DISCUSSION

Tea is the most popular and cheapest beverage produced from the young shoots, comprising two or three leaves and a bud of commercially cultivated tea plant. Among the various diseases of tea, blister blight is the major foliar disease which is responsible for huge economic losses, the tea produced from infected shoots is of poor quality (Chandra Mouli, 1983). In the present investigations two sites at different geographical locations viz. Castelton Tea Estate (1505m asl) Kurseong, Darjeeling hills and Hansqua Tea Estate (106m asl), Siliguri plains were selected for the occurrence of blister blight disease and to develop immunodiagnostic kits for early detection of this disease for proper management.

Weather conditions have profound influence on the development of the pathogen and disease incidence. The incidence of blister blight starts from the month of June, and is highest during August - September and then gradually decreases by December, in Castleton Tea Estate. Where as the incidence chart of Hansqua Tea Estate clearly shows blister blight occurrence from November - March, the maximum during January.

Debnath *et. al.* (1994) recorded blister blight disease on 17 tea cultivars in Darjeeling, West Bengal, India. The correlation between disease severity and morphological and anatomical characters was examined by them. Sugha *et. al.* (1994), undertook the survey of blister blight of tea in Himachal Pradesh and found out that properly managed tea plantations had higher levels of disease than poorly managed plantations. It was also observed that recently pruned bushes and those in the shade had higher levels of disease. Similarly, Wang Wuingshen Wang Q.S. (1994) studied the occurrence of blister blight in China. The disease was more prevalent in areas with an elevation of >700m. Investigation on the epidemiology of blister blight clearly pointed out the importance of weather parameters in starting an epidemic, since sufficient number of spores occurred in the atmosphere all through the year (Kerr and Shanmughanathan, 1966).

The habitat of pathogen which invades the aerial parts of the plants is immediately and profoundly influenced by weather. These pathogens usually reproduce abundantly and with the onset of favourable conditions spread rapidly from a minimum amount of initial inoculum (Rotem, 1978). The ability of a pathogen to survive during periods of adverse conditions enables it to carry over from one season to another. Atmospheric parameters influencing disease development are usually temperature, relative humidity, rainfall, plant density etc. It is possible to forecast the intensity of a disease by correlating the factors,

which are essential for the establishment of the pathogen in the host and its dissemination, with the meterological records for a given period. In Castleton Tea Estate, Darjeeling Hills, the disease incidence showed positive correlation with rainfall and relative humidity, leaf wetness is the most important factor for the germination of spores.

Rolando *et. al.* (1989) reported that the level of infection of *Eucalyptus* by *Puccinia psidii* varied with temperature, leaf wetness period and photo period. Higher disease intensity was observed at 20-25^{0}C after 24hours of wetness of leaf surface and disease was inversely correlated with leaf exposure during incubation period. Detailed analysis of the effect of rainfall variable on the epidemiology of *Phytophthora* blight of pepper was carried out by Bowers *et. al.* (1990). They obtained largest absolute direct effect by the culmulative amount of rainfall while the culmulative number of days with rainfall, the culmulative daily average temperature and chronological time had far lesser effect. Continuous leaf wetness of 11h is optimum for infection and maximum infection takes place in 8h leaf wetness. A thin film of water is more favourable for germination and hence when dew is formed in the evening, the spores germinate to bring about infection. Therefore, the epidemic of the disease is very common in areas lying above 700m from SL and in humid, foggy regions (Baby and Premkumar, 2000). On the contrary, the incidence of blister blight in Hansqua Tea Estate clearly shows that foggy conditions and high relative humidity has got a great contribution for the occurrences of the disease, because the correlation chart shows negative correlation with maximum temperature, minimum temperature and rain.

Flush shoots with well-developed sporulating lesions were collected from the experimental plots for the collection of spores in the laboratory. The size of the spores was measured using ocular and stage micrometer. Fungal plant pathogens invade host plant cells with a variety of specialized infection structures of which, appressorium is the most important infection structure (Hoch and Staples 1987).

Formation of appressoria being the first step in establishing the disease, the factors affecting this process are of vital importance in deciding the fate of the pathogen in initial stages (Purkayastha and Menon, 1981). In the present study, it has been observed that the basidiospores of *E. vexans* germinate readily in pH range of 5 to 8.8 and temperature 25^{0}C. Maximum percentage of germination as well as appressoria production takes place in pH 5.5 and pH 7.0.

Advances made in the formulation of concepts and techniques of modern, quantitative cell biology in recent years have paved the way for a basic understanding of the physiology and biochemistry of plant host pathogen interactions. Differences in physiological responses and morphological structures of various host genotypes affect their susceptibility or resistance to invasion and its consequences while similar variation in pathogens influence their growth rate and virulence (Loomis and Adams, 1983). The success or failure of infection is determined by the dynamic competition and the final out come is determined by the sum of favourable and unfavourable conditions for both the pathogen and host cells. At the same time the potential host may be able to detect or recognize a fungal pathogen and use the initial act of recognition to trigger a range of induced resistance (Callow, 1982, 1983; Purkayasth, 1994).

In the host pathogen interaction, therefore, the initial cellular recognition is followed by communication between its components. This exchange of information is generally mediated by soluble antigens located on or near the cell surface (Chakraborty, 1988). In the present study varietal resistance tests of 31 varieties of tea released by Darjeeling Tea Research Centre, Kurseong, Darjeeling; Tocklai Experimental Station, Jorhat, Assam; and UPASI Tea Research Centre, Valparai, Tamilnadu, against the blister blight pathogen, *Exobasidium vexans* was carried out by artificially inoculating the plants with *E.vexans* spores. Percentage infection of blister blight was recorded. Of the 31 varieties tested, AV2, TV18 and UP8 showed high susceptibility where as TV26, S449 and UP2 showed least susceptibility.

The significance of antigenic relationships between plant hosts and pathogenic organisms with regard to disease susceptibility has been recognized by many investigators. Parasitic relationship can only be established if the host recognizes the pathogen on one hand and the pathogen can overcome the various defence mechanisms of the host, on the other hand whenever an intimate and continuing association of cells of host and pathogen occur it has been observed that partners of this association have a unique serological resemblance to one another involving one or more antigenic determinants. The presence of cross reactive antigens (CRA) between plant host and their parasites and the concept that these antigens might be involved in determining the degree of compatibility in such interactions have been discussed by several authors (DeVay *et. al,* 1972; DeVay and Adler 1976;

Kalyana Sundaram, 1978; Chakraborty, 1988; Purkayastha, 1989; Purkayastha *et. al.* 1991; Purkayastha, 1994).

In the present study, leaf antigens of 31 tea varieties, 4 non hosts (*O. sativa, P. indica*, *T. patula* and *L. lucida*) and one non pathogen of tea (*F. graminearum*) were cross reacted separately with anti-*E. vexans* antiserum. (Polyspecific and Polyclonal). The presence of CRA between *E. vexans* and 14 tea varieties, CP1, K1/1, T-78, BS/7A/76, B777, AV2, TV18, TV25, TV27, TV28, UP8, UP9, UP17, UP26, was evident in immuno diffusion test. No common antigenic substance was found between *E. vexans* and 10 other varieties (TV26, TS449, TeenAli /17/1/54, T-135, TV23, TV29, TV30, BSS1, BSS2 and BSS3). However, weak precipitation reaction was observed with antigens of 7 tea varieties (P-1258, HV39, TV20, TV22, TV30, UP2 and UP3).

The occurrence of CRA and their involvements in various host parasite combinations have been demonstrated. These are cotton and *Verticillium alboatrum* (Charudattan and DeVay, 1972) Cotton and *Fusarium Oxysporum* f. sp. *vasinfectum* (Charudattan and DeVay, 1970; Kalyana Sundaram *et. al.*, 1975), sweet potato and *Ceratocystis fimbriatae* (DeVay *et. al.*, 1976), potato and *phytophthora infestans* (Palmerley and Callow, 1978, Alba and DeVay, 1985), Soybean and *Macrophomina phaseolina* (Chakraborty and Purkayastha, 1983), soybean and *Colletotrichum dematium* (Purkayastha and Banerjee, 1986), Soybean and *Myrothecium roridum* (Ghosh and Purkayastha, 1990, coffee and *Hemiliea vastafrix* (Alba *et. al.*, 1983), groundnut and *Macrophomina phaseolina* (Purkayastha and Pradhan, 1994); Tea and *Bipolaris carbonum* (Chakraborty and Saha, 1994); Tea and *E. vexans* (Chakraborty *et. al.*, 1997).

Enzyme linked immunosorbent assay is probably one of the most sensitive serological techniques for the detection of cross reactive antigens. (Alba and DeVay, 1985; Chakraborty and Saha, 1994). In the present study polyspecific *E. vexans* antisera was raised against blister infected tea leaves of Castleton Tea Estate and Hansqua Tea Estate and polyclonal antisera was raised against *E. vexans* basidiospores. The antisera obtained were purified to minimize non specific binding. At the beginning, the sensitivity of the assay was optimized. Homologous soluble antigens at a concentration as low as 25ng/ml could be detected in indirect ELISA by all the three antisera. Absorbance values decreased with increase in dilutions. Chakraborty *et. al.* (1996) also reported that antiserum raised against

Pestalotiopisis theae could detect homologus antigen at 25ng/ml. Antiserum dilution of upto 1:16,000 was effective for detections.

In the present study indirect ELISA readily detected CRA between tea leaf antigens and *E. vexans,* at a concentration of 1:250 antiserum dilution. Alba and DeVay (1985) also detected CRA in crude preparations and in purified preparations from mycelia of *Phytophthora infestans* (races 4 and 1.2.3.4.7) using antisera of potato cultivars King Edward and Pentland Dell at concentrations lower than 50µg/ml protein in indirect ELISA. Among the 31 tea varieties tested with antiserum of *E. vexans* (Polyspecific and polyclonal), very high absorbance values were obtained in case of AV2, TV18 and UP8, TV26, S449 and BSS3 showed very low absorbance values.

Visible outcome of a compatible host pathogen interaction may be obtained in many cases only after few days of infection, by which time the pathogen would be well established in the host tissues. In phytopathology studies, therefore, it is necessary to have techniques by which pathogen can be detected at a very early stage. Recent trends have developed highly specific techniques for the detection of pathogen at a very early stage (Hansen and Wick, 1993). Various formats of ELISA using polyclonal antiserum has found widespread application in plant pathology and are routinely used for detection and identification purposes (Clark and Adams, 1977; Clark, 1981; Lommel *et. al.* 1982; Sundaram *et. al.* 1991; Lyons and White, 1992; Chakraborty *et. al.* 1995; Chakraborty *et. al.* 1996; Chakraborty *et. al.* 2001a and 2001b).

In the present study, the differential response of twelve (12) tea varieties to *Exobasidium vexans* has been observed through Indirect ELISA of artificially inoculated tea leaves. Among the four Darjeeling varieties tested (CPI, S449, HV39 and AV2), AV2 showed highest ELISA absorbance for blister infected antigens. Tocklai variety 18 and UPASI-8 showed high susceptibility to blister blight. The artificially inoculated leaves (*in vitro conditions*) showed swelling and curling symptoms, blister formation (white postules) was absent in these cases. Gunasekera *et. al.* (1997) investigated the effects of ultraviolet B(UV-B:290-320nm) component of solar radiation on blister blight of tea, in field trials in SriLanka, using UV-screening filter materials held over a commercial crop. They found that exclusion of UV-B radiation by polyester C, 75-85% increased the number of translucent spots (immature sites of infection), but it had little or no effect on sporulation of

E. vexans. Basidiospores that were artificially inoculated on leaves and exposed to full or filtered solar radiation, had increased survival and germination, when UV-B wavelengths were removed. They suggested that the UV-B component of solar radiation plays an important role in the natural regulation of blister blight disease in the field.

In order to determine the earliest time of which infections could be detected, highly susceptible varieties AV2, TV18 and UPASI-8 were artificially inoculated with *E. vexans* spores and ELISA readings were taken after every 24 h interval for 12 days. Infection could be detected as early as 48 h after inoculation in susceptible varieties (AV2, TV18 and UP8). ELISA could successfully detect infection in leaf tissues much earlier than the appearance of the visible symptoms which generally appears after 12 days of inoculation with *E. vexans.* This is in confirmity with the results of several previous authors who have reported that ELISA could detect pathogens in tissues (Linfield, 1993; Jamaux and Spire, 1994). In experiments conducted over 20 days with *Fragaria vesca,* Mohan (1988) showed that ELISA positive material was detectable 6-8 days after inoculation with *P.fragariae* when the plants were apparently still healthy.

Results of various experiments of this study has established very definitely the importance of cross reactive antigens between host and pathogen in determining the response of the host to pathogen. This has also been supported by the works of several previous workers (DeVay and Adler, 1976;. Chakraborty and Purkayastha, 1983; Chakraborty and Saha, 1994b; Chakraborty *et. al.* 1995; 1997). It is also important in the studies on host parasite relationship to determine the cellular location of the CRA. For this purpose, in this study, fluorescence tests were conducted with loosened cells and cross sections of tea leaves, and basidiospores of *E. vexans.* Loosened cells were obtained from calli prepared from stem segments of TV18. Cross sections of healthy tea leaves (TV18 and AV2) and loosened cells were treated with anti-*E. vexans* antiserum followed by staining with FITC conjugated anti rabbit globulin specific goat antiserum. Bright fluorescence was observed in both the loosened cells and cross section of tea leaves (TV18 and AV2) treated with polyclonal antiserum. Treatment of infected leaf tissues with anti-*E. vexans* antiserum followed by FITC, labelled antibodies also showed bright fluorescence. DeVay *et. al.* (1981) determined the tissue and cellular location of major CRA shared by cotton and *F. oxysporum* f.sp. *vasinfectum;* Chakraborty and Saha (1994b) also showed the

cellular location of CRA showed by *Camellia sinensis* and *Bipolaris carbonum*, (Chakraborty *et. al.* 1995. Chakraborty *et. al.* 1997).

Detection of pathogen in host tissues using antibody based immunofluorescent technique has been reported by several previous authors (Warnock, 1973; Hornok and Jagicza, 1973; Reddy and Ananthanarayan, 1984). Dewey *et. al.* (1984) suggested, on the basis of immunofluorescence studies that chlamydospores, basidiospores and mycelia of *Phaseolus* Schweinitzii contained molecules antigenically related to species specific antigens secreted by mycelia grown in liquid culture. They also demonstrated the presence of mycelium and Chalmydospores in naturally and artificially infested soil samples, using this technique. Different test formats including indirect ELISA, Western blotting, dot blot and indirect immunofluorescence was assessed by Wakeham and White (1996) for their potential to detect resting spores of *Plasmodiophora brassicae* in soil.

The dot immunobinding technique has been found to be rapid and sensitive method for detection of viruses and plant pathogenic bacteria. Detection of fungal pathogens is a more recent application of these methods. Antiserum specificity obtained against fungal pathogen varied greatly in the studies done by Lange *et. al.* 1989. The antiseurm against *P. brassicae* resting spores used in their study showed no cross reaction with other common rest pathogens (*Pythium ultimum, R. solani,* and *F. oxysporum*), and did not cross react with resting spores of *Polymyxa betae,* which is also a member of the *Plasmodiophoraceae.* In the present study, antigens prepared from blister infected leaf of CTE, basidiospores collected from blister infected leaves (CTE), healthy tea leaves and artificially inoculated tea leaves with *E. vexans* were tested on nitrocellulose paper. Infected and artificially inoculated leaf antigens gave intense dots when compared to the healthy control confirming the presence of fungal pathogens.

Complex mixtures of antigens can be quickly and easily separated by high-resolution techniques such as sodium dodecyl sulfate-acrylamide gel electrophoresis using discontinuous buffer systems and two dimensional techniques. However, once separated in this manner, it has been difficult to determine which of the separated species reacted with a given antiserum. Several methods have been developed previously. Towbin *et. al.* (1979) overcame these problems by electrophoretically transferring the separated mixture onto nitrocellulose. 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 the alkaline phosphatase substrate 5-bromo-4-chloroindoxyle phosphate (BCIP) and nitro blue tetra zolium (NBT) to detect the precipitated indoxyl group. When the substrate 5-bromo 4-chloroindoxyl phosphate is used, the phosphate is cleaved by the enzyme and the indoxyl group precipitates. The hydroxyl group of the indigo then tautomerizes forming a Ketone, and under alkaline conditions dimerization occurs, forming a dehydroindigo. In the process of dimerizing, it releases hydrogen ions and reduces the nitro blue tetrazolium which precipitates, forming an intense blue deposition of diformazan. In the present study, healthy and blister infected tea leaf antigens were run for SDS PAGE analysis, transferred on to the nitrocellulose paper, probed with polyspecific and polyclonal antibody of *E. vexans* and treated with BCIP and NBT. Two types of colour reactions were observed in both the above cases. Healthy antigens showed higher CRA when probed with polyspecific antibody, where as polyclonal antibody did not recognize healthy antigens.

Although occurrence of blister blight disease was recorded more than a century ago regular and intensive control measures were practised only in the last 40 years. With the advent of the diseases, experiments were initiated to effectively combat the disease. Several workers have worked on the cultural operations and chemical control (fungicides) of the disease.

Venkata Ram, 1974a suggested the removal of shade trees to achieve more control on blister blight. However, regulation of shade by pollarding the branches wherever necessary was most useful. Several workers have worked on the chemical control of blister blight. Use of copper oxychloride or cuprus oxide, Nickle chlorides have been the best choice for protective control of the disease, (Venkata Ram and Chandra Mouli 1984). In tea recovering and pruning stage nickle chloride cannot be used due to its phytotoxicity on tender succulent shoots. (Venkata Ram, 1978). In order to find out alternative fungicides to copper oxychloride and nickle chloride many other fungicides both organic and inorganic were evaluated (Chandra Mouli, 1979a, Chandra Mouli and Premkumar, 1986). Of the many protectant therapeutant organic fungicides, only chlorothalonil and dithianon provided satisfactory control (Chandra Mouli and Premkumar, 1986). New lines of approach were

made by introducing systemic fungicides in blister blight disease control programme. By using systemic fungicides satisfactory disease control could be achieved even by less frequent sprays. However, the systemic fungicides have the limitation under severe wet weather conditions (Venkata Ram, 1974). Since basidiospores of the fungus failed to germinate in acidic pH, excellent disease control was obtained by spraying a very dilute acid water (pH 1.3) (Venkata Ram, 1979). Foliar application of Hexa conazole one of the systemic fungicides as recommended by UPASI, controls the disease, but has toxic side effects. In order to explore the possibilities of involving an efficient, eco-friendly alternative, in the present study few selective botanical pesticides were screened out of which *Azadirecta indica* and *Catharanthus roseus* were selected for further field evaluation at Castleton Tea Estate, Kurseong, Darjeeling. Foliar applications of plant extracts (20g/litr)and contaf 5E (1:10,000 dilution) twice at an interval of 10 days were sprayed to the tea bushes of the marked area. It is interesting to note that the incidence of blister blight was highest 88.3% in untreated plots, lowest (22.3%) was observed in plots sprayed with Hexaconazole. Blister blight incidence was noted as 45.6% in plots treated with *Catharanthus* spray (45.6% and 53.0% in neem spray). DAC-ELISA format was developed using polyclonal antibody raised against basidiospores of *E. vexans*. When ELISA results of leaf tissues collected from both treated and untreated plants exposed to natural inoculum after 15 and 30 days of spray schedule were compared, A 405 values were always reduced in treated leaf tissues than untreated ones. It indicates clearly that in the treated leaf tissues the establishment of the pathogen (*Exobasidium vexans*) was not successful due to the application of biocide. A major out come of the present study has been the development of immunodiagnostic kits of blister blight pathogen (*E. vexans*) using immunoassays, immunofluorescence, dot immunoblotting as well as western blotting at a very early stage of infection, have paved the way to take preventive measures in the field conditions there by minimizing the crop loss.