Discussion

Host pathogen interaction occurring in nature is one of the most complex biological processes and involves recognition between the host and the pathogen both at the cellular and subcellular level. A cell that reacts in a special way in consequence of an association with another cell or its products does so because it acquires information which is conveyed through chemical or physical signals in the process of recognition. Plants in nature have evolved a series of mechanisms by which they can defend themselves against the multitude of organisms attacking them. The versatile multicomponent defense is adequate to provide them protection against most of their potential pathogens; only a few of them can overcome this defense and cause disease (Sinha,1995). Varieties within the host species are resistant when they possess one or more resistant gene (s) and susceptible when they lack any such genes. The spatial and temporal deployment of plant defense responses involves the complex interplay of signal events, often resulting in superimposition of signalling processes (Graham and Graham ,1996). Inspite of lacking immune responses like animals, plants have nevertheless evolved immune mechanisms of various types by which they can counter the advance of foreign organisms. The result is that disease tends to be specific, a given pathogen usually infecting distinct range of host plants. To account for the observed specificity and degree of variability of host parasite system, the fungal receptors must have a high information content. Antigens located on near the cell surface are generally involved in information transfer and / or the maintenance of membrane integrity during the cell to cell interaction of host and parasites.

Pathogenicity test of *Sphaerostilbe repens* was initially carried out on twenty five tea varieties. Among all the tested varieties, TV-26 and T-17 were most susceptible while UP-9 and UP-8 were most resistant. Studies on pathogenicity are extremely useful for several purposes, mainly in understanding more clearly the mechanism of disease development and the way and degree to which host plant performance is altered (Loomis and Adams, 1983). Violet root rot disease was generally observed to occur in water logged area with poor drainage systems. Since the growth and infectivity of the pathogen depends on a number of environmental factors, the effect of some of the factors on mycelial growth of *S. repens* was investigated *in vitro*. Optimum growth of the fungus occurred after 24 days of growth, pH 5.0 and utilising complex carbon source such as starch or sucrose and preferred organic nitrogen sources. Rhizomorph

formation which is an important event in the pathogen life cycle *in vivo*, was also found to occur *in vitro* when the fungus was grown in certain media.

Polyclonal antibodies were raised against mycelial, cell wall and spore antigens of *S. repens* and these were used for determining the presence of cross reactive antigens (CRA) between tea varieties and *S. repens* as well as for the immunodetection of the pathogen in tea 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 *S. repens* 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 optimized 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 2ng/ml could be detected in indirect ELISA by antiserum raised against pooled mycelial suspensions of five *P. fragariae* races. He reported that this sensitivity was achieved because of the indirect ELISA method used; the direct double antibody sandwich form of ELISA (Voller *et. al.* 1976) detected antigens of protein concentrations greater than 50µg/ml. The indirect method used 'native' antibodies rather than antibody conjugates required in the direct assays and hence avoided masking or impairing of antigen binding sites by the conjugation procedure. Chakraborty *et. al.* (1996) also reported that antiserum raised against *Pestalotiopsis theae* could detect homologous antigens at 25ng/ml. Antiserum dilution of upto 1:16000 was effective for detection.

The presence of CRA among *S. repens* and tea varieties was evident in indirect ELISA, using PAb raised against mycelia, cell wall or spore antigen preparations of *S. repens* at a concentration of 40µg/ml with tea root antigens at a concentration of 100µgml. Though much difference was not observed in ELISA values among the different varieties T-17 the most susceptible variety exhibited the highest value. In general the reactivity of the tea varieties with the antibodies was quite less as evident from a generally low range of ELISA values. Alba & Devay (1985) also detected CRA in crude and in purified preparations from mycelia of *Phytophthora infestans*

with antisera of potato in concentration of lower than 50µg/ml protein using indirect ELISA. The presence of CRA several in host pathogen interaction has also been reported by a number of previous workers. e.g. soybean and *Myrothecium roridun* (Ghosh and Purkayastha (1990); groundnut and *Macrophomina phaseolina* (Purkayastha 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 *S. repens* was tested with othr fungal species. Results revealed that Pab of *S. repens* reacted to some extent with two entomopathogenic isolates *Beauveria* and *Metarrhizium* species. 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. Similar results with *P. fragariae* were also reported by Amouzon-Alladaye *et. al.* (1988). In their studies, antibodies obtained with a strain of *P. fragariae* detected two different strains of this pathogen in pure culture by DAS and DAC-ELISA. Non specific cross reactions with most fungi of the root flora of strawberry represented an advantage. Harrisen *et. al.* (1990) further reported that anit *P. infestans* γ globulin reacted strongly with extract of *P. erythroseptica* 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; Lommel *et. al.* 1982; Sundaram *et. al.* 1991; Lyons & White 1992 Chakraborty *et. al.* 1996 and Viswanathan *et. al.* 2000). In the present study antisera raised against mycelia or cell wall antigen preparation of *S. repens* were used for the detection of the pathogen in tea roots and

the response was more or less similar with both antisera. Infected tea root extracts generally had higher ELISA values than the healthy ones. Pab raised against spore antigens were however not very effective in pathogen detection. Holtz *et. al.* (1994) produced polyclonal antisera to both the cell wall fraction and the soluble cytosol fraction of *Thielaviopsis basicola*. Both indirect ELISA and fungal capture sandwich ELISA revealed no difference in specificity of the cell wall and the cytosol antisera and the antibodies to each immunising fraction reacted with the other fraction. 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 *S. repens* in tea root tissues could be detected by both DAC and DAS ELISA using PAb raised against either mycelial or cell wall antigens. It was observed that PAb of *S. repens* could also react with antigens from tea roots infected with other pathogens showing certain degree of cross reactivity.Since PAbs raised against *S. repens* 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 is 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 *S. repens* by DAC ELISA. Of the twenty five samples tested four samples from - plot no. 11 and 12 of Matigara Tea Estates , plot no. 8 of Bijoynagar T.E. and plot no. 5 of Bentaguri T.E. showed high A_{405} values while all the others had relatively low values. Thus it was possible to identify these soils as being contaminated with *S. repens* 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 balls/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 *S. repens* in soil.

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, conidial wall also plays an important role in recognition phenomenon. Hence in this study, the conidial wall was also characterized by agglutination test. Responses of conida to different lectin revealed that the surface components contained glucose,mannose and N-acetylgalactosamine residues. Further the presence of glycocongugates containing N-acetyl glucosamine was also established. Studies on the condial ell wall surface of *F. solani* and *F. oxysporum* by Cristinzio *et. al.* (1988) revealed that the surface contained α-D mannosyl and α-D galactosaminyl residues whereas other species tested such as *F. culmorum, F. graminearun, F. moniliformae, F. xylarioides, F. avenaceus* and *F. sambucinum* did not contain these residues in the outermost layer of the conidial wall. Glycoprotein nature of the material released from conidial wall of *Colletotrichum* graminicola was established by Mercure *et. al.* (1995) on the basis of studies with FITC conjugated lectins. They further determined glucose and mannose to be components of this glycoprotein.

Results of various experiments of this 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 the workers of several previous workers (DeVey and Adler, 1976; Chakraborty and Purkayastha, 1983; Chakraborty and Saha, 1994b). 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 *S. repens*. Cross sections of tea roots were treated with anti-*S. repens* PAb followed by staining with FITC conjugated anti rabbit globule specific goat antiserum. Bright fluorescence was observed in case of epiblema, cortical and endodermis region . So treatment of root sections with *S. repens* revealed that the CRA was concentrated mainly around the epiblema, cortical and endodermal zone. Treatment of mycelia and conidia of *S. repens* with homologous antiserum and FITC

showed a general fluorescence that was more intense on young hyphae and conidia. DeVay *et. al.* (1981a) determined the tissue and cellular location of major CRA shared by cotton and *F. oxysporum* f.sp. *vasinfestans.* On the basis of strong fluorescence obtained at the epidermis, cortex, endodesmis and xylem tissues, they suggested that the CRA determinants in roots have a general distribution. DeVay *et. al.* (1981b) also used FITC labelled antibodies for races of *P. infeotans,* to detect the CRA in potato leaf section. It was also reported by Chakraborty and Saha (1994b) 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 tea leaves was also established by Chakraborty *et. al.* (1995).

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 chlalmydospores, 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 virus and plant pathogenic bacteria. 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 pathogen (*Pythium ultimum, Rhizoctonia solani* and *F. oxysporum*) and did not cross react with resting spores of *Polymyxa betae,* which is also member of the Plasmodiophoraceae. In this study, antigen of mycelial, cell wall, spore, culture filtrate, amended soil, soil from infected plot, healthy and *S. repens* inoculated tea root, mycelial antigen of other tea root pathogens were prepared and tested on nitrocellulose paper against PAbs raised against mycelial, cell wall and spore of *S. repens* using fast red of NBT / BCIP as substrate. Antigens of homologous source,

soil of infected plot showed deep coloured dot. Infected tea root antigens also showed deep coloured dot when compared to healthy confirming the presence of fungal pathogen. Other tea root pathoges responded slightly or no cross reactivity with *S. repens.* Wakeham and White (1996) got positive detection of soluble components of the spore wall and whole resting spores of *P. brassicae* in PBST was seen at concentrations at or above $1x10^7$ and $1x10^6$ resting spores ml^{-1} resting spores ml^{-1} respectively. When the sonicated fraction of the resting spores was assayed, the limit of detection was $1x10^5$ resting spores ml^{-1} Identification of resting spores in artificially infested soil required a minimum concentration of $1x10^8$ spores gm^{-1} soil.

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 once separated in this way, it has been difficult to determine which of the separated species reacted with a given antiserum several method have been developed previously. Towbin (1979) *et. al.* overcame these problems be electrophoretically transferring the separated mixture onto nitrocellulose. Once attached to the nitrocellulose, the antigenicity of each of the separated species could be tested by treating the blot with antiserum and the bound antibody detected with radio labeled staphylococeal protein A or corresponding anti-antibody. Blake *et. al.* (1984) have described a method using alkaline phosphatase substrate 5-bromo 4-chloroindoxyl phosphate and nitroblue tetrazolium (NBT) to detect the precipitated indoxyl group. When the substrate BCIP is used, the phosphate is cleaved by the enzyme and indoxyl group precipitates. The hydroxyl group of the indigo then tautomerizes forming a ketone, and under alkaline conditions dimerization occurs, forming a dehydro indigo. In the process of dimerization it release Hydrogen ion and reduces the NBT which precipitates, forming a intense blue deposition of dimerization. For Western blot analysis, electrophoresis of the soluble mycelia proteins from *V. dahlia* and *F. oxysporum f. sp. vasinfectum* was performed in PAGE by Sundaram *et. al.* (1991). They confirmed the specificity of the antibodies prepared for *V. dahliae* mycelium extract reacted with the purified protein of *V. dahliae* antigens, but not with the *F. oxysporum* protein. In theb present study, antigens prepared from mycelial, cell wall, spore, culture filtrate of *S. repens,* infested soil, healthy and artificially *S. repens* inoculated root and 5 different soil fungi were prepared and

probed with *S. repens* PAb. The PAb of *S. repens* was found to very specific for detection of the pathogen in the soil, infected root tissues and in different isolates of fungi. Walsh *et. al.* (1996) also performd Western blotting using the raw serum of *Spongospora subterranean* spore balls.

The immunoenzymatic assay has several advantages over conventional bioassays in determining the colonization pattern of fungi in roots. It allows direct detection of the hyphae present on the root; the specificity of the staining of hyphae is dependent on the specificity of the serum. A specific fungal antiserum employed with an enzymatic staining technique was developed and proved to be a valuable tool in the study of root infecting fungi, for it permits their direct, selective observation in host tissue. Moreover this staining assay offers many advantages over fluorescence antibody techniques. It does not require the use of fluorescence microscope, and specimens can be viewed with a dissecting microscope. The increased field of view of dissecting microscope allows for the screening of more specimens in less time than is required with fluorescent techniques. It may be possible to sequentially stain different fungi in the same root tissue by using different enzymes linked to the antibodies and / or different diazo dyes. Geric and Huisman (1987) used this specific serological staining procedure to detect *Verticillium dahlie* on and in the root cortex of host with an alkaline phosphatase antirabbit IgG conjugant that hydrolyzed the substrate napthol-As-phosphate to a product that reacted with a diazonium salt. In 1988, Geric and Huisman again used this technique to determine the dynamic colonization of cotton roots by *V. dahlie* under field condition. They observed that the colony density of *V. dahlie* on roots varied with the distance of tissue from the root tip. Young and Andrews (1990) also used this immunocytochemical staining for *Athelia bombacina* for detection of hyphae of the fungus within apple leaf tissue, and used the stain to examine the effect of the antagonists on pseudothecial development of *Venturia inaequalis.* In the present study detection of *Sphaerostilbe repens* in tea root tissues was done using the Pab of *S. repens* reacting with alkaline phosphatase antirabbit IgG conjugate and napthol-AS-phosphate and fast blue BB salt as substrate. Rhizomorph entered through the lenticels below the bark of root-stem transition region which stained blue colour. Intense fungal colony as shown by the blue colour were also visible clearly within the cells of cortical tissue.

Consequent to the study on the detection of *S. repens* in root tissues and soil, experiments were conducted both *in vivo* and *in vitro* for the management of the disease. Since it is necessary to reduce the use of fungicide / pesticide in tea plantation it was decided to test the efficacy of biocontrol agents in disease management. For this purpose *Trichoderma harzianum* and *T. viride* were selected and experiments were conducted using these as a biocontrol agents. Both *T. harzianum* and *T. viride* inhibited the growth of *S. repens in vitro.* There are several reports on the ability of *T. harzianum* and *T. viride* to inhibit the growth of pathogen under *in vitro* condition. Ten 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 to be highly inhibitory to *F. oxysporum* f.sp. *lycopersici* in dual culture followed by *T. harzianum* (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* sp. Baby and ChandraMouli (1996) tested antagonistic potential of *Trichoderma* sp. and *Gliocladium virens* against primary root pathogens of tea *viz. Fomes noxius, P. hypolaterita, Rosellinia arcuata* and *Armillaria mellea in vitro.* In dual culture, the mycoparasites invariably invaded the pathogen, though there was variation in the rate and quantity of invasion. *T. harzianum* was found to be the best colonizer of *Poria* and *Armillaria* and *T. viride* colonized with *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 *Rosellinia.* Production of toxic metabolite(s) was more in *G. virens than Trichoderm.* Hazarika *et. al.* (2000) also tested the antagonistic effect of *Trichoderma harzianum* against *U. zonata,* causing claarcoal stump rot 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. harzianum, T. viride* and *T. virens* were tested by Hazarika and Das (1998) for their potential to suppress *Rhizoctonia solani.* Culture filtrate of *T. harzianum* and *T. virde* inhibited mycelial growth and sclerotial germination. Wheat bran substrate supported maximum growth of all isolates followed by firm yard manure and tea waste. Both *T. harzianum* 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. harzianum,* three isolates, viz. T8, T10 and T2 was 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 growth of *Sclerotinia sclerotiorum* was significantly suppressed by *Gliocladium virens* and *T. viride* significantly. Amongst fungal antagonists tested by Sharma and Sharma (2001), T*richoderma harzianum* and *T. viride* were found most effective in inhibiting mycelial growth of *Dematophora nectrix* in dual culture.

T. harzianum and *T. viride* were tested *in vitro* for their ability to reduce violet root rot intensity. Of the various delivery systems tested for this biocontrol agent, tea waste formulations were found to be most effective. Disease intensity was reduced by both *T. harzianum* and *T. viride* when tested under potted condition as well as in the field. This was observed in all tested varieties. This result is in conformity with that of Hazarika *et. al.* (2000) who reported that planting of tea seedlings after dipping roots in spore suspension of *T. harzianum* 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 mortality of plant increased to 62.2% when *T. harzianum* were applied us soil drench. Significant control of charcoal stump rot of tea with antagonistic microflora obtained previously by Borthakur and Dutta (1992) and Hazarika *et. al.* (1999). The role of *T. harzianum* and *T. viride* as biocontrol crops is well established. Sarker and Jayarajan (1996) reported that root rot of sesamum caused by *Macrophomina phaseolina* was significantly reduced to 12% by seed treatment with antagonist (*T.harzianum*) in comparison to untreated inoculated control plants where 62% disease incidence was noticed. Prasad *et. al.* (1999) found three *T. harzianum* isolates (PDBCTH-2, 7 and 8) and the *T. viirde* isolates (PDBCTV4) highly efficient in controlling root / collar rot of sunflower caused by *Sclerotium rolfsii.* Under green house condition PDBCTH-8 showed maximum disease control of 66.8% followed by PDBCTH-7 (66%), PDBCTV-4 (65.4%), PDBCTH-2 (61.6%) and were even superior than systemic fungicide.

In conclusion, it can be stated that violet root rot can cause severe damage to

tea plants, particularly to those growing in non-aerated soils with poor drainage. Detection, even by immunological methods, is not possible at very early stages, because the pathogen does not penetrate deep into the tissues. However, immunodetection can be used as an early tool for detection of *S.repens* in soil. Once detection is done before the appearance of symptoms , strategies for control can be formulated. In this study, effective formulations of *Trichoderma harzianum* and *Trichoderma viride* have been prepared, by which violet root rot can be controlled.