Immunodetection of *Fusarium graminearum*, *Trichoderma harzianum* and *Trichoderma viride* in soil, their interaction and possible role in the development of root disease of soybean

Thesis submitted for the Degree of Doctor of Philosophy in Science (Botany) of the University of North Bengal

Chaitali Basu, M. Sc.

Department of Botany
University of North Bengal

2002

UNIVERSITY OF NORTH BENGAL
Department of Botany

Professor B. N. Chakraborty
Ph.D., F.P.S.I

IMMUNO-PHYTOPATHOLOGY LABORATC
P.O. N.B.U. 734430, Siliguri, West Bengal,Ir
Phone: 0353-581411 (O), 0353-581347 (R)
Fax : 0353-581546, 0353-581739
E-mail : rubisha@dte.vsnl.net.in
bnc_nbu@rediffmail.com

This is to certify that Ms Chaitali Basu has carried out her research work under my supervision. Her thesis entitled **"Immunodetection of *Fusarium graminearum*, *Trichoderma harzianum* and *Trichoderma viride* in soil, their interaction and possible role in the development of root rot disease of soybean"** is based on her original work and is being submitted for the award of Doctor of Philosophy (Science) degree in Botany in accordance with the rules and regulations of the University of North Bengal.

(B.N.Chakraborty)

ACKNOWLEDGEMENT

I must pay deepest and heartiest gratitude and indatedness to my teacher guide Prof. B. N. Chakraborty, Dept. of Botany, University of North Bengal for his constant guidance, enthusiastic discussions, continuous encouragement and spontaneous help in every step of the present investigation.

I express sincere gratitude to Dr. (Mrs.) U. Chakraborty, Head of the Dept. of Botany NBU for her kind help and co-operation and also express my deep sense of gratitude to Prof. P. K. Sarkar and Prof. A. P. Das, Dept. of Botany for their encouragement in this field of work.

I am also indebted and must pay my greatest gratitude to my father Prof. P. K. Basu, Dept. of Botany, NBU and my mother Mrs. R. Basu for their blessings and inspiration to conduct and complete research work.

I am indebted to Dr. (Mrs) Banani Sarkar who was always with me while conducting research activity.

I record my thanks to Miss Monica Sharma and Mr. Debabrata Deb, Research Scholars and Anil Sharkar, field man . Immuno-phytopathology Lab., Dept. of Botany, NBU for their untiring help and good cooperation in the laboratory and field as well as during preparation of the manuscript

I am thankful to Mr. Sudhangshu Das, Computer Link, Shibmandir, for spending much time in connection with computer work required for the preparation of the theirs and also to Mr. Babul Biswas for the preparation of photographs.

Last but not the least mention must be made of my husband Dr. S. Sarkar, Chief Chemist, Tata Chromite mines Jaj pur, Orrisa, and my son Chandramouli Sarkar for their continuous co operations assistance and needful stimulation

Chaitali Basu

(Chaitali Basu)

Department of Botany
North Bengal University
Pin – 734430

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INTRODUCTION

Soybean [*Glycine max* (L.) Merill] is a major world crop. It is an ancient crop with hundreds of food, feed and industrial uses. Today, soybeans are grown to some extent in most parts of the world and are a primary source of protein and vegetable oil. Soybean products have become more important in formulating new, low cost nutritionally balanced high protein foods and beverages for human consumption. As soybean acreage has expanded throughout the world, diseases have increased in number and severity. One or more diseases can generally be found in fields wherever soybeans are grown. Fusarium root rot occurs in most soybean growing areas of the world and is considered potentially destructive in the tropics and subtropics. Root rot of soybean caused by *Fusarium graminearum* Schwabe has been reported by Agarwal and Sarbhoy (1978). The disease usually develops on seedlings and young plants. Older plants generally are less susceptible than younger ones. When the disease is severe, seedlings are stunted and weak. Infection is generally confined to the roots and lower stem. Cotyledons of diseased seedlings are chlorotic and later become necrotic. The lower part of the tap root system may be destroyed. The pathogen is usually confined to the cortex but vascular elements are invaded in advanced stages of disease. When soil moisture is low, infected seedlings or plants may wilt and in some instances, plants in an entire field may be wilted.

Increasing movement of plant material in world-wide trade coupled with restrictions on the use of plant protection chemicals has emphasized the need for phytosanitary measures for controlling the spread of economically important pathogenic organisms. Routine testing of large numbers of samples will, however, only be possible when specific sensitive and easy handle methods of diagnosis are available. Recent trends in detection of plant pathogenic fungi include the development of more rapid diagnostic techniques with high specificity for the target organism. These techniques can be used to detect fungi present in low amounts in and on plant tissue and, therefore, in many cases the pathogen can be detected at an early stage of disease development than was previously possible (Hansen and Wick, 1993; Werres and Steffens, 1994; Chakraborty and Chakraborty, 2002).

One of the most difficult and intriguing aspects in the study of biology is an understanding of the significant events of the interaction between plants and micro-organism at the cellular and subcellular level. The success or failure of infection is determined by dynamic competition and the final outcome is determined by the sum of favourable and unfavourable conditions for both pathogen and host cells. It is generally accepted that the cells recognize one another through pairs of complimentary structures on their surfaces. There is evidence that host-parasite compatibility is related to their antigenic similarly (Devay and Adler, 1976). The presence of cross-reactive antigens (CRA) between plant hosts 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 authors (Chakraborty, 1988; Purkayastha, 1994). Besides, recent trends in detection of plant pathogens include the development of more rapid diagnostic techniques with high specificity for the target organism. These techniques can be used to detect fungi, bacteria, and viruses present in low amounts in and on plant tissue and, therefore, in many cases the pathogen can be detected at an earlier stage of disease development than was previously possible. Some of these rapid, sensitive techniques are enzyme linked immunosorbent assay (ELISA), immunofluorescence (IF) and the polymerase chain reaction (PCR). A limited number of immunoassays have been developed for the detection of organisms in soil employing polyclonal, immunological reagents. An assay of detecting the cavity spot pathogen, *Pythium violea* has been developed as a practical method for determining the pre-sowing disease risk in fields (Manocha and Su, 1992). Of the assays tested by Wakeham and White (1996), indirect immunofluorescence appeared to be the most rapid and amenable assay for the detection in soil of low levels of resting spores of *Plasmodiophora brassicae*.

Although most plant pathogenic fungi can be detected by microscopy or other conventional means, serological techniques have advantage where (a) the fungus in question is not readily identified by morphological characteristics, (b) species identification is important and difficult by conventional means, (c) detection of root pathogens prior to development of foliar symptoms is necessary, (d) large numbers of samples must be processed for a particular disease for which conventional methods are time-consuming, (e) rapid, on-site detection is necessary for making disease management decisions for high-value crops, (f) regulations governing the use of pesticides require demonstration of the presence of a particular pathogen, (g) the fungus causes disease at low, difficult-to-detect populations in plant tissue, or (h) plant material is subject to quarantine regulation.

With the advent of immunoassay detection systems and their successful adaptation to the detection of root pathogen, the present investigation has been undertaken:

(a) to screen soybean varieties resistant to *Fusarium graminearum*,

(b) to prepare mycelial antigens from *F. graminearum*, *T. harzianum* and *T. viride* and raise polyclonal antibody against these mycelial antigen preparations;

(c) purification of antigen and antisera and analysis by immunoblotting;

(d) to determine serological cross reactivity between soybean roots and *F. graminearum* using ELISA

(e) to detect *F. graminearum* in soil and in soybean root tissues at an early stage of disease development using immunoassays.

(f) *In vitro* interaction studies of *F. graminearum* with *T.harzianum* and *T. viride*

(g) to apply T. harzianum and T. viride in soil for biological control of root rot of soybean

(h) to determine the changes in the population of the root pathogen after Trichoderma infestation in soil by ELISA and immunoblotting

LITERATURE REVIEW

Immunoassays are being used routinely for the detection of plant pathogens in vegetatively propagated plant material and seeds in conjunction with quarantine seed-testing, seed-certification, and pathogen-indexing programmes. While the majority of these programmes are directed toward the detection of viruses and bacteria, immunoassays have also been developed that detect fungi in seed and vegetative tissue. Tests such as Enzyme linked immunosorbent assay (ELISA), Indirect immunofluorescence assay (IFA), Dot blot and Western Blotting have demonstrated the sensitivity and specificity required to replace such time-consuming and expensive bioassays as indicator-plant inoculation, growing on tests, and dilution plating. These assays will help growers, crop-consultants and plant health care professionals from having to rely excessively on symptomatology and/ or time-consuming diagnostic procedures, and permit early detection of pathogens. This should be viewed as management tools. One critical application of immunoassay technology is the monitoring of pathogen spread in a crop system. Immunoassays have the potential to detect and quantify pathogen propagules in soil and other complex matrices. Direct detection and quantification of pathogens in soil may be more difficult to achieve as a result of low propagule counts, soil complexity, and other factors. However, highly sensitive tests using immunoassay and immunofluorescence combined with appropriate soil preparation measures will likely overcome some of these obstacles to provide information that can be used in making crop-management decisions.

A short and comprehensive review on (a) Immunodetection of plant pathogenic fungi and (b) Potentiality of biocontrol fungi for crop management have been presented below.

(A) Immunodetection of Plant Pathogenic fungi

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 untike humoral immunity or specific target of antibodies that commonly operates in animals. The serological cross reactivity between host and pathogen has been a subject of considerable interest to a number of workers and a number of reviews pertaining to this area have been published previously (Damian, 1964, Devey *et. al.*, 1967; Devay *et. al.*, 1972; Purkayastha, 1973; Devay and Adler, 1976; Damian 1979; Clark, 1981; Chakraborty 1988; Purkayastha, 1989; Purkayastha *et. al.*, 1991, Purkayastha, 1994). Detection of plant pathogenic fungi within host tissues by serological means is a relatively recent development in the field of plant pathology. The merit of this method of detection lies in its ability to detect very small amounts of pathogen in tissues which is generally not detected by conventional techniques. Recent reviews have been published by Hansen and Wick (1993), Werres and Steffens (1994) and Chakraborty and Chakraborty (2002).

Protein extracts of resistant and susceptible cabbage seedlings inoculated with *Fusarium oxysporum* f. sp. *conglutinans* were subjected to electrophoretic and immuno-chemical analysis. By electrophoresis in starch gel, 4 components were separated but no significant differences were observed in uninoculated or inoculated resistant seedling extracts. In contrast, immunochemical analysis with rabbit antisera revealed upto 7 components in extracts of infected susceptible cabbage, compared with 4 infected susceptible cabbage, compared with 4 components in healthy susceptible and healthy and inoculated resistant plants. The additional components detected in infected susceptible cabbage were not original fungus protein. They may have been formed either by the fungus after infection or more likely by the infected plant cell. On the basis of different immunological experiments it was suggested that these components were not merely breakdown products but antigenic substances (Probably proteins) which differed from the normally present substances in the healthy plants (Heitefuss *et. al.*, 1960). The relationship between antigenic substances produced by sweet potato in response to black rot infection caused by *Ceratocystis fimbriata* and the magnitude of disease resistance was pointed out by Uritani and Stahmann (1961). Tissue extracts of healthy sliced and black rotted sweet potato roots of several Japanese varieties showed precipitation lines with antisera towards corresponding extracts from an American variety (Sunny-side). Antigenic components designated as A and C were distributed in tissue extracts of all varieties. However, B and D were produced in response to the infection. The amount produced in several Japanese varieties was correlated with the degree of resistance. In healthy root tissue B and D seemed to be present in very small amounts and increased in response to simple injury or slicing but to a much lesser extent than that after infection.

The immunological responses of *Verticillium alboatrum* an *V. nigrescens* pathogenic to cotton were compared by Wyllie and DeVay (1970). On the basis of antigenic pattern *Verticillium* species were distinctly differentiated from one another. Defoliating strain of *V. albo-atrum* (T9) was shown to differ antigenically from the non-defoliating strain (SS4). It appeared to be more closely related serologically to the mildly virulent *V. nigrescens* isolates than was the defoliating T9 isolates.

Serodiagnostic methods for the differentiation between resistant and susceptible varieties of cotton infected with *Fusariam oxysporum* and *Citrus* sp. with *Phytophthora citrophthora* have been described by Abd-El, Rehim and Hashan (1970) and Abd-E1-Rehim *et al.* (1971a). Serological and immunoelectrophoretical investigation on watermelon varieties, resistant and susceptible 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 pathogen and seed globulins. It was noted that a_2b globulin fraction was present only in 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 dipoloid solopathogenic lines of *U. maydis* were used in serological studies. *Avena sativa* var. “Victory” and *Hordium vulgare* var. “California Mariout” 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 qualitatively significant serological difference among them.

Common antigens among four varieties of cotton (*Gossypium hirsutum*) and isolates of *Fusarium* and *Verticillium* species were compared by Charudattan and DeVay (1972). One antigenic substance was common among the varieties of cotton and isolates of *F. oxysporum* f. sp. *vasinfectum,* F. *solani* f. sp. *phaseoli, V. albo-alerum* and *V. nigresens.* Cotton varieties which were resistant or susceptible to *Fusarium* wilt shared the common antigen with both pathogenic and nonpathogenic isolates of *F. oxysporum* f. sp. *vasinfectum.* However, the common antigen was not shared between cotton and non-pathogenic isolates of *F. moniliforme.* In immunodiffusion tests five to eight precipitin bands were observed in homologous reactions; of these only one or two bands were common in heterologous reactions between the fungal and the cotton preparations. The common antigenic determinant shared by cotton and 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.

Antigenic affinity among the saline soluble proteins of *Triticum aestivum* and *Avena sativa* and soil borne fungus *Ophiobalus graminis* were determined by Abbott (1973). Single precipitin band in immuodiffusion test was formed when antisera of the wheat and Oat roots were allowed to diffuse with the antigens of *O. graminis.* Common antigen was also shared by both avirulent and virulent isolates of *F. oxysporhm* f. sp. *vasinfectum* with disease resistant and susceptible line of cotton. In all cases, the fungal isolates invaded and parasitized cortical tissues of cotton roots, but only those fungal isolates that caused disease become established in the vascular system (Kalyanasundaram *et al.*, 1978).

Rabbit antisera were raised against soluble extracts of *Phytophthora infestans* (race 4) and tubers of "Arran Banner" and "Golden wonder" potato cultivars showing field susceptibility and resistance respectively to late blight. Their antisera were then 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 hosts (mung-bean, pea, radish, cucumber and maize), nor between potatoes and the alternative pathogen, (*Fusarium solani*) and two non-pathogens (Ustilago *maydis* and *Phytophthora cinnamoni*)

Charudattan and DeVay (1981) isolated, purified to homogeneity and partially characterised a major cross reactive antigenic substance (CRA) from conidial culture of *Fusarium oxysporum* f. sp. *vasinfectum* common to roots of cotton (*Gossypium hirsutum*). The tissue and cellular location of the CRA and their possible role in host parasite compatibility has been subsequently described by Devay *et al.*, (1981). Indirect staining of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross reactions of cotton roots cut near or just below the root hair zone, the CRA was concentrated mainly around xylem elements, the endodermis and epidermal cells and was present throughout the cortex tissue. Protoplast prepared from cross sections of young cotton roots also contained the CRA which was concentrated in the region of plasmalemma. Treatment of conidia and mycelia of *F. oxysporum* f. sp. *vasinfectim* with asntiserum to cotton and indirect staining with FITC indicated that the CRA was mainly present in hyphal tips and in patch like areas on conidia.

Chakraborty and Purkayastha (1983) detected cross reactive antigen shared between soybean cultivars and *Macrophomina phaseolina* causing charcoal rot disease. Rabbit antisera were raised against root antigens of soybean cultivars (soymax and UPSM-19) and *M. phaseolina* isolate (M.P1) and tested against homologous and heterologous antigens following immunodiffusion test. When antiserum of *M. phaseolina* was reacted against its own antigens and antigens of susceptible soybean cultivars (Soymax, R-184), strong precipitation reactions were observed. In case of resistant cultivars, (UPSM-19 and DS-73-16) no such reactions were observed. Reciprocal cross reactions between antiserum of the resistant cultivar and antigens of three isolates of *M. phaseolina* also failed to develop even weak precipitation bands. Four antigenic substances were found to be common between the susceptible soybean cultivars and isolates of *M. phaseolina* in immunoelectrophoretic tests, but no common antigens were detected between resistant cultivars and the fungus. Common antigens were also detected in extracts of urediniospores of *Hemileia vastatrix* and in leaf and root extracts of coffee plant. An antigenic disparity was observed between coffee plants of physiologic group D and E. Common antigens shared between coffee plants and urediniospores of *H. vastatrix* and their possible involvement in such interaction were discussed by Alba *et. al.* (1983). Serological relationship between *Colletotrichum corchori* and jute cultivars (JRC-212) was detected by Bhattacharyya and Purkayastha (1985).

Heide and Swardegard-Peterson (1985) prepared rabbit antisera against soluble antigens extracted from *Erysiphe graminis* f.sp. *hordei* and barley (*Hordeum vulgare*). Antigens extracted from four near isogenic barley lines were cross reacted with the antisera of *E. graminis* f.sp. *hordei* which shared immunologically identical antgens.

Immunodiffusion, immunoelectrophoretic and cross immunoelectrophoretic analysis of rice antigens and their serological relationship between *Acrocylindrium oryzae* was determined by Purkayastha and Ghosal (1985). One precipitation band was observed when the antigen of *A. oryzae* was cross reacted with its own antiserum or against the antisera of four susceptible rice cultivars (Jaya, Ratna, IR-8, CR-126, 42-1). No precipitin band was detected between the antiserum of the resistant cv. Mahasuri and antigen preparation from three isolates of *A. oryzae* or between the antigens of resistant cvs. Mahasuri and Rusail and the antiserum of *A. oryzae.* 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 for potatoes cv. king Edward and cv. Pentland Dell by using an indirect enzyme linked immunosorbent assay (Alba and Devay 1985). They suggested that the fungal mycelia do not easily release cross reactive antigens (CRA) into synthetic media where they grow and most *P. infestans* CRA are thermolabile and can be concentrated by precipitation in the presence of 40% saturated amonium sulphate (SAS). An antigenic disparity was noticed when 40% SAS from *P. infestans* Race-4 mycelia preparation was assayed with asntisera for cvs. king Edward and Pentland Dell. The occurrence of CRA in *P. infestans* mycelium and their involvement in such interactions were discussed.

The common antigenic relationship between soybean cultivars and *Colletotrichun dematium* var. *treeneata* was ascertained following immunodiffusion, immunoelectrophoretic and crossed immunoelectrophoretic tests (Purkayastha and Banerjee 1986). 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*).

Antigens obtained from two isolates of *Macrophomina phaseolina,* a pathogen of groundnut, four non-pathogens of groundnut (viz. *Corticium sasaki, Colletotrichum lindemuthianum, C. corchori* and *Botrytis alii*) and five cultivars of *Arachis hypogea*

Cross reactivity of antiserum raised against *Phytophthora fragaria* with other *Phytophthora* species and its evaluation as a genus detecting antiserum has also been discussed by Mohan (1989). Antiserum of *P. fragariae* isolates (Anti-PfM) reacted strongly with antigens from several *Phytophthora* species. Some cross reactions with antigens from *Pythium* species are decreased by fractionating on an affinity column of sepharose 4B bound to extracts of *Fragaria vesca* roots infected with *P. fragariae.* The affinity purified anti-PfM retained its high cross reactivity with the various *Phytopthora* species Anti-PfM could not be made specific for *P. fragariae* because it was raised against components shown to be antigenically similar in all *Phytophthora* species tested. However, immunoblotting with the affinity purified anti-PfM produced distinct patterns for *P. fragariae, P. erythroseptica* and *P. cactorum.*

Kitagawa *et al.*, (1989) has also developed competitive types of two novel enzyme linked immunosorbent assays (ELISA) for specific detection of *Fusarium oxysporum* f.sp. *cucumerinum* as well as for general detection of ten strains of common *Fusarium* species that show specific pathogenicities to different plants. Antiserum against a strain of *F. oxysporum* f.sp. *cucumerinum* (F504) was elicited in rabbits, and a highly specific, sensitive and accurate ELISA for the homologous strains was developed by using the antiserum with B-D-galactosidase-labelled antirabbit IgG as the secondary antibody and cell fragments of the strain attached to amino-Dylark balls as the solid-phase antigens. This assay was specific for strain F504 and showed little cross reactivity with nine other strains of *Fusarium* species including strain 501 of *F. oxysporum* f. sp. *cucumerinum.* Strain F501 possess pathogenicity against cucumber similar to that of strain F504, although slight differences were observed between these two strains regarding their spore formation and pigment production.

A polyclonal antibody was prepared by immunizing rabbit with mycelial extract of *Phytophthora infestans* reacted in an enzyme linked immunosorbent assay with mycelial extracts of two *Phytophthora* species but not with those of ten were compared by immunodiffusion, immunoelectrophoretic and cross immunoelectrophoretic techniques for the presence of cross reactive antigens. Common antigens were found among the susceptible cultivars of groundnut and two isolates of *M. phaseolina,* but not between nonpathogens and groundnut cultivars. No antigenic similarity was found between non-pathogen and *M. phaseolina* isolates. Cross immunoelectrophoretic tests confirmed that at test one antigen was common between cvs. J-11 and TMV-2; Kadiri-71-1 and TMV-2 and kadiri-71-1 and isolates of *M. phaseolina* (Purkayastha and Ghosal 1987).

Changes in antigenic patterns were detected after chemical induction of resistance in susceptible soybean cultivar (soymax) to *Macrophomina phaseolina.* sodium azide (100μg/ml) altered antigenic patterns in cv.soymax and reduced charcoal rot disease (Chakraborty and Purkayastha 1987). Common antigenic relationship between susceptible rice cultivar (Jaya) and *Sarocladium orzae* could be altered by the application gibberellic acid (100μg/ml.) and sodium azide (100μg/ml). These chemicals reduced sheath rot disease of rice (Ghosal and Purkayastha, 1987).

Evaluation of antisera raised against pooled mycelial suspensions from five isolates (Pf-1, Pf-2, Pf-3, Pf-10 and Pf-11) representing five physiological races of *Phytophthora fragariae* for detecting the red core disease of strawberries by enzyme linked immunosorbant assay (ELISA) was done by Mohan (1988). Root extracts prepared from alpine strawberry *Fragaria vesca* and *F. ananassa* cv. Cambridge Favourite injected with any of the five isolates studied produced strong reaction in ELISA. In *F.vesca* ELISA-positive material was detectable 6-8 days after inoculation before macroscopic symptoms appeared. The cultivar Red Gauntlet, (resistant to Pf-1, 2 and 3 but susceptible to Pf-10 and II) reflected differential response in ELISA. The absorbance produced by extracts of plants infected with virulent isolates was significantly higher than that obtained with the corresponding extracts of plants inoculated with avirulent isolates. The ELISA test proved valuable in screening certified strawberry stock (Mohan, 1988).

other micro organisms found in potato. *P. infestans* mycelium in potato leaf tissue was readily detected by ELISA using either the plate trapped antigen or F(ab)2 antibody fragment techniques (Harrison *et al.,* 1980). Amount of mycelium in leaf extracts was estimated by comparing the values obtained in ELISA with those for known concentrations of *P. infestans* mycelium.

Cross reactive antigens shared by soybean cultivars and the different strains of *Myrothecium roridum* (M-1, ITCC-1143;ITCC-1409) were analyzed by Ghosh and Purkayastha (1990). Results of immunodiffusion revealed that common antigens were present only between virulent strain of *M.roridum* (M-1) and susceptible host cultivars (DS-74-24-2 and PK-327). No cross reactive antigen was detected in case of resistant cultivars (UPSM-19 and DS-73-16). Common antigenic relationship between soybean and *Colletotrichum dematium* var. *truncata* was also studied by Purkayastha and Banerjee (1990) using immunodiffusion, immunoelectrophoresis and indirect ELISA technique. Cross reactive antigens were detected between susceptible soybean cultivars and the virulent strain of *C. dematium* var. *truncata* but no cross reactive antigen was detected between soybean cultivars and avirulent pathogen (*C. dematium*) or non pathogen (*C. corchori*). Results of immunodiffusion and immunoelectrophoresis showed absence of common antigen between resistant cultivars (UPSM-19) and the pathogen while the results of indirect ELISA indicated the presence of common antigen between the two at a very low level. They compared antigenic patterns of untreated and cloxacillin treated soybean leaves which induced resistance of soybean against anthracnose disease. Disappearance of the antigen from cloxacillin treated leaves of susceptible soybean cultivar "Soymax" was correlated with alteration of disease reaction.

Monoclonal antibody (MAb) raised against haustorial complexes (VB10) isolated from pea leaves infected with the powdery mildew fungus *Erysiphe pisi* recognised a 45KDa N-linked glycoprotein which was specially located in the haustorial plasma membrane. This glycoprotein was clearly distinct from a previously characterised 62kDa plasma membrane which was also specially located in the haustorial plasma membrane. These antibodies were used, along with MAb VB7 which binds to a major 62kDa glycoprotein in the cell wall and plasma membrane of both haustoria and surface hyphae to label haustoria within epidermal strips from infected pea leaves using indirect immunofluorescence. Results showed that all these glycoproteins recognised by MAbs expressed early in haustorial development (Mackie *et al.*, 1993). Molecular differentiation in the extrahaustorial membrane of pea powdery mildew haustoria at early and late stage development was subsequently focussed by Roberts *et al.* (1993).

Polyclonal antiserum raised against a strain of *Fusarium oxysporum* f. sp. *narcissi* was tested by enzyme-linked immunosorbent assay. Antiserum raised to cell wall fractions gave better recognition than 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. Ten days after inoculation the pathogen was readily detected. in the most susceptible cultivar at points remote from the inoculation site. A direct correlation was observed between positive results in the enzymes linked immunosorbent assay and recovery of the pathogen on selected medium (Linfield, 1993).

Purkayastha and Pradhan (1994) observed that three strains of *Sclerotium rolfsii* were serologically different and their pathogenecities also differ markedly with host cultivars. Virulent strains showed common antigenic relationship with their respective susceptible host cultivars but not resistant cultivars. Antigenic change in a susceptible cv. AK-12-24 after treatment with a systemic fungicide kitazin was also evident. This change in host may be due to inactivation of a suppressor/ inhibitor gene for resistance by the fungicidal treatment. They suggested that the resistance could be induced in susceptible plants if specific antigens are eliminated by suitable treatment.

Chakraborty and Saha (1994) detected cross reactive antigens (CRA) shared between *Camellia sinensis* and *Bipolaris carbonum.* Antigens obtained from tea varieties, isolates of *B. carbonum* and non-pathogens of tea (*Bipolaris tetramera*) were compared by immunodiffusion, immunoelectrophoresis and enzyme-linked immunosorbent assay. CRA were found among the susceptible varieties (TV-9, 17 and 18) and isolates of *B. carbonum* (BC-1, 2, 3 and 4). Such antigens were not detected between isolates of *B. carbonum* and resistant varieties (TV-16, 25 and 26), non pathogens and tea varieties, as well as non pathogens and isolates of *B.carbonum.* Indirect staining of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross sections of leaves (TV-18), the CRA was concentrated mainly around epidermal cells. Treatment of mycelia and conidia of *B. carbonum* with antisera to leaves (TV-18) and indirect staining with FITC indicated the presence of CRA in the young growing hyphal tips and conidia.

Polyclonal antisera were also raised against mycelial suspension of *P theae* (isolate-pt-2) causal agent of grey blight disease and leaf antigens of Teen-Ali-17/1/54 and CP-1 and immunological tests were performed in order to detect CRA shared by the host and parasite. CRA were found among the susceptible varieties and isolates of *P. theae* (pt-1, 2 and 3). Such antigens were not detected between isolates of *P theae* and resistant varieties, *B tetramera* and tea varieties or isolates of *P theae.* Indirect staining of antibodies using FITC also indicated the presence of CRA in the epidermal cells and mesophyll tissue of tea leaves. CRA was evident in the young hyphal tips of the mycelia and on the setulae and appendages of the conidia of *P. theae* (Chakraborty *et al.*, 1995a).

Another serological experiment was performed by Chakraborty *et al.* (1996) by raising polyclonal antisera against leaf antigens to tea varieties (TV-18, Teen Ali 17/1/54 and CP-1) and mycelial antigens of *G. cingulata* (isolate GC-1) separately in white rabbits. CRA was found among the susceptible varieties and *G. cingulata* isolates. Such antigens were not detected between *G. cingulata* and resistant varieties of tea, non pathogens and tea varieties as well as *G. cingulata* and non-pathogens. In cross section of tea leaves (TV-18), the CRA was found to be concentrated in epidermal cells, mesophyll tissue and vascular elements.

Cross reactive antigens shared by *Fusarium oxysporum* and *Glycine max* were also detected using indirect immunofluorescence test by Chakraborty *et al.* (1997). For this, polyclonal antisera were raised against the mycelial suspension of *F. oxysporum* and root antigen of the susceptible soybean cultivar (UPSM-19). The immunoglobulin (IgG) fraction of those antisera were purified by ammonium sulfate precipitation and DEAE-Sephadex column chromatography. Antigens of susceptible cultivars showed higher absorbance values than resistant cultivars when tested against the purified anti *F. oxysporum* antiserum. Indirect fluorescence tests using FITC indicated that in cross-sections of roots of susceptible cultivars (UPSM-19) CRA were concentrated around xylem elements, endodermis and epidermal cells while in resistant varieties fluorescence was concentrated around epidermal cells.

Immunodetection of teliospores of *Telletia indica,* causal agent of Karnal bunt (KB) of wheat using fluorescent staining test were done by Gupta *et al.* (2000). Polyclonal antibodies were raised against teliospores in New Zealand white rabbits. The indirect immunofluorescence (IIF) test was developed using anti-teliospores serum and binding was monitored by goat-rabbit antibody conjugated to FITC. The standarization of IIF test was carried out by optimization of dilutions of anti-teliospores antibodies, fluorescent probe and exposure time. The teliospores of *T. indica* showed bright green, patchy and ring shaped fluorescence around the teliospore. The spore exhibited uniform distribution in discrete regions of spore probably in spore episporium. Similar fluorescence pattern in the teliospores of KB isolated from infected wheat seeds of cultivars HD 23328,UP 2338, PBW 393, WH 542, as well as RR 21 (susceptible cultivars) respectively, is an indication of the presence of similar antigenic configuration of teliospores. Again, they did not exhibit varition in the expression of teliospore associated molecular pattern during previous and subsequent years of infection.

Polyclonal antiserum raised against *T. indica* also reacted strongly in agglutination reaction with intact teliospores of pantnagar isolate. The wheat grains with different grades of infection could be readily detected by Seed Immunoblot Binding Assay (SIBA). The teliospores of Karnal bunt infected wheat seeds when kept for vigour testing on nitrocellulose paper, formed a coloured imprint after the paper was assayed. The SIBA developed should not only be a better indication of teliospores load on seed but also quality of seed in terms of vigour. The developed immuno detection method apparently proves to be useful in routine monitoring of wheat lots for the presence of Karnal bunt pathogen (Kumar *et al.*, 2000). Enzyme linked immunosorbent assay using PAb raised against *Colletotrichum falcatum* was performed in order to detect pathogen well before the symptom development. When 20 different sugarcane varieties were subjected to ELISA test after pathogen inoculation, it showed a clear variation in disease resistance among them as in field-testing. (Viswanathan *et al.*, 2000).

Immunological detection of *Sphaerostilbe repens*, *Trichoderma viride* and *Trichoderma harzianum* using DAC-ELISA formats have been demonstrated by Chakraborty *et al.* (2000) in order to develop strategies for management of violet root rot of tea. Polyclonal antibody based immunoassay for detecting *Fomes lamaoensis*, causing brown root rot disease of tea has also been developed (Chakraborty *et al.*, 2001a). Eight blood samples were collected and IgG were purified using DEAE cellulose. Immunodiffusion tests were performed in order to check the effectiveness of mycelal antigen preparations of *F. lamaoensis* for raising PAbs. Optimization of PAbs were done using indirect ELISA. Increased activity of PAbs against *F.lamaoensis* could be noticed from second bleedings, which continued upto fourth bledding. Root antigens prepared from healthy and artificially inoculated (with *F.lamaoensis*) tea plants (Teen Ali –17/1/54, TV-18, TV-22, TV-26, TV-27, TV-28, TV-30, S-449, BSS-2) were analysed following DAC-ELISA format. Such format was also used to detect the pathogen in infested soil. Young mycelia of *F. lamaoensis* gave bright fluorescence in indirect immunofluorescence tests using PAbs and FITC-conjugates of goat specific for rabbit globulin. Such immunological assays developed for detection of *F. lamaoensis* in rhizosphere of tea plantation can enable disease prevention at an early stage.

Immunodiagnostic kits were developed for detection of *Ustulina zonata,* causing charcoal stump rot disease, in the soil and tea root tissues. PAbs were raised separately against mycelial and cell wall antigens prepared from 10-day-old culture of *U. zonata*. Optimization of PAbs were done using indirect ELISA. Two different ELISA formats such as direct antigen coated (DAC) and double antibody sandwich (DAS) were tested to detect the pathogen in soil and artificially inoculated tea root tissues. Indirect immunofluorescence using PAbs and FITC-conjugates of goat specific for rabbit globulin were assessed for their potential to detect mycelia and spores in soil (Chakraborty *et al.*, 2001b)

Serological cross reactivity between *Glomerella cingulata* and *Camellia sinensis* were studied by Chakraborty *et al.* (2002b). PAbs were raised against antigen preparations from mycelia and cell wall of *G.cingulata* (isolate Gc-1), causal agent of brown blight of tea, mycelia of *Fusarium oxysporum* (non pathogen of tea) and leaf antigens of TV-18 and CP-1. CRA was found among the susceptible varieties of tea and isolates of *G. cingulata* (Gc-1, 2 and 3). Such antigens were not detected between resistant varieties of tea and isolates of *G. cingulata* (Gc-1, 2 and 3); non-pathogen (*F. oxysporum*) and tea varieties; isolates of *G. cingulata* and *F. oxysporum* and between non-host (*Glycine max*, *Cicer arietinum* and *Camellia japonicum*) and *G. cingulata.* Antisera raised against cell wall preparations gave better recognition than that against mycelial preparations as observed in ELISA test with antigens of tea leaves of different ages.

(B) Potentiality of Biocontrol fungi for crop management

It was as early as 1934 when Weindling showed that *Trichoderma* had the potentiality to be effective agents for biocontrol. Later, isolation of toxic principle from the culture filtrate of *Trichoderma* (Weindling and Emerson, 1936; Weindling, 1937, 1941) attracted the attention of plant pathologists to the importance of the genus related to the biological control of the disease. Isolation and identification of an antibiotic substance (Viridin) from *T. viride* by Brian and McGowan (1945) further raised hopes of controlling plant diseases by biological means. Since then, a dramatic increase in research efforts have been observed and several review articles (Papavizas and Lumsden, 1980; Schroth and Hancock, 1981; Kommedahl and Windels, 1981; Papavizas, 1985) have been published considering the use of specific microorganisms for the biocontrol of plant diseases. On the basis of the research findings on detailed aspects of *Trichoderma* species, two species mainly *T. harzianum* and T. viride are well recognised now a days all over the world as biocontrol agents.The present review will focus mainly on the potential for biocontrol of plant diseases by *Trichioderma* with special emphasis on its commercial uses.

Biocontrol by adding large amounts of *T. harzianum* its food base to soil is exemplified by the work of Wells *et al.* (1972). These researches were among the first to report the large-scale use of *Trichoderma* preparations on solid media for field control of *Sclerotium rolfsii* on tomato transplant. Their system, however, required large amounts of organic matter (4,200 kg per hectare) for disease control. Backmen and Rodriguez-Kabana (1975) grew *T. harzianum* on a commercial, insoluble diatomaceous earth granule impregnated with molasses and applied the granules by hand over rows of pea nuts at 112 or 140 kg per hectare 70 and 100 days after planting. At 140 kg per hectare, *T. harzianum,* significantly limited damage caused by *S. rolfsii* and increased yield over a three years period. Control by this method was equivalent to that obtained with PCNB. *Trichoderma* species, especially, *T. harzianum* grown on solid substrate have been tested with varying degree of success against the following diseases; white rot of onion caused by *Sclerotium cepivorum* (Abd.-ElMoity and Shatla, 1981; Abd-ElMoity *et al.*, 1982; Papavizas *et al.*, 1982), cucumber diseases and cotton wilt caused by *Veriticillium dahliae* (Fedorinchik *et al.*, 1975). *Rhizoctonia* damping off and blight of several crops caused by *Sclerotium* *rolfsii* (Chet *et al.*,1982 ; Elad *et al.*, 1980,1981a,b,1982a) and *Rhizoctonia* fruit rot of cucumber (Lewis and Papavizas, 1980).

Grosclaude *et al.* (1973) developed an indigenous method to apply conidia of the antagonist to wounds during cutting by means of special pruning shears. The antagonist applied by this method 48 hours in advance of inoculation with the pathogen protected two years old plum trees. Not only a preventive but also a curative treatment has been reported (Dubos and Richard, 1974) for the control of silver leaf disease with *T. viride* (Corke, 1978). The strain of *T. viride* used in these experiment does not produce antibiotics or enzymes and it is not allergenic (Richard, 1979). Attempts to suppress above ground diseases with *Trichoderma* have not been limited to wound applications. Tronsmo and Dennis (1977) were able to protect strawberry fruit against *Botrytis cinerea* and *Mucor mucedo* by spraying strawberry plants at early flowering, with aqueous suspensions of conidia of *T. viride.* In subsequent studies (Tronsmo and Tstaas, 1980), apple flowers sprayed with conidia of an isolate of *T. harzianum* capable of growing at low temperature had considerably lower incidence of dry eye spot in the field.

A greenhouse method for selecting biological agents to control *Rhizoctonia* rot of beans was developed by Cardoso and Echandi (1990). A soil amendment (SF-1) formulated by Huang and Kuhlman (1991a, b), when added at 1% (w/w) to soil, controlled more than 50% of damping off slash pine seedlings caused by *P. aphanidermatum, R. solani* in fumigated or non fumigated soils in the greenhouse. In soil amended with SF-21, the predominant fungus, *T. harzianum* was stimulated and the colony formation units increased and remained high for more than 50 days. Budge and Whipps (1991) applied glass house trials of *T. harzianum* for the biological control of *Sclerotinia sclerotiorum* in lettuce. Isolates of *Trichoderma* spp. were evaluated with two delivery methods suggested by Roiger and Jeffers (1991). The bioassay developed to evaluate isolates of *Trichoderma* spp. was effective.

The efficacy of antagonist *Trichoderma viride* in controlling the pathogenic activity of *Macrophomina phaseotina,* responsible for the dry root-rot of mung was evaluated by Kheri and Chandra (1991). The antagonist applied as seed coating reduced mortality due to *M. phascolina* from 19% to 8% in mung var T-44 and from 19% to 10% in var Pusa Baisakhi in unsterilized soil under green house conditions. The biocontrol efficacy of the antagonist showed an improvement in sterilized soil. The dry weight of shoots, grains and nodules showed an increase of 31.7%, 16.6% and 100% respectively in T-44 and 27%, 32.1% and 133.3% respectively in Pusa Baisakhi. Biological control of bean root rot disease caused by *Rhizoctonia solani* with *T. harzianum* (TC-11) was done by Liv (1991). Following in vitro dual culture technique and antibiosis bioassay, *T. harzianum* (TC 11) was shown to be strongly antagonistic to *R. solani.* Knudsen *et al.* (1991a, b) controlled various soil borne plant pathogens with the help of biocontrol fungus *T. harzianum.* They applied mycelial biomass of *T. harzianum* in alginate pellets with wheat bran.

Application of *Trichoderma* to seed was suggested as an alternative approach to introducing them into soil (Harman *et al.*, 1981). This method requires smaller amounts of biological material than in-furrow or broadcast applications. Control was achieved with conidia of several ultraviolet induced biotypes of *T. harzianum* (Papavizas *et al.*, 1982) and *T. viride* (Papavizas and Lewis, 1983). Improved yields were obtained in a *Rhizoctonia*-infested seed of corn and soybean treated with *T. harzianum* (Kommedahl *et al.*, 1981).

The use of *T. harzianum* as a seed treatment to control *R. solani* was found to be effective in field conditions (Elad *et al.*, 1982). Seed treatment with *T. viride* and *T. harzianum* was also found to be equally good for controlling damping of disease of tomato caused by *Pythium indicum* (Krishnamoorty and Bhaskaran, 1990), *Macrophomina phaseolina* infection on *Vigna mungo* (Shahzad *et al.,* 1991) and to control of root rot disease caused by *Fusarium solani* and *Rhizoctonia solani* on pea root (Diab *et al.*, 1990). Success of seed treatment has been observed to depend on the isolates used (Papavizas *et al.*, 1982; Papavizas and Lewis, 1983), the age of the seed inoculant (Kommendahl *et al.*, 1981), the kind of soil and its microbiota (Hader *et al.*, 1984) the inoculum potential of the pathogens in the soil (Wu, 1982). Biological control practices for direct protection of plants from pathogens involve the development of antagonistic microorganisms at the infection court before or after infection takes place. The role of *Trichoderma* as fungal antagonists is known for a long time. The treatment of *Trichoderma* on pruning cuts of fruit trees has prevented infection by canker causing pathogens. Sprays with *Trichoderma* in the field also reduced *Botrytis* rot of strawberries and of grapes at the time of harvest and storage (Agrios, 1988).

Spraying the spore suspension of *T. viride* on to sunflower was able to reduce *Sclerotinia sclerotiorum* rot in field (Wu, 1991). Sesan and Tica (1990) controlled grape vine pathogens (*Botrytis cinerea, Armillariella mellea, Phomopsis viticola*) by the application of *T. viride* on seedlings during their hot bed forcing period. Spraying of emulsion of *T. harzianum* spore to control *Alternaria cassiae* and *A. carassa* on *Cassia obtusifolia* was applied by A,sellen *et al.* (1991). Besides spraying, Tschen (1991) controlled stem rot of Chrysanthemum caused by *Rhizoctonia solani* by the application of *Trichoderma* as a coating material.

The mechanisms proposed in connection with the biocontrol of *T. viride* and *T. harzianum* are presumptive. Suggested mechanism for biocontrol by the two species are antibiosis, lysis, composition, and mycoparasitism (Papavizas and Lumsden, 1980, Ayers and Adams, 1981, Cook and Baker, 1983). Several toxic metabolites are produced *in vitro* by the two antagonists, and there is some evidence that such metabolites are produced in bits of organic matter in soil. *T. viride* produce gliotoxin on seed coats in soil and noninoculated pea seed planted in natural soil containing the antagonist had gliotoxin in the seed coats. Besides, *T. viride* also produce various inhibitory substances (Wu, 1991). Dry root rot of mung bean caused by *Macrophomina phaseolina* was reduced by the application of biocontrol agent *Trichoderma viride* isolates multiplied in organic substrates, such as coir pith, groundnut shell and press mud as row application in a acid soil condition (Raguchander *et al.*, 1993). Among the organic substrate, groundnut shell medium supported the production of maximum number of chlamydospores, better native *Rhizobium* modulation and higher yield. Sclerotial number and root rot incidence were greatly reduced in ground shell as compared to coir pith and press.

Arisan-Atac *et al.* (1995) characterised eleven strains of *Trichoderma viride*, 2 strains of putative teleomorph *Aypocrea rufa* and 9 of several other *Trichoderma sp.* by random polymorphic DNA amplification (RAPD) finger printing and screened for their ability to antagonize growth of European strains of the chest nut blight causing fungus *Cryphonectria parasitica*, using a dual-culture assay. The best strains were found in the species *T. harzionum, T. parecramosum*, and *T. viride*. A field experiment was conducted by Sankar and Jeyarajan (1996) to manage root rot of sesamum, caused by *Macrophomina, phaseolina*, by seed treatment with antagonists. *Trichoderma harzianum* of *T. viride* significantly reduced the root rot incidence to 10.1% and 12.8% respectively, compared to 60% incidence in the control plots. By seed treatment with *T. harzianum*, plants recorded a rhizosphere population of $35x10^3$ cfu/g. Carbendazim treatment did not increase the rhizosphere and soil population of antagonists. Soil population of *Trichoderma* spp. was maximum in all plots applied with *Trichoderma*, spp. High rhizospere/ soil ratio was recorded due seed treatment with antagonists. Seed treatment with *T. harzianum* significantly increased root length, shoot length, yield and oil content over the control.

Ten isolates of *Trichoderma* spp. were screened by Padmodaya and Reddy (1996) *in vitro* for their efficacy in suppressing the growth of *Fusarium oxysporum* f. sp. *Lycopersici*. *Trichoderma viride* was found highly inhibitory to *F. oxysporum* f. sp. *lycopersici* in dual culture followed by *T. harzianum* . Studies on production of volatile compounds by *Trichoderma* spp. revealed that *T. viride*, as effective in reducing radial growth. The same isolates also proved effective in reducing radial growth of *F. oxysporum* f. sp. *lycopersici* in a study on production of non-volatile compounds by *Trichoderma* spp.

T. viride, T. harzianum I and II, *T. hamatum* and *G. virens* were used biocontrol agents to manage the ginger rhizome rot disease caused by *Pythium aphanidermatum* and compared with fungicide mancozeb (Usman *et al.,* 1996). Two years of field trials showed that the isolate *T. harzianum* I was efficient in controlling the disease both in solarised and non-solarised pots. The disease incidence was less and the yield was high in both the years. *T. hamatum* was the second best in both the years. In general, the yield was higher in solarised pots in both the years but significant increase in yield was obtained in the second year only. The weed growth was also suppressed in the solarised plot to an extent of 40%.

Baby and Chandramouli (1996) tested antagonistic potential of *Trichoderma* spp. and *Gliocladium virens* against primary root pathogens of tea viz. *Fomes noxius, Poria hypolaterita, Rosellinia arcuata* and *Armillaria* and *T. viride* that of *Rosellinia. G.virens* colonized all the pathogens fairly well. The antagonists showed moderate to high antibiosis against all pathogens excepting *Rosellinia.G. virens* showed high antibiosis to *Rosillinia.*Production of toxic metabolite(s) was more in *G. virens* than in *Trichoderma.* The effect of biocontrol agents and plant and plant product on *Macrophomina phoseolina* causing charcoal rot of cowpea and other soil microorganisms was studied by Ushamalini *et al.* (1997). Soil application of neem cake @ 150kg/ha and Farmyard manure @ 10t/ha reduced the charcoal rot incidence significantly and increased the yield. Both *Trichoderma viride* and *T. harzianum* recorded a root rot incidence of 17.0% and 17.6% respectively as against 38.3% in control. Seeds soaked in 10% extracts of *Adenocalyma alliaceum* and *Vitex nubundo* showed 98.4 and 97.8% germination compared to 92.3% in control. Neem cake registered the maximum population of fungi, bacteria, actinomycets and minimum population of *M. phaseolina* both in the shizosphere and non-rhizosphere soil at the initial (20DAS) and later (60DAS) stages of crop growth while, the rhizosphere population of *T. viride* in both stage the crop was maximum in seeds treated with *T. viride* and in the non-rhizosphere region, neem cake and *T. harzianum* recorded the maximum population respectively at 20 and 60 DAS.

T. harzianum caused a great reduction in the infection level of damping-off and root-rot diseases and resulted in increased root weight both in pot and field experiments during two successive growing seasons (Abada, 1994). Eleven strains of *Trichoderma viride*, and 9 of several other *Trichoderma* sp. were characterized by random polymorphic DNA amplification (RAPD) finger printing and screened for their ability to antagonize growth of *Cryphonectria parasitica*, using a dual-culture assay. The best strains were found in *T. harzianum* and *T. viride*. The successful application of these strains against chestnut blight *in vivo* is demonstrated. (Arison-Atac *et al.*, 1995). Zimand *et al.*, (1996) observed that germination and germ-tube elongation of *Botrytis cinerea* on bean leaves were reduced in the presence of *T. harzianum* T39. A reduction of 20 to 50% in germ-tube biomass was observed 20 h after inoculation. This reduction in germination did not result in complete prevention of disease development on the leaves. The production of pectin-degrading enzymes by *B. cinerea* was measured up to 4 days after inoculation. They suggested that *T. harzianum* T39 acts by reducing the enzyme activities of the pathogen.

Hyphal interactions between *T. harzianum* and *Sclerotinia sclerotiorum* were investigated in dual culture and in sterilized soil, by light and scanning electron microscopy. In dual culture, partial degradation of the *S. sclerotiorum* cell wall was observed. In sterile soil, conidia of *T. harzianum* germinated and the developing mycelium made contact with that of *S.sclerotiorum* forming short branches and appressorium-like bodies which aided in holding and penetrating the host cell wall. *T. harzianum* conidia reduced the pre and post-emergence effect of

S. sclerotiorum in cucumber by 69 and 80%, respectively, and in lettuce by 46 and 72%, respectively. Hyphal mycoparasitism, rather than sclerotial parasitism, is suggested to be the mechanism by which *T. harzianum* controls *S. sclerotiorum* under these conditions (Inbar *et al.*, 1996).

Over 100 isolates of *T. harzianum* were obtained from soil samples and from the phylloplane of grape and orange. A sub sample of 48 isolates were tested and found to be antagonistic of *Botrytis cinerea* .The antagonistic activity of *T. harzianum* may be effective if it is integrated with other control practices, and may result in acceptable levels of disease control with reduced levels of pesticide use. (Latorre *et al.,* 1997). Numerous fungi and bacteria, including existing biocontrol strains with known activity against soil borne pathogens as well as isolates collected from the roots and rhizosphere of tomato plants growing in the field were tested for their efficacy in controlling *Fusarium* wilt of tomato. (Larkin and Fravel, 1998). Tomato seedlings were treated with the potential biocontrol agents in the greenhouse and transplanted into pathogen-infested field soil. Isolates of *G. virens* and *T. hamatum,* significantly reduced *Fusarium* wilt compared to disease controls.

Hervas *et al.* (1996) determined whether *Trichoderma harzianum,* applied alone or in combination to other biocontrol agents to chickpea cultivars 'ICCV 4' and 'PV 61' differing in their levels of resistance to Fusarium wilt, could effectively suppress disease caused by the highly virulent race 5 of *Fusarium oxysporum* f. sp. *ciceris*. Seeds of both cultivars were sown in soil amended with the three microbial antagonists, alone or in combination, and 7 days later seedlings were transplanted into soil infested with the pathogen. All three antagonistic microorganisms effectively colonized the roots of both chickpea cultivars, whether alone or incombination, and significantly suppressed *Fusarium* wilt development. In comparison with the control, the incubation period for the disease was delayed on average about 3 days and the final disease severity index and standardized area under the disease progress curve were reduced significantly between 14 and 33% and 16 and 42%, respectively, by all three microbial antagonists. The extent of disease suppression was higher and more consistent in 'PV 61' than in 'ICCV 4' whether colonized by *B. subtilis*, nonpathogenic *F. oxysporum*, or *T. harzianum*. The combination of *B. subtilis* + *T. harzianum* was effective in suppressing *Fusarium* wilt development but it did not differ significantly from treatments with either of these antagonists alone. In contrast, the combination of *B. subtilis* + nonpathogenic *F. oxysporum* treatment was not effective by either antagonist. Two isolates of *T. harzianum* were tested by Kapat *et al.* (1998) to determine their capacity to reduce the level of hydrolytic enzymes produced by *Botrytis cinerea* both *in vitro* and *in vivo*, and to inhibit infection caused by *B. cinerea.*

Mathew and Gupta (1998) studied potential of seven promising biocontrol agents (BCAs) *Chaetomium globosum, Coniothyrium minitans, Gliocladics virens Laltisaria arvalis Trichoderma hamatum Rhizoctonia solani* Kuhn causing root rot of French bean (*Phaseolus vulgaris* L.) under *in vitro* and glasshouse conditions. *In vitro* evaluation of BCAs by dual-culture method revealed that *T. harzianum* caused maximum inhibition, followed by *T. hamatum, T.viride* and *G. virens.* In pot experiments, *G. virens* and *T. harzianum* proved superior to other antagonists in reducting pre-emergence root rot to 6.7 and 13.3% respectively, as compared to 36.7% in control. *T. harzianum* was also effective to reduce post emergence root rot. Pre-inoculation of antagonists proved to be superior method to check post emergence root rot.

Hazarika and Das (1998) tested isolates of *Trichoderma harzianum, T. viride* and *T. virens* for their potential to suppress *Rhizoctonia solani,* the French bean root rot pathogen, under *in vitro* conditions. All isolates inhibited growth of *R.solani.* Culture filtrate of *T. harzianum* and *T. viride* inhibited mycelial growth and sclerotial germination. Wheat bran substrate supported maximum growth of all isolates followed by farm yard manure and tea. Both *T. harzianum* and *T.*

viride effectively controlled the bean rot disease when they were applied as seed and soil treatment.

Prasad *et al.* (1999) tested fourteen isolates of *Trichoderma* and *Gliocladium* species *in vitro* against *Sclerotium roffsii,* the causal organism of root/collar rot of sunflower. Two isolates of *T. viride,* four isolates of *T. harzianum,* one each of *T. hamatum T. polysporum,* inhibited mycelial growth of the pathogen significantly. Among *Trichoderma* species, *T. harzianum* isolates PDBCTH 2 gave 61.4% inhibition of mycelial growth followed by PDBCTH 8 (55.2%) and PDBCTH 7(54.9%). Complete inhibition of sclerotial germination was obtained with the culture filtrates of *T. harzianums* (PDBCTH 2, 7 and 8) and *T. pseudokoningu.* The three *T. harzianum.* siolates and the *T. viride* isolate (PDBCTV-4) were superior under greenhouse conditon with PDBCTH8 showing maximum disease control (66.8%) followed by PDBCTH7 (66.0%), PDBCTV4 (65.4%), PDBCTH2 (61.6%) and were even superior to the fungicide, captan.

Experiments were conducted to determine the influence of VAM fungi, *Rhizobium* sp. and *Trichoderma harzianum* individually as well as in combinations on the material attributes of *Acacia nilotica* seedlings (Rani *et al.*, 1999). The ability of *Trichoderma harzianum* to control the rotting of pepper (*Capsicum annuum*) plant roots caused by *Phytophthora capsici* was studied (Ahmed *et al.*, 1999). Interaction between the fungi was assessed *in vitro* on three culture media (V8c, Czapek and 2% water agar) and *in vivo* in plants grown in a substrate inoculated with *P. capsici* and *T. harzianum.* Studies on mutual antagonism in vitro showed that *P. capsici* was inhibited by *T. harzianum;* however, the intensity of inhibition differed according to the medium used, being greatest on Czapek. Analysis of the fungal populations in the plant growth substrate showed that *T. harzianum* consistently reduced that of *P. capsici* over time. This reduction in pathogen population was associated with a reduction in root rot between 24 and 76% although plant growth (dry weight) was still reduced by 21.2-24.7%, compared with the uninoculated control. In the absence of *T. harzianum* with the same pathogen inoculum level, the reduction in dry weight was 59.8–68.6% suggesting that *T. harzianum* reduced the damage.

Tomato is affected by many foliar and root diseases of which the soil borne pathogen, *Pythium aphanidermatum* inflicts considerable damage. Alice and Muthuswamy (1999) conducted an experiment to find the efficacy of biodegraded farmyard manure against the soil borne pathogen. To each 20 kg heap of decomposed farmyard manure and decomposed coir pith 100g *Trichoderma viride* commercial formulation (5g/ kg) were added after adjusting the moisture content to 50% w/w. It was thoroughly mixed and the heap was passed with red earth slurry at the sides. This was incubated for 30 days and was applied at a rate of 5g/ kg of pot soil. The following treatments viz. seed treatment (*T. viride* 4g/ kg of seed) along with soil application of biodegraded decomposed farm yard manure immediately after sowing, seed treatment along with soil application of biodegraded decomposed coir pith seed a week sowing significantly recorded 22.34, 22.34, 21.67 and 23.00% preemergence damping off incidence as against the control which recorded 48.67% disease incidence. Correspondingly there was increase in shoot vigour Index (720.89, 719, 78, 737, 04 and 744.75) and rot vigour Index (303.12, 299.3, 307.84 and 307.50). The control recorded a shoot length of 6.4cm and a root length of 2.9cm with a corresponding 331.59 and 148.85 shoot and root vigour Index. To conclude there is no difference in damping off incidence. Pertaining to the time of application viz., the soil application immediately after sowing and a week before sowing. The seed treatment with *T. viride* (4g/ kg of seed) along with soil application (5g/ kg of soil) of biodegraded decomposed farm yard manure and biodegraded coir pith was ineffective against *Pythium aphanidermatum*.

To test the efficacy of *Trichoderma viride* product in different carrier for root rot control of sunflower, three commonly available carriers viz., Talc, Lignite and Koline were selected by Mohan and Jayarajan (1999). *T. viride* was multiplied in yeast molasses medium. It was mixed with carrier at the rate of 500ml/kg Kilogram of carrier and this was used as stock culture for seed treatment. Four gram of carrier per kilogram of seeds was used in each treatment. Treated seeds were then sown in earthen pots at the rate of 25 seeds in each pot containing 10 kg of black soil. Seeds treated with captan (@ 4gm/kg seeds) used as comparative check and seeds without any treatment served as control. Before sowing, pods were inoculated with 500g of ten day old *Macrophomina phaseolina* culture multiplied in sand maize medium. Each treatments were replicated four times. The result revealed that seed treatment with *T. viride and Tale mixture* @4g/ kilogram significantly reduced the root rot incidence of sunflower and also recorded maximum yield. Other parameters like. *T. viride* population, R. S. ratio, plant height and root length also optimum in *T. viride* + Talc mixture combination. This was followed by *T. viride* and lignite combination. The potential use of native isolates of *Trichoderma viride* as bicontrol agent demonstrated the antagonistic activity against *Macrophomina phaseolina* intecting rice fallow black gram (Rettinassababdy *et al.,* 1999). Six native isolates of *Trichoderma viride* (TV-1, TV-2, TV-3, TV-4, TV-5 and TV-6) obtained from the rhizosphere regions of rice fallow black gram of Karaikal district, U.T. of Pondichery were screened in *vitro* against *M. phaseolina* by dual culture technique. Among the six different native isolates tested, TV-3 was identified as an efficient antagonist which not only inhibited the growth but also reduced the sclerotial size of *M. phaseolina.* Among eight antagonistic microorganisms tested for their efficacy in suppressing *Rhizoctonia bataticola* under *in vitro* conditions, *Trichoderma viride* and *T. harzianum* overgrew the test fungus (Prashanthi *et al.*, 2000). In pot culture experiments, seed treatment and soil drenching of *T. viride* and *P. fluorescencs* reduced the mortality of seedlings to a maximum extent. However, seed treatment was more effective than soil incorporation with the above bioagents. Growth of *Aspergillus niger* was inhibited maximum by *T.*

koningi, followed by *T. harzianum* and *T. hamatum* in *in vitro* conditions. In pot culture experiments and field trials, seed+soil treatment with *Trichoderma* spp. showed reduction in collar rot disease incidence and improved the growth parameters of groundnut viz. number of seeds per plant, 100 seed weight, shelling percent, harvest index, pot yield, dry shoot and root weight (Rao and Sitaramaiah, 2000).

In another experiment, antagonistic microflora, viz. *Trichoderma viride, Trichoderma harzianum, Gliocladium* virens, *Bacillus subtilis* and *Pseudomonas fluorescens* were evaluated against *Ustulina zonata,* causing charcoal stump rot of tea. All antagonistic microflora were most effective in inhibiting the mycelial growth of *U. zonata* in dual culture. Inhibitory activity of autoclaved culture filtrates was much less as compared to filter sterilized culture filtrates. Inoculation of these antagonists by seedling root dip and soil application in disease sick pots significantly reduced mortality of plants, besides increase in plant growth and dry matter production of tea plants. Maximum reduction in plant mortality and highest plant growth and dry matter production was recorded in *T. harzianum* and *B. subtilis* treated plots (Hazarika *et al.*, 2000).

Influence of talc-based formulations of *Trichoderma viride* and *Pseudomonas fluorescens* on damping-off disease, growth of chilli seedlings and population of *Pythium aphanidermatum* was studied by Manoranjitham *et al.* (2000a, b) under pot culture conditions. Seed treatment with *T. viride* (4g/Kg^{-1}) + *P. fluorescens* (5g Kg^{-1}) showed 7.00 and 12.50% of post emergence damping-off, respectively against 27.50 and 54.75% in control. The treatment also increased the shoot length, root length and dry matter production of chilli seedlings, and reduced the population of *P. aphanidermatum* from 16.75×10^2 cf U g^{-1} at 20 days after souling compared to 17.50×10^{-2} cfu g^{-1} and 17.08×10^2 cfu g^{-1} in control.

Manoranjitham *et al.* (2000a, b) further reported that soil application of *Trichoderma viride* and *Pseudomonas fluorescencs* effectively checked the preemergence and post-emergence damping off of tomato caused by *Pythium aphanidermatun* under pot culture experiments. Talc based formulation of antagonists significantly reduced the soil population of *Pythium* and increased the shoot length, root length and dry matter production of tomato seedlings.

Fungal isolates obtained from the rhizosphere of Wheat (*Tritium estivum*) Tomato (*Lycopersicon esculentum*), Brinjal (*Solanum melanogena*) and Diancha (*Sesbania sesban*) were used to suppress foot and root rot of barley caused by *Sclerotium rolfsii* (Bari *et al.*, 2000). Among, 250 fungal isolates, nine of the *Trichoderma* spp. six of *Fusarium* sp. and one of *Pythium* sp. showed positive ability to suppress the disease in barley. One isolate (TF-24) of *Trichoderma* sp. was most effective in increasing seed germination, growth promotion, and reducing the disease incidence of barley. Fungal isolates of *Trichoderma* sp. showed very strong antibiosis in both solid and liquid media against *S. rolfsii* than others.

Nalathambi *et al.* (2000) isolated some isolates of *Trichoderma* and *P. flourescens* from sugarcane under *in vitro* conditions. They reported that treatment with *P. flourescens* significantly reduced the seedling mortality besides better germination (40-50%), increased root and shoot length and seedling vigour than *Trichoderma* (15-20%) and fungicide application under mist chamber conditions. Association of identical bacterial colonies (10^2g) were isolated from surface sterilized root samples 7 weeks after soil application. Singh and Singh (2000) also observed that a local isolate of *T. harzianum* (ITCC No. 4542) directly attacked and lysed the mycelium and sclerotia of *Sclerotium rolfsii* when they grew the two fungi in dual culture in petriplates and soil. *T. harzianum* and *T. viride* treated sclerotia gave complete inhibition of schlerotial germination after 30 days of incubation in soil. In greenhouse experiment *T. harzianum* applied in the form of wheat bran culture of *S. rolfsii* infested soil gave as high as 85.54 and 83.55% disease control in first and second growth cycle of brinjal seedling respectively.

However, *T. viride* gave 86.18% and 85.63% disease control in first and second growth cycle of brinjal seedlings, respectively. The degree of disease control achieved increased in amount of biocontrol agents applied.

Mycelial and sclerotial growth of *Sclerotinia sclerotiorum* was significantly suppressed by *T. viride in vitro.* Collar rot disease in brinjal could be effectively controlled in pot test with unsterilized soil when the antagonists were applied before, after or simultaneously with *S. sclerotiorum* (Phookan and Challiha, 2000). Antagonistic fungi isolated from the rhizosphere of ginger were evaluated for biocontrol potential *in vitro* and *in vivo* against rhizome rot pathogen by Joseph and Sivaprasad (2000). Two isolates viz. *Trichoderma viride* and *Aspergillus fumigatus* significantly reduced disease incidence and pathogen build up. *T. viride* treated pots recorded disease intensity score of 0.3 and pathogen population of 26 cfu/ 50mg soil as against 7.7 and cfu of control. Further *T. viride* exhibited positive influence on plant growth. Gangopadhyay and Joshi (2000) used *T. harzianum* and *T. viride* in controlling root rot of cotton and chickpea in sick fields. Seed treatment with *Trichoderma* SD@4g 1kg seed provided 40-75% disease control against root rot due to *Macrophomina phaseolina* in these two crops. Eapen *et al.* (2000) isolated *Trichoderma* sp. from root and soil samples of cardamom. *T. harzianum* caused maximum suppression of nematodes, especially in native, non-sterile soil under green house conditions. All the isolates promoted the growth of cardamom seedlings, whether or not the plants were infested with root knot nematodes. A mixture of *Trichoderma* isolates when applied in two sick cardamom nurseries reduced the incidence of rhizome rot disease caused by *Pythium vexans* and *Rhizotonia solani* and root Knot nematode, population significantly.

Products to control soil borne pathogens such as *Sclerotinia, Pythium, Rhizoctonia* and *Fusarium* include *Coniothyrium minitans,* species of Gliocladium, *Trichoderma, Streptomyces* and *Bacillus* and nonpathogenic

Fusarium. Products containing *Trichoderma, Ampelomyces quisqualis, Bacillus* and *Ulocladium* are being developed to control the primary foliar diseases, *Botrytis* and powdery mildew (Paulitz and Belanger, 2001).

T. harzianum and *Alcaligenes* sp. strain AMB8 applied alone or in combination significantly reduced the incidence of *Phytophthora capsici* induced nursery rot disease of black pepper (Anith and Man Mohandas, 2001). The bacterial strains were able to survive on the stem cutting of black pepper, which is used as planting material. Combined inoculation had no effect on the population dynamics of the fungal or bacterial antagonist. The biocontrol agents also improved the root and shoot growth of the plants in the nursery. Eight isolates of *T. harzianum* were isolated from soils of different betelvine plantations of West Bengal on modified *Trichoderma* specific medium (TSM) and were tested by D'souza *et al.* (2001) for their cultural, morphometric characters and antagonistic potential against four major fungal pathogens of betelvine. Seed and seedlings rot complex of soybean caused predominantly by *Rhizoctonia solani, Sclerotium rolfsii, Macrophomina phaseolina* and *Fusarium* sp. in a major obstacle in increasing soybean production in many countries. It is very difficult to manager these pathogens as their nature of survival is both through the formation of sclerotia, Chlamydospores and saprophytic phase on soil organic matter. Biological control has emerged as an alternatives and promising means for management of such type of diseases. Biocontrol agents like *G. virens* and *T. harzianum* antagonise pathogens by antibiosis, competition, mycoparasitism or other of direct exploitation (Pant and Mukhopadhyay, 2001).

Amongst fungal antagonists tested by Sharma and Sharma (2001), *T. harzianum* and *T. viride* were found most effective in inhibiting mycelial growth of *Dematophora necatrix* in dual culture. Pre-inoculation application of *T. harzianum* and *T. viride* reduced seedling mortality in pot experiment. Disease incidence was significantly reduced when *T. harzianum* and *T. viride* were added naturally infested soil 15 days prior to seed sowing. Arya and Kaushik (2001) studied the efficacy of six species of *Trichoderma*, against the forest tree nursery damping of fungi, namely *Fusarium oxysporum, Pythium aphanidermatum* and *Rhizoctonia solani* both *in vitro* and *in vivo.* Evaluation of the fungal antagonists by dual culture method revealed that *T. viride* caused maximum inhibition of all the three pathogens followed by *T. harzianum, T. hamatum* and *T. longibrachiatum.*

MATERIALS AND METHODS

[A] Plant Materials

[i] Source of seeds :

The seeds of 8 varieties (viz. Macs-58, NRC-12, PK-416, NRC-7, PK-564, JS-335, Soymax and UPSM-19) of soybean [*Glycine max* (L) Merrill] were obtained from the Pulses and Oil seeds Research Station, Berhampore, West Bengal and stored at 20°C and also at room temperature (30±2°C). The seeds were disinfected with 'Agrosan-GN' in order to avoid microbial decomposition during storage. It is necessary to mention that about 60-80% of the seeds of those varieties lost their viability after one year. Hence, seeds were procured from the seeds Research Station every year.

[ii] Growth of plants :

Healthy soybean seeds were treated with 0.1% $HgCl_2$ for 2 minutes to remove superficial contaminants, washed several times with sterile distilled water and sown in earthen pots (10 seeds/pot of 25cm diam) containing non infested sandy soil (soil : sand =1:1). The plants were grown in the Phytopathological Experimental Garden, Department of Botany of the University of North Bengal under natural conditions of daylight and temperature (26– 34°C). The pots were watered daily with ordinary tap water. The plants were grown during March to October. Usually 15-day-old plants were used for inoculation throughout the investigation except otherwise stated.

[B] Fungal Culture

[i] Source of culture :

A virulent strain of *Fusarium graminearum* Schwabe was obtained from the Division of Mycology and Plant Pathology, Indian Agricultural Research

Institute, New Delhi. This isolate (Fg1) was used for raising polyclonal antibody after completion of Koch's postulate. Another isolate of *F. graminearum* (Fg2) was obtained from the fungal culture collection of Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal, which was originally isolated from naturally infected roots of soybean grown in the field of pulses and oil seeds Research Station, Berhampore. Third isolate of *F.graminearum* (Fg3) was isolated from naturally infected soybean roots (soymax).

Trichoderma harzianum (Th-1) and *Trichoderma viride* (Tv-1) were obtained from Indian Type Culture Collection, Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi. Isolates of *T. harzianum* (Th-2) and *T. viride* (Tv-2) were collected from Department of Plant Pathology, Uttar Banga Krishi Viswavidyalaya, Cooch Behar.

The following five fungal isolates were also used in this study: *Sphaerostilbe repens, Ustulina zonata, Rosellinia arcuata, Sclerotium rolfsii, Sclerotinia sclerotiorum* These were obtained from fungal culture collection of Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal.

[ii] Completion of koch's postulates :

Soybean seeds were surface sterilized with 0.1% $HgCl_2$ solution for 5 minutes, washed with sterile distilled water and sown in pots containing sandy soil previously infested with conidia and mycelia of *F. graminearum.*

The pathogen was reisolated from infected roots after 20 days of inoculation into potato-dextrose agar (PDA) slants, examined after 15 days of incubation (at 28°C) and the identity of the organism was confirmed after comparing it with the stock culture already made available for the purpose.

[iii] Maintenance of stock cultures :

Those three fungi (*F. graminearum, T. harzianum* and *T. viride*) were subcultured on PDA slants. After 15 days, the cultures were stored under three different conditions (5°C, 20°C and 30°C) in sterile liquid paraffin. Apart from weekly transfer for experimental work, the cultures of *F. graminearum* isolates were also examined at a regular interval to test the pathogenicity.

[iv] Assessment of mycelial growth

(a) Solid media : To assess mycelial growth of *F. graminearum, T. harzianum* and *T. viride* in solid media, the fungi were first-grown in Petridishes each containing 20ml of PDA and incubated for 7 days at 30°C. Agar block (4mm. diam.) containing the mycelia was cut with a sterile cork borer from the advancing zone of mycetial mat and transferred to each petridish containing 20ml of sterilized solidmedia. Following solid media were used for assessment of growth:

1. Potato Dextrose Agar Media [PDA]

Peeled potato	—	40gm
Dextrose	—	2gm
Agar Agar	—	2gm
Distilled water	—	100 ml

2. Richard's solution

Potassium nitrate	—	10g
Potassium dihydrogen phosphate	—	5 g
Magnasium sulphate	—	2.5g
Ferric chloride	—	Pinch
Sucrose	—	30g
Distilled water	—	1000ml

(1) and (2) used for *F. graminearum, T. herzianum* and *T. viride.*

3. Soybean agar media

Soybean seed dust	—	8gm
Agar Agar	—	2gm
Distilled water	—	100ml

(3) used for *F. graminearum*

4. **Peptone Rose Bengal Agar**

Peptone	—	5gm	KH_2PO_4	—	1gm
Dextrose	—	10gm	$MgSO_4, 7H_2O$	—	0.5gm
Agar Agar	—	20gm	Rose Bengal	—	30mg
Distilled Water	—	1l	Formal dehyde	—	200ppm

[30-100mg. streptomycin was added after auto elaving]

(4) A selective medium for *T. herzianum* and *T. viride*

(b) Liquid media : To assess the mycelial growth of *F. graminearum, T. harzpanum* and *T. viride* were first grown in petridishs separately, each containing 20ml of PDA medium and incubated for 4 days at 30°C. From the advancing zone of the mycelial mat, agar block (4mm diameter) containing the mycelia, was cut with a sterilized cork borer and transferred to each Ehrlenmeyer flask (250ml) containing 50ml of sterilized Richard's medium [KNO_3, 10.0g; K_2HPO_4, 5.0g; $MgSO_4$, $7H_2O$, 2.5g; Feclz, 0.02g; Sucrose, 30.0g; Distilled water 1 l] for a desired period at 30^0C. Finally, the mycelia were strained through muslin collected in aluminium foil cup of known weight dried at 60°C for 96 hours, cooled in a desiccator and weighed.

[C] Preparation of inoculum and inoculation technique

[i] Sick pot : Sick pot method as described by Nene *et al.* (1981) was adopted with modification. Pots (size 9" diam.) were filled with sandy soil (1:1). Naturally infected as well as artificially inoculated (with *F. graminearum*) soybean plants were chopped into small pieces and these were incorporated uniformly in the surface soil of those pots and kept for one month. After the said period of incubation, 10 seeds each of the different soybean cultivars were separately sown in each pot. Control sets were maintained by sowing soybean seeds in non infested sandy soil.

On other hand, sand maize meal medium was prepared by mixing river bed sand and maize meal in the ration of 9:1 respectively. At first all sand bags were properly sterilized in autoclave (2 times). Then sterilized sand and maize meal mixed together and the ratio was 9:1. Then 25ml water was add in each plastic bag which contain 100gm mixture (sand maize meal). After that all plastic bags were properly plugged and wrapped with paper. Then again sterilized in autoclave (20 lb 15 minutes). After cooling each bag was inoculated with *F. graminearum* and incubated at 28°C for 10 days. The sand maize meal culture was thoroughly mixed with non-infested sandy soil in each pot pots were water and kept as such for 15 days. Surface sterilized soybean seeds were sown in each pot and disease intensity was assessed.

[ii] **Water culture :** Fifteen surface sterilized (with 0.1% $HgCl_2$ solution for 5 min.) soybean seeds of each cultivar were sown in each pot containing autoclaved sandy soil. The seedlings were nursed until their further transfer.

F. graminearum was grown in potato dextrose broth (100ml broth/250ml) at 30°C on a shaker (8h. each day) for 10 days. Entire contents of a flask was diluted with sterile distilled water to get the final inoculum dilution of 2.5% (approx. 6.5×10^5 spores/ml). In each sterilized glass tube (150x15mm) 20ml of inoculum was poured. Subsequently 15 days old seedlings were up rooted *from* the experimental garden. The root system was washed thoroughly in running tap water, then rinsed twice in sterilized distilled water. The seedling was transferred into each tube and plugged with cotton. After every two days sterilized distilled water was added to the tubes in order to make up the loss. In control tubes one seedling in each tubes was transferred.

[D] The assessment of disease intensity

Plates were examined after 10, 20, 30 days of inoculation. Disease intensity was assessed on the basis of percentage loss in dry weight of roots as described by Chakraborty and Shil (1989). After desired period of incubation the plants were uprooted, washed, dried at 60°C for 96h and weighed. Root rot index was calculated in the following way : on the basis of percentage loss in dry weight of root in relation to control, they were graded into to groups and a value was assigned to each group (viz. 1– 10%. loss in weight = 1, 11–15% = 2, 26–50%=3, 51–75%=4, 76–100%=5). The root rot index in each case was quotient of the total values of the replicate roots and the number of roots (i.e. number of plants).

[E] Extraction of total soluble protein

[i] Root protein : Soluble protein were extracted from healthy and *F. graminearum* infected roots of soybean cultivars as well as from the mycelia of *F. graminearum* following the method as described by Chakraborty and Saha (1994). Seeds of soybean cultivars were grown in earthen pots containing *Fusarium* infested soil as well as in sterilized soil separately. Healthy and infected plants were up rooted after two week intervals, washed with cold water and kept at –15°C for 1h. Finally, roots (taken their fresh weight) were crushed with sea-sand in mortar and pestle in cold (4°C) and stored at –15°C for 1h and homogenized with 25ml of .05M Na phosphate Buffer (pH 7.2) at 4°C. Homogenate was strained through cheese cloth and then was centrifuged (12,100g) at 4°C for 1h and known quantity of ammonium sulphate was added to it for 100% precipitation kept at 4°C for overnight and centrifuged (10,000g) for 15min at 4°C precipitate was dissolved in the same extractive buffer (pH7.2) and dialysed against 0.005 M Na phosphate Buffer solution for 24 h at 4°C. During this period 10 changes were given. The dialysate (i.e. soluble protein) was used for gel electrophoretic study.

[ii] Mycelia protein : To extract soluble mycelial protein, *F. graminearum, T. harzianum* and *T. viride* were grown separately in sterilized liquid medium (100ml distilled water, Dextrose 2gm, Peeled potato 40gm) 10 days at 30±1°C.

Mycelia were collected and taken their fresh weight. After that Mycelia were washed with 0.2% NaCl solution, rewashed with sterile distilled water and crushed in cold (4°C), stored –15°C for 2h. Rest of the procedure was as described for root protein preparation. The soluble proteins were used for gel electrophoretic study.

[F] Purification of mycelial protein by SAS fractionation

After crushing Mycelia they were stored –15°C for 2h. and then centrifugation takes place. After centrifugation ruperhatent part (crude protein) were collected. Then known amount of ammonium sulphate (70gm ammonium sulphate required for 100ml solution) was added to it for 100% precipitation. It was added slowly and thoroughly mixed with magnetic stirrer and kept at 4°C for over night. Then it was centrifuged (10,000g) to 15 minutes at 4°C. The pallate was dissolved in the some extractive buffer (pH 7.2) and dialyses against 0.005 M Na phosphate buffer solution for 24h at 4°C. During this period 10 changes were given. The buffer solution, out side the bag was tested time to time with $Brcl_2$ to understand complete elimination of ammonium sulphate within the bag. After dialysis dialysate was used for gel electrophoretic study.

[G] Protein Estimation

The soluble protein were estimated following the method of Lowry *et al.* (1951). Initially an alkaline mixture was prepared by mixing of 0.5ml of 1% $CuSO_4$, 0.5ml of 2% sodium potassium tartarate, 50ml of 2% $Na_2 CO_3$ dissolved in 0.1N NaOH.

Finally, reaction mixture was prepared by mixing 0.1ml of the protein sample, 0.9ml water and 5ml of Folin-phenol solution (Folinphenol : water =1:1) was added after 15-20 minutes reaction. Then again incubated for 15-20 minutes.

In case of blank, water was used instead of protein sample. At the end of the incubation optical density value of each sample was determined by systronics photoelectric colorimater (720nm). Quantity of protein was estimated following the standard curve made with bovine serum albumin (BSA).

[H] SDS-Polycrylamide Gel Electrophoresis of Soluble Protein

[i] Preparation of Gel : *Stock solutions :* For the preparation of gel, the following stock solutions were initially prepared as described by Laemmli (1970).

(a) 30% Acrylamide

Acrylamide	—	29gm.
Bis-acrylamide	—	1gm
Distilled water	—	100ml

[Filtered and stored at 4°C]

(b) 1.5 M Tris [Lower gel buffer]:

Tris	—	18.18 gm.
Distilled water	—	100 ml.

[pH was adjusted to 8.8]

(c) Tris 1M [Upper gel buffer]

Tris	—	6.06gm
Distilled water	—	100ml

[pH was adjusted to 6.8 with HCl]

(d) 10% APS [Ammonium peroxidisulphate]

Ammonium peroxidisulphate	—	·1gm
Distilled water	—	1gm

[Freshly prepared each time]

[ii] Slab gel preparation : For slab gel preparation two glass plates were washed with dehydrated alcohol and dried. Then 1 mm thick spacers were placed between the glass plates at the 2 edges and the 2 sides of glass plates were sealed with gel sealing tape and kept in the gel casting unit.

Preparation of Resolving and stacking Gel :[For MINI — GEL Apparatus]

		Stacking (5%)	Resolving (10%)
Distilled water	—	2.1ml	2.85ml
30% Acrylamide	—	0.5ml	2.55ml
Tris buffer (pH–6.8)	—	0.38ml (pH 6.8)	1.95ml
10% SDS [Sodium dodecyl sulphate]	—	0.03g	0.075g
10% APS [Ammonium peroxidisulphat	—	0.003g	0.003g
TEMED	—	3ml	7.5ml

Resolving gel solution was prepared (as described) and cast very slowly and carefully up to a height. The gel was over layered with water and kept some times for polymerization. Then stacking gel solution was prepared as mentioned above. After poly merization of resolving gel, water overlay was decanted off and comb was placed, stacking gel solution was poured carefully up to a high over the resolving gel and over layered with water. Finally the gel was kept for 30 minutes for polymerization.

[iii] Sample preparation : Sample was prepared by mixing the protein with sample buffer whose composition was as follows :

1M Tris buffer (pH 6.8)	—	12.5ml.
10% SDS	—	2.3gn
Glycerol	—	13gm.
β – Mereaptoethanol	—	5ml
Distilled water	—	100ml
Bromophenol blue	—	0.005g.

At first, 34 μl of each sample protein was taken in each tube and 16 μl of sample buffer was mixed in each tube. All the tubes were floated in boiling water bath for 3 minutes. After cooling 40μl of samples were applied per well in case of slab gel. Along with the samples, protein marker was also taken in a separate tube prepared and loaded.

[iv] **Electrophoresis :** For electrophoresis the electrode buffer was prepared as follows :

[0.025M Tris, 0.192 Glycine]

Tris	—	3.02gm.
Glycine	—	18.8 gm.
Distilled water	—	1000ml

[pH was adjusted to – 8.3]

10% sodium dodecyl sulphate (SDS) – 10ml.

In this case 2mA current was applied per well up to resolving gel and then 3 mA was applied for 3h until the dye reached at the bottom of the slab gel.

[v] **Fixing :** For fixing the fixer solution was prepared as follows :

Glacial acetic acid	—	10ml
Methanol	—	20ml
Distilled water	—	70ml

The entire gel was removed from the glass plates and then the stacking portion was cut off from the resolving gel. After that the gel was soaked for over night for fixing.

[vi] **Staining :** The staining solution was prepared as follows :

Coomassil blue R_{250}	—	250 mg
Methanol	—	45ml.
Glacial acetic acid	—	10ml
Distilled water	—	45ml

The mixture was filtered with Whatman No.1. Then after fixing, the gel was stained by staining solution for 4 hrs at 37°C. and finally soaked with destaining (Methanol-45ml, Distilled water-45ml, acetic acid-10ml) until the background became clear. Rf values of individual bands were determined.

[I] Source and Maintenance of Rabbits for Serological Work

Male rabbits used for immunological works were supplied by animal supplier. All of them were of Australian strain and white in colour. The initial weight of the rabbits varied from 1.8kg to 2.4kg and their age varied from 9-11 months, before experimentation. The rabbits were kept in separate cages (60cmx45cmx30cm) attached with metal trays at the bottom and placed in a well ventilated cleaned animal room.

Each rabbit was supplied with 20g carrot (*Daucus carota*); 50g soaked gram (*Cieer arietinum*) 50-70g grass (*Cynodon dactylon*), 4-5 leaves of cauliflower (*Brassica oleracea* L. var. *botrytis*) daily. Along with the food, fresh water was supplied proportionately with the age of rabbits. Under new environmental conditions the rabbits were kept under close observation at least for a week before immunization.

[J] Preparation of antigens

[i] Extraction and purification of Fungal protein : Discs (4mm) of mycelium were transferred to Ehrlenmeyer flasks (250ml) each containing 50ml of sterilized liquid medium (g/L Distilled water; sucrose, 30g; KNO_3, 10g; KH_2PO_4 5g; $MgSO_4$, $7H_2O$, 2.5g and $FeCl_3$, 0.02g)and incubated for 15 days at 30±1°C. For extraction of soluble proteins (i.e. antigens) three type of mycelial mats (*F. graminearum, T. herzianum* and *Iviride*) were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water separately. Then fresh weights were taken. Washed mycelia were homogenized with 0.05M sodium phosphate buffer (pH 7.2) in a mortar and pestle in the presence of sea sand. Homogenates were kept overnight at 4°C and then centrifuged (15000g) for 30 minutes at 4°C. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at 4°C. After this period the mixture was centrifuged (15000g) for 30 min at 4°C, the supernatant dissolved in

0.05M sodium phosphate buffer (7.2). The preparation was dialysed for 48h against 1l of 0.005M phosphate buffer (7.2) with ten changes. After dialysis the antigens were stored at –20°C until required.

[ii] **Estimation of soluble protein :** The protein contents of fungal antigen preparations were determined following the method as described by Lowery *et al.* (1951) using bovine serum albumin as the standard.

[K] Preparation of antisera

[i] **Immunization :** Before immunization, normal sera were collected from each rabbit. Antisera were raised in separate rabbits against antigen preparation of mycelia of *F. gramenearum,* mycelia of *T. herzianum* and mycelia of *T. viride.* 0·5ml of antigen was mixed with the same amount of Frreund's complete adjuvant for emulsification. After surface sterilization with absolute alcohol that mixture was injected to the rabbit. First two injection completed with complete adjuvant at 7 days intervals. From third injection antigen (0·5ml) was mixed with Freund's incomplete adjuvant (0·5ml) was mixed with Freund's in complete adjuvant (0·5ml). This dose repeated at 7 days intervals for 4 consecutive weeks. Five days after the last injection the blood samples were collected.

[ii] **Bleeding :** Bleeding of rabbit was performed by ear vein puncture. In order to bleed rabbit or to handle them during injection, the animal was taken out from the cage, placed on its back; on the wooden boards (measuring 60cmx30cmx1cm, fixed in a 60° position) with the neck in the triangular gap and the head below the boards legs were tied to the screws and thus the body was fixed.

The hairs were removed from the vein on the ear with the help of a razor and disinfected with rectified spirit. After irritation of the ear with xylene an incision was made with a sharp sterilized blade on the border vein of the ear and about 10ml of the blood samples were collected in a sterile glass graduated tube.

After collection of desired quantity of blood all precautions were taken to stop the flow of blood from the punctured area of the ear. The blood samples were kept as such for 1h at 30°C for clotting. In order to avoid loss of serum included in the clot, it was loosened from the glass surface by turning a sterile wooden stick around the glass near the glass wall. Finally, normal sera as well antisera were clarified by contrifugation (5000 r.p.m for 10 minutes at 4°C) and distributed in small sterile vial and stored at –20°C for further used.

[L] Determination of titre value

Titre of antisera against the homologous antigens and titres of antigens against homologous antisera were determined following immunodiffusion technique as described by Ouchterlony (1967) and Clausen (1969). A constant amount (5μl) of undiluted antiserum or antigen was placed in the central well, while diluted antigens or antiserum (diluted with normal saline 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128) were pipetted into the outer well. Diffusion was allowed for 48-72h. at 25°C in a humid chamber. Titre was expressed as the reciprocal of the highest dilution of antiserum or antigen which reacted with antigen or antiserum gigging precipitation lines.

[M] Immunodiffusion test

[i] **Preparation of agar slides :** The Glass slides (5cm×5cm) were degreased successively in 90% (v/v) ethanol; ethanol: diethylether (1:1v/v) and ether, then dried in hot air oven and sterilized inside the petridish each containing one slide. A conical flask containing Tris-barbiturate buffer (pH 8.0) was placed in a boiling water bath; when the buffer was hot, 0.9% agarose was mixed to it and boiled for the next 15 minutes. The flask was repeatedly shaken thoroughly in order to preparation of absolutely clear molten agarose which was mixed with 0.1% (w/v) sodium azide (a bacteriostatic agent). The molten agarose was poured in glass slides (10ml/slide) and kept 15 minutes for solidification. After that 3-7 wells were cut out with a sterilized cork borer (7-8 mm diam.) at a distance of 2 cm from the central well.

[ii] Diffusion : Agar gel double diffusion test was performed following the method of Ouchterlony (1967). The antigens and undiluted antisera (50μl/well) wells and diffusion was allowed to continue in a moist chamber for 48-72h at 25°C. Precipitation reaction was observed in the agar gel only in cases where common antigens were present.

[iii] Washing, staining and drying 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 stained with 0.5% amido black (0.5g amido black, 5g $HgCl_2$, 5ml glacial acetic acid, 96 ml distilled water) for 5 minutes at room temperature.

After staining slides were washed thrice in distaining 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°C.

[N] Indirect Enzyme linked Immunosorbent Assay (Indirect ELISA) : Direct antigen coated ELISA (DACELISA)

[i] Preparation of buffers : For ELISA following buffers were prepared.

1. Antigen coating buffer [Carbonate-bicarbonate buffer 0.05M, pH-9.6]

Stocks

A.	Sodium Carbonate	—	5·2995g.
	Distilled water	—	1000ml
B.	Sodium bicarbonate	—	4.2g
	Distilled water	—	100ml

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

2. **Phosphate Buffer Saline** [0.05MBS, pH 7.2]

Stocks

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

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

3. **0.15M Phosphate buffersaline - Tween** [0.15MPBS Tween, pH 7.2]

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

4. **Antisera dilution buffer** [0.15MPBS - Tween pH 7.2]

In 0.15 M PBS - Tween, pH 7.2, 0.5% bovine serum albumin (BSA) was added.

5. **Substrate**

1mg PNPP powder as added in 1ml 1% diethanolamine.

6. **Stop solution**

0.3N NaOH solution was used to stop the reaction.

[iii] Method of DAC ELISA for soil test : The ELISA (plate trapped antigen (PTA) ELISA or DAC ELISA) was performed following the method as described by Walsh *et al.*, 1996. Different types of soil (i.e., field soil, pot soil, spiked soil) were grounded with pestles and mostens and soils antigens were diluted with coating buffer. The diluted antigens were loaded (200μl/well) in a Nunc 96 well ELISA plate. After loading plate was incubated overnight for 16h at 6°C. Then plate was washed four times under running tap water and once with PBS-Tween and each time plate was shaken dry subsequently antiserum was diluted (1:500) in antisera dilution buffer and loaded (200ml/well) to each well and incubated at room temperature for 2h. After further washing 200µl/well of antirabbit IgG goat antiserum labelled with alkaline phosphatase (sigma chemicals USA) diluted (1:10,000) in PBS-T containing BSA (0.5% weight/vol) was added and incubated at room temperature for 3h. Then plate was again washed, dried and loaded with 200µl of p-nitrophenyl phosphate substrate in each well and incubated for 30-45 min. colour development was stopped by adding 50µl/well of 0.3N NaOH solution and absorbance was determined in an ELISA reader (Cambridge Tech. Inc. USA) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

[O] Fluorescence antibody staining and microscopy

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

[i] **Mycelia :** Fungal mycelia were grown in liquid Richard's medium as described earlier. After four 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, pH 7.2 and incubated for 30 minutes at 27°C. Then mycelia were washed thrice with PBS-Tween (pH 7.2) as mentioned above and treated with goat antirabbit IgG (whole molecule) conjugated with flurescin isothiocyanate (sigma) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 30 min at 27°C. After incubation mycelia were washed thrice in PBS (pH 7.2) and mounted in 10% glycerol. A coverglass was placed on mycelia and sealed. Then slides were observed and photographs were taken.

[ii] **Fungal spores :** Fungal spores were collected from 15 day-old culture and a suspension of this was prepared with PBS, pH 7.2. Spore suspensions were taken in microcentrifuge tubes and centrifuged at 3000g for 10min and the PBS

supernatant was discarded. Then 200µl of diluted (in PB5 pH 7·2, 1:125) was added into the microcentrifuge tube and incubated for 2h at 27°C. After incubation tubes were centrifuged at 3000g for 10min. and the supernatant was discarded. Then the spores were rewashed 3 times with PBS-Tween pH 7.2 by centrifugation as before and 200µl of goat antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and the tubes were incubated in dark at 26^0C for 1h. After the dark incubation excess FITC-antisera was removed by repeated washing with PBS-Tween pH 7.2 and the spores weremounted on glycerol jelly and observed under Leica microscope equipped with 1-3 UV-fluorescence filter. Photographs were taken as described before.

[P] Western blotting

Blot transfer was done in four steps

[i] Antigen and antisera preparation : Soluble proteins were extracted from healthy and infected root of soybean plants and also from *Trichoderma viride, T. harzianum* and Fusarium graminearum (i.e., three types of fungal mycelia). The process of immunization and preparation of antisera were described earlier.

[ii] SDS PAGE analysis : SDS PAGE analysis of total soluble protein was performed as described previously.

[iii] Transfer process : *Preparation of transfer buffer :* (Towbin), 25mM Tris. 192 mM glycine in 20% Reagent grade Methanol, pH 8.3.

[Tris - 3.03gm; Glycine – 14.4gm; 200ml Methanol — volume make up to 1 litre]

SDS Gel electrophoresis was carried out in a mini Gel unit. Following gel run it was transferred to Towbin buffer and equilibrated for 1hr. The transfer unit was attached to a power pack. The presoaked filter paper was placed on the platinum anode and air bubbles were rolled out with a glass rod over the pre wetted membrane was placed followed by the gel and finally on top again another presoaked filter paper was placed. The cathode was placed on the sandwich and pressed. The unit was run for 1hr. at 15 volts constant voltage. After the run the membrane was dried for 1hr. and preceded for immunological probing.

[iv] Immunoblotting : Blocking was done by 5% non fat dried milk and 0.02% sodium azide in 0.15M PBS pH 7.2 with 0.02% Tween-20 in a heat sealable plastic bag and kept for 1hr. with occasional shaking. Antibody was added (1:40) to the blocking solution and incubated in plastic bag at 4°C overnight. All the processes were done by occasional shaking. The nitro cellulose membrane was washed properly in 200ml of 150 mM NaCl, 50mM Tris HCl, pH 7.5 to remove azide and phosphate from filter before enzyme coupled reactions Enzyme was added (1:10,000 in alkaline phosphatase buffer and kept for 1hr. at room temperature. The membrane was washed in 150mM NaCl, 50mM Tris HCl, pH 7.5 and substrate was added (66μl NBT+ 33μl BCIP + 10ml of Alkaline phosphatase buffer). The reaction was monitored carefully and when bands were observed of the desired intensity the paper was transferred to a tray of 200μl of 0.5 EDTA, pH 8.0 in 50ml of 0.15M PBS.

[Q] Dot Blot

Dot blot was performed following the method suggested by Lange and Heide (1989) with some modification. Following buffers were used for dot blot :

(i) Carbonate-bicarbonate (0.05M, pH–9.6) Coating buffer.

(ii) Tris buffer saline (10mM, pH–7.4) with 0.9% NaCl and 0.05%. Tween-20 for washing.

(iii) Blocking buffer — 10% casein hydrolysate in 0.05M Tris, 0.5M NaCl, 0.5% Tween-20, pH-10.3.

Nitrocellulose membrane (Millipore, H5SMO5255, 7cmx10cm, Pore size-0.45μm, Millipore Corporation, Bedford) was first cut carefully into the required size and placed inside the template 2μl of coating buffer (carbonate-bicarbonate buffer) was loaded in each well of the template over the NCM and kept for 25 min to dry. Following this 2μl of test samples (antigen samples) were loaded into the template wells over the NCM and kept for 3hrs at room temperature. Template was removed and blocking of the NCM was done with 10% non-fat dry milk (casein) prepared in TBS for 30 min dry milk (casein) prepared in TBS for 30 min.

Antibody (1:125) was added directly in the blocking solution and further incubated at 4°C for overnight. The membrane was then washed several times in TBS-Tween-20 (pH 7.4). Enzymatic reactions were done by treating the NCM membrane with Alkaline-Posphatase Conjugate (1:10;000) for 2hrs at 37°C. This was followed by washing for 25 min. In TBS-Tween Substrate [1 tablet each of Tris buffer and Fast Red (sigma chemicals) dissolved in 20ml double distilled water) was next added and colour developed noted. Finally reaction was stopped by floating the NCM in deionzed water.

EXPERIMENTAL

Part – 1 : Pathogenicity test of *Fusarium graminearum* on different soybean varieties

Pathogenicity of *Fusarium graminearum* (isolate Fg. 1) has tested on eight varieties of soybean (Viz Macs–58, MRC–12, PK– 416, NRC–7, PK–564, JS–335, Soymax and UPSM–19). Methods of inoculation and disease arrestment have seen described in detail under materials and methods and results are given in **Table–1 and Fig 1.** Healthy and inoculated plants were uprooted after 10, 20 and 30 days of inoculation and percentage loss in dry weight of roots were determined and root rot index of infected roots were computed. Young plants showed initially wilting symptom which was followed by necrosis **(Plate–1).** Infection was very much prominent in the root system in its advance stage. Percentage loss in dry weight of roots as well as root rot index were low at the initial stage of infection but increased with time in some varieties. After 20 days of inoculation, maximum plants died in these varieties. On the basis of severe infection in potted condition **(Plate–1, Fig–1)** as well as in the sick plot **(Plate–2)** these varieties were considered has highly susceptible towards *F. graminearum* infection. In the field condition plants were grown in healthy and sick plots. Such sick plots were maintained with inoculums of *F. graminearum* prepared in sand maize meal medium and *F. graminearum* infected soybean roots (Soymax).

It appears from the result given in **Table–1, Fig-1** and **Plates 1 & 2** that among the eight soybean varieties tested against *F. graminearum* under glass house and field conditions, Soymax was found to be highly susceptible while UPSM – 19 was found to be resistant.

Three other verities (viz., Macs–58, PK–564 and TS–335) were also found to be susceptible **(Fig-1).** Maximum loss in weight of roots were noticed in Soymax and Macs–58 within 10 days of inoculation while only 4% loss was estimated in UPSM–19 under similar condition. In all other varieties disease index reached upto 50–75% loss in dry weight of roots after 30 days of inoculation. Disease incidence could be determined only after 10 days of inoculation following sand maize meal culture or sick plot inoculation method. Hence, water culture method was adopted for inoculation and detection of biochemical changes, as well as serological tests. In this case direct response of *F. graminearum* could be detected as early as 24–48 hr. of inoculation of 15 day-old soybean plants (Soymax) in relation to distilled water control. After 96 hr. of inoculation yellowing of leaves, witting of plants and discolouration of roots were visible in Soymax, Macs–58, JS–335 and PK–564.

Table – 1

Pathgenicity test of *Fusarium graminearum* on different Soybean varieties

Varieties	% loss in dry weight [a] (Days after inoculation)			Root rot index of infected roots [b] (Days after inoculation)		
	10	20	30	10	20	30
Macs – 58	14.5	28.2	58.6	1.4	2.5	3.6
NRC – 12	10.3	23.2	37.7	1.0	1.8	3.1
PK – 416	9.2	14.6	32.3	1.0	1.8	3.0
NRC – 7	6.3	16.9	33.5	1.5	2.2	2.8
PK – 564	11.2	26.4	45.7	1.0	1.9	3.5
JS – 335	12.8	36.4	48.2	1.2	2.1	3.9
Soymax	19.2	45.7	68.5	1.8	3.6	4.9
UPSM – 19	4.0	10.8	12.9	1.0	1.3	1.7

a = In relation to control
b = Average of 50 plants/ variety
Root rot index :
1 – 10 % loss in wt. = 1;
11 – 25% loss in wt. = 2
26 – 50% loss in wt. = 3
51 –75% loss in wt. = 4
76 – 100% loss in wt. = 5

Plate 1 (A-E): Intact Plant (*Glycine max*) inoculation, (A) Healthy, (B-E) Artificially inoculated with *F.graminearum.* (A&B) Soymax; (C) JS-335; (D) Macs-58; (E) PK-564

Plate 2 (A-C) Field grown Soybean plants (Soymax). (A&B) Healthy plants; (C) Plants grown in sick plot infested with *F.graminearum*

Pathogenicity test of *Fusarium graminearum* on different Soybean varieties

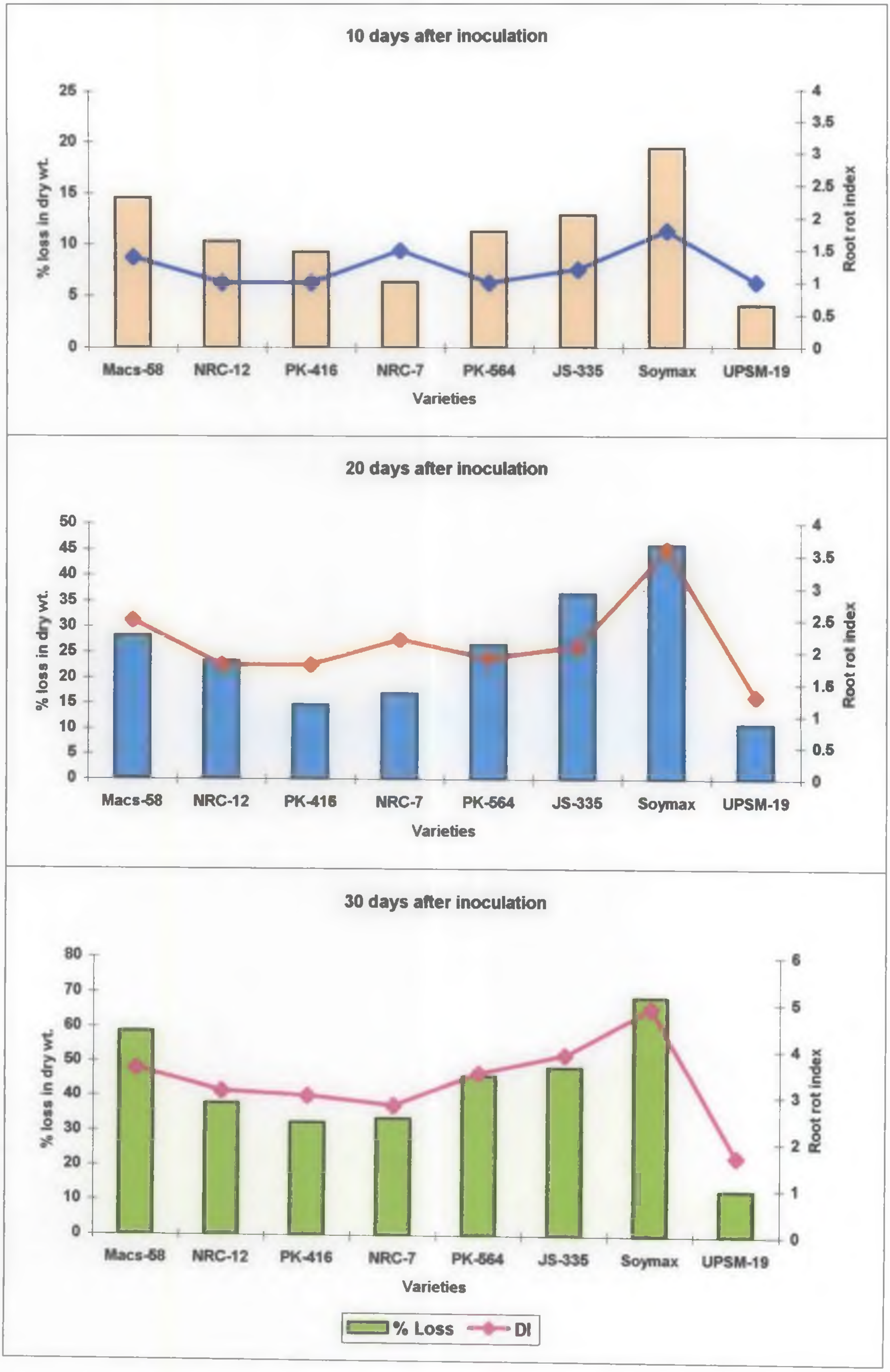

Fig: 1

Part – II : Cultural Conditions affecting growth of *F. graminearum* isolates.

Three isolates *F. graminearum* **(Fg–1, Fg–2 and Fg–3)** were used in the prevent investigation for serological test. These isolates were collected from different localities and used after completion of Koch's postulate. Mycelial growth behaviours and conidia structure of these three isolates of *F. graminearum* has been presented in **Plate–3 (Figs A-I)**. The colonies of *F. graminearum* isolates were very fast growing on potato dextrose agar medium, reaching 9 cm diam. within 5 days at 28°C, greyish rose to livid red to crimeson after becoming vinaceous with brown tinge, aerial mycelium floccose, somewhat lighter coloured and becoming brown. Sporulation often scarce, densely branched conidiophores occurring besides solitary phialides, Phialides doliforan, 10–15 × 3.8 – 4.5μm. Conidia slender falcate, moderately curved, with pointed and curved apical and pedicallate based cells, mostly 5–6 septate and 40–60 × 4.0–5.8 μm. Chlamydorpores scarce and often completely absent, mostly intercalary and in chains 10–12 μm diam. Germination of conidia on glass slides were observed after 20 hr of inoculation at 25°C.

Three isolates of *F. graminearum* **(Fg.–1, Fg.–2** and **Fg.–3)** were further grown in Richard's medium to determine the rate of mycelial growth, optimum pH and temperature. These isolates were grown in the said medium for a period upto 30 days at 28 ± 2°C.

The mycelial growths of the isolates were recorded after 5,10, 15, 20, 25 and 30 days. Maximum growth of *F. graminearum* was observed after 15 days of incubation and then rate of growth declined **(Table – 2).**

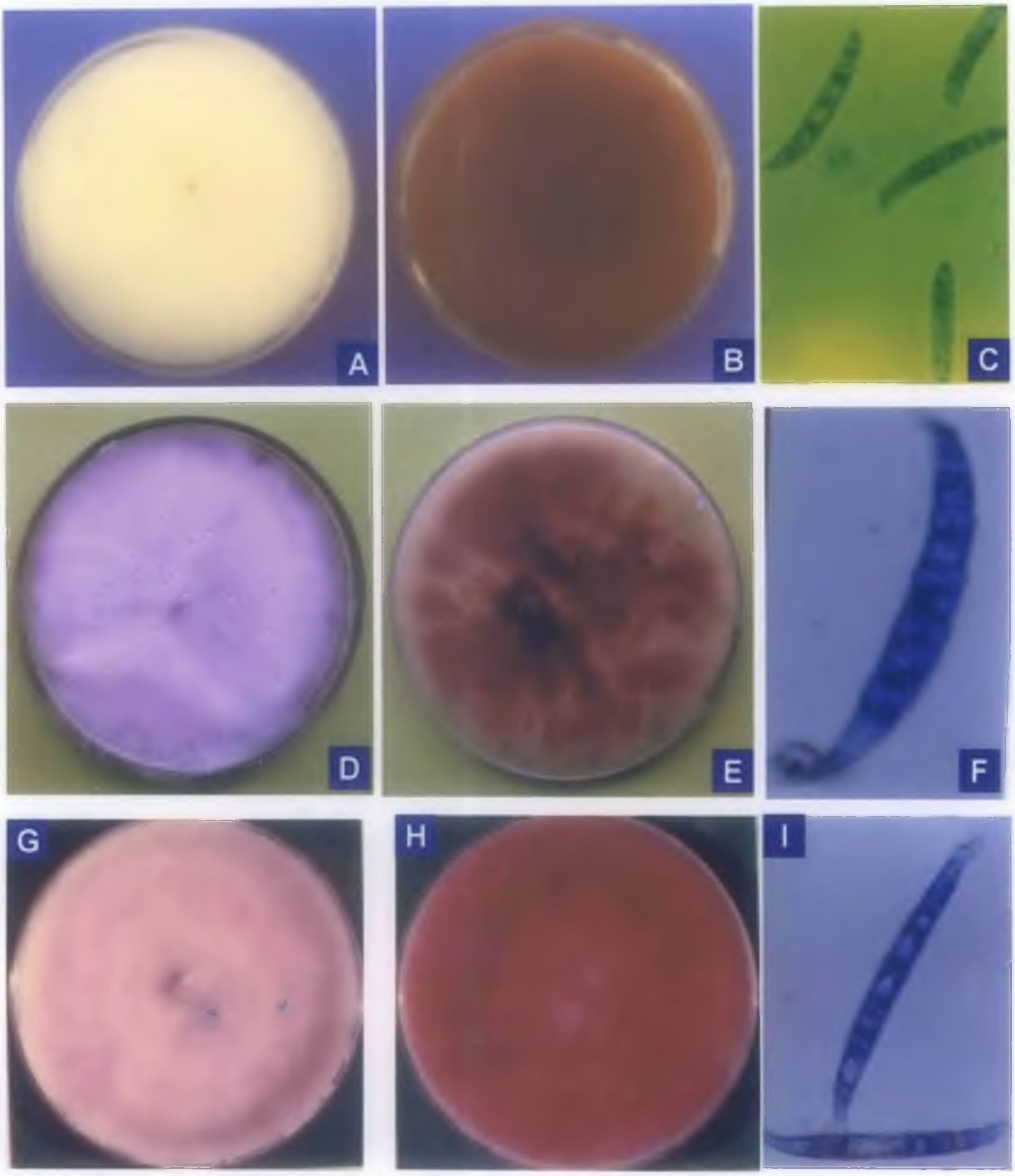

Plate 3 (A-I): Mycelial growth of *F. graminearum* isolates on Potato Dextrose Agar media. Front view (A,D&G), back view (B,E&H) and Macroconidia X 850 (C,F&I). Isolates Fg-3 (A-C), Fg-1(D-F), Fg-2 (G-I)

Table – 2

Effect of incubation period on the mycelial growth of *F. graminearum* isolates.

Incubation period	Average mycelial dry wt (mg) [a]		
	Fg. – 1	Fg.–2	Fg. – 3
5	244.8 ± 5.2	189.5 ± 2.2	226.5 ± 2.5
10	427.6 ± 4.2	376.8 ± 4.1	410.3 ± 3.3
15	672.3 ± 3.6	527.3 ± 2.9	625.8 ± 4.6
20	585.3 ± 2.8	493.5 ± 3.8	521.6 ± 2.9
25	542.8 ± 2.4	438.7 ± 4.3	493.8 ± 4.7
30	459.6 ± 5.9	406.3 ± 2.5	412.7 ± 3.3

Temperature – 28 ± 2°C
a – Average of 3 replicates.

In order to determine the optimum pH for the growth of *F. graminearum* isolates, buffer solution with pH values ranging from 4 to 8 were prepared by mixing KH_2PO_4 and K_2HPO_4 each at 0.03 M concentration. The pH of the medium was adjusted using N/10 NaOH or N/10 HCL to obtain the corresponding range of pH values. Richard's medium and phosphate buffer were sterilised by autoclaving for 15 mints at 15 lb pressure and equal part of buffer and medium were mixed before use.

Each flask (250 ml) containing 50 ml of the medium was inoculated with mycelial block (4 mm dia) of *F. graminearum* isolates and incubated for 15 days at 28 ± 2°C. The results are given in Table–3. It appears from the results that *F. graminearum* grew over a range of pH 5.5–7.0 and showed optimum growth at pH 6.5. Mycelial growth increased upto pH 6.5 and then gradually declined.

Table – 3

Effect of different Ph on the mycelial growth of *F. graminearum* isolates.

pH	Average mycelial dry wt. (mg) [a]		
	Fig. – 1	Fig. – 2	Fig. – 3
4.0	345.0 ± 3.3	310.7 ± 2.8	302.0 ± 2.3
4.5	422.8 ± 2.9	418.3 ± 3.3	437.3 ± 3.7
5.0	487.5 ± 4.1	479.5 ± 3.2	466.6 ± 2.5
5.5	523.8 ± 2.2	511.7 ± 3.7	535.3 ± 3.3
6.0	535.0 ± 2.9	530.0 ± 2.8	552.8 ± 2.8
6.5	572.9 ± 3.3	566.5 ± 4.3	596.3 ± 4.1
7.0	505.6 ± 4.6	510.2 ± 4.4	527.5 ± 2.9
8.0	268.2 ± 2.8	296.4 ± 3.3	307.3 ± 4.8

Incubation period – 15 days
Temperature - 28 ± 2°C
a = Average of 5 replicates

In the next experiment, *F. graminearum* isolates were grown in Richard's medium adjusting the optimum pH 6.5 for 15 days at different temperatures ranging from 2°C to 40°C. Results **(Table – 4)** revealed that *F. graminearum* grew over a wide range of temperature, however, maximum growth was noted at 30°C and then there was gradual decline.

Table – 4

Effect of different temperature on the mycelial growth of *F. graminearum* isolates

Temperature (°C)	Average mycelial dry wt.(mg) [a]		
	Fig. – 1	Fig. – 2	Fig. – 3
20	377.6 ± 4.3	345.0 ± 2.9	317.8 ± 4.2
25	516.5 ± 2.7	527.3 ± 4.9	542.9 ± 3.7
30	642.8 ± 4.4	592.3 ± 3.2	608.5 ± 2.8
35	407.2 ± 3.3	411.5 ± 2.8	397.8 ± 3.6
40	142.9 ± 4.5	108.8 ± 3.6	125.6 ± 2.2

a =Average of 5 replicates
pH of medium – 6.5
Incubation period – 15 days.

Part – III : *In vitro* and *in vivo* interaction between *F.graminearum* and *Trichoderma* sp.

***In vitro* test**

Trichoderma harzianum and *Trichoderma viride* isolates were selected for *in vitro* interaction study as well as *in vivo* test. Microscopic observation of *T. harzianum* revealed that it is fast growing, hyaline, colonies bearing repeatedly branched conidiophores in tuft with divergence, often irregularly flask shaped phialides. These grow best in daylight and in the dark they quickly loose the capacity to sporulate. Colonies were reaching over 9cm diameter in 5 days at 20°C. Conidia are subglobose to short oval, measuring approximately 2.8 × 3.2 µm **(Plate 4)**. The colonies of *T. viride* have been observed to reach 4.5 –7.5cm diameter in 5 days at 20°C. Conidiophores are typically pyramidally branched i.e., short branches occurring near the tip and longer ones with repeated branching in the lower part. Phialides are arranged in divergent groups of 2-4 slender and irregularly bent. Conidia are almost globose, 3.6-4.5 μm diam. and 4.8 μm long, most distinctly are roughened **(Plate 4)**.

Antagonistic properties of *T. harzianum* and *T. viride* were studied through dual palte method. Mycelial discs of 4mm diameter cut from the margin of 4 day-old cultures of both test pathogen (*F.graminearum*) and antagonists were placed opposite to each other on PDA in petriplates (9cm dia.). The distance between inoculum blocks was 6cm. Control sets were prepared both for *F. graminearum* as well as for *T. harzianum* and *T. viride.* Five replications of each interaction were studied. These were incubated at 30°C. Three isolates of *F. graminearum* were separately paired with *T. harzianum* and *T. viride*. Results have been presented in Plate 5 (Figs A–J). Within 72 hr *T. viride* overgrow the pathogen and lysed it over a period of time. While *T. harzianum* formed an inhibition zone around it though pathogen also was not able to grow further.

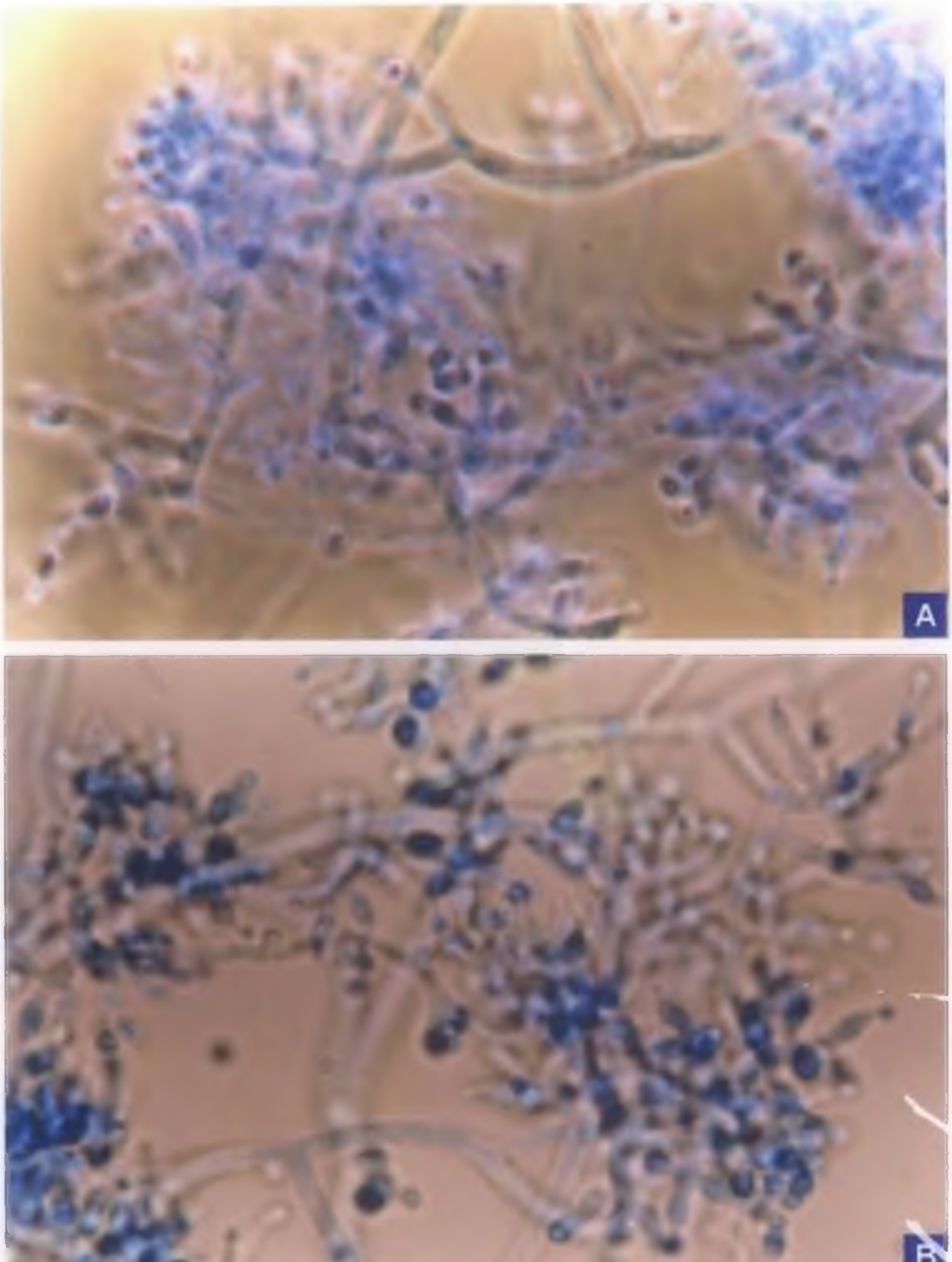

Plate 4 (A & B): Mycelia and conidia of *T.viride* and *T.harzianum*

***In vivo* test**

In order to find out the efficacy of these biocontrol fungi to manage the root rot disease of soybean, experiments were designed both in glasshouse condition as well as in field conditions. Two strains each of *T. harzianum* and *T. viride* were tested for this purpose. The experiments in both cases were set up with the following treatments; (a) *F. graminearum* (b) *T. harzianum* (c) *T. viride* (d) *F. graminearum* + *T. harzianum*, (e) *F. graminearum* + *T. viride* and (f) control plants without any treatment. Application of biocontrol fungi in soil was done at least 10 days before inoculation with pathogen. Ten replicates of each treatment were taken. Highly susceptible variety Soymax, JS-335 and PK-564 were selected for this study. Results **(Table-5)** revealed that both the biocontrol fungi were found to be very effective in reducing root rot disease. Population of *F. graminearum* was also determined in the various treatments after application of biocontrol fungi.

Table 5:

Effect of *Trichoderma harzianum* and *Trichoderma viride* on the development of root rot disease of Soybean.

Variety	**Root rot index of infected roots [a]**		
	Treatments		
	F. graminearum	*F. graminearum* + *T. harzianum*	*F. graminearum* + *T. viride*
Soymax	4.6	1.3	1.9
JS – 335	3.8	1.0	2.4
PK – 564	3.6	1.8	1.2

30 days after inoculation with F. graminearum
a = in relation to control
Root rot index – 1 – 10% loss in at – 1
11 – 25% loss in at – 2
26 – 50% loss in at – 3
51 – 75% loss in at – 4
76 – 100% loss in at – 5

Part IV : SDS-PAGE analyses of soluble proteins of *F.graminearum*, *T.harzianum* and *T.viride*

Mycelial proteins were prepared and purified from three isolates of *F. graminearum* **(Fg-1, Fg-2 and Fg-3)**, two isolates of *T. harzianum* (Th-1 and Th-2), and two isolates of *T.viride* (Tv-1 and Tv-2). These preparations were considered as antigens which were used for immunization purpose. Before that soluble proteins were analysed by SDS-Polyacrylamide gel electrophoresis. Gels were stained and molecular weight of each proteins were determined using molecular marker. Protein pattern of three isolates of *F. graminearum*, two isolates of *T. harzianum* and two isolates of *T. viride* and molecular weights have been presented in **Table – 6** and **Plate 5 (Figs. A–C)**.

Table : 6

SDS-PAGE analysis of soluble proteins of *F. graminearum, T. harzianum* and *T. viride*

Organism	Isolates	Molecular weight (kDa)
F. graminearum	Fg-2	72.4, 65.6, 42.0, 39.5, 35.2, 31.1, 29.0, 25.2, 21.0, 19.5
F. graminearum	Fg-1	67.3, 65.1, 62.6, 59.1, 39.5, 31.1, 29.0, 23.4, 22.8, 20.0, 18.5, 17.1, 15.2
F. graminearum	Fg-3	78.3, 70.2, 68.0, 65.1, 63.2, 54.4, 39.5, 37.4, 31.1, 29.0
T. harzianum	Th-1	69.1, 56.3, 50.2, 40.9, 43.0, 40.1, 39.5, 32.5, 29.0, 26.2, 24.1, 23.9, 23.5, 21.1, 20.0, 19.9, 18.7, 16.1, 15.2, 14.1, 13.2, 12.1
T. harzianum	Th-2	68.0, 62.8, 58.1, 52.3, 40.9, 43.0, 40.1, 39.5, 37.8, 29.0, 26.8, 25.3, 24.2, 23.5, 21.9, 20.0, 19.1, 18.5, 16.0, 15.2, 14.1, 13.3, 12.0
T. viride	Tv-1	59.1, 52.3, 40.2, 43.0, 41.6, 39.8, 37.8, 27.6, 23.5, 21.2, 19.5, 15.2, 14.7, 13.2
T. viride	Tv-2	43.0, 41.6, 37.8, 27.6, 21.2, 19.5, 14.3, 13.2

Part V : Immunodiffusion test

In the present investigation attempts have been made to raise polyclonal antibody against three fungi viz. *F. graminearum* (soybean root rot pathogen), two potential biocontrol fungi (*T. harzianum* and *T. viride*). In all cases three bleedings were collected and IgG were purified. Effectiveness of each antigen preparation in raising antibodies were initially checked using agar gel double diffusion techniques. The precipitin reaction as developed in agar plates were analysed by proper staining. Immunodiffusion pattern developed after homologous reaction between antigen and antibody of *F.graminearum*, *T. harzianum* and *T. viride* have been presented in **Plate 6 (Figs D, E, and G).** Strong positive reactions were observed in all the reactions. These batches of polyclonal antibodies (PAbs) were used for further immunological tests. Besides the PAb raised against *F. graminearum* was tested against soybean root antigens. Strong precipitin reactions were observed when root antigen preparation of Macs-58, NRC-12, PK-416, PK-564 and JS-335 were cross reacted with PAb of *F. graminearum* **(Table-7)**. However, no common precipitation reaction could observed in case of UPSM 19 when tested against IgG preparations of all three blood samples. Weak precipitin reactions were observed with NRC–7 when tested against IgG preparations of all three blood samples. Reciprocal cross reaction with PAb of soybean root antibody also failed to develop even weak precipitin reaction as evident in **Plate 5 (Figs F, H and I)**. PAbs raised against *F. graminaerum*, *T. harzianum* and *T. viride* were further used for the development of immunodiagnostic kits for detection of pathogen in soybean root tissue and soil as well as for detection of biocontrol fungi in soil. For such experiments initially enzyme linked immunosorbant assay using direct antigen coated (DAC)-ELISA formats were standardized.

Table 7

Detection of cross reactive antigens shared between *F. graminearum* and Soybean varieties.

Root antigen	PAb of *F. graminearum*		
	1st bleeding	2nd bleeding	3rd bleeding
Macs – 58	±	+	+
MRC – 12	±	±	+
PK – 416	±	±	+
NRC – 7	±	±	+
PK – 564	+	+	+
JS – 335	+	+	+
Soymax	+	+	+
UPSM – 19	—	—	—

\+ = Common precipitin band present
± = Weak precipitin reaction
— = Common precipitin band absent.

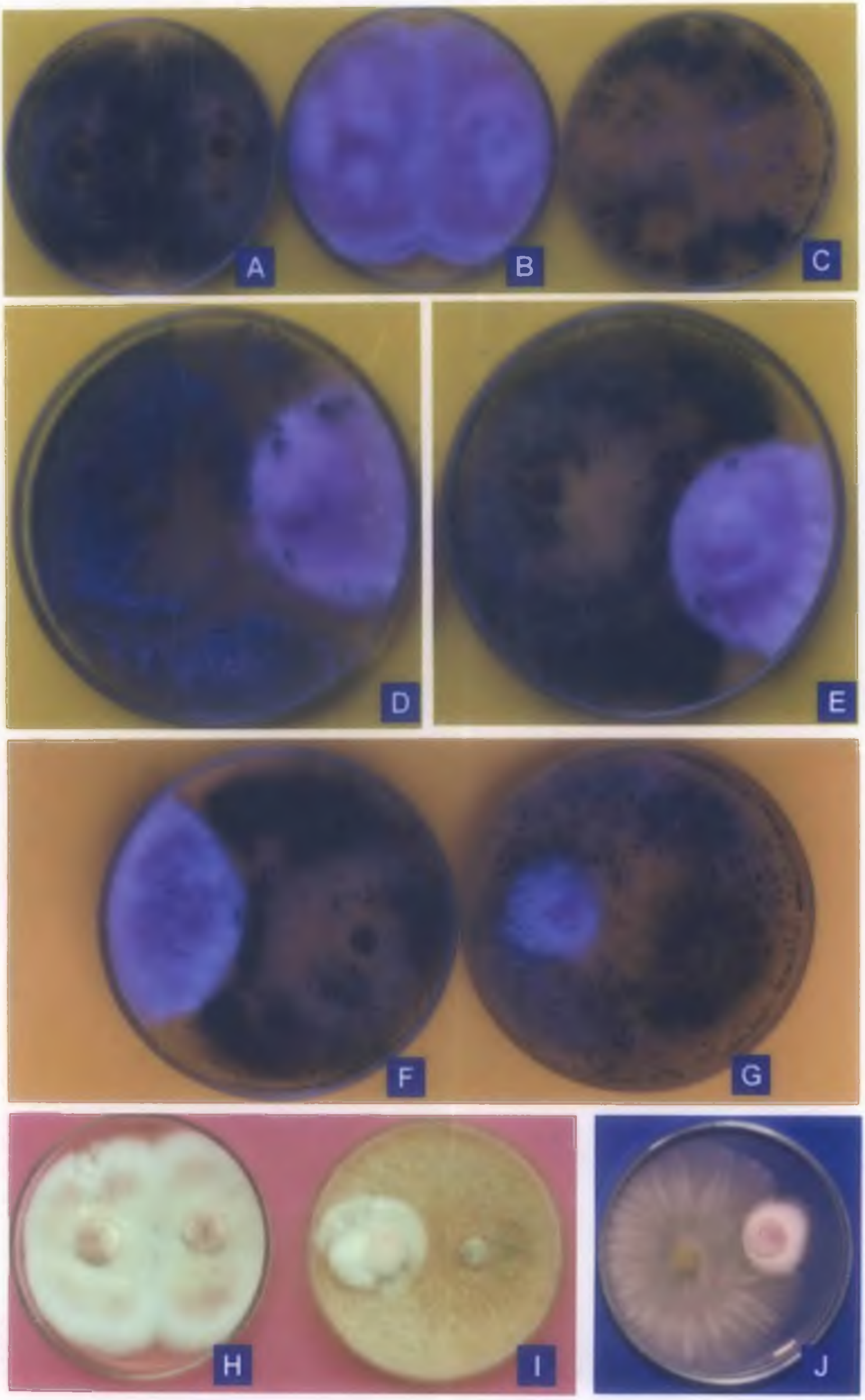

Plate 5 (A-J): Homologous pairing of *F.graminearum* (B&H), *T.harzianum* (A) and *T.viride* (C). Pairing of *F.graminearum* with *T.harzianum* (D,E & J), and with *T.viride* (F,G & I).

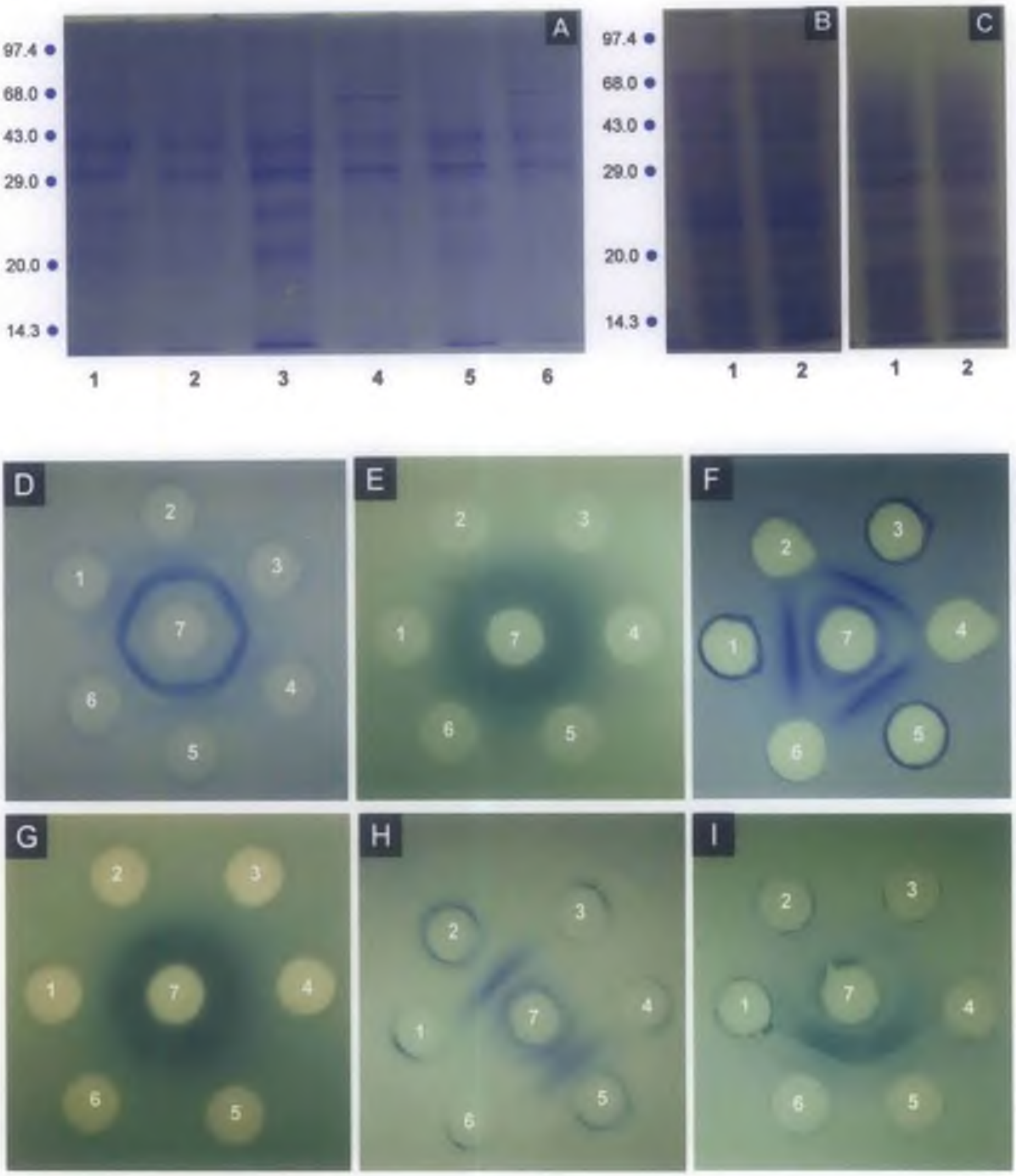

Plate 6 (A-C): SDS-PAGE analysis of mycelial proteins of (A)*F.graminearum* isolates Fg-2 (lanes 1& 2), Fg-1 (lane 3) & Fg-3 (lanes 4-6) ; (B)*T.harzianum* isolates Th-1 (lane 1) & Th-2 (lane 2) and (C)*T.viride* isolates Tv-1 (lane 1) & Tv-2 (lane 2). **(D-I)**: Agar gel double diffusion test. Central wells (7) were loaded with PAbs of *F.graminearum* (D, F, H & I), *T.harzianum* (E) and *T.viride* (G). Peripheral wells (1-6) were loaded with homologous antigens (D, E & G) and heterologous antigens (F,H&I).

Part – VI : Optimisation of ELISA

Polyclonal antibodies were raised separately against *F. graminearum* (isolate Fg.-1), *T. harzianum* (isolate Th.–1) and *T. viride* (isolate Th.-1). In each case three bleedings were collected and IgG, were purified. IgG for first, second and third bleeding of each of those three fungi were used for optimisation following formats of direct antigen coated enzyme leaked immunosorbant assay. In all cases two variables, dilution of antigen extract and dilution of PAbs were considered. Enzyme dilution was kept constant at 1 : 10,000.

PAbs of F. gramicenum :

Antigen dilution: Using two dilutions (1 : 125 and 1 : 250) of purified IgG of first, second and third bleedings and eight different concentrations of mycelial antigens of *F. granineanum* (isolate Fg.-1) DAC-ELISA was performed. Detail results have been presented in Tables 8 - 10 and Fig 2

Table : 8

DAC–ELISA reaction with various mycelial antigen concentrations of *F. graminearum* and homologous PAb (First bleeding).

Antigen Concentration (ng/ ml)	Absorbance at 405 nm	
	PAb of *F. graminearum* Dilutions	
	1 : 125	**1 : 250**
62.5	0.262 ± 0.04 [a]	0.204 ± 0.02 [a]
125	0.275 ± 0.02	0.258 ± 0.07
250	0.372 ± 0.05	0.289 ± 0.05
500	0.481 ± 0.02	0.345 ± 0.02
1000	0.622 ± 0.04	0.497 ± 0.04
2000	0.873 ± 0.02	0.622 ± 0.02
4000	1.136 ± 0.02	0.885 ± 0.03
8000	1.403 ± 0.02	1.267 ± 0.02

± = Standard Error. a = Mean of three experiments.

Table -9

DAC–ELISA reaction with various mycelial antigen concentrations of *F. gramineanum* and homologues PAb (Second bleeding).

Antigen Concentration (mg/ml)	Absorbance at 405 n m PAb of F. gramineanum Dilutions	
	1 : 125	1 : 250
62.5	0.327 ± 0.04 [a]	0.225 ± 0.03 [a]
125	0.370 ± 0.02	0.262 ± 0.06
250	0.497 ± 0.03	0.369 ± 0.02
500	0.592 ± 0.02	0.541 ± 0.04
1000	0.746 ± 0.01	0.682 ± 0.02
2000	0.886 ± 0.01	0.933 ± 0.03
4000	1.164 ± 0.04	1.062 ± 0.01
8000	1.621 ± 0.09	1.451 ± 0.04

± = Standard Curve. .   a = Mean of three experiments.

Table -10

DAC–ELISA reaction with various mycelial antigen concentrations of *F. gramineanum* and homologous PAb (Third bleeding).

Antigen Concentration (ng/ ml)	Absorbance at 405 nm PAb of *F. gramineanum* Dilutions	
	1 : 125	1 : 250
62.5	0.334 ± 0.02 [a]	0.235 ± 0.02 [a]
125	0.398 ± 0.04	0.252 ± 0.05
250	0.482 ± 0.05	0.349 ± 0.02
500	0.572 ± 0.04	0.477 ± 0.04
1000	0.698 ± 0.03	0.611 ± 0.02
2000	0.792 ± 0.04	0.732 ± 0.03
4000	0.987 ± 0.02	0.932 ± 0.02
8000	1.365 ± 0.03	1.293 ± 0.05

± = Standard Error.   a = Mean of three experiments.

Optimization of mycelial antigen concentrations of *F.graminearum* by DAC-ELISA

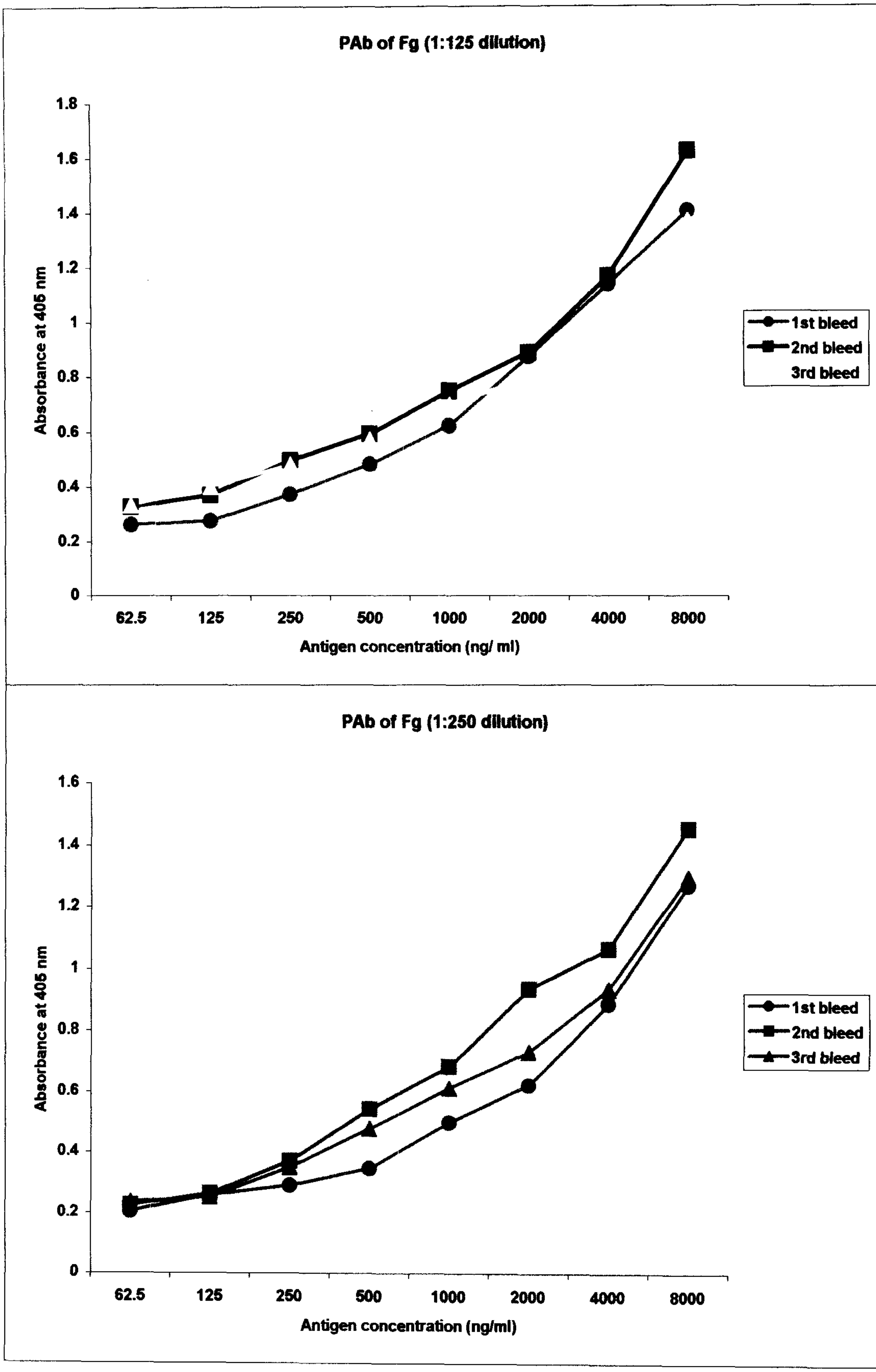

Fig: 2

Antisera dilution :

PAbs raised against mycelial antigen of *F. gramineaum* were diluted ranging from 1 : 125 to 1 : 16000 and tested against its homologous antigen at a concentration of 8µs/ml. Absorbance values in ELISA decreased from 1 : 125 dilution to 1 : 16000 dilution. The reactions were performed using all three bleedings. Results are shown in Tables 11-13 and Fig 3

Table : 11

DAC – ELISA reaction with various dilutions of PAb of *F. gramineanum* (first bleeding) and homologous antigen.

PAb of *F. gramineanum* (First bleeding) Dilutions	Absorbance at 405 mm
	Antigen concentration (8µs/ml)
1 : 125	0.997 ± 0.08
1 : 250	0.796 ± 0.02
1 : 500	0.514 ± 0.03
1 : 1000	0.482 ± 0.04
1: 2000	0.367 ± 0.04
1 : 4000	0.323 ± 0.02
1 : 8000	0.316 ± 0.01
1 : 16000	0.225 ± 0.02

± = Standard Error.
Average of three experiments.

Table –12

DAC – ELISA reaction with various dilutions of PAb of *F. gramineanum* (Second bleeding) and homologous antigen.

PAb of *F. gramineanum* (Second bleeding) Dilutions	Absorbance at 405 mm
	Antigen concentration (8µs/ml)
1 : 125	1.278 ± 0.03
1 : 250	0.996 ± 0.02
1 : 500	0.785 ± 0.02
1 : 1000	0. 589 ± 0.04
1: 2000	0.472 ± 0.03
1 : 4000	0.417 ± 0.04
1 : 8000	0.358 ± 0.02
1 : 16000	0.249 ± 0.02

± = Standard Error.
Average of three experiments.

Table –13

DAC – ELISA reaction with various dilutions of PAb of *F. graminearum* (Third bleeding) and homologous antigen.

PAb of *F. gramineanum* (Third bleeding) Dilutions	Absorbance at 405 mm
	Antigen concentration (8µs/ml)
1 : 125	1.235 ± 0.04
1 : 250	1.112 ± 0.02
1 : 500	0.942 ± 0.01
1 : 1000	0. 778 ± 0.02
1: 2000	0.645 ± 0.04
1 : 4000	0.416 ± 0.03
1 : 8000	0.350 ± 0.02
1 : 16000	0.282 ± 0.03

± = Standard Error.
Average of three experiments.

Optimization of PAb concentrations of *F.graminearum* by DAC-ELISA

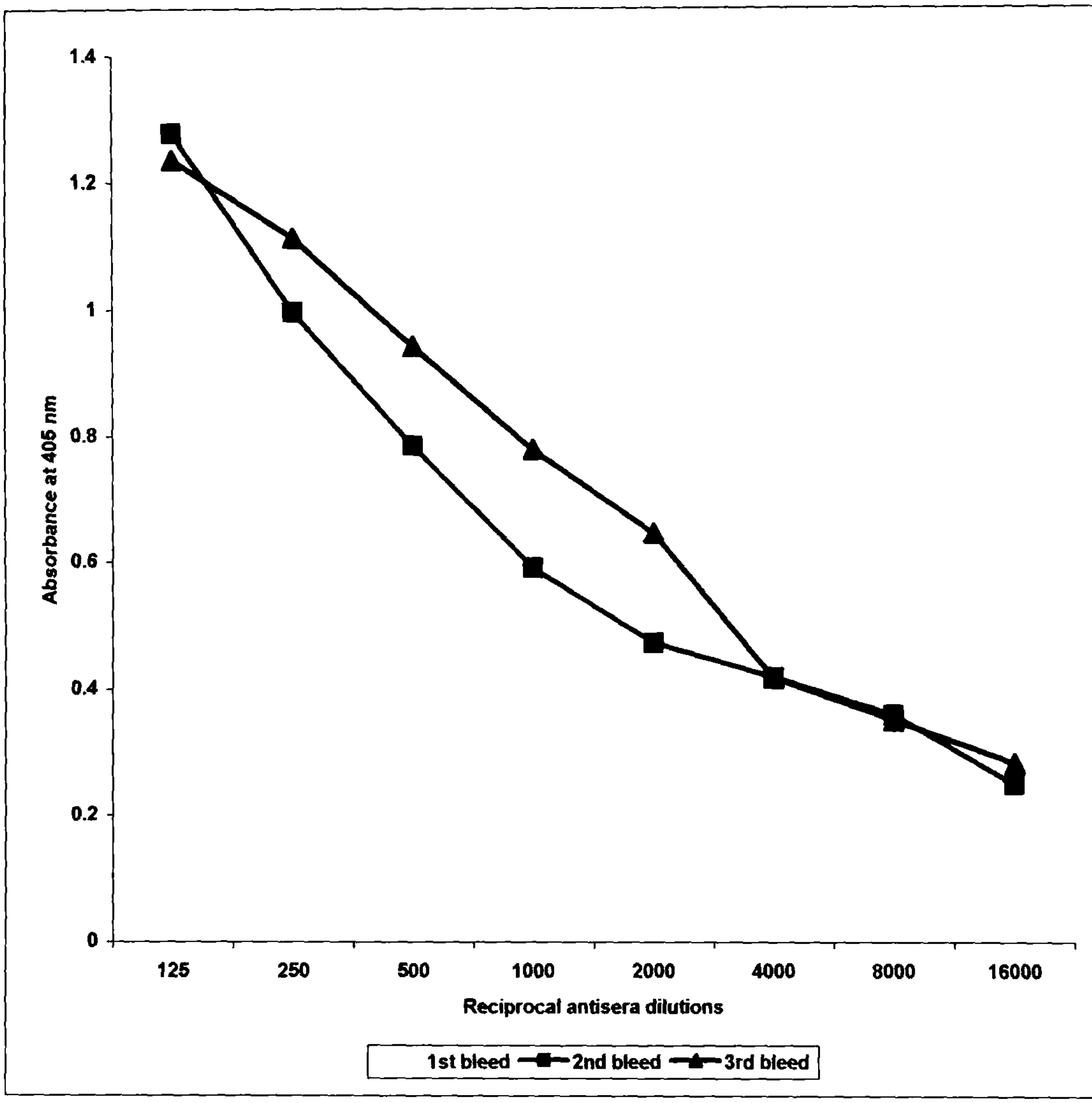

Fig: 3

PAb of *T. harzianum*

Polyclonal antibody was raised against mycelial antigen prepared from *T. harzianum* (isolate Th–1). In this case also three bleedings were collected and IgG were purified.

Antigen dilution:

Using two dilutions (1 : 125 and 1 : 250) of purified IgG of first, second and third bleeding and eight variable concentrations of mycelial antigens prepared from *T. harzianum* (isolate Th –1) DAC – ELISA was performed. Detail results have been presented in Tables 14 – 16 and Fig 4

Table : 14

ELISA reaction with various mycelial antigen concentrations of *T. harzianum* and homologous PAb (First bleeding).

Antigen Concentration (ng/ml)	Absorbance at 405mm PAb of *T. harzianum* dilution	
	1 : 125	1 : 250
62.5	0.375 ± 0.08 [a]	0.289 ± 0.07 [a]
125	0.412 ± 0.05	0.322 ± 0.03
250	0.432 ± 0.05	0.389 ± 0.04
500	0.562 ± 0.02	0.404 ± 0.03
1000	0.811 ± 0.04	0.598 ± 0.04
2000	0.952 ± 0.02	0.658 ± 0.02
4000	1.145 ± 0.03	1.121 ± 0.02
8000	1.282 ± 0.02	1.186 ± 0.03

± = Standard Error. a = Mean of three experiments.

Table : 15

ELISA reaction with various mycelial antigen concentrations of *T. harzianum* and homologous PAb (Second bleeding)

Antigen Concentration (mg/ml)	Absorbance at 405nm PAb of *T. harzianum* Dilution	
	1 : 125	1 : 250
62.5	0.396 ± 0.02 [a]	0.314 ± 0.03 [a]
125	0.481 ± 0.03	0.342 ± 0.04
250	0.472 ± 0.01	0.398 ± 0.02
500	0.619 ± 0.02	0.493 ± 0.02
1000	0.692 ± 0.05	0.587 ± 0.03
2000	0.821 ± 0.03	0.642 ± 0.04
4000	0.922 ± 0.02	0.806 ± 0.03
8000	1.203 ± 0.01	0.993 ± 0.02

± = Standard Error.
a = Mean of three experiments.

Table : 16

ELISA reaction with various mycelial antigen concentrations of *T. harzianum* and homologous PAb (Third bleeding).

Antigen Concentration (ng/ml)	Absorbance at 405mm PAb of *T. harzianum* Dilution	
	1 : 125	1 : 250
62.5	0.284 ± 0.02 [a]	0.208 ± 0.04 [a]
125	0.297 ± 0.03	0.202 ± 0.04
250	0.330 ± 0.01	0.231 ± 0.02
500	0.386 ± 0.02	0.242 ± 0.03
1000	0.456 ± 0.02	0.382 ± 0.04
2000	0.498 ± 0.03	0.398 ±
4000	0.660 ± 0.04	0.513 ± 0.02
8000	0.756 ± 0.02	0.664 ± 0.01

± = Standard Error.
a = Mean of three experiments.

Optimization of mycelial antigen concentrations of *T.harzianum* by DAC-ELISA

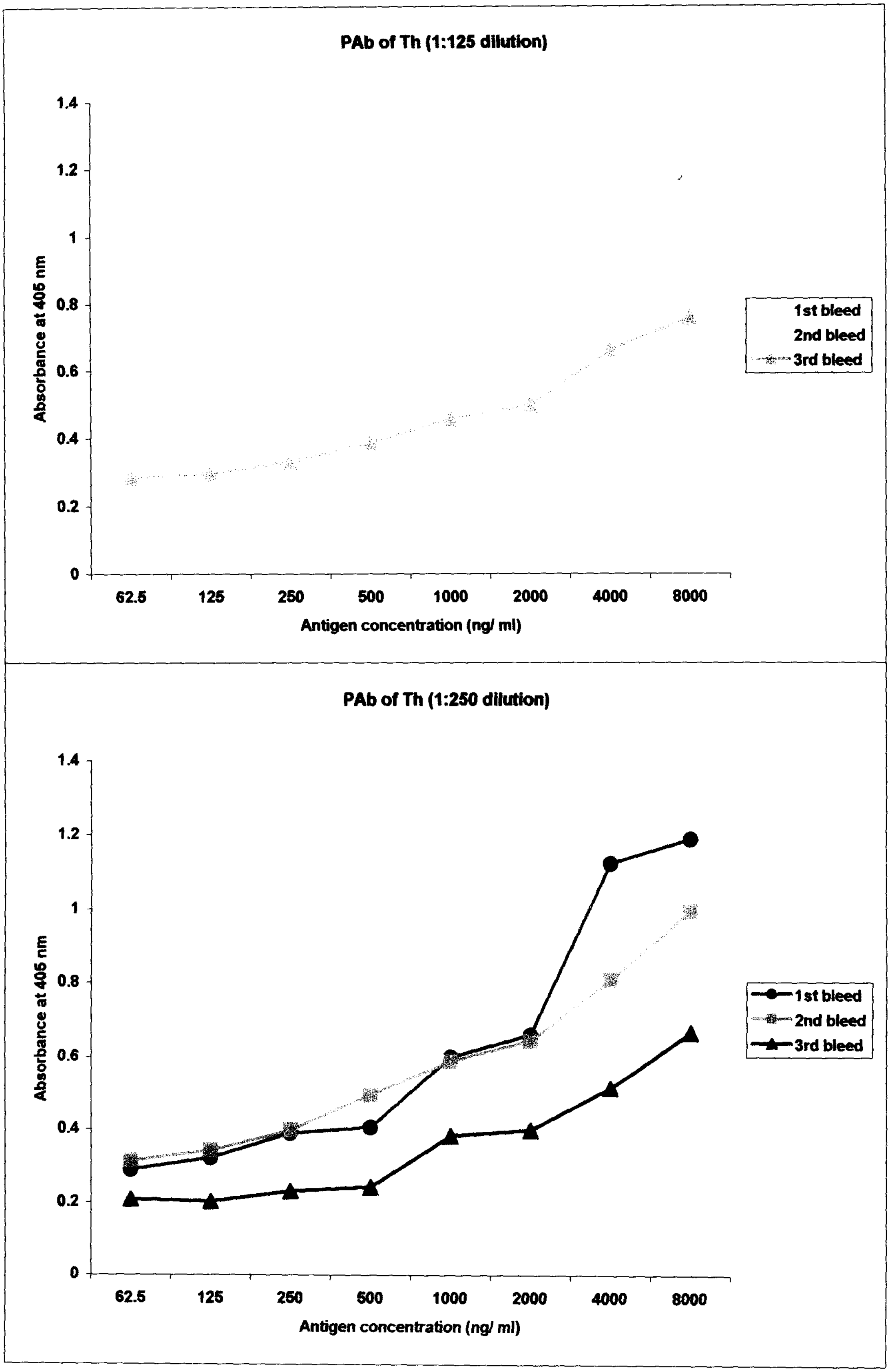

Fig: 4

Antsera dilution:

PAbs raised against mycelial antigen of *T. harizianum* were diluted ranging from 1 : 125 to 1 : 16000 and tested against its homologous antigen at a concentration of 8µm/ml. Absorbance values in ELISA decreased from 1 : 125 dilution to 1 : 16000 dilution. The reactions were performed using all three bleedings. Results are given in Tables17 - 19 and Fig. 5

Table : 17

DAC-ELISA reaction with various dilution of PAb of *T. harzianum* (First bleeding) and homologous antigen.

PAb of *T. harizianum* (First bleeding) Dilutions	Absorbance at 405 mm
	Antigen concentration (8µg/ml)
1 : 125	1.126 ± 0.03
1 : 250	1.025 ± 0.04
1 : 500	0.868 ± 0.02
1 : 1000	0.580 ± 0.02
1: 2000	0.427 ± 0.04
1 : 4000	0.332 ± 0.02
1 : 8000	0.263 ± 0.08
1 : 16000	0.254 ± 0.05

± = Standard Error.
Average of three experiments.

Table : 18

DAC-ELISA reaction with various dilution of PAb of *T. harzianum* (Second bleeding) and homologous antigen.

PAb of *T. harizianum* (Second bleeding) Dilutions	Absorbance at 405 mm
	Antigen concentration (8µg/ml)
1 : 125	1.072 ± 0.02
1 : 250	0.912 ± 0.05
1 : 500	0.762 ± 0.04
1 : 1000	0.665 ± 0.02
1: 2000	0.489 ± 0.01
1 : 4000	0.394 ± 0.01
1 : 8000	0.349 ± 0.03
1 : 16000	0.296 ± 0.05

± = Standard Error.
Average of three experiments.

Table : 19

DAC-ELISA reaction with various dilution of PAb of *T. harzianum* (Third bleeding) and homologous antigen.

PAb of T. harizinum (Third bleeding) Dilutions	Absorbance at 405 mm
	Antigen concentration (8µg/ml)
1 : 125	0.669 ± 0.05
1 : 250	0.553 ± 0.04
1 : 500	0.482 ± 0.04
1 : 1000	0.411 ± 0.02
1: 2000	0.358 ± 0.03
1 : 4000	0.308 ± 0.01
1 : 8000	0.278 ± 0.01
1 : 16000	0.264 ± 0.02

± = Standard Error.
Average of three experiments.

Optimization of PAb concentrations of *T.harzianum* by DAC-ELISA

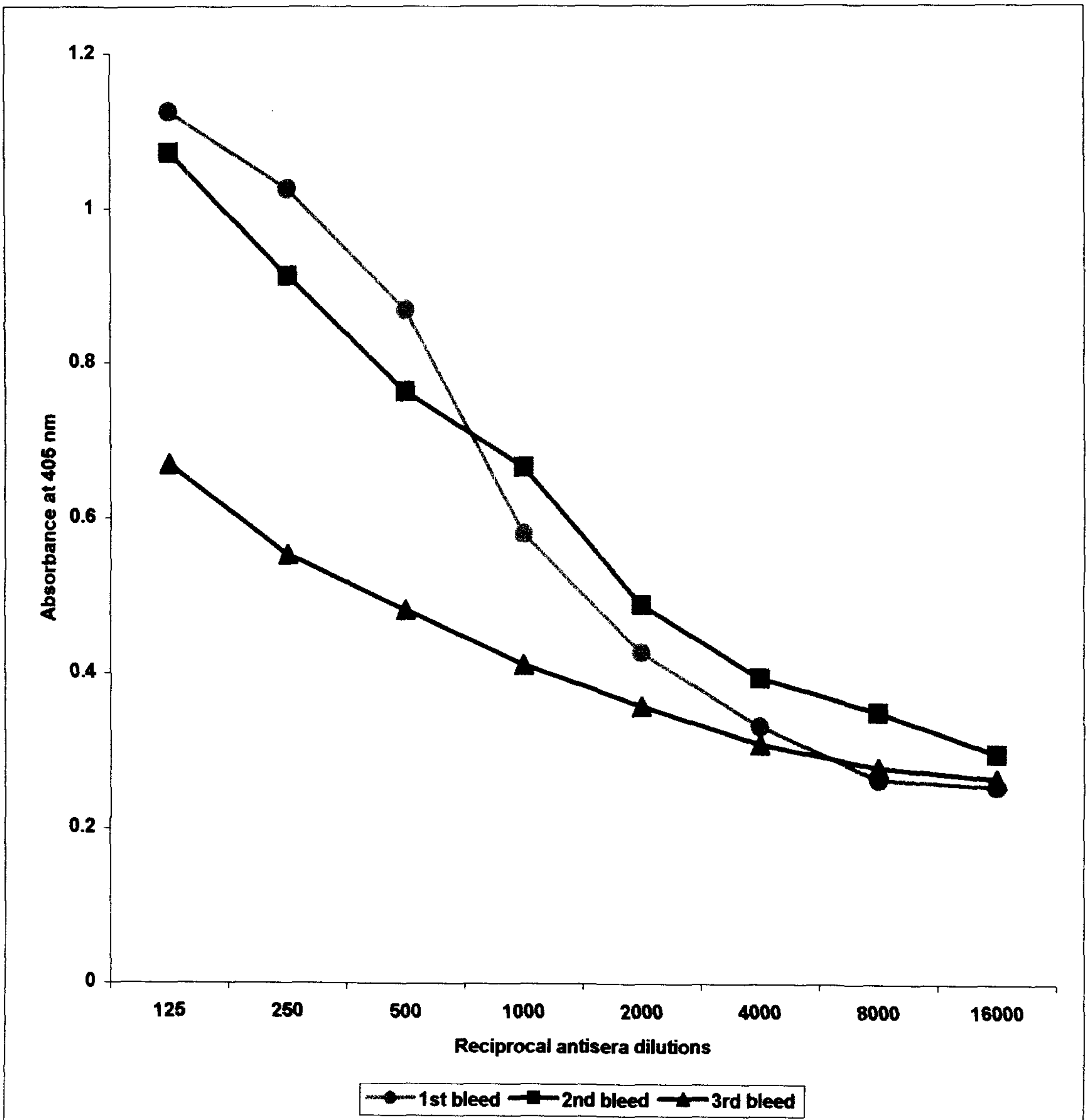

Fig: 5

PAb of *T. Viride*

Polyclonal antibody was also raised against mycelial antigen preparation of *T. viride* (isolate Tv–1). Purified IgG of three bleedings were used for optimisation purpose.

Antigen dilution :

Two dilutions (1 : 125 and 1 : 250) of purified IgG of first, second and third bleedings and eight variables concentrations of mycelial antigen prepared from *T. viride* (isolate Tv–1) were used for ELISA test. Results are given in Table 20 - 22 and Fig 6

Table : 20

ELISA reaction with various mycelial antigen concentrations of *T. viride* and homologous PAb (First bleeding)

Antigen concentration (ng/ ml)	Absorbance at 405 nm PAb of *T. viride* Dilutions	
	1 : 125	1 : 250
62.5	0.261 ± 0.02 [a]	0.212 ± 0.08 [a]
125	0.398 ± 0.03	0.259 ± 0.02
250	0.417 ± 0.02	0.332 ± 0.04
500	0.512 ± 0.01	0.356 ± 0.05
1000	0.598 ± 0.04	0.484 ± 0.02
2000	0.782 ± 0.02	0.592 ± 0.07
4000	0.899 ± 0.01	0.787 ± 0.05
8000	1.134 ± 0.03	1.005 ± 0.02

± Standard error

a = Mean of three experiments

Table : 21

ELISA reaction with various mycelial antigen concentrations of *T. viride* and homologous PAb (Second bleeding)

Antigen concentration (mg/ ml)	Absorbance at 405 nm PAb of *T. viride* Dilution	
	1 : 125	1 : 250
62.5	0.248 ± 0.05 [a]	0.226 ± 0.09 [a]
125	0.376 ± 0.02	0.263 ± 0.04
250	0.421 ± 0.02	0.312 ± 0.03
500	0.537 ± 0.03	0.356 ± 0.02
1000	0.598 ± 0.04	0.452 ± 0.06
2000	0.633 ± 0.03	0.583 ± 0.02
4000	0.782 ± 0.02	0.662 ± 0.03
8000	1.005 ± 0.01	1.978 ± 0.02

± Standard error,

a = Mean of here experiments

Table : 22

ELISA reaction with various mycelial antigen concentrations of *T. viride* and homologous PAb (Third bleeding)

Antigen concentration (ng/ ml)	Absorbance at 405 nm PAb of *T. viride* Dilutions	
	1 : 125	1 : 250
62.5	0.235 ± 0.04 [a]	0.219 ± 0.02 [a]
125	0.386 ± 0.04	0.245 ± 0.03
250	0.432 ± 0.02	0.337 ± 0.03
500	0.516 ± 0.05	0.512 ± 0.02
1000	0.597 ± 0.03	0.545 ± 0.01
2000	0.698 ± 0.04	0.583 ± 0.01
4000	0.795 ± 0.08	0.696 ± 0.02
8000	1.825 ± 0.05	1.785 ± 0.06

± Standard error

a = Mean of here experiments

Optimization of mycelial antigen concentrations of *T.viride* by DAC-ELISA

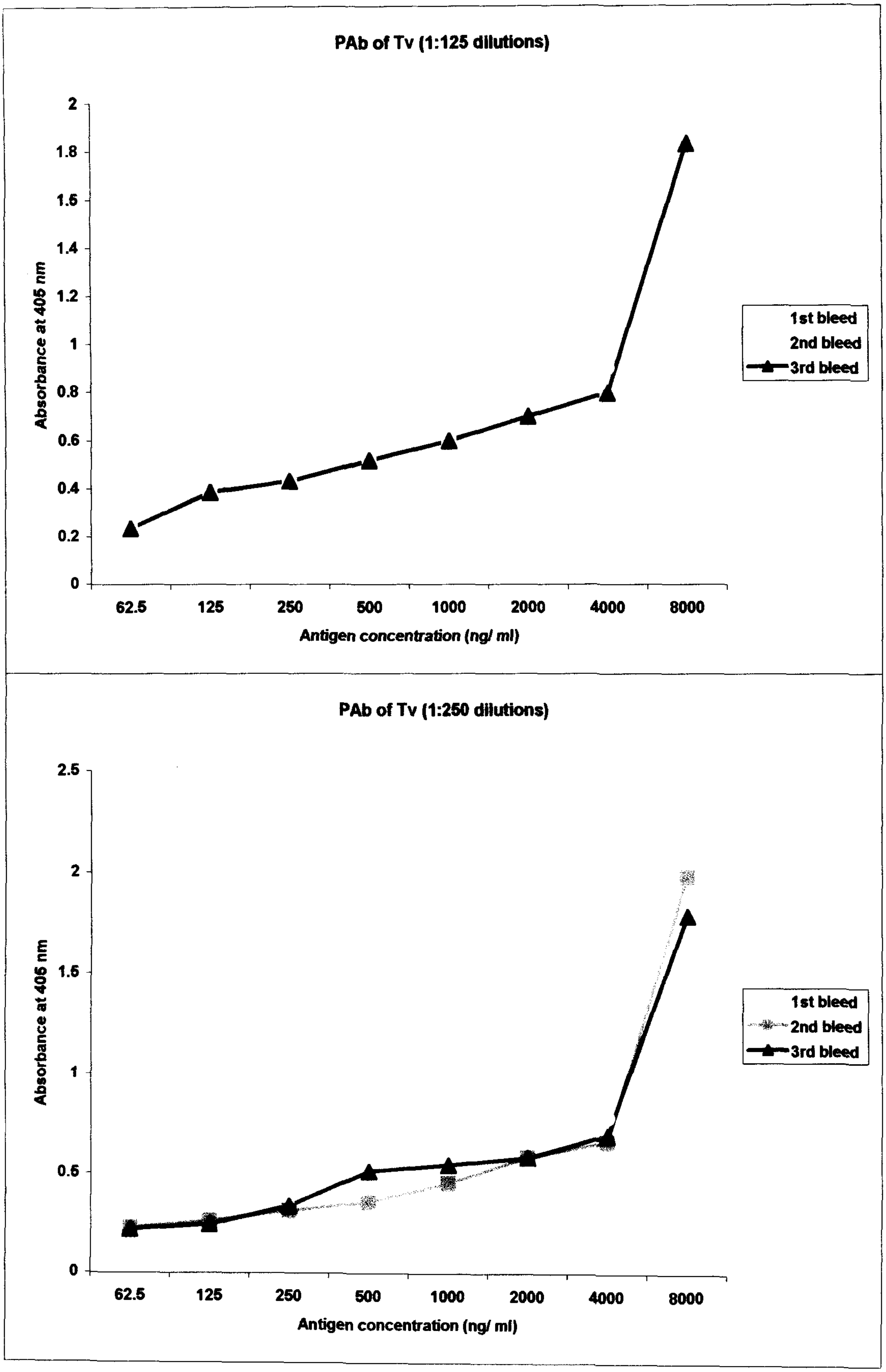

Fig: 6

Antisra dilution

PAbs raised against mycelial antigen of *T. viride* were diluted ranging from 1 : 125 to 1 : 16000 and tested against its homologous antigen at a concentration of 8µg/ ml. Absorbance value in ELISA decreased from 1 : 125 dilution to 1 : 16000 dilution. The reactions were performed using all three bleedings. Results are given in Tables 23 - 25 Fig. 7

Table : 23

ELISA reaction with various dilutions of PAb of *T. viride* (First bleeding) and homologous antigen.

PAb of *T. viride* (First bleeding) Dilutions	Absorbance at 405 nm Antigen concentration (8 µg/ml)
1 : 125	1.036 ± 0.05
1 : 250	0.975 ± 0.04
1 : 500	0.862 ± 0.03
1 : 1000	0.618 ± 0.03
1 : 2000	0.587 ± 0.05
1 : 4000	0.489 ± 0.04
1 : 8000	0.382 ± 0.02
1 : 16000	0.217 ± 0.01

± Standard error,
Average here experiments

Table : 24

ELISA reaction with various dilutions of PAb of *T. viride* (Second bleeding) and homologous antigen.

PAb of *T. viride* (Second bleeding) Dilutions	Absorbance at 405 nm Antigen concentration (8 µg/ml)
1 : 125	1.212 ± 0.01
1 : 250	0.972 ± 0.05
1 : 500	0.816 ± 0.02
1 : 1000	0.769 ± 0.02
1 : 2000	0.537 ± 0.03
1 : 4000	0.446 ± 0.04
1 : 8000	0.389 ± 0.02
1 : 16000	0.234 ± 0.02

± Standard error,
Average here experiments

Table : 25

ELISA reaction with various dilutions of PAb of *T. viride* (Third bleeding) and homologous antigen.

PAb of *T. viride* (Third bleeding) Dilutions	Absorbance at 405 nm Antigen concentration (8 µg/ml)
1 : 125	0.865 ± 0.05
1 : 250	0.732 ± 0.02
1 : 500	0.663 ± 0.04
1 : 1000	0.538 ± 0.02
1 : 2000	0.436 ± 0.01
1 : 4000	0.289 ± 0.05
1 : 8000	0.217 ± 0.03
1 : 16000	0.193 ± 0.02

± Standard error,
Average of three experiments

Optimization of PAb concentrations of *T.viride* by DAC-ELISA

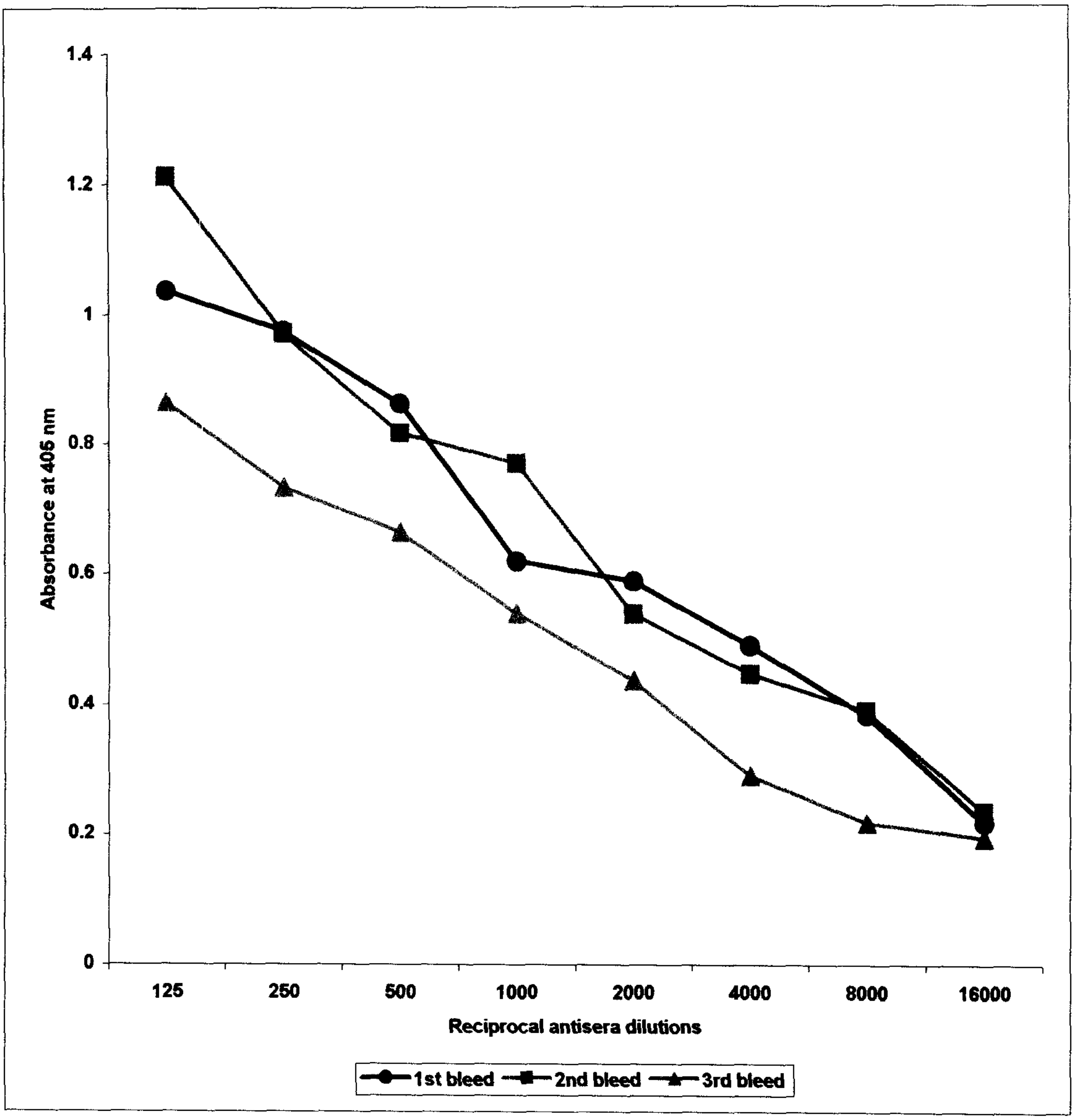

Fig: 7

Part VII: Detection of cross-reaction antigen shared between.

F. graminearum, and Ghycine max.

Indirect ELISA could readily detected cross reactivity between pathogen antisera and host tissues. Cross reactive antigens have in several host pathogen combinations been shown to be the determinants of susceptibility and resistance. In the present study pathogenicity test of eight Soybean varieties revealed different responses ranging from high susceptibility to high resistance. In order to determine whether resistance or susceptibility could be correlated with cross reactive antigens, ELISA tests were carried out with antigens of all varieties and PAb of *F. graminearum,*. The ELISA reactivity of different Soybean varieties against PAbs of the pathogens was determined and the results were compared with those of the pathogenicity tests. Results have been presented in Table–26.

Table: 26

DAC–ELISA response of healthy root antigen of Soybean varieties, non-host and non-pathogens of Soybean

Antigen	PAb of *F. graminearum*
Soybean varieties	
Macs – 58	0.892 ± 0.02
MRC – 12	1.112 ± 0.05
PK – 416	1.103 ± 0.04
MRC – 7	1.038 ± 0.02
PK – 564	1.246 ± 0.03
JS – 335	1.196 ± 0.02
Soymax	1.033 ± 0.05
UPSM – 19	0.863 ± 0.02
Tea root	
Tv – 9	0.433 ± 0.05
F. graminearum	
Isolate Fg – 1	1.836 ± 0.05
Fg –2	1.933 ± 0.02
Fg – 3	1.735 ± 0.08
Non pathogen of Soybean	
G. cingulate	0.383 ± 0.02
P. theae	0.411 ± 0.05

Part VIII : Detection of *F. graminearum* in Soybean root tissues using ELISA.

Healthy Root antigens were prepared from eight Soybean varieties. These were used for ELISA test using PAb of *F. graminearum*.

Plants were inoculated artificially wilt *F. graminearum* and harvested root samples were used for extraction of rot antigens from Soybean varieties. These were analysed using DAC – ELISA formats. Results have been presented in Table–27.

Besides one of the most susceptible varieties *Soymax* were inoculated and following inoculation 24 hr. after each internal antigens were prepared both from healthy and inoculated roots.

Results have been presents in Table–28. It indicates that DAC–ELISA can detect the pathogen entry within 48 hr. after inoculation.

Table: 27

DAC-ELISA responses of healthy and inoculated Soybean root against PAb of *F. graminearum.*

Varieties	PAb of *F. graminearum*	
	H	I
1. Macs - 58	0.937 ± 0.05	1.208 ± 0.05
2. NRC - 12	1.026 ± 0.07	1.334 ± 0.02
3. PK - 416	1.115 ± 0.08	1.418 ± 0.03
4. NRC – 7	1.034 ± 0.04	1.376 ± 0.02
5. PK – 564	1.235± 0.02	1.571 ± 0.01
6. JS – 335	1.192 ± 0.07	1.652 ± 0.05
7. Soymax	1.186 ± 0.06	1.873 ± 0.02
8. UPSM - 19	0.983 ± 0.05	1.106 ± 0.06

± = Standard error

Average of 5 replicates.

Table: 28

Indirect ELISA reaction of PAb of *F. graminearum* with healthy and artificially inoculated Soybean root antigen at different period.

Time after inoculation (in days)	PAb of *F. graminearum* (1 : 250 dilution)	
	H	I
1	0.680 ± 0.01	0.681 ± 0.03
2	0.639 ± 0.08	0.736 ± 0.05
3	0.641 ± 0.01	0.830 ± 0.02
4	0.656 ± 0.02	0.918 ± 0.05
5	0.671 ± 0.02	1.108 ± 0.03
6	0.650 ± 0.08	1.121 ± 0.09
7	0.622 ± 0.03	1.201 ± 0.01
8	0.680 ± 0.04	1.212 ± 0.03
9	0.625 ± 0.04	0.455 ± 0.04
10	0.631 ± 0.03	1.351 ± 0.01

± = Standard error
Average of 5 replicates.

Part IX: Detection of *F. graminearum, T. harzianum* and *T. viride* in soil using western blot and Dot blot.

Identification of *F. graminearum T. harzianum* and *T. viride* propagules in artificially infected and non-infected root rhizosphere soil was carried out through dot immunobinding reaction. Soil antigens were prepared from soil samples from *F. graminearum, T. harzianum* and *T. viride* amended soil and different location and Dot blot analysis was done. Results have been presented in Plate–7 (Figs–D-H). Positive reactions on blots indicate the presence of propagules. When confected antigen and soil antigen was seen in electrophoresis and transferred in nitrocellulose blot and probed with respective PAbs clear indication was observed regarding positive reaction of antigens with its antibody as reflected in Plate 7 (Figs A- C).

Soil samples of rhizosphere of different treatments were collected at a depth of 7–9 inches from soil surface. *F. graminearum* was evaluated through DAC – ELISA by reacting the antigens from collected soils after 30 days of pathogen inoculation with the PAbs of *F. graminearum, T. harzianum* and *T. viride.* Control set was prepared from uninfected soil is control plants.

In DAC–ELISA results from soil (treated with *F. graminearum, T. harzianum* and *T. viride* reacted with PAb of *F. graminearum* showed significantly lower absorbance values than that of soil antigen treated with *F. graminearum* alone. This indicated that population of *F. graminearum* soil had been reduced by the biocontrol agents.

Table: 29

ELISA values of Soil antigens of different treatment with PAbs of *F. graminearum, T. harzianum* and *T. viride.*

Antigen source	Source of PAbs		
	F. graminearum	T. harziarum	T. viride
Uninfected Soil	0.372 ± 0.09	0.283 ± 0.02	0.297 ± 0.03
Treatment			
F. graminearum + *T. harzianum*	0.492 ± 0.02	0.937 ± 0.04	0.463 ± 0.05
F. graminearum + *T. viride*	0.487 ± 0.02	0.462 ± 0.02	0.836 ± 0.02
F. graminearum	0.935 ± 0.03	0.340 ± 0.02	0.417 ± 0.06
T. harzianum	0.427 ± 0.02	0.838 ± 0.04	0.422 ± 0.03
T. viride	0.363 ± 0.03	0.332 ± 0.02	0.817 ± 0.02

Sample Collected after 20 days of inoculation
PAb dilution 1 : 500
± = Standard error.

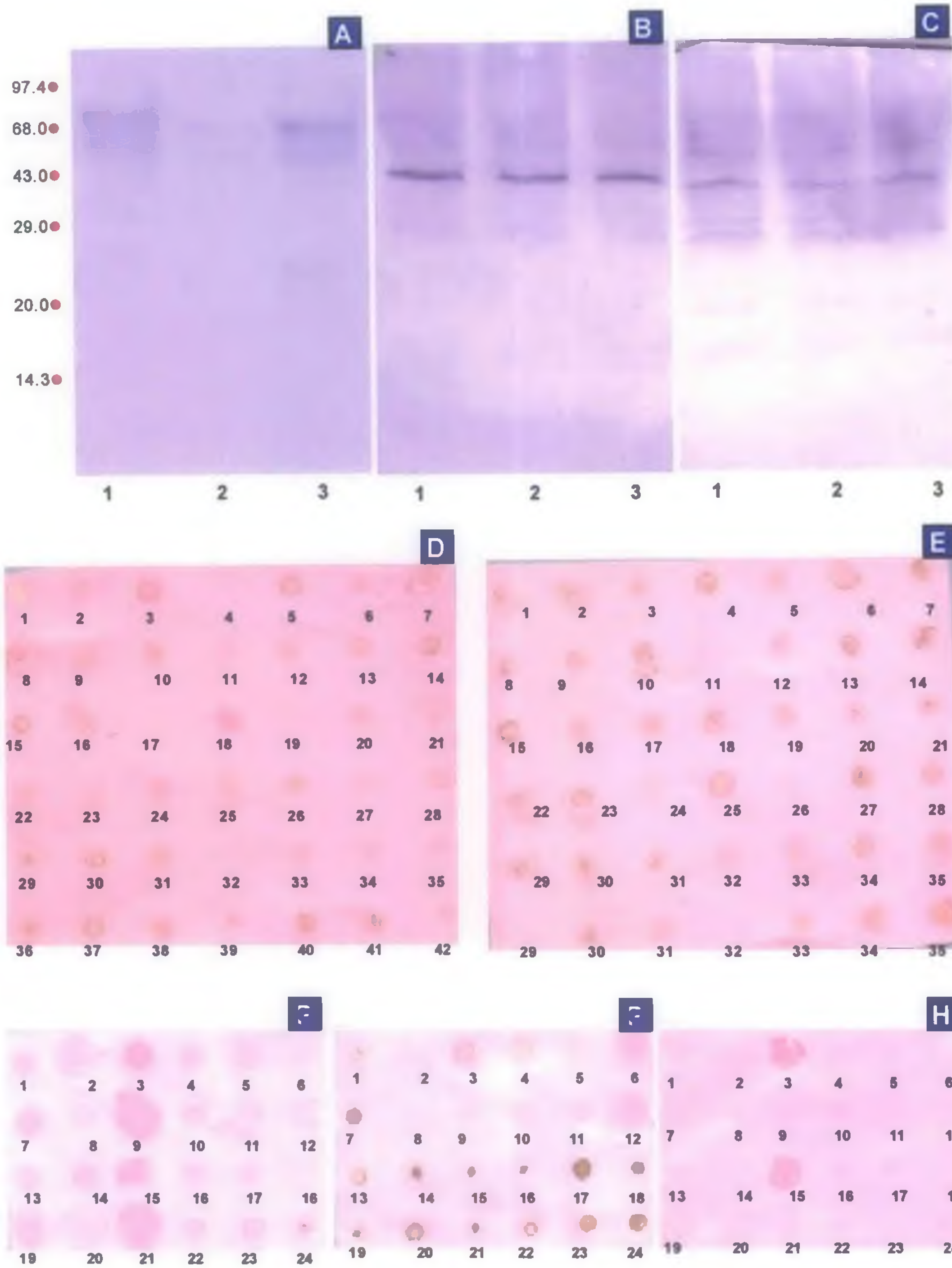

Plate 7 (A-H): [A-C]: Western blot analysis of fungal antigen using homologous PAb of (A) *F.graminearum* (lanes 1&3: Fg-1, lane 2: Fg-2), (B) *T.harzianum* (lanes 1-3) and (C) *T.viride* (lanes 1-3: Tv-1). [D-H]: Dot immunobindings of antigens and antibody on nitro-cellulose paper. (D,E&F) PAb of *F.graminearum*, (G) PAb of *T.harzianum* and (H) PAb of *T.viride*.(D&E) Soil antigens of various fields (1-42); (F) mycelial antigen of *F.graminearum* isolate Fg-2 (1, 7, 13, 19), isolate Fg-1 (3, 9, 15, 21), Fg-3 (4, 10, 16, 22), *T.harzianum* (5,11,17,23), *T.viride* (6,12,18,24); (G) Mycelial antigen of Th-1 (1-6), Th-2 (7-12), soil (13-24); (H) Mycelial antigen of Tv-1 (3), Tv-2 (15), soil (1,2,4-8,10-14,16-18).

Part X: Indirect immunofluorescence of hypae and conidia of *F. graminearum*, *T. harzianum* and *T. viride*.

Fluorescent antibody labelling with fluorescein isothiocyanide (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. Present study reports the use of indirect immunofluorescent test using polyclonal antibodies of *F. graminearum*, *T. harzianum* and *T. viride* as well as mycelia of *F. graminearum*, *T. harzianum* and *T. viride* in rhizosphere soil. Mycelia and soil preparation were photographed under UV fluorescence and the intensity of bright apple-green fluorescence indicated the positive reaction. Pre-immune sera did not show reactivity with the mycelia of *F. lamaoensis*, *T. harzianum* and *T. viride* followed by FITC and mycelia was not auto-fluorescent. Examination of mycelia treated with homologous PAbs of mycelia and stained indirectly with FITC indicated strong fluorescence throughout the mycelia, specially in young hyphal tips. Results have been presented in Plates – 8, 9 and 10.

Amended soil preparation was done for immunofluoroscence study as described under materials and methods. Microscopic observation UV-fluorescence revealed that presence of strongly fluorescing mycelia. Thus immunofluorescence could be used to detect the pathogen in soil.

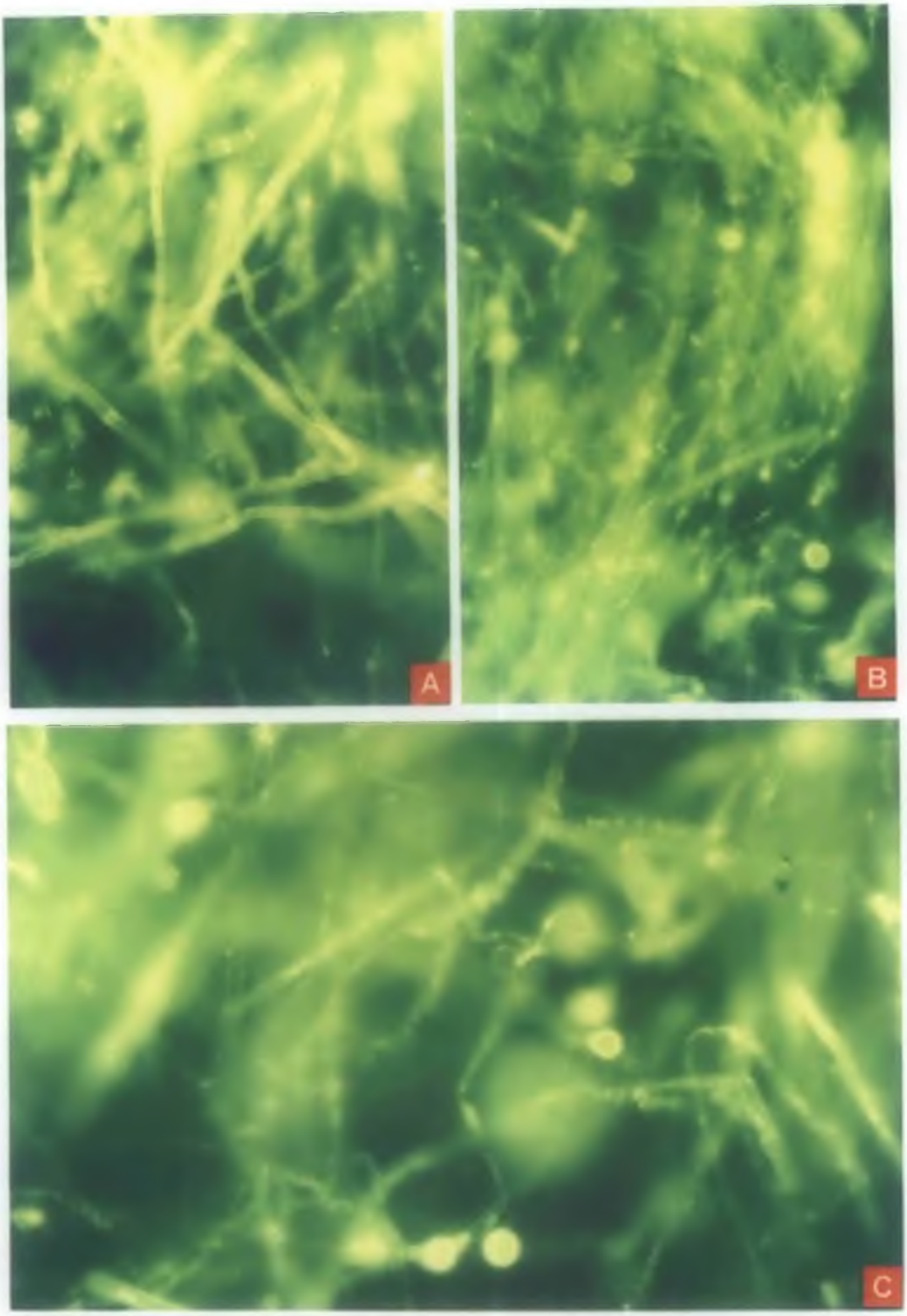

Plate 8 (A-C): Indirect immunofluorescence of hyphae and conidia of *F.graminearum* treated with PAb of Fg-1 and stained with FITC antibodies of goat specific for rabbit globulin. (A) Isolate Fg-2; (B) Isolate Fg-3; (C) Isolate Fg-1

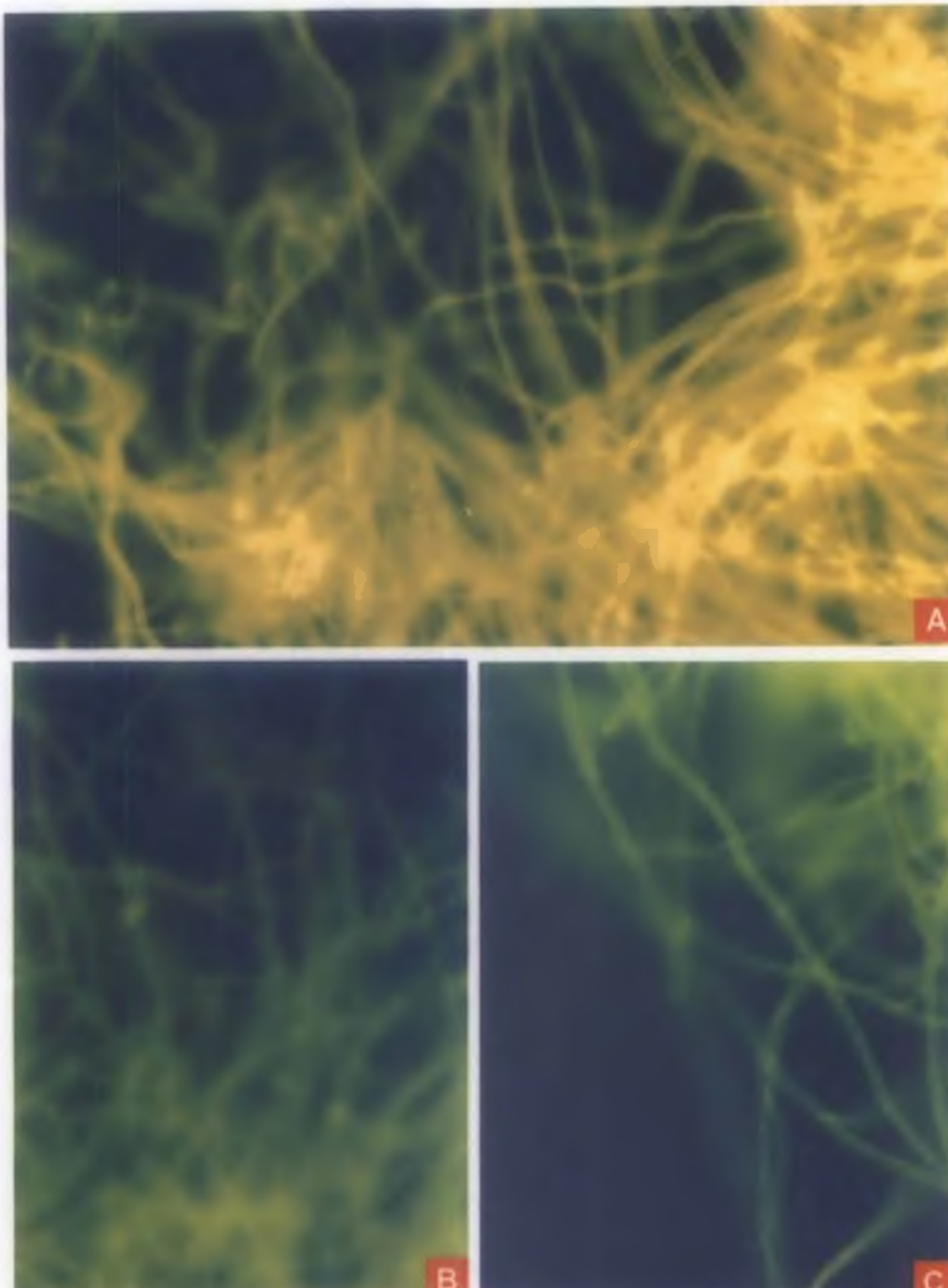

Plate 9 (A-C): Indirect immunofluorescence of hyphae of *T.harzianum and T.viride* treated with homologous PAbs and stained with FITC antibodies of goat specific for rabbit globulin. (A) IsolateTh-1; (B) Isolate Tv-1; (C) Isolate Th-2

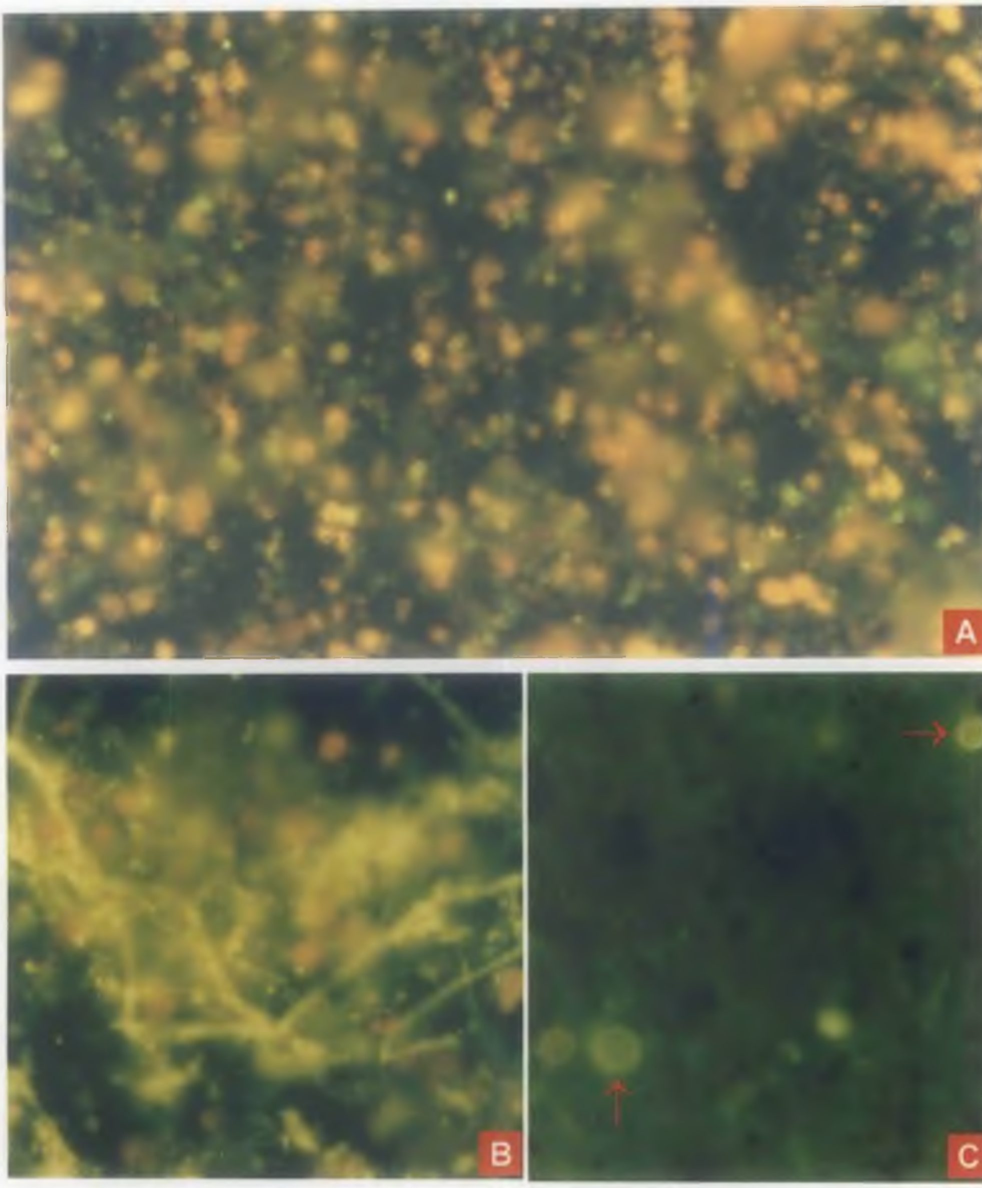

Plate 10 (A-C): Immunofluorescence of conidia of *T.harzianum* (A), *T.viride* (B) and chlamydospores of *F.graminearum* (C) treated with homologous PAbs and stained with FITC antibodies of goat specific for rabbit globulin

DISCUSSION

Host parasite interactions are generally initiated in nature by the fungal spores since they come in contact with the host cells at the first instance. Therefore, they play a very good recognition phenomenon. The presence 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 reviewed earlier by several authors (De Vay *et al.*, 1972; DeVay and Adler, 1976; Kalyansundaram, 1978; Chakraborty, 1988; Purakayastha, 1989).

In the present study root antigens of eight soybean varieties and isolates of *F. graminearum* (Fg1 and Fg2) were cross reacted . Antigens from two non pathogens of soybean, viz. *Glomerella cingulata* and *Pestalotiopsis theae* were also considered for serological comparisons. It is significant to note that in immunodiffusion test susceptible varieties shared the common antigens with both the isolates of *F. graminearum* (Fg1 and Fg2) tested. However, antigenic disparity was noticed in cross-reaction with antigens and antisera of resistant variety and the isolates of the pathogen.

The preparation and treatment of antigens are most important because most antigens are labile and easily denatured. The selections of test animal as well as the 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).

The present study has established very definitely the importance of cross-reactive antigens between host and pathogen in determining the responses of the host to pathogen. This has also been supported by works of several previous workers (Chakraborty and Purakayastha, 1983; Purakayastha *et al.*, 1991, Chakraborty and Saha, 1994; Chakraborty *et al.*, 1999). It is also important in studies on host parasite relationship to determine the cellular location of the CRA.

For this purpose in this study fluorescence tests were conducted with cross section of tea roots as well as mycelia and conidia of *F. graminearum*. Cross sections of soybean roots were treated with anti- *F. graminearum* followed by staining with FITC conjugated antirabbit globule specific goat antiserum. Bright fluorescence was observed in case of epiblema region, and cortical cell.

Treatment of root sections with *F. graminearum* revealed that the CRA was concentrated mainly around cortical zone. Treatment of mycelia and conidia of *F. graminearum* with homologous antiserum and FITC showed a general fluorescence that was more intense on young hyphae. DeVay *et al.* (1981) determined the tissue and cellular location of major CRA shared by cotton and *F. oxysporum* f. sp. *vasinfectum*. On the basis of strong fluorescence obtained at the epidermis, cortex, endodermis and xylem tissues, they suggested that the CRA determinants in roots have a general distribution. DeVay *et al.* (1981) also used FITC labelled antibodies for races of *P. infestans*, to detect the CRA in potato leaf section. It was also reported by Charkraborty and Saha (1994) that CRA between tea and *B. carbonum* were mainly present in the hyphal tips and in patch like areas on the condia, mycelium and mainly around epidermal cells and mesophyll tissues of the leaf. The cellular location of CRA between *P. theae* and tealeaves was also established by Chakraborty *et al.* (1995).

In the present investigation using antibodies indirectly labelled with fluorescein isothiocyanate (FITC) the location of CRA in cross sections of soybean roots of resistant (UPSM-19) and susceptible (Soymax) cultivars and fungal cells (*F. graminearum)* has been established. Treatment of mycelia and conidia of *F. graminearum* with antiserum to roots of cv. Soymax and using indirect staining with FITC indicated that CRA was mainly present in young hyphal tips and in patch like areas on conidia. The results are also in conformity with the work of DeVay *et al.* (1981a) involving treatment of conidia and mycelia of *Fusarium oxysporum* f. sp. *vasinfectum* with antisera to cotton.

The tissue and cellular location of major CRA shared by cotton and *Fusarium oxysporum* f. sp. *vasinfectum* was determined by DeVay *et al.* (1981a). Cross sections of young cotton roots with antiserum to *F. oxysporum* f. sp. *vasinfectum* and followed by an antirabbit globulin specific goat antiserum FITC conjugate exhibited strong fluorescence at the epidermis and xylem tissues indicating a general distribution of CRA determinants in roots. FITC labelled antibodies for races of *P. infestans* were also used to detect CRA in potato leaf sections (DeVay *et al.,* 1981). In cross section of tea leaves (TV-18), cellular location of CRA shared by *B. carbonum* was evident mainly around epidermal cells (Chakraborty & Saha, 1994).

Polyclonal antibodies were raised against mycelial antigens of *F. graminearum* and these were used for determining the presence of cross reactive antigens (CRA) between soybean varieties and *F. graminearum* as well as for the immunodetection of the pathogen in root tissues and in soil. Since enzyme linked immunosorbent assay (ELISA) has proved to be one of the most sensitive serological techniques, PAbs raised against *F. graminearum* were used in ELISA test for pathogen detection. Since ELISA is a very sensitive technique and non-specific binding interferes with the actual antigen-antibody reaction, initially PAbs were purified and IgG fractions were used in all further tests. Prior to other tests, the sensitivity of assay was optimised and the minimum detectable antigen concentration and optimum IgG concentration were determined in homologous reactions. Positive results were obtained with very low concentration of both antigens and IgG. It was reported by Mohan (1988) that a concentration of *Phytophthora* antigens,as low as 2μg/ ml could be detected in indirect ELISA by antiserum raised against pooled mycelial suspensions of five *P. fragariae* races. Chakraborty *et al.* (1996) also reported that antiserum raised against *Pestalotiopsis theae* could detect homologous antigens at 25 μg/ml. Antiserum dilution of upto 1:16000 was effective for detection.

The presence of CRA among *F. graminearum* and soybean varieties was evident in indirect ELISA, using PAb raised against mycelial antigen preparations of *F. graminearum* at a concentration of 40 μg/ml with soybean root antigens at a concentration of 100 μg/ml. Though much difference was not observed in ELISA values among the different varieties Soymax the most susceptible variety exhibited the highest value. Alba & DeVay (1985) also detected CRA in crude and in purified preparations from mycelia of *Phytophthora infestans* with antisera of potato at concentration lower than 50 μm/ml protein using indirect ELISA. The presence of CRA in several host pathogen interaction has also been reported by a number of previous workers. e. g. Soybean and *Myrothecium roridun* (Ghosh and Purakayastha (1990): Groundnut and *Macrophomina phaseolina* (Purakayastha and Pradhan (1994); Tea and *Bipolaris carbonum* (Chakraborty and Saha, 1994); Tea and *Pestalotiopsis theae* (Chakraborty *et al.*, 1995) and Tea and *Glomerella cingulata* (Chakraborty *et al.* (2002). Cross reactivity of the PAb raised against *F. graminearum, T.harzianum* and *T.viride* were tested with other fungal species. Results revealed that PAb of *F. graminearum* reacted to some extent with two entomopathogenic isolates *Beauveria* and *Metarrhizium* species.

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 & 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 detect CRA in semi purified mycelial preparation of *B. carbonum* at concentrations ranging from 5-25 μm/ml with antiserum dilution 1:125. In cross-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 & Saha, 1994). Based on these findings it can be assumed that indirect ELISA may serve as an important technique to detect cross-reactive antigens, even in those interactions where conventional serological techniques have failed to detect (Johnson, 1962; Caroll *et al.*, 1972). Mohan (1989) showed that antisera raised against mycelial suspension of *Phytophthora fragariae* (PfM) reacted strongly with antigens from several *Phytophthora* species. He observed that anti-PfM could not be made specific for *P. fragariae* because it was raised to components shown to be antigenically similar in all *Phytophthora* sp. tested. Harrisen *et al.* (1980) further reported that anit *P. infestans γglobulin* reacted strongly with extract of *P. ervthroseptica* in DAC-ELISA but not with extracts of nine unrelated fungi or a culture of bacterium *Erwinia carotovora,* all of which were saprophytes of pathogens of potato.

With the advent of more sensitive techniques like ELISA, detection of plant pathogens in host tissues is now possible even when the pathogen concentration in host tissues in very low or when visible symptoms have not yet developed. This offers a definite advantage over classical techniques and is thus gaining an importance for pathogen detection purposes. Various formats of ELISA using polyclonal antisera has found wide spread application in plant pathology and are routinely used for detection and identification purposes (Clark, 1981; Chakraborty *et al.*, 1996 and Viswanathan *et al.*, 2000). Viswanathan *et al.* (2000) reported that presence of *Colletotrichum falcatum* in sugarcane tissues could be detected by ELISA. They reported that when twenty different sugarcane varieties were subjected to ELISA test after pathogen inoculation a clear variation in disease resistance was seen. They suggested that this technique could be reliably used to screen sugarcane genotypes for red rot resistance at an early stage. In the present study presence of *F. graminearum* in soybean root tissues could be detected by DAC-ELISA using PAb raised against mycelial antigens. It was observed that PAb of *F. graminearum* could also react with antigens from roots infected with other pathogens showing certain degree of cross reactivity. Since PAbs raised against *F. graminearum* could detect the presence of the pathogen in root tissues, it was decided to determine the efficacy of the PAb in detecting the specific pathogen in the soil. Detection of specific pathogen in soil equally or more important than detecting the pathogen in the root tissues. Detection of specific pathogens in soil requires very sensitive techniques, which would make it possible to differentiate between the various microorganisms. Use of serological techniques most specifically ELISA are gaining importance in such studies. In the present study, initially antigens prepared from soil collected from various tea estates were tested against PAb of *F. graminearum* by DAC-ELISA. Of the twenty-five soil samples tested four samples showed high A_{405} values while all the other had relatively low values. Thus it was possible to identify these soils as being contaminated with *F. graminearum*. Wakeham and White (1996) reported the ability of polyclonal antisera of *Plasmodiophora brassicae* to detect the presence of the pathogen in soil. In another study Walsh *et al.* (1996) reported serological detection of spore balls of *Spongospora subterranea* and its quantification in soil. They reported that the antiserum could detect about 100 spore ball/gm soil but discrimination of spore ball levels appear to be better for concentration greater than 2000/gm soil. There was a quantifiable relationship between concentration of spore balls and ELISA values. In the present study, using spiked soil, the ELISA values decreased with decrease in concentration of spores. Thus ELISA showed potential for detection of *F. graminearum*, *T. harzianum* and *T. viride* in soil.

Detection of pathogen in host tissues using antibody based immunoflurescent technique has been reported by several previous authors (Warnock, 1973; Hornok and Jagicza, 1973; Reddy and Anantanarayanan, 1984). Dewey *et al.* (1984) suggested, on the basis of immunofluorescence studies that chlamydospores, basidiospores and mycelia of *Phaeolus schweinitzii* contained molecules antigenicaly related to species specific antigens secreted by mycelia grown in liquid culture. They also demonstrated the presence of mycelium and chlamydospores in naturally and artificially infested soil samples, using this technique. *Phytophthora* could be detected in soil by immunofluorescence antibody technique (Watabe, 1990).

The dot immunobinding technique has been found to be rapid and sensitive method for detection of fungal pathogens is a more recent application of this method. Antiserum specificity obtained against fungal pathogen varied greatly in the studies done by Lange *et al.* (1989). The antiserum against *Plasmodium brassicae* used in their study showed no cross reaction with other common rest pathogen (*Pythium ultimum, Rhizoctonia solani and F. oxysporum.* In this study, antigen of mycelia, amended soil, soil from infected plot, healthy and *F. graminearum* inoculated soybean root, mycelial antigen of biocontrol fungi were prepared and tested on nitrocellulose paper against PAbs raised against *F. graminearum*, *T.harzianum*, and *T. viride* using fast red or NBT/ BCIP as substrate. Antigens of homologous source, soil of infected plot showed deep coloured dot. Infected soybean root antigens also showed deep coloured dot when compared to healthy confirming the presence of fungal pathogen. Wakeham and White (1996) got positive detection of soluble components of the spore wall and whole resting spores of *P. brassicae* in PBST.

Complex mixture of antigens can be separated by high-resolution techniques such as sodium dodecyl acrylamide gel electrophoresis using discontinuous buffer systems and two-dimensional techniques. However one separated in this matter, it has been difficult to determine which of the separated species reacted with a given antiserum. Several methods have been developed previously. Towbin *et al.* (1979) overcame these problems by electrophoretically transferring the separated mixture onto nitrocellulose. The PAbs of *F. graminearum*, *T.harzianum* and *T. viride* are very much specific for detection of the pathogen in the soil, infected root tissues and in different isolates of fungi. Walsh *et al.* (1996) also performed Western blotting using the raw serum of *Spongospora subterranean* spore balls.

Consequent to the study on the detection of *F. graminearum* root tissues and soil, experiments were conducted both *in vivo* and *in vitro* for the management of root rot disease. For this purpose *Trichoderma harziamum*

T. viride were selected and experiments were conducted as a biocontrol agents Both inhibited the growth of *F. graminearum in vitro.* There are several reports on the ability of *T. harziamum and T. viride* to inhibit the growth of pathogen under invitro condition. 10 isolates of *Trichoderma* species were screened by Padmodaya and Reddy (1996) in *in vitro* for their efficacy in suppressing the growth of *Fusarium oxysporum* f. sp. *lycopersici, Trichoderma viride* (H) was found highly inhibitory to *F. oxysporum* f. sp. *lycopersici* in dual culture follwwed by *T. harziamum* (A. P.). Studies on production of volatile compounds by *Trichoderma* species revealed that *T. virde* (H), *T. viride* (A. P.) and *Trichoderma* sp. (D) as effective in reducing radial growth of *F. oxysporum* f. sp. *lycopersici* in a study on production of non-volatile compounds by *Trichoderma* spp. Baby and ChandraMouli (1996) tested antagonistic potential of *Trichoderma* spp. and *Gliocladium virens* against primary root pathogens of tea viz *Fomesnoxius, P. hypolaterita, Rosellinia arcuata and Armillaria* and *T. viride* that of *Rosellinia G. virens colonozed all the pathogens excepting Rosellinia. G. virens* showed high antibiosis to *Rsosellnia.* Production of toxic metabolitel(s) was more in *G. virens* than *Trichoderma* Hazarika *et al.* (2000) also tested the antagonistic effect of *Trichoderma harzianum* against *U. zonata,* causing charcoal stump root of tea in dual culture method. Both antagonists were most effective in inhibiting the mycelial growth of *U. zonata.* Assam and Tamil Nadu isolates of *T. harziamum, T. viride* and *T. biride* were tested by Hazarika and Das (1998) for their potential to suppress *Rhizoctonia solani.* Culture filtrate of *T. harziamum and T. virde* inhibited mycelial growth and scolerotial germination. Wheat bran substrate supported maximum growth of all isolates followed by firm yard and tea. Both *T. harziamum and T. viride effectively* controlled the bean rot disease when they were applied as seed and soil treatment. In dual culture of 11 isolates of *T. harziamum* three isolates, viz. T8, T10 and T2 were effective against *Sclerotium rolfsii,* the causual agent of stem rot of groundnut and they overgrew the pathogen up to 92%, 85% an 79% respectively, in *vitro.*

Phookan and Chaliha (2000) reported that the growth of *Sclerotinia sclerotiorum* was significantly suppressed by *Gliocladim virens* and *T. viride* significantly. Amongst fungal antagonists tested by Sharma and Sharma (2001), *Trichoderma harziamum* and *T. viride* were found most effective in inhibiting mycelial growth of *Dematophoranectrix* in dual culture.

T. harziamum and *T. viride* were tested in vivo for their ability to reduce violated root rot intensity. Of the various delivery systems tested for this biocontrol agents, tea waste formulation were found t be most effective. Disease intensity was reduced by both *T. harziamum* and *T. viride* when tested under potted conditions as well as in the field. This was observed in all tested varieties. This research are in conformity with that of Hazarika *et al.* (2000). They reported that planning of tea seedlings after dipping roots in spore suspension of *T. harziamum* reduced 56.6% mortality of plant due to *U. zonata* infection. This was also obtained with *T. viride* and *G. virens.* However, they observed that the reduction of morality of plant increased to 62.2% when *T. harziamum* were applied as soil drenceh.

The role of *T. harziamum* and *T. viride* bio control crops is well established. Sarker and Jayarajan (1996) reported that *Seasamum* caused by *Macrophomina phaseolina* was significant and 12.8% respectively compared to 60% incidence in the control pots Prasad *et al.* (1999) found 3 *T. harziamum* isolates (PDBCTH-2, 7 and 8) and the *T. viride* isolate (PDBCTV-4) were highly efficient in controlling root / collar rot of sunflower caused by *Sclerotium rolfsii. Trichoderma* spp. are common antagonists found in almost all the soils. Many isolates produced volatile and non-volatile antibiotics. The most effective antagonists from literature are species of *T. harziamum, T. longibrachiatum and T. viride.* In *Trichoderma* mycoparasitism is one of the main mechanism(s) involved in biocontrol followed by competition and antibiosis. Excretion of extracellular enzymes viz. cellulase chitinase, etc., exhibited by *Trichoderma* spp. which were found a good source for the lysis of the cell walls of the pathogen.

Efficacy of two isolates of *T. longibrachiatum* against *R. solani* was reported by Sreenivasaprasad and Manibhushanrao (1990a, b).

Studies on the mycoparasitism of the antagonists reveals that the hyphae of *T. longibrachiatum* form small hook/peg like structures pressing on the host, coil loosely around the host hyphae directly or produce small branches that coiled tightly. On the other hand, with *T. virens*, the host hyphae intermingled; certain of the host hyphae cells, which were vacuolated, became empty. Further, the hyperparasite coil around the host hyphae and adhere through small appresoria-like structures which even penetrate them at certain points (*Manibhulhanrao et al.*, 1989b).

Baby (1992) further studied the biocontrol potential of *T. virens* (Tv1) by incorporating into soil the wheat-bran-saw dust (WBSD) preparation (15-d) at 10 to 25 g/kg soil and obtained 10 and 57% protection respectively. Incidentally, isolate Th1 showed strong inhibition to the pathogen *R. solani*. Hence, the quantity of commercial products of *Trichoderma* kg/ha can be determined based on the *R. solani* populations in soil. Recently, Jeyarajan and Nakkeeran (1995) discussed about the exploitation of a commercial product of *Trichoderma*. This product for field use could be safely stored for 75-d at a constant temperature of 20-30°C.

Kumaresan and Manibhushanrao (1991) studied the hyperparasitic potential of *T. longibrachiatum* isolates (Th 1 and Th 2) against *R. solani* to understand the associated mechanism(s) involved in the biocontrol efficacy of the antagonists. The isolate Th 1 showed extensive hyperparasitism leading to wall lysis and the protection against ShB fungus was more in *in vitro* studies, while Th 2 isolate surpassed Th 1 in rendering protection to rice plants under green house conditions. This ability of Th 2 might be due to more aggressive coiling of host hyphae. It is pertinent to mention here that in biocontrol strategy, any interference by direct introduction might not yield the microorganisms with the ecological balance of the soil microflora, might not yield the expected result over a long-term basis. However, the ecological balance of soil saprophytes can be manipulated by a suitable organic amendment to promote a specific group of soil microflora.

Mycoparasitism of rice ShB fungus, *R. solani* by *Trichoderma* spp. and *Gliocaldium* spp., is well known (Baby and Manibhushanrao, 1996a.b.) Among the many potential antagonistic soil fungi, *G. virens* and *T. longibrachiatum* of late have been used as biocontrol agents as they have been termed as presumptive and potential mycoparasites. Of late, the genus *Gliocladium* was merged with *Trichoderma,* hence *G. virens* is referred as *T. virens* in the subsequent literature.

It is generally opined that combination of two or more systems such as fungal antagonists, organic amendments, PAB and systemic fungicides provide high level control than that could be achieved with each method alone. This would expose the soil saprophyte (ShB fungus) to a 'double / triple barrier' whereby the resistance would protect the fungicide from a tolerant isolate of the pathogen and the fungicide would protect the resistance from novel compatible virulence. While the organic manure will increase the residential or introduced antagonists this will again reduce the level of primary inoculums as well as incidence of ShB disease.

A comprehensive monographic study on Rice Sheath blight disease was published by Manibhushanrao (1995) with emphasis on various methods of integrated control. *Trichoderma* species are known to have greater tolerance to a broad spectrum of fungicides are able to tolerate treated soil more rapidly than other soil competitors and are also effective against a wide spectrum of plant pathogens under various conditions.

As early as 60 years ago, it was opined that *Trichoderma* might serve as a potential biocontrol agent against soil-borne pathogenic fungi (Weindling and Emerson, 1936), and it has become an established fact in the following years (Baker, 1989; Adams, 1990; Lynch *et al.* 1991; and Haran *et al., 1996*) *Trichoderma* spp. are capable of effectively controlling a range of pathogens as well as being rhizospehere competent. They also provide varied levels of biocontrol of important soil-borne pathogens viz. *R. solani, Verticillium* spp.

Phythium spp. (Sivan and Chet, 1986 and Devaki *et al., 1992).* The efficacy of *Trichoderma* spp. as biocontrol agents of groundnut stem rot and root-rot diseases were reported (Sreenivasaprasad and Manibhushanrao, 1993).

In the damping off sugar beet caused by *P. aphanidermatum,* control was augmented by using *T. harzianum* and metalaxyl seed treatment (0.1%) simultaneously. However, the antagonist or metalaxyl alone did not yield the level of control (Mukhopadhyay and Chandra, 1986). When *Rhizoctonia ataticola* infested soil was infested with wild and tolerant strains of *T. harzinum* significantly lower disease incidence occurred to the control and other treatments. Significant reduction was observed under both the conditions (Vyas and Khare, 1986). *Trichoderma* sp. with outstanding ability to adapt to extreme conditions of temperature, soil and moisture and pesticides (fungicides) are used by various scientists in India.

Growth enhancement by the fungal antagonist *Trichoderma* spp. is an added advantage of biocontrol agents. In many of the claims from researchers from India, this potential is exploited and the reasons attributed are either due to the phytosanitizing effect or elimination of minor pathogens and clolonizers or due to growth promotive effect (Bagyaraj *et al.,* 1979, Elavarasan, 1989 and Kumaresan and Manibhushanrao, 1991).

Trichoderma spp. is a potential biocontrol agent for control of soil-borne diseases of small cardamom, the queen of species in India. Cardamom is affected severly by *Phytophthora meadi* causing capsule rot, rhizome rot caused by *Phythium vexans and R. solani. Trichoderma vifide and T. harzianum* proved to be effective under nurseries and in plantations (Joseph Thomas *et al., 1996).* Indian scientists are visualizing the commercial formulations of *Trichoderma* s for control of plant diseases. Coffee husk or tea waste and these two in combinations with farmyard manure supported luxuriant growth *T. viride.*

The biological control of important diseases of crop plants like pearl millet, rice, sunflower, maize and cash crop mulberry caused by *Sclerospora grtaminicola* can effectively be controlled using *Trichoderma viride, T. harzianum, Chaetomium globosum, Aspergillus niger* and *Bacillus subtilis* (Shishupala, 1988 and Shishupala and Shetty, 1989).

Due to the outstanding performance of *Trichoderma* spp. in the control of broad spectrum of plant pathogens, it is essential to improve the biocontrol potential through strain improvement. In India, a continuous attempt to release improved strains through genetic manupulation, protoplast fusion, DNA transformation, producing mutants etc., are in progress. The development of protoplast fusion technique allows recombination in the progeny with different characteristics from two or more parental strains. *Trichoderma* strains developed through protoplast fusion showed very high biocontrol potency and pesticide resistance. These strains exhibited changes at phenotypic and genotypic levels. Thus, development of fungicide tolerant / resistant strains can be successfully used in association with IPM concept (Viji *et al.*, 1993).

Application of wheat bran saw dust (WBSD) preparation of *Trichoderma* spp. has been useful to control several soil-borne, root-infecting pathogens under field conditions (Baby and Manibhushanrao, 1993). This study should be extended on a priority basis to rice ShB control. A suitable, cheap and freely available substrate is recommended for India marginal farmers. This concept is termed as transferring expertise from lab-to land or reaching the unreached. In another study, Sivan *et al.*, (1984) found that a mixture of peat and wheat bran was a much better substrate. Various kinds of formulations can be made which not only ensure survival but also promote activity of the biocontrol agent with the appropriate substrate (Chet *et al.*, 1979; and Sivan *et al.*, 1984). Though *Trichoderma* spp. especially *T. virens* and *T. harzianum* formulations are available in the market, the commercialisation is not popularised. In India, scientists need to work more to bring the commercial forms of microbial antagonists come out as viable and feasible alternative to bio-pesticides useful to farmers.

SUMMARY

1. A review of literature pertaining to this investigation has seen presented.

2. Materials used in this investigation and experimental procedures followed have been discussed in detail.

3. Pathogenicity of *Fusarium graminearum* was tested on eight varieties (Macs – 58, NRC – 12, PK – 416, MRC – 7, PK – 564, JS – 335 Soymax, UPSM – 19) of Soybean. Among these Soymax appeared to be highly susceptible and UPSM – 19 was found to be resistant.

4. Cultural Conditions affecting growth of *F. graminearum* were studied with special reference to their growth variable pH and temperature.

5. Polyclonal antebodies were raised against antigen preparations from mycelia of *F. graminearum*, *T. harzianum* and *T. viride*. Sulphate precipitation followed by DENE – cellulose chromatography. IgG obtained in each case was used for different immunoenzymatic tests.

6. To check the effectiveness of PAbs, agar gel double diffusion tests were performed using crude antibody as well as purified IgG obtained from three different bleedings. Strong precipitin reactions were observed in homologous cross-reaction of each case.

7. Optimisation of ELISA using PAbs of *F. graminearum, T. harizianum, T. viride* at variable concentrations were performed. ELISA values decreased with the decrease of antigen concentrations ranging from 62.5 to 8000 ng/ml.

8. DAC – ELISA tests were performed using PAbs raised against *F. graminearum* against root antigens prepared from eight varieties of Soybean, non pathogen and non host major cross reactive antigens.

9. Detection of *F. graminearum* in artificially inoculated Soybean root tissues using DAC – ELISA was performed. Pathogen detected in artificially inoculated plant using DAC – EISA format.

10. SDS – PAb analysis of soluble proteins of *F. graminearum, T. harzianum and T. viride* were done.

11. Using PAbs of *F: graminearum, T. harizianum* and *T. viride*, western blot analysis of these antigen preparations was done.

12. Dot blots were carried out to detect population of *F. graminearum, T. harizianum* and *T. viride.*

13. Indirect immunofluoresence study of hyphae and using PAbs of *F. graminearum* and FITC was carried out. Apple green fluorescence was observed in young hyphae.

14. Hyphae and conidia of *T. harzianum* was treated with homologous PAb and labelled with FITC for fluorescence.

15. Immunofluorescence study of *T. viride* hyphae and conidia were done using PAb and FITC conjugates. Yong hyphae gave strong fluorescence.

16. In vitro interaction was done between *F. graminearum* isolates and *T. harzianum* and *T. viride* isolates separately. Strong inhibition was observed.

17. In vitro study with biocontrol agent was done using three selected Soybean varieties. Isolates of *T. harzianum* and *T. viride* reduced disease markably.

18. Immunodetection of *F. graminearum, T. harzianum* and *T.* viride using variable immunonoassays viz DAC – ELISA, DOT – Blot, western blot and indirect immunofluorescence. was done.

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