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Serological detection of *Fusarium graminearum* Schwabe causing wilt disease of *Glycine max* (L.) Merill and its management

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Among eight varieties of soybean (*Glycine max*) tested for screening resistance towards *Fusarium graminearum* causing root rot disease, Soymax was found to be highly susceptible while UPSM-19 was found to be resistant. Three other varieties (viz. Macs-58, PK-564 and JS-335) were also found to be susceptible. Cultural characteristic of three isolates of *F. graminearum* (Fg1, Fg2, Fg3) *in vitro* revealed optimum growth after 15 days of incubation at 30^{0}C and at pH 6.5. Polyclonal antibody (PAb) was raised in white male rabbit against mycelial antigen of *F. graminearum* (isolate Fg1) and immunoglobulin (IgG) was purified. Effectiveness of raising antibody against the pathogen was checked by agar gel double diffusion test and confirmed by dot immunobinding assay. Optimization of antigen and antibody concentration was done using plate trapped antigen coated enzyme linked immunosorbent assay (PTA-ELISA). Immunodetection of *F. graminearum* in soybean root (Soymax) artificially inoculated with pathogen using PTA-ELISA format was developed. Treatment of mycelia and conidia of *F. graminearum* with homologous PAb and reacted with fluorescein isothiocyanate (FITC) showed a strong apple green fluorescence which was more intense on young hyphal tips. Following *in vitro* interaction study with one of the potential isolates of *Trichoderma harzianum* against *F. oxysporum*, field evaluation of this biocontrol agent was done by selecting three highly susceptible soybean varieties (Soymax, JS-335 and PK-564) against *F. graminearum. T. harzianum* was found to be very effective in reducing root rot disease index in all three soybean varieties tested.

Keywords : Dot immunobinding assay, *Fusarium graminearum*, Indirect immunofluorescence PTA-ELISA, soybean, *Trichoderma harzianum*

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is a major crop with hundreds of food, feed and industrial uses. Soybean seed, being a primary source of protein and vegetable oil, its products have become more important in formulating new, low cost nutritionally balanced high protein food 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 caused by *Fusarium graminearum* Schwabe occurs in most soybean growing areas of the world and is considered potentially destructive in the tropics and subtropics. The disease usually develops on seedlings and young plants. Older plants 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 cholotic 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. Among predominant species causing Fusarium head blight (FHB) one of the most destructive diseases of wheat (*Triticum aestivum*), *Fusarium graminearum* has been reported to be the most pathogenic (Saharan, 2023).

Increasing movement of plant materials in world-wide trade coupled with restrictions on the use of

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plant protection chemicals has emphasized the need for phytosanitary measures for controlling the spread of phytopathogens. Effective and stringent biosafety and biosecurity to safeguard of Indian Agriculture have been emphasized by Dubey (2022). 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 microorganisms include the development of more rapid diagnostic techniques with high specificity for the target organism (Chakraborty and Chakraborty , 2021; Preethi and Umesha, 2025).

Trichoderma spp. having multiple beneficial traits are being considered as nature's 'boon' to human beings, which if utilized suitably can lead to sustainable eco-friendly agriculture, replacing or supplementing the use of chemicals. *Trichoderma* has been exploited for biocontrol against phytopathogens . Genetic relatedness among *Trichoderma harzianum*, *T. asperellum* and *T. erinaceum* and their evaluation for management of Sclerotial blight of *Vigna radiata* (Dey *et.al*. 2020) and induced immunity developed by *Trichoderma* spp. in plants (Chakraborty *et.al*. 2020) have been elucidated. Rice health management by *Trichoderma* spp. and induction of phytoalexin accumulation have been demonstrated (Mukherjee, 2023; Khati and Chakraborty, 2023).

In the present study, polyclonal antibody based serological detection of *Fusarium graminearum* was developed using various immunological formats such as Agar gel double diffusion test, dot immunobinding assay, plate trapped antigen coated enzyme linked immunosorbent assay (PTA-ELISA) and indirect immuofluorescence. Attempts have also been made for *in vitro* interaction study with one of the potential isolates of *Trichoderma harzianum* against *F. oxysporum*, and subsequently field evaluation of this biocontrol agent for developing management strategies of root rot disease of soybean .

MATERIALS AND METHODS

Source of seeds

The seeds of eight varieties (Viz. Macs-58, NRC-12, PK-416, NRC-7, PK-564, JS-335, Soymax and UPSM-19) of soybean (*Glycine max*) were obtained from the Pulses and Oil seeds Research Station, Berhampore, West Bengal an stored at 20^0C and also at room temperature (30± 2^0C). 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 Seed Research Centre every year during the experimental periods.

Fungal culture

A virulent strain of *Fusarium graminearum* schwabe was obtained from the Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi. This isolate was assigned as Fg1 after completion of Koch's postulate and was used for raising polyclonal antibody. 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 plants 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).

Growth of plants

Healthy soybean seeds were treated with 0.1% $HgCl_2$ for 2 min. to remove superficial contaminants, washed several times with sterile distilled water and sown in earthen pots (10 seeds /pot of 10" dia) containing non-infested sandy soil (soil: sand 1:1). The plants were grown in the Phytopathological Experimental garden of the Department under natural conditions of day light and temperature (26-34^{0}C). The pots were watered daily with ordinary tap water. The plants were grown during March to October.

Assessment of mycelial growth

The fungal isolates of *F. graminearum* (Fg1, Fg2 and Fg3) were first grown in Petridishes each containing 20 ml of potato dextrose agar (PDA) medium and incubated for 7 days at 30^{0}C. Agar

block (4 mm dia) containing the mycelia was cut with a sterile cork borer from the advancing zone of mycelial mat and transferred to each Petridish containing 20 ml of sterilized PDA medium. Three isolates of *F. graminearum* were also grown in Richard's solution to determine the rate of mycelia growth, optimum pH and temperature. 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 0.03M concentration. The pH of the medium was adjusted using N/10 NaOH or N/10 HCL to obtain the corresponding range of pH values. Each flask (250ml) containing 50 ml of the medium was inoculated with mycelia block (4 mm dia) of *F. graminearum* isolates and incubated for 15 days at 28±2^{0}C.

Preparation of inoculum and inoculation techniques

Sick pot method as described by Nene *et al*. (1981) was adopted with modification. Earthen pots (10" dia) 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 and subsequently 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 the other hand, river bed sand and maize meal (9:1) were mixed and 25 ml water was added in each bag and sterilized two times (20lb 15 min). After cooling each bag was inoculated with *F. graminearum* and incubated at 28^{0}C for 10 days. The sand maize meal culture was thoroughly mixed with non infested sandy soil (1:1) kept in pots, watered and kept as such for 15 days. Surface sterilized soybean seeds were sown in each pot and disease intensity was assessed.

Disease assessment

Plants were examined after 10, 20, 30 days of inoculation. Disease intensity was aseessed on the basis of percentage loss in dry weight of roots as described by Chakraborty and Shil (1989). After desired period of incubation plants were uprooted, washed, dried at 60^{0}C for 96 h and weighed. Root rot index was calculated on the basis of percentage loss in dry weight of root in relation to control and they were graded into five groups and a value was assigned to each group (viz. 1-10% loss in weight = 1; 11-25% =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).

Extraction and estimation of soluble protein

Soluble mycelial proteins of *F. graminearum* were extracted following the technique as described by Chakraborty *et al*. (1995). Estimation of soluble protein was done following the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) as standard.

In vitro and in vivo study

Antagonistic properties of *Trichoderma harzianum* against *F. graminearum* was studied using dual plate method. Mycelial discs of 4 mm dia cut from the margin of 4-day-old cultures of both test pathogen (*F. graminearum*) and antagonist were placed opposite to each other on PDA in Petriplates (9"dia). The distance between inoculum block was 6 cm. Control sets were prepared both for *F. graminearum* as well as for *T. harzianum*. Five replications of each interaction were studied at 30^{0}C. In order to find out the efficacy of this biocontrol fungus (*T. harzianum*) to manage the root rot disease of soybean, experiments were designed both in glasshouse condition as well as in field conditions. Application of *T. harzianum* in soil was done at least 10 days before inoculation with pathogen.

SDS-PAGE analysis of total soluble protein

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of protein profile of *F. graminearum* was carried out following the method described by Sambrook *et. al*. (1989).

Preparation and purification of antigens

Mycelial and root antigens were prepared using the method as dscribed by Chakraborty and Purkayatha (1983) antigens were further purified

by 100% ammonium sulphate precipitation, dialyzed and stored at -20^{0}C. Soil antigen for microplate trapping and nitrocellulose blotting were prepared following the method of Walsh *et. al.,* (1996). Soil (1 g) was crushed in 2 ml of 0.05M sodium carbonate bicarbonate buffer (pH 9.6) in mortar and pestle and kept overnight at 4^{0}C. Subsequently it was centrifuged at 10,000 rpm for 10 min. Supernatant was used as antigen for experimental purposes.

Production of antibody

Polyclonal antibody (PAb) against mycelia antigen of *F. graminearum* (isolate Fg1) was raised in white male New Zealand rabbit using the techniques as described by Chakraborty *et.al.* (1995). Normal sera were collected from each rabbit before immunization. Antigen (1 ml) mixed with 1 ml Freund's complete adjuvant was intramuscularly injected first week followed by incomplete adjuvant weekly upto 12 weeks. Blood samples were collected by marginal ear vein puncture, 3 days after the first six immunization, kept at 37^{0}C for 1 h for clotting, followed by centrifugation at 5000 rpm for 10 min at room temperature. Purification of IgG was done as using method of ion exchange chromatography on a DEAE cellulose column as described by Clausen (1988). The concentration of IgG was determined of the selected fraction's absorbance at 280 nm and 260 nm using a standard formula (Jayaraman, 1996).

Agar gel double diffusion test

Agar gel double diffusion tests were performed using antigen and antiserum following the method of Ouchterlony (1967). The slides were cleaned, stained with Coomassie blue (R-250, Sigma), destained, dried and photograph taken.

Plate trapped enzyme linked immunosorbent assay (PTA-ELISA)

Mycelial antigen of *F. graminearum* isolates and root antigens of soybean varieties were diluted with coating buffer and loaded in 96 wells ELISA plate. PTA-ELISA format as suggested by Chakraborty *et al*. (1995) was followed. The plate was incubated at 25^{0}C for 4 h and washed with running tap water and twice with PBS tween. After that 200 ml blocking reagent was added to each well, further incubated at 25^{0}C for 1 h and washed again as mentioned earlier. Purified IgG raised against mycelial antigen of *F. graminearum* (isolate Fg1) was diluted (40 mg/ml) and 200 ml loaded in each well and incubated at 4^{0}C overnight. After a further washing, antirabbit IgG goat antiserum labeled with Alkaline phosphatase (diluted 10,000 times in PBS) was added (200 ml) to each well and kept in dark for 1 h. Colour development was stopped by adding 50 ml/well of NaOH (3N) solution and the absorbence was determined in an ELISA Reader (Labsystem multiscan) at 405 nm. Absorbance values in well not coated with antigens were considered as blank.

Dot immunobinding assay (DIBA)

Dot immunobinding assay using antigen and PAb of *F. graminearum* was performed on nitrocellulose membrane (NCM; Milipore, pore size – 0.45 m) as described by Lange *et. al*. (1989). The colour intensity of the dots as developed with Fast Red (Sigma) was noted.

Indirect immunofluorescence test

Indirect immunofluorescence was performed following the method of Chakraborty *et.al*. (1995). Mycelia and conidia of *F. graminearum* were washed with PBS tween (pH 7.2) and treated separately with normal sera and with PAb of *F. graminearum* diluted with PBS (3:10) and incubated for 1 h in room temperature. Specimens were washed again as before and treated with goat antirabbit (whole molecule) conjugated with fluorescein isothiocyanate (FITC) (Sigma) diluted with PBS (1:40) and incubated in dark for 45 min at room temperature. After incubation specimens were washed thrice and mounted in 10% glycerol. Then slides were examined and photographed using a Leica Leitz Biomed Microscope with fluorescence optics fitted with I-3 ultraviolet filter.

RESULTS

Pathogenicity test of F. graminearum

Pathogenicity of *F. graminearum* (isolate Fg1) was tested under glass house and field conditions

on eight varieties of soybean (viz. Macs-58, MRC-12, PK-416, NRC-7, PK-564, JS-335, Soymax and UPSM-19. 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 (Fig 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 as well as in the sick plot (Fig 2), Soymax was found to be highly susceptible while UPSM-19 was found to be resistant. Three other varieties (viz. Macs-58, PK-564 and JS-335) were also found to be susceptible. 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 (Fig.3).

Table 1. Effect of incubation period on the mycelial growth of *F. graminearum* isolates.

Incubation period (Days)	Average mycelial dry wt. (mg)[a]		
	Fg1	Fg2	Fg3
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

[a] Average of 5 replicates

Table 2. Effect of different pH on the mycelial growth of *F. graminearum* isolates

pH	Average mycelial dry wt. (mg)[a]		
	Fg 1	Fg 2	Fg3
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	534.0 ± 2.8	530.0 ± 2.8	552.8 ± 2.8
6.5	572.9 ± 3.3	566.5 ± 4.3	576.3 ± 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^{0}C
[a] Average of 5 replicates

Cultural conditions affecting growth of F. graminearum isolates

Mycelial growth behaviour on PDA medium and conidia structure of three isolates of *F. graminearum* has been presented in Fig. 4 (A-I). They were very fast growing on PDA medium, reaching 9 cm dia within 5 days at 28^{0}C, grayish rose to livid red to crimson 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 X 3.8 – 4.5 mm. Conidia slender falcate, moderately curved with pointed and curved apical and basal cells, mostly 5-6 septate and 40-60 x 4.0-5.8 mm. Chalmydospores scarce and often completely absent, mostly intercalary and in chains 10-12 mm dia. Germination of conidia on glass slides were observed after 20 h of incubation at 25^{0}C. Three isolates (Fg1, Fg2, Fg3)

Table 3. Effect of temperature on the mycelial growth of *F. graminearum* isolates

Temperature (^{0}C)	Average mycelial dry wt. (mg) [a]		
	Fg1	Fg2	Fg3
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	529.3 ± 3.2	608.5 ± 2.8
35	407.2 ± 3.3	411.5 ± 2.8	397.8 ± 3.6
40	149.9± 4.5	108.8± 3.6	125.6 ± 2.2

[a] Average of 5 replicates
pH of medium 6.5
Incubation period 15 days

Table 4. SDS-PAGE analysis of soluble proteins of *F. graminearum* isolates

F. graminearum Organism Molecular weight (kDa)	Isolates
Fg1	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
Fg2	72.4, 65.6, 42.0, 39.5, 31.1, 29.0, 25.2, 21.0, 19.5
Fg3	78.3,70.2, 68.0, 65.1, 63.2, 54.4, 39.5, 37.4, 31.1, 29.0

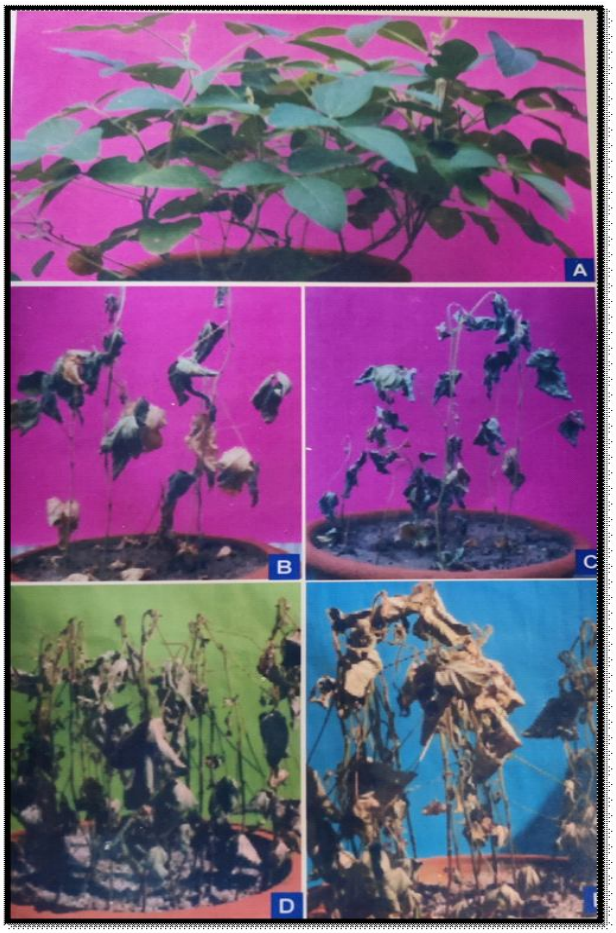

Fig. 1(A-E): Soybean plant (*Glycine max*) (A) Healthy,(B-E) Artificially inoculated with *F. graminearum*.(A&B) Soymax; (C) JS-335; (D) Macs-58; (E) PK-564

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

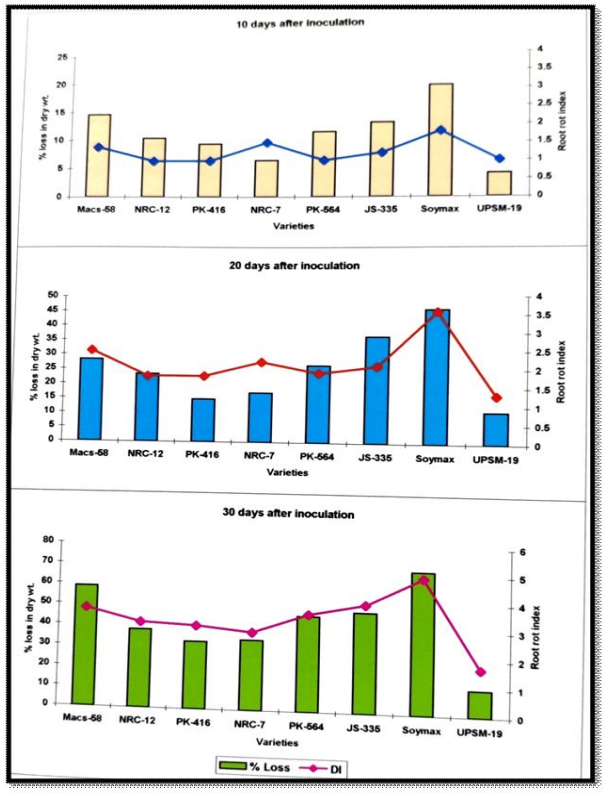

Fig. 3. Pathogenicity test of *Fusarium graminearum* on different soybean varieties

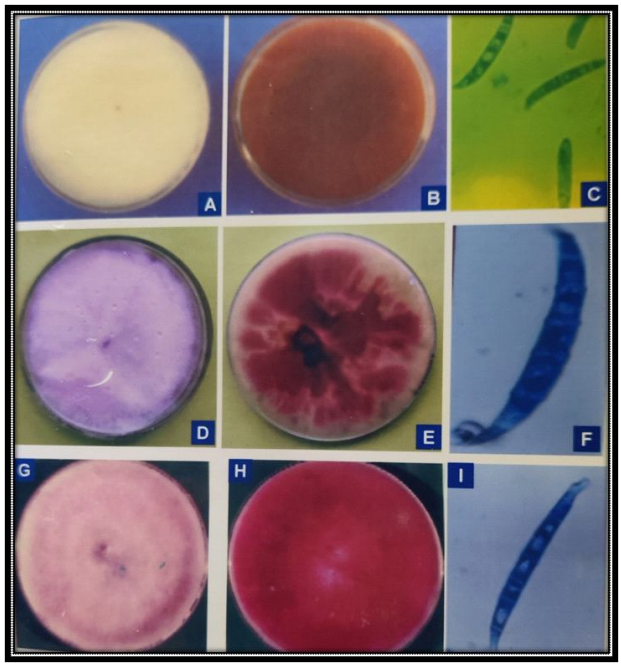

Fig. 4 (A-I): Mycelial growth of *F. graminearum* isolates on Potato Dextrose Agar medium. Front view (A,D,G), back view (B,E, H) and Macroconidia X850 (C,F,I). Isolates Fg3 (A-C), Fg 1(D-F), Fg2 (G-I).

of *F. graminearum* were further grown in Richard's medium for a period upto 30 days at 28±2^{0}C to determine the rate of mycelia growth, optimum pH and temperature. The mycelia growth of isolates were recorded after 5,10,20,25 and 30 days. Maximum growth was observed after 15 days of incubation and then rate of growth declined (Table 1). Mycelial growth of three isolates were studied from 4 to 8 pH. It appears from the results (Table 2) 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. Further, *F. graminearum* isolates were grown in Richard's medium adjusting the optimum pH 6.5 for 15 days at different temperatures ranging from 20^{0}C to 40^{0}C. Results (Table 3) revealed that *F. graiminearum* grew over a wide range of temperature, however, maximum growth was noted at 30^{0}C and then there was gradual decline.

SDS-PAGE analyses of soluble proteins of F. gramenearum isolates

Mycelial proteins were prepared from three isolates of *F. graminearum* (Fg1, Fg2 and Fg3) and purified. Soluble proteins were analysed by SDS-Polyacrylamide gel electrophoresis. Molecular weight of proteins resolved on gel for three isolates of *F. graminearum* have been presented in Fig .5 and Table 4.

Antigen antibody reaction in Immuno-diffusion test

Effectiveness of mycelial antigen of *F. graminearum* (isolate Fg1) for raising polyclonal antibody (PAb) was checked by homologous cross reaction following immunodiffusion test. Control set using normal sera and mycelial antigen of pathogen was negative. Strong precipitin reactions were evident (Fig 6 A) in homologous reaction. When mycelial antigens of three pathogen isolates (Fig1, Fg2, Fg3) along with mycelial antigens of non pathogens of soybean (*Glomerella cingulata, Pestalotiopsis theae* and *Corticium invisum*) were reacted in agar gel double diffusion test against PAb of *F. graminearum* , strong reactions were observed against all three pathogen isolates but no precipitin reactions were evident against all three non pathogens of soybean (Fig 6 B).

Dot immunobinding assay

PAb raised against *F. graminearum* (isolate Fig.1) exhibited strong colour reaction on nitrocellulose paper (Fig. 7, lane 3) in its homologous reaction with mycelial antigen. Mycelial antigens of two other isolates of *F. graminearum* (Fg2, Fg3) also showed positive colour reactions (Fig. 7, lane 1 and lane 2). In reciprocal cross reaction with PAb of *F. graminearum* and root antigens extracted from artificially inoculated highly susceptible two soybean varieties (Soymax and Macs-58) also exhibited positive colour reaction (Fig 7, lane 4 and lane 5).

Optimization of antigen and PAb concentrations using PTA-ELISA format

Optimization of PAb raised against *F. graminearum* was done using PTA-ELISA format considering two variables i.e. concentration of antigen and concentration of PAb (IgG). In each case three bleedings were collected and IgG were purified. IgG for first, second and third bleedings of *F. graminearum* were used for optimization. Doubling dilution of mycelial antigen ranging from 62.5 to 8000 ng/ml tested against 1:125 and 1: 250 dilution of PAb of Fg1. ELISA values decreased with the decrease of antigen concentration but the values were quite high indicating the range of detection could be much lower (Fig. 8). Double dilution of IgG purified from PAbs obtained from three bleedings were also tested against homologous antigens at a concentration of 10 mg/ml. Absorbance values decreased from 1:125 to 1:16000 dilutions. However, absorbance values increased with the different bleedings (Fig.9).

Detection of F. graminearum in soybean root tissue using PTA-ELISA

Root antigens were prepared from healthy plants of eight varieties of soybean. Plants were artificially inoculated with *F. graminearum* and 10 days after inoculation plants were uprooted and root antigens were prepared from inoculated plants of each varieties. These were analysed

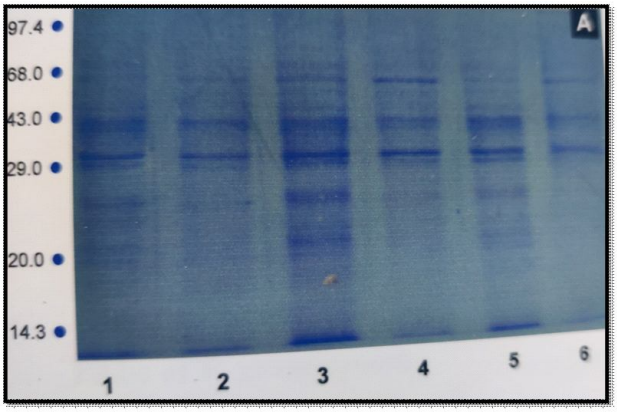

Fig. 5: SDS-PAGE analysis of mycelia proteins of *F. gramineraum* isolates Fg2 (lanes 1&2), Fg1(lane 3) and Fg3 (Lanes 4-6)

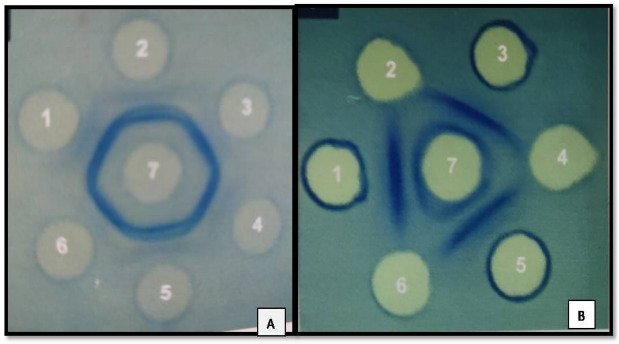

Fig. 6. Agar gel double diffusion test. Central wells (7) loaded with PAb of *F. graminearum* (A&B) (A) Peripheral wells (1-6) were loaded with homologous antigens (B) Peripheral wells were loaded with mycelial antigens of pathogen isolates (1) Fg1, (3) Fg2,(5) Fg3,Mycelial antigens of non pathogens (2) *G.cingulata*, (4) *P. theae*, (6) *C. invisum*

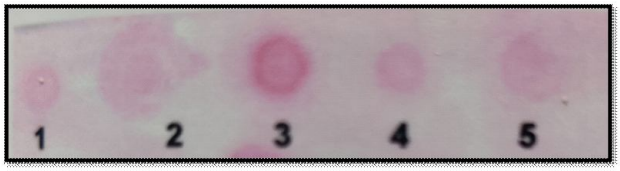

Fig. 7. Dot immunobinding assay of mycelial antigens of *F. graminearum* isolates (1) Fg2, (2) Fg3, (3) Fg1, Artificially inoculated soybean root antigens of (4) Soymax, (5) Macs58 and PAb of *F. graminearum* on nitrocellulose paper.

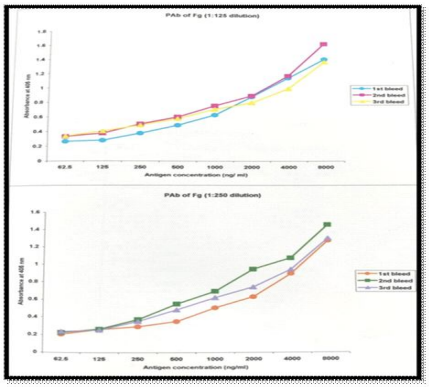

Fig. 8. Optimization of mycelial antigen concentration**s** of *Fusarium graminearum* using PTA-ELISA format

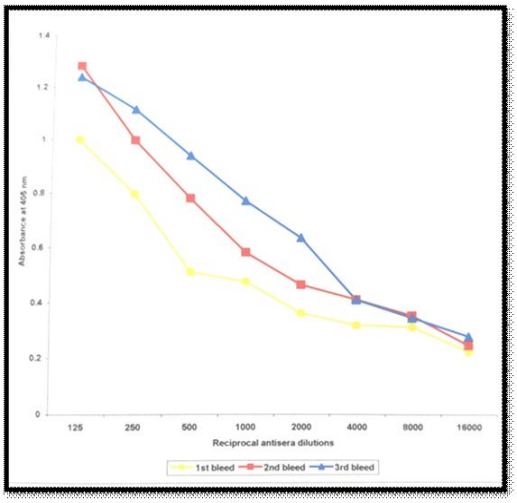

Fig. 9 : Optimization of PAb concentrations of *Fusarium graminearum* using PTA-ELISA format

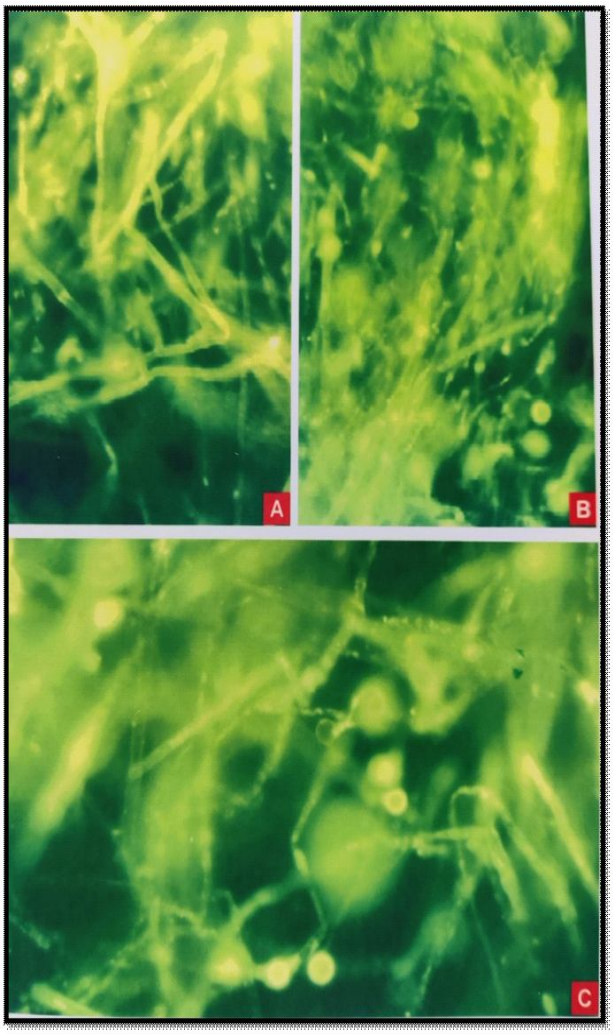

Fig. 10. Indirect immunofluorescence of hyphae and conidia of *F. graminearum* treated with PAb of Fg1 and stained with FITC antibodies of goat specific for rabbit globulin. (A) Isolate Fg2, (B) Isolate Fg3, (C) Isolate Fg1.

using PTA-ELISA format. Results have been presented in Table 5. Besides, one of the highly susceptible variety (Soymax) plants were artificially inoculated with the pathogen and root antigens were prepared from inoculated plants 24 h after inoculation and continued up to 10 days following inoculation. Each time healthy plant root antigens were prepared and ELISA reactions were performed using PAb of *F. graminearum* (1:250 dilution). Absorbance values were recorded and results have been presented in Table 6.

Table 5. PTA-ELISA responses (A 405 nm) of healthy and inoculated root antigens of soybean varieties against PAb of *F. gramineraum*

	PAb of *F. grammineraum*	
Soybean varieties	Healthy root antigen	Inoculated root antigen
Macs 58	0.637 ± 0.06	1.208 ± 0.05
NRC 12	0.696 ± 0.02	1.334 ± 0.02
PK 416	0.703 ± 0.04	1.418± 0.03
NRC 7	0.617 ± 0.07	1.376 ± 0.02
PK 564	0.699 ± 0.03	1.571 ± 0.01
JS 335	0.642 ±0.05	1.652 ±0.05
Soymax	0.705 ±0.02	1.987 ±0.04
UPSM 19	0.683 ± 0.05	1.106 ±0.06

Average of 5 replicates; ± = Standard error

Table 6 . PTA-ELISA responses (A 405 nm) of PAb of *F. gramineraum* with healthy and artificially inoculated root antigens of Susceptible variety (Soymax) at different interval

Time after inoculation (Days)	PAb of *F. graminearum* (1:250 dilution) Root antigen	
	Healthy	Inoculated
1	0.680 ±0.02	0.736 ± 0.03
2	0.649 ± 0.08	0.781 ± 0.05
3	0.651 ± 0.07	0.830 ± 0.04
4	0.656 ± 0.03	0.918 ± 0.02
5	0.671 ± 0.03	1.108 ± 0.05
6	0.688 ± 0.02	1.121 ± 0.09
7	0.712 ± 0.04	1.206 ± 0.06
8	0.697 ± 0.06	1.312 ± 0.03
9	0.776 ± 0.02	1.451 ± 0.04
10	0.782 ± 0.08	1.752 ± 0.06

Average of 5 replicates; ± = Standard error

Indirect immunofluorescence of mycelia and conidia of F. graminearum

Mycelia and conidia of *F. graminearum* did not show autoflorscence nor did it fluoresce when treated with normal serum followed by reaction with FITC. Treatment of mycelia and conidia of *F. graminearum* with homologous PAb and reacted with FITC showed a strong apple green fluorescence which was more intense on young hyphal tips while conidia and chlamydospore showed a general fluorescence throughout the surface (Fig 10).

In vitro interaction of T. harzianum against F. graminearum and its field evaluation for management of root rot of soybean

Microscopic observation of *Trichoderma harzianum* revealed hyaline, colonies bearing repeatedly branched conidiophores in tuft with divergence, often irregularly flask shaped phialides. Grow best in daylight and in the dark fungus quickly loose the capacity to sporulate. Colonies were reaching over 9 cm diameter in 5 days at 20^0C. Conidia are subgloboe to short oval, measuring approximately 2.8 X 3.2 mm. *In vitro* interaction of *T. harzianum* against *F. graminearum* (isolates Fg1, Fg2, Fg3) have been presented in Fig 11. *T. harzianum* formed an inhibition zone around the *F. graminearum* and pathogen was not able to grow further. Field

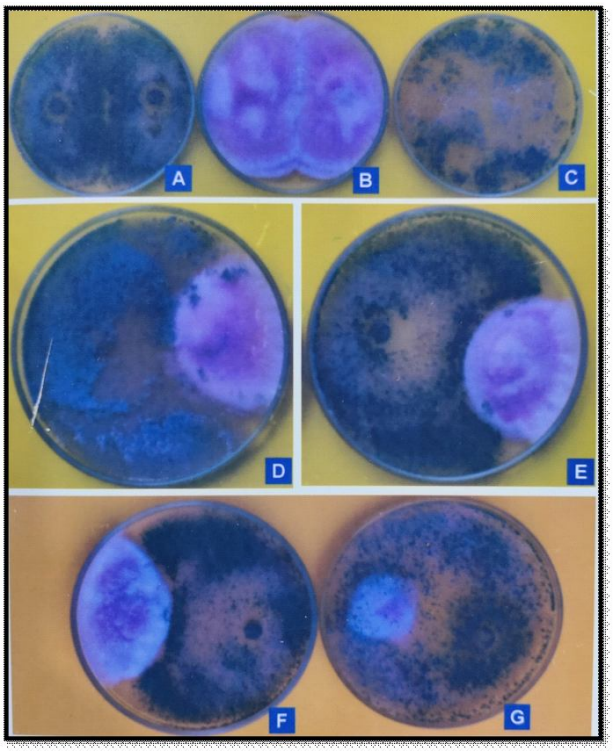

Fig.11. Homologous pairing of *Trichoderma harzianum* (A&C), *Fusarium graminearum* (B). *In vitro* interaction of *T. harzianum* with *F. graminearum* isolates Fg1 (D&E), Fg2 (F) and Fg3(G).

Table 7. Effect of *Trichoderma harzianum* on development of root rot disease of soybean

Soybean Variety	Root rot index of infected roots [a] *Treatments*	
	F. graminearum	*T. harzianum* + *F. graminearum*
Soymax	4.6	1.3
JS 335	3.8	2.2
PK564	3.6	1.5

[a] In relation to control- 30 days after inoculation with *F. graminearum*

Root rot index : 1-10% loss =1
11-25% loss =2
26-50% loss =3
51-75% loss = 4
76-100% loss = 5

evaluation of the *T. harzianum* (BCA) was done using three highly susceptible soybean varieties (Soymax, JS-335 and PK-564) following soil application of biocontrol agent (BCA) 10 days prior to inoculation with *F. graminearum* (isolate Fg1). Ten replicates of each treatment were taken. Root rot index of infected roots were calculated after 30 days of inoculation. Results (Table 7) revealed that *T. harzianum* was found to be very effective in reducing root rot disease index in all three soybean varieties tested.

DISCUSSION

Among eight soybean varieties tested for screening resistance towards *F. graminearum,* Soymax was found to be highly susceptible while UPSM-19 was found to be resistant. Three other varieties (viz. Macs-58, PK-564 and JS-335) were also found to be susceptible. Since the growth and infectivity of the pathogen (*F. graminearum*) depends on a number of environmental factors, effect of some of those factors on mycelial growth of three isolates of *F. gramineraum* (Fg1, Fg2, Fg3) were investigated. Maximum growth was observed after 15 days of incubation and then rate of growth declined. *F. graminearum* grew over a range of pH 5.5 – 7.0 and showed optimum growth at pH 6.5 while the pathogen grew over a wide range of temperature, however, maximum growth was noted at 30^0C and then there was gradual declined.

Detection of phytopathogens causing plant diseases using various serological formats are gaining ground in case of fungal diseases. Both disease detection and diagnosis of plant disease is rapidly increasing by using immunological formats (Chakraborty and Chakraborty, 2021). Effectiveness of mycelial antigen of *F. graminearum* in raising antibody was evaluated using agar gel double diffusion test and dot immunobinding assay. Development of polyclonal antibody based immunodetection of *Curvularia lunata* causing leaf blight of *Persea bombycina* (Acharya *et. al.* 2021) and *Sphaerostilbe repens* causing violet root rot disease of *Camellia sinensis* (Das *et. al*., 2022) using serological assays have also been demonstrated as rapid and sensitive method for pathogen detection. Effectiveness of mycelial antigens of *Ustulina zonata* (Chakraborty *et.al.* 2002a), *Fomes lamaoensis* (Chakraborty *et.al.* 2002b), *Bipolaris sorokiniana* (Chakraborty *et. al*. 2016), *Dreschlera oryzae* (Khati and Chakraborty, 2019) and *Sclerotium rolfsii* (Bhagat and Chakraborty, 2020) in raising PAb against these fungal pathogens were also evident as a strong colour reaction on nitrocellulose paper in dot immunobinding assay. Since ELISA is a very sensitive technique and non-specific binding interferes with the actual antigen-antibody reaction, initially PAb raised against *F. gramineraum* (isolate Fg1) was purified and IgG fractions were used in all immunoassays. Initially optimization of minimum detectable antigen concentration and optimum IgG concentration were determined in homologous reactions using PTA-ELISA formats and this format has also been used for detection of *F. gramineraum* in healthy and artificially inoculated soybean root tissues. Mycelia and conidia of *F. gramineraum* when treated with homologous PAb and reacted with FITC labeled antibodies of goat specific for rabbit globulin, strong fluorescence was evident in young hyphae and conidia. Similarly, treatment of mycelia and spore of *Exobasidium vexans* causing blister blight of tea (Chakraborty and Sharma, 2007) and other phytopathogens (Chakraborty and Chakraborty, 2021) with their respective homologous PAbs followed by FITC labeling developed bright apple green fluorescence which was more intense on young hyphal tips and spore wall. Serological detection of *Aspergillus niger*, a dominant storage fungus isolated from seeds of *Vigna radiata*, *Cajanus cajan* and *Lens culinaris* has been worked out

which would be of help in seed storage mamagement (Palit *et. al*. 2023).

One of the potential isolates of *Trichoderma harzianum* inhibited mycelia growth of *F. oxysporum,* when interacted *in vitro.* When field evaluation of this biocontrol agent was done by selecting three highly susceptible soybean varieties (Soymax, JS-335 and PK-564) against *F. graminearum, T. harzianum* was found to be very effective in reducing root rot disease index in all three soybean varieties tested. Induced immunity developed by *Trichoderma* spp. in plants by accumulation of defense enzymes such as β-1,3,glucanase, chitinase and peroxidase have been illustrated by Chakraborty *et.al*. (2020). *Trichoderma* strains obtained from tree bark have been utilized for health improvement of rice crop (Mukherjee, 2023). Resistance induced in rice plants following application of *Trichoderma harzianum* against *Drechslera oryzae* and accumulation of phytoalexin has been demonstrated by Khati and Chakraborty (2023).

DECLARATION

Conflict of Interest. Authors declare no conflict of interest.

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