzyme, dilution of the antiserum and dilution of the antigens were optimized. In all cases, homologous ELISA reaction using antigens of *S.rolfsii* was carried out as described under Materials and Methods.

4.8.3.1.1. Enzyme dilution

In this experiments, keeping the antigen (10μg/ml) and antiserum dilution (1:125) constant, different dilutions of alkaline phosphatase was used. Dilution ranged from 1:10,000 to 1:40,000. On the basis of results 1:10,000 of alkaline phosphatase was used in all further experiments.

4.8.3.1.2. Antiserum dilution

Antiserum dilutions ranging from 1:125 to 1:16,000 were tested against homologous antigen at a concentration of 10μg/ml. Absorbance values in ELISA decreased from the dilution of 1:125 to 1:16,000 (Fig.12). An absorbance value of 1.81 was obtained at 1:125 dilution which was 3 times of that obtained at 1:16,000 dilution (Table 32). 1:250 dilution was selected for further experiments.

Table 32 : ELISA reaction with various dilution of anti-*S. rolfsii* antiserum and homologous antigen

Antiserum dilution	Absorbance at 405 nm
1:125	1.81± 0.032
1:250	1.68± 0.015
1:500	1.65±0.003
1:1000	1.54±0.014
1:2000	1.27±0.002
1:4000	1.17±0.016
1:8000	0.86±0.001
1:16000	0.61±0.002

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

4.8.3.1.3. Antigen dilution

Doubling dilutions of S.rolfsii mycelial antigen ranging from 8000 to 62.5 ng/ml were tested against two antiserum dilutions (1:125 and 1:250). ELISA values increased with the concomitant increase of antigen concentration (Table 33). Concentrations as low as 62.5ng/ml could be easily detected by ELISA at both antisera dilutions (Fig. 13).

4.8.3.2. Comparison of ELISA reactivity among antigens of different soybean varieties against antiserum of *S.rolfsii*

Among six varieties of soybean tested for varietal resistance tests against *S.rolfsii* , differential responses were obtained. Certain varieties exhibited high susceptibility, others were moderately susceptible. Conventional techniques for determination of host resistance or susceptibility are being replaced by more rapid and sensitive modern serological techniques. It was therefore considered worthwhile to detemine the ELISA reactivity of different soybean varieties against antiserum of the pathogen.

Effect of dilution of anti-*S.rolfsii* antiserum on ELISA reaction with homologous mycelial antigen

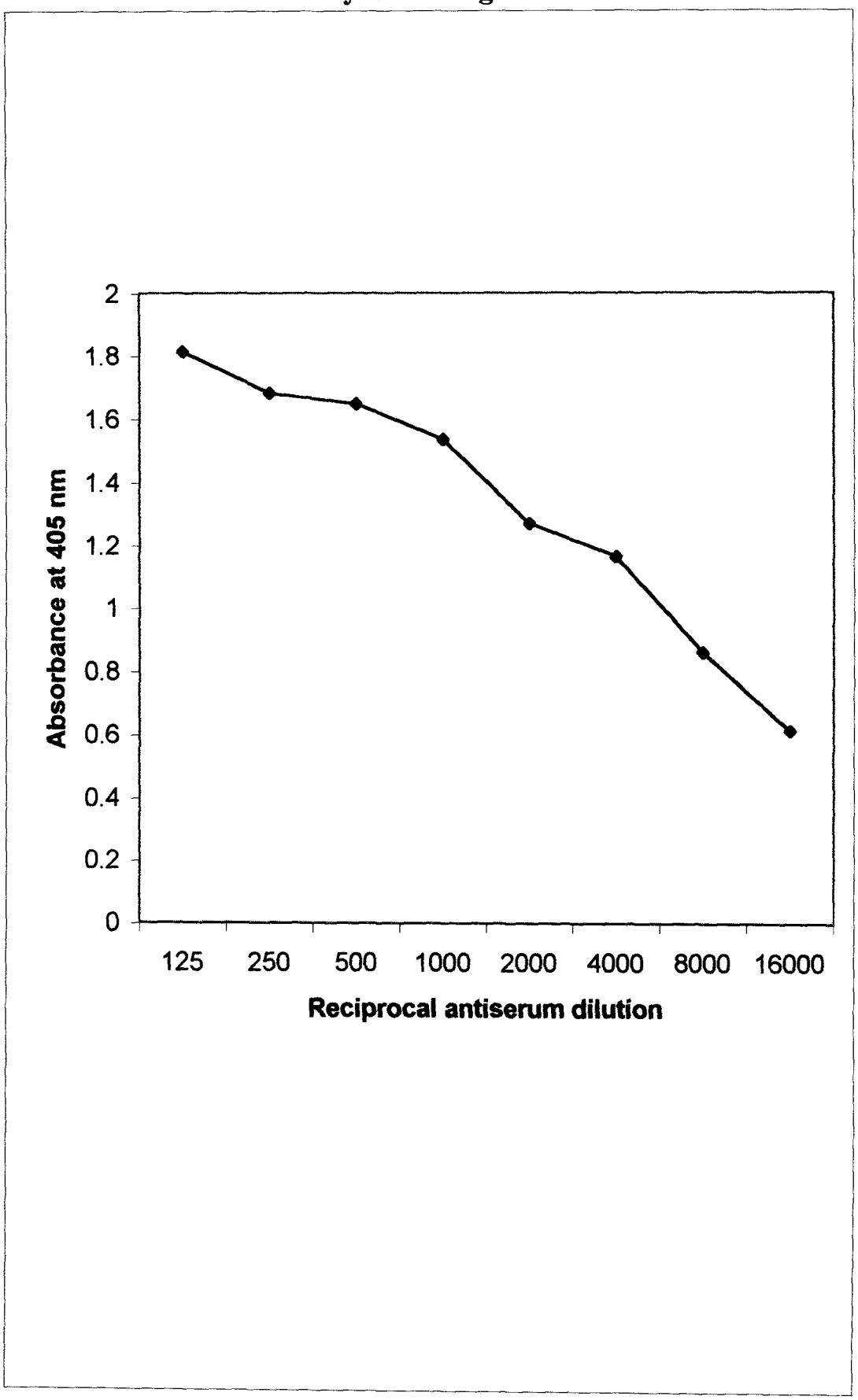


Fig.12

Table 33 : ELISA reaction with various concentration of mycelial antigen of *S. rolfsii* and homologous antiserum

Antigen dilution (ng/ml)	Absorbance at 405 nm	
	Antisera dilution (1:125)	Antisera dilution (1:250)
8000	2.380±0.112	2.803±0.122
4000	2.110±0.010	1.940±0.102
2000	1.985±0.065	1.798±0.0045
1000	1.823±0.026	1.727±0.033
500	1.808±0.015	1.511±0.022
250	1.710±0.032	1.436±.116
125	1.660±0.077	1.310±0.049
62.5	1.591±0.045	1.250±0.551

Anti -*S. rolfsii* antiserum dilution 1:125 and 1:250

Enzyme dilution = 1: 10,000 ; ± = Standard error

Antigens were prepared from soybean roots of six varieties, six isolates of the pathogen (*S.rolfsii*) , as well as two non –pathogens. All these antigens at a concentration of 40 µg/ml were tested by ELISA against purified antiserum of *S.rolfsii,* except antigens of the *S.rolfsii* isolates, which were used at a concentration of 10 µg/ml. In all caes, experiments were repeated under similar concentrations. Results (Table 34) revealed that absorbance values in ELISA varied with the different varieties. The different isolates of the pathogen tested also showed reactivity with the antiserum of the pathogen. Highest absorbance value, however, was obtained in the homologous reaction. Absorbance values for normal serum controls were below the corresponding test values.

Effect of dilution of mycelial antigen of *S.rolfsii* on ELISA reaction with homologous antiserum

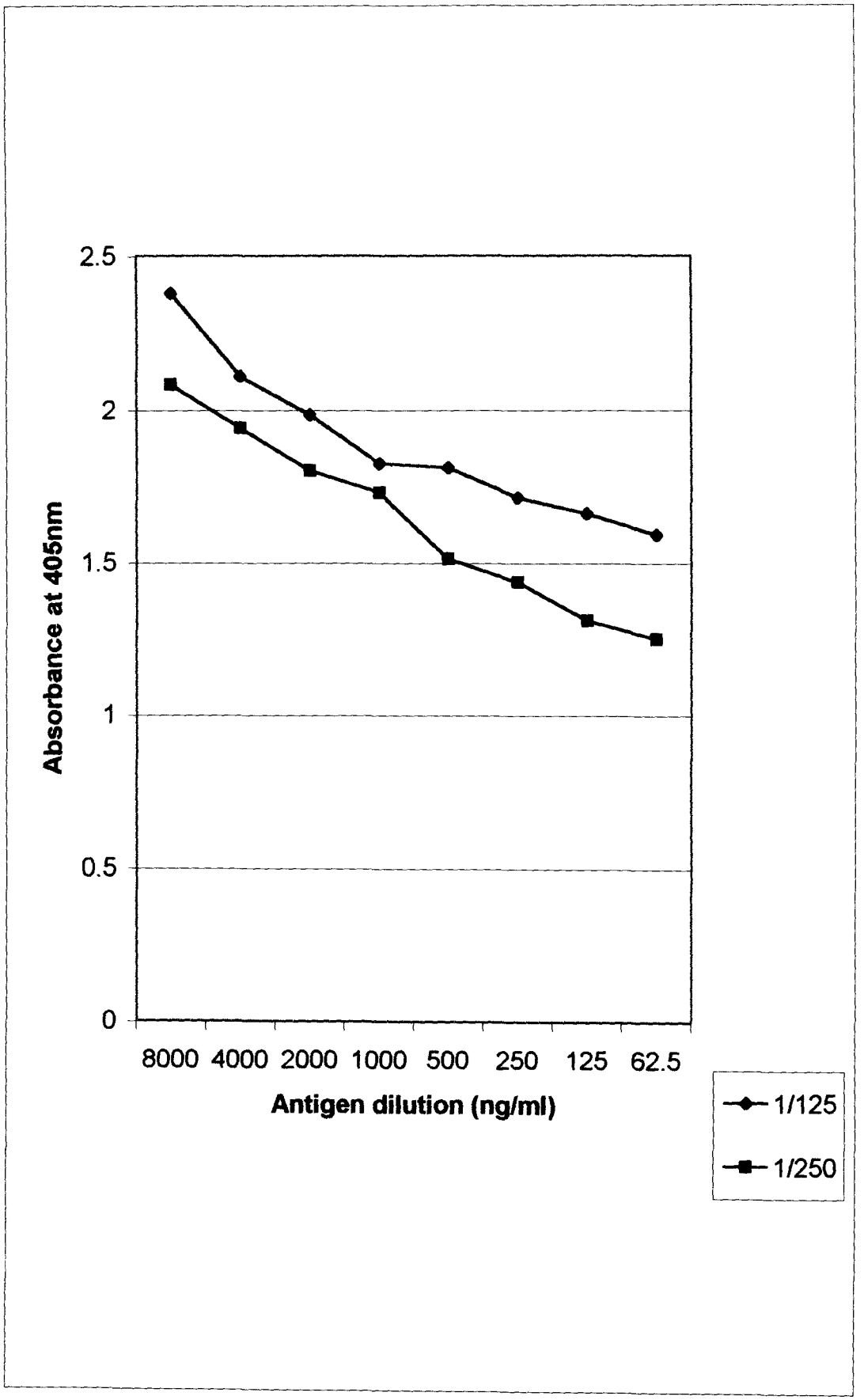


Fig.13

Table 34 : Indirect ELISA values (A 405) of soybean root antigens and mycelial antigen (pathogen and non-pathogen) reacted with antiserum of *S. rolfsii*.

Antigens of host and parasite	Absorbance at 405 nm
Host (soybean root)	
PK-262	0.988±0.021
Bragg	0.922±0.045
NRC-7	0.866±0.049
Pusa-16	0.997±0.027
J-80	1.032±0.031
Macs-58	1.124±0.033
Pathogen (*S. rolfsii*)	
Isolate 1	1.983±0.023
Isolate- 2	1.880±0.015
Isolate –3	1.795±0.026
Isolate – 4	1.696±0.022
Isolate – 5	1.772±0.037
Isolate – 6	1.894±0.028
Non-pathogen	
Fomes lamaoensis	0.542±0.011
Ustulina zonata	0.441±0.016

Anti *S. rolfsii* antiserum 1: 250 dilution.

Antigen concentration 10 μg/ml.

± Standard error

4.9. Detection of *S.rolfsii* in artificially inoculated soybean root tissues by DAC-ELISA

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 infected antigen extracts indicates the measure and extent of infection. Initially, in the previos experiments, it was observed that cross reactive antigens are present between *S.rolfsii* and susceptible soybean varieties. In this experiment, six soybean varieties were artificially inoculated with *S.rolfsii.* It has been observed in pathogenicity tests, that well established symptoms of *S.rolfsii* infection appeared on aboveground level within 10-12 days after inoculation. Therefore, root antigens were prepared from healthy and infected (artificially inoculated with *S.rolfsii*) soybean plants (varieties PK262, Bragg, NRC7, Pusa16, J80 and Macs58) , 12 days after inoculation with *S.rolfsii.* Concentration of root antigen and dilution of anti-*S.rolfsii* antiserum were 40 μg/ml and 1:250 respectively. Results are presented in Fig.14. Absorbance values for antigen prepared from inoculated roots of all varieties were higher than their respective healthy root antigens.

4.10. Cellular location of CRA using Immunofluorescence

Fluorescent antibody labellig with fluorescein isothiocyanate (FITC) is known to be one of the powerful techniques to determine the cell or tissue location of major cross reactive antigens (CRA) shared by host and parasite. In the presen study, following immunodiffusion, immunoelectrophoresis, DAC-ELISA, the presence of CRA shared by *Glycine max* and *Sclerotium rolfsii* has been detected. It was decided to determine the tissue and cellular location of CRA in root tissues soybean varieties as well as mycelia and sclerotia of *S.rolfsii.* Detailed methods of antibody staining of root sections and mycelial preparations have already been described in Materials and Methods. Photographs were taken under UV-fluorescence.

ELISA reactions of anti-*S.rolfsii* antisera with healthy and inoculated soybean plants

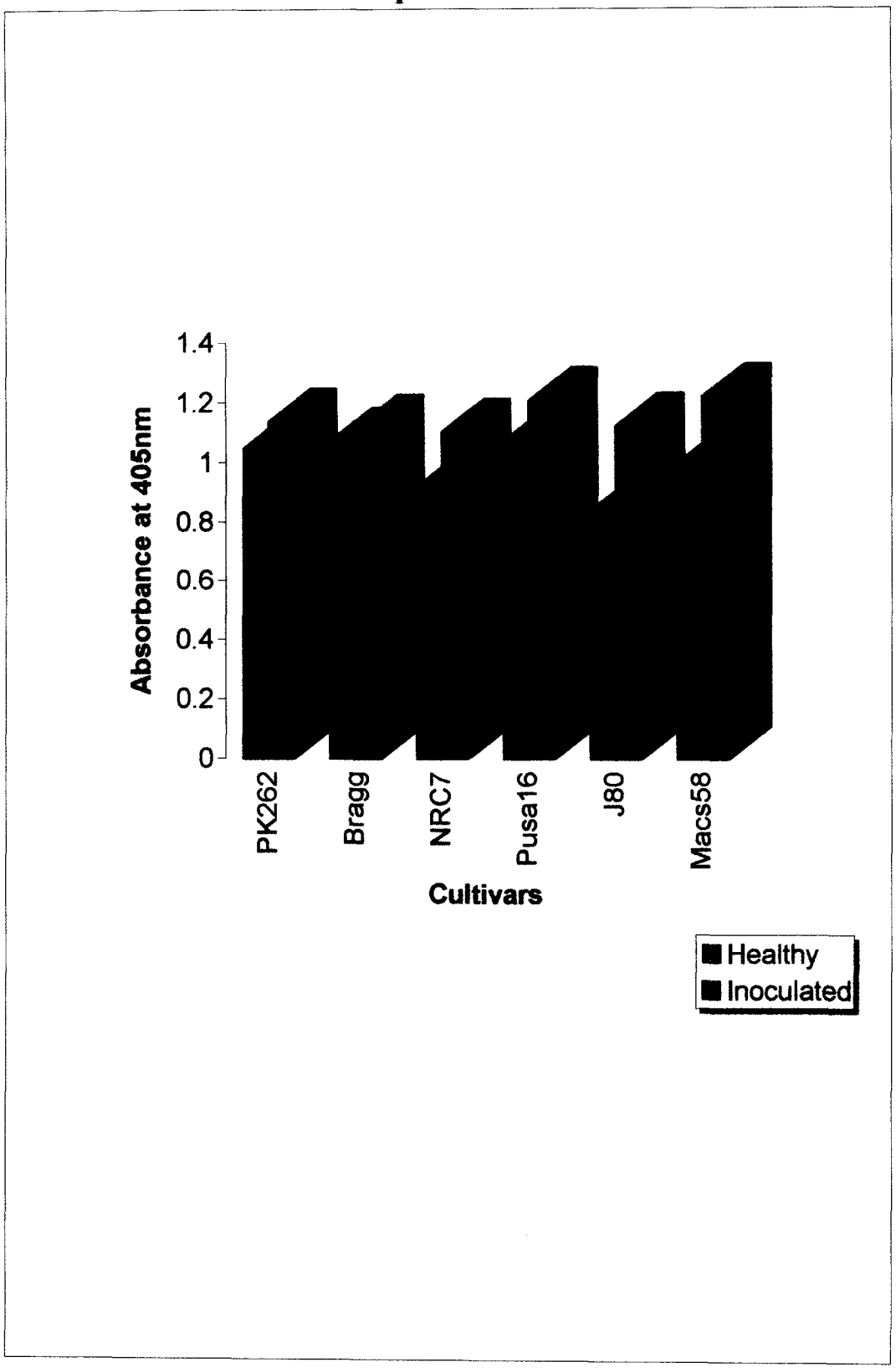


Fig.14

4.10.1 Root tissue

Cross sections of soybean roots (variety- Bragg and Macs-58) were treated separately with normal serum, homologous and pathogen antisera, then reacted with FITC. Root sections exhibited a natural autofluorescene under UV-light on the cuticle. Same observations were noted when the root sections were treated with normal serum and labelled with FITC. Root sections treated with antiserum of Macs-58 and then reacted with FITC, developed bright fluorescence which was distributed throughout the root tissues. Of much significance was the strong reaction of anti-*S.rolfsii* antiserum with root tissues of two soybean varieties (Macs-58 and Bragg). CRA was concentrated mainly around epidermal and cortical cells in case of Bragg (Plate 10 B), whereas in case of Macs-58, CRA was distributed throughout the epidermal, cortical tissues (Plate 11 A & B) and also vascular tissues (Plate 12 A & B).

4.10.2 Mycelia and Sclerotia

Mycelia and sclerotia of *S.rolfsii* were not auto-fluorescent nor did they fluoresce when treated with normal serum followed by FITC. Treatment of mycelia and sclerotia of *S.rolfsii* with homologous antiserum and FITC showed a general flurescence that was more intense on young hyphal tips (Plate 13) and on the germinated sclerotia (Fig.14 A&B).

4.11. Serological changes associated with induction of resistance in soybean plants

In the present investigation attempt was made to induce disease resistance in soybean plants (variety-Macs58) applying twelve chemicals belonging to three separate groups i.e. metal salts, growth regulators and biological compound (chitosan). Among the tested chemicals cupric chloride and ferric chloride were found to be highly effective in reducing disease intensity. Consequent changes in glyceollin level due to induction of resistance were determined. Similarly alteration in antigenic patterns after chemical induction of resistance by cupric chloride were also worked out since both are believed to be associated with plant disease resistance.

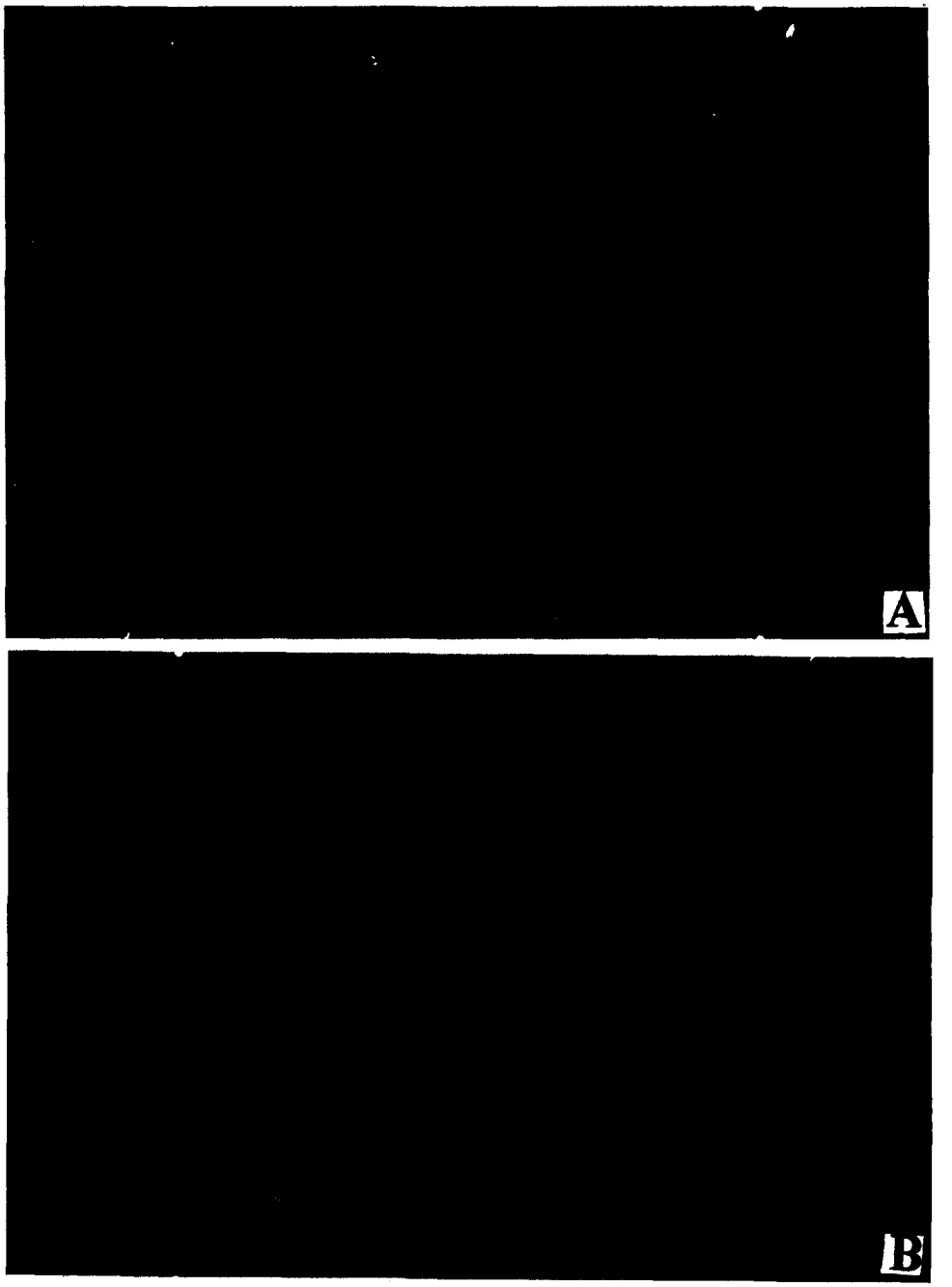


Plate 10 (A & B): T.S. of soybean root (Variety - Bragg)
(A)- Unstained; (B) Treated with antiserum of *S.rolfsii* and FITC antibodies of goat specific for rabbit globulin

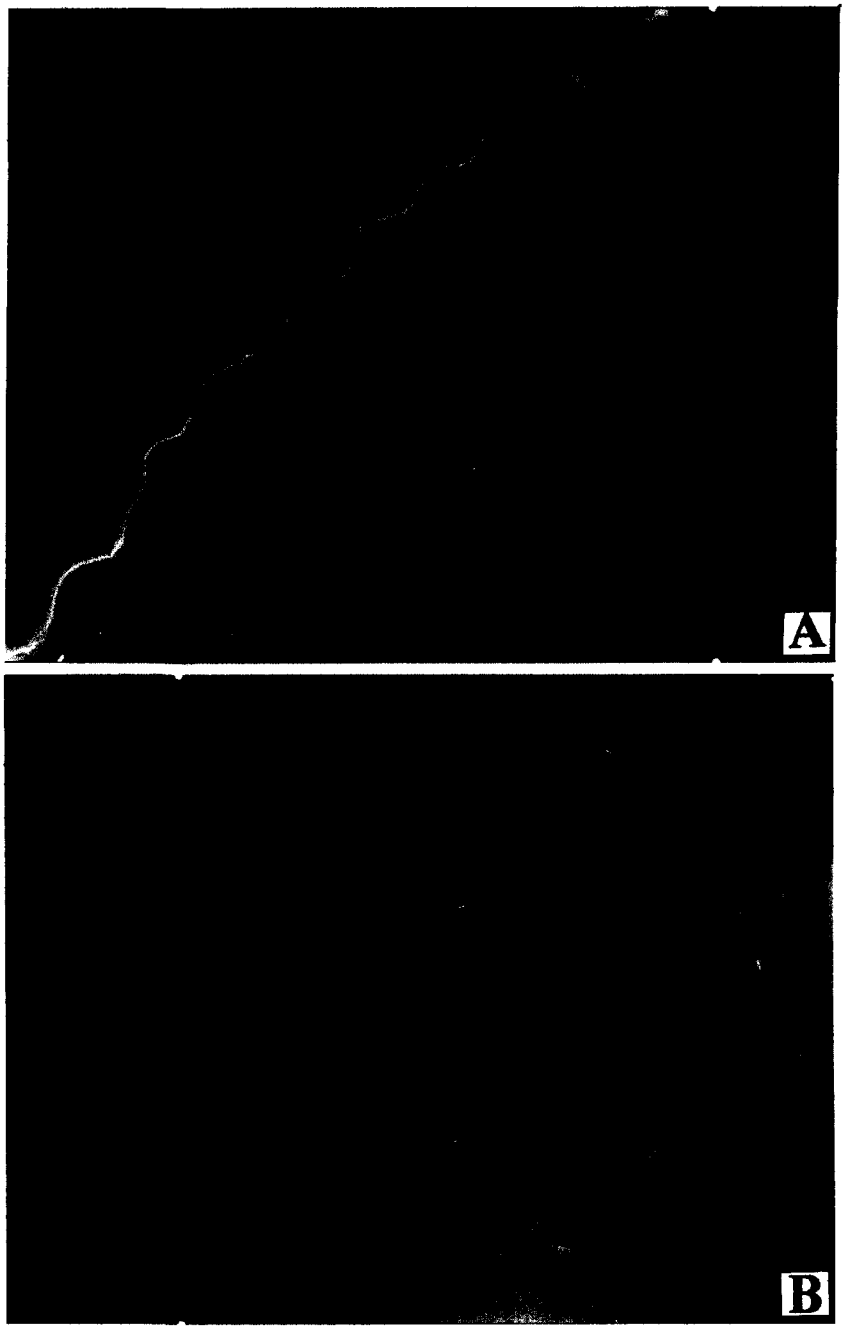


Plate 11 (A & B): Fluorescent antibody staining of soybean root tissues (Variety- Macs58) for cross reactive antigens shared with *S.rolfsii*. Root sections treated with antiserum of *S.rolfsii* and FITC antibodies of goat specific for rabbit globulin

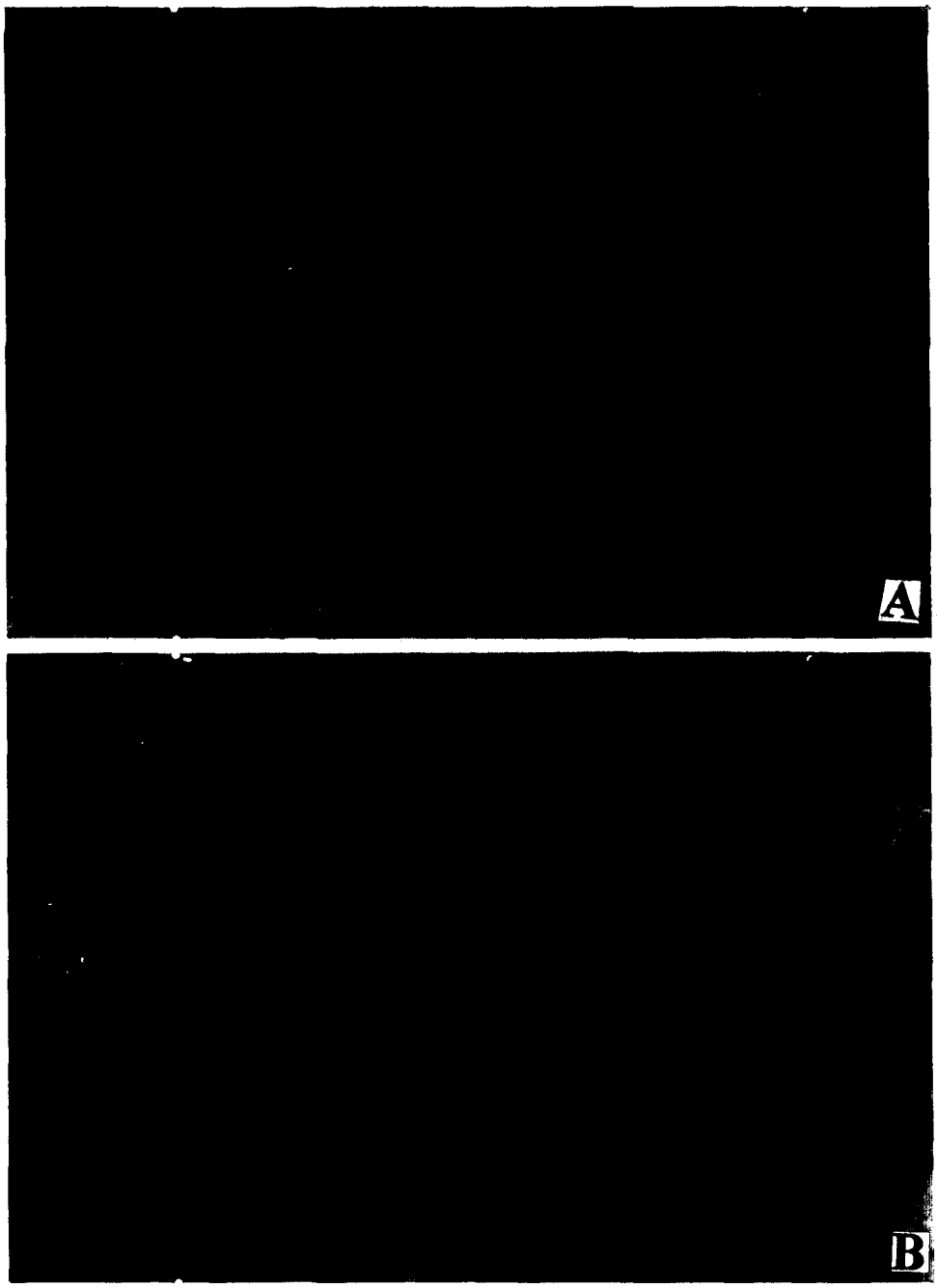


Plate 12 (A & B): T.S. of collar region of soybean plants (Variety- Macs58) treated with antiserum of *S.rolfsii* and FITC antibodies of goat specific for rabbit globulin

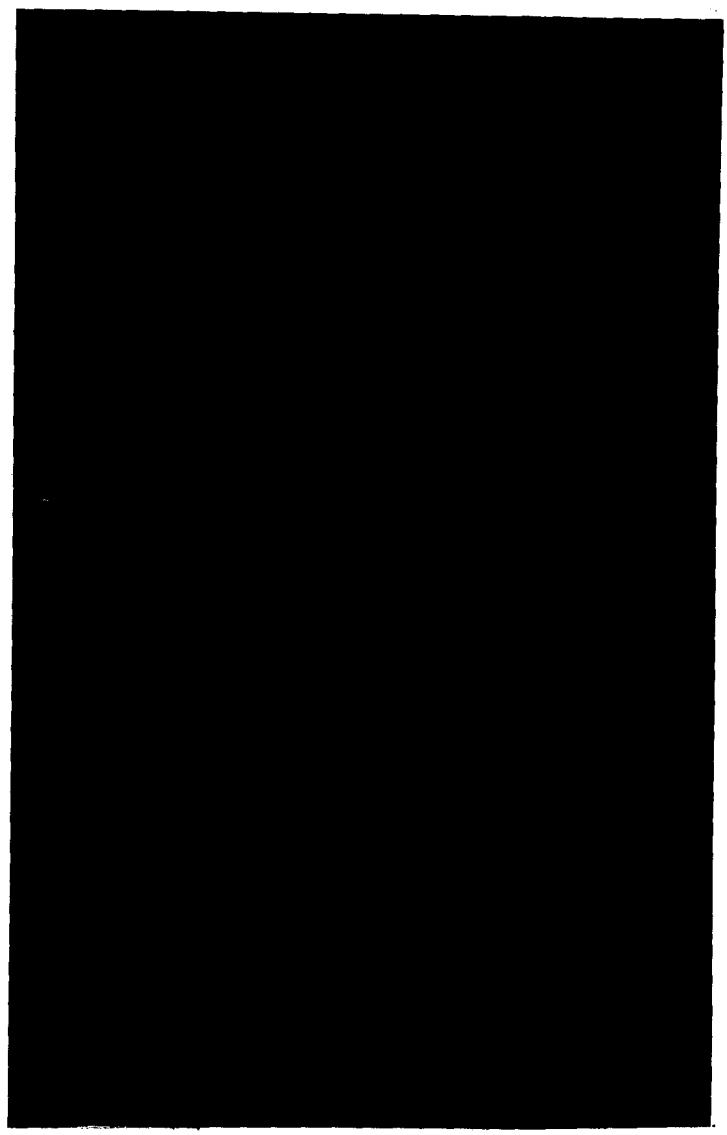


Plate 13: Mycelia of *S.rolfsii* treated with antiserum of *S.rolfsii* and FITC antibodies of goat specific for rabbit globulin

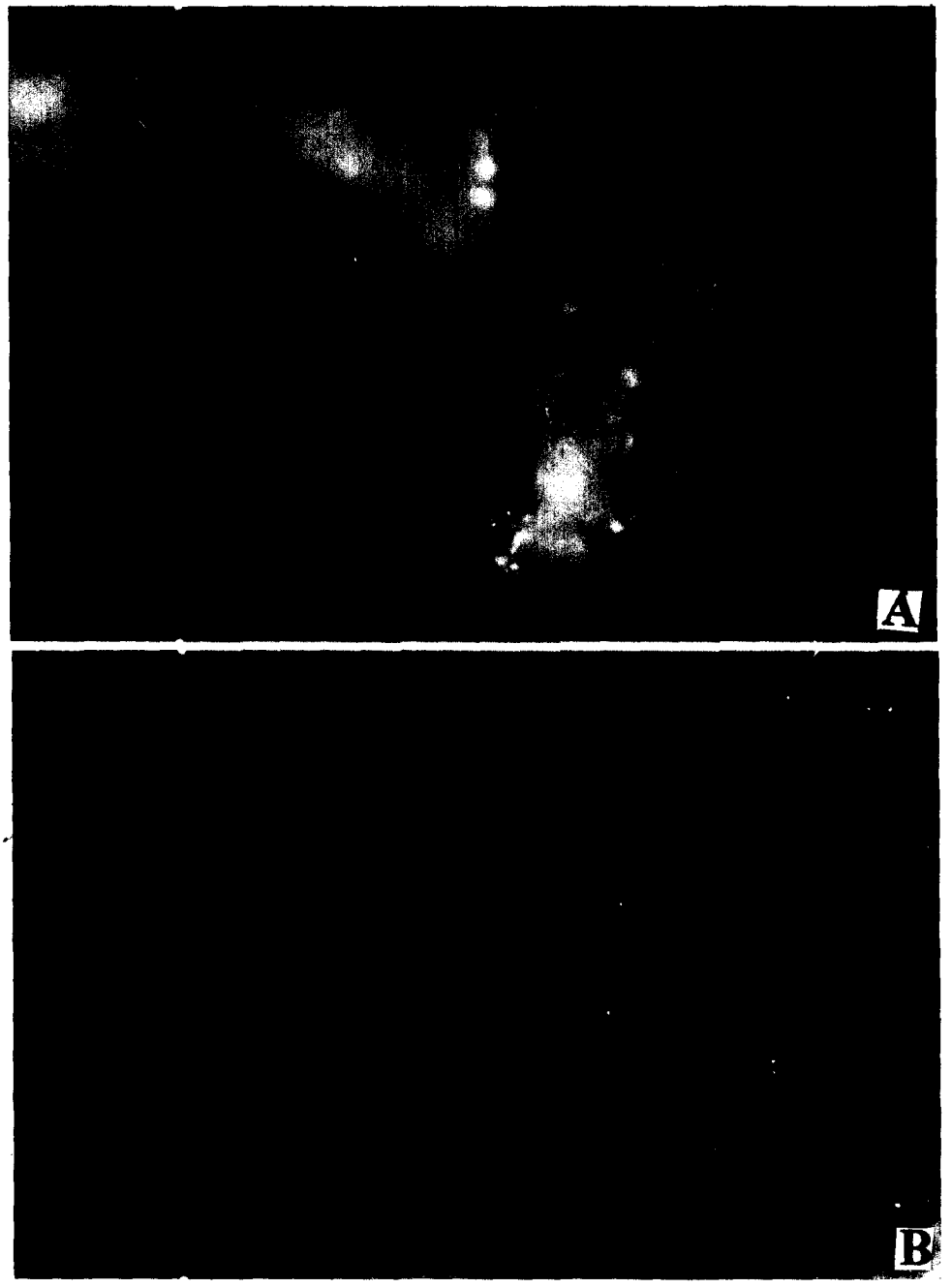


Plate 14 (A & B): Germinated sclerotia treated with antiserum of *S.rolfsii* and FITC antibodies of goat specific for rabbit globulin

To study the consequent changes in antigenic patterns in cupric chloride treated soybean plants (Macs-58), agar gel double diffusion tests were performed with antisera raised against untreated and cupric chloride treated soybean root(Macs-58) antigens as well as antiserum of *S.rolfsii.* against both homologous and heterologous antigens. Results (Table- 35) reveal that strong precipitin reactions occurred when antiserum of *S.rolfsii* was reacted against its own antigens as well as root antigens of untreated susceptible soybean variety- Macs-58. However, antigens of treated soybean roots showed no precipitin reaction. Reciprocal cross reaction between antiserum of treated roots and the pathogen antigens also failed to develop even weak precipitin bands.

Table 35: Immunodiffusion tests of antigens of *S.rolfsii* and soybean root tissues before and after treatment with cupric chloride

Antigens	**Antisera**		
	Macs-58 (Untreated)	**Macs-58 (Treated)***	***S.rolfsii***
Soybean variety (Macs – 58)			
Untreated	+	+	+
Treated*	±	+	-
Pathogen			
S.rolfsii	+	-	+

- *Plants treated with cupric chloride (10^{-3}M)
- Common precipitin band: + = present ; ± weak band ; - = band absent

The presence or absence of common antigens between host and parasite could be confirmed by the immunodiffusion test, but it was 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 following immunoelectrophoresis.

Table 36 : Immunoelectrophoretic analysis of antigens of *S.rolfsii* and soybean root tissues before and after treatment with cupric chloride

Antigens	No. of precipitin lines with host and parasite antisera		
	Macs-58 (Untreated)	Macs-58 (Treated)*	*S.rolfsii*
Soybean variety (Macs – 58)			
Untreated	3	1	2
Treated*	1	2	0
Pathogen			
S.rolfsii	2	0	4

*Plants treated with cupric chloride (10^{-3}M)

The effectiveness of each antigen extract in raising antibodies was checked by homologous reactions. The homologous patterns formed by antigens and antisera of *S.rolfsii* , untreated soybean roots and treated soybean roots contained four, three and two precipitin lines respectively (Table 36) When antigens of untreated roots and the fungal antigens were cross reacted with antiserum of Macs-58 (untreated), only two precipitin bands were visible for the pathogen as common antigen. However, cupric chloride treated root antigens shared only one precipiin line with untreated root antigens. But no common antigenic substances could established with treated root antigens and pathogen antisera or vice versa . It appears that these observed antigenic changes owing to cupric chloride treatment have some significance in the resistance of soybean to *S.rolfsii.*

Discussion

In nature plants survive in the face of attack by many microbial organisms that threaten their survival and attempt to use them as a food source by employing several layers of defense responses. In addition to specific defense responses based on so-called R-genes, against certain strains of a pathogen, plants have broad spectrum defense responses which are preformed, such as surface waxes, or that can be induced locally or systemically by biotic or abiotic agents in nature (Oostendorp *et.al*, 2001). Induced resistance in plants has been the subject of considerable research over the past two decades with the discovery that many pathogens or chemical compounds may be used to elicit host defense mechanisms leading to reduced pathogen attack. Pathogen–produced elicitors are considered to be the primary signals responsible for the induction of plant defense reactions. Chitin, the main wall component of many filamentous fungi, and chitosan, the deacetylated derivative of chitin, have been shown to be potential elicitors of several plant defense responses including lignification. Seedlings raised from pearl millet seeds (HB3) which were soaked in 0.4% chitosan for 12h showed resistance against downy mildew disease (Shivkumar, 2000).

In the present investigation, a series of experiments have been performed using twelve chemicals belonging to three separate groups, viz. eight metal salts(cupric chloride, ferric chloride, lithium sulphate, manganese sulphate, sodium molybdate, magnesium sulphate, zinc chloride and barium sulphate), three growth regulators (IAA, 2,4-D and 2,4,5-T) and one biological compound, chitosan with a view to alter disease reaction against *Sclerotium rolfsii* in susceptible soybean variety (Macs-58). Apart from this, the effect of different concentrations of the above chemicals on sclerotial germination of *S.rolfsii* was also studied. Among the tested chemicals cupric chloride and ferric chloride were found to be highly effective in reducing disease intensity. The use of nickel chloride as foliar spray to tea plants for the control of blister blight caused by *Exobasidium vexans* was demonstrated by Venkataram (1961). Host sensitization as a factor in induction of resistance in rice

(cv. Dharial) against *Drechslera oryzae* by seed treatment with phytoalexin inducers have also been reported by Sinha and Hait (1982). When rice seedlings were treated separately with sodium selenite, lithium sulphate, cycloheximide and thioglycolic acid and challenge inoculated with *D.oryzae*, disease symptoms reduced markedly.

Many plant pathogenic fungi are known to produce a range of cell wall degrading enzymes that macerate plant cell wall, including pectolytic enzymes such as pectin methylestarase, polygalacturonase and pectate lyase that may have important roles in the infection process for the development of disease symptoms (Bateman and Basham, 1976; Walton,1994;Chilosi and Magro, 1998). Pectolytic enzymes are often produced in culture and during plant infection sequentially as multiple isoenzymes and may constitute a catabolic pathway for the complete degradation of pectic polysaccharides, the process being initiated by constitutive forms (Chilosi and Magro, 1997). The importance of such enzymes in pathogenicity is supported by the ability of purified enzymes to reproduce disease symptoms (Bateman, 1968; Barash et.al., 1984; Holtz and Knox-Davies, 1985) and by the correlation of the pectolytic enzyme level with the extent of damage to the plant (Olsson, 1989; Wijesundera *et.al.,* 1989; Cleveland and Cotty, 1991; Bayen *et.al.,* 1997). Moreover there are evidences of involvement of pectolytic enzymes in pathogenic mutants (Wattad et.al., 1995), antibodies blocking enzymes (Wattad et.al., 1994), chemicals like tannic acid and rufianic acid (Martin and Grossman,1972).

Significant increases in calcium and magnesium levels in the infected tissue imply induced polygalacturonase activity. Bateman and Miller (1966) reported such changes in resistant bean plants, when infected with *Rhizoctonia solani* as a defence response. In this opinion such accumulation leads to transformation of enzyme-sensitive cell wall pectic components at and around the lesion site to less soluble, enzyme tolerant calcium and magnesium pectates and this acts as dynamic resistant factor. Similar report is also available for *Rhizoctonia solani* infection of groundnut (Reddy *et.al.*,1988). Calcium may further help in host defense by promoting biosynthesis of callose, a cell wall component (Kauss, 1987) and phytoalexin (Kurosaki *et.al*, 1987), both known to be components of plants dynamic defense.

Calcium did not, however, any such effect on *Fusarium solani* infection of bean (Kendra and Hadwiger, 1987) and pod rot of ground nut due to *Pythium myriotylum* and *Rhizoctonia solani* (Filonow *et.al,* 1988).

Lignification has been observed in many plant species following attempted infection by pathogenic organisms such as fungi, bacteria, viruses and nematodes. There is strong evidence that lignification is an important mechanism for disease resistance (Carver et.al., 1994; Mauchmani and Slusarenko, 1996). In the same way that lignified tissue in the host plant such as endodermis, sclerenchymatous fibres etc. seem to prevent further ingress by certain fungal pathogens (Bishop and Cooper, 1983), induced lignin deposition at or around the site of penetration would also be expected to limit the pathogen by providing a physical barrier to mechanical penetration through cell wall. Lignification of cell walls bordering lesions may play an important role in limiting lesion expansion (Stockwell and Hanchey, 1987). Ride (1980) reported that lignified papillae and haloes from wheat leaves inoculated with *Botrytis cinerea* are highly resistant to degradation *in vitro* by various fungal species. The resistance is also supported by experiments testing the release of carbohydrate by fungal culture filtrates from lignified walls. Lignified cell wall could also constitute a barrier preventing free nutrient movement and therefore help to starve a pathogen. Lignin precursors themselves might exert a toxic effect on pathogens or, by binding two fungal cell walls, make them more rigid and an impermeable, thus hindering further growth or uptake of waterband nutrients. Coniferyl alcohol, for example, is highly toxic *in vitro* for fungi at low concentrations (Hammerschmidt and Kuc, 1982). Increase levels of lignins were reported in various crop plants such as muskmelon infected with *Colletotrichum lagenarium* (Touze and Rossignol,1977), potato infected with *Phytophthora infestans* (Friend, 1981), cucumber against *Spherotheca fuliginea* (Bashan and Cohen, 1983) and *Cladosporium cucumerinum* (Hammerschmidt, 1984). Involvement of lignin and callose during systemic acquired resistance in pearl millet against downy mildew pathogen *Sclerospora graminicola* has been demonstrated by Satyan (2000). Deposition of lignin and callose were found in significant amounts in resistant and

induced resistant than in the suscepible pearl millet cultivar. It was also observed that in resistant cultivar, lignification and deposition of callose occurred earlier than the susceptible cultivar. At the region of papilla formation, pathogen development was completely arrested.

The physiological/biochemical basis of resistance of plants to fungal pathogens has been associated with both preformed and infection induced antimicrobial compounds (VanEtten *et.al.*, 1994). However, the expression of resistance (i.e. defense) in most plant-pathogen interactions cannot be explained by the presence of preformed inhibitors. Most research on resistance mechanisms has shown that the plant uses defenses that are activated after infection to stop pathogen development. Many biochemical changes occur in plants after infection, and some of these have been associated with the expression of defense because they have activity against pathogens *in vitro*. Matern and Kneusel (1988) proposed rapid synthesis of phenolics following infection to be an important first line defense in plants.

Changes in host physiology following infection or induced resistance is often associated with an activation of oxidase activity and a post infectional increase in the level of such enzyme is a common phenomenon in diseased tissue, more so in an incompatible interaction. The activation of polyphenol oxidase would seem to be important in that it can oxidise phenolics to quinones which may be more fungitoxic. The infected resistant tisssue shows in many cases a higher oxidase activity than the infected susceptible tissue as also the healthy one. Such observations have led to the speculation that stimulated polyphenoloxidase activity possibly contribute to the resistance of plant against the pathogen. Various observation on the role of polyphenoloxidase in host resistance have been summarised by Sinha (1989).Greater increase in polyphenoloxidase activity in resistant than in susceptible plant was reported for soybean infected with *Phytophthora megasperma* f.sp. *glycinea* (Lazarovits and Ward, 1982). Hait and Sinha (1987) observed that seed treatment with cystein and sodium salanite protected rice from brown spot disease was associated with greater polyphenol oxidase activity in treated plants which responded

to inoculation with *Helminthosporium oryzae* with more pronounced increases in response to inoculation. Protection of bean plants from chocolate spot disease treating with wyerone was correlated with increased level of polyphenoloxidase and peroxidase activity (Tarrad *et.al.*, 1993).The effect of two resistance inducing chemicals, viz., sodium selenite (10^{-5}M) and zinc sulphate (10^{-4}M) on phenylalanine ammonialyase (PAL), peroxidase (PO) and polyphenol oxidase(PPO) activities in rice plant infected with *Rhizoctonia solani* was studied (Bhattacharyya and Roy,2000).

In the present investigation, it has been clearly established that wet seed treatment with some metal salts can induce strong protective effects in soybean plants against *Sclerotium rolfsii* infection. Such induced effects are mostly correlated with an increased biosynthesis of phenolics and stimulated oxidase activity at and around the site of host-pathogen interaction. It is well known that PAL is the first enzyme of the phenyl propanoid pathway and considered as the key enzyme in the regulation of the flux of the phenyl propanoid compounds such as lignin and their derivatives and also appeared to be associated with hypersensitive reaction. In the present study, PAL, PO and PPO activities were more in infected plants than in healthy plants, and the treated soybean plants had a higher activity in comparison to susceptible check. Though all of the treatments recorded higher post infection levels of PO activity, the three more effective treatments recorded much higher levels than the lesser effective treatments like lithium sulphate and sodium molybdate. Increased PO activity is presumed to be associated with enhanced lignification, an important mechanism for limiting the spread of the pathogen. Though a decreased trend of PAL activity was recorded as time progressed, the treated plants showed significantly higher activity even after seven days of inoculation.The plants treated with cupric chloride, the most effective treatment, elicited the maximum increase in PAL activity following inoculation and also led to the highest post-infection level. There is a strong correlation between activation of PAL and the production of both lignin and a range of phenolic compounds in resistant interactions of soybean plants with *Phytophthora* species (Mohr and Cahill, 2001).

One type of biochemical response that is strongly associated with defense is the accumulation of phytoalexins (Daniel and Purkayastha, 1995). In the present investigation, at the onset, pathogenicity test of *Sclerotium rolfsii* was carried out on six soybean varieties. Among the tested varieties, Macs-58 was found to be highly susceptible while PK-262 and Bragg were moderately susceptible. As soybean varieties showed differential responses towards *S.rolfsii*, it was considered worthwhile to detect the level of phytoalexin (glyceollin) accumulation in those varieties in response to fungal infection. It appears from the experimental results that PK-262 and Bragg contained more glyceollin (377-392 μg/g fresh wt. tissue) than Macs-58 (189 μg/g fresh wt. tissue).

Disease resistance of several crop plants have been correlated with the rate of production of phytolaexin (Hammerschmidt, 1999). The majority of studies of resistance and susceptibility in soybeans to *Phytophthora megasperma* f.sp. *glycinea* have dealt with infection of root and hypocotyl region. This system has attracted attention because it has provided a model system for study of the production and role of a phytoalexin (glyceollin) in a gene-for-gene system (Bhattacharyya and Ward,1987). In another study it has been confirmed that resistant and susceptible responses can also be differentiated in leaves. However, responses of leaves were found to be greatly influenced by age and stage of development. All leaves became resistant as they aged, and very young unfolding trifoliate leaves were susceptible even in resistant cultivars (Ward, 1989). Evidence that glyceollin, the pterocarpan phytoalexin from soybean, occur in four isomeric forms (glyceollin I-IV) was established by Burden and Bailey (1982). Of these, glyceollin IV has been isolated in minor amounts only, from cotyledons treated with $CuCl_2$ and no evidence that it play a significant role in the resistance response has been provided. Glyceollin I-III are all inhibitory to mycelial growth and zoospore germination of *P. megasperma* f.sp.*glycinea* and have been demonstrated to accumulate in significant amount in soybean tissues (Bhattacharyya and Ward, 1985). The proportions of the three isomers reported by different authors have varied considerably. Glyceollin-I predominated in roots treated with $CuSO_4$ or hypocotyl treated with $AgNO_3$. In cotyledons, glyceollin I and III have been reported to occur in roughly equal amounts

following treatment with $CuCl_2$. In leaves infiltrated with sodium iodoacetate, glyceollin-III was reported to be the main constituent. Although these reports suggest that there are major differences in the ability of soybean organs to synthesize three isomers, the possibility remains that the differences result from a combination of different experimental conditions and the use of different eliciting agents. None of these studies has examined the relationship of the accumulation of three isomers to resistance and susceptibility in the host-pathogen interaction.

The aforesaid statements indicate that phytoalexin has a role in disease resistance in plants. The differential response of soybean varieties towards *Sclerotium rolfsii* could probably be attributed to their capacity to accumulate more glyceollin. Induced changes in disease reactions by the application of diverse group of non-conventinal chemicals capable of inducing phytoalexin production have been elucidated by several research workers (Sinha, 1984; Chakraborty and Purkayastha, 1987; Rouxel *et.al.*, 1989; Purkayastha, 1994). It was reported by Keen *et.al.*,(1981) that sodium iodoacetate acts as an abiotic elicitor of glyceollin in primary leaves of cv. Harosoy soybeans and that it is associated with the resistance expression. Copper sulphate, sodium nitrate and chloram-phenicol were found to be effective in inducing capsidiol production in *Capsicum annum* (Watson and Brooks, 1984). Accumulation of increased levels of glyceollin following treatment with sodium azide was reported by Chakraborty and Purkayastha (1987). Rouxel *et.al.*,(1989) reported the accumulation of phytoalexin in $CuCl_2$ and $AgNO_3$ treated leaves of *Brassica juncea.* It seemed highly interesting, therefore, to induce changes in disease reactions in susceptible soybean variety (Macs-58) by the application of selected non-conventional chemicals.

Reduction in disease symptoms in susceptible soybean variety (Macs58) after the treatment with $CuCl_2$ and/or $FeCl_2$ may be correlated with the higher accumulation of glyceollin in treated plants. Results revealed that treatment with $CuCl_2$ and $FeCl_2$ induced a high level of glyceollin (485µg/g fresh wt and 332µg/g fresh wt) after challenge inoculation with the pathogen (*S.rolfsii*) in comparison to the untreated inoculated plants(190µg/g fresh wt.). The results of the present study as well as all the above reports, therefore, point to the ability of certain chemicals to induce

protection, which in some cases, could be due to direct fungitoxic effects of these chemicals on the pathogen, or it could be due to the activation of certain metabolic process within the host cells leading to the production of greater amounts of antifungal compounds (phytoalexins).

The genetic information contained in nucleic acid is expressed in the cell via protein synthesis.Several proteins function as enzymes in the metabolic pathways which synthesize or break down cellular components. When plants are infected by pathogens, the protein in the penetrated plant cells are changed chemically and physically. Some enzymatic proteins are also produced in penetrated cells by pathogens themselves. Thus, qualitative and quantitative changes of proteins are related to both plants and pathogens (Uritani, 1971).

In the present investigation, it was noticed that protein content increased in the infected roots of susceptible cultivars (Macs-58, J-80). Increased protein level was also detected after infection of susceptible bean leaves by *Uromyces phaseoli.* Similar findings were reported by other workers (Tomiyama,1966; Daly,1972; Ouchi *et.al.*,1974).They suggested that protein configuration changes in the host may induced the host's accessibility to the pathogen which is related by susceptibility. The great accumulation of protein in susceptible host after infection could be attributed to the total proteins of both host and parasite. However , it is difficult to separate the relative contribution of host and parasite to the total protein content.Sometimes protein content of the host after inoculation remains unchanged but their isozymes pattern may change. Changes in protein patterns in barley leaves after inoculation *with Erysiphe graminis* f. sp. *hordei* could be detected by polyacrylamide gel electrophoresis (PAGE) study but there is no change in protein content of mildew infected barley leaves in comparison with healthy leaves (Johnson *et. al.*, 1976).

In the present study, protein patterns of healthy and *S.rolfsii* infected roots, collar region and leaves of susceptible soybean variety (Macs-58) as well as mycelia of *S.rolfsii* were evaluated by SDS-PAGE. Protein preparation from collar region of susceptible soybean variety (Macs-58) inoculated with *S. rolfsii* exhibited 2-3

additional protein bands, in relation to their healthy control. This is in conformity with the work of Uritani and Stahmann (1961) who reported that sweet potato infected by *Ceratocystis fimbriata* developed new proteins both in resistant and susceptible varieties. Five new isozyme bands such as acetyl esterase, acid phosphatase, malate dehydrogenase, succinate dehydrogenase and peroxidase were detected in the susceptible line of barley after inoculation with *Erysiphe graminis* f. sp. *hordei* (Sako and Stahmann,1972). Differential changes in soluble leaf protein of tomato after inoculation with virulent and avirulent races of *Cladosporium fulvum* were determined by Dewit and Bakker (1980).

The pesence of cross-reactive antigen (CRA) between plant host and their parasites and the concept that these antigens might be involved in determining the degree of compatibility in such interactions have been demonstrated by several workers (De Vay *et.al.*,1972; DeVay and Adler, 1976; Kalyansundaram, 1978; Chakraborty, 1988; Purkayastha, 1989; Purkayastha *et. al.*,1991). In the present study root antigens of 6 soybean varieties (Macs-58, PK-262, Bragg, NRC-7, Pusa-16, J-80) and 6 isolates of *S. rolfsii* were cross reacted seperately with antisera of *S.rolfsii.* Reciprocal cross reaction was also caried out with antisera of host (Macs-58). Antigens from two non-pathogens of soybean, viz. *Fomes lamaoensis, Sphaerostilbe repens* were also considered for serological comparisons. It is significant to note that in immunodiffusion test susceptible soybean varieties shared the common antigens with the different isolates of *S. rolfsii* tested. Antigens of non-pathogens failed to develop any precipitin band. Immunoelectrophoretic analysis with antigen and antisera preparation from soybean roots and *S. rolfsii* also substantiated the results of immunodiffusion tests.

Several earlier studies have also implicated the importance of common antigens in host-pathogen compatibility. Presence of CRA has been demonstrated in various host-parasite combinations such as flax and *Melamspora lini* (Doubly et.al.,1960), cotton and *Verticilium albo-atrum* (Charudattan and DeVay, 1972), cotton and *Fusarium oxysporum* f. Sp. *vasinfectum* (Charudattan and DeVay,1970; Kalyansundaram *et.al.*,1975), sweet potato and *Ceratocystis fimbriata* (DeVay

et.al.,1967), potato and *Phytophthora infestance* (Palmerley and Callow, 1978, Alba and DeVay, 1985), soybean and *Macrophomina phaseolina* (Chakraborty and Purkaystha, 1983), soybean and *Colletotrichum dematum* var. *truncata* (Purkayastha and Banerjee, 1986), soybean and *Myrothecium roridum* (Ghosh and Purkayastha, 1990), jute and *Colletotrichum corchori* (Bhattacharyya and Purkayastha, 1985). Coffee and *Hemilea vastatrix* (Alba et.al.,1983), ground nut and *Macrophomina phaseolina* (Purkayastha and Ghosal, 1987), Carrot and *Pythium violae* and *Pythium sulcatum* (Lyons and White, 1992), ground nut and *Sclerotium rolfsii* (Purkayastha and Pradhan, 1994), tea and *Bipolaris carbonum* (Chakraborty and Saha, 1994), tea and *Pestalotiopsis theae* (Chakraborty et.al., 1995), soybean and *Fusarium oxysporum* (Chakraborty et.al.,1997).

Present result also support the findings of previous workers. Purkayastha and his co-workers have examined various host-pathogen / non-pathogen combinations including cultivars of soybean, rice, jute, pegion pea, bean, groundnut to find out their serological relationship with some fungal pathogens as well as non-pathogens following agar-gel double diffusion and immunoelectrophoretic tests. More than 50% combinations exibited cross-reactive antigen (CRA) between host and pathogens. No such CRA could be detected between resistant host and their respective pathogens. However, at a very low concentration CRA was detected between resistant host (soybean) and *Colletotrichum dematium* following enzyme linked immunosorbent assay (Purkayastha and Banerjee, 1990).

Conventional serological techniques have sometimes failed to detect cross reactive antigens in some host-parasite interactions such as wheat and *Puccinia graminis* var. *tritici* (Johnson, 1962), alfalfa and *Corynebacterium insidosum* (Caroll *et.al.*,1972). The preparation and treatment of antigens are most important because most antigens are labile and easily denatured. The selection of test animals and amount of antigen for immunization purpose are also important since too much material may reduce antibody formation. Moreover, a number of factors such as age of plant tissue, culture of microbes and methods of extraction of antigen have profound influence on the yield of antigenic substance and this may account for the failure to detect common antigens as suggested by DeVay and Adler (1976). Alba

et.al.,(1983) showed that uredinispores of *Hemilea vastatrix* shared common antigenic determinent with coffee plant, in contrast to their previous conclusions made with the same host-parasite system (Alba *et.al.*, 1973). This agreement was attributed to the low concentrations of antigenic preparations used in the earlier investigation.

Enzyme linked immuno sorbent assay has proved to be one of the most sensitive serological technique in detecting CRA at very low concentration (Alba and DeVay, 1985; Mohan, 1988; Chakraborty and Saha, 1994).In the present study the presence of CRA among *S.rolfsii* and soybean cultivars was evident in indirect ELISA using antigen and antisera of host and parasite and goat antirabbit IgG conjugate. Indirect ELISA readily detected CRA in semipurified mycelial preparation at concentrations ranging from 10-25 µg protein/ml with antiserum dilution of 1/125 and 1/250. Antigenic preparation (10µg/ml) from *S.rolfsii* exhibited higher absorbance value at 405 nm with antiserum of susceptible soybean cultivar (Macs-58). Higher absorbance value was also noticed in the reciprocal cross-reactions involving antiserum of the *S.rolfsii* and antigenic preparation of cv.Macs-58 (40 µg/ml). Since the indirect ELISA test made under the same condition and with at least three replications of each combination, it appears that this observed antignic disparities as reflected in their OD values developed as a result of antigen antibody reaction have some significance in the basic compatibility of host (*Glycine max*) and pathogen (*S. rolfsii*).

CRA was also detected in crude preparations and purified preparations from mycelia of *Phytophthora infestans* (races 4 and 1,2,3,4,7) with antisera of potato cvs. King Edward and Pentland Dell in concentrations lower than 50 µg protein/ml (Alba and DeVay, 1985) using indirect ELISA. Antiserum raised against *Phytophthora fragariae* detected homologous soluble antigen at protein concentrations as low as 2 µg/ml (Mohan, 1988). Indirect ELISA could also readily detected CRA in semipurified mycelial preparation of *B. carbonum* (isolate BC-1) higher absorbance value was deteced than the reaction with antiserum of susceptible tea variety (TV-18)

with antigenic preparation from *B. carbonum* (isolate BC-1) higher absorbance value was detected than the reaction with resistant variety (TV-26) of tea (Chakraborty and Sinha, 1994). Based on these findings it can be assumed that indirect ELISA may serve as an important technique to detect cross-reactive antigens, to determine their properties and to investigate their properties and to investigate their possible role in host-parasite interactions, even in those interactions where conventional serological techniques have failed to detect (Johnson, 1962; Caroll *et.al.*, 1972).

The involvement of CRA in host-parasite compatibility has been discussed by several authors (DeVay *et.al.*, 1967; DeVay and Adler,1976; Chakraborty, 1988; Lyons and White, 1992) and is strongly supported by results of the present investigation. These results are also in conformity with those of previous workers (Palmerly and Callow,1978; Alba *et.al.*, 1983; Alba and DeVay, 1985) who suggested that not all CRA contribute towards host-parasite compatibility but rather that only certain key CRA are important.

Recent trends in detection of plant pathogenic fungi include the development of more rapid diagnostic techniques with high specificity for the target organisms. The technique can be used to detect fungi present in low amount in or on plant tissue and therefore, in many cases, the pathogen can be detected at an earlier stage of disease development than was previously possible. Results of both pathogenicity tests and cross reactivity tests between *S.rolfsii* and soybean varieties revealed the differential responses of the different varieties towards the pathogen. Following this, the ability of the antisera raised against mycelial antigens of *S.rolfsii* to detect infection in artificially inoculated soybean roots were tested using DAC-ELISA formats. The antisera could detect infections in all varieties irrespective of their susceptibility or resistance in other tests. A number of previous workers have also successfully detected pathogenic fungi within host tissue (Hansen and Wick, 1993).

The cellular location of the CRA is also important in determining the nature of host-pathogen interaction. In order to determine the cellular location of CRA, fluorescence studies were conducted with cross sections of soybean roots as well as

mycelia and sclerotia of *S.rolfsii*. Bright fluorescence were observed on the epidermal and cortical tissues of the soybean roots as well as on the young hyphal tips and the sclerotia. The major CRA shared by cotton and *F.oxysporum* f.sp.*vasinfectum* was determined by De Vay *et.al* (1981). Cellular location of major CRA shared between tea and foliar fungal pathogens have also been described [Chakraborty and Saha, 1994 ; Chakraborty *et.al.* 1995].

In the present investigation the changes in antigenic patterns were also detected in susceptible soybean variety (Macs-58) after chemical induction of resistance. One common antigenic substance was found to be missing from the uninoculated cupric chloride treated soybean roots. This change increased the antigenic disparity between treated roots and the parasite and consequently the resistance of soybean to *S.rolfsii* increased to a considerable extent. In this situation cupric chloride appeared to act as a "conditioner" of the plant cells which responded to infection by producing glyceollin at a greater rate than they would normally. Selection of parasite by host or vice versa may be controlled to some degree by fortuitous homologies of their genomes. Where there is a similarity for synthesis of cortical cell component, a compatible relationship would result, whereas lack of homology would either repress metabolic processes or trigger the formation of metabolites which are toxic to cells of both host and parasite. If CRA have a functional role, other than in recognition phenomena, it probably will be found in the infective process and be subject to the over-riding effects of substances such as phytoalexins or other inhibitory substances already present in host tissues or induced by parasitic microorganisms.

Research on chemically induced disease resistance with the commercially available activators, and a large number of studies with various biological systems and experimental agents has led to a dramatic increase in our knowledge about the various defense signaling pathways in plants. Of these pathways, the salicylic acid dependent SAR pathway seems to be the most robust to exploited for practical crop protection. With this knowledge and with the pathway mutant sets available in Arabidopsis, it will be much easier in the future to determine quickly whether novel

disease control chemicals with suspected inducing activity do in fact have primary targets in the plant. This will also help the optimal utilization of the complex interactions between the various signaling pathways for practical crop protection. The experience with the chemical plant activators available, so far, suggests that some basic inducible broad spectrum defense responses are preserved across the plant kingdom. Chemical activation of disease resistance in plants represent an additional option for growers to protect their crops from losses due to plant diseases.

A synthetic resistance activator must fit the same stringent set of criteria concerning environmental and toxicological safety and reliability under practical conditions and it must be commercially interesting for agrochemical producers, farmer and supplier. Integration into existing crop management schemes or development of new crop management programmes may be possible with this novel tool of induced plant defense.

Summary

(1) A review of literature has been presented in connection with biochemical changes following chemical treatment and serological relationship between host and parasite.

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

(3) Pathogenicity test of *Sclerotium rolfsii* on different soybean varieties (e.g. PK-262, Bragg, NRC-7, J-80, Pusa-16 and Macs-58) was done. Among the six soybean varieties, Macs-58 was found to be highly susceptible which was selected for induction of resistance.

(4) Accumulation of glyceollin from six soybean varieties were detected after 24 and 48h of inoculation with *S. rolfsii.* Higher accumulation of glyceollin was noticed after 48h of inoculation. PK-262 and Bragg contained more glyceollin (377-392 μg/g fresh wt. tissue) than Macs-58 (189 μg/g fresh wt. tissue).

(5) A series of experiments have been performed using twelve chemicals belonging to three separate groups, viz. metal salts (cupric chloride, ferric chloride, lithium sulphate, manganese sulphate, sodium molybdate, magnesium sulphate, zinc chloride and barium sulphate); growth regulators (IAA, 2,4-D, and 2,4,5-T) and biological compound (chitosan) in order to induce resistance in soybean plants (highly susceptible variety – Macs58). Among the tested chemicals cupric chloride and ferric chloride were found to be highly effective in reducing disease intensity.

(6) Fungitoxicity assay using selected metal salts, growth regulators and chitosan, on sclerotial germination of *S.rolfsii* were done.

(7) Biochemical changes associated with induction of disease resistance in soybean plants by non-conventional chemicals with special reference to phenol content, enzyme activities such as peroxidase, polyphenol oxidase, pectolytic enzymes, phenylalanine ammonia lyase as well as calcium and magnesium levels were determined.

(8) Maximum and minimum increase in phenol level (total phenol and orthodihydroxy phenol) was recorded in cupric chloride and sodium molybdate treated plants respectively.

(9) Polyphenol oxidase and peroxidase activities were higher in treated inoculated plants than untreated inoculated plants. However, pectolytic enzyme activity in treated inoculated plants was significantly less than untreated inoculated plants. Ca^{++} and Mg^{++}content were also related to pectolytic enzyme activity. Reduction of pectolytic enzyme reduced its activity in rotting tissue, was linked with greater accumulation of Ca^{++} and Mg^{++}.

(10) The plants treated with cupric chloride, the most effective treatment, elicited maximum increase in PAL activity following inoculation with *S.rolfsii.*

(11) Accumulation of glyceollin in susceptible soybean variety – Macs-58 before and after alteration of disease reaction by cupric chloride and ferric chloride were detected. Both the chemicals induced glyceollin synthesis in uninoculated soybean plants. $CuCl_2$ and $FeCl_3$ induced high level of glyceollin (485 and 332 µg/g fresh wt. tissue respectively) after challenge inoculation with the pathogen (*S.rolfsii*) in comparison to the untreated inoculated plants.

(12) Mycelial protein of *S. rolfsii* and different parts of inoculated soybean plants were estimated and analysed by SDS-PAGE. Old culture of mycelia contained more protein than the young. Leaves contain more protein than roots. Soluble proteins prepared from collar region of soybean plants (Macs-58) inoculated with *S.rolfsii* exhibited 2-3 additional bands, in comparison to their healthy control.

(13) Polyclonal antisera were raised against antigen preparations from mycelia of *S. rolfsii* and soybean roots (Macs-58).

(14) In agar gel double diffusion tests antiserum of *S. rolfsii* and Macs-58 reacted with antigens of different isolates of *S. rolfsii* and soybean varieties. Strong precipitin reaction occurs between pathogen antigen and host antisera as well as host antigen and pathogen antisera.

(15) Antigenic comparison among six soybean varieties and six isolates of the pathogen and two non pathogens using anti- *S.rolfsii* antiserum were done following conventional set up for immunoelectrophoresis. Susceptible soybean varieties shared the common antigens with the different isolates tested.

(16) Optimum conditions for ELISA reactions with anti-*S.rolfsii* were determined. An antiserum dilution 1:125 and an enzyme (alkaline phosphatase) dilution of 1: 10,000 were optimum. Antigen upto a concentration of 10μg/ml were detected in homologous reaction by ELISA.

(17) Detection of pathogen (*S. rolfsii*) in artificially inoculated soybean root tissues using DAC-ELISA formats, were developed.

(18) Cellular location of cross reactive antigens(CRA) shared by host(*Glycine max*) and parasite (*S.rolfsii*) using fluorescein isothiocyanate (FITC) was determined. Cellular location of CRA in mycelia and sclerotia of *S.rolfsii* were also studied. Major CRA was concentrated on the epidermal and cortical tissues of soybean roots and young hyphal tips of the pathogen.

(19) Alteration in antigenic patterns after chemical induction of resistance by cupric chloride in susceptible soybean plants were detected using immunodiffusion and immunoelectrophoretic tests. These observed antigenic changes owing to $CuCl_2$ treatment have some significance in the resistance of soybean to *S.rolfsii.*

(20) Implication of the results have been discussed.

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