Chapter VI Biochemical alterations induced in Taro in response to Phytophthora colocasiae infection

6.1 Introduction

Two kinds of defense responses are developed in plants: pre-existing (passive defense mechanisms) represented by a waxy cuticle or reservoirs of antimicrobial compounds and active responses, induced by pathogen attack (Hutcheson, 1998; Lebeda et al., 2001). The initiation of reactive oxygen species (ROS) production is one of the first events following the recognition of a pathogen by the plant (Baker and Orlandi, 1995). ROS, a collective term for radicals and other non-radical but reactive species derived from oxygen, have been implicated in numerous developmental and defence responses in plant cells (Low and Merida, 1996; Lamb and Dixon, 1997). ROS may induce lipid peroxidation, which has been detected as an early event in the HR, and may also damage or modify DNA and proteins (Adam et al., 1995).

Various enzyme systems may be involved in ROS metabolism. Neutrophil-analogous, membrane-bound NADPH oxidase (Levine et al., 1994) and cell wall peroxidase (POX) (Bolwell et al., 1995; Gross et al., 1997) belong to an important enzyme system generating H$_2$O$_2$. It has been proposed that a rapid increase in either intracellular H$_2$O$_2$ is involved in the induction and/or execution of hypersensitive reaction (HR) (Low and Merida, 1996; Bestwick et al., 1997). H$_2$O$_2$ is required for the cross-linking of plant cell wall components as a part of the structural defence response (Lamb and Dixon, 1997). The production of H$_2$O$_2$ may also lead to the development of an
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antimicrobial environment within the apoplast (Peng and Kuc, 1992). POX is also capable of reducing the level of H$_2$O$_2$, e.g. during H$_2$O$_2$-dependent polymerisation of hydroxycinnamyl alcohols (lignin biosynthesis) (Monties et al., 1989) and H$_2$O$_2$-dependent cross-linking of cell wall proteins such as hydroxyprolinerich glycoproteins and proline-rich proteins (Bestwick et al., 1995).

Within hours these events are followed by a broad spectrum of metabolic modifications that include: (a) stimulation of the phenylpropanoid and fatty acid pathways, (b) production of defense-specific chemical messengers such as salicylic acid (SA) or jasmonates, and (c) accumulation of components with antimicrobial activities such as phytoalexins and pathogenesis related (PR) proteins (Hutcheson, 1998).

Besides these, plant phenolics, carbohydrates and amino acids have received considerable attention in relation to disease resistance. In fact, the metabolic pathways of these substances are interconnected: phenolic and amino acid pathways use products of carbohydrate metabolism as their precursors. During host-pathogen interaction, amino acids may act as substrate for the pathogen (Titarenko et al., 1993) or they may have a fungistatic effect through their involvement in metabolic reactions associated with disease resistance, e.g. the synthesis of specific proteins related to the infection (Schmelzer et al., 1989; Graham et al., 1990). Results reported on saccharide metabolism during plant infection are contradictory. Lukens (1970) and Gibbs and Wilcoxson (1972) found that saccharides have little or no effect on fungal development. On the other hand, Jeun and Hwang (1991) reported that carbohydrates increase the severity of the infection and that they may serve as easily metabolized carbon substrates for the pathogen. The antimicrobial property of phenolic compounds and especially those of the flavonol group is well documented (Smith and Banks, 1986; Hahlbrock and Scheel, 1989). Isoflavanoids with antimicrobial activity have been characterized in *Medicago* species (Latunde-Dada
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The pterocarpan medicarpin has been implicated in the resistance of Alfalfa to *Colletotricum trifolii* and several leaf spot diseases (Kessmann *et al*., 1990; Ebel and Cosio, 1994). This antimicrobial role of phenolics is based on indirect evidence, such as correlations between the timing of phytoalexin accumulation in resistant and susceptible interactions and the potency of phenolics as antimicrobial agents *in vitro*.

Taro is one of the oldest cultivated crops grown for its edible corms and leaves (Kuruvilla and Singh, 1981; Coates *et al*., 1988). Taro corms and leaves are also accredited to have medicinal value in reducing tuberculoses, ulcers, pulmonary congestion and fungal infection (Misra and Sriram, 2002). The Food and Agriculture Organization estimates that 9.1 million Mt of corms are produced annually on a surface of 2 million ha, but this largely underestimates production as few countries keep reliable figures (http://faostat.fao.org). Leaf blight has become a limiting factor for taro production in all taro growing-countries including India causing yield loss of 25-30% (Thankappan, 1985; Misra and Chowdhury, 1997). The disease starts with the onset of monsoon and continues till the end of monsoon. It appears as a small spot on the taro leaves that later coalesce and whole leaf is destroyed within 20 d.

This work is focused on study of the early and late events of biochemical activities during *P. colocasiae*-taro interaction and to examine whether changes in these biochemicals during the development of the infection are related to the tolerance to *P. colocasiae*.

### 6.2 Materials and Methods

**6.2.1 Plant materials and *P. colocasiae* inoculation experiment**

Two taro lines, cv. Telia and its near-isogenic line cv. Muktakeshi were used as the leaf blight susceptible and resistant cultivars respectively. Both the cultivars were grown from tubers in pots containing soil and *Trichoderma* enriched compost. *P. colocasiae* spore was generated by using the method Mishra *et al.* (2008b). For infection, taro leaves of same
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age were inoculated on their abaxial surfaces with 50 μL of *P. colocasiae* sporangial suspension containing approximately 500 sporangia or with water as control and the symptom were allowed to develop.

6.2.2 Sampling of Taro leaves

The progress of leaf blight disease on the taro leaves was followed periodically. Samples of leaves with lesions were collected for analysis at the following four stages of lesion development and decay of leaves.

**Stage: 1** The early stage of infection (2 d after inoculation): This was characterized by the appearance of water-soaked lesions on the leaves.

**Stage: 2** The intermediate stage of infection (5-6 d after stage one): Yellowing of young leaves was initiated.

**Stage: 3** Late stage of infection (7-10 d after inoculation): This was characterized by increase in the intensity of blackening and decay of leaves. The yellowing of leaves persist and spread further uniformly.

**Stage: 4** Very late stage of infection (10-20 d after inoculation): This was characterized by the destruction of entire leaf, total yellowing of all leaves, wilting and death of the plants.

For control, healthy leaves were collected for analysis at periods corresponding to each of the stages of the infested plants.

6.2.3 Biochemical experiments

(i) Preparation of plant crude extracts.

Plant leaves were cut at one day of intervals from the stage 1 and leaves were thoroughly washed and leaf blight infected tissues consisting of a 1 cm section of leaf tissues were sampled. The enzyme extracts were prepared according to method described by Hahlbrock and Ragg (1975). Briefly, leaf blight infected tissues (1 g) were ground in a pestle and mortar on ice with 5 mL of 50 mM phosphate buffer (pH 6.0) containing 0.15
% polyvinylpyrrolidone, 1 mM EDTA, 1 mM dithiothreitol (DTT). Debris was removed by centrifugation (4°C, 15 min, 15 000 g) and crude enzyme extracts were stored at -80 °C until assay. Intact plants of the same age were used as a control, and were kept separately from infected plants.

(ii) Enzyme activity assays.

Peroxidase (POD; E.C 1.11.1.7) activity was assayed in the supernatant by the method of van Gestelen et al. (1997). The 3.0 mL reaction volume contained 50 mM phosphate buffer (pH 6.0), 100 mM 3, 5-dichloro-2-hydroxybenzenesulfonic acid, 10 mM 4-aminoantipyrine, and 150 µL of enzyme extract (approx. 0.05 mg mL⁻¹ protein). After incubation at 25°C for 5 min, the reaction was started by addition of 30 ml of 100 mM H₂O₂ and the monitoring of the reaction followed at A₅₁₀ nm. The enzyme activity was calculated using the molar extinction coefficient of 2.47 mM⁻¹ cm⁻¹. The specific activity was expressed as µmol min⁻¹ mg⁻¹ protein. A standard curve was made with horseradish peroxidase (HRP).

The activity of L-phenylalanine ammonia-lyase (PAL; E.C. 4.3.1.5.) was determined by the method of Zucker (1965). Aliquot of 0.3 mL of the crude extract was mixed with 1.9 mL boric acid-borax buffer (0.2 mol L⁻¹, pH 8.8). The reaction mixture was thermostatized at 36°C. The reaction started after the addition of 0.6 mL L-phenylalanine (0.1 mol L⁻¹). After 15 min of incubation at a constant temperature, spectrophotometric reading was recorded at 290 nm. The enzyme activity was calculated based on the molar extinction coefficient of t-cinnamic acid (E₉₀ =104 mM⁻¹cm⁻¹). One unit (U) of enzyme activity was defined as the amount of enzyme forming 1 pmol of transcinnamic acid from L-phenylalanine min⁻¹ mg⁻¹ of protein.

Total β-1,3-glucanase (E.C.3.2.1.6) activity was assayed colorimetrically using the laminarin dinitrosalicylic method according to Ji and Kuc (1995). The 1.0 ml reaction
mixture consisted of 5 mg laminarin, 100 mM sodium acetate buffer, pH 5.5 and 0.3 mL of the enzyme crude extract. The reaction mixture was incubated for 1 h at 37°C, 0.5 ml of dinitrosalicylic reagent that was prepared according to (Fischer and Kohtes, 1951) was added followed by heating. The absorbance of the resulting colored solutions was recorded at 560 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol reducing sugar per h under standard assay conditions.

(iii) Protein concentration.

Protein concentration was determined using bovine serum albumin as a standard by the method of Bradford (1976).

(iv) Extraction and measurement of total phenols

Total phenols were extracted according to the method described by Ndoumou et al. (1996) with some modifications. One gram of sampled tissue was extracted twice with 50 ml of 70% methanol at room temperature for 15 min with constant shaking. The methanolic extracts of the same sample were combined, filtered with Whatman no. 1 paper and the methanol was evaporated at 40°C under vacuum using a rotary evaporator. The aqueous phase obtained was adjusted to 70 ml with distilled water, and the pigments were removed by adding 50 ml of 40% (NH₄)₂SO₄, 1.4 ml of 80% H₃PO₄ and 50 ml of petroleum ether. This process was repeated thrice. The ether phases were discarded and the aqueous phases of the same sample combined and extracted four times with 50 ml ethyl acetate. The aqueous phases were discarded and the organic phases of the same sample were combined, dried by addition of 5 g MgSO₄ and filtered after 5 min with Whatman no.1 paper. The salt residue was discarded and the clear organic phase was dried at 40°C under vacuum using a rotary evaporator. The dried extract of soluble phenolic compounds was dissolved in 2 ml of pure methanol. Total amount of phenols was determined by using Folin-Ciocalteu’s phenol reagent. Purified phenol extracts were
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diluted 800 times in pure methanol. For assay, 5 ml of 0.2 N Folin-Ciocalteu reagent was added to 4 ml of diluted phenol extract and the reaction mixture was stored at 50°C for 30 min before measuring the absorbance at 765 nm. Total phenol content was expressed as µg of phenol per gm. of leaf.

(v) Determination of Total Sugars

Total sugar content was determined using the anthrone method of Yemm and Willis (1954). Sampled leaves were dried at 60°C, grinded with glass powder and suspended in distilled water. After filtration from cheesecloth, the samples were suspended in distilled water and filtered through Whatman No.1 filter paper. One ml of the filtrate was incubated with 5 ml anthrone solution (0.12 g anthrone in 100 ml 6.5 M H$_2$SO$_4$) at 90°C for 10 min. The absorbance of the green product was measured at 630 nm (Beckman DU-68 spectrophotometer). Glucose equivalents were calculated from a standard curve obtained with pure analytical grade glucose.

6.2.4 Statistics

Experimental design was completely randomized and consisted of three independent experiments. All tests for significance were conducted at the p≤0.05 level. The software MSTAT-C was used for statistical analysis (1989).
Figure 6.1 Time-course activity of peroxidase in infected and uninfected taro leaves of resistant and susceptible variety at different intervals (days after inoculation of *P. colocasiae*). Vertical bar represent means of three separate experiments ± standard error.

Figure 6.2 Time-course activity of β-1,3-glucanase in infected and uninfected taro leaves of resistant and susceptible variety at different intervals (days after inoculation of *P. colocasiae*). Vertical bar represent means of three separate experiments ± standard error.
Figure 6.3 Time-course activity of L-phenylalanine ammonia-lyase in infected and uninfected taro leaves of resistant and susceptible variety at different intervals (days after inoculation of *P. colocasiae*). Vertical bar represent means of three separate experiments ± standard error.

Figure 6.4 Total phenol content in infected and uninfected taro leaves of resistant and susceptible variety at different intervals (days after inoculation of *P. colocasiae*). Vertical bar represent means of three separate experiments ± standard error.
Figure 6.5 Total sugar level in infected and uninfected taro leaves of resistant and susceptible variety at different intervals (days after inoculation of *P. colocasiae*). Vertical bar represent means of three separate experiments ± standard error.
6.3 Results and Discussions

A typical analysis of plants infected by pathogen has revealed changes in the enzyme activities of both the plant and the fungus and in metabolite concentrations within the infected host tissues. However, due to the intricate nature of host-pathogen interactions, it is an exceedingly difficult task to delineate the individual change caused by the host or pathogen. In this study, initially the progression of the disease was accompanied in taro leaves under controlled conditions. After inoculation and up to 2 d there were no symptoms on any of the inoculated leaves, suggesting a period of *P. colocasiae* establishment and after the establishment of leaf blight disease, an attempt was made systematically to analyze changes in a number of key plant biochemical parameters for taro infected with *P. colocasiae* during the various stages of the disease process and to correlate those changes with disease symptoms.

6.3.1 Time-course activity of peroxidase in taro plant infected with *P. colocasiae*

The induction of PO activity has been repeatedly reported in several plant species in response to pathogen infection (Lee and Lee, 2000; Baysal *et al.*, 2003; Mlickova *et al.*, 2004; Mohamed and Hasabo, 2005; Bindschedler *et al.*, 2006). The peroxidase activity, in general increases under different stress conditions like wounds, fungi infections, salinity, water stress and nutritional disorders, inducing also the lignin increment and production of ethylene and induce the increase of the production of phenols oxidized at the cell wall (van Huystee, 1987; Schallenberger, 1994). This activity, suggests a cell effort for the establishment of a physiochemical barrier, able to isolate the infected area (Urs and Dunleavy, 1975). In the present study, the induction of PO activity in *P. colocasiae*-infected taro leaf of resistant and susceptible cultivars over their uninfected controls has been revealed (Fig. 7.1). The highest induction was recorded at the fifth day after the inoculation of *P. colocasiae*, where approximately 2-fold increase over uninfected counterparts was observed in resistant variety of taro. However, not much
substantial increase in enzyme activity was observed in the susceptible cv. ‘Telia’ throughout the infection. The enhancement of PO in taro leaf of resistant cv. upon *P. colocasiae* infection herein was in agreement with that reported for resistant cvs of muskmelon *Cucumis melo* upon infection with *Pseudoperonospora cubensis* (Reuveni *et al.*, 1992), in sugarcane upon *Colletotrichum falcatum* invasion (Sundar *et al.* 1998), *Cucumis sativus* upon inoculation with cucumber downy mildew *P. cubensis* (Lebeda and Dolezal, 1995; Lebeda *et al.*, 2001), green bean upon infection with *Uromyces appendiculatus* (Siegrist *et al.*, 1997), where a positive correlation between the enhancement of PO activity and the degree of plant resistance towards the pathogen was recorded.

**6.3.2 Time-course activity of PR-proteins in taro plant infected with *P. colocasiae***

Plants respond to the presence of microbial pathogens by *de novo* synthesis of certain proteins often referred to as pathogenesis-related (PR) proteins. β-1,3-glucanase and PAL are the representatives of the PR proteins. The induction of such enzymes occurs in different plant species in response to fungal attacks (Hanselle and Barz, 2001; Kapoor *et al.*, 2003; Zhao *et al.*, 2005). The results showed that β-1,3-glucanase markedly induced in *P. colocasiae* infected taro leaf of resistant cv. Muktakeshi over its uninfected counterparts. While, it is slightly induced in such leaves of susceptible cv. Telia (Fig. 7.2). The maximum increase in infected taro leaves of resistant and susceptible cvs was found to be 4 and 2.0 fold respectively at 5 day after the appearance of symptom. Similarly, β-1,3-glucanase has been induced in resistant cv. of celery infected with *Fusarium oxysporum* (Krebs and Grumet, 1993) and chickpea upon infection with the fungal pathogen *Ascochyta rabiei* (Hanselle and Barz, 2001) with 2.0 and 5.5-fold increase over control respectively. Furthermore, Baysal *et al.* (2003) have used the alterations in the activity of such enzyme as a molecular marker of resistance in tomato seedlings against bacterial canker caused by *Clavibacter michiganensis*. The enzyme β-
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1,3-glucanases has been reported to have a role in defense against invading fungal pathogen because of their potential to hydrolyze fungal cell wall polysaccharides, β-1,3-glucan (Kang and Buchenouer, 2002). From these results, we can conclude that the higher induction of β-1,3-glucanase in resistant (cv. Muktakeshi) than susceptible one (cv. Telia) is suggestive of its role in disease resistance against *P. colocasiae*.

Some studies (Hahlbrock and Schell, 1989; Campbell and Ellis, 1992) point the enzyme PAL as the precursor of the lignin biosynthesis, phenols, flavonoids and phytoalexines by plant tissues, related to the plant response system against microorganisms, insects and other stress factors. The results showed that PAL markedly induced in *P. colocasiae* infected taro leaf of resistant cv. Muktakeshi over its uninfected counterparts after 4 d of appearance of symptom. While, it is slightly induced in such leaves of susceptible cv. Telia (Fig. 7.3). The observed result with two cultivars reflects their pathogen defense capacity against *P. colocasiae*.

The potential of the Muktakeshi cultivar is clear, presenting increase of activity of PR-proteins in applied inoculation of leaf blight pathogen and it seems to be the most responsive genotype against *P. colocasiae*.

### 6.3.3 Total phenols assay

Comparative analysis of the genotypes and *P. colocasiae* infection showed that the phenol content was highest in the resistant variety than the susceptible variety, by 1.8 fold. The infection leads to the marked increase in phenol content in resistant cultivar compared to susceptible and their control counterparts. One day after the appearance of leaf blight symptom, infected resistant taro cultivar showed 1.6 fold increase in phenol content, while susceptible cultivar showed 1.5 fold increase in phenol content (Fig. 7.4). This result indicates that the inhibitory effect of phenolics on *P. colocasiae* development and also depends on its level in plant tissue of different genotypes. In fact, in infected plants, phenolics are substrates for the synthesis of compounds involved in disease
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resistance, like pterocarpan phytoalexins and hydroxycinnamic acid esters (Dixon and Lamb, 1990), and for the production at or near the infection site of bioresistant phenylpropanoid polymers (lignins and suberins) which act to scar over the wound and or as a barrier to the penetration or the propagation of the pathogen (Ebel and Grisebach, 1988; Davin and Lewis, 1992). This finding again supports the claim that Muktakeshi cultivar is resistant variety for taro leaf blight.

6.4.4 Total Sugar assay

Our results show that the highly susceptible cultivar of taro contains more soluble carbohydrates than the resistant variety. In the susceptible cultivar, the total sugar content decreased more rapidly in the infected leaves than the non inoculated control leaves. This may be attributed to their utilization by the fungus for its growth. These results are in agreement with those reported on Botryosphaeria apple rot (Hwang, 1983), on charcoal rot of sorghum (Patil et al., 1985), and on Phytophthora blight of pepper (Jeun and Hwang, 1991). During the infection, the total sugar content decreased in the Telia cultivar, while it remained almost constant in Muktakeshi variety. It seems that total sugar variation during infection is genotype-dependent, and that in the resistant the fungus does not utilize sugars of this group for its growth (Fig. 7.5).

Overall, the observed biochemical alterations associated with the infection suggest that, in the first stage, the plant uses unspecific mechanisms to try to eliminate the fungus, such as an increase in phenolic. However, this mechanism seems not to be sufficient to avoid the disease and a number of biochemical changes, such as increased levels of Peroxidase, PR-proteins and decrease in sugars suggests that a cascade of events has been triggered to cause the death of the infected tissue. The major question remaining is how the fungus causes or modulates these apparent responses in the plant? Therefore, to verify these findings and advance an understanding of this complex pathogen–host interaction as well as the detailed analysis of gene expression of both pathogenicity factors and host
response factors will be required. Along these lines, gene expression is currently being analyzed in two cDNA libraries generated from resistant and susceptible cultivars after inoculations with \textit{P. colocasiae} in an attempt to understand the gene expression during pathogenesis.