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Differential Induction of Phenylpropanoid Metabolites in Suspension-Cultured Cells of Sugarcane by Fungal Elicitors

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The effect of elicitors isolated from the mycelial walls of *Colletotrichum falcatum* (the red rot pathogen of sugarcane) and from *C. lindemuthianum* (a non-pathogen) in suspension-cultured cells of sugarcane was studied. Both the elicitors induced the synthesis of enzymes of the phenylpropanoid pathway such as PAL, TAL and 4CL and also resulted in the enhanced accumulation of phenolics. However, a specific induction of the defense parameters at higher levels was recorded in suspension cells treated with the pathogen elicitor and no such differential response was observed in the case of the non-pathogen elicitor. Elicitor induced necrosis and browning of cells were observed which suggests an additional evidence that elicitors simulate pathogen infection and thus provide a valuable reason that study on elicitor induced responses may be useful in understanding the host defense mechanisms against the red rot pathogen at molecular level.

Keywords: *Colletotrichum falcatum, Colletotrichum lindemuthianum,* elicitor, phenylalanine ammonia lyase, tyrosine-ammonia lyase, 4 coumarate CoA ligase, phenolics.

Sugarcane (*Saccharum officinarum* L.) is one of the important commercial crops grown under a wide range of agroclimatic conditions. Red rot disease caused by the fungus *Colletotrichum falcatum* Went. [Perfect stage: *Glomerella tucumanensis* (Speg.) Arx and Muller] is the major constraint to sugarcane cultivation (Alexander and Viswanathan, 1996). In recent years concerted efforts have been invested in gaining a thorough understanding of the red rot resistance mechanism operating at molecular level (Ramesh Sundar and Viswanathan 1997; Ramesh Sundar et al., 1998; Ramesh Sundar et al., 1999; Ramesh Sundar et al., 2001).

Plants possess resistance genes, which are pathogen specific and are generally ineffective against other pathogens. This suggests a very specific molecular signaling reaction between an avirulence gene product produced by the pathogen (elicitor) and a resistance gene product (a receptor) in the plant (Dixon and Lamb, 1990). Specific recognition events are followed by the activation of a number of cellular responses associated with expression of resistance phenotype (Dixon and Harrison, 1990). The major differences between susceptible and resistant interactions may be the time, amount and speed with which the defense genes are expressed (Vidhyasekaran, 1998). Plants are able to recognize fungal

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pathogens by their secreted products, so-called elicitors. Defense responses are induced upon perception of either specific or non-specific elicitors. Specific elicitors are products of pathogen avirulence genes and are hypothesized to be recognized by the products of corresponding, pathogen-race specific resistance genes (Linden et al., 2000).

Elicitors are signal molecules and elicit synthesis of phytoalexins (Nelson et al., 1994), phenolics (Ryerson and Heath, 1992), lignin (Vance et al., 1980), pathogenesis-related proteins (Knogge et al., 1991) and hydroxy-proline rich glycoproteins (Templeton and Lamb, 1988). A considerable number of fungal elicitors have been well characterized and were used to investigate the early molecular events involved in the expression of disease resistance in response to pathogens (Scheel and Parker, 1990; Basse and Boller, 1992). Suspension-cultured cells and fungal elicitors have been used to detect defense genes in plants. At the molecular level, the elicitation response in cell cultures exhibits many phenomena, which may be important in intact plant-pathogen interactions. It is a useful model system for studies on the nature, selectivity, regulation and coordination of gene expression associated with induced resistance (Dixon, 1986). Cultured plant cells respond to fungal elicitor in a manner comparable to intact plant-pathogen systems. Suspension-cultured cells have been widely used by many workers to study the host-pathogen interaction at molecular and cellular level (Felix et al., 1991; Velazhahan and Vidhyasekaran, 2000). By inducing over production of defense-responsive chemicals at early stages of infection process, the susceptible varieties can be made resistant by genetic engineering techniques. These elicitors may be the key to enhance disease resistance in genetically engineered plants, which proves to be an emerging strategy for crop disease management.

To facilitate biochemical studies of induced defense reactions, sugarcane suspension cultured cells were developed and the elicitor molecule isolated from the cell wall of the red rot pathogen *C. falcatum,* were used to search for defense responsive phenylpropanoid mechanisms. A non-pathogenic elicitor isolated from the mycelial cell walls of *C. lindemuthianum* was used to study the specificity of the pathogen elicitor isolated from *C. falcatum* towards sugarcane cells. The possible induction of defense responsive phenylpropanoid mechanisms such as phenylalanine ammonia-lyase (PAL), 4CL, TAL and phenolics in sugarcane cell cultures by the fungal elicitors has been investigated in this study.

Material and Methods

Fungal culture

The sugarcane red rot fungus *C. falcatum* was isolated from the red rot infected sugarcane internodes. Monoconidial culture of the fungus was multiplied on oatmeal broth under room temperature (28±2 °C). The non-pathogen elicitor was derived from *C. lindemuthianum,* which was isolated from anthracnose infected field bean pods and grown in Potato Dextrose broth under similar growing conditions.

Isolation, partial purification and characterization of cell wall elicitor

The fungal mycelial mats were harvested from 8-day-old liquid cultures. The mycelia were homogenized using 5 ml of water g^{-1} wet weight. The filtrate was collected using a coarse sintered glass funnel and the residue obtained on the filter saved. The residue was homogenized three more times in water, once in a mixture of chloroform and methanol (1:1) and finally in acetone. This preparation, when air-dried, represents the fraction referred to as the mycelial walls. Elicitors were extracted from the mycelial walls by suspending 1 g of walls in 100 ml of distilled water and autoclaving for 20 min. at 120 °C. The autoclaved suspension was filtered through a coarse sintered glass funnel. The filtrate was then clarified by centrifugation and concentrated to 10 ml under reduced pressure (Anderson-Prouty and Albersheim (1975). The crude elicitor solutions were assayed for hexose by the anthrone method (Dische, 1962) and the protein content was determined by the method of Bradford (1976). The crude elicitors were purified by gel filtration using Sephadex G-200. Individual 3 ml fractions were collected using a Bio-Rad automated econosystem. The fractions corresponding to carbohydrate and protein peaks were pooled (Ramesh Sundar, 1999). The elicitation activity of the pooled peak fractions was tested by assessing their ability to induce PAL, 4CL, TAL activities and phenolic synthesis in suspension-cultured cells of sugarcane.

Suspension-cultured sugarcane cells

Inner two whorls of young leaves of the sugarcane cultivar CoC671 was used as the tissue explant for the initiation of callus on MS basal medium (Murashige and Skoog, 1962), supplemented with 1.0 mg l^{-1} of 2,4-dichlorophenoxy acetic acid. Development of friable embryogenic calli was observed after 3 weeks and the same was maintained in subculture medium (MS basal medium supplemented with 2,4-D 2 mg l^{-1} of medium) (Liu et al., 1984). Rapidly growing embryogenic cell suspensions were established from one month old embryogenic calli as described by Ho and Vasil (1983). Sugarcane cell suspensions were transferred to a fresh medium and kept in shaking for 6 days prior to treatment with 1 ml of filter sterilised cell wall elicitor. Controls were treated with 1 ml of sterilised distilled water. Immediately following the addition of elicitor, cultures were returned to standard growth conditions. Samples were collected at different time intervals for analysis.

Determination of PAL activity

Sugarcane suspension-cultured cells (1 g) were homogenised in 5 ml of 0.1 mol/l sodium borate buffer (pH 7.0) containing 0.1 g insoluble polyvinylpolypyrrolidone (PVP). The extract was filtered through cheese cloth and the filtrate was centrifuged at 20,000 × *g* for 35 min. The supernatant served as an enzyme source. PAL activity was determined spectrophotometrically as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm as described by Dickerson et al. (1984). The reaction mixture consisted of 0.4 ml of enzyme extract, 0.5 ml of 0.1mol/l borate buffer (pH 8.8) and 0.5 ml of 12 mmol/l L-phenylalanine in the same buffer and incubated for 30 min at 30 °C. In reference cell,

0.4 ml of enzyme extract was taken along with 1.0 ml of borate buffer. The amount of *trans*-cinnamic acid synthesised was calculated using its extinction coefficient of 9630 M^{-1} cm^{-1}. Enzyme activity was expressed on a fresh weight basis n mol min^{-1} g^{-1}.

4-Coumarate: CoA ligase (4CL)

4CL was extracted by the method described by Dickerson et al. (1984). Samples of sugarcane suspension-cultured cells (1 g) were added to the cold suspension of PVP containing 0.1 M Tris HCl (pH 7.8), 20 mM mercaptaethanol and 25% glycerol (v/v). Immediately before the extraction, an additional 7 µl of mercaptaethanol was added to the PVP and buffer and the supernatant was used in the enzyme assay. The CoA ligase activities were determined using the spectrophotometric assay described by Knobloch and Hahlbrock (1975). The absorbance changes were measured at 333 nm using a Hitachi 200-20 spectrophotometer. The extinction coefficient for 4-coumarate CoA, 23 10^3 m^{-1} cm^{-1} (Gross and Zenk, 1966) was used to calculate the amount of produce synthesised per minute. Activity was expressed on fresh weight basis (n mol 4-coumarate CoA min^{-1} g^{-1} tissue).

Tyrosine-ammonia lyase (TAL)

Sugarcane suspension-cultured cells (1 g) were homogenised in 3 ml of 0.1 M sodium borate buffer (pH 7.0) containing 30 µg insoluble polyvinyl polypyrolidone (PVP). The homogenate was filtered through 4 layers of cheese cloth. The filtrate was centrifuged at 20,000 × *g* for 30 min. at 4 °C. The supernatant served as the source of enzyme extract. The reaction mixture consisted of 50 µl enzyme extract + 2.45 ml of 1 M sodium borate buffer (pH 8.8) + 0.5 ml of 2 mM tyrosine. The absorbance values were read spectrophotometrically at 290 nm. The TAL activity was expressed as change in absorbance (in units) min^{-1} g^{-1} fresh weight (Dickerson et al., 1984).

Estimation of phenolic content

Phenolic content of sugarcane suspension-cultured cells was estimated as per the procedure given by Zieslin and Ben-Zaken (1993). One g of tissue was homogenized in 10 ml of 80% methanol and agitated for 15 min. at 70 °C. One ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin-Ciocalteau reagent (1N), and the solution was kept at 25 °C. After three min. 1 ml of saturated solution of Na_2CO_3 and one ml of distilled water was added and the reaction mixture was incubated for 1 h at 25 °C. The absorption of the developed blue colour was measured using a Hitachi 200-20 spectrophotometer at 725 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reaction with a phenol solution (C_6H_6O) and expressed as catechol equivalents g^{-1} tissue weight.

Testing the browning reaction of suspension-cultured cells due to elicitor treatment

Browning of elicitor-treated cells was observed from 0 to 24 h after treatment. The intensity of browning was expressed in terms of increasing +++ symbol.

Changes in pH of suspension-cultured cells due to elicitor treatment

The changes in pH of the suspension in which cultured-cells were treated with elicitors were recorded from 0 to 24 h after treatment. pH values of the cell suspension was observed in an orion pen type pH meter.

Results

PAL activity was induced in sugarcane suspension cells due to treatment with elicitors isolated from *C. falcatum* and *C. lindemuthianum,* the pathogen and the non-pathogen, respectively. There was a significant induction of higher levels of PAL activity due to treatment with the pathogen elicitor when compared to that of the non-pathogen elicitor. A significant increase in PAL activity was observed from 24 h after elicitor treatment and it continued to increase throughout the experimental period in case of *C. falcatum* elicitor and not so in case of *C. lindemuthianum. C. falcatum* was able to induce more than 3 times of PAL activity when compared to the control. However, *C. lindemuthianum* could induce up to 34% of PAL activity as that of elicitor untreated control *(Fig. 1).*

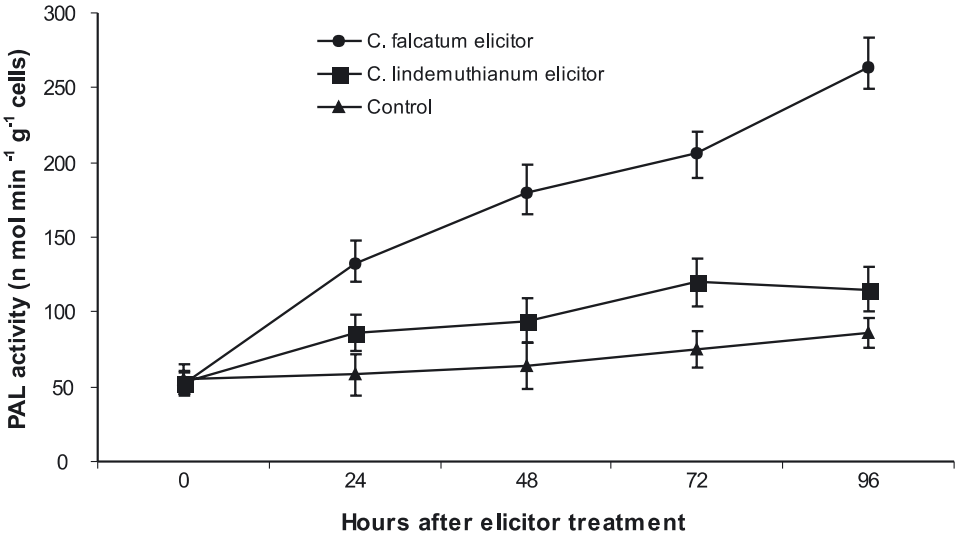

Fig. 1. Induction of PAL activity in sugarcane suspension-cultured cells treated with elicitors.
Data are means of three independent samples.
Vertical bars indicate standard deviation of three replications

Induction of 4-CL was observed in sugarcane suspension-cultured cells on treatment with elicitors isolated from *C. falcatum* and *C. lindemuthianum. C. falcatum* elicitor triggered a steep rise in the level of 4-CL activity by 24 h after treatment and was extended up to 96 h after elicitor treatment. It was observed that the enzyme 4-CL was found to be constitutively present in sugarcane suspension cells and on treatment with elicitors a differential response could be recorded between the pathogen and a non-pathogen elicitor *(Fig. 2).*

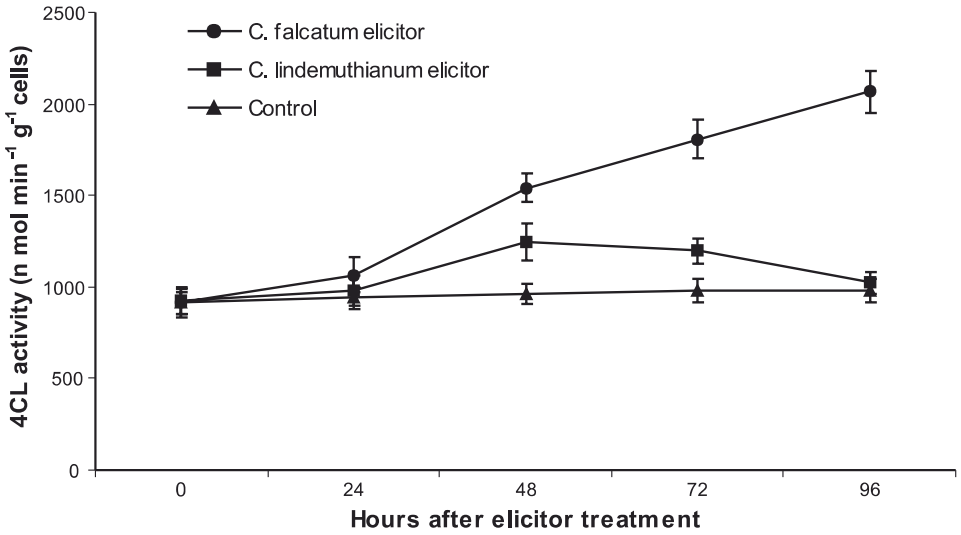

Fig. 2. Changes in 4-coumarate Co A ligase (4CL) in elicitor treated suspension-cultured cells of sugarcane.
Data are means of three independent samples.
Vertical bars indicate standard deviation of three replications

C. falcatum and *C. lindemuthianum* elicitors were found to significantly induce higher levels of TAL in suspension-cultured cells of sugarcane. The increasing trend was similar up to 72 h after treatment. *C. falcatum* elicitor triggered an increase in the level of TAL activity throughout the experimental period. However, a decrease in the induction level was noticed in cells treated with *C. lindemuthianum* from 72 h after treatment. The results suggest that sugarcane cells respond specifically to treatment with the elicitor isolated from the pathogen *C. falcatum* when compared to that of the non-pathogen *C. lindemuthianum (Fig. 3).*

Accumulation of phenolics was recorded due to treatment with elicitors *(Fig. 4).* A significant higher level of induction of phenolics was observed due to treatment with *C. falcatum* elicitor when compared to that of the elicitor isolated from *C. lindemuthianum,* though the trend seemed to be similar. Interestingly, a constant level of constitutive phenolics was found in elicitor untreated control cells without much of changes in their level of accumulation over a period of experimental time.

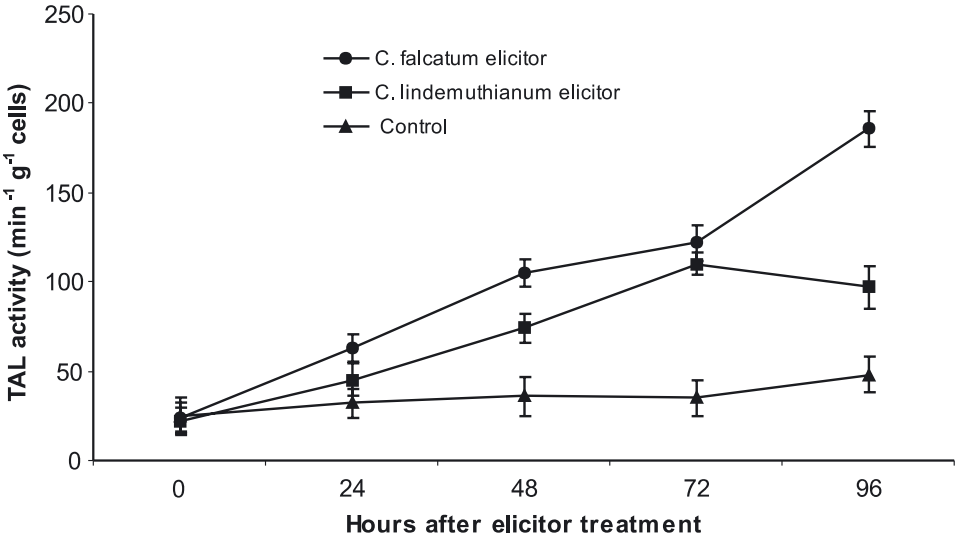

Fig. 3. Changes in tyrosine ammonia lyase (TAL) activity in elicitor treated suspension-cultured cells of sugarcane.
Data are means of three independent samples.
Vertical bars indicate standard deviation of three replications

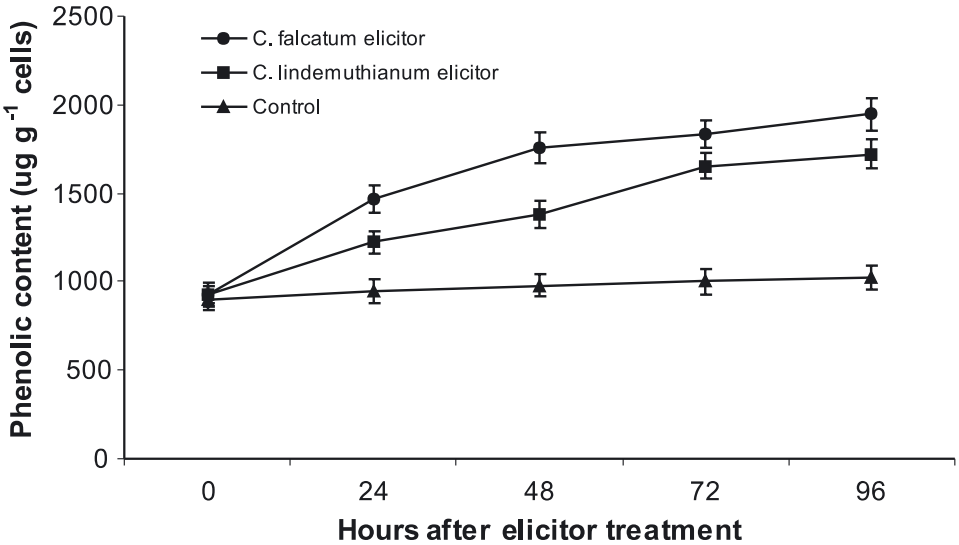

Fig. 4. Induction of phenolics in elicitor treated suspension-cultured cells of sugarcane.
Data are means of three independent samples.
Vertical bars indicate standard deviation of three replications

Both the elicitors induced tissue browning of the treated suspension cells starting from 4 h after treatment and the intensity continued to increase throughout the experiment period of 24 h. There was no significant difference in the intensity of necrosis between the two elicitors used in this study *(Table 1).* pH of the suspension cells was not much altered due to elicitor treatment *(Table 2).*

Table 1

Reaction of sugarcane suspension-cultured cells to treatment with elicitors isolated from *Colletotrichum falcatum* and *C. lindemuthianum*

Hours after elicitor treatment	Intensity of browning*			
	C. falcatum elicitor		*C. lindemuthianum* elicitor	
	Control	Treated	Control	Treated
0	–	–	–	–
4	–	+	–	+
12	–	++	–	++
24	–	+++	–	+++

+, ++ and +++ indicate increasing intensity of browning of cells
– No browning
* Data are means of three replications

Table 2

Changes in pH of suspension-cultured cells of sugarcane in response to treatment with elicitors isolated from *Colletotrichum falcatum* and *C. lindemuthianum*

Hours after elicitor treatment	pH of the cell suspension*			
	C. falcatum elicitor		*C. lindemuthianum* elicitor	
	Control	Treated	Control	Treated
0	5.8	5.8	5.8	5.8
4	5.8	5.4	5.8	5.2
12	5.2	5.7	5.0	5.6
24	5.1	5.8	4.8	5.6

* Data are means of three replications

Discussion

Plant physiological research has exploded in several areas focused on secondary metabolites. Plants have been shown to increase secondary metabolite concentrations following pathogen attacks as ways to defend the plant against predation, increasing the plant's fitness in the community. Secondary metabolites derived from phenylpropanoid metabolism play a decisive role for the signal exchange between pathogens and plants (Rickauer et al., 1997). Suspension-cultured cells and pathogen derived elicitors has been

proven as model systems in understanding the host-pathogen interaction at molecular level. The elicitor molecules mimic the pathogen response and thus are used to assess the responsive defense mechanism in plants (Fakuda, 1997).

The biochemical and molecular details of the relationship between elicitor activity and pathogen specificity on host plants are poorly understood. An attempt was made in this study to compare the induction of phenylpropanoid metabolites in suspension-cultured cells due to treatment with elicitors isolated from the red rot pathogen *C. falcatum* and the non-pathogen *C. lindemuthianum.* Both the elicitors induced phenylpropanoid metabolites in suspension-cultured cells of the sugarcane cultivar CoC 671. However, *C. falcatum* elicitor specifically induced higher levels of phenylpropanoid metabolites and no such differential induction was observed with *C. lindemuthianum* elicitor treatment.

Host specificity was identified in elicitors from *Phytophthora megasperma* f. sp. *glycinea* (Keen et al., 1983), *C. lindemuthianum* (Tepper et al., 1989) and *Rhyncosporium secalis* (Hahn et al., 1993). The detection of elicitors with different potential activities supports the model that there are general non-host resistance mechanisms as well as other systems that are more specific and functional in race-cultivar interactions (Tepper and Anderson, 1984). However, in spite of several attempts, host specific elicitors could not be identified in most of the pathogens.

The phenylpropanoid pathway is an extension of the shikimic acid pathway creating secondary metabolites in the presence of PAL. In the presence of greater concentrations of PAL, a well-studied activating enzyme, more phenylalanine is converted to secondary metabolites. Phenylpropanioids serve as low molecular weight antibiotics, phytoalexins, insect repellents and signal molecules in plant microbe interactions; they also function as complex, polymeric constituents of surface and support structures such as suberin, lignin and other cell wall components (Hahlbrock and Scheel, 1989).

PAL activity increased due to treatment with both the elicitors. However, suspension-cultured cells of sugarcane accumulated relatively higher levels of PAL due to *C. falcatum* elicitor treatment. PAL is the key enzyme of the phenyl propanoid pathway required for the synthesis of phenolics, phytoalexins and lignins, the three key factors responsible for disease resistance (Vidhyasekaran, 1988). PAL activity is believed to determine the biosynthetic flux through the phenylpropanoid pathway and so higher PAL activity produces concomitant increase in the levels of phenolics and peroxidases (Orr et al., 1993). PAL activity was found to be higher in sugarcane varieties resistant to red rot than the susceptible ones due to the pathogen inoculation (Singh et al., 1993). Mc Ghie et al. (1997) observed that in response to *Pachymetra chaunorhiza* elicitor treatment in suspension-cultured cells of susceptible variety of sugarcane, the Pal activity showed a large and sustained increase at 24 h after addition of elicitor. It supports the assumption that elicitor treatment closely mimics a true infection (Fritzemeier et al., 1987). Similar results of induced PAL activity has been reported in bean cells following treatment with *Colletotrichum lindemuthianum* elicitor treatment (Edwards et al., 1985), in alfalfa cell cultures treated with an elicitor from *C. lindemuthianum* (Dalkin et al., 1990), in poplar cells treated with elicitors from *Phytophthora megasperma* f. sp. *glycinea* and *Fusarium oxysporum* (Monze de Sa et al., 1992), in rice cells treated with *Rhizoctonia solani* elicitor

(Velazhahan and Vidhyasekaran, 2000) and in mungbean leaves and suspension cells treated with *Macrophomina phaseolina* elicitor (Ramanathan and Vidhyasekaran, 1997).

4CL activity was differentially induced by *C. falcatum* elicitor and a non-specific induction by the *C. lindemuthianum* elicitor was also recorded. 4CL is another key enzyme, which along with PAL coordinately gets induced in many incompatible host-pathogen interactions resulting in resistant reaction. 4CL activates the phenylpropanoid acids to their CoA thiol esters and is considered to be a significant regulatory enzyme of phenolic biosynthesis (Dickerson et al., 1984). Activation of 4CL by fungal elicitors has also been reported. Induction of 4CL activity was correlated with increased resistance to pathogen infection (Cramer et al., 1985). Similarly treatment of plant cell cultures with active fungal elicitors induced the accumulation of 4CL mRNAs (Kuhn et al., 1984). Transient induction of 4CL activity was reported by Davis and Ausubel (1989) in suspension-cultured cells of *Arabidopsis* with maximum levels at 8 to 10 h after elicitor treatment. 4CL activity increased significantly from 6 h after *R. solani* elicitor treatment on suspension-cultured cells of rice (Velazhahan and Vidhyasekaran, 2000).

Coordinate induction of PAL and 4CL mRNAs occur in response to pathogen infection (Esnault et al., 1987). Similarly treatment of plants or plant cell cultures with fungal elicitors induced the accumulation of PAL and 4CL mRNA (Kombrink and Hahlbrock, 1986). In the present study it was observed that suspension-cultured cells of sugarcane responded to both the fungal elicitors by inducing both PAL and 4CL. Similar observations on the coordinated induction of PAL and 4CL was reported by Velazhahan and Vidhyasekaran (2000) on rice due to *Rhizoctonia solani* elicitor treatment and in mungbean cells due to *Macrophomina phaseolina* elicitor treatment by Ramanathan and Vidhyasekaran (1997).

In the present study, induction of tyrosine ammonia lyase (TAL) was observed in elicitor treated suspension-cultured cells of sugarcane. TAL activity paves way for a secondary entry into the phenyl propanoid pathway effectively bypassing the 4 hydroxylation of cinnamic acid to p-coumaric acid. The flux of material via the TAL route is thought to be secondary and most metabolites enter the general phenyl propanoid pathway through PAL. Not surprisingly, there is very limited data available on TAL mediated phenyl propanoid pathway (Barber and Mitchell, 1997). In coordination with PAL, the activity of TAL was found to have specific activity in some of the disease resistant sugarcane varieties (Madan et al., 1995).

The definite role of phenolic intermediates in the active expression of resistance has been critically reviewed (Nicholson and Hammerschmidt, 1992). The accumulation of phenolic substance in response to pathogen infection has been reported by several authors (Arora, 1983; Vidhyasekaran, 1988). Induced synthesis of phenolic compounds is a common phenomenon of host-pathogen interactions and specific phenols have been implicated in host resistance (Southerton and Deverall, 1990). Elicitor induced synthesis of phenolics have also been reported by many workers. Early and enhanced synthesis of phenolics was reported to occur within 1 h after elicitor treatment in suspension-cultured cells of tomato (Vera-Estrella et al., 1992), within 6 h in *C. lindemuthianum* elicitor treated bean cells (Anderson et al., 1991) and within 8 h in elicitor treated tobacco cell cultures (Godiard et

al., 1991). Since the production of phenolic compounds depend upon PAL and 4CL activity (Colligne and Slusarenko, 1987) increased phenolic synthesis in sugarcane leaves and suspension-cultured cells might be due to increased activity of PAL and 4CL and that phenols could have long been associated with passive and active defense responses.

Early and rapid browning of suspension-cultured cells of the susceptible cultivar may be considered as an indication of the relatively high sensitivity of the susceptible cells to elicitor treatment. This result gives additional evidence that elicitors mimic and simulate pathogen infection and thus provide a valuable reason that study of elicitor induced responses may be relevant.

Our study yielded a number of defense responsive mechanisms that will not only serve as molecular probes but also contribute to our understanding of host defense mechanisms against the red rot fungus. Future research should address the time, location and concentration of phenyl propanoid metabolites as well as their relationship to other putative defense responses.

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