# Experimental

# Chapter I

## 4.1. Studies on morphology and physiology of *Lasiodiplodia theobromae*

*Lasiodiplodia theobromae* affects roots, stems and leaves of tea plants of all ages (Sarmah, 1960; Chandramouli, 1999; Singh, 2005). It particularly causes severe damage in the nurseries of tea gardens where seedlings are raised. Conidia were initially hyaline, unicellular and sub-ovoid to ellipsoidal. Mature conidia were two-celled, cinnamon to dark brown, thick walled and often with longitudinal striations, 18-30 × 10-15 µm in size (Sutton, 1980; Khanzada *et al.*, 2004). This fungus has a wide host range. It attacks more than 280 species of plants in different parts of the world (Domsch *et al.*, 1980; Sutton, 1980). Mycelia colonies were initially white, soon becoming black and fast-spreading with immersed and superficial, branched, septate mycelium. Shiny black pycnidia were produced on the surface. The morphological characters for pycnidia and conidia were similar (Sutton, 1980) (Plate VI). For conducting studies on behaviour of the fungus, it was necessary to culture the fungus in artificial media. Inoculum production in the form of spores was found difficult in the routine fungal culture media. A literature search showed that studies on the environmental and nutritional requirements of *L. theobromae* are limited. The present work was therefore undertaken to study the effects of culture media, temperature, pH and light on mycelial growth and sporulation of *L. theobromae*.

### 4.1.1. Effect of different liquid medium on growth and sporulation of *Lasiodiplodia theobromae*

Growth and sporulation of *L. theobromae* was studied on potato dextrose broth (PDB), oat meal broth (OMB), potato carrot broth (PCB), yeast extract glucose broth (YEGB) and malt extract broth (MEB) media (40ml) in 250 ml conical flasks. In addition, some of the media were supplemented with root extract of tea plants. Procedures of media preparation have been described in section-3.4 of materials and methods.

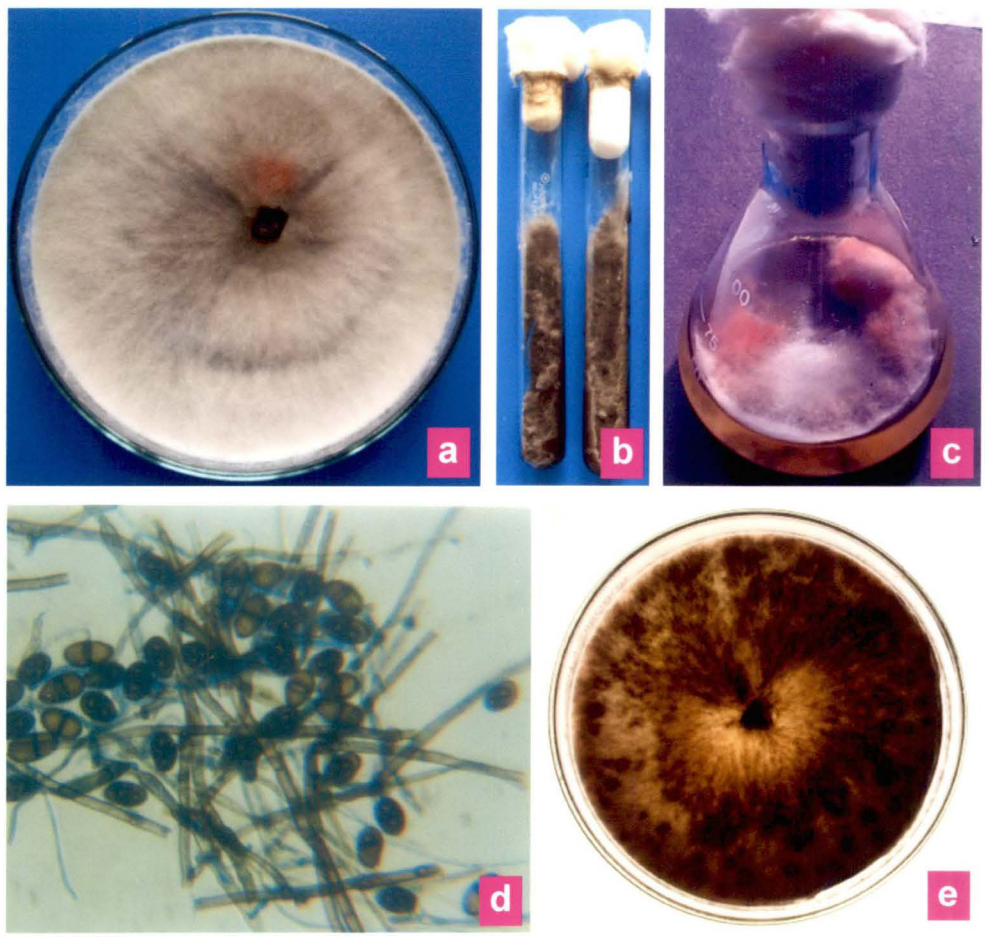


**PLATE VI**

**fig. a :** Culture of *Lasiodiplodia theobromae* releasing pink coloured pigment in petridish.

**fig. b :** Culture of *L. theobromae*.

**fig. c :** Culture of *L. theobromae* in potato dextrose broth (PDB) with pink coloured pigment.

**fig. d :** Sporulated mycelia of *L. theobromae*.

**fig. e :** Shiny black pycnidia on the surface of mycelial mat of *L. theobromae*.

From the results (Table 6), it was evident that PDB-RE (potato dextrose broth supplemented with root extract) was best for both growth and sporulation of *Lasiodiplodia theobromae*. Mycelial dry weight was recorded as 360mg after 20 days and sporulation was excellent after 25 days of incubation on PDB-RE medium. In PDB, mycelial dry weight was found 320 mg after 20 days of incubation but sporulation was found poor. Next to PDB, MEB-RE (Malt extract broth supplemented with root extract) also showed good mycelial growth and sporulation. Non-supplimented PCB recorded the least mycelial growth and no sporulation. The results revealed that in general, sporulation was remarkably high when *L. theobromae* was grown in root extract supplemented media in comparison to the non-supplemented media. Mycelial growth also increased marginally when PDB, OMB and PCB were supplemented with root extract. However, YEGB and MEB did not show any difference in mycelial growth between supplemented and non-supplemented media, but there was no sporulation in the non-supplimented media. Addition of root extract increased sporulation significantly especially in MEB-RE media. Thus the result indicated that root extract supplementation is not necessary for *in vitro* mycelial growth of *L. theobromae*, but it remarkably enhanced the sporulation.

### 4.1.2. Effect of Light on growth and sporulation *of Lasiodiplodia theobromae*

Light plays an important role on chemo-physiological process of living organism directly or indirectly for completion of its life cycle. From the results (Table 7) it was found that light had no significant effect on mycelial growth of *L. theobromae*, which was found to be equally good under complete light, complete dark and alternate 12h light and dark conditions. But sporulation was noticed excellent after 10 days when the fungus was grown under complete light condition. However, under complete dark conditions, sporulation was poor and delayed until 20 days. Overall results indicated that there was little variation in mycelial growth under different light conditions, but light induced sporulation.

**Table 6:** Mycelial growth and sporulations of *Lasiodiplodia theobromae* in different liquid media.

| Medium of growth | Incubation period | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 5 days | | 10 days | | 15 days | | 20 days | | 25 days | |
| | Mwt* | Spn** | Mwt | Spn | Mwt | Spn | Mwt | Spn | Mwt | Spn |
| PDB-RE | 150.0 ±5.77 | - | 200.0 ±11.4 | + | 350.0 ±5.77 | ++ | 360.0 ±8.35 | +++ | 335.0 ±9.45 | +++ + |
| PDB | 145.0 ± 2.88 | - | 225.0 ±8.88 | - | 280.0 ±5.56 | - | 320.0 ±7.48 | + | 295.0 ±4.56 | + |
| OMB-RE | 120.0 ±5.77 | - | 125.0 ±2.89 | + | 150.0 ±5.64 | + | 185.0 ±6.45 | ++ | 220.0 ±5.87 | ++ |
| OMB | 85.00 ±3.66 | - | 95.00 ±3.66 | - | 100.0 ±4.56 | - | 135.0 ±4.86 | - | 165.0 ±7.45 | - |
| PCB- RE | 25.00 ±2.88 | - | 85.00 ±2.00 | - | 125.0 ±4.64 | + | 170.0 ±4.84 | ++ | 210.0 ±7.56 | ++ |
| PCB | 15.00 ±2.89 | - | 30.00 ± 2.66 | - | 95.00 ±3.64 | - | 120.0 ±4.98 | - | 165.0 ±5.43 | - |
| YEG- RE | 140.0 ±11.4 | - | 170.0 ±7.54 | - | 225.0 ±8.76 | + | 295.0 ±7.27 | + | 320.0 ±7.43 | + |
| YEG | 120.0 ± 5.77 | - | 135.0 ±5.77 | - | 270.0 ±5.77 | - | 305.0 ±9.45 | - | 295.0 ±7.88 | - |
| MEB- RE | 145.0 ±3.66 | - | 185.0 ±5.66 | - | 230.0 ±6.67 | + | 280.0 ±8.67 | ++ | 315.0 ±9.28 | +++ |
| MEB | 122.± 5.92 | - | 165.0 ±8.67 | - | 210.0 ±6.56 | - | 285.0 ±8.56 | - | 324.0 ±9.41 | - |

*Mwt = Mycelial dry weight,

**Spn = Sporulation, - = Nil; + = Poor; ++ = Fair; +++ = Good; ++++ = Excellent

RE = Tea root extract supplemented, PDB = Potato dextrose broth, OMB = Oatmeal broth, PCB = Potato carrot broth, YEG = Yeast extract glucose broth, MEB = Malt extract broth. Incubation temperature = 28 ± 1°C

Mean of three replications; Data after ± indicates standard error values.

**Table 7:** Effect of different light condition on growth and sporulation of *Lasiodiplodia theobromae* in PDB-RE medium.

| Different light condition | Incubation period | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 5 days | | 10 days | | 15 days | | 20 days | | 25 days | |
| | Mwt* (mg) | Spn** | Mwt (mg) | Spn | Mwt (mg) | Spn | Mwt* (mg) | Spn | Mwt (mg) | Spn |
| Light (24h light) | 165.0 ±6.75 | - | 210.0 ±8.56 | + | 265.0 ±5.89 | ++ | 290.0 ±7.89 | +++ | 325.0 ±9.46 | ++++ |
| Dark (24h dark) | 175.0 ±4.65 | - | 225.0 ±7.87 | - | 260.0 ±5.34 | - | 320.0 ±9.45 | + | 335.0 ±8.61 | ++ |
| Normal (Alternate 12h light | 180.0 ±6.76 | - | 230.0 ±6.56 | - | 270.0 ±6.73 | + | 340.0 ±8.46 | ++ | 320.0 ±9.84 | ++ |

*Mwt = Mycelial dry weight,
**Spn = Sporulation, - = Nil; + = Poor; ++ = Fair; +++ = Good; ++++ = Excellent,
Incubation temperature = 28 ± 1°C
PDB-RE = Tea root extract supplemented potato dextrose broth
Mean of three replications; Data after ± indicates standard error values.

### 4.1.3. Effect of temperature on growth and sporulation of *Lasiodiplodia theobromae*

Results presented in figure (Fig. 3) indicate that *L. theobromae* was capable of growing at temperature that ranges 8°C to 36°C. Best growth was recorded at 28°C while no growth was observed at temperature 40°C and above.

### 4.1.4. Effect of pH on growth and sporulation of *Lasiodiplodia theobromae*

pH plays an important role on physiology of any organism. Growth and sporulation of *L. theobromae* was studied at different pH. It was found that *L. theobromae* was able to grow within a wide range of pH, from 3.5 to 8.0 (Fig. 4). The fungus however, failed to grow in alkaline environment, beyond pH 8.0. The optimum pH for growth was recorded at the range of pH 5.5 to 6.5. The results indicated that slightly acidic pH to neutral pH was optimum for the growth of *L. theobromae*.

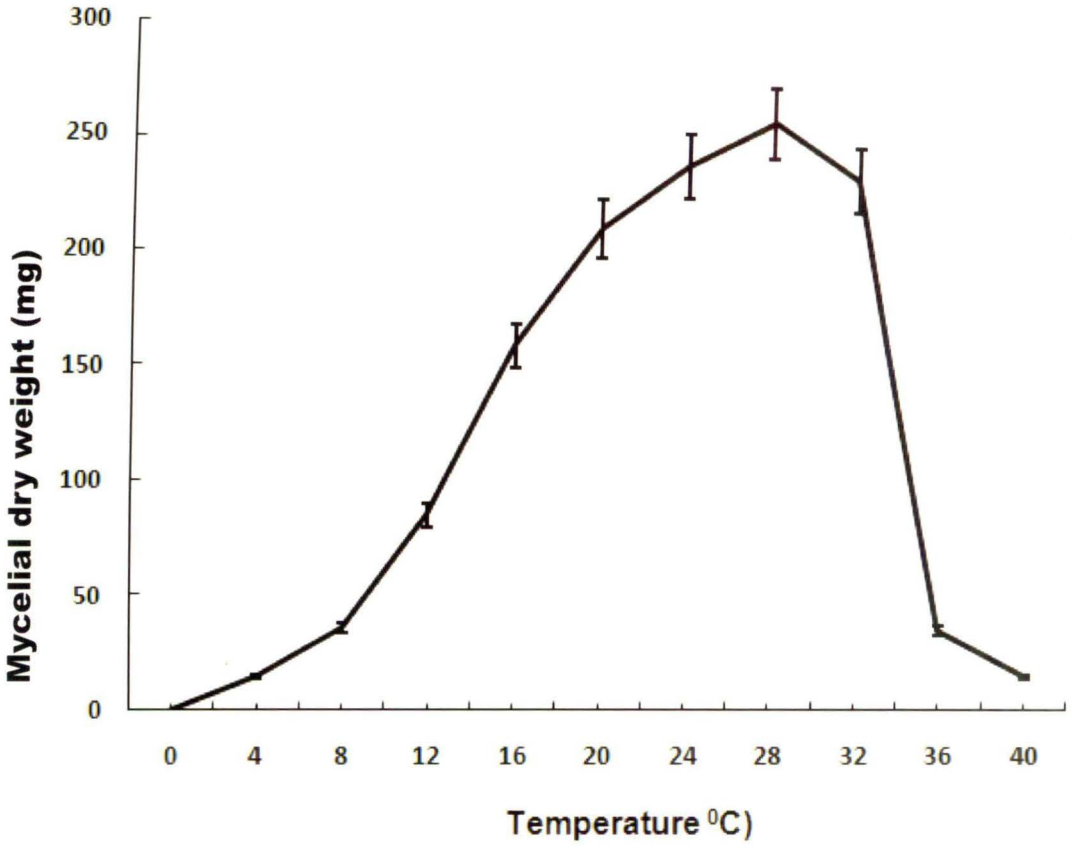


**Fig. 3:** Effect of different temperature on mycelial growth of *Lasiodiplodia theobromae* in potato dextrose broth supplemented-tea root extract (PDB-RE) medium after 15 days of incubation.

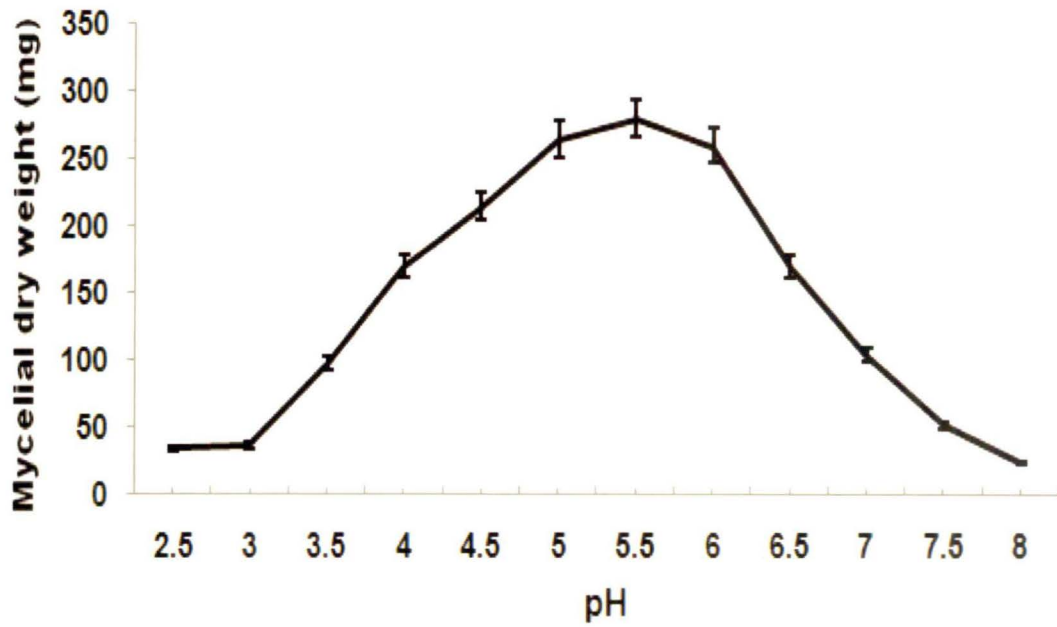


**Fig. 4:** Effect of different pH on mycelial growth of *Lasiodiplodia theobromae* in potato dextrose broth supplemented-tea root extract (PDB-RE) medium after 15 days of incubation.

# Chapter II

## 4.2. Pathogenicity of *Lasiodiplodia theobromae* in different tea varieties

Differential pathogenicity of a fungus to different varieties gives us information about the degree of susceptibility or resistance of a particular variety to a particular pathogen. In the present study, pathogenicity of *L. theobromae* was tested separately on roots and leaves of seven different tea seed varieties (TS-464, TS-491, TS-506, TS-520, TS-489, TS-449 and TS-463).

### 4.2.1. Pathogenicity on leaves

Pathogenicity was studied following the whole plant inoculation method as described in the materials and methods (section 3.6.1). Ten plants of each seed varietiy (raised through seed germination) were artificially inoculated with *Lasiodiplodia theobromae* in triplicate sets (Plate VII: fig. a & b). Symptoms appeared on leaves after 4, 8, 12 and 16 days of inoculation which were assessed following the disease assessment procedure as mentioned in the materials and methods (Section 3.6.3). Results (mean foliar disease index) were calculated and have been presented in Table 8.

From the results (Table 8 & Fig. 5) it was found that TS-449 was most susceptible to *Lasiodiplodia theobromae*. It showed mean foliar disease index/plant of 12.42 after 16 days. On the contrary, TS-491 was most resistant against *Lasiodiplodia theobromae* showing mean foliar disease index/plant of 3.20. Next to TS-449 variety, TS-464 and TS-520 were also found to be susceptible showing mean foliar disease index/plant of 10.20 and 9.8 respectively after 16 days of inoculation. TS-506 and TS-489 were found to be moderately resistant showing mean disease index/plant of 5.90 and 5.30 respectively after 16 days of inoculation. A variety was considered susceptible when it produced mean foliar disease index/plant of more than 9.0 while a variety was considered highly resistant when it produced mean foliar disease index/plant of less than 5.0. However the varieties showing mean foliar disease index/plant of 5.0 to 9.0 were considered as moderately resistant after 16 days of inoculation. Graphical representation of mean foliar disease index data after 16 days of inoculation has been shown in Fig. 5.

**Table 8:** Pathogenicity test of *Lasiodiplodia theobromae* on tea seedlings of seven different varieties (based on mean foliar disease index).

| Tea variety | Days after inoculation | | | |
|---|---|---|---|---|
| | 4 days | 8 days | 12 days | 16 days |
| | Mean foliar disease index/plant* | Mean foliar disease index/plant | Mean foliar disease index/plant | Mean foliar disease index/plant |
| TS-449 | 1.54±0.3 | 5.54±0.4 | 9.64±0.5 | 12.42±0.5 |
| TS-464 | 1.28±0.8 | 3.61±0.3 | 6.01±0.4 | 10.20±0.3 |
| TS-520 | 1.00±0.3 | 2.92±0.08 | 6.34±0.3 | 9.80±0.5 |
| TS-506 | 0.85±0.07 | 2.28±0.25 | 5.10±0.2 | 5.90±0.03 |
| TS- 489 | 0.69±0.25 | 2.12±0.07 | 3.90±0.08 | 5.30±0.2 |
| TS-463 | 0.52±0.35 | 1.64±0.06 | 3.10±0.02 | 4.25±0.05 |
| TS-491 | 0.50±0.05 | 1.42±0.03 | 2.70±0.05 | 3.20±0.06 |
| CD of 5% | 0.45 | 0.78 | 1.25 | 2.5 |

*Mean of three replications; Data after ± indicates standard error values.

**Data presented on the basis of 10 plants per treatment.

### 4.2.2. Pathogenicity on roots

Pathogenicity of *L. theobromae* was further studied on seven tea seed varieties following root inoculation technique. Details of the technique have been described in materials and methods (Section-3.6.2). Fifteen plants (raised through seed germination in 2 plastic pots) of each variety were artificially inoculated in triplicate sets with *Lasiodiplodia theobromae*. Symptoms appeared on root after 4, 8, 12 and 16 days of inoculation which were assessed following root disease assessment procedure as mentioned in materials and methods (Section-3.6.4). Results (mean root disease index/plant) were calculated and have been presented in Table 9.

From the results (Table 9 & Fig. 6) it was observed that TS-449 showed mean root disease index/plant of 59.5 after 16 days of inoculation. Among the seven

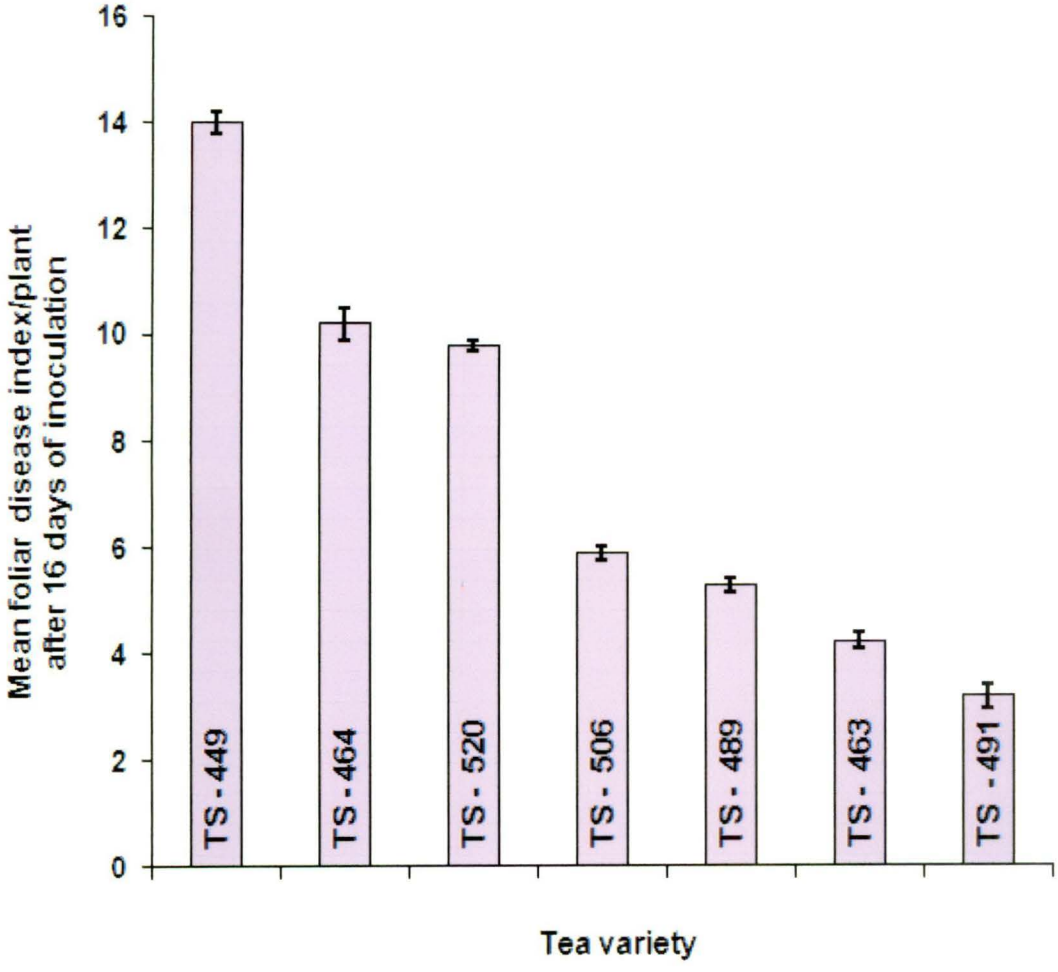


**Fig. 5:** Pathogenicity test of *Lasiodiplodia theobromae* on leaf of tea seedlings of seven different varieties ( based on mean foliar disease index).

varieties tested, TS-449 was most susceptible to *L. theobromae*. TS-491 was most resistant against *L. theobromae* showing mean root disease index/plant of 17.5. Two other varieties TS-464 and TS-520 were also found to be susceptible showing mean root disease index/plant of 35.8 and 31.6 respectively after 16 days of inoculation. TS-506 and TS-489 were found to be moderately resistant showing mean root disease index/plant of 28.2 and 21.7 respectively after 16 days of inoculation. Graphical representation of mean root disease index data after 16 days of inoculation has been shown in Fig. 6. A variety was considered susceptible when it produced mean root disease index/plant of more than 30.0 while a variety was considered highly resistant when it produced mean root disease index/plant of less than 20.0. The varieties showing mean root disease index/plant of 30.0 to 20.0 were considered as moderately resistant after 16 days of inoculation.

**Table 9:** Pathogenicity test of *Lasiodiplodia theobromae* on tea seedlings of seven different varieties (based on mean root disease index).

| Tea variety | Inoculation period | | | |
|---|---|---|---|---|
| | 4 days | 8 days | 12 days | 16 days |
| | Mean root disease index/plant | Mean root disease index/plant | Mean root disease index/plant | Mean root disease index/plant |
| TS-449 | 15.5±0.8 | 23.7±0.7 | 48.2±2.5 | 59.5±2.5 |
| TS-464 | 12.7±0.5 | 15.6±0.6 | 22.5±1.5 | 35.8±1.5 |
| TS-520 | 10.4±0.6 | 16.7±0.4 | 18.8±1.4 | 31.6±0.9 |
| TS-506 | 9.5±0.5 | 15.7±0.8 | 14.6±0.8 | 28.2±1.2 |
| TS- 489 | 8.3±0.7 | 9.5±0.5 | 10.8±0.6 | 21.7±1.6 |
| TS-463 | 8.2±0.5 | 8.5±0.4 | 9.6±0.3 | 17.9±0.6 |
| TS-491 | 6.9±0.4 | 7.7±0.3 | 9.2±0.6 | 17.5±0.8 |
| CD of 5% | 1.6 | 2.4 | 2.8 | 3.6 |

*Mean of three replications; Data after ± indicates standard error values.

**Data presented on the basis of 15 plants per treatment.

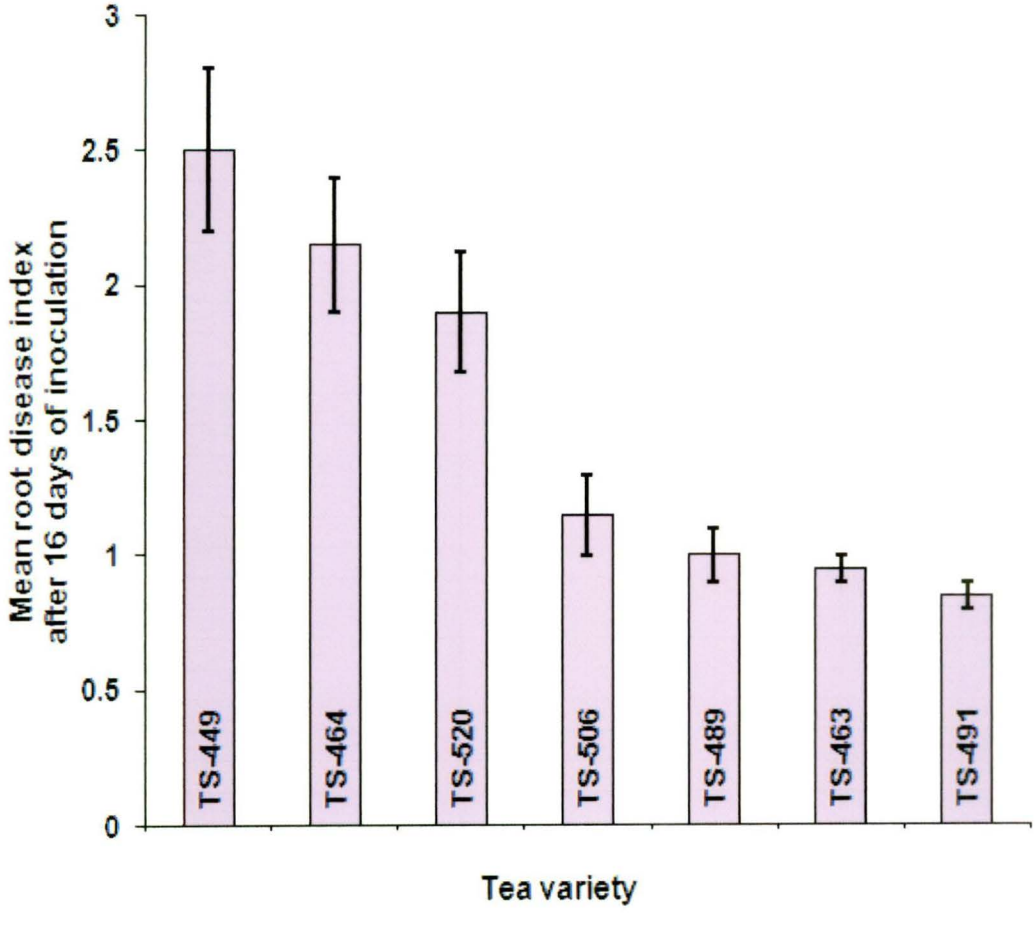


**Fig. 6:** Pathogenicity test of *Lasiodiplodia theobromae* on roots of tea seedlings of seven different varieties ( based on mean root disease index).




**PLATE VII** **Healthy and infected tea plants.**

**fig.a:** Healthy tea plants of TS-449 in experimental glass chamber (Control).

**fig.b:** Tea plants (TS-449) artificially infected with *L. theobromae* in glass chamber.

**fig.c:** Nursery tea plants (TS-449) naturally infected with *L. theobromae*.

# Chapter III

## 4.3. Serological studies

Serological methods are most sophisticated methods for detection of cross-reactive antigens between different host-pathogen interactions. The level of cross reactive antigens present in the interactions (between different host varieties and a virulent pathogen) can be exploited for determining differential resistance of different varieties against a pathogen. Several workers have shown that the possibility of susceptibility is greater when cross-reactive antigens are more (Alba *et al.*, 1983; Purkayastha and Banerjee, 1990; Chakraborty and Saha, 1994). In the present study five different techniques (immunodiffusion, immunoelectrophoresis, indirect enzyme linked immunosorbent assay, immunofluorescence and immuno gold-silver enhancement) were performed to detect cross-reactive antigens (CRAs). The level of cross-reactive antigens was used to determine susceptible and resistant cultivars. The results of the serological studies were compared with that of conventional pathogenicity test.

In the present study, root antigens of seven different tea seed varieties and of two different fungi were prepared. The details of the antigen preparation procedures have been described in section-3.7 of materials and methods. The antigens of respective varieties were designated by lowercase letter 'a' suffixed with codes of tea varieties (i.e. 449 for TS-449, 489 for TS-489, 520 for TS-520, 491 for TS-491, 463 for TS-463, 464 for TS-464, 506 for TS-506). Codes of fungi were 'Th' for *Trichoderma harzianum* and 'Lt' for *Lasiodiplodia theobromae*. Antisera of different tea varieties and fungi were designated by upper case letter 'A' suffixed with respective codes of the tea varieties or fungi. Three different antisera (449A, 491A, LtA) and normal sera (NS) were used in the present study. Procedures of preparation of the antisera and normal sera have been discussed in the materials and methods (Section-3.9.1).

### 4.3.1. Serological relationship between different tea varieties and pathogen (*Lasiodiplodia theobromae*) by agar gel double diffusion

The agar gel double diffusion method as described by Ouchterlony (1958) was followed to determine the relationship between host and pathogen. Detailed of the immunodiffusion technique has been described in materials and methods section 3.9.1. Semi quantitative estimation of antibody activity of the three different antisera (449A, 491A, LtA) against their homologous antigens was performed. Titre values of the antigens (449a, 491a and Lta) against their homologous antisera were also determined. Results are presented in table 10. Immunodiffusion of seven different antigens (449a, 489a, 520a, 491a, 463a, 464a, 506a) and antigens of two different fungi (Lta and Tha respectively of pathogen and non-pathogen of tea) were performed against three different antisera (449A, 491A, LtA) and the results are shown in Table 11 and Plate VIII.

From the results noted in Table 11, it was found that common antigenic relationship were present not only in case of homologous reactions (i.e. between antigen and antisera of *L. theobromae*, antigen and antisera of TS-449 and between antigen and antisera of TS-491) but also between antigens of different tea varieties (464a and 491a) against antisera 449A (Plate VIII: fig.b) and also antigens like 489a, 463a and 520a against antisera 491A (Plate VIII: fig.c). Precipitation reactions in the form of arcs were also found in case of heterologous reactions (tea antigens of 506a, 449a and 464a against antisera of *L. theobromae* (LtA)). However, almost no precipitation was found between 491a and 463a against LtA (Plate VIII:fig.a). Cross reactive antigens were present in heterologous combination when Lta (antigen of pathogen) was reacted with 449A (antisera of susceptible variety). But when Tha (antigen of non-pathogen) reacted with 449A (antisera of susceptible variety), no precipitation arcs were observed (Plate VIII: fig.b). In heterologous combination (Plate VIII: fig.c), when Lta (antigen of pathogen) was reacted with 491A (antisera of resistant variety), there was no visible precipitin arc. Similarly, antigen of non-pathogen (Tha) when reacted with 491A (antisera of resistant variety) no precipitation arc was discernible. There was also no precipitation arc when normal sera (NS) reacted with different antigens.

**Table 10:** Semi quantitative estimation of antigens and antisera of tea varieties and *Lasiodiplodia theobromae.*

| Host and Pathogen | Titre of antigen against homologous antiserum | Titre of antiserum against homologous antigens |
|---|---|---|
| **Host Variety** | | |
| TS-449 | 8 | 16 |
| TS-491 | 8 | 16 |
| **Pathogen** | | |
| *L. theobromae* | 16 | 32 |

Incubation time, 72h
Temperature, 25±1°C

**Table 11:** Common antigenic relationship between tea seed varieties and *Lasiodiplodia theobromae* (Based on agar gel double diffusion test).

| Antigen of pathogen, host and non-pathogen | Antisera of pathogen and host | |
|---|---|---|
| | *L. theobromae* (LtA) | TS-449 (449A) |
| Pathogen | | |
| *L. theobromae* | ++++ | ++ |
| Susceptible varieties | | |
| TS-449 (449a) | +++ | ++++ |
| TS-464 (464a) | +++ | ++++ |
| Resistant verities | | |
| TS-491 (491a) | – | ++ |
| TS-463 (463a) | – | ++ |
| Non-pathogen | | |
| *Trichoderma harzianum*(Tha) | – | – |

Common precipitin band absent (-); faint (+); prominent (++); few (+++); many (++++)

The codes of different antigens and antisera are in the parenthesis.

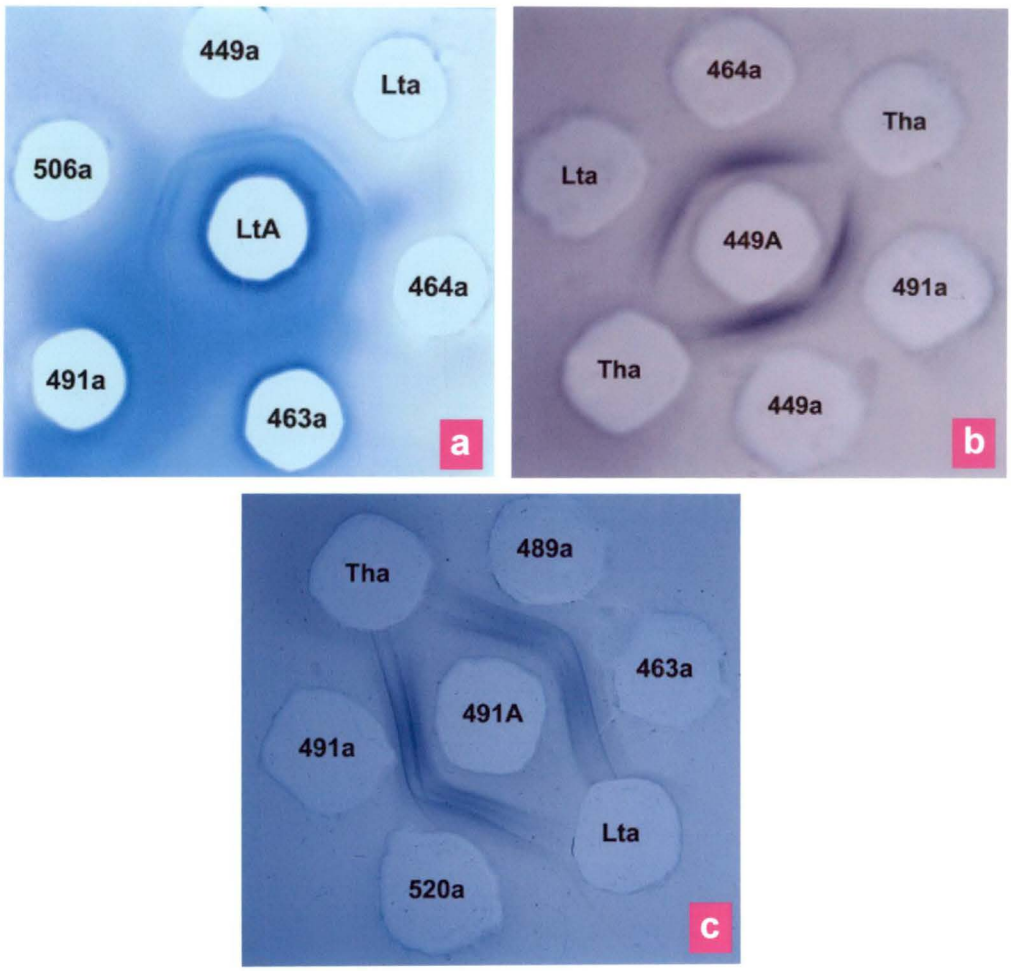


**PLATE VIII**

**Agar gel double diffusion test using different antigens and antisera.**

**fig. a :** Different tea root and pathogen antigen used in the wells are TS-560 (506a), TS-449 (449a), TS-464 (464a),TS-463 (463a), TS-491 (491a) and *Lasiodiplodia theobromae* (Lta) were placed in the peripheral wells and antisera of LtA in the central well.

**fig. b :** Different tea root, pathogen and non-pathogen antigen used in the wells are TS-449 (449a), TS-464 (464a), TS-491 (491a), *Lasiodiplodia theobromae* (Lta) and *Trichoderma harzianum* (Tha) were placed in the peripheral wells and antisera of TS-449 (449A) in the central well.

**fig. c :** Different tea root, pathogen and non-pathogen antigen used in the wells are of TS-489 (489a), TS-463 (463a), TS-520 (520a), TS-491 (491a), *Trichoderma harzianum* (Tha)) and *Lasiodiplodia theobromae* (Lta) in the peripheral wells and antisera of TS-491 (491A) in the central well.

### 4.3.2. Serological relationship between different tea varieties and test pathogen (*L. theobromae*) by immunoelectrophoresis

A combination of electrophoresis and radial immunodiffusion in agar gel is called immunoelectrophoresis. Immunoelectrophoresis was performed following the method as described in materials and methods section 3.9.2. Seven different antigens of tea (449a, 464a, 520a, 506a, 489a, 463a, 491a), two antigens of fungi (Lta and Tha) and two antisera (449A and LtA) were used for the study.

From Fig.7 and Table 12 & 13, it was evident that antigens 463a and 491a shared four precipitin arcs when reacted with the antisera 449A, antigens 506a and 520a shared three precipitin arcs when reacted with the antisera 449A but antigens 489a and 463a showed two precipitin arcs when reacted with the antisera 449A. Similarly, when antisera of *L. theobromae* (LtA) were subjected to react with antigens (449a and 464a) three precipitin arcs were found. But antigens 506a and 520a showed two precipitin arcs with the same antisera. No arc was found between LtA and either of the antigens 463a and 491a. Precipitin arc was also absent between antisera LtA and antigen Tha.

### 4.3.3. Indirect enzyme linked immunosorbent assay (ELISA) between *L. theobromae* and different tea seed varieties.

The different format of enzyme linked immunosorbent assays (ELISA) has become popular as a diagnostic tool in a practical form (Bom and Boland, 2000; Kennedy *et al.* 2000, Sumarah and Miller, 2005; Babitha *et al.*, 2006). The indirect-ELISA technique is one of the most sensitive serological techniques to detect and quantify low concentration of antigen. It has been clearly pointed out by several authors (Chakraborty and Saha, 1994; Kratka *et al.*, 2002; Dasgupta *et al.*, 2005) on the basis of their findings that indirect-ELISA can serve as a useful technique to detect cross-reactive antigens, which determine the susceptibility or resistance of a host. Therefore, it was considered worthwhile to study cross-reactive antigens between *L. theobromae* and tea seed varieties by using indirect- ELISA format.In the present study antigens (prepared from roots, leaves and stems) of seven different tea seed varieties (TS-464, TS-491, TS-506, TS-520, TS-489, TS-449 and TS-463) were tested against three different antisera (449A, 491A and LtA).

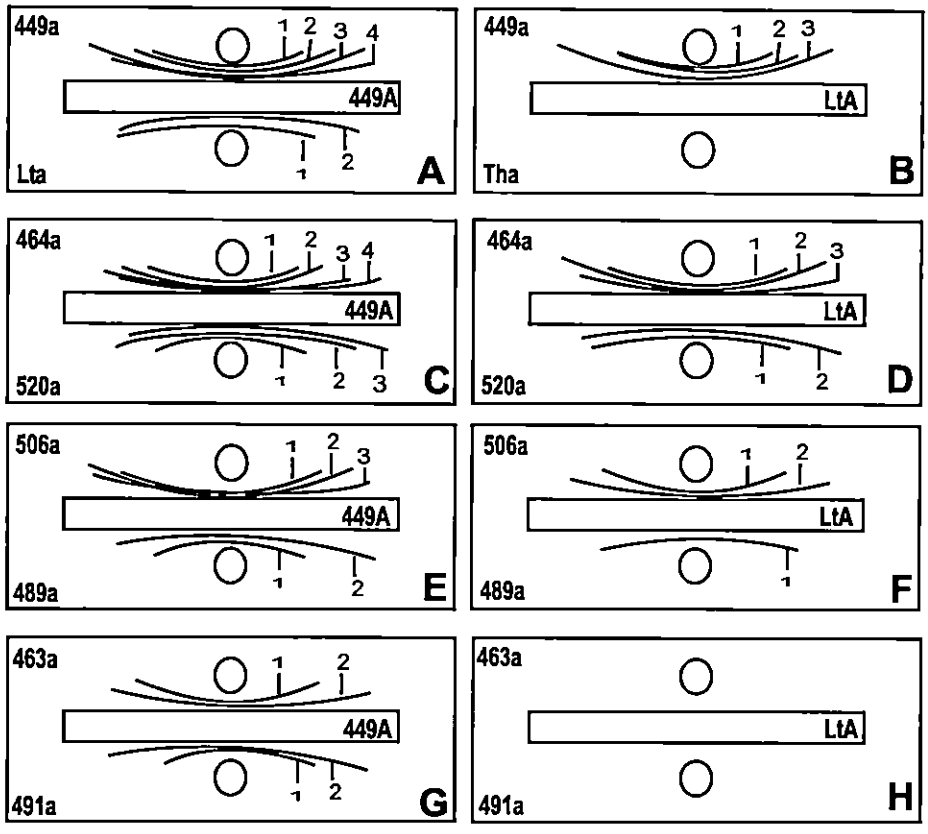


**Fig.7(A-H):** Immunogram showing immunoelectrophoresis patterns of antigens and antisera. Different antigens used in the peripheral wells are of TS-449 (449a), TS-464 (464a), TS-520 (520a), TS-489 (489a), TS-463 (463a), TS-491(491a),*Trichoderma harzianum* (Tha) and *Lasiodiplodia theobromae* (Lta). Different antisera used in the central rectangular troughs are of TS-449 (449A) and *L. theobromae* (LtA)

**Table 12:** Immunoelectrophoresis test of antigens and antisera of tea varieties and *Lasiodiplodia theobromae*.

| Antigen of pathogen, host and non-pathogen | Antisera of *L. theobromae* (LtA) | | | | Antisera of TS-449 (449A) | | | |
|---|---|---|---|---|---|---|---|---|
| | Precipitin arcs | | | | Precipitin arcs | | | |
| | 1st | 2nd | 3rd | 4th | 1st | 2nd | 3rd | 4th |
| Susceptible variety | | | | | | | | |
| TS-449 (449a) | + | + | + | - | + | + | + | + |
| TS-464 (464a) | + | + | + | - | + | + | + | + |
| Resistant variety | | | | | | | | |
| TS-491 (491a) | - | - | - | - | + | + | - | - |
| TS-463 (463a) | - | - | - | - | + | + | - | - |
| Non-pathogen | | | | | | | | |
| *Trichoderma harzianum* (Tha) | - | - | - | - | - | - | - | - |

Common precipitin band present (+)
Common precipitin band absent (-)
The codes of different antigens and antisera are in the parenthesis.

**Table 13:** Comparison of precipitation arcs found in immunoelectrophoresis of tea seed varieties (susceptible and resistant), pathogen (*Lasiodiplodia theobromae*) and non-pathogen (*Trichoderma harzianum*).

| Antigens of pathogen, host and non-pathogen | Total no. of precipitin arcs Antisera of pathogen and host | |
|---|---|---|
| | *L. theobomae* (LtA)* | TS-449 (449A) |
| Susceptible variety | | |
| TS-449 (449a) | 3 | 4 |
| TS-464 (464a) | 3 | 4 |
| Resistant variety | | |
| TS-491 (491a) | 0 | 2 |
| TS-463 (463a) | 0 | 2 |
| Non-pathogen | | |
| *T. harzianum* (Tha) | 0 | 0 |

*The codes of the different antigen and antisera are in the parenthesis.

In the present study antigens (prepared from roots, leaves and stems) of seven different tea seed varieties (TS-464, TS-491, TS-506, TS-520, TS-489, TS-449 and TS-463) were tested against three different antisera (449A, 491A and LtA). The antigen codes of tea varieties were further prefixed with uppercase letter 'R','L' or 'S' to designate antigens prepared from root, leaf or stem respectively. Three different dilutions of antigens (5µg protein/ml, 10µg protein/ml and 20µg protein/ml) and two different dilutions of antisera (1/125 and 1/250) were used. The detail procedure of indirect-ELISA as well as the preparation of antigens and antisera has already been discussed in the materials and methods (Sections-3.10, 3.7 & 3.8). An ELISA reader determined the absorbance of all the combinations at 492 nm and the results are given in Table 14, 15 &16.

From Table 14, it was found that root antigens of different tea varieties showed differential absorbance with three different antisera in indirect-ELISA. Antisera (449A) of susceptible variety, TS-449, showed maximum absorbance with homologous antigen and minimum with root antigen (R491a) of resistant variety TS-491. When antisera (LtA) of *L. theobromae* were used, it showed maximum absorbance with the antigen (R449a) of susceptible variety but minimum with the antigen (R491a) of resistant variety. Similarly when antisera (491A) of resistant variety reacted with homologous antigen, maximum absorbance was found but minimum absorbance was recorded when 491A reacted with antigen R449a.

Leaf antigens of different tea varieties also showed differential absorbance with different antisera during indirect-ELISA. Antisera (449A) of susceptible plant showed maximum absorbance with leaf antigen (L449a) of susceptible plant and minimum in case of leaf antigen (L491a) of resistant plant. When antiserum (LtA) of *L. theobromae* was used, it showed maximum absorbance with the antigen (L449a) of susceptible plant, but minimum with the antigen (L491a) of resistant plant. Maximum absorbance was recorded when antiserum (491A) of resistant tea seed variety TS-491, reacted with homologous antigen L491a. The same antisera (491A) showed minimum absorbance when reacted with antigen L449a (Table 15). Similar results were also found from Table 16, where tea stem antigens of different tea varieties showed absorbance with three different antisera used during the study. Antisera (449A) of susceptible plants showed maximum absorbance with stem antigen (S449a) of susceptible tea variety and minimum in case of stem antigen

**Table 14:** Indirect ELISA ($A_{492}$) result of different combination of antigens (Twenty one antigens) of root of seven varieties and of *Lasiodiplodia theobromae* against three different root antisera of TS-491, TS-449 and *L. theobromae*.

| Antigen (Root antigen) | µg protein /ml | Normal sera (NS) and antisera (AS) of susceptible and resistant tea varieties | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | TS-449 (449A) | | | TS-491 (491A) | | | *L. theobromae* (LtA) | | |
| | | NS 1/125 | AS 1/125 | AS 1/250 | NS 1/125 | AS 1/125 | AS 1/250 | NS 1/125 | AS 1/125 | AS 1/250 |
| TS-491 (R491a) | 20 | 0.038 | 0.350 | 0.261 | 0.045 | 0.920 | 0.715 | 0.043 | 0.135 | 0.117 |
| | 10 | 0.031 | 0.395 | 0.187 | 0.034 | 0.863 | 0.676 | 0.037 | 0.126 | 0.103 |
| | 5 | 0.026 | 0.328 | 0.125 | 0.027 | 0.770 | 0.568 | 0.028 | 0.100 | 0.090 |
| TS-463 (R463a) | 20 | 0.028 | 0.590 | 0.394 | 0.024 | 0.838 | 0.600 | 0.032 | 0.151 | 0.121 |
| | 10 | 0.027 | 0.460 | 0.201 | 0.022 | 0.780 | 0.586 | 0.028 | 0.137 | 0.116 |
| | 5 | 0.024 | 0.383 | 0.170 | 0.023 | 0.759 | 0.479 | 0.027 | 0.120 | 0.113 |
| TS-489 (R489a) | 20 | 0.025 | 0.684 | 0.496 | 0.024 | 0.759 | 0.558 | 0.029 | 0.176 | 0.153 |
| | 10 | 0.023 | 0.513 | 0.333 | 0.021 | 0.735 | 0.404 | 0.026 | 0.150 | 0.128 |
| | 5 | 0.022 | 0.437 | 0.277 | 0.020 | 0.650 | 0.381 | 0.025 | 0.143 | 0.109 |
| TS-506 (R506a) | 20 | 0.026 | 0.691 | 0.500 | 0.031 | 0.681 | 0.460 | 0.031 | 0.196 | 0.166 |
| | 10 | 0.024 | 0.588 | 0.487 | 0.027 | 0.630 | 0.389 | 0.024 | 0.170 | 0.150 |
| | 5 | 0.023 | 0.478 | 0.370 | 0.023 | 0.561 | 0.326 | 0.025 | 0.143 | 0.112 |
| TS-520 (R520a | 20 | 0.025 | 0.771 | 0.681 | 0.025 | 0.574 | 0.360 | 0.028 | 0.278 | 0.249 |
| | 10 | 0.023 | 0.62 | 0.508 | 0.022 | 0.427 | 0.233 | 0.025 | 0.193 | 0.167 |
| | 5 | 0.021 | 0.54 | 0.481 | 0.023 | 0.322 | 0.219 | 0.024 | 0.188 | 0.132 |
| TS-464 (R464a) | 20 | 0.028 | 0.792 | 0.734 | 0.024 | 0.413 | 0.270 | 0.031 | 0.385 | 0.343 |
| | 10 | 0.027 | 0.666 | 0.619 | 0.022 | 0.319 | 0.228 | 0.028 | 0.281 | 0.255 |
| | 5 | 0.020 | 0.584 | 0.540 | 0.021 | 0.224 | 0.197 | 0.025 | 0.259 | 0.213 |
| TS-449 (R449a) | 20 | 0.028 | 0.878 | 0.782 | 0.038 | 0.373 | 0.291 | 0.028 | 0.535 | 0.438 |
| | 10 | 0.025 | 0.729 | 0.624 | 0.026 | 0.299 | 0.219 | 0.027 | 0.380 | 0.391 |
| | 5 | 0.024 | 0.635 | 0.502 | 0.023 | 0.191 | 0.155 | 0.024 | 0.352 | 0.270 |
| *L. theobromae* ( Lta) (Pathogen) | 20 | 0.025 | 0.646 | 0.420 | 0.036 | 0.283 | 0.154 | 0.028 | 0.765 | 0.589 |
| | 10 | 0.024 | 0.536 | 0.360 | 0.027 | 0.121 | 0.117 | 0.027 | 0.675 | 0.421 |
| | 5 | 0.023 | 0.346 | 0.257 | 0.018 | 0.108 | 0.065 | 0.020 | 0.612 | 0.345 |
| *T. harzianum* (Tha) (Non-Pathogen) | 20 | 0.026 | 0.146 | 0.120 | 0.038 | 0.133 | 0.114 | 0.025 | 0.117 | 0.108 |
| | 10 | 0.024 | 0.126 | 0.110 | 0.028 | 0.121 | 0.097 | 0.023 | 0.097 | 0.086 |
| | 5 | 0.020 | 0.116 | 0.097 | 0.012 | 0.108 | 0.045 | 0.020 | 0.068 | 0.058 |

* Codes of antigens and antisers are in the parenthesis.

**Table 15:** Indirect ELISA ($A_{492}$) result of different combination of antigens (Twenty one antigens) of leaf of seven varieties and of *Lasiodiplodia theobromae* against three different antisera of TS-491, TS-449 and *L. theobromae.*

| Antigen (Leaf antigen) | µg protein/ml | Normal sera (NS) and antisera (AS) of susceptible and resistant tea varieties | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | TS-449 (449A) | | | TS-491 (491A) | | | *L. theobromae* (LTA) | | |
| | | NS 1/125 | AS 1/125 | AS 1/250 | NS 1/125 | AS 1/125 | AS 1/250 | NS 1/125 | AS 1/125 | AS 1/250 |
| TS-491 (L491a) | 20 | 0.031 | 0.351 | 0.265 | 0.031 | 0.950 | 0.722 | 0.025 | 0.113 | 0.072 |
| | 10 | 0.027 | 0.283 | 0.252 | 0.027 | 0.725 | 0.672 | 0.023 | 0.066 | 0.052 |
| | 5 | 0.023 | 0.240 | 0.210 | 0.023 | 0.633 | 0.595 | 0.021 | 0.043 | 0.046 |
| TS-463 (L463a) | 20 | 0.025 | 0.464 | 0.324 | 0.025 | 0.856 | 0.696 | 0.028 | 0.123 | 0.120 |
| | 10 | 0.022 | 0.386 | 0.317 | 0.022 | 0.682 | 0.573 | 0.027 | 0.101 | 0.106 |
| | 5 | 0.023 | 0.387 | 0.301 | 0.023 | 0.539 | 0.522 | 0.020 | 0.081 | 0.082 |
| TS-489 (L489a) | 20 | 0.024 | 0.561 | 0.397 | 0.024 | 0.773 | 0.613 | 0.028 | 0.236 | 0.207 |
| | 10 | 0.022 | 0.427 | 0.381 | 0.022 | 0.670 | 0.495 | 0.025 | 0.187 | 0.160 |
| | 5 | 0.021 | 0.420 | 0.328 | 0.021 | 0.447 | 0.392 | 0.024 | 0.143 | 0.126 |
| TS-506 (L506a) | 20 | 0.038 | 0.668 | 0.449 | 0.038 | 0.615 | 0.558 | 0.025 | 0.240 | 0.212 |
| | 10 | 0.026 | 0.517 | 0.394 | 0.026 | 0.535 | 0.486 | 0.022 | 0.219 | 0.182 |
| | 5 | 0.023 | 0.470 | 0.319 | 0.023 | 0.441 | 0.321 | 0.023 | 0.182 | 0.135 |
| TS-520 (L520a | 20 | 0.029 | 0.722 | 0.593 | 0.024 | 0.546 | 0.457 | 0.024 | 0.369 | 0.349 |
| | 10 | 0.026 | 0.633 | 0.474 | 0.022 | 0.480 | 0.339 | 0.022 | 0.332 | 0.234 |
| | 5 | 0.025 | 0.524 | 0.417 | 0.023 | 0.345 | 0.269 | 0.021 | 0.278 | 0.171 |
| TS-464 (L464a) | 20 | 0.031 | 0.885 | 0.622 | 0.024 | 0.494 | 0.414 | 0.038 | 0.383 | 0.382 |
| | 10 | 0.027 | 0.766 | 0.512 | 0.021 | 0.450 | 0.240 | 0.026 | 0.331 | 0.346 |
| | 5 | 0.023 | 0.694 | 0.492 | 0.020 | 0.312 | 0.195 | 0.023 | 0.306 | 0.325 |
| TS-449 (L449a) | 20 | 0.025 | 0.921 | 0.674 | 0.031 | 0.412 | 0.365 | 0.028 | 0.526 | 0.466 |
| | 10 | 0.022 | 0.825 | 0.674 | 0.031 | 0.412 | 0.365 | 0.028 | 0.526 | 0.466 |
| | 5 | 0.023 | 0.858 | 0.513 | 0.027 | 0.363 | 0.220 | 0.025 | 0.426 | 0.386 |
| *L. theobromae* ( Lta) (Pathogen) | 20 | 0.022 | 0.640 | 0.419 | 0.039 | 0.287 | 0.158 | 0.028 | 0.765 | 0.589 |
| | 10 | 0.021 | 0.532 | 0.357 | 0.025 | 0.129 | 0.115 | 0.027 | 0.675 | 0.421 |
| | 5 | 0.020 | 0.344 | 0.243 | 0.022 | 0.102 | 0.061 | 0.020 | 0.612 | 0.345 |
| *T. harzianum* (Tha) (Non-Pathogen) | 20 | 0.028 | 0.143 | 0.128 | 0.042 | 0.137 | 0.117 | 0.025 | 0.117 | 0.108 |
| | 10 | 0.021 | 0.121 | 0.116 | 0.026 | 0.124 | 0.092 | 0.023 | 0.097 | 0.086 |
| | 5 | 0.018 | 0.112 | 0.095 | 0.016 | 0.098 | 0.040 | 0.020 | 0.068 | 0.058 |

* Codes of antigens and antisers are in the parenthesis

**Table 16**: Indirect ELISA ($A_{492}$) result of different combination of antigens (Twenty one antigens) of stem of seven varieties and of *Lasiodiplodia theobromae* against three different root antisera of TS-491, TS-449 and *L. theobromae*.

| Antigen (Stem antigen) | µg protein /ml | Normal sera (NS) and antisera (AS) of susceptible and resistant tea varieties | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | TS-449 (449A) | | | TS-491 (491A) | | | *L. theobromae* (LTA) | | |
| | | NS 1/125 | AS 1/125 | AS 1/250 | NS 1/125 | AS 1/125 | AS 1/250 | NS 1/125 | AS 1/125 | AS 1/250 |
| TS-491 (S491a) | 20 | 0.031 | 0.206 | 0.176 | 0.038 | 0.768 | 0.698 | 0.025 | 0.194 | 0.158 |
| | 10 | 0.027 | 0.147 | 0.103 | 0.026 | 0.602 | 0.569 | 0.022 | 0.155 | 0.111 |
| | 5 | 0.023 | 0.102 | 0.087 | 0.023 | 0.590 | 0.450 | 0.023 | 0.115 | 0.094 |
| TS-463 (S463a) | 20 | 0.025 | 0.281 | 0.231 | 0.025 | 0.656 | 0.535 | 0.031 | 0.213 | 0.207 |
| | 10 | 0.022 | 0.160 | 0.134 | 0.023 | 0.434 | 0.405 | 0.027 | 0.162 | 0.148 |
| | 5 | 0.023 | 0.139 | 0.120 | 0.021 | 0.418 | 0.381 | 0.023 | 0.146 | 0.132 |
| TS-489 (S489a) | 20 | 0.024 | 0.345 | 0.287 | 0.028 | 0.612 | 0.482 | 0.025 | 0.242 | 0.285 |
| | 10 | 0.022 | 0.232 | 0.176 | 0.027 | 0.387 | 0.335 | 0.022 | 0.217 | 0.224 |
| | 5 | 0.021 | 0.186 | 0.164 | 0.020 | 0.312 | 0.275 | 0.023 | 0.154 | 0.196 |
| TS-506 (S506a) | 20 | 0.038 | 0.387 | 0.342 | 0.028 | 0.534 | 0.323 | 0.024 | 0.287 | 0.327 |
| | 10 | 0.026 | 0.278 | 0.235 | 0.025 | 0.324 | 0.286 | 0.022 | 0.224 | 0.286 |
| | 5 | 0.023 | 0.215 | 0.169 | 0.024 | 0.276 | 0.215 | 0.021 | 0.208 | 0.223 |
| TS-520 (S520a | 20 | 0.029 | 0.451 | 0.429 | 0.025 | 0.488 | 0.303 | 0.038 | 0.536 | 0.357 |
| | 10 | 0.026 | 0.383 | 0.281 | 0.022 | 0.315 | 0.265 | 0.026 | 0.482 | 0.315 |
| | 5 | 0.025 | 0.321 | 0.232 | 0.023 | 0.210 | 0.175 | 0.023 | 0.356 | 0.247 |
| TS-464 (S464a) | 20 | 0.038 | 0.497 | 0.434 | 0.024 | 0.426 | 0.287 | 0.029 | 0.558 | 0.412 |
| | 10 | 0.026 | 0.425 | 0.345 | 0.022 | 0.289 | 0.189 | 0.026 | 0.524 | 0.398 |
| | 5 | 0.023 | 0.386 | 0.312 | 0.021 | 0.147 | 0.120 | 0.025 | 0.482 | 0.386 |
| TS-449 (R449a) | 20 | 0.029 | 0.585 | 0.518 | 0.025 | 0.320 | 0.233 | 0.025 | 0.579 | 0.444 |
| | 10 | 0.026 | 0.615 | 0.454 | 0.022 | 0.132 | 0.115 | 0.023 | 0.551 | 0.439 |
| | 5 | 0.025 | 0.706 | 0.574 | 0.023 | 0.106 | 0.097 | 0.021 | 0.463 | 0.428 |
| *L. theobromae* ( Lta) (Pathogen) | 20 | 0.038 | 0.634 | 0.465 | 0.024 | 0.165 | 0.128 | 0.028 | 0.765 | 0.589 |
| | 10 | 0.026 | 0.567 | 0.412 | 0.022 | 0.143 | 0.109 | 0.027 | 0.675 | 0.421 |
| | 5 | 0.023 | 0.487 | 0.356 | 0.021 | 0.127 | 0.086 | 0.020 | 0.612 | 0.345 |
| *T. harzianum* (Tha) (Non-Pathogen) | 20 | 0.023 | 0.148 | 0.122 | 0.039 | 0.135 | 0.118 | 0.025 | 0.117 | 0.108 |
| | 10 | 0.021 | 0.125 | 0.113 | 0.027 | 0.124 | 0.098 | 0.023 | 0.097 | 0.086 |
| | 5 | 0.018 | 0.111 | 0.099 | 0.014 | 0.107 | 0.045 | 0.020 | 0.068 | 0.058 |

* Codes of antigens and antisers are in the parenthesis.

(S491a) of resistant tea variety. When antisera (LtA) of *L. theobromae* were used, it also showed highest absorbance with the antigen (S449a) of susceptible tea variety, but minimum with the antigen (S491a) of resistant variety. Antisera (491A) of resistant tea variety TS-491, showed maximum absorbance when reacted with antigen S491a, but minimum when reacted with antigen S449a. From the ELISA ($A_{492}$) results it was clear that cross-reactivity was higher between pathogen and susceptible tea varieties than pathogen and resistant varieties. Results obtained from the combinations showed that the absorbance values of normal serum in control were lower than the corresponding test values.

### 4.3.4. Comparative analysis of ELISA and Cross reactive antigens (CRA)

Higher absorbance value in indirect ELISA corresponds to presence of higher CRA. Presence of higher CRA indicate compatibility and susceptibility whereas lower ELISA values indicate presence of lower CRA and indicate resistance. Accordingly indirect-ELISA value reflected the presence of CRA (10µg/ml) in different tea varieties like TS-464 (R464a), TS-491 (R491a), TS-506 (R506a), TS-520 (R520a), TS-489 (R489a), TS-463 (R464a) and TS-491 (R491a) against the antisera (1:125) of *L. theobromae* (LtA). Results have been presented graphically in (Fig.8 & 9). From the figure it was evident that R449a showed maximum absorbance while R491a showed minimum absorbance. Hence, it may be concluded that TS-449 was most susceptible and TS-491 was most resistant to the pathogen *L. theobromae* than the other tea varieties used in the present study. Susceptibility of the cultivars proportionally increased with increasing CRA among host and pathogen. Resistance and susceptibility determined by conventional pathogenicity test (Table 8 & 9) were in conformity with that of CRA-based pathogenicity.

### 4.3.5. Immunofluorescence for cellular location of antigens and cross-reactive antigens

Immunofluorescence is the method for observing cellular location of antigens by the use of fluorescent-labeled antibodies (Goldsby et al., 2003). A fluorescent compound has the property of emitting light of certain wavelength when it is excited by exposure to light of shorter wavelength. The emitted light from the antibodies can be viewed with a fluorescence microscope equipped with a UV light source. It was also found

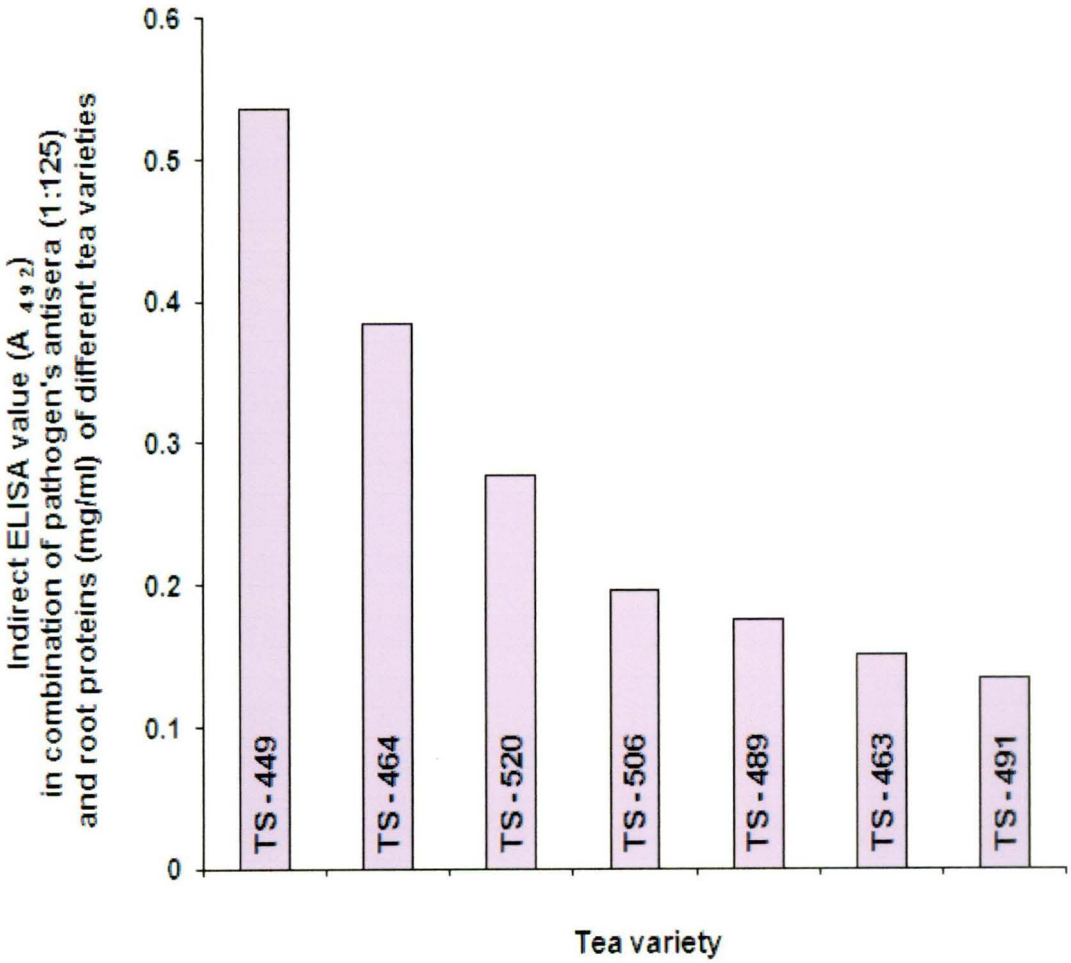


**Fig. 8:** Indirect ELISA value ($A_{492}$) in combination of pathogen's antisera (1:125) and root proteins (10µg/ml) of different tea varieties.

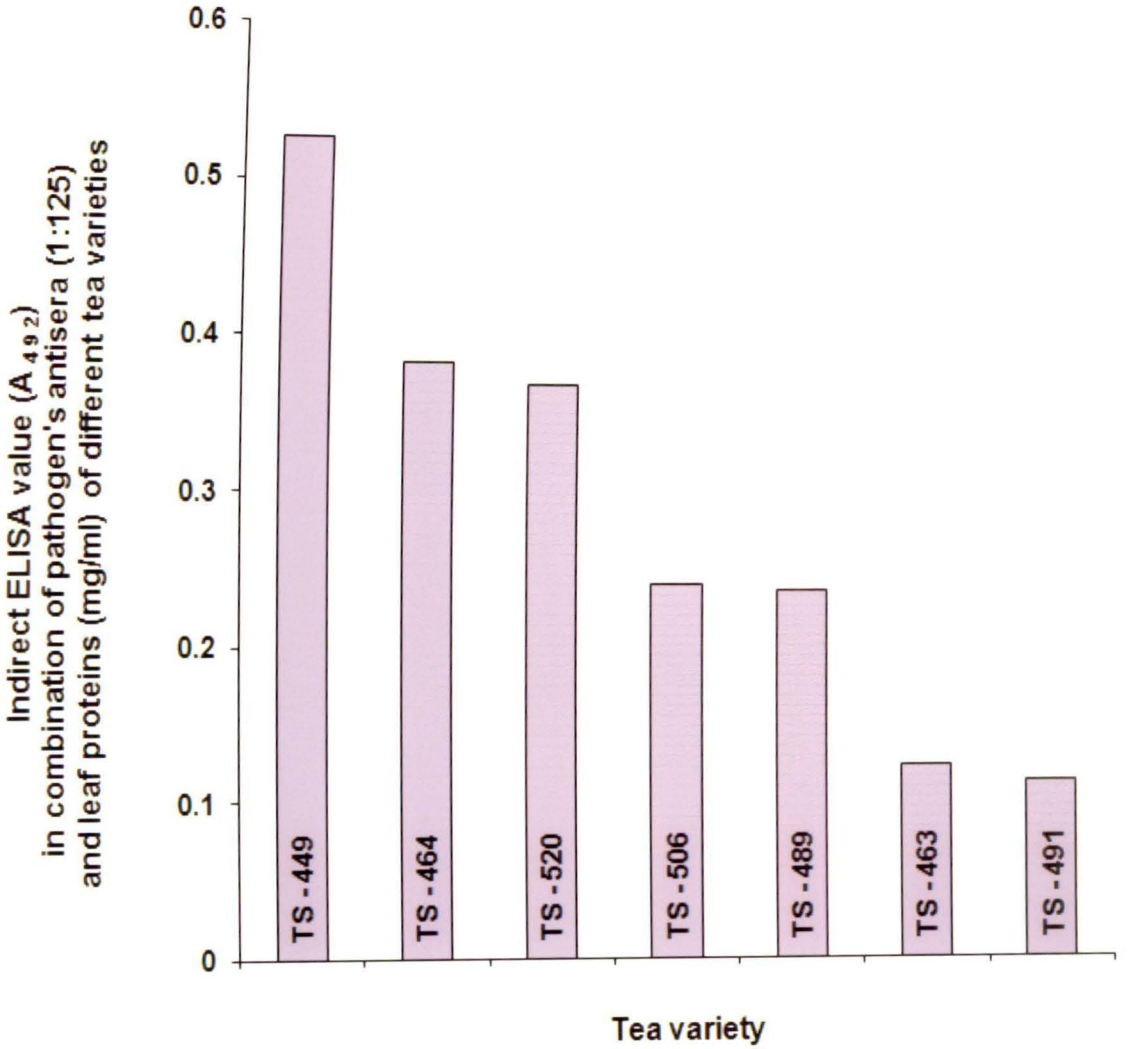


**Fig. 9:** Indirect ELISA value ($A_{492}$) in combination of pathogen's antisera (1:125) and leaf proteins (10µg/ml) of different tea varieties.

that cross-reactive antigens were varied in different parts like stem, leaf and root of plant against the antisera raised against the test pathogen. To find out tissue and cellular location of cross-reactive antigens using the antisera of test pathogen, immunofluorescence study was performed following the method of De Vay *et al.* (1981). Detailed method has been discussed in materials and methods (Section-3.11.1).

In the present study, roots of six months old plants of two different varieties, resistant (TS-491) and susceptible (TS-449) were collected from experimental garden for immunofluorescence study. Two different types of antisera, 449A and LtA were used for the study. From the observation of unstained root sections under UV-fluorescence microscope, a faint natural autofluorescence in the outer walls of the epidermal cells were revealed (data not shown). A similar type of fluorescence was also observed in the root sections when treated with normal serum followed by fluorescein isothiocyanate (FITC) labelling. Root sections of TS-449 (used as *in situ* antigen source) showed bright fluorescence in the epidermal region, cortical cells and xylem elements when it was treated with antisera (449A) of respective seed variety (i.e. homologous antiserum) and indirectly labelled with FITC (Plate IX: Fig. a). In heterologous reactions i.e. when root sections of susceptible tea variety (TS-449) were treated with antisera of *L. theobromae* (LtA) and indirectly labelled with FITC, strong bright fluorescence was observed (Plate IX: fig. b). Comparatively less fluorescence was found when mycelial mat of *L. theobromae* (used as *in situ* antigen source) were treated with antisera (491A) of resistant tea variety followed by indirect labelling with FITC (Plate IX: fig. c).

### 4.3.6. Immunogold labelling for cellular location of antigens and cross-reactive antigens

Immunogold labelling and electron microscopy is a widely used tool for detection of cellular location of antigens (Lee *et al.*, 2000; Trillus *et al.*, 2000; Nahalkova *et al.*, 2001). To visualize immunogold labels in light microscope, silver enhancement is essential. Colloidal gold labels are normally visible only under electron microscope. Silver enhancer enhances the colloidal gold label by precipitation of metallic silver to give high contrast signal visible under light microscope.

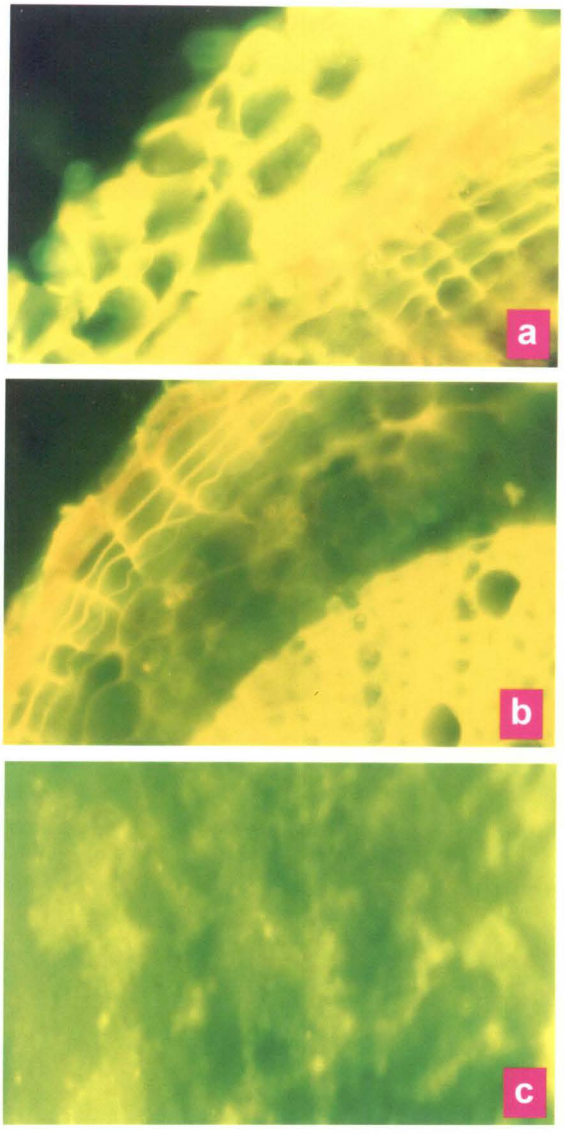


**PLATE IX**

**Fluorescein isothiocyanate (FITC) antibody staining of tea root tissues for detection of cross-reactive antigens and homologous antigens.**

**fig. a :** Root section of TS-449(449a) treated with antisera of TS - 449 (449A) and FITC-conjugated antirabbit goat antisera.

**fig. b :** Root section of TS-449(449a) treated with antisera of *Lasiodiplodia theobromae* (LtA) and FITC-conjugated antirabbit goat antisera .

**flg. c :** Mycelial mat (Lta) treated with antisera of tea root of TS-491(491A) and FITC-conjugated antirabbit goat antisera.

Earlier serological experiments like immunodiffusion, immunoelectrophoresis indirect ELISA and immunofluorescence studies clearly indicated the presence of cross reactive antigens (CRA) between tea varieties and *L. theobromae*. To find out cellular location of cross-reactive antigens in stem, leaf and root of tea varieties against the antisera of pathogen, immunogold-labelling and silver enhancement studies were performed. Thin sections (of roots, leaves and stems) of one susceptible (TS-449) and one resistant (TS-491) variety were used as *in situ* antigen source. To detect cross-reactive antigens in the tea varieties, antisera of TS-449 (449A) and *L. theobromae* (LtA) were used. The detailed procedure has been described in materials and methods (Section-3.11.2).

From the results (Plate X: fig. a-e) it was found that the stem sections of TS-449 treated with antisera (449A) of TS-449 showed strong silver precipitation in epidermal region, cortical cells and xylem elements, but when it was used to treat stem of TS-491, it showed less silver precipitation. Similarly when antiserum of *L. theobromae* (LtA) was used to treat stem sections of susceptible tea variety, TS-449, it showed strong silver precipitation in the above mentioned regions, but showed less precipitation when used to treat stem sections of resistant tea variety, TS-491. Similarly, from the results (Plate XI: fig. a-e) it was also found that leaf-sections of TS-449 treated with the antisera (449A) of TS-449 showed strong silver precipitation in epidermal region, mesophyll tissue and xylem elements, but precipitation was considerably less when it was used to treat leaves of TS-491. Similarly when antisera of the pathogen, *L. theobromae* (LtA) was used to treat leaf-sections of susceptible tea variety TS-449, it showed darker silver precipitation in the above mentioned regions, in comparison to treatment on resistant tea variety TS-491. Almost same results were found when root sections were treated by the same technique (Plate XI: fig. c & d). There was no precipitations (Plate X: fig. e) when normal sera was used to treat leaf-sections of TS-449 and TS-491.

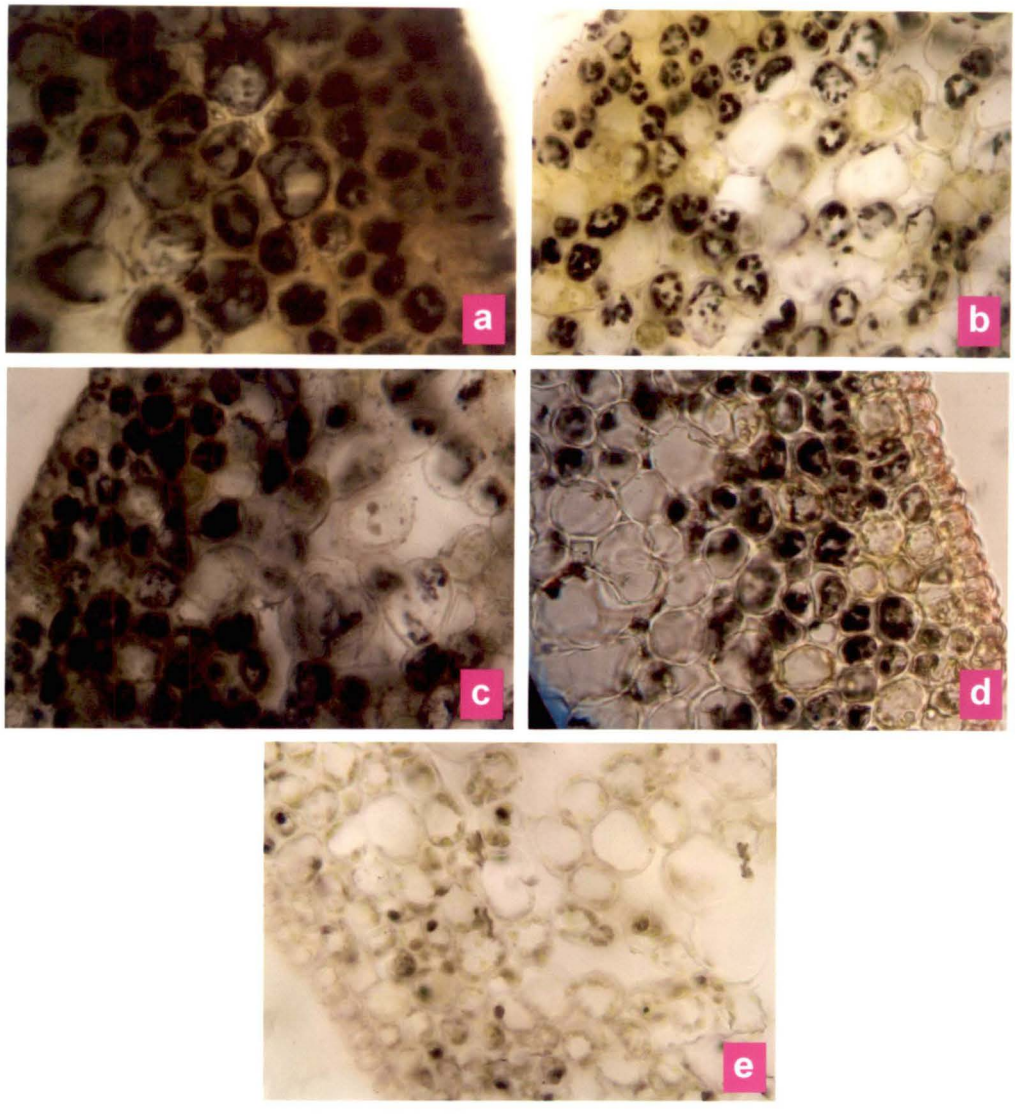


**PLATE X**

**Immunogold labeling and silver enhancement of tea leaf tissues for detection of cellular location of cross-reactive antigens and homologous antigens.**

**fig.a** : Stem tissue (TS-449) treated with antisera of TS-449 (449A) and immunogold labelled IgG.

**fig.b** : Stem tissue (TS-491) treated with antisera of TS-449 (449A) and immunogold labelled IgG.

**fig.c** : Stem tissue (TS-449) treated with antisera of *Lasiodiplodia theobromae* (LtA) and immunogold labelled IgG.

**fig.d** : Stem tissue (TS-491) treated with antisera of *Lasiodiplodia theobromae* (LTA) and immunogold labelled IgG.

**fig.e** : Stem tissue (TS-449) treated with normal sera of rabbit and immunogold labelled IgG.

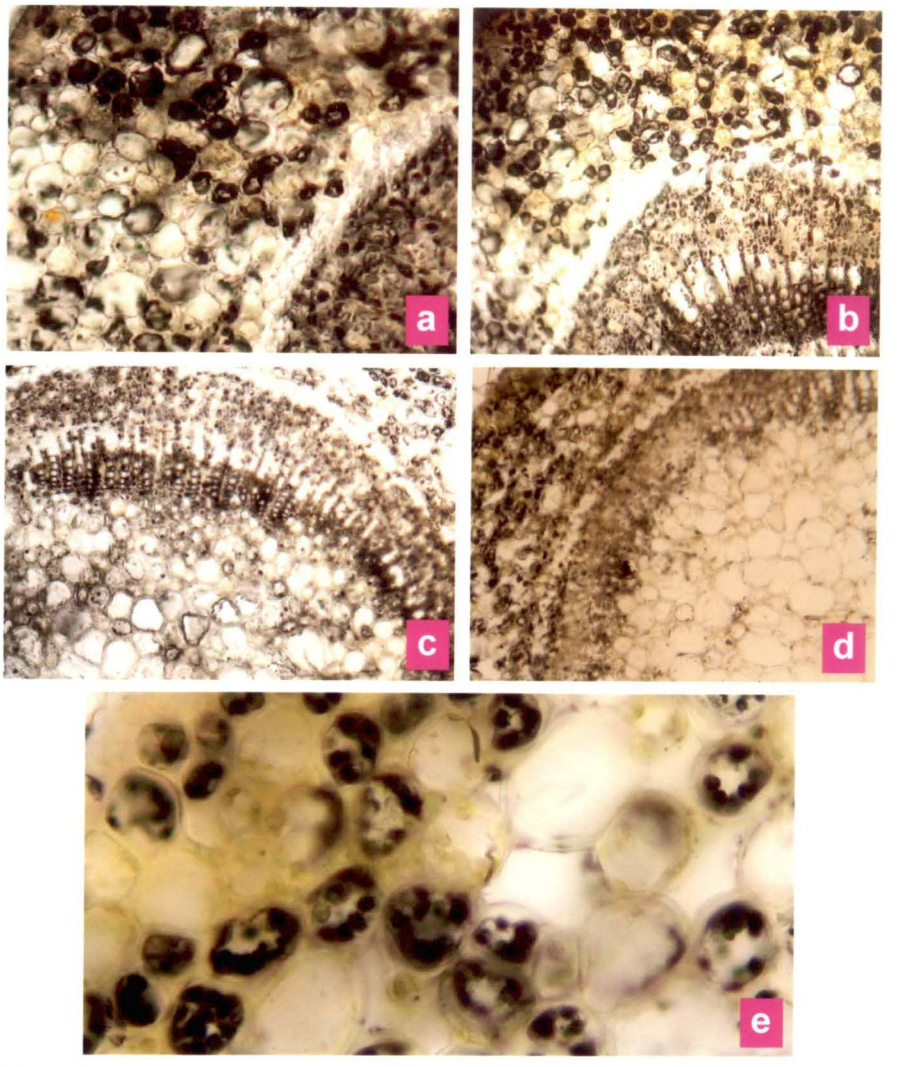


**PLATE XI**

**Immunogold labeling and silver enhancement of tea leaf tissues for detection of cellular location of cross-reactive antigens and homologous antigens.**

**fig.a :** Leaf tissue (TS-449) treated with antisera of *Lasiodiplodia theobromae* (LTA) and immunogold labelled IgG.

**fig.b :** Leaf tissue (TS-491) treated with antisera of *Lasiodiplodia theobromae* (LTA) and immunogold labelled IgG.

**fig.c :** Root tissue (TS-449) treated with antisera of *Lasiodiplodia theobromae* (LtA) and immunogold labelled IgG.

**fig.d :** Root tissue (TS-491) treated with antisera of *Lasiodiplodia theobromae* (LTA) and immunogold labelled IgG.

**fig.e :** Stem cell showed black coloured intracellular location of cross reactive actigens by immunogold labelled IgG.

# Chapter IV

## 4.4. Studies on defense related enzyme: phenylalanine ammonia-lyase (PAL)

It is known that phenylalanine ammonia-lyase (PAL) play a key role in the phenyl-propanoid pathway which catalyses the conversion of L-phenylalanine to trans-cinnamic acid in the first step that lead to the synthesis of defense related compounds like lignin, phytoalexins such as isoflavonoids, and coumarins (Dixon and Lamb, 1990; Mahadevam and Sridhar, 1996). Induced systemic resistance is involved in the expression of enzymes encoded in phenyl-propanoid pathway. PAL activity has been reported to increase by exogenous application of BABA (Newton *et al.*, 1997), aqueous leaf extract of *Azadirachta indica* (Paul and Sharma, 2002) and Benzothiadiazole (Gorlach *et al.*, 1996).

### 4.4.1. PAL activity with abiotic inducers

In the present study, different abiotic inducers (Nickel chloride, Salicylic acid and Jasmonic acid) were used to study PAL activity in susceptible tea seed variety (TS-449). Fresh young (six months old) plants were used for induction of PAL. A corresponding control set was also maintained for each treatment. Twenty five tea plants were taken for each treatment. The detailed procedure of application of abiotic inducers has been discussed in materials and methods (Section-3.12.1) and the procedure of enzyme assay has been discussed in Section 3.13.

About three to four fold increase in PAL activity was observed after 4 d in JA and SA treated plants, while $NiCl_2$ treated plants showed only marginal increase (Table 17, Fig. 10). Treated plants that were challenge inoculated with *L. theobromae* recorded higher enzyme activity than treated-uninoculated plants. Maximum induction of PAL was observed in JA treated plants followed by SA. $NiCl_2$ produced minimum induction (about 2 fold) in PAL activity. Untreated plants inoculated with *L. theobromae* showed slight increase in enzyme activity after 4 d of inoculation. PAL activity increased gradually until $4^{th}$ day following challenge-inoculation by *L. theobromae* and then declined. No change in activity was recorded in the untreated-uninoculated control sets.

**Table 17:** Phenylalanine ammonialyase (PAL) activity in seedlings of TS-449 pre-treated with abiotic inducers followed by challenge-inoculation of *Lasiodiplodia theobromae.*

| Treatments | PAL activity (µmol $min^{-1}g^{-1}$ fresh weight tissue) | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 2.5± 0.02 | 2.6± 0.04 | 2.5± 0.04 | 2.6± 0.05 | 2.7± 0.02 |
| *L.theobromae* | 2.5± 0.04 | 3.4± 0.05 | 3.6± 0.06 | 3.2± 0.03 | 3.0± 0.04 |
| Nickel chloride | 2.6± 0.02 | 2.8± 0.03 | 3.2± 0.04 | 3.6± 0.06 | 3.5± 0.05 |
| Nickel chloride + *L.theobromae* | 2.4± 0.02 | 3.2± 0.03 | 4.7± 0.04 | 5.6± 0.06 | 5.8± 0.02 |
| Salicylic acid | 2.64±0.03 | 3.66±0.05 | 5.68±0.03 | 6.72±0.06 | 7.73±0.03 |
| Salicylic acid + *L.theobromae* | 2.67±0.02 | 4.72±0.04 | 6.73±0.06 | 7.88±0.04 | 8.79±0.07 |
| *Jasmonic acid* | 2.64±0.03 | 4.7± 0.05 | 5.6± 0.06 | 7.8± 0.04 | 8.8± 0.02 |
| Jasmonic acid + *L. theobromae* | 2.67±0.02 | 5.6± 0.05 | 6.8± 0.05 | 8.4± 0.04 | 9.6± 0.07 |

Mean of three replications; Data after ± indicates standard error values.

### 4.4.2. PAL activity with biotic inducers

In the present study, *Trichoderma harzianum* and *Trichoderma virens* were used as biotic inducers for increased expression of PAL activity. Six month old susceptible tea plants (TS-449) were used for the purpose. The detailed procedure of application of biotic inducers in order to trigger plants for induction has been discussed in materials and methods (Section-3.12.2). Twenty five tea plants were taken for each treatment. Corresponding control sets were also maintained.

From the results (Table 18 & Fig. 11) it was found that tea plants treated with *T. virens* and *T. harzianum* showed increased PAL activity. *T. harzianum* produced higher activity than *T. virens* in treated-uninoculated plants but on challenge inoculation by *L. theobromae*, *T. virens* treated plants showed higher PAL activity. In untreated-uninoculated control plants, PAL activity remained unchanged after 4 days of treatment.

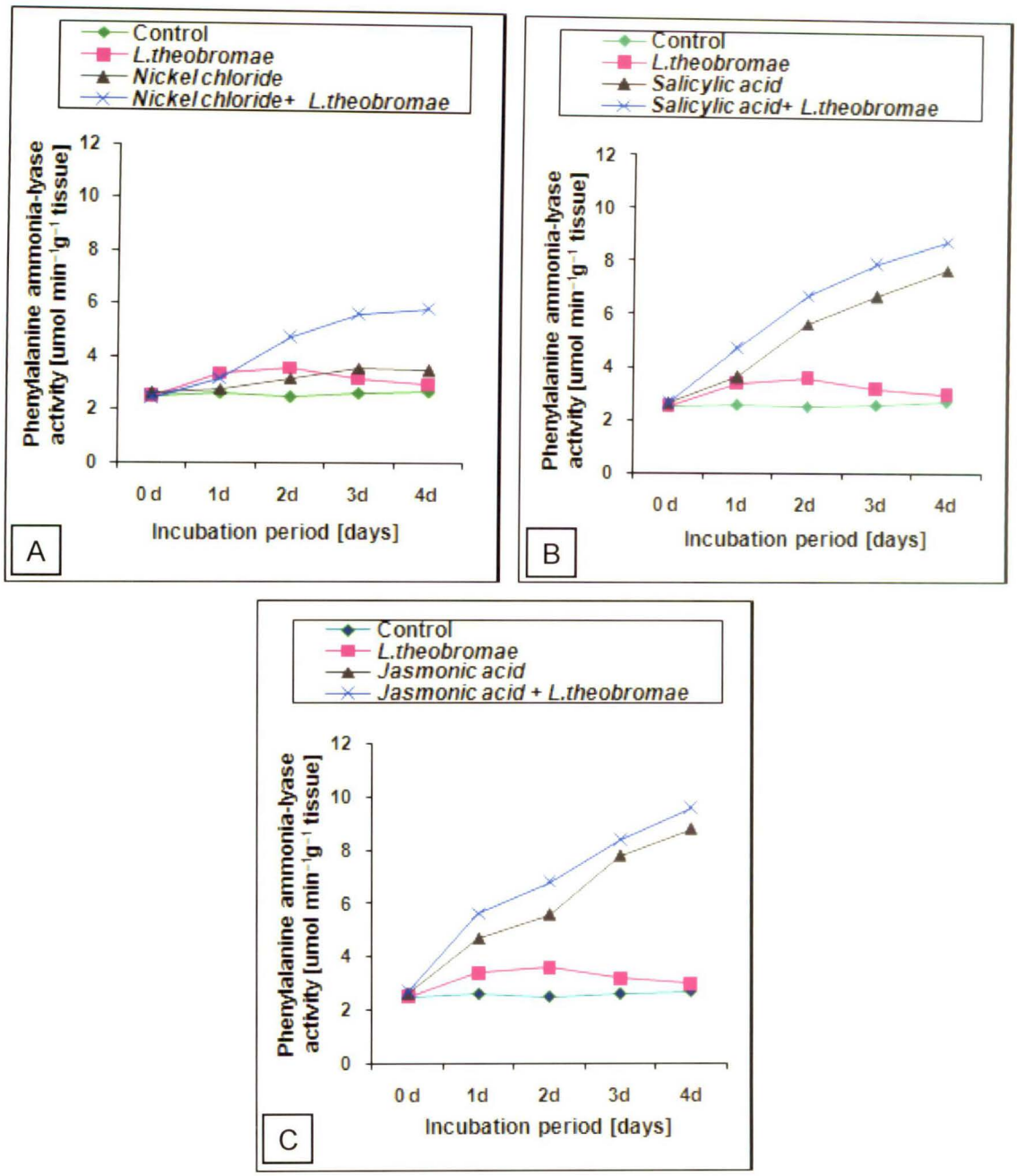


**Fig. 10:** Phenylalanine ammonia lyase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with Nickel chloride.

B. Treated with Salicylic acid.

C. Treated with Jasmonic acid.

**Table 18:** Phenylalanine ammonia lyase (PAL) activity in seedlings of TS-449 pre-treated with biotic inducers followed by challenge-inoculation of *Lasiodiplodia theobromae.*

| Treatments | PAL activity (µmol $min^{-1}g^{-1}$ fresh weight tissue) | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 2.5± 0.02 | 2.6± 0.04 | 2.5± 0.04 | 2.6± 0.05 | 2.7± 0.02 |
| *L.theobromae* | 2.5± 0.04 | 3.4± 0.05 | 3.6± 0.06 | 3.2± 0.03 | 3.0± 0.04 |
| *T. harzianum* | 2.4± 0.04 | 2.8± 0.03 | 3.7± 0.04 | 4.2± 0.05 | 3.8± 0.02 |
| *T. harzianum +L.theobromae* | 2.4± 0.03 | 2.8± 0.03 | 3.7± 0.04 | 4.5± 0.05 | 4.8± 0.04 |
| *T. virens* | 2.4± 0.02 | 3.2± 0.02 | 3.8± 0.05 | 4.0± 0.03 | 2.3± 0.02 |
| *T. virens +L.theobromae* | 2.3± 0.02 | 3.7± 0.04 | 4.2± 0.02 | 4.8± 0.03 | 5.4± 0.03 |

Mean of three replications; Data after ± indicates standard error values.

### 4.4.3. PAL activity with phyto-extracts

Four different aqueous leaf extracts (*Azadirachta indica*, *Acalypha indica*, *Catharanthus roseus* and *Jasminum jasminoides*) were used to induce PAL activity in tea plants. The details procedures of application of phyto-extracts have been discussed in materials and methods (Section-3.12.3). Control sets (untreated-uninoculated control and untreated-inoculated control) were also maintained for each treatment. Twenty five tea plants were kept for each treatment.

From the results (Table 19 & Fig. 12) it is evident that *Azadirachta indica* leaf extract is the most potential inducer of PAL activity among all the tested botanicals. Plants treated with the other three leaf extracts (*Acalypha indica*, *Jasminum jasminoides* and *Catharanthus roseus*) also showed significant increase in enzyme activity. In general, the challenge-inoculated plants showed higher activity than uninoculated but treated plants. Untreated-uninoculated control plants showed little change in enzyme activity.

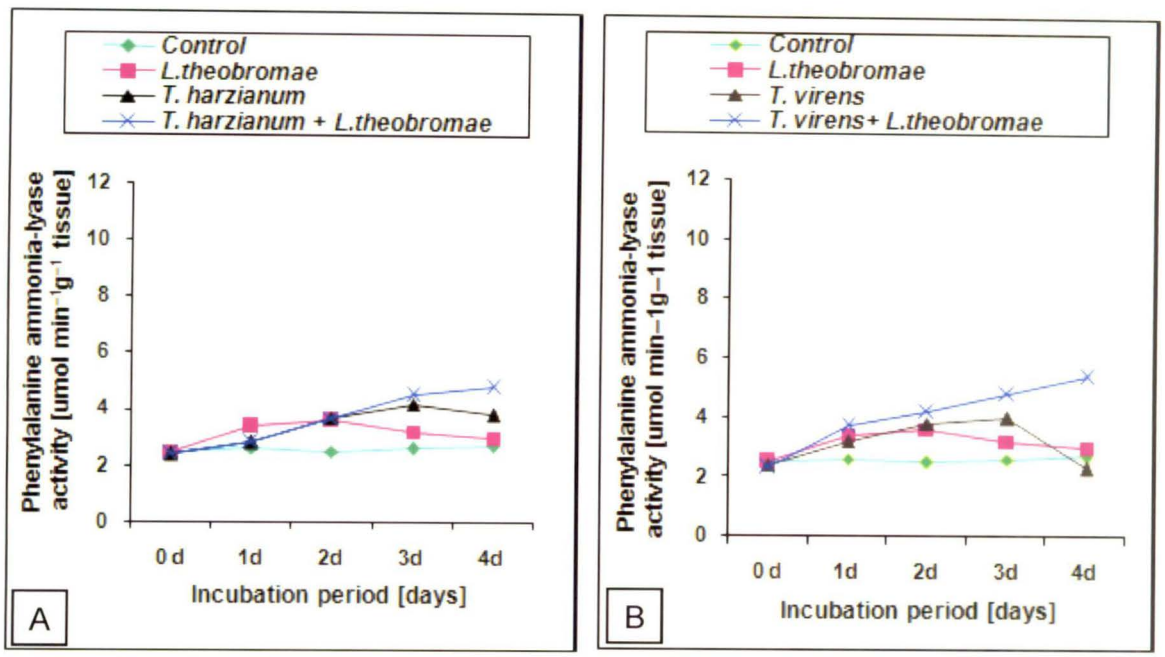


**Fig. 11:** Phenylalanine ammonia lyase activity in treated and treated-inoculated seedlings of TS-449.

A.Treated with *Trichoderma harzianum.*

B.Treated with *Trichoderma virens.*

**Table 19:** Phenylalanine ammonia lyase (PAL) activity in seedlings of TS-449 pre-treated with phyto-extracts followed by challenge-inoculation of *Lasiodiplodia theobromae.*

| Treatments | PAL activity (µmol $min^{-1}g^{-1}$ fresh weight tissue) | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 2.5± 0.02 | 2.6± 0.04 | 2.5± 0.04 | 2.6± 0.05 | 2.7± 0.02 |
| *L.theobromae* | 2.5± 0.04 | 3.4± 0.05 | 3.6± 0.06 | 3.2± 0.03 | 3.0± 0.04 |
| *Azadirachta indica* | 2.80± 0.02 | 3.40± 0.03 | 5.14± 0.05 | 5.73± 0.03 | 5.54± 0.05 |
| *Azadirachta indica* + *L.theobromae* | 2.70± 0.02 | 3.88± 0.04 | 5.80± 0.03 | 7.71± 0.06 | 7.08± 0.06 |
| *Acalypha indica* | 2.70± 0.03 | 4.08± 0.02 | 6.20± 0.03 | 5.80± 0.04 | 5.44± 0.05 |
| *Acalypha indica* + *L.theobromae* | 2.50± 0.02 | 4.16± 0.03 | 6.86± 0.04 | 6.52± 0.03 | 6.80± 0.04 |
| *Catharanthus roseus* | 2.50± 0.03 | 3.53± 0.02 | 3.86± 0.04 | 4.24± 0.06 | 4.54± 0.05 |
| *Catharanthus roseus* + *L.theobromae* | 2.70± 0.05 | 4.24± 0.06 | 4.85± 0.04 | 5.24± 0.03 | 6.55± 0.05 |
| *Jasmoinum jasminoides* | 2.68± 0.04 | 3.25± 0.05 | 4.45± 0.05 | 4.85± 0.03 | 4.24± 0.02 |
| *J. jasminoides* + *L.theobromae* | 2.70± 0.04 | 3.80± 0.03 | 5.85± 0.02 | 6.25± 0.02 | 6.65± 0.06 |

Mean of three replications; Data after ± indicates standard error values.

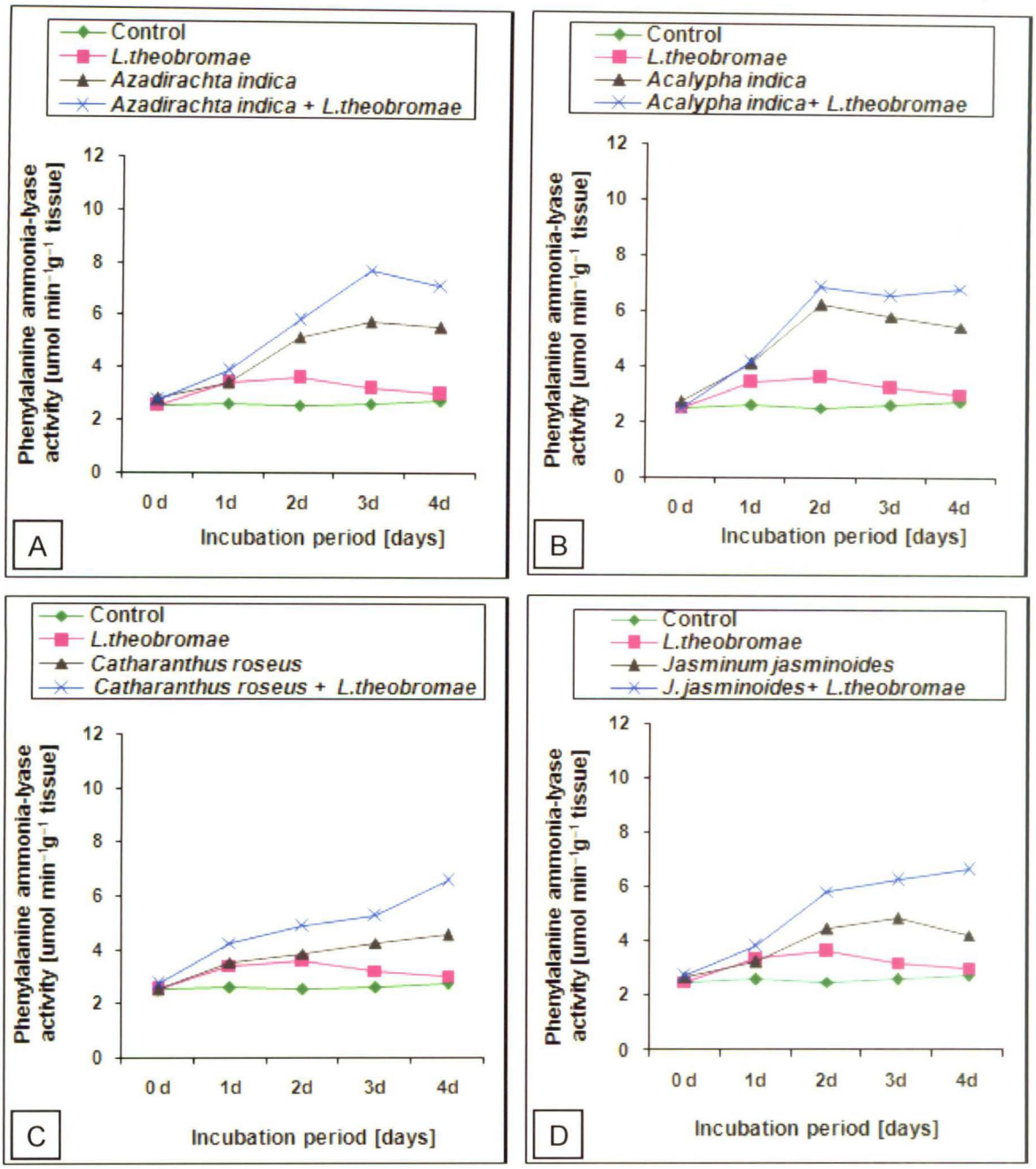


**Fig. 12:** Phenylalanine ammonia lyase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with *Azadirachta indica* leaf extract.

B. Treated with *Acalypha indica* leaf extract.

C. Treated with *Catharanthus roseus* leaf extract.

# Chapter V

## 4.5. Studies on defense related enzyme: polyphenol oxidase (PPO)

Polyphenol oxidase (PPO) is one of the defense related enzymes related to phenyl-propanoid pathway. PPO acts on tannin in phenol form and convert tannin in quinone form, which has inhibitory activity against phytopathogenic microorganisms (Mahadevan and Sridhar, 1996). It has been reported that PPO activity can be significantly increased by application of SA (Meena *et al.*, 2001).

### 4 .5.1. PPO activity with abiotic inducers

A Susceptible variety (TS 449) was used for induction of PPO by abiotic inducers ($NiCl_2$, SA and JA). The detailed procedure of application has been discussed in materials and methods (Section-3.12.1). Enzyme activity was measured and recorded at every 24 h following treatment until the $4^{th}$ day after which the activity was found to decline. Methods of measuring the enzyme activity have been discussed in materials and methods (Section-3.14).

It was evident from the results presented in Table 20 & Fig.13 that activity of PPO increased almost three fold in SA and JA treated plants that were challenge inoculated by *L. theobromae.* The uninoculated but treated plants showed a low increase in enzyme activity which was less than the activity showed by inoculated but untreated plants. $NiCl_2$ treated plants showed an increase in enzyme activity similar to untreated inoculated plants, but challenge inoculation caused reduction in enzyme activity. Control set showed no significant change in PPO activity.

### 4.5.2. PPO activity with biotic inducers

Induction of PPO activity was studied following exogenous application of biotic inducers (*T. harzianum* and *T. virens*) following methods described in materials and methods (Section-3.12.2).

From the results (Table 21 & Fig. 14) it was found that *T. harzianum* was a better inducer of PPO activity than *T. virens*. Maximum activity was showed by treated plants (*T. harzianum*) that were challenge inoculated with *L. theobromae*. In *T. virens* treated plants, no significant change in activity occurred following challenge

inoculation. Enzyme activity in control sets remained almost unchanged throughout the duration of experiment.

**Table 20:** Polyphenol oxidase (PPO) activity in seedlings of TS-449 pre-treated with abiotic inducers followed by challenge-inoculation of *Lasiodiplodia theobromae*.

| Treatments | PPO activity = K x (ΔA $min^{-1}$) μmol $min^{-1}$ $g^{-1}$ fresh weight issue (K= 0.272 for polyphenol oxidase) | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 2.72± 0.03 | 2.81± 0.04 | 3.08± 0.03 | 3.33± 0.03 | 3.54± 0.02 |
| *L.theobromae* | 2.72± 0.4 | 3.16± 0.02 | 3.50± 0.02 | 4.24± 0.03 | 5.54± 0.02 |
| Nickel chloride | 2.84± 0.03 | 3.54± 0.03 | 3.86± 0.02 | 4.78± 0.05 | 5.34± 0.04 |
| Nickel chloride + *L.theobromae* | 2.90± 0.02 | 4.34± 0.05 | 4.86± 0.03 | 5.64± 0.04 | 4.86± 0.06 |
| Salicylic acid | 2.74± 0.02 | 2.85± 0.04 | 3.25± 0.03 | 3.64± 0.04 | 3.85± 0.03 |
| Salicylic acid + *L.theobromae* | 2.70± 0.02 | 3.55± 0.03 | 3.90± 0.04 | 4.45± 0.03 | 6.52± 0.02 |
| Jasmonic acid | 2.64± 0.02 | 4.86± 0.04 | 5.42± 0.03 | 5.75± 0.04 | 4.86± 0.03 |
| Jasmonic acid + *L.theobromae* | 2.73± 0.04 | 4.94± 0.02 | 5.94± 0.03 | 6.25± 0.05 | 6.45± 0.04 |

Mean of three replications; Data after ± indicates standard error values.

### 4.5.3. PPO activity with phyto-extracts

Four different aqueous leaf extracts (*Azadirachta indica*, *Acalypha indica*, *C. roseus* and *J. jasminoides*) were used to induce PPO activity in tea plants. The detailed procedures of application of phyto-extracts in order to induce PPO in tea plants have been discussed in materials and methods (Section-3.12.3).

Results (Table 22 & Fig. 15) revealed an overall two to three fold increase in PPO activity in treated plants. Challenge inoculation with *L. theobromae* caused significant increase in enzyme activity in treated-inoculated sets. Maximum induction

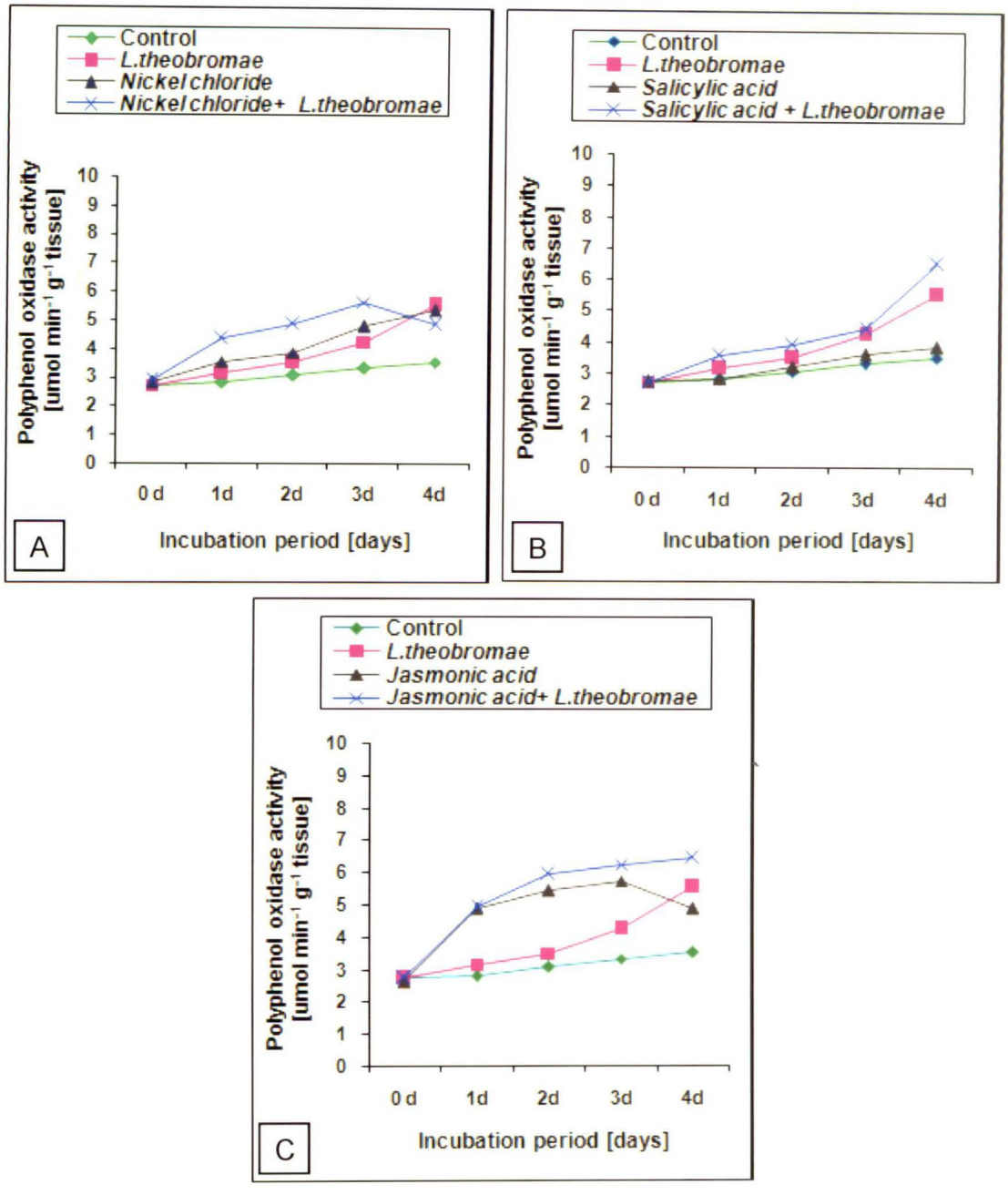


**Fig. 13:** Polyphenol oxidase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with Nickel chloride.

B. Treated with Salicylic acid.

C. Treated with Jasmonic acid.

was caused by *Acalypha indica* leaf extracts. Untreated-uninoculated sets showed no change in PPO activity.

**Table 21:** Polyphenol oxidase (PPO) activity in seedlings of TS-449 pre-treated with biotic inducers followed by challenge-inoculation of *Lasiodiplodia theobromae.*

| Treatments | PPO activity = K x (ΔA $min^{-1}$) μmol $min^{-1}$ $g^{-1}$ fresh weight issue (K= 0.272 for polyphenol oxidase) Days after inoculation | | | | |
|---|---|---|---|---|---|
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 2.72± 0.03 | 2.81± 0.04 | 3.08± 0.03 | 3.33± 0.03 | 3.54± 0.02 |
| *L. theobromae* | 2.72± 0.4 | 3.16± 0.02 | 3.50± 0.02 | 4.24± 0.03 | 5.54± 0.02 |
| *T. harzianum* | 2.73± 0.03 | 3.5± 0.04 | 3.8± 0.02 | 4.8± 0.02 | 5.6± 0.03 |
| *T. harzianum* + *L.theobromae* | 2.70± 0.03 | 4.24± 0.04 | 4.85± 0.02 | 5.45± 0.03 | 6.80± 0.02 |
| *T. virens* | 2.57± 0.02 | 2.89± 0.03 | 3.75± 0.04 | 4.25± 0.05 | 5.65± 0.03 |
| *T. virens* + *L. theobromae* | 2.65± 0.02 | 3.75± 0.04 | 5.45± 0.03 | 6.55± 0.02 | 5.45± 0.03 |

Mean of three replications; Data after ± indicates standard error values.

### 4.5.4. Study of polyphenol oxidase isoform patterns

Most of the defense related enzymes have several isozymes. Some isozymes are present constitutively while some other isozymes are known to be inducible. Inducible isozymes are of great importance in the study of induced resistance in plants as they are indicators of resistance induction. In the present study, it was considered worthwhile to find inducible isozymes, if any, of three different enzymes such as polyphenol oxidase, β-1,3-glucanase and peroxidase. The detailed procedure of total soluble protein separation by polyacrylamide gel electrophoresis and PPO enzyme specific staining technique have been discussed in materials and methods (Section-3.18 & 3.19.1 respectively).

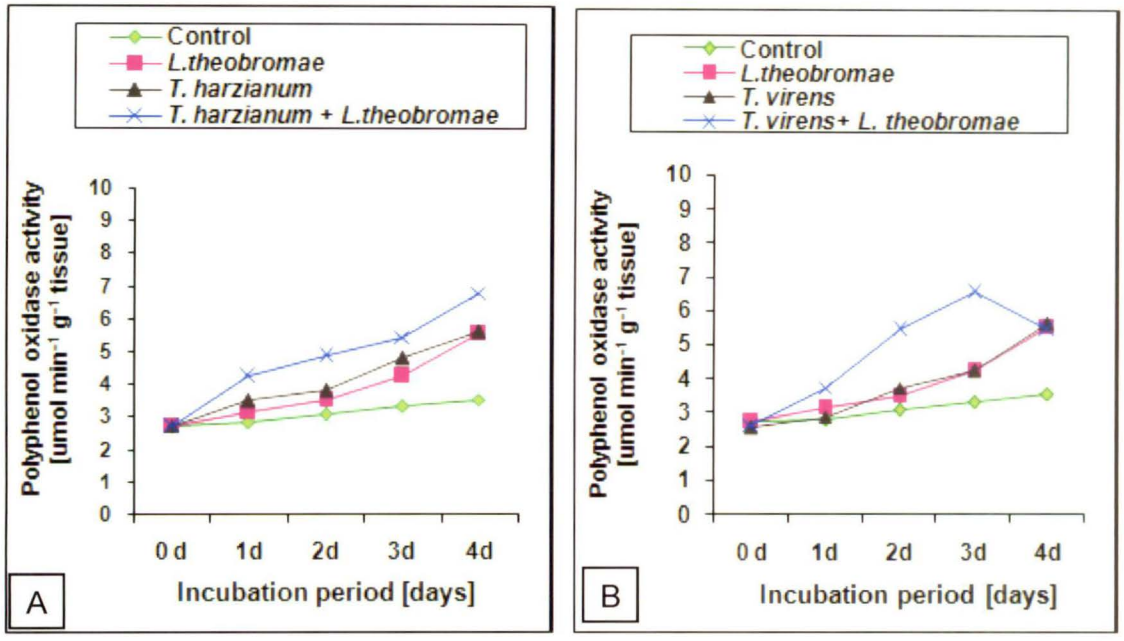


**Fig. 14:** Polyphenol oxidase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with *Trichoderma harzianum.*

B. Treated with *Trichoderma virens.*

**Table 22:** Polyphenol oxidase activity in seedlings of TS-449 pre-treated with phyto-extracts followed by challenge-inoculation of *Lasiodiplodia theobromae*

| Treatments | PPO activity = K x (ΔA $min^{-1}$) μmol $min^{-1}$ $g^{-1}$ fresh weight tissue (K= 0.272 for polyphenol oxidase) | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 2.72± 0.03 | 2.81± 0.04 | 3.08± 0.03 | 3.33± 0.03 | 3.54± 0.02 |
| *L.theobromae* | 2.72± 0.4 | 3.16± 0.02 | 3.50± 0.02 | 4.24± 0.03 | 5.54± 0.02 |
| *Azadirachta indica* | 2.80± 0.02 | 3.40± 0.03 | 5.14± 0.05 | 5.73± 0.03 | 5.54± 0.05 |
| *Azadirachta indica* + *L.theobromae* | 2.70± 0.02 | 3.88± 0.04 | 5.80± 0.03 | 7.71± 0.06 | 6.82± 0.06 |
| *Acalypha indica* | 2.70± 0.03 | 4.08± 0.02 | 6.20± 0.03 | 5.80± 0.04 | 5.44± 0.05 |
| *Acalypha indica* + *L.theobromae* | 2.50± 0.02 | 4.16± 0.03 | 6.86± 0.04 | 6.52± 0.03 | 7.08± 0.04 |
| *Catharanthus roseus* | 2.50± 0.03 | 3.53± 0.02 | 3.86± 0.04 | 4.24± 0.06 | 4.54± 0.05 |
| *Catharanthus roseus* + *L.theobromae* | 2.70± 0.05 | 4.24± 0.06 | 4.85± 0.04 | 5.24± 0.03 | 6.55± 0.05 |
| *Jasmoinum jasminoides* | 2.68± 0.04 | 3.25± 0.05 | 4.45± 0.05 | 4.85± 0.03 | 4.24± 0.02 |
| *J.jasminoides* +*L.theobromae* | 2.70± 0.04 | 3.80± 0.03 | 5.85± 0.02 | 6.25± 0.02 | 6.65± 0.06 |

Mean of three replications; Data after ± indicates standard error values.

Polyacrylamide gel electrophoresis (PAGE) followed by enzyme specific staining of PPO was performed to visualize the expression of different isozymes in pre-treated and challenge-inoculated (by *L. theobromae*) susceptible tea plants (TS-449). Pre-treatments were done by four different inducers viz. *Azadirachta indica, Acalypha indica, Jasminum jasminoides* and salicylic acid. PPO isozyme bands of different intensity as well as of different $R_f$ (relative front) values were found when treated with different inducers (Plate XII). Altogether four different isozyme bands were visible ($R_f$ = 0.09, 0.30, 0.40 and 0.50). Among these, two isoforms of $R_f$ 0.09 and 0.05 were expressed constitutively in all the experimental and control sets. But in plants pre-treated with *Acalypha indica* aqueous leaf extract and salicylic acid, the

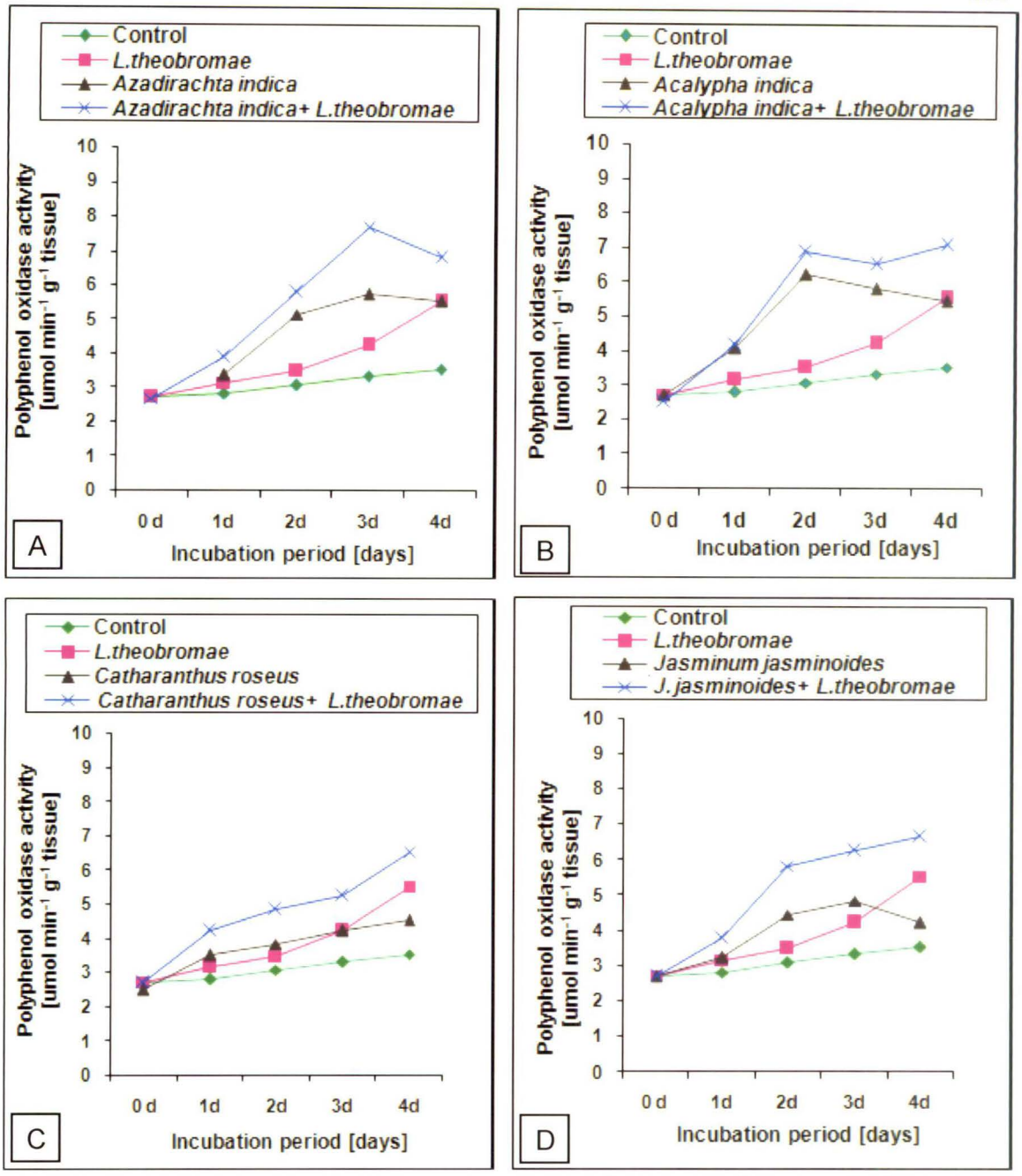


**Fig. 15:** Polyphenol oxidase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with *Azadirachta indica* leaf extract.

B. Treated with *Acalypha indica* leaf extract.

C. Treated with *Catharanthus roseus* leaf extract.

D. Treated with *Jasminum jasminoides* leaf extract.

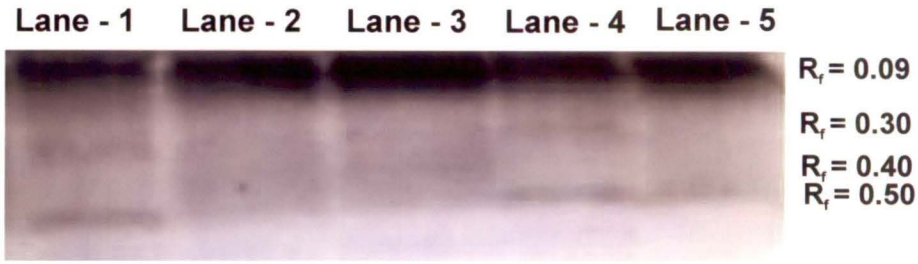


**PLATE XII**

**Polyphenol oxidase isoform pattern was determined on susceptible tea seedlings (TS-449) treated with different inducers following challenge-inoculation by *Lasiodiplodia theobromae* using native Polyacrylamide gel electrophoresis.**

**Lane-1:** Treated with *Azadirachta indica* aqueous leaf extract.

**Lane-2:** Treated with *Acalypha indica* aqueous leaf extract.

**Lane-3:** Treated with salicylic acid.

**Lane-4:** Treated with *Jasminum jasminoides* aqueous leaf extract.

**Lane-5:** Untreated-uninoculated (control).

isozyme having $R_f$ 0.09 was expressed at higher levels in comparison to untreated-uninoculated control. A very low intensity band of $R_f$ 0.40 was expressed in *Acalypha indica* leaf extract and SA treated plants.

Plants treated with leaf extracts of *Azadirachta indica,* and *Jasminum jasminoides* (Lane 1 and lane 4 respectively) showed three isoforms of $R_f$ 0.09, 0.30 and 0.50. Untreated-uninoculated control plants (Lane 5) showed two bands of $R_f$ 0.09 and 0.50.

# Chapter VI

## 4.6. Studies on defense related enzyme: β-1,3-glucanase

Among the pathogenesis related proteins, β-1,3-glucanase plays an important role in plant defense against phyto-pathogenic fungi. β-1,3-glucanase hydrolyse β-1,3-glucans present in chitin, embedded in matrix (Lawrence *et al.*,1996). Higher β-1,3-glucanase activity in some resistant plants and low in the susceptible plants has been reported by Kini *et al.*, 2000.

### 4.6.1. β-1, 3-glucanase activity with abiotic inducers

Activity of β-1-3-glucanase was determined in tea plants by abiotic inducers ($NiCl_2$, SA and JA). The procedures of application of abiotic inducers and challenge-inoculation have been discussed in materials and methods (Section -3.12.1). The procedure for enzyme assay has been discussed in materials and methods (Section-3.16).

A maximum of five-fold increase in enzyme activity was found in SA treated plants (Table 23 & Fig.16). Activity was slightly more when treated plants were challenge inoculated by *L. theobromae*. A continuous increase in β-1,3-glucanase activity was observed in SA treated or treated-inoculated plants until fourth day. But in JA and $NiCl_2$ treated plants, it declined after the third day. Untreated but inoculated plants also showed rise in enzyme activity. However, the increase was much less compared to treated-inoculated sets. In control sets no significant increase in enzyme activity was found.

### 4.6.2. β -1, 3-glucanase activity with biotic inducers

*T. harzianum* and *T. virens* were used as biotic inducers to induce β-1,3-glucanase activity in tea plants. The detailed procedure of application has been discussed in materials and methods (Section-3.12.2).

From the results (Table 24 & Fig. 17) it was found that tea plants treated with *T. virens* and *T. harzianum* and challenge-inoculated by *L. theobromae* showed approximately five fold increase in enzyme levels in comparison to control. Enzyme

**Table 23:** β-1,3-glucanase activity in seedlings of TS-449 pre-treated with abiotic inducers followed by challenge-inoculation of *Lasiodiplodia theobromae*.

| Treatments | β-1,3-glucanase activity (nmol $min^{-1}mg^{-1}$ fresh weight tissue) | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 10± 0.6 | 12± 0.5 | 10± 0.7 | 10± 0.6 | 10± 0.7 |
| *L.theobromae* | 10± 0.5 | 14± 0.7 | 17± 0.5 | 20± 0.8 | 23± 0.5 |
| Nickel chloride | 10± 0.4 | 24± 0.6 | 26± 0.4 | 25± 0.8 | 20± 0.4 |
| Nickel chloride + *L.theobromae* | 10± 0.3 | 18± 0.4 | 33± 0.7 | 42± 0.9 | 28± 0.8 |
| Salicylic acid | 10± 0.6 | 20± 0.7 | 35± 0.8 | 45± 0.8 | 50± 0.7 |
| Salicylic acid + *L.theobromae* | 10± 0.6 | 23± 0.6 | 33± 0.7 | 47± 0.8 | 54± 0.9 |
| Jasmonic acid | 10± 0.4 | 14± 0.6 | 17± 0.5 | 16± 0.4 | 12± 0.6 |
| Jasmonic acid + *L.theobromae* | 10± 0.3 | 15± 0.5 | 21± 0.7 | 27± 0.7 | 25± 0.8 |

Data are the mean of three replicates; Data after ± indicates standard error values.

**Table 24:** β-1,3-glucanase activity in seedlings of TS-449 pre-treated with biotic inducers followed by challenge-inoculation of *Lasiodiplodia theobromae*.

| Treatments | β-1,3-glucanase activity (nmol $min^{-1}mg^{-1}$ fresh weight tissue) | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 10± 0.6 | 12± 0.5 | 10± 0.7 | 10± 0.6 | 10± 0.7 |
| *L.theobromae* | 10± 0.5 | 14± 0.7 | 17± 0.5 | 20± 0.8 | 23± 0.5 |
| *T. harzianum* | 10± 0.4 | 20.45± 0.3 | 28.63±0.4 | 35.20±0.5 | 38.6± 0.6 |
| *T. harzianum* + *L.theobromae* | 10± 0.3 | 25.32± 0.4 | 35.52± 0.3 | 42.30± 0.2 | 45.2± 0.4 |
| *T. virens* | 10± 0.6 | 20.42± 0.3 | 25.56± 0.5 | 32.65± 0.4 | 37.4± 0.3 |
| *T. virens* + *L.theobromae* | 10± 0.6 | 18.45±0.3 | 36.40± 0.4 | 45.53± 0.5 | 50.5± 0.3 |

Data are the mean of three replicates; Data after ± indicates standard error values.

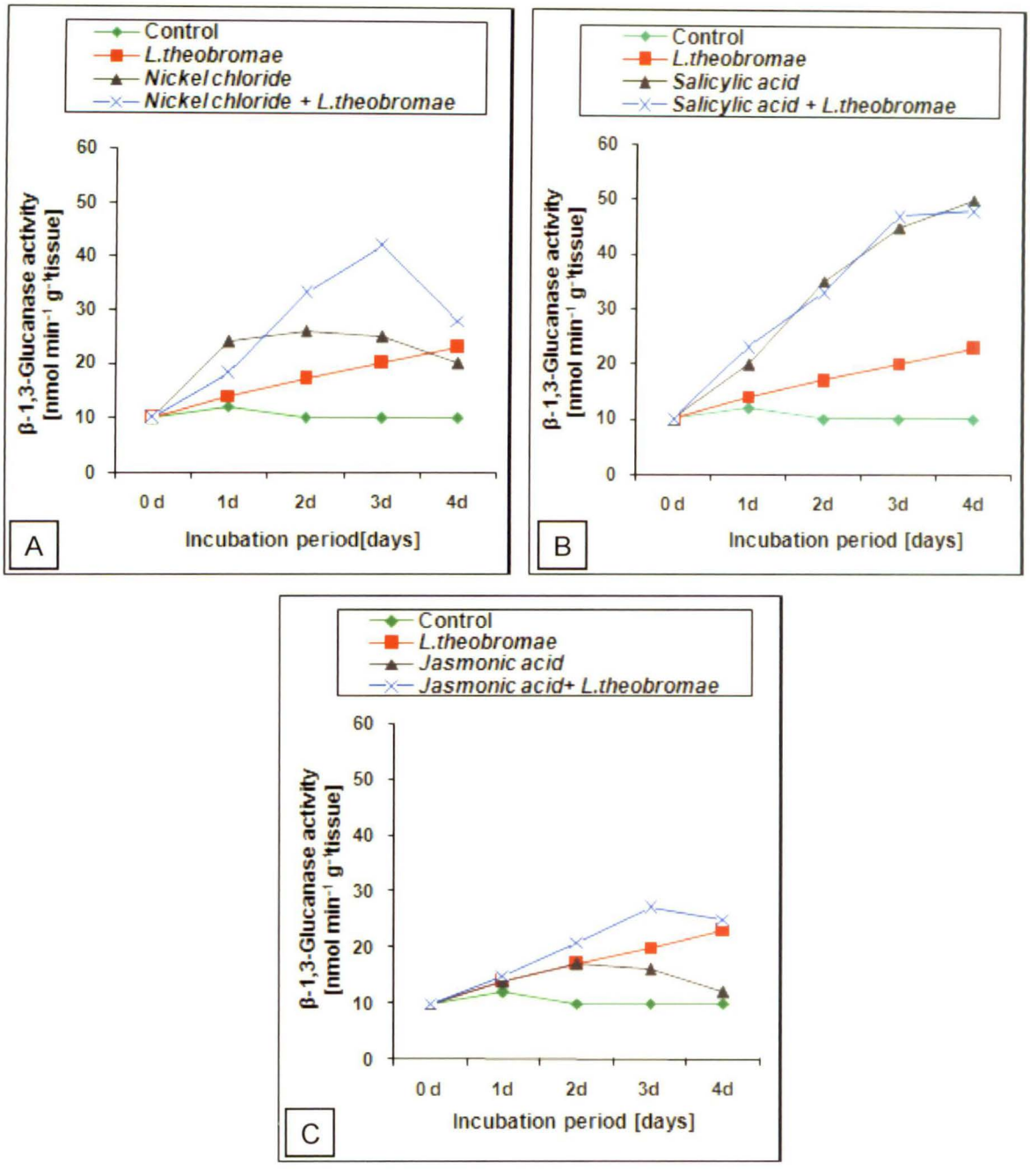


**Fig. 16:** β-1,3-Glucanase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with Nickel chloride.

B. Treated with Salicylic acid.

C.Treated with Jasmonic acid.

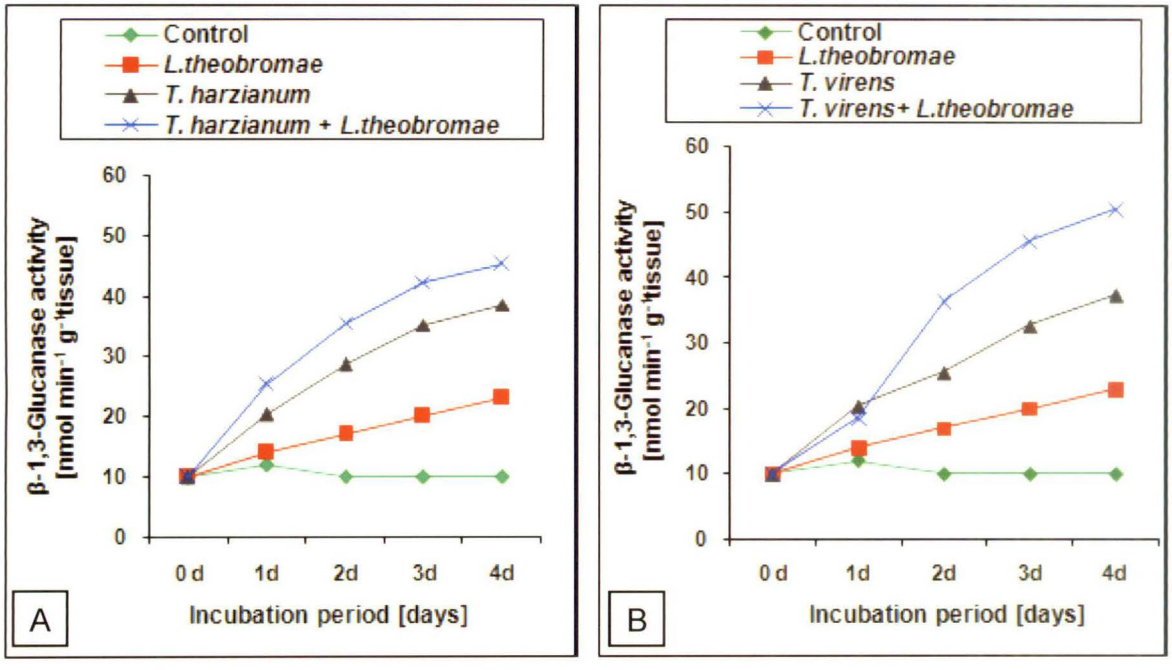


**Fig. 17:** β-1,3-glucanase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with *Trichoderma harzianum.*

B. Treated with *Trichoderma virens.*

activity continued to increase until fourth day in all treatments. Both inducers produced similar rise in activity in treated and treated-inoculated sets. However, plants which were challenge inoculated showed higher activity than non-challenged plants.

### 4.6.3. β-1, 3-glucanase activity with phyto-extracts

Induction of β-1,3-glucanase activity was studied following exogenous application of botanical inducers (leaf extracts of *Azadirachta indica*, *Acalypha indica*, *C. roseus* and *J. jasminoides*) in tea plants (TS-449) following methods described in materials and methods(Section-3.12.3).

From the results (Table 25 & Fig. 18) it was found that plants treated with *Acalypha indica* leaf extract and challenge-inoculated by *L. theobromae* showed maximum five-fold increase in β-1,3-glucanase activity after third day of treatment after which the activity declined. Leaf extracts of *Azadirachta indica* produced minimum increase in activity among the other three plants tested. Control plants did not show any significant change in enzyme levels throughout the duration of the experiment.

### 4.6.4. Study of β-1,3-glucanase isoform patterns

Study of β-1,3-glucanase isozymes were carried out in one susceptible (TS-449) and one resistant (TS-491) tea variety following pre-treatment separately with five different inducers (*Acalypha indica, Catharanthus roseus, T. virens, T. harzianum* and SA) and challenge-inoculation by *L. theobromae*. Procedure of total soluble protein separation by PAGE and β-1,3-glucanase enzyme specific staining technique have been discussed in materials and methods (Section 3.18 & 3.19.2 respectively).

Separation of β-1,3-glucanase isozymes in polyacrylamide gels are shown in Plates XIII and XIV. Differential expression of β-1,3-glucanase isozymes were found in both the tested varieties(Plate XIII). Three different isozyme bands were visible with different $R_f$ values (0.1, 0.35 and 0.45.) in each variety. Among these, two isoforms of $R_f$ 0.1 and 0.35 were found as constitutive. But they were expressed at higher levels in treated-inoculated plants in comparison to untreated-uninoculated and treated-inoculated controls as evident from their differential band intensity in

both the varieties. An inducible β-1,3-glucanase isozyme ($R_f$ 0.45) was found to express in treated-uninoculated (Plate XIII) and treated-inoculated (Plate XIV) plants.

**Table 25:** β-1,3-glucanase activity in seedlings of TS-449 pre-treated with different phyto-extracts followed by challenge-inoculation of *Lasiodiplodia theobromae.*

| Treatments | β-1,3-glucanase activity (nmol $min^{-1}mg^{-1}$ fresh weight tissue) | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 10± 0.6 | 12± 0.5 | 10± 0.7 | 10± 0.6 | 10± 0.7 |
| *L theobromae* | 10± 0.5 | 14± 0.7 | 17± 0.5 | 20± 0.8 | 23± 0.5 |
| *Azadirachta indica* | 10± 0.5 | 12± 0.7 | 17± 0.8 | 25± 0.7 | 20± 0.6 |
| *Azadirachta indica* + *L.theobromae* | 10± 0.4 | 17± 0.6 | 20± 0.7 | 23± 0.7 | 20± 0.6 |
| *Acalypha indica* | 10± 0.6 | 23± 0.8 | 28± 0.7 | 35± 0.9 | 40± 0.8 |
| *Acalypha indica* + *L.theobromae* | 10± 0.7 | 27± 0.6 | 42± 0.8 | 49± 0.8 | 48± 0.8 |
| *Catharanthus roseus* | 10± 0.6 | 33± 0.5 | 38± 0.8 | 42± 0.9 | 34± 0.8 |
| *Catharanthus roseus* + *L.theobromae* | 10± 0.5 | 27± 0.6 | 35± 0.7 | 47± 0.7 | 35± 0.8 |
| *Jasminum jasminoides* | 10± 0.4 | 18± 0.5 | 26± 0.6 | 32± 0.4 | 31± 0.3 |
| *J. jasminoides* + *L. theobromae* | 10± 0.2 | 23± 0.5 | 28± 0.3 | 32± 0.4 | 27± 0.5 |

Data are the mean of three replicates; Data after ± indicates standard error values.

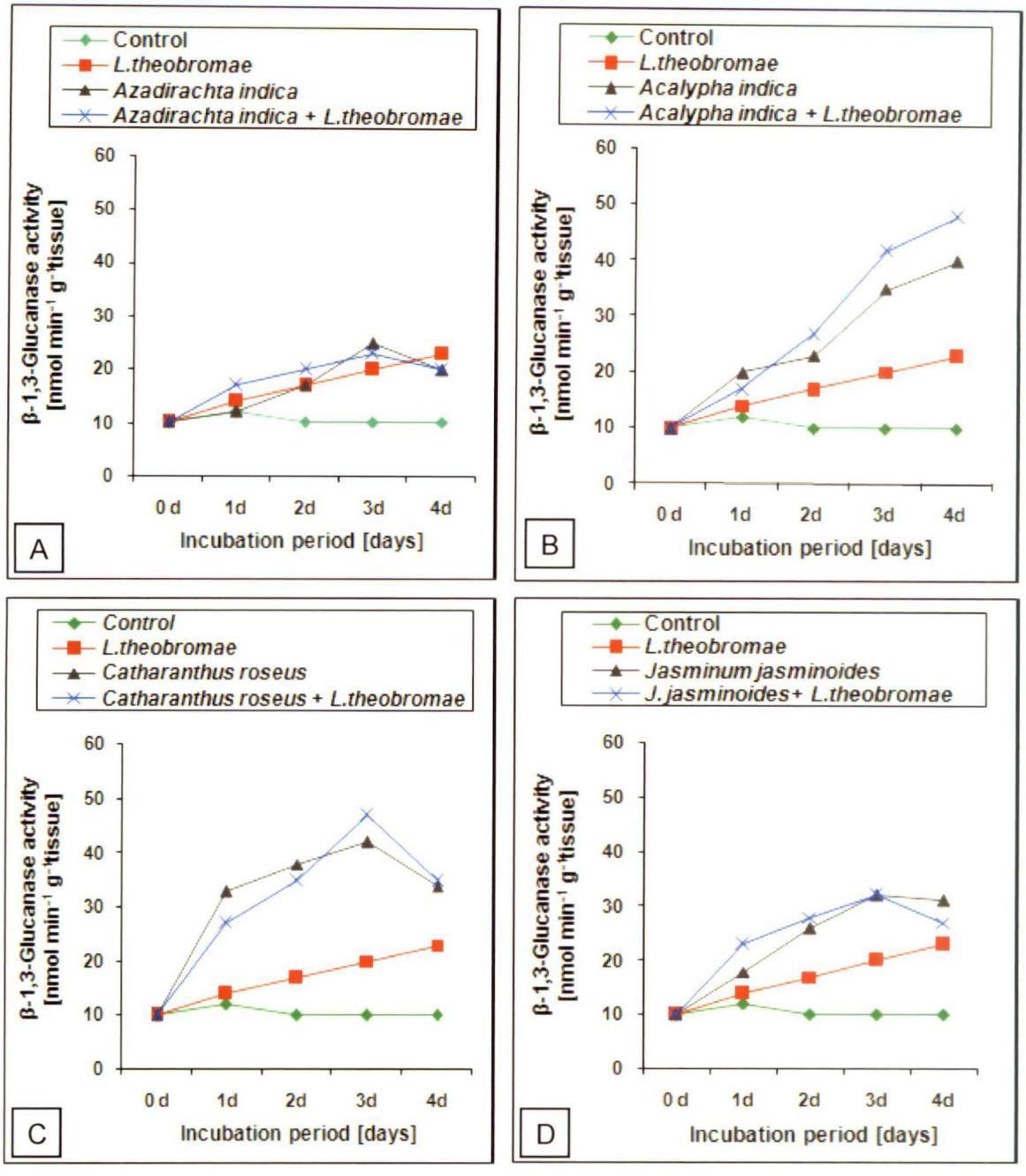


**Fig. 18:** β-1,3-glucanase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with *Azadirachta indica* leaf extract.

B. Treated with *Acalypha indica* leaf extract.

C. Treated with *Catharanthus roseus* leaf extract.

D. Treated with *Jasminum jasminoides* leaf extract.

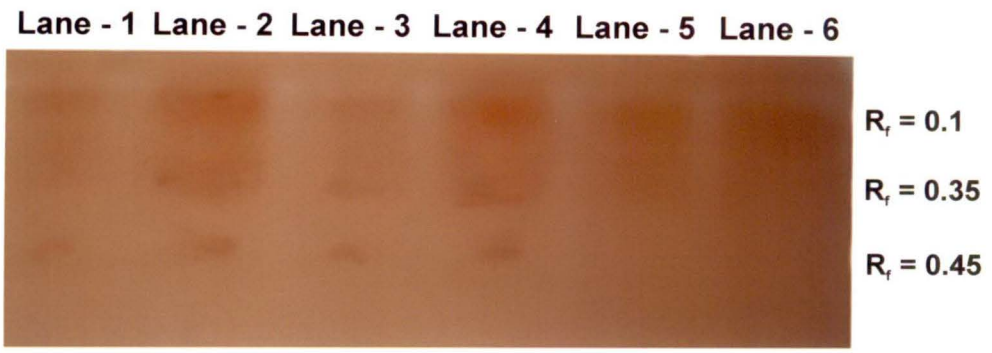


**PLATE XIII**

**β -1, 3-glucanase isozyme pattern was studied on two different tea varieties using Polyacrylamide gel electrophoresis.**

**Lane-1:** Plants (TS-491) inoculated by *Lasiodiplodia theobromae.*

**Lane-2:** Plants (TS-491) treated with *Acalypha indica* and inoculated by *Lasiodiplodia theobromae.*

**Lane-3:** Plants (TS-449) inoculated by *Lasiodiplodia theobromae.*

**Lane-4:** Plants (TS-449) treated with *Acalypha indica* and inoculated by *Lasiodiplodia theobromae.*

**Lane-5:** Plants (TS-491) untreated-uninoculated (control).

**Lane-6:** Plants (TS-449) untreated-uninoculated (control).

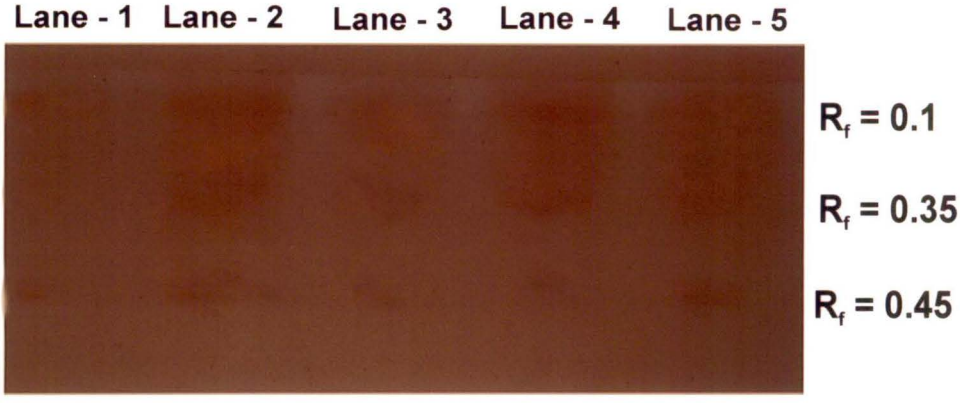


**PLATE XIV**

**β - 1, 3- glucanase isoform pattern was studied on susceptible tea plants (TS-449) treated with different inducers following challenge-inoculation by *Lasiodiplodia theobromae***

**Lane-1:** Plants inoculated by *Lasiodiplodia theobromae.*

**Lane-2:** Plants treated with *Trichoderma virens.*

**Lane-3:**Plants treated with C*atheranthus roseus* leaf extract.

**Lane-4:** Plants treated with salicylic acid.

**Lane-5:** Plants treated with *Trichderma harzianum.*

# Chapter VII

## 4.7. Studies on defense related enzyme: Chitinase

Many phytopathogenic fungi contain chitin as major structural cell wall component (Wessels and Sietsma, 1981). Chitinases commonly known as plant hydrolases, are the key defense enzymes for plant protection against fungal pathogens. Chitinase degrade chitin present in fungal cell-wall components. Several authors have demonstrated the activity of chitinase as growth inhibitor of fungi (Mauch *et al.*, 1988; Alirio *et al.*, 1992; Elzen and Cornelissen, 1993). One of the most studied defense responses is the induction of pathogenesis-related proteins (PRs). Among the PRs, chitinase is of particular interest. Hence, induction of chitinase activity was taken into consideration in the present study.

### 4.7.1. Chitinase activity with abiotic inducers

Three different abiotic inducers ($NiCl_2$, SA and JA) were used to elicit defense response in tea seedlings (TS-449). The level of chitinase activity was studied in different treatments. The detailed procedure of inducer application has been discussed in materials and methods (Section 3.12.1) and the procedure of determining enzyme activity has been discussed in materials and methods (Section 3.15.1).

Results (Table 26 & Fig.19) indicated that nickel chloride treated plants showed maximum chitinase activity on third day following challenge inoculation with *L. theobromae*. Challenge inoculated plants showed higher activity than uninoculated plants. SA also produced good increase in activity in treated plants. Chitinase activity was found to be highest on the third day in the treated sets after which it declined. Untreated-inoculated plants also recorded increase in chitinase activity which was highest on the second day. The control sets did not show any significant change in activity.

**Table 26**: Chitinase activity in seedlings of TS-449 pre-treated with abiotic inducers followed by challenge-inoculation of *Lasiodiplodia theobromae*.

| Treatments | Chitinase activity (mg GlcNac $g^{-1}$ $h^{-1}$ fresh weight tissue) | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 3.0± 0.04 | 3.2± 0.02 | 3.5± 0.01 | 3.0± 0.02 | 3.0± 0.03 |
| *L.theobromae* | 3.2± 0.02 | 4.0± 0.03 | 5.3± 0.03 | 4.2± 0.02 | 3.5± 0.03 |
| Nickel chloride | 3.0± 0.02 | 6.0± 0.03 | 5.0± 0.02 | 4.4± 0.03 | 4.0± 0.02 |
| *Nickel chloride + L.theobromae* | 3.0± 0.04 | 5.5± 0.06 | 6.8± 0.05 | 8.0± 0.06 | 7.4± 0.07 |
| *Salicylic acid* | 3.3± 0.06 | 4.5± 0.05 | 6.5± 0.7 | 5.4± 0.06 | 4.8± 0.05 |
| Salicylic acid + *L.theobromae* | 3.3± 0.05 | 4.2± 0.06 | 6.2± 0.05 | 7.2± 0.08 | 5.5± 0.07 |
| *Jasmonic acid* | 3.0± 0.02 | 3.4± 0.03 | 3.9± 0.02 | 4.2± 0.04 | 3.6± 0.05 |
| Jasmonic acid + *L.theobromae* | 3.0± 0.04 | 3.8± 0.04 | 4.5± 0.03 | 5.8± 0.02 | 3.5± 0.05 |

Data is the mean of three replicates; Data after ± indicates standard error values.

### 4.7.2. Chitinase activity with biotic inducers

*Trichoderma harzianum* and *Trichoderma virens* were used as biotic inducers to induce chitinase activity. Six month old susceptible tea plants (TS-449) were used for the purpose. Application procedure of biotic inducers has been discussed in materials and methods (Section 3.12.2).

Results (Table 27 & Fig. 20) revealed that *T. harzianum* is a better inducer of chitinase activity than *T. virens*. Enzyme levels showed a peak on the third day with an overall two to three fold increase in comparison to control. Challenge inoculation with *L. theobromae* increased chitinase levels considerably in both treatments.

### 4.7.3. Chitinase activity with phyto-extracts

Aqueous leaf extracts of *Azadirachta indica*, *Acalypha indica*, *C. roseus* and *J. jasminoides* were applied exogenously in order to induce chitinase activity in tea plants (TS 449). The procedures of application have been discussed in materials and methods (Section 3.12.3).

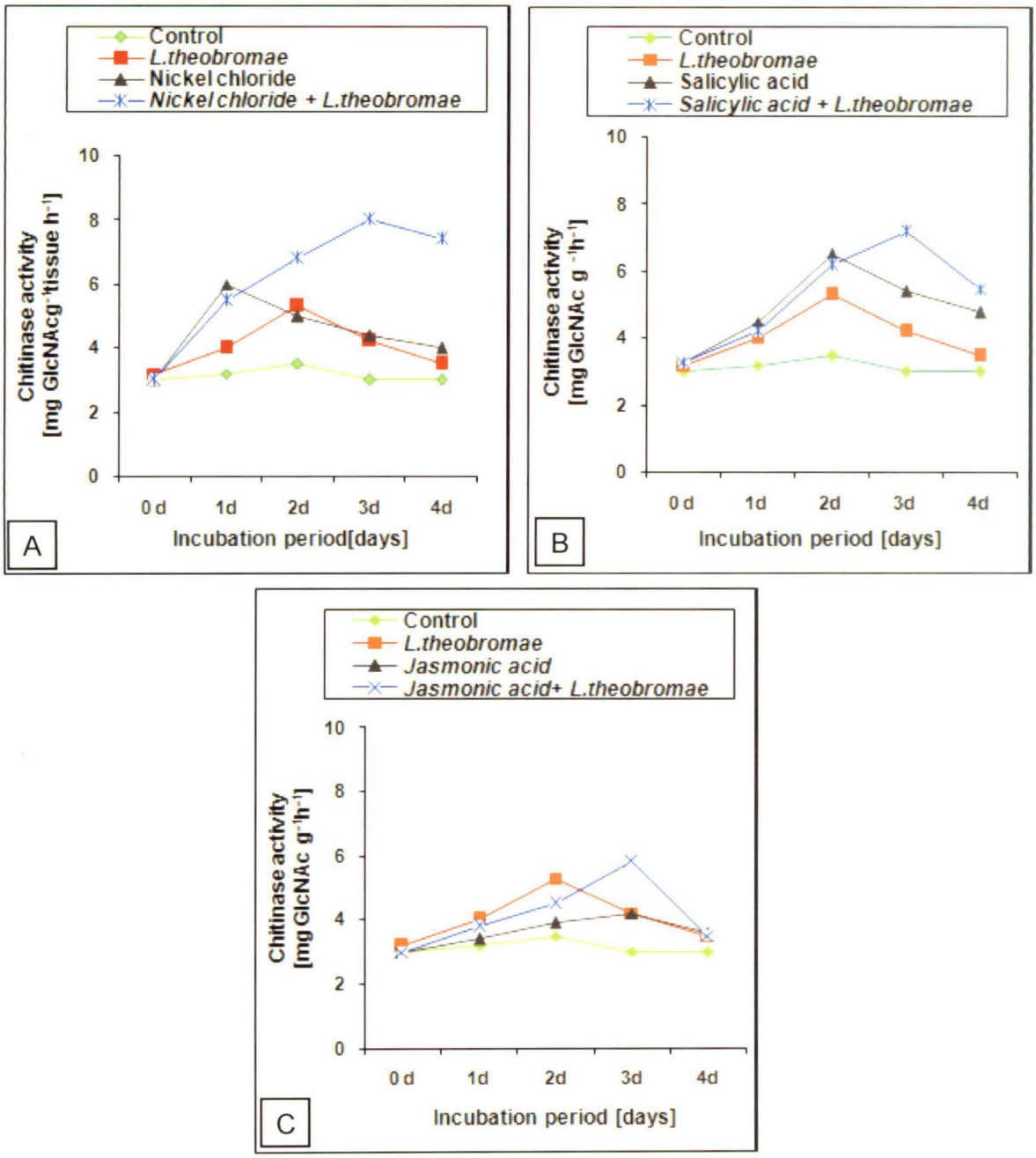


**Fig. 19:** Chitinase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with Nickel chloride.

B. Treated with Salicylic acid.

C. Treated with Jasmonic acid.

**Table 27:** Chitinase activity in seedlings of TS-449 pre-treated with biotic inducers followed by challenge-inoculation of *Lasiodiplodia theobromae*.

| Treatments | Chitinase activity ( mg GlcNac $g^{-1}$ $h^{-1}$ fresh weight tissue) | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 3.0± 0.04 | 3.2± 0.02 | 3.5± 0.01 | 3.0± 0.02 | 3.0±0.03 |
| *L.theobromae* | 3.2± 0.02 | 4.0± 0.03 | 5.3± 0.03 | 4.2± 0.02 | 3.5±0.03 |
| *T. harzianum* | 3.4± 0.05 | 4.2± 0.05 | 4.8± 0.07 | 5.5± 0.06 | 5.8± 0.06 |
| *T. harzianum* + *L.theobromae* | 3.4± 0.04 | 5.2± 0.05 | 6.6± 0.08 | 8.7± 0.09 | 7.5± 0.08 |
| *T. virens* | 3.5± 0.06 | 3.9± 0.04 | 4.3± 0.05 | 4.8± 0.06 | 4.2± 0.07 |
| *T. virens* + *L.theobromae* | 3.5± 0.06 | 4.5± 0.07 | 5.8± 0.06 | 6.5± 0.07 | 5.6.±0.06 |

Data is the mean of three replicates; Data after ± indicates standard error values.

There was a general two fold increase in chitinase levels in all treatments. The plants pretreated with leaf extract of *Acalypha indica* showed maximum chitinase induction which showed a peak after three days of treatment. This was higher than pathogen induced rise in chitinase levels. Highest activity was recorded in treated-inoculated sets. Untreated and untreated control set showed no increase in chitinase activity. *Azadirachta indica* was also a suitable inducer of chitinase, which showed similar increase in chitinase levels. The other two tested extracts also induced the enzyme with respect to control.

### 4.7.4. Study of chitinase activity by chitin supplemented plate method

Tea plants of seven varieties (TS-464, TS-491, TS-506, TS-520, TS-489, TS-449 and TS-463) were induced by two different selected inducers (extract of *Acalypha indica and* nickel chloride). After treatment, chitinase was extracted following the method as described in the materials and methods (Section-3.15.1). Details of the technique have also been discussed in materials and methods (Section-3.15.2). Diameters of lytic zones along with intensity of the lytic zones

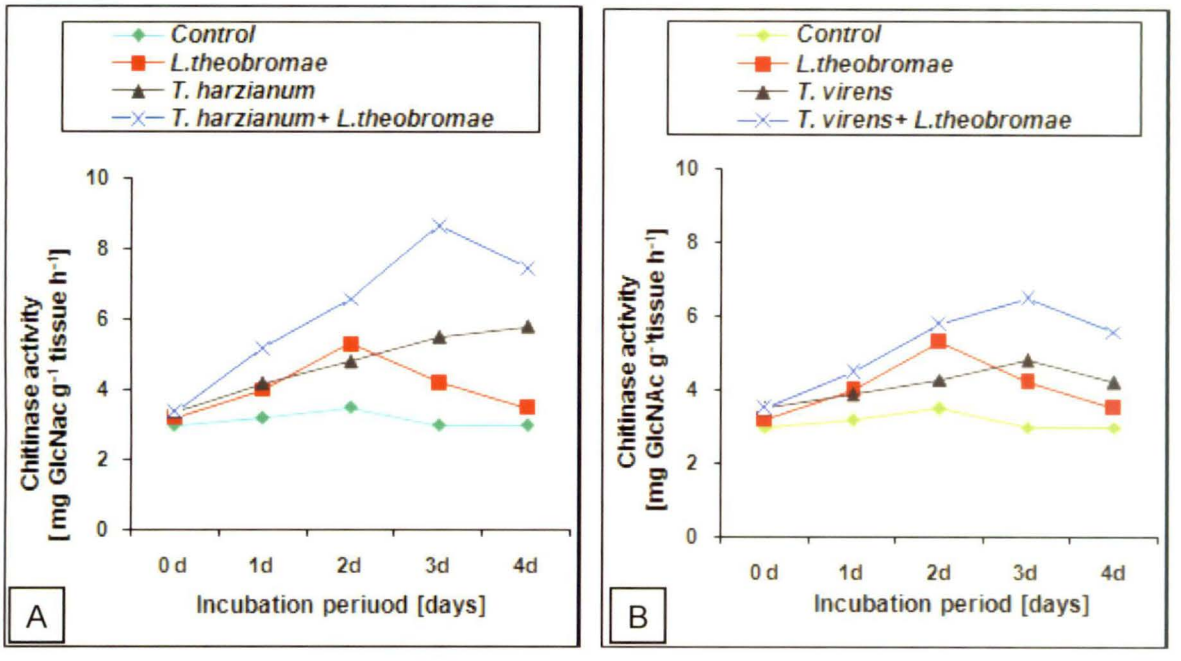


**Fig. 20:** Chitinase oxidase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with *Trichoderma harzianum.*

B. Treated with *Trichoderma virens.*

**Table 28:** Chitinase activity in seedlings of TS-449 pre-treated with different phyto-extracts followed by challenge-inoculation of *Lasiodiplodia theobromae*.

| Treatments | Chitinase activity (mg GlcNac $g^{-1}$ $h^{-1}$ fresh weight tissue) | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 3.0± 0.04 | 3.2± 0.02 | 3.5± 0.01 | 3.0± 0.02 | 3.0± 0.03 |
| *L.theobromae* | 3.2± 0.02 | 4.0± 0.03 | 5.3± 0.03 | 4.2± 0.02 | 3.5± 0.03 |
| *Azadirachta indica* | 3.2± 0.04 | 3.5± 0.05 | 4.4± 0.06 | 6.0± 0.05 | 4.5± 0.07 |
| *Azadirachta indica* + *L.theobromae* | 3.2± 0.05 | 3.9± 0.04 | 5.8± 0.06 | 6.5± 0.07 | 5.4± 0.06 |
| *Acalypha indica* | 3.3± 0.04 | 4.5± 0.07 | 5.8± 0.06 | 6.0± 0.07 | 4.3± 0.05 |
| *Acalypha indica* + *L.theobromae* | 3.2± 0.04 | 4.6± 0.06 | 6.4± 0.06 | 6.5± 0.07 | 6.0± 0.06 |
| *Catharanthus roseus* | 3.0± 0.05 | 4.6± 0.07 | 5.2± 0.06 | 5.8± 0.04 | 4.5± 0.05 |
| *Catharanthus roseus* + *L.theobromae* | 3.0± 0.05 | 3.5± 0.04 | 4.8± 0.06 | 6.5± 0.07 | 5.5± 0.07 |
| *Jasminum jasminoides* | 3.2± 0.04 | 3.5± 0.05 | 4.5± 0.07 | 5.0± 0.03 | 4.7± 0.04 |
| *J.jasminoides* + *L.theobromae* | 3.7± 0.05 | 4.2± 0.03 | 5.8± 0.06 | 5.2± 0.02 | 4.5± 0.03 |

Data is the mean of three replicates; Data after ± indicates standard error values.

produced on plates were considered as indicator of chitinase activity in different varieties. It was evident from the results (Table 29 & Plate XV & XVI) that diameter of lytic zone was 26.5mm with very high intensity grade in *Acalypha indica* leaf extract treated and inoculated susceptible plants. Under similar condition resistant plants showed lytic zone of 23.0 mm in diameter with moderately high intensity grade. Other tested varieties showed lytic zone diameter between 23.5mm and 24mm. Intensity grade ranged between high to very high. Similarly, nickel chloride treated and challenge-inoculated susceptible plants showed lytic zone of 25.3mm and very high intensity grade. Resistant plants treated with nickel chloride and challenge-inoculated with *L. theobromae* showed chitinase activity of 24.8mm in diameter with very high intensity grade. Although variation of the diameters of the lytic zones were

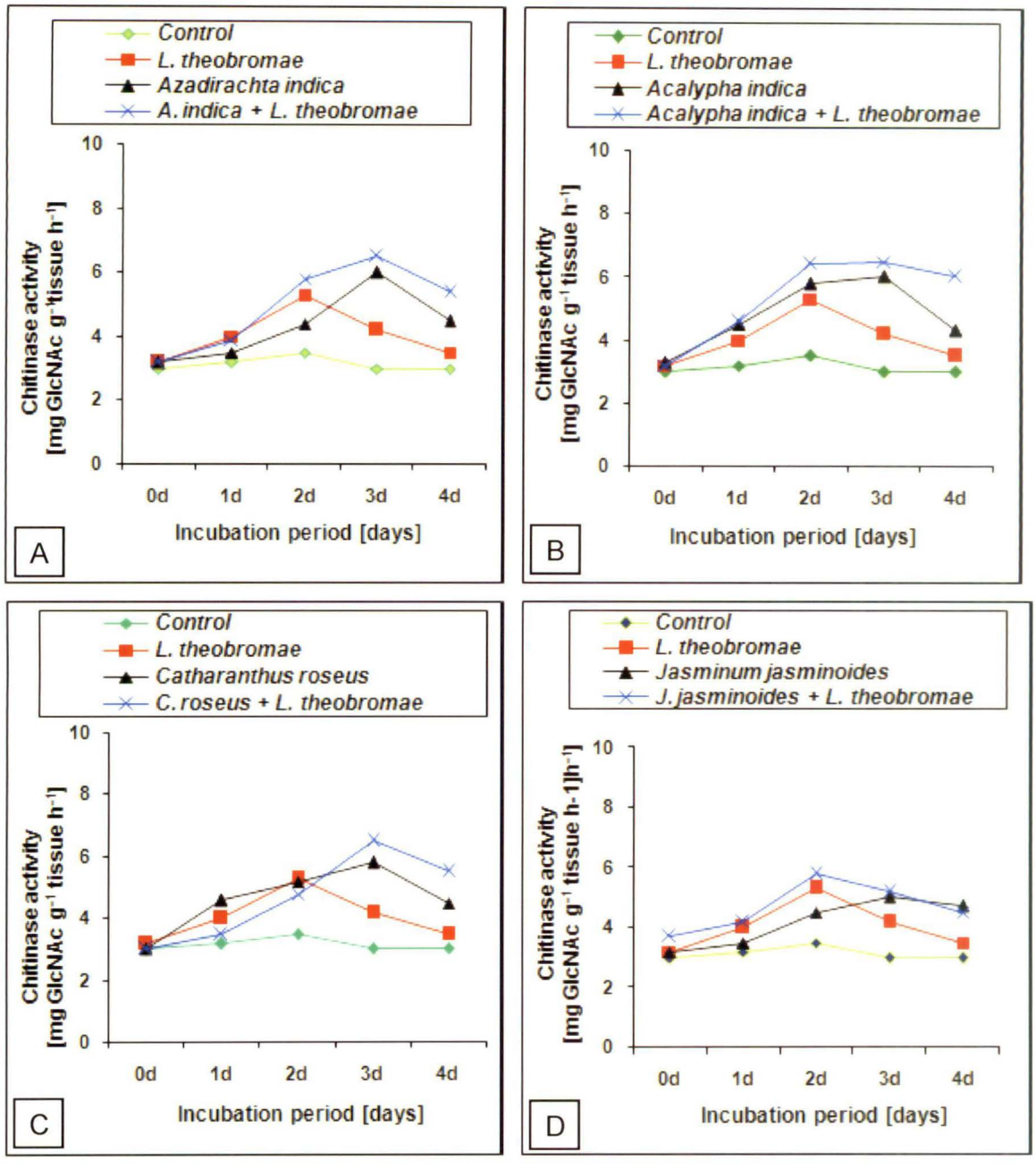


**Fig. 21:** Chitinase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with *Azadirachta indica* leaf extract.

B. Treated with *Acalypha indica* leaf extract.

C. Treated with *Catharanthus roseus* leaf extract.

D. Treated with *Jasminum jasminoides* leaf extract.

**Table 29:** Chitinase activity of treated-inoculated tea plants of different varieties.

| Treatments | Diameter (mm)* of lytic zones in different tea seed varieties (enzyme activity grade)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | TS- 449 | TS- 464 | TS- 520 | TS- 506 | TS- 489 | TS- 463 | TS- 491 |
| Control | 20.0 (+) | 20.6 (+) | 21.2 (+) | 20.5 (+) | 20.0 (+) | 20.5 (+) | 22.0 (+) |
| *L.theobromae* | 22.5 (++) | 22.4 (++) | 22.0 (++) | 22.6 (++) | 22.0 (+) | 22.0 (+) | 22.5 (+) |
| *Acalypha indica* | 25.0 (++++) | 25.0 (+++) | 24.5 (++) | 23.5 (++) | 22.8 (++) | 22.0 (++) | 23.7 (++) |
| *A. indica* + *L.theobromae* | 26.5 (++++) | 24.0 (++++) | 23.6 (++++) | 23.5 (++++) | 23.0 (+++) | 23.5 (+++) | 23.0 (+++) |
| *Nickel chloride* | 23.8 (+++) | 23.6 (++) | 23.4 (++) | 23.2 (++) | 23.5 (+++) | 23.6 (++) | 23.6 (++) |
| *Nickel chloride* + *L. theobromae* | 25.3 (++++) | 24.2 (++++) | 24.0 (++++) | 24.0 (++++) | 23.6 (+++) | 24.7 (+++) | 24.8 (+++) |

*Diameter of the petriplates = 90mm.
**Enzyme activity expressed by intensity grades of the lytic zones: + = very low, ++ = low, +++ = high, ++++ = very high.

not very significant but the intensity of the lytic zones significantly differed from the control set which produced a very low intensity grade

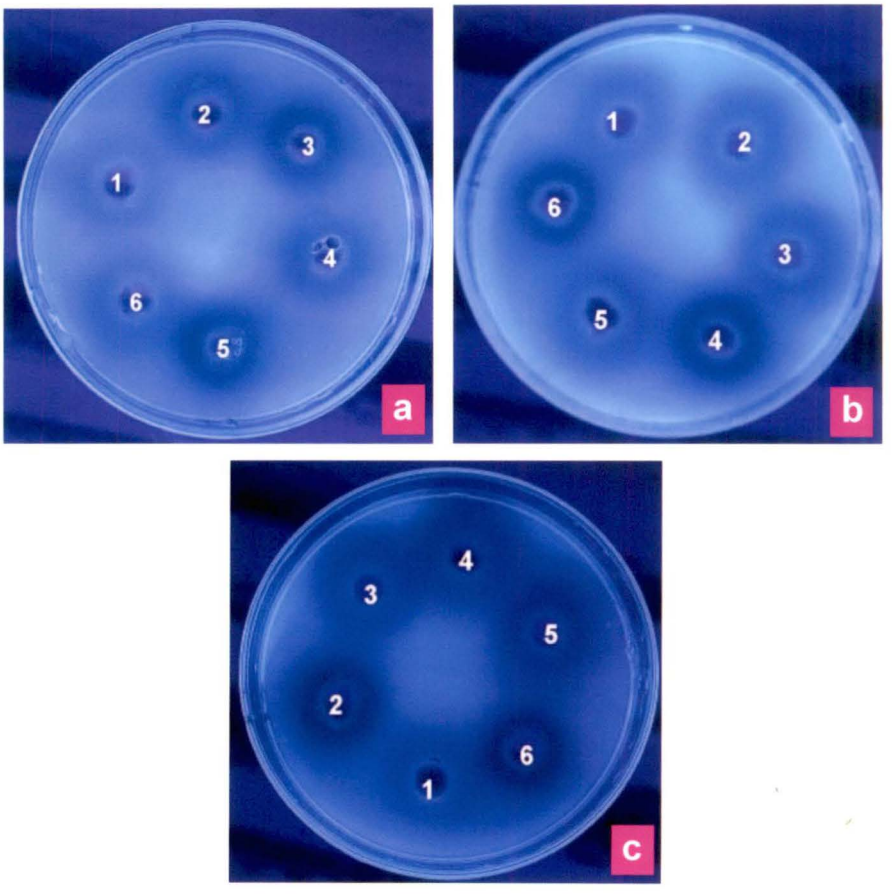


**PLATE XV**

**Chitinase activity in differentially treated tea plants in chitin supplemented plates.**

**fig. a:** TS-489: 1= Control, 2 = Nickel chloride, 3 = Nickel chloride + *L. theobromae*, 4 = *L. theobromae*, 5 = *Acalypha indica* + *L. theobromae*, 6 = *Acalypha indica*.

**fig. b:** TS-463: 1= Control, 2 = *L. theobromae*, 3 = Nickel chloride, 4 = Nickel chloride + *L. theobromae*; 5 = *Acalypha indica*, 6 = *Acalypha indica* + *L. theobromae*.

**fig. C:** TS-491: 1= Control, 2 = *L. theobromae*, 3 = Nickel chloride, 4 = Nickel chloride + *L. theobromae*, 5 = *Acalypha indica*, 6 = *Acalypha indica* + *L. t heobromae*.

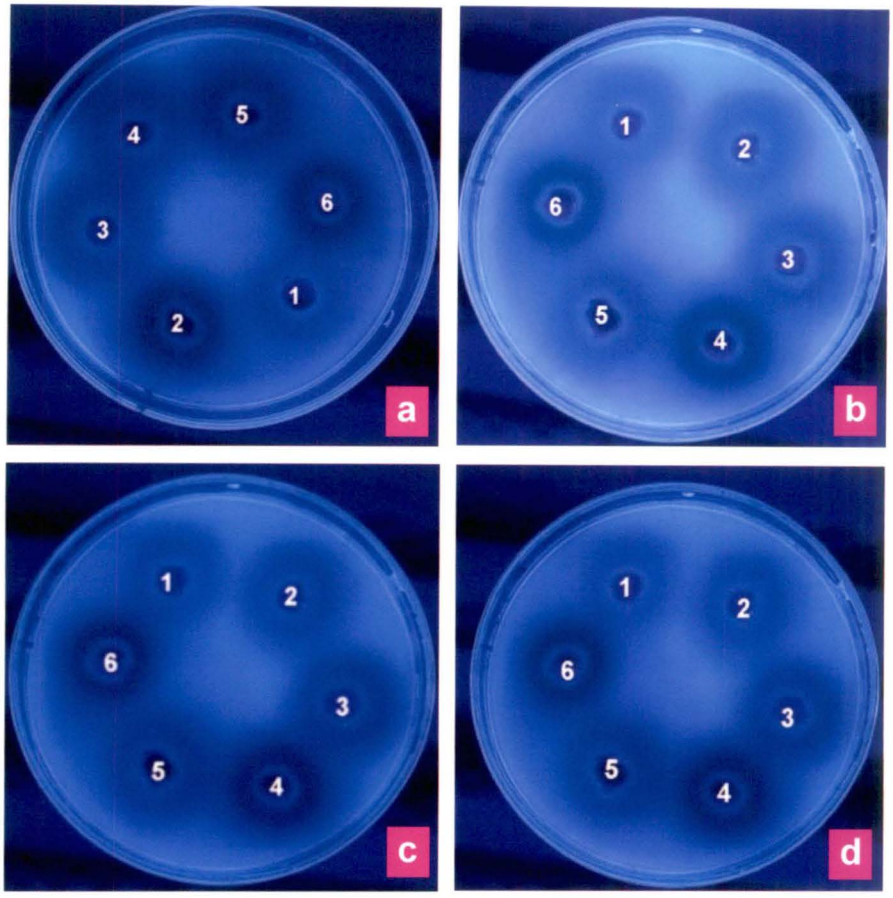


**PLATE XVI**

**Chitinase activity in differentially treated tea plants in chitin supplemented plates**

**fig.a:** TS-449:1=Control, 2=Nickel chloride + *L. theobromae*, 3 = Nickel chloride, 4 = *L. theobromae*, 5 = *Acalypha indica*, 6 = *Acalypha indica* + *L. theobromae*.

**fig. b:** TS-464: 1= Control, 2 = *L. theobromae*, 3 = Nickel chloride, 4 = Nickel chloride + *L. theobromae*; 5 = *Acalypha indica*, 6 = *Acalypha indica* + *L. theobromae*.

**fig. c:** TS-520: 1= Control, 2 = *L.theobromae*, 3 = Nickel chloride, 4 = Nickel chloride + *L. theobromae*; 5 = *Acalypha indica*, 6 = *Acalypha indica* + *L. theobromae*.

**fig. d:** TS-506: 1= Control, 2 = *L. theobromae*, 3 = Nickel chloride, 4 = Nickel chloride + *L.theobromae*; 5 = *Acalypha indica*, 6 = *Acalypha indica* + *L. theobromae*.

# Chapter VIII

## 4.8. Studies on defense related enzyme: peroxidase

Peroxidase is a stress related defense enzyme, induced in plants under various environmental changes such as heavy metals, salts, temperature (Kiwan and Lee, 2003), air pollution, (Lee *et al.*, 2000). Salicylic acid acts as an endogenous signal in the induction of systemic acquired resistance (SAR) (Gaffney *et al.*, 1993) and produces pathogenesis-related proteins (PRs) like peroxidase (PR-9) along with chitinase (PR-3) and β-1,3-glucanase (PR-2). Like the other defense related enzymes, peroxidase was also studied for its role, if any, in tea. The detailed procedures of enzyme extraction and assay have been discussed in materials and methods (Sections 3.17). Peroxidase activity was induced by abiotic, biotic and also by plant extracts. Details of application procedures of application of different inducers have been discussed in the materials and methods (Section 3.12).

### 4.8.1. Peroxidase activity with abiotic inducer

Nickel chloride, SA acid and JA were used as abiotic inducers to induce resistance in tea plants (TS-449) following which the changes in levels of peroxidase activity was studied. The detailed procedure of application has been discussed in materials and methods (Section-3.12.2).

From the results (Table 30 & Fig. 22) it was observed that $NiCl_2$ is the best inducer of peroxidase activity which increased approximately 2.5 fold on the third day after challenge inoculation with *L. theobromae*. Untreated plants that were inoculated with *L. theobromae* showed only marginal increase in enzyme activity. SA and JA treated plants showed similar increase in enzyme levels which increased further on challenge inoculation. Untreated-uninoculated control sets showed no change in peroxidase levels.

### 4.8.2. Peroxidase activity with biotic inducers

*T. harzianum* and *T. virens* were used as biotic inducers on tea plants (TS-449) and the level of peroxidase was studied.

**Table 30:** Peroxidase activity in seedlings of TS-449 pre-treated with abiotic inducers followed by challenge-inoculation of *Lasiodiplodia theobromae*.

| Treatments | Peroxidase activity ($\Delta A_{420}$ min $^{-1}$ g $^{-1}$ fresh weight tissue) 1 unit = 0.001 Absorbance | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 60± 2 | 62± 3 | 60± 3 | 60± 2 | 60± 4 |
| *L.theobromae* | 60± 3 | 66± 4 | 81± 4 | 83± 5 | 85± 3 |
| Nickel chloride | 60± 7 | 68± 6 | 115± 7 | 135± 10 | 140± 13 |
| Nickel chloride+ L.theobromae | 60± 6 | 85± 7 | 135± 13 | 155± 14 | 135± 11 |
| *Salicylic acid* | 60± 7 | 75± 8 | 84± 8 | 95± 9 | 114± 7 |
| Salicylic acid + *L.theobromae* | 60± 5 | 78± 7 | 90± 8 | 114± 9 | 135± 7 |
| *Jasmonic acid* | 60± 7 | 78± 3 | 82± 2 | 94± 5 | 106± 4 |
| Jasmonic acid + *L. theobromae* | 60± 6 | 84± 5 | 98± 6 | 112± 3 | 85± 5 |

Data is the mean of three replicates; Data after ± indicates standard error values.

From the results (Table 31 & Fig. 23) it was found that tea plants treated with both T. *virens* and *T. harzianum* showed nearly three-fold increase in enzyme activity after challenge-inoculation by *L. theobromae*. Maximum activity was observed on the 4$^{th}$ day. Unchallenged plants showed lower levels of peroxidase than challenge-inoculated plants. Control sets did not show any change in enzyme activity.

### 4.8.3. Peroxidase activity with phyto-extracts

Peroxidase activities were measured after pre-treatment of tea plants with aqueous leaf extracts of *Azadirachta indica*, *Acalypha indica*, *C. roseus* and *J. jasminoides*).

From the results (Table 32 & Fig. 24) it was found that plants treated with *Acalypha indica* leaf extract and challenge-inoculated by *L. theobromae* showed maximum increase in peroxidase levels. Activity gradually increased and reached a

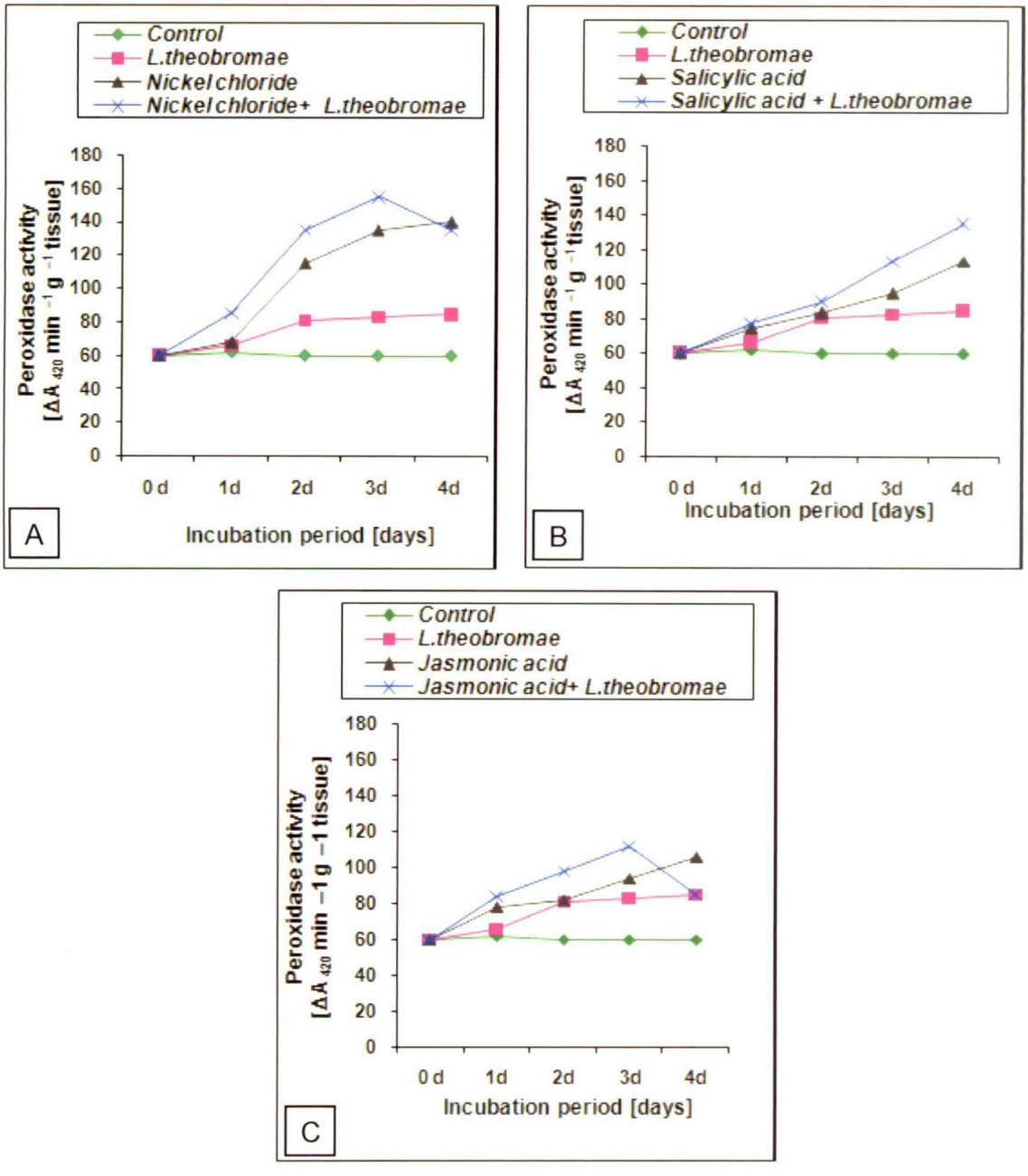


**Fig. 22:** Peroxidase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with Nickel chloride.

B. Treated with Salicylic acid.

C.Treated with Jasmonic acid.

peak on the third day with approximately 2.5 fold increases in comparison to control. Challenge inoculated plants showed higher enzyme activity than uninoculated but treated plants.

**Table 31:** Peroxidase activity in seedlings of TS-449 pre-treated with biotic inducers followed by challenge-inoculation of *Lasiodiplodia theobromae.*

| Treatments | Peroxidase activity ($\Delta A_{420}$ $min^{-1}$ $g^{-1}$ fresh weight tissue) 1 unit = 0.001 Absorbance | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 60± 2 | 62± 3 | 60± 3 | 60± 2 | 60± 4 |
| *L.theobromae* | 60± 3 | 66± 4 | 81± 4 | 83± 5 | 85± 3 |
| *T. harzianum* | 60± 5 | 80± 7 | 128± 8 | 132± 8 | 136± 9 |
| *T. harzianum* + *L.theobromae* | 60± 4 | 115± 6 | 142± 8 | 156± 8 | 162± 12 |
| *T. virens* | 60± 6 | 75± 8 | 118± 9 | 137± 8 | 138± 8 |
| *T. virens* + *L. theobromae* | 60± 6 | 85± 7 | 126± 7 | 158± 8 | 163± 7 |

Data is the mean of three replicates; Data after ± indicates standard error values.

### 4.8.4. Study of peroxidase isoform patterns

Peroxidase isoform patterns were studied in tea plants (TS-449) separately treated with two different inducers (*Azdirachta indica, Acalypha indica*) and the treated plants were challenge-inoculated. It was found that plants treated with *Azdirachta indica* and *Acalypha indica* showed prominent peroxidsae isoforms with two major bands (Plate XVII).

The peroxidase isozyme analysis was performed following procedures described in materials and methods (Section 3.19.3). After 3 days, two major bands (approx mass 38KDa and 33 KDa) were observed in all treatments. Staining intensity was more for the slower migrating isozyme in case of untreated-inoculated plants.

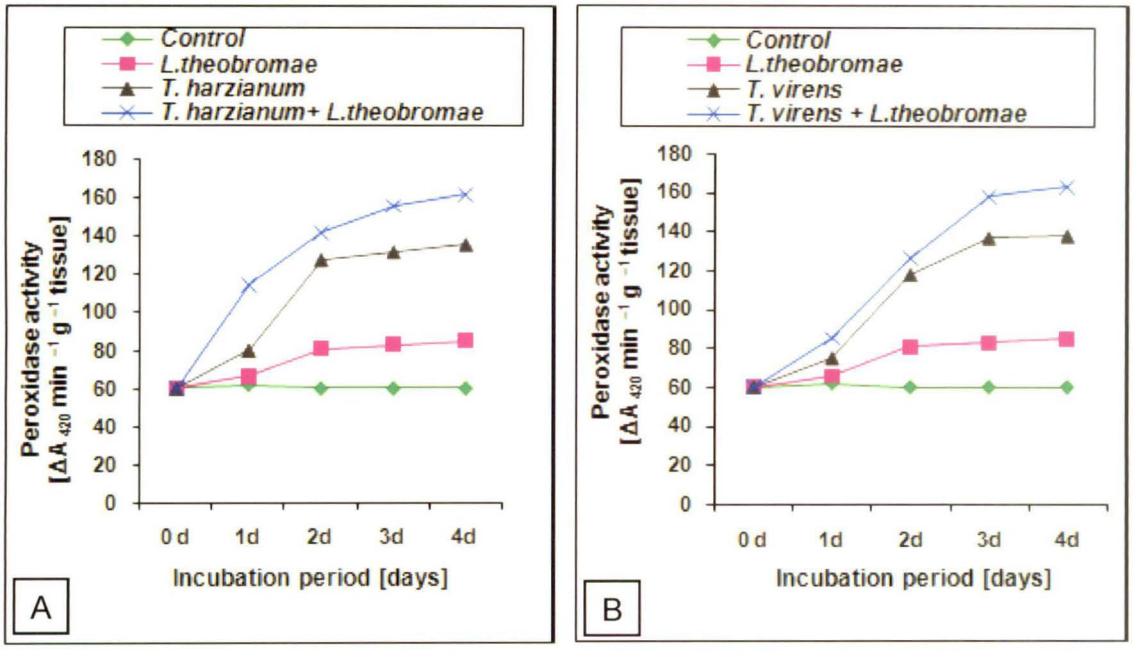


**Fig. 23:** Peroxidase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with *Trichoderma harzianum.*

B. Treated with *Trichoderma virens.*

**Table 32:** Peroxidase activity in seedlings of TS-449 pre-treated with phyto-extracts followed by challenge-inoculation of *Lasiodiplodia theobromae*.

| Treatments | Peroxidase activity ($\Delta A_{420}$ min $^{-1}$ g $^{-1}$ fresh weight tissue) 1 unit = 0.001 Absorbance | | | | |
|---|---|---|---|---|---|
| | Days after inoculation | | | | |
| | 0 d | 1d | 2d | 3d | 4d |
| Control | 60± 2 | 62± 3 | 60± 3 | 60± 2 | 60± 4 |
| *L.theobromae* | 60± 3 | 66± 4 | 81± 4 | 83± 5 | 85± 3 |
| *Azadirachta indica* | 60± 7 | 68± 7 | 110± 8 | 145± 12 | 105± 8 |
| *Azadirachta indica* + *L.theobromae* | 60± 6 | 75± 7 | 125± 9 | 140± 13 | 115± 8 |
| *Acalypha indica* | 60± 7 | 75± 8 | 95± 7 | 145± 12 | 105± 8 |
| *Acalypha indica* + *L.theobromae* | 60± 6 | 70± 7 | 135± 7 | 155± 7 | 128± 7 |
| *Catharanthus roseus* | 60± 7 | 64± 6 | 81± 9 | 95± 8 | 70± 8 |
| *Catharanthus roseus* + *L.theobromae* | 60± 6 | 75± 8 | 125± 8 | 145± 13 | 140± 12 |
| *Jasminum jasminoides* | 60± 4 | 65± 6 | 73± 5 | 82± 4 | 75± 6 |
| *J. jasminoides* + *L.theobromae* | 60± 7 | 72± 6 | 82± 7 | 95± 8 | 106± 9 |

Data is the mean of three replicates; Data after ± indicates standard error values.

While both isozymes were less intense in control and treated-uninoculated sets, a particular band of molecular mass approximately 33 KDa showed maximum intensity in the treated-inoculated leaf samples. The peroxidase isozyme induced by pathogen infection appeared to be different from that induced following treatment with leaf extract and then challenge-inoculated with pathogen. Expression of the 33 KDa isozyme did not increase much following treatment with phyto-extract but increased dramatically when these treated plants were inoculated with *L. theobromae*.

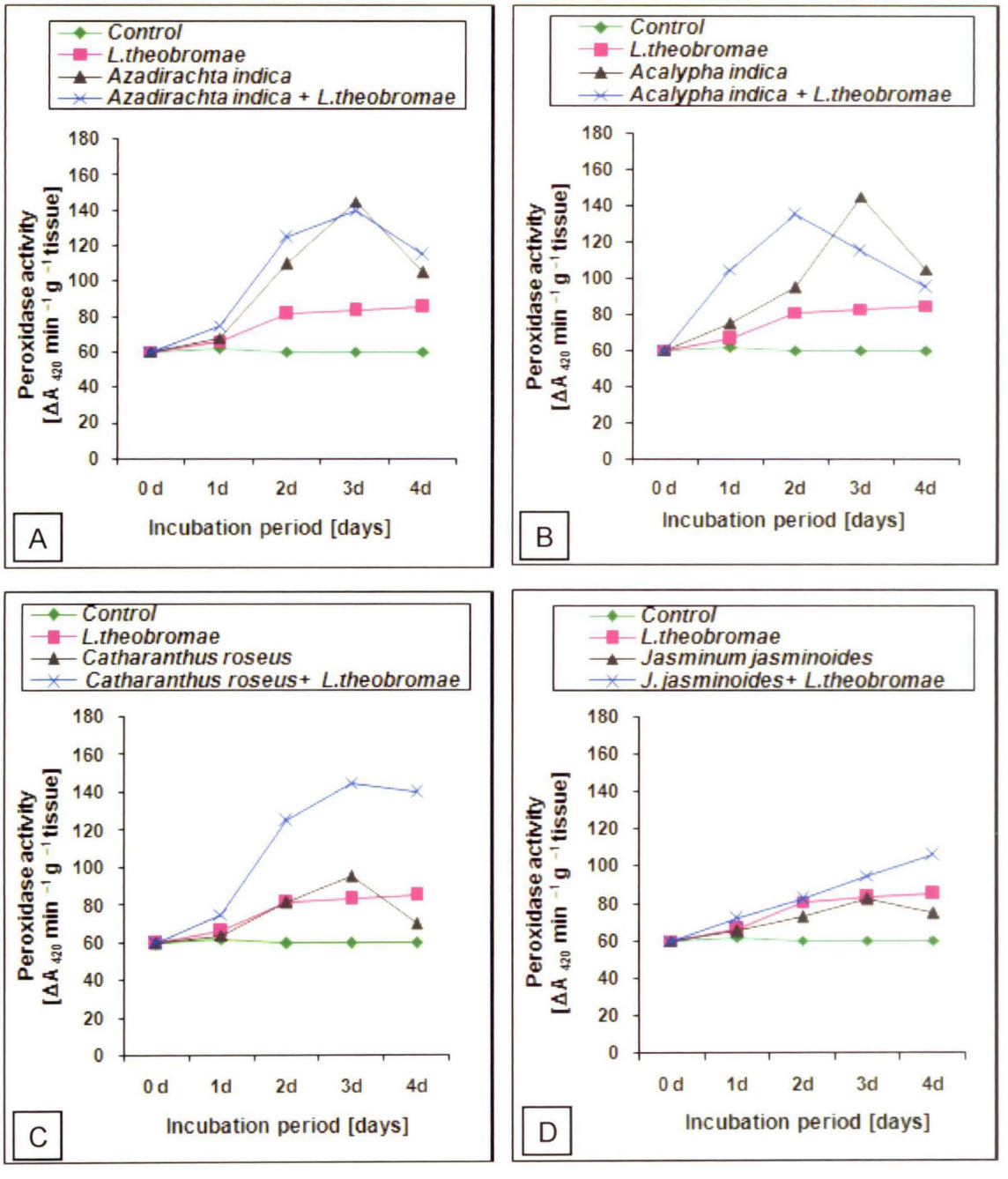


**Fig. 24:** Peroxidase activity in treated and treated-inoculated seedlings of TS-449.

A. Treated with *Azadirachta indica* leaf extract.

B. Treated with *Acalypha indica* leaf extract.

C. Treated with *Catharanthus roseus* leaf extract.

D. Treated with *Jasminum jasminoides* leaf extract.

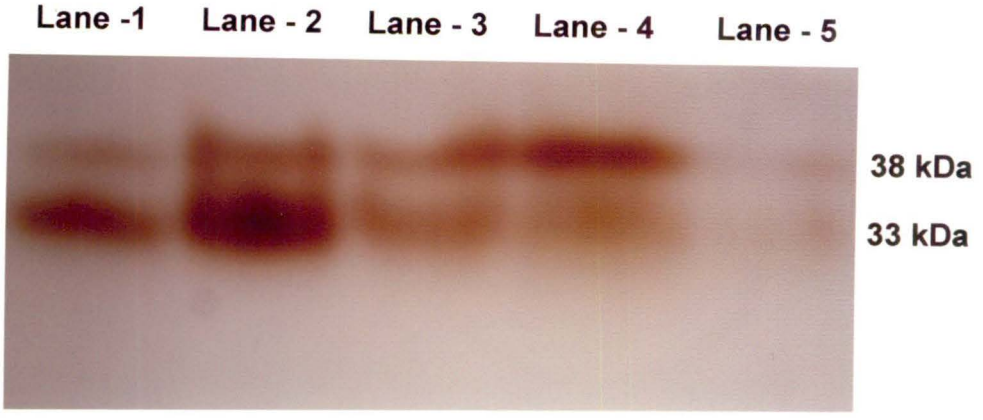


**PLATE XVII**

**Peroxidase isoform pattern studied on susceptible tea seed variety (TS-449) treated with different phyto-extracts following challenge-inoculation with *Lasiodiplodia theobromae.***

**Lane-1:** Treated with *Azadirachta indica* aqueous leaf extract and inoculated.

**Lane-2:** Treated with *Acalypha indica* aqueous leaf extract and inoculated.

**Lane-3:** Treated with *Acalypha indica* aqueous leaf extract.

**Lane-4:** Untreated and inoculated.

**Lane-5:** Untreated-uninoculated (Control).

# Chapter IX

## 4.9. Foliar application of inducers, for controlling diplodia disease caused by *L. theobromae* in tea plants

Tea seedlings (TS-449) were treated with several inducers (plant extracts/abiotic inducer/biotic inducer) separately and challenge-inoculated by *L. theobromae*. In control set, sterile distilled water was sprayed instead of inducer. Mean foliar disease index / plant were calculated after 4, 8, 12 and 16 days of inoculation following the methods described by Sinha and Das (1972). Procedures of inducer application, foliar inoculation technique and assessment of disease have been discussed in materials and methods (Section 3.12, 3.6.1 & 3.6.3 respectively). The results were noted in Tables 33, 34 & 35.

From the results (Table 33) it was evident that all the four phyto-extracts, when applied, showed significant reduction in mean foliar disease index/plant. It may be noted here that none of the tested leaf extracts showed antifungal activity towards *L. theobromae* when tested *in vitro* (data not shown). Tea plants treated with *Azadirachta indica* aqueous leaf extracts showed maximum control of the disease with mean foliar disease index of only 15.3 followed by *Acalypha indica* with mean foliar disease index of 16.3 after 16 days of inoculation. *C. roseus* and *J. jasminoides* treated plants also showed significant reduction in disease index. Among the abiotic inducers (Table 34), SA and $NiCl_2$ treated plants exhibited lower mean foliar disease index (16.7 and 21.7 respectively) values in comparison to untreated-inoculated (control), where mean foliar disease index was found to be 67.5 after 16 days. Similarly, challenge inoculation of plants by *L. theobromae* was performed after treatment by two biotic inducers, *T. virens* and *T. harzianum* (Table 35). Plants treated with both inducers showed reduction in mean foliar disease index (16.8 and 24.7 respectively) in comparison to control plants.

**Table 33**: Disease incidence following application of aqueous leaf extracts in susceptible tea seedlings (TS-449) against *Lasiodiplodia theobromae.*

| Treatments | Mean foliar disease index/plant* | | | |
|---|---|---|---|---|
| | 4 days | 8 days | 12 days | 16 days |
| *L.theobromae* | 14.6±0.05 | 25.7±0.04 | 52.4±0.08 | 67.5±0.02 |
| *Az. indica* leaf extract+ *L.theobromae* | 4.3±0.06 | 7.4±0.02 | 12.5±0.07 | 15.3±0.03 |
| *Acalyopha indica* leaf extract+ *L.theobromae* | 5.3±0.05 | 8.4±0.07 | 13.6±0.03 | 16.3±0.06 |
| *Cathatanthus soreus* leaf extract+ *L.theobromae* | 8.4±0.06 | 12.3±0.03 | 15.7±0.05 | 18.5±0.02 |
| *Jasminum jasminoides*+ *L.theobromae* | 9.2±0.04 | 15.4±0.06 | 23.5±0.04 | 32.6±0.06 |
| CD at 5% | 4.09 | 4.30 | 3.80 | 3.20 |

*Mean of three replicates; Data after ± indicates standard error values.

**Table 34:** Disease incidence following application of abiotic inducers in susceptible tea seedlings (TS-449) against *Lasiodiplodia theobromae.*

| Treatments | Mean foliar disease index/plant* | | | |
|---|---|---|---|---|
| | 4 days | 8 days | 12 days | 16 days |
| *L. theobromae* | 14.6±0.05 | 25.7±0.04 | 52.4±0.08 | 67.5±0.02 |
| Salicylic acid + *L.theobromae* | 4.5±0.02 | 7.2±0.03 | 12.6±0.05 | 16.7±0.06 |
| Nichel chloride + *L.theobromae* | 5.6±0.06 | 8.5±0.04 | 15.8±0.03 | 21.7±0.02 |
| Jasmonic acid + *L.theobromae* | 7.6±0.03 | 12.7±0.04 | 16.8±0.07 | 22.5±0.05 |
| CD at 5% | 4.12 | 3.70 | 3.15 | 2.80 |

*Mean of three replicates; Data after ± indicates standard error values.

**Table 35:** Disease incidence following application of biotic inducers in susceptible tea seedlings (TS-449) against *Lasiodiplodia theobromae*.

| Treatments | Mean foliar disease index/plant* | | | |
|---|---|---|---|---|
| | 4 days | 8 days | 12 days | 16 days |
| *L.theobromae* | 14.6±0.05 | 25.7±0.04 | 52.4±0.08 | 67.5±0.02 |
| *Trichoderma virens* + *L.theobromae* | 6.5±0.03 | 8.2±0.05 | 12.7±0.04 | 16.8±0.02 |
| *Trichoderma harzianum* + *L.theobromae* | 7.8±0.06 | 12.9±0.04 | 16.8±0.05 | 24.7±0.03 |
| CD at 5% | 3.25 | 4.86 | 4.20 | 3.42 |

*Mean of three replicates; Data after ± indicates standard error values.