

# Molecular Basis of Red Rot Resistance in Sugarcane

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## ABSTRACT

Red rot of sugarcane caused by *Colletotrichum falcatum* Went is one of the devastating diseases of sugarcane causing significant loss to sugarcane production in India and other Asian countries. Complex polyploidy and lack of information on inheritance to red rot in sugarcane make breeding for red rot resistance more difficult. Hence researchers have studied the mechanism of red rot resistance in sugarcane in detail. Initial studies based on biochemical tools identified oxidative enzymes and red rot pigments in disease resistance. Further studies revealed the role of pathogenesis-related (PR) proteins and 3-deoxyanthocyanidin phytoalexins especially luteolinidin and apigeninidin in red rot resistance. Recent studies using semiquantitative RT-PCR after pathogen inoculation from sugarcane cultivars varying in red rot resistance, revealed differential accumulation of transcripts of the flavanoid biosynthetic pathway like coumarate-4-hydroxylase, chalcone synthase, chalcone reductase, flavanoid 3'-5' hydroxylase and flavanoid glycosyl transferase and this transcript analysis, further confirmed the role of sugarcane phytoalexins in red rot resistance. Similarly the role of PR- proteins like chitinase and  $\beta$ -1,3-glucanase was established at the transcript level. Detailed molecular studies using differential display (DD)-RT-PCR identified expression of more number of differentially expressed transcripts during the host-pathogen interaction. Full length sequences of many potential transcripts were identified and are being characterized. Also to identify specific proteins involved in host resistance, proteomic approach has been attempted by optimizing sample preparation from stalk tissues, 2-D electrophoresis (2-DE), down-stream processing of identified spots and bioinformatics. Several resistance associated proteins spots were identified and they are being analyzed critically. Overall, application of molecular techniques was found to be useful in identifying transcripts/proteins involved in host defence. Further studies are in progress to validate their specific involvement in red rot resistance.

**Keywords:** 2-D electrophoresis, *Colletotrichum falcatum*, differential display, PR-proteins, phytoalexins, red rot, resistance mechanism

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## INTRODUCTION

Sugarcane (*Saccharum* spp. hybrids) is a major cash crop cultivated in more than 23 million ha in tropical and subtropical regions of the world, producing up to 1.6 billion metric tons of crushable stalks in the year 2009 and accounts for nearly 60% of the total sugar produced in the world. Sugarcane meets ~60% of sweetener's requirement globally. India is the second largest producer of sugarcane in the world and the crop is cultivated in almost all the states occupying an area of 4.42 million ha with an annual production of ~285 million tonnes of canes in the year 2009 (<http://faostat.fao.org>). More than 488 sugar mills crush about 50-60% of the canes produced to manufacture crystal sugar in the country. About 30-40% of the produced canes are utilized to manufacture *gur* and *khandsari* sugar as an alternative sweetener in thousands of village industries in India. Also significant portion of the canes are crushed for extraction of juice for domestic consumption and about

10% canes are used as seed canes for planting (Sundara 1998). Sugarcane production in various regions of the country is affected by different biotic and abiotic stresses. Among the biotic constraints, red rot a fungal disease caused by *Colletotrichum falcatum* Went (Perfect state: *Glomerella tucumanensis* (Speg) Arx Muller) is the major constraint affecting cane production in most of the sugarcane growing areas. Similarly sugarcane production is significantly affected by red rot in different countries like Pakistan, Bangladesh, Thailand, Australia, Fiji, USA etc. The disease occurs in 77 countries representing all the sugarcane growing continents (Singh and Lal 2000; Viswanathan 2010).

The disease was first recorded in Java during 1893 (Went 1893) and in India during 1901 (Barber 1901). Series of disease epidemics in the sub-tropical India had devastated cane cultivation in entire Gangetic plains and Punjab till 1960s. Later the disease spread to tropical India and caused severe damage to the crop and the disease was responsible for the elimination of many commercial varieties

in India through the disease epidemics which occurred in different regions (Viswanathan and Samiyappan 1999; Viswanathan 2010). Severe epiphytotics on cv. 'CoC 671' the wonder cane of tropical region, during 1980s and 1990s have caused extensive damage to cane plantations in thousands of hectares in the Peninsular India. Likewise many important commercial varieties viz., 'BO 3', 'BO 10', 'BO 11', 'BO 17', 'BO 54', 'Co 312', 'Co 419', 'Co 453', 'Co 658', 'Co 785', 'Co 997', 'Co 1148', 'Co 1158', 'Co 6304', 'Co 7805', 'CoC 671', 'CoC 92061', 'CoJ 64', 'CoS 8436', 'CoSe 95422' etc were lost due to the disease in the last 100 years (Fig. 1). At present the disease is prevalent in most of the sugarcane growing states in the country at varying intensities (Viswanathan 2010).

## Symptoms

Planting of infected setts results in failure of germination or death of germinated seedlings. Such seedlings exhibit discolouration of foliage or drying of whorl (Fig. 2). Characteristic symptoms of red rot are noticed during cane formation stage. Infected canes either singly or in clumps show sudden yellowing of leaves and later the leaves gradually dry (Fig. 3). Such isolated clumps or clumps in patches give no external evidence of any diseased condition except varying intensities of rind discolouration (Fig. 4). Splitting of canes longitudinally reveal specific red rot symptoms inside the stalk tissues as reddening of internal tissues with white spots which are usually elongated at right angles to the long axis of the stalk (Fig. 5). Further, infected cane may show the red discolouration throughout the length of the stalk and longitudinal cavities containing either mycelium may develop and later the affected tissues turn muddy, shrink and dry out (Fig. 6). At later stages, the pathogen comes out as pinkish sporulation at rind tissues, growth rings and leaf scars and fruiting bodies of the fungus (acervuli) on the rind tissues. The pathogen does not cause any foliar symptoms except discrete brown or red lesions of varying sizes on the mid ribs (Fig. 7). These lesions may coalesce to form long lesions that extend the entire length of the leaf or they may remain as a series of unconnected lesions.

## Impact of the disease

The pathogen affects the economically valuable stalk tissues hence a limited infection can bring about drastic changes in the juice quality. The disease affected cane gives poor sugar recovery because of impaired sucrose metabolism. The red rot infection reduced total carbohydrates in the diseased canes and the reduction was more in the highly susceptible varieties (Agnihotri *et al.* 1989). Moreover, the pathogen produces abundant quantities of acid invertases which break the sucrose into glucose and fructose for the consumption by the pathogen. Higher production of acid invertases in the highly susceptible varieties was recorded upon pathogen infection as compared to resistant varieties (de Silva *et al.* 1977). Pathogen infection also results in increased levels of total soluble salts, acidity, reducing sugars and gum and simultaneously decrease in pH, sucrose and purity of cane juice in affected canes (Singh and Waraich 1977). Similarly, our studies have revealed that pathogen infection has drastically reduced Brix, sucrose percentage, purity and commercial cane sugar (CCS) per cent in the diseased canes. The affected canes recorded 25 to 75% reduced sucrose content as compared to the healthy canes (Viswanathan and Samiyappan 1999). During the milling process, mixing of juice from healthy and diseased canes results in spoilage of entire juice due to inversion of sucrose.

In subtropical India, severe infection of red rot can cause two third of cane stalks produced (Chona 1980). Severe red rot epidemics in Peninsular India during 1990s caused losses of 30-50% in cane yield in varieties like 'Co 6304', 'CoC 671', 'CoC 85061', 'CoC 86062', 'CoC 92061', 'CoSi 86071', etc. However, yield losses of upto 100% were found in different factory regions (Viswanathan



**Fig. 1** Severe red rot infected sugarcane: crop exhibiting extensive drying of foliage and death of canes. **Fig. 2** Young sugarcane plant exhibiting yellowing of leaves in the whorl due to red rot infection. **Fig. 3** Red rot infection in grown up sugarcane. Affected sugarcane clump shows characteristic foliage discoloration and drying. **Fig. 4** Red rot infected cane showing discoloured patches on cane rind tissue. **Fig. 5** Internal symptoms of red rot. Reddening of internal tissues with white spots inside the canes. **Fig. 6** Internal symptoms of red rot. Mycelial growth of the pathogen in the pith cavities. **Fig. 7** Red rot symptoms on sugarcane leaves. Coalesced midrib lesions covering entire mid rib of the infected leaf. **Fig. 8** Progress of red rot lesion inside the stalks 60 days after pathogen inoculation by plug method in sugarcane genotypes varying in disease resistance. Restricted lesion in resistant (R) genotype as compared to the extended lesions covering many internodes in the susceptible (S) genotype. Arrows indicate the site pathogen inoculation. **Fig. 9** Progress of red rot lesion on the nodes 10 days after pathogen inoculation by nodal swabbing method in sugarcane genotypes varying in disease resistance. Restricted or no lesion in resistant (R) genotype as compared to the extended lesions on nodes in the susceptible (S) genotype. **Fig. 10** Differential accumulation of red rot pigments in sugarcane varieties varying in red rot resistance. Methanolic extract from resistant tissue turns reddish due to release of anthocyanin pigments from the pathogen inoculated tissue (left) whereas, it remains colourless due to non-release of such pigment induction from susceptible tissue after pathogen inoculation (right). All photos unpublished.

*et al.* 1997; Alexander and Viswanathan 2002; Viswanathan *et al.* 2002). Ahmad *et al.* (1986) from Pakistan reported 28.5% losses in cane weight at initial infection by the pathogen and it reaches 82.7% when the disease intensity increased to 75%. They observed 30-87% loss in extraction of juice and 30-74% loss in recoverable sugar. At 40% infection level in Nigeria, more than 60% of the sucrose content of the juice had been converted to invert sugars like glucose and fructose (Olufolaji 1999). Outbreak of red rot-wilt complex disease caused by *Colletotrichum falcatum* and *Fusarium moniliforme* resulted in loss to a tune of 20 m dollars (US) to sugarcane in Thailand. The loss in cane yield varied between 34.6 and 73.7% in plant crop and 100% in ratoon crops, respectively. Among the two pathogens *C. falcatum* was shown to play a more significant role in disease severity than *F. moniliforme* (Pliansinchai 1999). Studies of Satyavir *et al.* (2002) in Haryana in India revealed that red rot infection reduces 7.1-32.5% in extraction, 7.4-38.7% in polarity, 0.5-8.3% in purity co-efficient, 7.8-39% in commercial cane sugar and increase of 19.2-40.95% in reducing sugars.

### Pathogen

Both anamorphic (*Colletotrichum falcatum* Went, Family: Melanconiaceae, Class: Coelomycetes) and teleomorphic (*Glomerella tucumanensis* (Speg.) Arx & Muller) stages of the fungus have been reported to cause the disease. However, anamorphic stage is the most commonly observed under Indian conditions. Sugarcane is the principal and most preferred host of *C. falcatum* and it does not have any other hosts in the field.

### Breeding for red rot resistance

In the early decades of the last century, breeding programmes in India were aimed at identifying varieties for sub-tropical India, to replace poor yielding Indian sugarcane *Saccharum barberi*. The breeding and selection process gave emphasis to adaptability, yield and quality improvement and resistance to red rot utilizing *S. officinarum*, *S. barberi* and the wild species *S. spontaneum*. These efforts resulted in the release of outstanding varieties from Sugarcane Breeding Institute (SBI) which were high yielding and tolerant to major disease, red rot. But recurrent outbreaks of red rot in epidemic forms resulted in the replacement of varieties regularly. The evolution of new races of the pathogen is the major factor for the breakdown of resistance in the well adapted varieties, hence the span of many varieties is reduced in the endemic region. Although genetics of inheritance of red rot resistance is not well established considerable progress has been made in the production of red rot resistant varieties in the past several years. Presently combining red rot resistance and economic traits is only a matter of chance, which could not be made consistent. There is also incomplete information on the parental material for resistance to major pathotypes of *C. falcatum*.

Extensive studies on sources of resistance have been carried out at SBI. These studies indicated that *S. spontaneum* has a large number of clones with red rot resistance. In *S. officinarum*, resistance sources were found to be low. Resistance in *S. barberi*, *S. sinense* and *S. robustum* to red rot is low although resistance in *S. barberi* is better than the other two species (Srinivasan and Alexander 1971; Alexander and Rao 1976; Alexander 1995). Sugarcane is highly heterozygous and complex polyploidy with meiotic irregularities. Thus information on inheritance to red rot resistance is practically lacking. Studies on the inheritance of red rot resistance have shown indiscriminate distribution of resistance or susceptibility among the progenies (Chona 1980). Even a susceptible  $\times$  susceptible cross has given some resistant seedlings and *vice versa*. Thus, it is essential to know about the combinability of the parents for developing red rot resistant lines. Biparental crossing involving a resistant parent is used for obtaining desired resistance in

the progeny. The commercial and near commercial clones are screened for red rot resistance, before their introduction in the field. Similarly, inter-specific hybrids and basic species in the sugarcane germplasm are screened for disease resistance regularly to prevailing pathogenic flora and are being utilized as parental clones in breeding programmes.

In the absence of clear-cut information on inheritance of red rot resistance, exact mechanism governing red rot resistance in sugarcane has not been understood, clearly. However, two kinds of resistance viz., the structural or mechanical that is static and the physiological or biochemical, which is dynamic, play a part in the defences of the plant against the pathogen. Only limited information is available on this area of work in sugarcane but for the work done at SBI, Coimbatore. This chapter summarizes these two mechanisms of red rot resistance with more emphasis on the recent findings on physiological resistance.

### Mechanical resistance

Mechanical or structural resistance refers to structures or modifications in the plant tissue, which mechanically restrict or prevent the entry or spread of the pathogen in the host tissues. The resistance offered by the nodes is apparently linked with the mechanical resistance to the red rot pathogen. It is attributed to several reinforcements of the cellular components such as relative thickness of the epidermis, cuticle and rind, relative abundance of vascular bundles specifically below the rind portion, nodal tissue with lignified thick walled sclerenchyma and the presence of cross walls in the xylem vessels. The mechanical resistance functions in two ways: the one against the extension of the primary lesion (nodal transgression) and the other against the occurrence of secondary lesions (serial spots). It has been found that younger nodes have more continuity as compared to older ones and probably this facilitates faster rate of red rot development in younger tissues.

Some of the workers have earlier suggested that the genotypes with more open vascular bundles through the nodes are susceptible as in *Fusarium* susceptibility (Varma and Mittal 1949). Jaglan *et al.* (1995) reported significant variability in continuity of vessels in internodes of different clones. Although, they could not correlate this with their relative red rot resistance, they found resistant varieties like 'Co 7314' and 'CoS 767' recorded the least continuity of vessels through nodes whereas they found moderate continuity in moderately susceptible cultivar 'Co 1158'. They found all the highly susceptible clones show significantly higher continuity than these three cultivars. However, this concept of morphological resistance is not acceptable since the pathogen does not sporulate in the xylem vessels of standing canes. In addition, spread of conidia multiplied in the white spots or cavities to xylem vessels is not possible since the cells have been damaged severely. Further, all the features which govern the morphological resistance in many of the disease resistant varieties have not been brought out. Most of the early varieties which were under cultivation, were once rated as resistant to red rot and subsequently they succumbed to the pathogen, most probably due to a new variant of *C. falcatum*. In such varieties mechanical resistance must be completely absent since, the variety did not undergo any change in its nodal characters and the new pathotypes do not exhibit any drastic changes in their morphology to adapt to the host architecture. Resistance to pathogen penetration at nodal region or other regions through morphological features can be considered as a trait in this resistance. Since the author witnessed many clones, which are highly susceptible in artificial inoculation, still resist the pathogen attack when inoculated through nodal region, whorl or root indicating that certain morphological features restrict pathogen entry, which is crucial for colonization on the nodal region and subsequent internodal passage. This supports the existence of mechanical resistance partially if not completely (Viswanathan 2010).

Most of the varieties identified for commercial cultiva-



tion possess some levels of resistance. The varieties which possess broad based resistance i.e. resistance against many pathotypes are expected to survive in the field for longer years in the field. In the history of sugarcane cultivation in India almost all the varieties succumbed to the red rot pathogen in the endemic region. Longevity of the varieties is the difference among them. Varieties like 'CoS 767' survived for nearly 20 years in the endemic region of Northern India. Although it succumbed to a new pathotype it still remains resistant to many other pathotypes.

An intriguing part of host-pathogen interaction is the differential interaction between the host and different pathotypes. Detailed studies on the pathogenicity of the pathogenic flora at different locations revealed existence of 11 major pathotypes in the country (Viswanathan 2010). The author has found a differential behaviour in many varieties like 'Co 1148', 'Co 7717', 'Co 8021', 'Co 7805', 'Co 86032', 'CoS 767' etc against the pathotypes. This information clearly suggests that selective adaptation of the pathogenic strains to host genotypes.

### Biochemical resistance

The mechanical defence mechanism determines the extent of longitudinal expansion of the lesion. On the other hand, physiological resistance mechanism governs lateral (and within the internode, longitudinal) extension of the lesion in the ground tissue. Apart from controlling the tissue destruction, the latter has repercussions on the physiological effects of the tissue. In addition to the sealing-off of fungal hyphae, inversion of sucrose caused by their enzymes is also restricted. In this context, Srinivasan and Bhat (1961) suggested while devising new 0-9 scale for rating red rot resistance that such resistance is doubly advantageous and emphasized greater attention on physiologic resistance than mere mechanical resistance as done previously on the basis of only average lesion length. They stressed that mechanical resistance as measured by the number of nodes crossed is highly variable while physiologic resistance as indicated by the nature of the lesion is surely more dependable. In view of the fact that physiological processes as well as structural equipment both of which are inherited, together determine the outcome of the host-parasite interaction. They felt that both types of resistance deserve to be taken into account, while laying greater emphasis on the dynamic aspect.

The pathogenic fungus is a hemibiotroph and the fungal hypha penetrates the host cells in the progressive phase of the disease by forming a minute penetration peg. These pegs can be seen as a constriction in the hypha, which expands to the normal hypha diameter immediately on reaching the other side of the cell wall. When the pathogen is spreading through the tissue, the hydrolytic enzymes polygalacturanase (PG), pectin methyl esterase (PME) and cellulases ( $C_x$  and  $C_{\alpha}$ ) enzymes are not detected. Additionally it was also found that extract of red rot lesion showing resistant reaction inhibited PG, PME and  $C_x$  enzymes produced by the pathogen, however, extracts from susceptible category had a lower degree of inhibitory effect (Srinivasan 1969). This suggests that the pathogen gains entry through mechanical pressure and enzyme produced by the pathogen also degraded by the host defence machinery during the parasitic phase. Sugars possibly inhibit production of PG, PME,  $C_1$  and  $C_x$  in the early stages of infection by *C. falcatum* in a susceptible variety and it is only when the supply of sugars is exchanged *in vivo* that the hydrolytic enzymes are elaborated. At a time there is proliferation of hyphal branches in the tissues, there is rapid death of host cells and parasitic hyphae become intercellular and this coincides with the appearance of hydrolytic enzymes. White spots and cavities appear in the ground tissues and this marks the end of the parasitic phase and the beginning of the saprophytic phase of the fungus. Hydrolytic enzymes do not appear to play a part in pathogenesis however they have a role in the saprophytic phase of the fungus which follows the parasitic phase.

Different biochemical features that impart resistance in a particular genotype have been reported. After the pathogen infection there is a reaction or a change in host cells in advance of the invading pathogen. The protoplasm in the affected area changes its colour and a gummy dark material oozes out of the cells and fills the intercellular spaces. Because of the presence of soluble pigment which is absorbed by the cell walls the infected area turns red. This results in reddening of fibrovascular tissues for several inches from the centre of infection. Pathogen advancement is checked temporarily in this zone of inhibition or stopped. In highly susceptible varieties infected by a compatible virulent strain, white spot account for major area inside the stalk tissues than a less virulent strain. White spots do not appear in resistant varieties or a variety infected by non compatible strain where the affected tissues have various intensities of red colour depending on the intensity of the resistant reaction (Srinivasan and Bhat 1961). Also hydrolytic enzyme produced by the pathogen was detected only in white spots, regions of a temporary harmony in host-pathogen interaction. Later studies have reported on the role of phenolic compounds in red rot resistance. This may be due to presence of antifungal compounds in the red pigment or by plugging of the pits in the cell walls by the gummy material. This process takes place in advance of infection and seals off further spread of the pathogen in adjoining tissues (Edgerton 1955; Srinivasan and Bhat 1961). Gum formation may also take place in susceptible varieties, but to a lesser extent and usually after the tissue has been invaded. Srinivasan and Bhat (1961) termed this as 'hyper-sensitive gummy reaction'. The nature of resistance depends on how rapidly the counteracting changes occur or how soon the red zone around the infected area develops. In resistant varieties the lesion may remain very small whereas in susceptible varieties they may extend entirely across the stalk (Fig. 8). On the nodal region pathogen makes infection on leaf scar, root primordial, bud and growth ring. Similar to the host behaviour inside the internodal tissues, resistant varieties record dark lesions confined to a few millimeters, where as in susceptible varieties it colonizes entire nodal region and the lesions spread vertically on both directions. Also the lesions are straw colour with limited reddish pigmentation (Fig. 9). This action of the cane tissues, especially in resistant varieties may be due to the action of the first invaded cells by taking up the red colour. When this happens the lesion remains very small and often the reaction is so offensive that the advancing mycelium is killed before the reaction it has a chance to spread materially. Mycelium advances slowly into the red zone.

Experience of Srinivasan (1969) revealed that isolations made from restricted dark lesions yielded the pathogen only rarely, while a large majority of transplant from the prominent white spots and the mottled ground tissue readily yielded the pathogenic culture. The presence and characters of white spots appear to be related generally to the width of the lesion and prominent white spots are indicative of poor resistance on the part of the host. Histological examination of previous workers showed that in the white spots the pathogen enters temporarily into a harmonious relationship with the host, characteristic of the more evolved type of parasite.

### 1. Phenolics and oxidative enzymes

Some workers (Rao *et al.* 1968; Wilson and Srivastava 1970) reported higher quantities of phenols in resistant and moderately resistant varieties. Similarly they also found that the resistant varieties maintained a higher level of polyphenol oxidase (PPO) activity before and after pathogen inoculation as compared to susceptible ones. However, further studies revealed that there was no correlation between total phenolic content and degree of resistance to red rot (Singh *et al.* 1976; Godshall and Lonergan 1987). These workers have found that in resistant varieties, the level of total phenols increased after infection and it maintained, while in

susceptible varieties, the level of phenolic content dropped after an initial increase. Srinivasan (1969) suggested that phenolics and their oxidation products are involved in this inhibitory process.

Number of host enzymes like peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and tyrosine ammonia-lyase (TAL) were reported to be associated with resistance in many sugarcane varieties. PPO and other oxidative enzymes have been related to resistance in several host pathogen interactions and are produced as an adaptive enzyme when phenolic compound appear in the tissue. The magnitude and rapidity of the production and release of phenolic compounds is known to be of paramount importance in disease resistance. Studies of Srinivasan (1969) clearly established that PPO activity was two fold in expressed healthy juice of completely resistant variety than the highly susceptible ones. Such juice samples inhibited conidial germination of the pathogen and this might possibly due to higher PPO in the extracted juice. In infected tissues, PPO activity was several times than in a susceptible tissue. PPO activity and resistance appear to be correlated and to be related to the degree of incompatibility between host tissue and pathogenic strain. The avirulent strain executed intense PPO activity in each of the host varieties inoculated with it but the others did so only in varieties that are resistant to them. PPO has been shown to be present in the reddened areas. Srivastava and Solomon (1990) found higher levels of PO and PPO in disease resistant mutants.

Higher activities of PAL and TAL involved in phenyl propanoid pathway are reported to be involved in red rot resistance (Madan *et al.* 1991). Studies of Singh *et al.* (1993) revealed that per cent increase in phenolic concentration was higher in cvs 'CoS 7918' and 'CoS 8315' which are resistant as compared to other varieties. They also found a significant increase in PPO and peroxidase (PO) activities in the early stage of (4-6 days) stalk infection. Similarly PAL activity was also higher in moderately resistant (MR) varieties as compared to susceptible varieties even before pathogen inoculation. After inoculation, PAL activity increased gradually up to 5 days and then declined. Here also the MR varieties showed higher enzyme activities. Detailed studies on PO levels in sugarcane varieties varying in red rot resistance revealed that resistant varieties like 'BO 91' and 'CoS 767' have multifold of this enzyme as compared to the susceptible varieties like 'CoC 671' and 'CoC 86062' (Viswanathan *et al.* 1996a). Comparison of PO isozyme profile after pathogen inoculation at different intervals revealed more prominence of PO isozymes on the fourth and the sixth days after pathogen inoculation. Eight days after pathogen inoculation, intensity of PO isozymes reduced. In susceptible and moderately susceptible cultivars the intensity of PO isozymes reduced 10 days after pathogen inoculation whereas, in resistant cultivars the intensity was maintained throughout the study period (Thirupathiraja *et al.* 2004). All these studies have been done with tissues from intact cane tissues. Cellular level enzyme activity in sugarcane varieties varying in disease reaction showed that calli from a resistant variety 'BO 91' recorded very high levels of PO, PAL and TAL than the cells of 'CoC 671', which is susceptible. The pathogen toxin treatment elicited significantly higher levels of PO in the callus tissues of 'BO 91' than in 'CoC 671'. Additionally induction of the defence enzymes was rapid in resistant cells. Among the enzymes, induction of PAL, TAL and PPO was not rapid as that of PO upon pathogen toxin treatment. Further studies on electrolyte leakage revealed that a more pronounced loss of electrolytes occur in the callus cells of 'CoC 671' than in 'BO 91' (Ramesh Sundar and Viswanathan 1997; Ramesh Sundar *et al.* 1998, 1999). The susceptible variety suffers more damage in their cells due to pathogen toxin activity and that is reflected in electrolyte release to the medium.

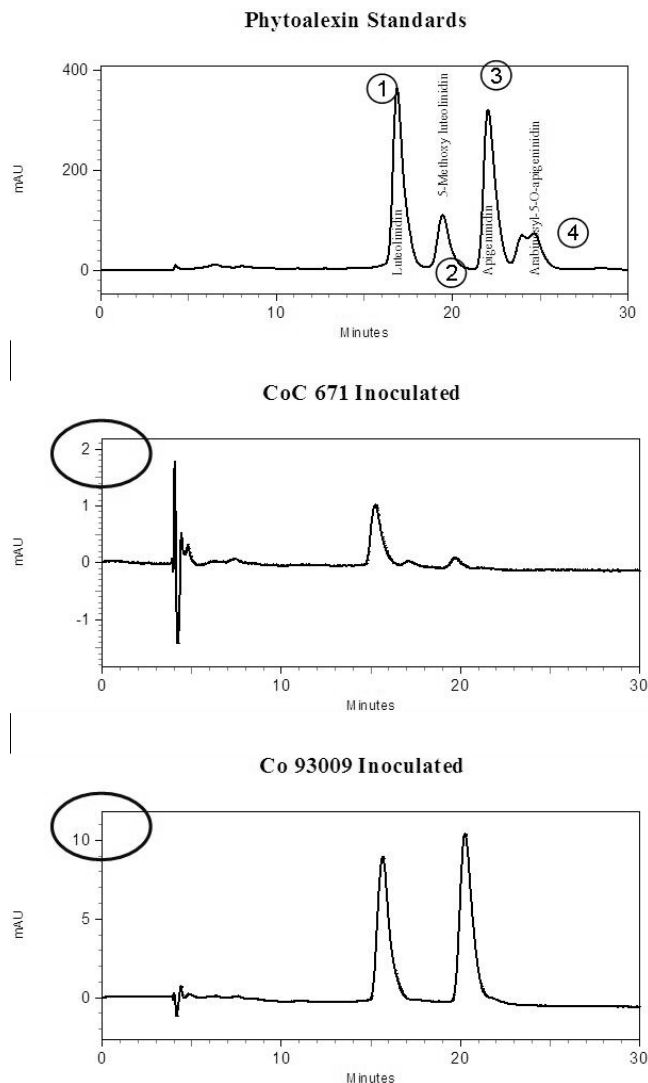
## 2. Phytoalexins

The red substance released in cells and intercellular spaces

near the invading hyphae in the infected cane stalks is referred as red rot pigment (RRP). The RRP's formed in response to *C. falcatum* infection in sugarcane inhibited conidial germination *in vitro* and slowed mycelial growth (Godshall and Loneragan 1987; Viswanathan *et al.* 1996b). Fractionation of the RRP's showed presence of several compounds and some of them are phenolics. Godshall and Loneragan (1987) detected RRP's between 24 and 48 h in the infected susceptible host and found conidial germination *in vitro* takes place much before the pigment accumulation. Hence, the pigment may not be effective in stopping the initial germination. The RRP's from resistant cane tissues showed seven compounds, while in susceptible cultivar 'CoC 671' has shown only four of them in thin layer chromatography (Viswanathan *et al.* 1996b). The loss of three pigment fractions in the susceptible cultivar after pathogen infection indicates that the invading pathogen in the stalks may destroy these resistance-contributing fractions.

In the chromatographic analysis, Godshall and Loneragan (1987) found no qualitative differences between the resistant and susceptible varieties, before or after infection. Our studies have shown the qualitative differences i.e. absence of three fractions in susceptible cv 'CoC 671'. Studies conducted at SBI and Godshall and Loneragan (1987) established the differential patterns of pigment accumulation between resistant and susceptible genotypes after pathogen infection. Godshall and Loneragan (1987) found that in resistant variety pigment localized at the site of inoculation at a concentration of 1200 µg/g tissue, with little spread of pigment into the surrounding tissue whereas susceptible show a diffuse pigmentation throughout the pith with a lower concentration of 600 µg/g tissue. Studies conducted at the SBI also very clearly indicated that pigment accumulation concentrated in higher quantities around the site of inoculation in resistant varieties whereas the susceptible tissues its concentration is low that too in diffused pattern (Fig. 10). In resistant variety, the pigmentation restricted within 5 cm from the point of inoculation whereas in the susceptible variety it extended beyond that in diffused concentration. While screening for red rot resistance, the author noticed confinement of red rot lesion within the inoculated internode in resistant types and the lesions are dark reddish. In susceptible it is quite opposite i.e. the lesions are progressive and pale reddish in colour, which indicate a low level of pigment synthesis. These studies are the firm evidences to show a resistant mechanism operating in sugarcane against *C. falcatum*. Godshall and Loneragan (1987) identified luteolinidin as the major component in RRP and it appeared 24 to 48 hours after infection in cane tissues. Other components were luteolin, chlorogenic acid and a glucoside of luteolinidin, which appeared later, and they could not identify other three fractions. Fractionation of the pigments by high performance liquid chromatography (HPLC) revealed the presence of 3-deoxyanthocyanidin compounds, which were identified as luteolinidin, apigeninidin and caffeic acid ester of 5-O-apigeninidin (Viswanathan *et al.* 1996c, 1996d). In resistant interaction, these compounds present at much higher concentration whereas in susceptible or compatible reaction they were completely absent or present in very low concentration. Among the three compounds, luteolinidin level was the highest and present in all the varieties tested. apigeninidin also present in all the varieties but in low concentration. The third fraction was found only in one among four varieties.

The resistant variety may resist pathogen mediated phytoalexin degradation, thereby arresting the pathogen growth in the canes after pathogen inoculation. This was confirmed by inoculation of partially purified pathogen toxin in sugarcane varieties varying in red rot resistance. Here induction of phytoalexins was recorded in both varieties. An important finding is that the susceptible variety accumulated reasonable amount of these phytoalexins when toxin was used for inoculation but in response to pathogen inoculation the same variety fail to synthesize phytoalexins probably due to their degradation by the pathogen. However,



**Fig. 11 HPLC profile showing differential accumulation of 3-deoxyanthocyanidin.** Unpublished figure.

here also resistant variety recorded multifold phytoalexins synthesis than in the susceptible variety (Viswanathan *et al.* 1996d). These findings clearly demonstrated that the phytoalexins were accumulated only in incompatible host pathogen interactions and compatible interactions had no such phytoalexins or with trace quantities. These three compounds were identified as phytoalexins in sorghum and *C. sublineolum* interaction a host pathogen interaction close to sugarcane and *C. falcatum* (Snyder *et al.* 1991).

Further studies with a set of host differentials proved that there is accumulation of anthocyanin compounds in incompatible interactions and no such induction/accumulation in compatible interactions (Viswanathan *et al.* 2000a; Viswanathan 2001). Earlier studies of Brinkler and Seigler (1991) reported a piceatannol compound as sugarcane phytoalexins but no further studies have been done to prove it. Recent studies of Malathi *et al.* (2008) conclusively proved that 3-deoxyanthocyanidin compounds act as sugarcane phytoalexins and probably govern resistance in sugarcane to red rot. The classical incompatible interaction of pathotypes from 'Co 1148' and 'Co 7717' on the host cultivars is very much demonstrated earlier at SBI (Padmanaban *et al.* 1996). This was further elaborated by quantifying phytoalexins in such interactions. The variety 'Co 1148' accumulates 3-deoxyanthocyanidins in incompatible interaction with Cf7717, which results in resistant reaction. However, the same variety fails to synthesize phytoalexins in case of matching pathotype Cf1148 infection. Similar behaviour of 'Co 7717' to Cf1148 and Cf7717 pathotype interactions was proved. Information on phytoalexin induction

in susceptible varieties by phytotoxin also amply demonstrates the role of phytoalexin in red rot resistance in sugarcane. Though previous studies have been unsuccessful in pinpointing biochemical basis of disease resistance, these studies at SBI have shown the possible role of phytoalexins in red rot resistance. Recent studies conducted at SBI with phytoalexin standards also gave further evidence on the differential accumulation of luteolinidin and apigeninidin compounds (Malathi *et al.* 2008). Very high level of phytoalexins was recorded in 'Co 93009' a resistant cultivar as compared to traces of phytoalexin fractions in 'CoC 671' (Fig. 11).

### 3. Pathogenesis related (pr) proteins

Accumulation of PR-proteins is an important phenomenon of plant defence responses upon infection by pathogens. Many of the PR-proteins have been purified and the purified proteins exhibited strong antifungal activity against many fungal pathogens under *in vitro* conditions. Induction of high levels of these proteins in host plants reduced disease development in many crop plants. Specific involvement of many proteins in disease resistance has resulted in isolation and cloning of these PR-proteins and subsequently, developing transgenic plants expressing PR1a, PR-2 ( $\beta$ -1,3-glucanase), PR3 (chitinases) and PR5 (thaumatin-like proteins) in various crop plants. Such transgenic plants expressing foreign genes showed enhanced resistance to fungal pathogens. Detailed studies on PR-proteins in red rot resistance showed that constitutive activities of chitinase and  $\beta$ -1,3-glucanase were higher in disease resistant varieties as compared to susceptible varieties. Upon pathogen inoculation, the resistant variety accumulated higher hydrolytic enzymes as compared to susceptible cultivars (Viswanathan and Samiyappan 1999). This information suggests a possible role of these enzymes in red rot resistance.

Further studies were conducted to identify the PR-proteins using Western blot technique in a set of resistant and susceptible varieties differing in resistance the disease (Viswanathan *et al.* 2005). The red rot resistant cv 'Co 93009' showed differential induction of four chitinase proteins with molecular mass ranging from 34-39 kDa after pathogen inoculation in leaf tissues. The intensity of these proteins increased with time from 6 to 42 h after inoculation. In susceptible variety 'CoC 671' induction of a 35-kDa chitinase protein was recorded. In stalk tissues induction of a 35-kDa chitinase protein was recorded 24 hr after inoculation in resistant variety whereas, in susceptible plants such induction was delayed. Similarly, early induction of 43.0- and 37.5-kDa TLPs by 24 h was observed in response to pathogen inoculation in the resistant variety whereas in the susceptible variety such induction was less intense and could be seen 8<sup>th</sup> and 9<sup>th</sup> days post inoculation. These studies give a clear indication that the PR-proteins may be an important defense mechanism operating in sugarcane against red rot as in other host-pathogen interaction.

### 4. Mechanism of induced systemic resistance

Earlier studies have clearly established the induction of systemic resistance in sugarcane against red rot by fluorescent pseudomonads (Viswanathan and Samiyappan 2002a, 2008). These studies gave evidences of enhanced resistance in disease susceptible cultivars like 'CoC 671', 'CoC 90063', 'CoC 92061', etc. under controlled and field conditions. Further studies were conducted in detail on the mechanism of induced resistance by *Pseudomonas* strains against the disease. Involvement of different PR-proteins such as  $\beta$ -1,3-glucanases, chitinases and thaumatin-like proteins (TLPs) was found to be associated with *Pseudomonas*-mediated induced resistance (Viswanathan and Samiyappan 2001a; Viswanathan *et al.* 2003b). Studies of Viswanathan *et al.* (2003b) have also shown strong anti-fungal activities of sugarcane chitinases purified from systemically protected stalk tissues against *C. falcatum*. Their results clearly de-

monstrated that bacterium treated disease susceptible sugarcane is able to restrict disease development to a level equivalent to moderately resistant varieties and many PR-proteins are involved in that ISR. In addition, enzymes of phenyl-propanoid pathway and oxidative pathway were also found to be involved in ISR (Viswanathan and Samiyappan 2002b). Characterization of *Pseudomonas* strains revealed that production of different metabolites/antibiotics such as salicylic acid, auxins, siderophores, pyocyanine, pyoluteorin and 2,4-diacetyl phloroglucinol and hydrolytic enzyme chitinase contribute to suppression of *C. falcatum*, induced resistance and growth promotion in sugarcane (Viswanathan and Samiyappan 2001b, 2004, 2006).

## Molecular basis of red rot resistance

### 1. Red rot resistance at the molecular level in sugarcane

Plants under are constant threat of infection by pathogens armed with a diverging array of effector molecules to colonize their host. Plants have in turn evolved a sophisticated detection and response systems that decipher pathogen signals and induce appropriate defences (Feys and Parker 2000). Recent genetic analysis involving plant mutants defective in resistance response to the invading pathogens has revealed a number of distinct, but interconnecting, signaling networks that are under both positive and negative control. These pathways operate atleast partly through the action of small signaling molecules such as salicylate, jasmonate and ethylene. The interplay of signals probably allows the plant to fine-tune defense responses in both local and systemic tissue. At SBI we have taken up both genomic and proteomic studies work to understand the host pathogen interaction of sugarcane-*C. falcatum* at molecular level. In genomics author's group has employed reverse transcriptase - polymerase chain reaction (RT-PCR), differential display RT-PCR, reverse northern and rapid amplification of cDNA ends (RACE) and subtraction suppression hybridization (SSH) to identify and characterize the resistance/defense genes involved in red rot resistance.

### 2. Pathogen recognition by the host plant

Plants are able to recognize fungal pathogens by their secreted products, referred as 'elicitors'. These elicitor molecules are signal molecules and elicit synthesis of phytoalexins, phenolics, lignin, PR-proteins, hydroxyl-proline rich glycoproteins and other defense molecules in host plant and the host recognizes the pathogen from several non-pathogens. Defence 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 resistant genes. Detailed studies were carried out to purify and characterize elicitors from *C. falcatum* and to study their role in recognition of *C. falcatum* by sugarcane.

A high molecular weight elicitor was isolated from the mycelial walls of *C. falcatum* and partially purified by gel filtration. The elicitor is characterized as a glycoprotein and the activity of elicitor resides in the carbohydrate moiety. The partially purified elicitor induced the accumulation of phenolics and the activities of phenylalanine ammonia-lyase (PAL) and peroxidase (POX) in sugarcane leaves and suspension-cultured cells. Sugarcane cells in culture responded to *C. falcatum* elicitor in a manner similar to sugarcane leaves (Ramesh Sundar *et al.* 2002a). Similarly, induction of hydrogen peroxide ( $H_2O_2$ ), reactive oxygen species ( $O_2^-$ ), lipoxygenase, lipid peroxidation, superoxide dismutase (SOD) and catalase was also observed in cell suspension cultures of sugarcane. A rapid outburst and a spurt in the generation of active oxygen species especially  $H_2O_2$  was observed indicating an early molecular event in recognition of the pathogen by the host cells. However, higher levels of the suppressor enzymes viz., catalase and SOD were found

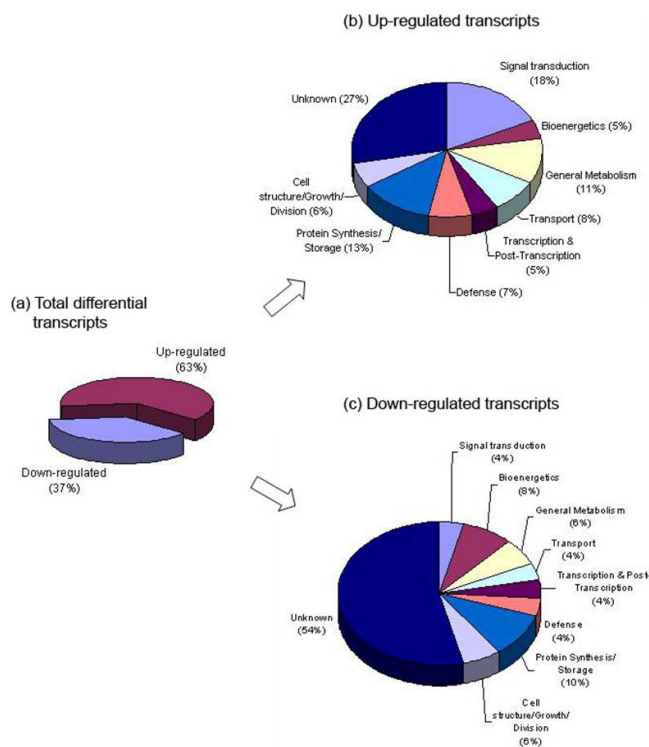
to be maintained throughout in the cells of sugarcane suspension cultures without any elicitor treatment (Ramesh Sundar *et al.* 2002b). When elicitor isolated from *C. falcatum* was compared with *C. lindemuthianum* a non-pathogen elicitor differential induction of POX isoforms in suspension-cultured cells of sugarcane cv. 'CoC 671' was found (Ramesh Sundar and Vidhyasekharan 2003a).

For studying the defense gene activation in sugarcane, the intact plant-pathogen system may not be ideal because the time course of pathogen infection cannot be monitored precisely. Cell cultures instead of whole plants and elicitors instead of live pathogens are found to be suitable models to study the defense gene activation in bean, rice and in sugarcane because of their high degree of reproducibility, rapid experimental cycles. Further, each cell in the culture is uniformly exposed to the elicitor preparation and hence the response of cells is relatively uniform. Preliminary results of the study conducted at SBI has provided insight into the mechanisms regulating the pathogen recognition at the interface which facilitates further elaboration of inducible defense response against *C. falcatum* in sugarcane (Ramesh Sundar and Vidhyasekharan 2003a, 2003b). These studies confirmed that the elicitor molecules from *C. falcatum* are responsible for specific recognition of the pathogen by the host resistance is determined by the rapidity of the downward signaling of defense pathway. Probably variation in initiation of signaling process between the resistant and susceptible genotype determines the pathogen colonization and disease development.

### 3. Gene expression studies

To understand genome complexity of sugarcane, a large scale expressed sequence tag (EST) programme known as 'SUCEST' was taken up recently in Brazil. More than 2,60,000 cDNA (complimentary DNA) clones were partially sequenced from 26 standard cDNA Libraries generated from different sugarcane tissues. They annotated 43,141 assembled sequences and found 50% of the putative identified sugarcane genes coding for protein metabolism, cellular communication/signal transduction, bioenergetics and stress responses. Vettore *et al.* (2003) found 80 SUCEST sugarcane assembled sequences (SASs) encoded proteins with clear similarity to the NB-ARC domain, which is characteristic of one of the major classes of disease resistance genes (*R* genes). The database contained more than 200 SAs encoding WRKY transcription factor domains, which have been implicated in the defense gene regulation in plants. They also found other genes related to defense responses like chitinases,  $\beta$ -1,3-glucanases, chalcone synthases, chalcone isomerases, isoflavone reductases, hydroxylproline-rich glycoproteins, proline-rich proteins, catalases, superoxide dismutases etc. have been putative orthologs in sugarcane, which indicates a high conservation of defense strategies among plants. The wealth of information generated in the SUCEST database promises exciting prospects for the scientists involved in sugarcane improvement and other crops. Many transcripts including the disease resistance and defense responses ESTs will be a basic resource for the understanding of the biology of this complex polyploid plant. This information may facilitate in identifying gene(s) involved in disease resistance in sugarcane.

The author's group has carried out detailed studies on identifying specific transcripts induced during host pathogen interaction to identify candidate genes involved in red rot resistance. After pathogen inoculation total RNA were isolated from sugarcane stalk tissues and were reverse transcribed to cDNA for expression analysis. Expression of the transcripts, the level of expression and the interval at which a particular gene expression etc, were analyzed using the custom made primers. The following transcripts R30, chitinase, metallothionein, receptor protein kinase (RPK) and reversibly glycosylated protein (RGP) were differentially expressed in resistant and susceptible varieties and their sequences shared sequence similarity with disease resis-



**Fig. 12** Relative abundance of differential display transcripts identified from *C. falcatum* inoculated sugarcane (cv 'Co 93009'), categorized based on their putative functions. Unpublished figure.

tance genes in other crops. The results also showed an early induction of defense/resistant gene(s) in the resistant variety and in case of susceptible variety, the induction was delayed significantly (Viswanathan *et al.* 2009a). In further studies, they employed differential display (DD)-RT-PCR a powerful technique to identify more transcripts involved in pathogen recognition, signal transduction and plant defense in sugarcane. Differential display technique revealed ~450 transcripts to be differentially expressed upon pathogen inoculation in sugarcane. About 202 transcripts were selected for their down-regulation and 243 transcripts were selected for their up-regulation upon pathogen inoculation. After homology searches the expressed sequence tags (ESTs) with a match in the databases (both characterized and uncharacterized) were categorized into eight groups based primarily on putative function. The total up-regulated and down-regulated transcripts were 63% and 37% respectively, indicating a higher percentage of transcripts that seems to be induced in response to pathogen inoculation. ESTs with a match in the databases were categorized into eight groups based primarily on putative function. Among the known proteins, the signal transduction group included the highest percentage of up-regulated sequences, followed by protein synthesis and storage, general metabolism, transport, defense, cell structure/growth/division, transcription/post transcription and bioenergetics. The important transcripts identified include 14-3-3-like protein, Senescence-associated protein DH, xylanase inhibitor protein 1 precursor, Putative chitinase, Leucine-Rich Repeat family protein, F-box domain containing protein, UMP/CMP kinase-a and Putative hydroxyproline-rich glycoprotein (Viswanathan *et al.* 2008a) (Fig. 12). Similarly, DD-RT-PCR was conducted with sugarcane cell lines treated with an elicitor molecule identified from red rot pathogen and similar transcripts were found to be differentially regulated upon elicitor treatment (Rahul 2010). Further northern and reverse northern blot analyses using radioactive  $\alpha$  [ $^{32}$ P]dCTP confirmed differential expression of potential DD transcripts (Viswanathan 2008; Viswanathan *et al.* 2008b, 2009b). This approach would be more useful to identify the potential transcripts during molecular interaction between the host and the pathogen. Recently Gupta *et al.* (2009) identified 85 clusters

of expressed gene tags (ESTs) that preferentially express upon *C. falcatum* infection, which were previously unreported. By real time RT-PCR profiling of selected EST clusters they identified several sugarcane clusters that show differential expression in response to biotic and abiotic stress conditions. In addition to six resistant gene analogues, (RGAs) You-Xiong *et al.* (2007) have isolated a full-length *R* gene (TIR-NBS-LRR) homologue gene termed *SNLR* from NCo376, a smut resistant variety in mainland China. They have characterized its expression profile in response to treatments with *Sporisorium scitamineum* (Syd.) M. Piepenbr., M. Stoll & Oberw 2002 causing smut, salicylic acid and  $H_2O_2$  by real-time RT-PCR. Further studies in this area of work will be more rewarding and would lead to identifying several candidate genes involved in disease resistance in sugarcane. Using cDNA-AFLP, recently they established differential gene expression in sugarcane during sugarcane-smut pathogen interaction. They found 40 transcript-derived fragments (TDFs), 34 newly induced plus six with obvious upregulated expression after infection (Que *et al.* 2011).

#### 4. Phytoalexin pathway transcripts

Since we have established the role of 3-deoxyanthocyanidin phytoalexins in red rot resistance, the genes involved in the flavanoid biosynthetic pathway like coumarate-4-hydroxylase (C4H), chalcone synthase (CHS), chalcone reductase (CHR), flavanoid 3'-5' hydroxylase (F3'5'H) and flavanoid glycosyl transferase (FGT) were further studied to establish relation between transcript accumulation and differential accumulation of phytoalexins in the resistant and susceptible cultivars (Rahul 2010). The pathogen inoculation enhanced their expression of C4H especially in resistant variety which may have positive correlation with their ability to synthesize various downstream compounds like phenolics and flavonoid compounds as reported earlier from various biochemical studies. Further along the phenylpropanoid pathway after the conversion of 4-coumaryl CoA to flavone, the flavone and isoflavonoid pathway is initiated. The CHS and CHR are key enzymes of phenyl-propanoid pathway diverting the substrate, naringenin chalcone to the flavanoid and isoflavonoid branches of the phenyl-propanoid pathway that synthesizes the precursor of a large number of secondary metabolites, including proanthocyanidins, anthocyanins, flavones, flavonols and isoflavonoid-phytoalexins among others (Zabala *et al.* 2006). Maximum induction of CHS was found in resistant variety, whereas in susceptible variety this induction was minimal. The F3'5'H transcript was found to be induced upon pathogen inoculation in the leaves of both the varieties and the flavanoid 3'5'-hydroxylase enzyme functions at an important branch point between flavonol and anthocyanin synthesis, as is evident from studies in petunia (*Petunia hybrida*), and potato (*Solanum tuberosum*). Induction of F3'5'H leads to the synthesis of flavanoids, flavanols, flavanones and phenolics. Reports of plant molecular responses to elicitor or pathogenic infections have pinpointed increase in activity of several genes of the phenyl-propanoid pathway leading to the synthesis of phenyl-propanoid metabolites, lignin and flavanoids. In a classical work done by Clive *et al.* (1999) the accumulation of phytoalexins in sorghum leaves and mesocotyl tissue after infection with *Colletotrichum sublineolum* (causing anthracnose) was established. After penetration of the fungus into the host cell it was restricted within infected regions by 72 h and an intense reddish pigmentation was observed by 36 h in the resistant cultivar whereas the susceptible variety the phytoalexin appears only by 48 h with proliferation of the pathogen. The HPLC profile of the phytoalexin from the resistant variety showed presence of luteolinidin, 5-methoxyluteolinidin, apigeninidin and the caffeic acid ester (CAE) of arabinosyl 5-O-apigeninidin, while susceptible variety showed presence of only two fractions apigeninidin and CAE of arabinosyl 5-O-apigeninidin. Their temporal studies revealed the accumulation of



CHS and PR-10 to occur by the 24 h in the resistant variety, while the same occurred only by the 36 h in the susceptible cultivar. Also the level of PR-10 transcript accumulation was lower in the susceptible cultivar than that in the resistant cultivar. Timely expression of the specific transcript at the right place in sufficient quantity is a pre-requisite in the development of resistance in a host system. Our studies have clearly shown the differential pattern of spatial accumulation of transcripts of phenylpropanoid pathway in resistant and susceptible varieties after inoculation of *C. falcatum* in sugarcane.

In a spatial study, transcripts derived from DD-RT PCR and genomic scanning studies were validated for their differential induction in sugarcane by semi-quantitative RT-PCR. Gene specific forward and reverse primers were designed for the selected defense related transcripts viz., xylanase inhibitor, glucanase, basal antifungal peptide, POX and isoflavone reductase. After pathogen inoculation in the stalk tissue, spatial expression was carried out in different parts viz., inoculated and adjacent internodes, root, apical meristem and third leaf from the top in resistant and susceptible varieties and it established differential regulation of 9 of the 11 transcripts.

### 5. Rapid amplification of cDNA ends (RACE)

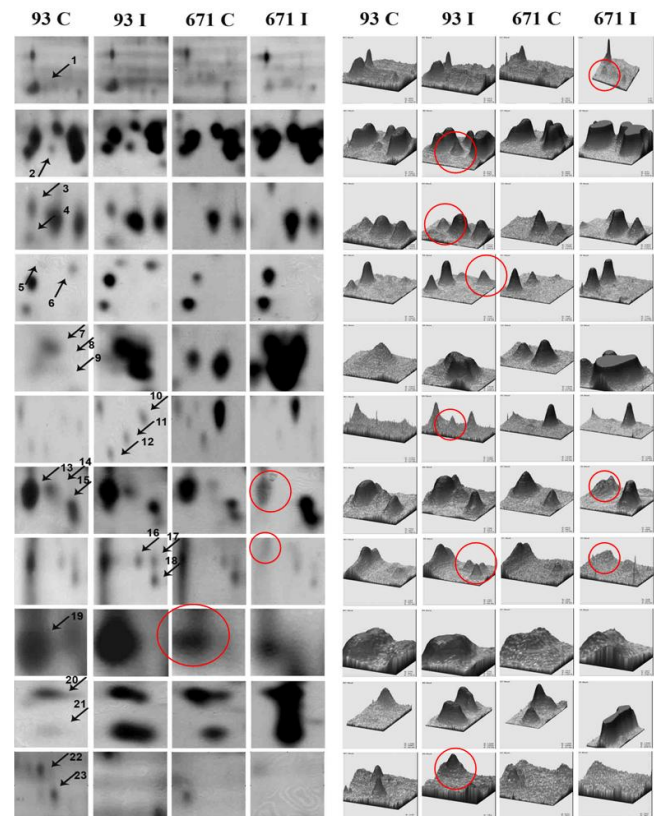
The potential defense related transcripts were selected for RACE analysis to clone the full length cDNAs. Compatible primers were designed at the 3' side using the available partial sequence information and used in the RACE protocol. Using RNA ligase-mediated RACE we have isolated full length sequences of the following four genes viz. 14-3-3 like protein, chitinase, xylanase inhibitor and basal antifungal peptide (Viswanathan *et al.* 2009b). After full length isolation of 14-3-3 like protein by RACE-PCR it has been characterized using bioinformatics tools. The CDD search in the NCBI database revealed the presence of conserved 14-3-3 super family domain, 5'UTR (1-86bp), ORF (87-857 bp) and 3'UTR (858-1094 bp) in the full length sequence of 1094 bp. The 14-3-3 homologues are known to mediate signal transduction by binding to phosphoserine-containing proteins. They are involved in growth factor signalling and also interact with MEK kinases.

### 6. Characterization of sugarcane chitinase

Differential expression of sugarcane chitinase in stalk tissues during pathogenesis of *C. falcatum* was established by RT-PCR studies in a set of resistant and susceptible cultivars and the result was confirmed by reverse northern analysis. In further studies full length of the gene was isolated and bioinformatic analysis was done to identify its functional domains and to predict the three dimensional structure of full length chitinase sequence. Translated sequences revealed the typical characteristics of family 19 glycosyl hydrolase, class I/IV chitinase starting with a signal peptide and ending with a signature domain. Phylogenic study grouped sugarcane chitinase in class IV, based on major deletions in catalytic domain. The close structural template 2DKV (Rice class-I chitinase) was successfully used for the prediction of sugarcane chitinase 3D model (Rahul 2010). The chitinase gene was cloned in an expression vector and studies are in progress to purify the expressed protein and to assess its biological activities.

### 7. Proteomics approach

The term proteome refers to the complete set of proteins that are specified by the genome, and analogous to genomics, proteomics describes the study and characterization of this complete set of proteins present in a cell, organ or organism at a given time. Genome-level studies (genomics) reveal or suggest what could theoretically happen, whereas the proteome-level investigations (proteomics) provides insights into the actual players involved in mediating specific



**Fig. 13 Identification of proteins (rings) involved in red rot resistance in sugarcane through 2-dimensional gel electrophoresis.** Left: Differentially induced proteins in the gel; Right: Graphical view of the expressed proteins. Unpublished figure.

cellular processes. In addition, the study of proteins introduces another level of complexity at the level of the post-translational modification (PTM) and the biological relevance of such modifications. These changes in PTM during the growth and development of organisms (including plants) or in response to stress (including disease) cannot be deduced from studies investigating genome sequences and/or transcript abundance. Such changes can only be deciphered through proteomics and it is a powerful tool in understanding which proteins are present in particular tissue under given conditions and it is one of the fastest growing areas of biological research. In addition to the enzymes, transport, and regulatory proteins many proteins contribute in cascades of reactions leading to the metabolites involved in disease resistance. This makes the proteome an essential target for studying metabolic pathways.

Information on the clearly identified proteins during host-pathogen interactions and defense signaling in sugarcane is lacking to understand disease resistance mechanism(s). Hence we have standardized a protocol for extraction of proteins for 2-DE from the rigid, fibrous sugarcane stalk tissues to identify specific proteins involved in resistance to red rot in sugarcane tissues for the proteome analysis. Among the five methods tested, 2D cleanup-phenol method was found to be the most suitable for producing high number of good quality spots and reproducibility. Thirty protein spots commonly present in three methods were selected and subjected to *LD*-IT-TOF-MS/MS analysis and a reference map has been established for sugarcane stalk tissue proteome. This is the first study on sugarcane stalk proteome analysis which possibly will show a new light on unexplored areas of sugarcane proteome research (Ramesh Sundar *et al.* 2010). In host pathogen interaction studies the number of protein spots was found to be higher ( $335 \pm 7$ ) in the resistant cultivar after 12 h of pathogen challenge whereas the inoculated susceptible cultivar had the lowest number of protein spots ( $280 \pm 3$ ). More than 250 protein spots that were detected in stalk tissues by pro-

teomic analysis showed reproducible abundance within replications. Approximately 50 protein spots were additionally induced in the resistant cultivar upon pathogen inoculation whereas ~ 24 proteins have disappeared in susceptible cultivar (Fig. 13). These studies have established that proteome analysis has the potential to provide significant insights into the molecular events that occur during sugarcane-*C. falcatum* interactions and this is the first attempt to standardize proteome analysis and to identify proteins involved in red rot resistance in sugarcane (Viswanathan *et al.* 2008a). Further studies using peptide mass finger printing would characterize the up/down regulated proteins and their role(s) in red rot resistance will be established.

## CONCLUSION

Overall, the studies conducted at SBI revealed definite role of 3-deoxyanthocyanidin phytoalexins and certain PR-proteins in red rot resistance. Although they are not the primary determinants of disease resistance they have been well established as potential antifungal weapons used by the plants to arrest the invading pathogen. Ongoing studies in our lab on identifying molecular basis of red rot resistance would reveal clear understanding of the molecular communication between the host and the pathogen that ultimately decides disease resistance. The newly identified transcripts will be good candidates for in-depth analysis to elucidate *C. falcatum*-responsive pathways in sugarcane and facilitate genetic manipulation to tailor this crop for tolerance to red rot and other diseases. Also disease resistance genes identified in our studies that showed differential regulation can provide preferred targets for breeding or to engineer durable disease resistance in sugarcane. Our studies on creating subtractive libraries to identify more resistance associated genes are in progress and detailed analyses of gene functions of the newly identified genes would help better understanding of the host resistance mechanisms underlying the response of sugarcane plant to different biotic and abiotic stresses.

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