Materials & Methods

3. Materials and Methods

3.1. Plant Material

3.1.1. Collection

Twenty five tea varieties collected from three experimental station of India viz. (a) Darjeeling Tea Research Centre, Kurseong, West Bengal (b) Tocklai Experimental Station, Jorhat Assam and (c) United Planter's Association of South India (UPASI) Tea Research Station Valparai, Tamilnadu, being maintained in the Tea Germplasm Bank of the Department of Botany, University of North Bengal were used for experimental purposes.Among these, seven were Darjeeling varieties (S-449, HV-39, T-135, AV-2, CP-1, BS/7A/76 and P-1258), eleven Tocklai varieties (Teen Ali 17/1/54, TV-9,22, 23, 25, 26, 27,28, 29 and six UPASI varieties (UP-2,UP-3, UP-8, UP-9, UP-26 AND BSS-2).

3.1.2. Propagation

Tea plants were propagated by cutting. Soil preparation is most important in propagation technique and hence, care was taken to prepare the soil well. Sandy soil (75% sand and 25% soil) with a pH ranging 4.5-4.9 was used . Soil pH was adjusted applying 2% aluminium sulphate solution followed by leaching with water to remove excess aluminium sulphate.

Polythene sleeves (8"x6") were filled up with the prepared soil and stacked in rows in bed and watered thoroughly. Beds were arranged in two rows, eleven in each row. Two hundred and fifty cuttings of 25 varieties were allowed for rooting in individual sleeves after dipping them in rooting hormone. Sleeves of each bed were covered with polythene cloche and the whole setup was kept under a green agro house (Plate 2,figs A-D).

3.1.3. Maintenance of tea sleeves in nursery

After the removal of the polythene cloche, the sleeves were treated with nursery mixture (nutrient) as suggested by Ranganathan and Natesan (1987). The composition of the nursery mixture is as follows :

Ammonium Sulphate	–	8 parts by weight.
Ammonium Phosphate-Sulphate(16:20)	–	35 parts by weight
Potassium Sulphate	–	15 parts by weight
Magnesium Sulphate	–	3 parts by weight
Zinc Sulphate	–	3 parts by weight

Thirty grams of the above mixture was dissolved in 1 litre of water. Each sleeve was treated with 50ml of the nutrient mixture upto 12 months once in 15 days.

3.1.4. Plantation

In the experimental plot simazine @75g / 20 l. water and Glyophosphate @1:200 were used for weed control (Borpujari and Banerjee, 1994). Then pits (1½' x 1½' x 1½') were dug at the intervals of 2' between plants and 3.5' between row to row. Planting mixtures were prepared in the ratio of 4.5 kg of well rotten dry cattle manure, 30 kg. rock phosphate, 30 kg. super phosphate and 2.5g phorate [O,O-diethyl S- (ethylthiomethyl) phosphor dithoioate]. At the bottom of each pit, rock phosphate was placed following which half portion was covered with cattle manure-soil mixture. Phorate was mixed with a portion of excavated soil and was applied approximately 5cm below the ground level. Following the soil preparation, the plants were inspected, selected and brought to the experimental garden and planted in the prepared soil. For experimental purposes, seedlings were also transferred to earthen pots (12" dia). Soil mixture was prepared in the same ratio as above and each pot was filled with the mixture. Ten month old seedlings with well-developed shoot and root system were transferred from the sleeves to the pots. These were then maintained in the pots with regular watering.

The mature plants (1 year above were maintained by applying a soluble mixture of N,P,K consisting of 10kg urea (46%N),20 kg ammonium phosphate (11% P_2O_5) 8kg murate of potash(60% K_2O) in the soil. Miraculin (7ml/ 10L) was sprayed at regular intervals for good growth of bush.

Only tipping was done once in year to promote lateral branching in young plants (three years) but in case of mature plants two year of deep pruning cycle was maintained.

Plate 2 (Figs. A-E) : Nursery grown tea saplings

3.2. Fungal Cultures

3.2.1. Source of Cultures

Sphaerostilbe repens B & Br. was obtained from Tocklai Experimental Station Jorhat, Assam. The culture was maintained on PDA (potato dextrose agar) medium by regular subculturing. Other pathogens used for the experimental purposes mentioned table 1.

Table 1 : List of fungal isolates used

Species	Host	Source
Sphaerostilbe repens	*Camellia sinensis* (L).O. Kuntze	Tocklai Experimental Station Jorhat, Assam
Ustulina zonata	*Camellia sinensis*	Tocklai Experimental Station Jorhat, Assam
Fomes lamaoensis	*C. sinensis*	Tocklai Experimental Station, Jorthat,Assam
Rosellinia arcuata	*C. sinensis*	UPASI Tea research Station, Valparai
Poria hypobrumea	*C. sinensis*	UPASI Tea research Station,Valparai
Fusarium oxysporum	*Glycine max*	Indian Agricultural Research Institute New Delhi
Metar hizium anisopliae 892	*Scolytus scolytus*	Immuno-Phytopathology Laboratory Dept. of Botany, N.B.U.
M. anisopliae-140	*Scolytus scolytus*	Immuno-Phytopathology Laboratory Dept. of Botany, N.B.U.
Beauveria bassiana 2028	*Scolytus scolytus*	Immuno-Phytopathology Laboratory Dept. of Botany, N.B.U
B. bassiana-135	*Scolytus scolytus*	Immuno-Phytopathology Laboratory Dept. of Botany, N.B.U
Trichoderma viride	Rhizosphere soil of *C. sinensis*	Isolated from Matigara Tea Estate
T. harzianum	Rhizosphere soil of *C. sinensis*	Isolated from Hansqua Tea Estate
Sclerotium rolfsii		
S.rolfsii-1	*Glycine max*	UBKV, Cooch Behar
S. r. - 2	*Cajanus cajan*	UBKV, Cooch Behar
S.r. 3	*Pisum sativum*	UBKV, Cooch Behar
S.r. 4	*Tagetes patula*	UBKV, Cooch Behar

3.2.2. Assessment of mycelial growth

Mycelial growth was assessed both on solid media and liquid media for various experimental purposes.

3.2.2.1. Solid media

To assess the growth of *S. repens* in solid media, the fungus was first grown in petridishes, each containing 20ml of PDA and incubated for 8 days at room temperature. Agar Block (6mm dia) containing the mycelia was cut with a sterile cork borer from advancing zone of mycelial mat and transferred to each petridish containing 20ml of sterilized solid media. The different solid media used for assessment of growth were prepared following the method of Dhingra and Sinclair (1985).

- Potato dextrose agar (PDA)

Peeled potato	–	40.00g
Dextrose	–	2.00g
Agar	–	2.00g
Distilled water	-	100ml

- Patato sucrose agar (PSA)

Peeled potato	-	40.00g
Sucrose	-	2.00g
Agar	-	2.00g
Distilled water	-	100ml

- Richards agar (RA)

KNO_3	–	1.00gn
KH_2PO_4	–	0.50g
$MgSO_4, 7H_2O$	–	0.25gn
Sucrose	–	3.00g
Agar	–	2.00g
Distilled water	–	100ml

- Carrot Juice Agar (CJA)

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

- Elliot's Agar (EA)

KH_2PO_4	–	.136g
$MgSO_4, 7H_2O$	–	0.050g
$Na_2 CO_3$	–	.106g
Dextrose	–	.500g
Asparagine	–	.10g
Agar	–	1.5g.
Water	–	100ml.

- Czapek Dox agar (CDA)

$NaNO_3$	-	0.20gn
K_2HPO_4	-	0.10g
Kcl	-	0.05g
$FeSo_4. 7H_2O$	-	0.05g
Sucrose	-	30.00g
Agar	-	2.00g
Distilled water	-	100ml

- Yeast extract dextrose agar (YDA)

Yeast extract	–	0.75g
Dextrose	–	2.0g
Agar	–	1.5g
Distilled water	–	100ml

All the petriplates were then incubated at 30^0C and colony diameter and sporulation behaviour of the fungi were studied at 3 days interval for 12 days.

3.2.2.2. Liquid media

The fungus was first allowed to grow in petridishes containing 20ml of PDA and were incubated at 30^0C for 7 days. From the advancing zone, the mycelial block (6mm) was cut with a sterilized cork borer and transferred to each Ehrlenmeyer flask (250ml) containing 50 ml of sterilized liquid midia. For assessment of growth in liquid media Richard's medium was used in all experiments with desired modification. And in all cases mycelia were strained through muslin cloth, collected in aluminium foil cup of known weight, dried at 60^0C for 96h, cooled in dessicator and weighed.

3.3. Inoculation technique

3.3.1. Preparation of Inoculum

(a) Pathogen

The inoculum of *S. repens* was prepared in sand maize meal media and in tea root pieces. Sand maize meal (SMM) media was prepared following the method described by Biswas and Sen (2000). SMM medium (150 gm) in autoclavable plastic bag (maize meal: washed sterilized soil : water = 1:9:1.5) was sterilized at 120 lb for 20 min. Inoculum in tea root pieces was prepared following the method of Hazarika *et. al.* (2000). First root and rootlets were cut into small pieces (1½"). Root pieces (6-7) were taken in 250 ml flask and pieces were dipped into water for 2h and autoclaved in the same way. On the next day water was decanted. All media were inoculated with *S. repens.*

(b) Biocontrol agents

Inoculum of *Trichoderma* species prepared in several media- wheat bran media (wheat-bran : sand-1:1,and 25ml of water for 150g of inoculum in each poly packet), saw dust media (saw dust and water), tea waste media (tea waste and water).Media were autoclaved and inoculated as above.

3.3.2. Inoculation of healthy tea plants

3.3.2.1. Pot grown plants

Pathogen

Two year old tea plants were planted in earthen pots (12") containing 5 kg soil and allowed to be established. Regular watering was done for two weeks and then 100 g of *S. repens* inoculum was added carefully in the rhizosphere of each plant. Disease assessment was done after 10, 20 and 30 days of inoculation.

Biocontrol agents

Inoculation with *Trichoderma* sp. was also done as described above, but atleast 10 days prior to inoculation with *S repens*. Experiments were designed considering different treatments as as follows : a) pathogen(*S repens*) only b) *T. harzianum*, c) *T. viride*, d) *S repens*+ *T. harzianum* e) *S repens* + *T. viride*, f) healthy plants. Two year old tea plants (Teen Ali-17/1/54) were taken in this experiment. For each treatment three replications were taken.

3.3.2.2. Field grown plants

Inoculation and different treatments of field grown plants was same as described for potted plants, except that, in this case, 300 g inoculum was added in each pit, After inoculation plants watered and maintained. Disease assessment was done after 15,30 and 45 days of inoculation.

3.4. Disease assessment

Disease intensity of violet root rot was assessed on the basis of above ground and underground symptoms together on a scale of 1-6: 0-no disease; 1-plants look sick and root surface starts roughening in patches; 2-most of the leaves withered or looking yellow,light black patches with rough surface appear on root; 3-defoliation starts with random yellowing, roots inky black with random patches; 4-random defoliation, upto 70% roots become black; 5-total defoliation,70-85% blackening of roots and 6-total defoliation with drying of shoots, 85-100% blackening and drying of roots.

3.5. Soluble protein

3.5.1. Extraction

3.5.1.1. Mycelia

Mycelial protein was prepared following the method of Chakraborty and Saha (1994). Initially the inoculum (6mm disc containing mycelium) was transferred to 250 ml Ehrlenmeyer flask each containing 50ml of sterilized liquid Richard's medium and incubated for 7 days at 30 $\pm 1^0$C. For extraction of soluble protein, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (25g fresh wt.), were homogenized with 0.05 M sodium phosphate buffer (7.2) supplemented with 10mM sodium metabisulphite and 0.05mM magnesium chloride in mortar with pestle in the presence of sea sand. Cell homogenates were kept overnight at 4^0C and then centrifuged (10000rpm) for 15 min. at 4^0C. This was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at 4^0C. Different fraction of mycelial protein, 0-20%, 20-40%, 40- 60 , 60-80% and 80-100% was also collected following the method Jayaraman (1996). After this period the mixture was centrifuged (10000rpm) for 15 min at 4^0C, the precipitate was dissolved in 5ml 0.05 M sodium phosphate buffer (pH=7.2). The preparation was dialysed for 72h. through cellulose tubing (Sigma chemical Co.USA) against 1L of 0.005 M sodium phosphate (pH=7.2) with ten changes. Then the dialysed material was stored at -20^0C and used as antigen for the preparation of antiserum and other experiments.

3.5.1.2. Root protein

Roots collected from the experimental garden were washed thoroughly and cut into small pieces. Roots were then weighed and crushed in mortar with pestle using sample buffer (1.M tris (pH6.8) 5ml; 10mM β mercaptoethanol 0.5ml; 10% SDS-2ml and 7ml H_2O). At the time of crushing sea sand and insoluble PVPP of equal weight was used. The root slurry was centrifuged at 10000 rpm for 30min at 4^0C. The supernatent was immediately used for SDS-PAGE analysis.

3.5.2. Estimation

Soluble proteins were estimated following the method as described by Lowry *et. at.* (1951). To 1ml of protein sample 5ml of alkaline reagent (0.5ml of 1% $CuSO_4$ and 0.5ml of 2% sodium potassium tartarate added to 50ml of 2% Na_2CO_3 in 0.1(N) NaOH)

and incubated at room temperature; and then 0.5ml of Folin Ciocalteau reagent (diluted 1:1 with distilled water) was added and again incubated for 15 min for colour development following which absorbance was measured at 720nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.6.3. SDS-PAGE analysis of total soluble protein

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for the detailed analysis of protein profile following the method of Laemmli (1970).

3.6.1. Preparation of stock solutions

The following stock solutions were prepared :

A. Acrylamide and N'N' - methylene bis acrylamide

A stock solution containing 29% Acrylamide and 1% bis acrylamide was prepared in warm water. As both of them are slowly deaminated to acrylic and bis acrylic acid by alkali and light, the pH of the solution was kept below 7.0 and the stock solution was filtered through Whatman No. 1 filter paper and was kept in brown bottle, stored at 4^0C and used within one month.

B. Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

C. Tris buffer

(a) 1.5M Tris buffer was prepared for resolving gel, (pH adjusted to 8.8 with conc. HCl and stored at 4^0C) for use.

(b) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer (pH adjusted to 6.8 with concentrated HCl and stored at 4^0C).

D. Ammonium Persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

E. Tris-Glycine electrophoresis buffer

This running buffer consists of 25mM Tris base, 250mM glycine (pH-8.3) and 0.1% SDS. A 1xSolution can be made by dissolving 3.02g Trisbase, 18.8g glycine and 10ml of 10% SDS in IL of distilled water.

F. SDS Loading buffer

This buffer contains 50mM Tris HCl (pH-6.8), 10mM β mercaptoethanol, 2% SDS, 0.1% bormophenol blue, 10% glycerol. A 1x solution was made by dissolving 0.5ml of 1M Tris buffer (pH 6.8), 0.5 ml of β mercaptoethanol (14. 4M), 2ml of 10% SDS, 10mg bromophenol blue, 1ml glycerol in 6.8ml of distilled water.

3.6.2. Preparation of Gel

Slab gels (mini, plate size 8cm x 10cm) were prepared for the analysis of protein patterns by SDS-PAGE. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5mm thick spacers were placed between the glass plates at the three sides, and were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing stock solutions in the following order and poured leaving sufficient space of the stacking gel (Comb+1cm). Composition of solutions were as follows :

	Solutions	Amount
1.	Distilled water	2.95 ml
2.	30% Acrylamide mix	2.50 ml
3.	1.5 M Tris (pH 8.8)	1.90 ml
4.	10% SDS	0.075 ml
5.	10% APS	0.075 ml
6.	TEMED	0.003 ml

12% resolving gel was prepared mixing the stock solutions in following order.

	Solutions	Amount
1.	Distilled water	2.45 ml
2.	30% Acrylamide mix	3.00 ml
3.	1.5 M Tris (pH 8.8)	1.9 ml
4.	10% SDS	0.09 ml
5.	10% APS	0.07 ml
6.	TEMED	0.003ml

The gel was immediately overlayered with isobutanol and kept for polymerization for 1h. After polymerization of the resolving gel, isobutanol was poured off and washed with water to remove any unploymerized acrylamide. Then stacking gel solution (5%) was prepared by mixing the stock solutions in the following proportion. Composition of 5% stacking gel was as follows:

Solutions		Amount
1.	Distilled water	2.1 ml
2.	30% acrylamide mix	0.5 ml
3.	1M Tris (pH 6.8)	0.38 ml
4.	10% SDS	0.03 ml
5.	10% APS	0.03 ml
6.	TEMED	0.003 ml

Stacking gel solution was poured over the resolving gel and comb was inserted immediately and overlayered with water. The gel was kept for 30 minutes. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was then mounted in electrophoresis apparatus. Tris-glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom was removed very carefully with a bent syringe.

3.6.3 Sample preparation

Sample (34µl) was prepared by mixing the sample protein with 1xSDS gel loading buffer (16µl) in cyclomixer. All the samples were floated in boiling water bath for 3min, the denature the protein sample. The samples were immediately loaded in a pre-determined

order into the bottom of the wells with a 100 T micropippet syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging in molecular weight from high to low (Phosphorylase b-97,400; Bovine serum Albumin-68,000 Ovalbumin-43,000; Carbonic Anhydrase 29,000 Soybean tripsin inhibitor 20,000; Lysozyme 14,300 daltons) was treated as the other samples and loaded in separate well.

3.6.4 Electrophoresis

Electrophoresis was performed at constant 18mA current for a period of 2 hrs 15min. until the dye front reached the bottom of the gel.

3.6.5. Fixing and staining

After electrophoresis, the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid : methanol : water (10:20:70) for overnight. The staining solution was prepared by dissolving 250mg of Coomassie brilliant blue (Sigma R 250) in 45ml methanol. After the stain was completely dissolved, 45ml of water and 10ml of glacial acetic acid were added. The prepared stain was filtered through Whatman no 1. filter paper.

The gel was removed from fixer and stained in this staining solution for 4h at 37^0C with constant shaking at a very low speed. After staining, the gel was finally destined in destainer solution containing methanol, water and acetic acid (4.5:4.5:1) at 40^0C with constant shaking until back ground became clear.

3.7. Preparation of antigen

3.7.1. Fungal antigen

3.7.1.1. Mycelia

Mycelial antigen was prepared following the method of Chakraborty and Purkayastha (1983) described earlier in soluble protein extraction procedure.

3.7.1.2. Cell wall

Isolation of cell wall from *S. repens* was done following the method of Keen & Legrand (1980). Mycelial mat of 9 day old culture was collected on filter paper using Buchner funnel and 40g of fresh packed cells were ground with water (4ml/g) for 1min in a high speed blender The resulting slurry was then disrupted in homogenizer for 1 min at

5^0C. The mixture was centrifuged for 1 min at 2000 rpm, the supernatant fluids discarded, and the sedimented walls washed with sterile distilled water (10ml/g) and pelleted by several centrifugation until the supernatant fluids were visually clear. Finally, the isolated cell walls were dissolved in 0.05M phosphate buffer (pH) and kept at -20^0C until further requirement. This cell wall antigen was used for immunization.

3.6.1.3. Spore antigen

Twelve day old cultures of *S. repens* growing in Richard's broth were shaken well and culture filtrate strained with muslin cloth. Culture filtrates were then centrifuged at 5,000 r.p.m. (4^0C) for 5min. Spores were washed 3 times in PBS (0.05M, pH 7.0) and stored inchilled at 4^0C for 30min. and then spores were crushed in mortar with pestle at 4^0C and mixed again in cyclomixer, and after centrifugation at 10,000 rpm (4^0C) for 10 min supernatant was used as spore antigen for immunization.

3.7.1.4. Culture filtrate

Twelve days old culture filtrate free from spore was saturated with 100% ammonium sulphate under constant stirring and kept overnight at 4^0C. Further steps of culture filtrate protein preparation was as in case of mycelia.

3.7.2. Root antigen

Antigens from healthy and infected roots were prepared following the method of Chakraborty and Purkayastha (1983. with modification. Roots were collected from the experimental pots and field and thoroughly washed in water and cut into pieces. Root pieces were weighed and homogenised in grinder with 0.05 M sodium phosphate buffer containing 2mM PVP-10 (Soluble), 10mM sodium metabisulphite and 0.5 mM magnesium chloride. Insoluble PVPP was also added during homogenization. The homogenate was then kept at 4^0C overnight. Following this, the slurry was once again crushed in mortar with pestle and centrifuged at 10,000 r.p.m. at 4^0C and supernatant was used as antigen.

3.7.3. Soil antigen

Soil antigen for microplate trapping and blotting were prepared following the method of Walsh *et. al.* (1996). 1gm of soil was crushed in 2 ml of 0.05µl sodium carbonate-bicarbonate buffer (pH 9.6) in mortar with pestle and kept overnight at 4^0C. Next day it

was centrifuged at 10.000 rpm for 10min. Supernatant was used as antigen for experimental purposes.

3.8. Cellwall characterization

3.8.1. Preparation of cell wall extract

To get mycelial wall extract from isolated cell wall, method described by Brown & Kimmins (1977) has been followed. Isolated cell walls (2g) were suspended in 80ml ice cold 0.1 (N) NaOH by blending in a chilled mixer cup at full speed for 30 seconds. The suspension was stirred in ice bath for 15h and then centrifuged at 8000g for 10min. After centrifugation, the precipitate was washed with 50ml ice cold water and the supernatant was neutralized to 7.0 by adding 1(N) HCl slowly with continued stirring at 0^0C. The neutralized supernatant was finally dialysed against distilled water for 48h. with 12 hourly changes and concentrated with polyethylene glycol 6000 (PEG-6000), which was then used as crude mycelial wall extract for SDS-PAGE analysis followed by Western blotting.

3.8.2. Protein estimation

Protein content of cell wall extracted from *S. repens* mycelia was estimated following the method as describe above in 3.5.2.

3.8.3. Carbohydrate estimation

Carbohydrate in extracted cell wall was estimated following Anthrone method. To 8ml of cell wall extract 1ml each of 0.3M $Ba(OH)_2$ and 5% $ZnSO_4$ e was added. The mixture was incubated for 10min. Following centrifugation, the supernatant was taken. Into the supernatant (2.0ml) 1.8ml of distilled water and finally 6ml of Anthrone reagent (200mg Anthrone powder dissolved in 100ml of concentrated H_2SO_4) was added. Then it was kept in a boiling water bath for 15min, cooled and absorbance was noted at 620nm. Glucose was used as a standard during the estimation of carbohydrate.

3.8.4. SDS-PAGE analysis

All the steps for SDS-PAGE analysis were same as described above , only fixing and staining were done in different ways. After electrophoresis gel containing mycelial and cell was protein with replica one was fixed in solution 1 (25% isopropanol in 10% acetic acid) or in fixer solution II (40% ethanol in aqueous 5% acetic acid) for protein and

carbohydrate staining overnight. Gels from fixer soulution 1 were stained with commassie blue R250 and then destained as describe earlier. Gels from fixer solution II were stained with periodic acid-Schiffs (PAS) reagent as described by Segrest and Jackson (1972) with modification. Details of PAS staining solution and the procedure was as follows :

At first five following solution A-E were prepared.

Solution A : Anhydrous sodium acetate (0.84gm and hydroxylamine hydrochloride (10g) were dissolved in 90 ml distilled water. Glacial acetic acid (0.54 ml) was added to it final volume was adjusted to 100ml with distilled water.

Solution B : Periodic acid (1.0gm) and anhydrous sodium acetate (0.82g) were dissolved in distilled water and final volume was made 100ml.

Solution C : 1.5g basic Fuschin was dissolved in 500ml boiling distilled water, filtered at 55^{0}C, cooled to 40^{0}C, 25ml 2N HCl was added and finally 3.75g $Na_2S_2O_5$ (Sodium metabisulphite) was added, agitated rapidly and allowed to stand stoppered in refrigerator for 6h, 1.2g charcoal was mixed to it vigorously for 1min. filtered rapidly and stored in refrigerator.

Solution D (Prepared before use) : 10% (w/v) sodium metabi sulphite (5ml) and 2N HCl (5ml) was dissolved in 90ml distilled water before use.

Solution E (Prepared before use) : 10% (w/v) sodium metabi sulpite (5ml) and 2 N HCl (5ml) was dissolved in 90ml distilled water and 20ml glycerol was added to it before use.

Procedure : Gels were soaked in solution 'A' for 15min then washed in running tap water. Next the gels were soaked in solution B for 15 min, washed in running tap water for 10 min. and then transferred to solution 'C' (diluted 1:1 with distilled water just before use) for washed three times for 1h. each time in solution 'E'.

3.8.5. Binding of FITC labelld concanavalin - A

Binding of fluorescent labeled concanavalin A to mycelia, cell wall as well as spore was done by the method as described by Keen and Legrand (1980). Initially mycelia and spores were incubated for 20 min. in 0.85% Nacl in 0.01M potassium phosphate buffer,

pH 7.4 containing 1mg/ ml. fluorescein isothiocyanate (FITC) labeled concanavalinA (ConA Sigma Chemicals). The hyphae or the spores were then washed thrice with saline solution by repeated low speed centrifugation and resuspension. For control sets these were incubated with lectin supplemented with 0.25M α-methylmannoside. All preparations were viewed under Leica photomicroscope equipped with epi-fluorescence optics (BP450-490 exciting filter, RKP-520 Beam splitting mirror, 515 suppression filter) Photographs were taken by Leica WILD MPS 32 camera 800 ASA film.

3.9. Agglutination response of spores

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

3.9.1. Preparation of spore suspension

Agglutination tests were done with ungerminated spores. For this, fungus was allowed to grow in liquid RM medium for 12 days at 28 ± 2°C . Spores were collected from the above grown culture as described before (3.6.1.3) in PBS to a concentration of approximately $5x10^6$/ml. The spore suspensions were used immediately after preparation.

3.9.2. Agglutination test

Ungerminated spore suspension (10µl) were kept in a moist chamber at room temperature for various incubation times (upto 2h). During incubation, slides were gently swirled several times to ensure maximum cellular contact. Agglutination of spore was observed under Leica Leitz Biomed microscope in bright field and arbitrarily scaled from 'O' (no-agglutination) to "4" (maximum agglutination).

3.10. Serology

3.10.1. Rabbits and their maintenance

New Zealand white male rabbits were used to raise antisera separately against initially, the body weights were recorded and were observed for at least one week inside the cages

before starting the immunization schedule. They were regularly fed with green grass, soaked gram seeds, green vegetables etc. twice a day. After each bleeding they were given saline water for 3 consecutive days and cages were cleaned everyday in the morning for better hygenic conditions.

3.10.2. Immunization

Before immunization, normal sera were collected from each rabbit. Separate rabbits were intramascularly injected once a week at 7 days interval with 1ml antigen mixed with 1ml of Freund's complete adjuvant (Difco, USA) for first two injections followed by incomplete adjuvant upto 12 weeks.

3.10.3. Bleeding

Bleeding was performed by marginal ear vein puncture, 3 days after the first six injections, and then after every fourth injection. In order to handle the rabbits during bleeding, they were placed on their backs on a wooden board fixed at a angle of 60^0. The neck of the rabbit was held tight in triangular gap at the edge of the board, and the body was fixed in such a way that the rabbit could not move during the bleeding. The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol, the ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5 to 10ml of blood samples were collected in sterile graduated glass tube.

After collection, all precautionary measures were taken to stop the flow of the blood from the puncture. The blood samples were kept as such at 37^0C for 1hr for clotting, following which, the clot was loosened with a sterile needle. Finally, the serum was clarified by centrifugation (2000g for 10min at room temperature) and distributed in 1ml vials and stored at- 20^0C.

3.10.4. Purification of IgG

3.10.4.1. Precipitation

IgG was purified as described by Clausen (1988). Crude antiserum (2ml) was first diluted with two volume of distilled water and an equal volume of 4M ammonium sulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16h at 22^0C. The precipitate

thus formed was collected by centrifugation at 10,000g at 22^0C for 1hr. Then the precipitate was dissolved in 2ml of 0.02M sodium phosphate buffer, pH 8.0.

3.10.4.2. Column preparation

Eight gram of DEAE cellulose (Sigma Co USA) was suspended in distilled water for overnight. The water was poured off and the DEAE cellulose was suspended in 0.005M phosphate buffer (pH 8.0) and the washing was repeated for 5 times. The gel was then suspended in 0.02M phosphate buffer, (pH 8.0) and was transferred to a column (2.6cm in diameter and 30cm high) and allowed to settle for 2hr. After the column material had settled, 25ml of buffer (0.02M sodium phosphate, pH8.0) washing was given to the column material.

3.10.4.3. Fraction collection

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02M to 0.3M. The initial elution buffer(1) was 0.02M sodium phosphate buffer (pH 8) whereas final elution buffer(2) was 0.3M sodium phosphate buffer(pH 8.0) . The buffer was applied in a flask on which one rubber connection from its bottom was supplying the column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column, buffer (2) was soacked into buffer (1) thereby producing a continuous raise in molarity. Ultimately, 40 fractions each of 5ml were collected and the optical density values were recorded at 280nm using UV VisSpectrophotometer (DIGISPEC-200GL) .

3.10.4.4. Estimation of IgG concentration

IgG concentration was estimated as described by Jayaraman (1996). Absorbance was taken at 280nm and 260nm and then concentration of IgG was calculated by the following formula : Protein concentration (mg/ml) = 1.55XO.D. 280nm – 0.76X O.D. 260nm.

3.11. Immunodiffusion tests

3.11.1. Preparation of agarose slides

Glass slides (5cm x 5cm) were degreased in 90% (v/v) ethanol, ethanol: diethylether (1:1) and then dried in hot air oven. After drying, plates were sterilized. Agarose gel was

prepared in Tris barbiturate buffer, pH 8.6 at 90^0C, 0.9% agarose (Sigma, USA) was added into the buffer and placed on a water bath and stirred till the agarose solution became clear. Into the clear agarose solution 0.1% (w/v) sodium azide was added. For gel preparation, 10ml of molten agarose was added per slide, after pouring, it was kept for solidification and then wells were cut with a sterilized cork borer (6mm dia).

3.11.2. Diffusion

Agar gel double diffusion tests were performed following the method of Ouchterlony (1967). The antigens and undiluted antisera (100µl/well) were pipetted directly into the appropriate wells and diffusion allowed to continue in moist chamber for 27hr at 25^0C.

3.11.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water for 2h and then aqueous NaCl solution (0.9% NaCl + 0.1% NaN_2) for 72h with 6 hourly changes to remove unreacted antigens and antiserum widely dispersed in the agarose. Then slides were stained with coomassie blue (R 250) for 10 min at room temperature. After staining, slides were destained with destain solution (methanol : water : acetic acid (45:45:10) with changes until the background became clear. Finally, the slides were washed with distilled water and dried in hot air oven for 3h at 50^0C.

3.12. Enzyme linked immunosorbent assay (ELISA)

The following buffers were prepared following the method as described by Chakraborty *et. al.* (1995).

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

Stocks

A. Sodium Carbonate – 5.2995g in 1000ml dist water

B. Sodium bicarbonate – 4.2g in 1000ml dist. water.

160ml of stock A was mixed with 360 ml of stock B and pH was adjusted to 9.6

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

Stocks

A. Sodium dihydrogen phosphate – 23.40g in 1000ml dist. water

B. Disodium hydrogen phosphate – 21.2940 in 1000ml dist water.

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

3. 0.15M Phosphate buffer Saline Tween (0.15M PBS – Tween, pH 7.2).

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

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

0.05M Tris, 0.135M NaCl, 0.0027M KCl

Tris - 0.657g

Nacl - 0.81g

KCl - 0.223g.

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

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

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

6. p-Nitrophenyl phosphate (Himedia) 1mg/ml in substrate buffer (1.0% [w/v] diethanolamine, 3mM NaN_3 ph 9.8).

3.12.1. Direct antigen coated (DAC) ELISA

ELISA was performed following the method as described by Chakraborty *et. al.* (1995). Plant and fungal antigens were diluted with coating buffer and the antigens were loaded (200µl/well) in 96 welled ELISA plate (Costar EIA/RIA, strip plate USA) arranged in 12 rows in a (cassette) ELISA plate. After loading, the plate was incubated at 25^0C for 4 hrs. Then the plate was washed 4 times under running tap water and twice with PBS Tween and each time shaken to dry Subsequently, 200µl of blocking reagent was added and incubated at 25^0C for 1hr. After incubation, the plate was washed as mentioned earlier. Puriified IgG was diluted in antisera dilution buffer and loaded (200µl/well) to each well and incubated at 4^0C overnight. After a further washing, antirabbit IgG goat antiserum labelled with Alkaline Phosphatase (diluted 10,000 times in PBS) was added to each well (200µl/well) and incubated at 37^0C for 2hrs. The plate was washed, dried and loaded with 200µl of p-Nitrophenyl Phosphate substrate in each well and kept in dark for 60 mins. Colour development was stopped by adding 50µl/well of 3N NaOH solution and

the absorbance was determined in an ELISA Reader (Labsystem, Multiskan) at 405mm. Absorbance values in wells not coated with antigens were considered as blanks.

3.12.2. Double antibody sandwich ELISA

Conjugation of alkaline phosphatase with γ-globulin :

Labelling of γ globulin with alkaline phosphatase has been done following glutaraldehyde one-step method. 5mg of Alkaline phosphatase enzyme was thoroughly mixed with 2mg of IgG. The total volume was made upto 5ml with PBS. It was kept at room temperature for 30min. with occasional shaking. Following this, 0.2% gluteraldehyde was added to the mixture and was again incubated for 2h with gentle stirring. Finally it was dialysed 3 times against 500ml ½ strength of PBS. After dialysis, bovin serum albumin (about 10mg/ml) + 0.02% NaN_3 was added and stored at 4^0C until required.

DAS ELISA was performed following the method of Brill *et. al.* (1994). Antisera of *S. repens* was diluted in coating buffer and loaded (200µl/well) in each well of ELISA plate. The plate was incubated for 4h at 37^0C and washed 4 times by flooding the wells with PBS-Tween, plate was shaken to dry. Then 200µl of blocking agent (1% BSA and 3mM NaN_3) was added to each well to overcome the nonspecific binding and plate was incubated at 25^0C for 1h. After blocking, plate was washed as mentioned earlier. Then test samples were added to empty well (200µl/well) and incubated overnight at 4^0C. On the next day plate was washed as before and alkaline phosphatase tagged IgG diluted in PBS, pH 7.2 was added to each well and incubated for 4h at 25^0C. Then plate was washed and 200µl pNPP substrate (1mg/ml) was added to each well and incubated for 90min. at 25^0C in dark. Colour development was stopped by adding 50µl/well of 3N NaOH solution. Absorbance values were recorded at 405 nm in an ELISA reader (Labsystems, Multiskan).

3.12.3. Competition ELISA

Competition ELISA was carried out in 96 well ELISA plate (Labsystems Finland) following the method as described by Lyons &White (1992). 3 sets were prepared each with 3 replicas for *S. repens, Trichoderma harzianum* and *T. viride*. All wells of one set was loaded with 100µl *S. repens* antigen, another with *Trichoderma harzianum* antigens and the 3rd set with *T. viride* antigen. The antigens were diluted in PBS before loading. Following incubation at 4^0C overnight, the plates were washed four times in running tap

water and twice in PBS-Tween, and were dried. Blocking of non-specific binding was achieved with 5% BSA in PBS, 200µl/well for 1h. The plates were washed and dried as before and stored at 4^0C until required. Test antigens were prepared from tea rhizosphere soils inoculated with *S. repens, T. harzianum* and *T. viride*, either simply or in different combination. These samples were added (75µl/well) to wells of 3 ELISA plate sets. To each of these wells, 75µl of *S. repens* or *T. harzianum* or *T. virde* IgG (40µg/ml) was added. The plates were incubated at 37^0C for 1h on a shaker. (The shaker was also used for all subsequent stages). At the end of this period 100µl of antigen / antiserum mixture was transferred to the corresponding wells on the pre-prepared test plates. Unreacted antiserum was added to control wells and the plates were incubated for 45 min. The plates were washed and 100µl antirabbit IgG alkaline phosphatase conjugated antiserum was added to all wells of the plates. Following 30min. incubation at 37^0C plates were washed and 100µl of p-Nitrophenyl phosphate substrate (1mg/ml) was added to each well. After a further 40 min. incubation in dark absorbance values were read at 405nm in an ELISA reader (Labsystems, Multiskan).

3.13. Immunoblotting

3.13.1. Dot-Blot

Dot-blot was performed following the method suggested by Lange *et. al.* (1989). 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, 0.5 NaCl, 0.5% Tween-20, pH-10.3

Nitrocellulose membrane (NCM; millipore, H5SMO 5255, 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 mins to dry. Following this 2µl of test samples (antigen samples) were loaded into the template wells over the NCM and kept for 3 hrs 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. Polyclonal antibody (IgG) of *S. repens* (1:500) was added directly in the blocking solution and further incubated at 4^0C 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 Phosphatase Conjugate (1:7500) for 2h a 37^0C. This was followed by washing for 25 min. in TBS-Tween. Substrate [1 tablet each of Tris buffer and Fast Red (Sigma chemicals)or NBT/BCIP tablet (Sigma) dissolved in 10 ml double distilled water] was next added and colour development noted. Finally, reaction was stopped by floating the NCM in deionized water. Colour development was categorized on the intensity of the dots.

3.13.2. Western blotting

Western blotting has been performed following the method of Sambrook *et. al.* (1989).

Stock Solutions

[A] All the solutions for SDS-Gel preparation for western blotting were as mentioned earlier in SDS-Gel electrophoresis

[B] Transfer buffer (Towbin buffer)

25 mM Tris, 192mM glycine in 20% reagent grade Methanol, pH 8.3.

Tris – 3.03g.

Glycine – 14.4 g.

200ml Methanol (adjusted to 1 lit. with dist. water)

[C] Phosphate buffer saline (0.15M) pH 7.2) PBS was made as mentioned previously.

[D] Blocking solution :

Casein hydrolysate – 5% in PBS

Sodium Azide – 0.02%

Tween -20 – 0.02%

[E] Antibody dilution : PBS = 14 ml; Blocking solution = 6 ml.

[F] Washing buffer (50mM Tris Cl, 150mM NaCl) pH-7.5.

Tris – 6.07 gm

NaCl – 8.78 gm

Distilled water upto 1 lit. made.

[G] Alkaline phosphatase buffer (100mM Tris -HCl, 100mM NaCl, 5mM $MgCl_2$ pH-9.5)

Tris – 12.14gm pH adjusted

NaCl – 5.84gm

$MgCl_2$ – 1.015gm

Double distilled water made upto 1 lit.

[H] Enzyme

Alkaline phosphatase tagged with antirabbit goat IgG alkaline phosphatase buffer enzyme diluted in (1:10000).

[I] Substrate :

5mg NBT in 100µl of 70% N,N, dimethyl formamide

2.5mg BCIP in 50µl of 100%, N,N, dimethyl formamide

Substrate solution was prepared by adding 66µl NBT and 33µl BCIP

10ml alkaline phosphatase buffer

[J] Stop solution : (0.5M EDTA in PBS) pH 8.0.

EDTA sodium salt - 0.0372gm in 200µl water PBS - 50 ml.

Procedure

Extraction of protein : Protein extraction was done as described earlier.

3.13.2.1. SDS-PAGE

SDS-PAGE was carried out as mentioned earlier.

3.13.2.2. Transfer process

Following the gel run, it was transferred to Towbin buffer and equilibrated for 1h. The filter paper (Bio-RAD, 2.5mm thickness) and nitrocellulose membrane (BIO-RAD, 0.45µm) were cut as same size of the gel and soaked in Towbin buffer for 20min. The transfer process was done in Trans-Blot SD Semi-Dry Transfer cell (BIO-RAD) through BIO-RAD power pack. The presoaked filter paper was placed on the platinum anode of the Semi-dry cell and prewetted membrane was placed over the filter paper. Bubbles between the filter paper and membrane were rolled out with a glass rod. Gel was carefully placed on the membrane and bubbles were removed as above and finally another presoaked

filter paper was placed on the top. The cathode was placed on the sandwich and pressed. The unit was run for 45min at 14 volt. After the run the membrane was dried for 1h and proceeded for immunological probing.

3.13.2.3. Immunoprobing

Blocking was done by 5% non fat dried milk in a heat sealable plastic bag and kept for 90min. with occasional shaking. Subsequently the membrane was put in antibody solution (diluted in PBS and blocking solution, 1:40) and incubated in plastic bag at 4^0C overnight. All the processes were done with occasional shaking. Next day the membrane was washed thrice in 250ml PBS. Final washing was done in Tris-Cl (50mM Tris, 150ml NaCl pH=7.5) to remove azide and phosphate from the filter before enzyme coupled reactions. Enzyme was added (1:1000 in alkaline phosphatase buffer) and kept 1h at room temperature. After enzyme incubation, membrane was washed thrice again in Tris-buffer saline, and 10ml substrate was added. The reaction was monitored carefully and when bands were observed up to the desired intensity the filter was transferred to a tray of stop solution.'

3.14. Fluorescence antibody staining and microscopy

3.14.1. Mycelia

Fungal mycelia were grown in liquid Richard's medium as described earlier. After 4 days of inoculation young mycelia were taken out from the flask and kept in eppendrof tube. After washing with PBS (pH 7.2), mycelia were treated with normal sera or antisera diluted (3:10) PBS, (pH 7.2) and incubated for 1h. at room temperature. Then mycelia were washed thrice with PBS-Tween (pH 7.2) as mentioned above and treated with goat antirabbit IgG (whole molecule) conjugated with fluorescein isothiocyanate (sigma) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45min at room temperature. After incubation, mycelia were washed thrice in PBS (ph 7.2) and mounted in 10% glycerol. A cover glass was placed on mycelia and sealed. Then slides were observed and photographed under both phase-contrast and UV fluorescence condition using Leica Leitz Biomed microscope with flourescence optics equipped with ultra violet (UV) filter set 1-3.

3.14.2. Spores

Fungal spores were collected from 12 days old culture and a suspension of this was prepared with PBS, pH-7.2. Spore suspensions were taken in micro-centrifuge tubes and

centrifuged at 3000g for 10min and the PBS supernatant was discarded. Then 60µl of IgG diluted in PBS (1:1) was added to the spores and incubated for 90 min at room temperature. After incubation, tubes were centrifuged at 3000g. 10min. and supernatant was discarded. The spores were rewashed 3 times with PBS-Tween (pH 7.2) by centrifugation as before and 40µl of goat antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and incubated in dark for 1h. After the dark incubation excess FITC antisera was removed by repeated washing with PBS Tween pH 7.2 and the spores were mounted on 10% glycerol jelly and observed under Leica microscope, equipped with 1-3 UV-fluorescence filter. Photographs were taken as described before.

3.14.3.Cross-section of tea roots

Initially, cross sections of healthy and infected tea roots were cut and immersed in phosphate buffer saline (PBS) pH 7.2. These sections were treated with normal serum or antiserum diluted in PBS (1:40) and incubated for 1h. at room temperature. After incubation, sections were washed thrice with PBS-Tween (pH 7.2) for 15 min and transferred to 40µl of diluted (1:40) goat antiserum specific of rabbit globulins and conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30min in dark. After that sections were washed thrice with PBS-Tween as mentioned above and then mounted on a grease free slide with 10% glycerol. Flourescence of the root sections were observed using Leica Leitz Biomed microscope with fluorescence optics equipped with UV-filter set 1-3. Tissue section were photographed under both phase contrast and UV-fluorescent conditions for comparison of treatment.

3.15. Immunocytochemical staining

The combinatin of immunochemical and histochemical techniques is feasible for the development of a staining procedure capable of locating specific fungi in host tissue. Subsequent to location of CRA in tea root tissues by immunocytochemical studies, and detection of *S. repens* by ELISA and immunoblotting, immuonocytochemical staining was performed to determine the specific location of *S.repens* in tea root tissues. For this, the method of Young and Andrew (1990) was followed. In this process the root sectinos of infected roots were made and incubated in 1% BSA solution for 15 minutes to prevent nonspecific binding of antibodies to root tissue. The root sections were rinsed (30s) three times with washing solution. Then sections were incubated for 1hr.at 37^0C on a rotary

shaker in *S. repens* PAb at a dilutin of 1:100. After incubation the root segments were washed as above, then incubated for 1hr. at 37^0C on a rotary shaker in goat antirabbit IgG (1:5000) dilution in direct ESLISA buffer containing 0.1% BSA) conjugated with alkaline phosphatase (Sigma). After incubation, the roots were washed as before, incubated in naphthol-AS-phosphate plus fast blue BB substrate solution for 40 minutes at 37^0C in the dark. Substrate solution consisted of 0.15 gm naphthol-AS-phosphate (Sigma) dissolved in 2.5ml of N-N-dimethyl formamide (Sigma), which was added to 500ml of Tris buffer (17g of Tris in 500ml distilled water, pH 9.1), this solution considered as stock solution. The staining solution, prepared immdiately before use, consisted of 1mg of fast blue (Himedia) and 5µl of 0.1M $MgCl_2$ added per ml of stock solution and filtered through Whatman No.1 filter paper. After washing the sections in PBS, were mounted in glycerol jelly and observed under bright field of the microscope (Leica Leitz Biomed) and photographs were taken in bright field.