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Induced Resistance – A Potential Supplementary Strategy for the Management of Red Rot in Sugarcane

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ABSTRACT

Induced resistance (IR) is the phenomenon by which a plant exhibits an increased level of resistance without changes of its basic genetic constitution. IR utilizes the plant's own defense mechanisms to restrict pathogen development. Resistance can be induced locally on the pre-treated parts of the plant as well as systemically on untreated parts and is then called systemic acquired resistance (SAR). Several chemicals such as salicyclic acid, acibenzolar-S-methyl, iso-nicotinic acid, jasmonic acid, etc. are known to induce resistance in certain plants, but only a few of them fulfil the criteria for being real plant activating compounds. The introduction of novel synthetic signal molecules is thought to be the first step for the integration of the principle of IR in a modern environmentally friendly plant protection concept. Plant diseases account for a considerable proportion of crop loss worldwide. In sugarcane, red rot disease caused by the fungus *Colletotrichum falcatum* is one of the important pathogens causing significant loss to the growers and the sugar industry. Many of the commercially superior varieties are out of cultivation because of either high levels of red rot susceptibility or breakdown of resistance, which is attributed to the emergence of new virulent pathotypes. In addition to breeding for durable red rot resistance, other viable options need to be explored for successful management of the disease. In this background, exploitation of the inherent SAR potential of highly successful commercial varieties, otherwise susceptible to red rot is a viable option to manage the disease. With the advent of tools of biotechnology, it has now become possible to understand better the molecular basis of IR and to regulate defense genes for augmenting disease resistance in crop plants. This review attempts to comprehensively present all the available information on inducing disease resistance in sugarcane, with a special focus on the red rot disease.

Keywords: BTH, *Colletotrichum falcatum*, elicitor, systemic acquired resistance (SAR)
Abbreviations: BABA, β-aminobutyric acid; **BTH**, benzothiadiazole; **ET**, ethylene; **HR**, hypersensitive response; **INA**, iso nicotinic acid; **IR**, induced resistance; **ISR**, induced systemic resistance; **JA**, jasmonic acid; **PAMP**, pathogen associated molecular patterns; **PGPR**, plant growth-promoting rhizobacteria; **PR**, pathogenesis related; **PRP**, pathogenesis related protein; **RGA**, resistant gene analogue; **SA**, salicylic acid; **SAR**, systemic acquired resistance; **SCMV**, sugarcane mosaic virus

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INTRODUCTION

Red rot caused by the fungus *Colletotrichum falcatum* Went (perfect state *Glomerella tucumanensis* (Speg.) Arx and Muller) is considered as the major constraint for sugarcane production in India. Releasing disease-resistant varieties has been the prime management strategy to contain the disease. Breeding for red rot resistance has been complicated by the frequent emergence of new pathogenic variants, which overpower the resistant variety (Viswanathan *et al*. 2003a). Most of the attempted plant protection chemicals have failed to cure the disease under field conditions. Obviously,

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Invited Review

there is a demand for new procedures that supplement the use of resistance breeding and fungicidal control in sugarcane plant protection. To a certain degree, susceptible plants can be altered to enhance their level of resistance against pathogens without genetic manipulation. Such enhancement of resistance in response to an extrinsic stimulus, without a known alteration of the genome is called ''Systemic Acquired Resistance (SAR)'' (Hammerschmidt 1999).

"Induced resistance" (IR) can be defined as an increased expression of natural defence mechanism in plants against various types of pathogens, provoked by a range of factors: pathogens causing hypersensitive necrotic reaction; avirulent or attenuated pathogenic strains; elicitors/pathogen associated molecular patterns (PAMPs) of pathogenic origin (glucans, proteins, lipids, etc.), abiotic elicitors, including synthetic harmless chemical products, such as 2,6-dichloroisonicotinic acid (INA), β-aminobutyric acid (BABA), benzothiadiazole or benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH), etc. IR, being based on the expression of latent genetic information present in plants, does not underlie genome alterations (mutations, introgression of foreign genetic material), thus enhancing its biological safety (Edreva 2004).

"IR" is expressed in plants both locally, at sites of infection (caused by pathogens) or treatment with elicitors, and systemically, when the effect manifests itself outside the affected sites. The induction of local resistance (hypersensitivity) is characterized by a rapid death of plant cells and thus localizing the infection. It is believed that a signal formed at sites of primary infection is transported to other portions of the plant, where defense reactions are induced. This form of IR was termed "SAR". The term was first proposed to designate the resistance observed in the upper leaves of tobacco (*Nicotiana tabaccum* L.), following the appearance of necrotic spots on the lower leaves infected by the tobacco mosaic virus (TMV) (Ross 1961). SAR is characterized by long-lasting effects against a broad spectrum of pathogens, including viruses, bacteria, fungi, and oomycetes (Stitcher *et al*. 1997).

SAR vs ISR

Plants resist attack by pathogens and herbivorous insects through constitutive and inducible defences. Based on differences in signalling pathways and spectra of effectiveness, different types of IR have been defined. SAR occurs in distal plant parts following localized infection by a necrotizing pathogen. It is controlled by a signalling pathway that depends upon the accumulation of salicylic acid (SA) and the regulatory protein NPR1. In contrast, induced systemic resistance (ISR) is induced by selected strains of non-pathogenic plant growth promoting bacteria (PGPR). ISR functions independently of SA, but requires NPR1 and is regulated by jasmonic acid (JA) and ethylene (ET) (Walters and Heil 2007).

ISR is a phenomenon, whereby resistance to infectious disease is systemically induced by localized infection or treatment with microbial components or products or by a diverse group of structurally unrelated organic and inorganic compounds (Kuc and Joseph 2001). Rhizobacteria-mediated ISR has been demonstrated against fungi, bacteria, and viruses in *Arabidopsis*, bean, carnation, cucumber, radish, tobacco, and tomato under conditions, in which the inducing bacteria and the challenging pathogen remained spatially separated. Non-pathogenic rhizobacteria can induce a systemic resistance in plants, which is phenotypically similar to pathogen-induced SAR (Van Loon *et al*. 1998).

Although the extent of IR attained by SAR and ISR can be similar, ISR usually affords lesser protection than SAR. In Arabidopsis, SA, JA and ET are involved to different extents in basal resistance against specific pathogens. Basal resistance against the oomycetous pathogen *Peronospora parasitica* and to *Turnip crinkle virus* (TCV) seems to be controlled predominantly by a SA-dependent pathway. In contrast, basal resistance against the fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea* was reduced only in JA and ET insensitive mutants, and not in *NahG* plants (Thomma *et al*. 1998). Interestingly, basal resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 was found to be affected in both *NahG* plants and in JA and ET-response mutants (Pieterse *et al*. 1998). Similarly, a combination of SA, JA, and ET-mediated defenses, governed resistance to *Xanthomonas campestris* pv. *armoraciae*, suggesting that basal resistance against this pathogen is controlled by a combined action of these three signals. This is in line with the conclusion of Cameron *et al*. (1994), stating that a hypersensitive response (HR) contributes to the level of resistance achieved. It is not clear yet, whether ISR is as broad-spectrum as in the case of SAR. Preliminary findings suggest that resistance induced by PGPR can be further boosted by application of SA, suggesting that bacteria do not activate the full spectrum of responses induced by pathogens. Indeed, pathogenesis-related proteins are induced only by a few PGPR strains among those capable of inducing resistance, and no evidence is available that these bacteria stimulate the plant to produce antimicrobial compounds, such as phytoalexins. In fact, these bacteria appear completely harmless, do not cause any symptoms and, yet, induce substantial resistance against different pathogens. In contrast, strong induction of SAR requires a necrotizing pathogen and is associated with the full range of defense reactions characteristic of a HR. However, the sugarcane red rot pathogen *C. falcatum* being a hemibiotroph warrants a detailed investigation on the priming induced response. The situation obviously confounds the search for the basic molecular-genetic mechanisms that underlie the induced state. It can now be said definitively that accumulation of PRs is not a pre-requisite for the induction of resistance. Nevertheless, because of the antipathogenic actions of at least some among the PRs, those are likely to contribute to the protective state against challenging pathogens. Together with other SA-induced antipathogenic activities, PRs could be responsible for the higher level of IR associated with SAR and tissue necrosis (Bargabus *et al*. 2004).

SYSTEMIC SIGNALLING AND MECHANISM OF RESISTANCE

Plants are often simultaneously challenged by pathogens and insects capable of triggering an array of responses that may be beneficial or detrimental to the plant. The efficacy of resistance mechanisms can be strongly influenced by the mix of signals generated by biotic stress as well as abiotic stress such as drought, nutrient limitation or high soil salinity. An understanding of their biochemical nature and knowledge of the specificity and compatibility of the signalling systems that regulate the expression of inducible responses could optimize the utilization of these responses in crop protection. Signalling conflicts and synergies occur during a plant's response to pathogens and insect herbivores, and much of the research on defense signalling has focused on salicylate- and jasmonate-mediated responses. Pathogens and insects, in a general sense, can elicit many of the same responses in plant foliage, albeit the temporal and spatial expression in relation to the onset of attack may differ widely depending on the attacking agent (Bostock *et al*. 2005).

Plant defense in response to microbial attack is regulated through a complex network of signalling pathways that involve three signalling molecules: SA, JA and ET. The SA and JA signalling pathways are mutually antagonistic. This regulatory cross talk may have evolved to allow plants to fine-tune the induction of their defenses in response to different plant pathogens (Kunkel and Brooks 2002). The important roles of SA and JA in defense signalling have become known largely through studies of SAR to pathogens (in the case of SA), and through studies of wound/herbivore IR to insects (in the case of JA). JA and its derivatives, collectively called jasmonates (JAs), have emerged as impor-

tant signals in the regulation of plant responses to pathogenic and beneficial microorganisms. The complex interplay of JAs with the alarm signals - SA and ET provides plants with a regulatory potential that shapes the ultimate outcome of the plant-microbe interaction (Pozo *et al*. 2005).

Although much of this work has been achieved with agronomic species, such as tobacco and cucumber as models, the more recent studies in *Arabidopsis* with many available mutants now in both signal generation and perception are fast revealing critical elements within the transduction paths. Cross-talk in defense signalling has emerged as an important and lively field in plant science research. With the mutants and chemical elicitors now available to manipulate responses in *Arabidopsis*, tomato and other species, and the keen interest in the topic by many research groups worldwide, progress should be rapid in defining points of synergy and of vulnerability in the pathways that govern plant responses to external stresses. Mutants in signal generation and perception are providing focus to the search for the critical elements in defense signal transduction (Delaney 2000).

Much progress has been made recently in elucidating the mechanism of SAR. Using the model plant *Arabidopsis*, it was discovered that the "isochorismate (ICS) pathway" is the major source of SA during SAR. In response to SA, the positive regulator protein NPR1 moves to the nucleus, where it interacts with TGA transcription factors (TFs) to induce defense gene expression, thus activating SAR. An overview of the understanding of the involvement of SA in plant resistance has been reviewed by Vasyukova and Ozeretskovskaya (2007). SA acts as a signal molecule in the SA-dependent pathway. The so-called salicylate burst, observed in tissues of plants after stress, increases their resistance. The mechanism whereby SA induces plant resistance depends on the ability of this compound to inhibit the enzymes of the antioxidant system of plants, which results in the accumulation of active oxygen species and the expression of defense genes. Wildermuth *et al*. (2001) established that SA is synthesized from chorismate by means of ICS, and that SA made by this pathway is required for local acquired resistance (LAR) and SAR responses by way of cloning and characterizing an Arabidopsis defence-related gene (SID2) as defined by mutation. Progress has been made in identifying key components and bioactive derivatives of SA-signalling pathways. SA signalling is mediated by both NPR1-dependent mechanisms and NPR1-independent mechanisms (Shah 2003), though the former pathway is better understood. SA-induced redox changes lead to the reduction of NPR1 from cytosolic, disulfide-bound oligomers to active monomers. NPR1 monomers nuclear localise and interact with the TGA class of basic leucine zipper TFs leading to the expression of a plethora of SA-dependent genes. Emerging evidence suggests that WRKY TFs participate extensively in SA defence responses, downstream or concomitant with NPR1, both as activators and repressors of SA transcription (Wang *et al*. 2006). Effector-mediated suppression of SA defences are now well established, however understanding the mechanism by which these diverse effectors link to and perturb SA signalling remains a significant challenge (Loake and Grant 2007).

Exciting data suggests that the mobile signal for SAR might be a lipid molecule (Durrant and Dong 2004). Phospholipid signalling is an important component in eukaryotic signal transduction pathways. In plants, it plays a key role in growth and development as well as in responses to environmental stresses, including pathogen attack. The involvement of both phospholipases C and D in early responses to the treatment of *Brassica napus* plants with the chemical inducers of SAR: SA, BTH and with the inducer mediating the ISR pathway, methyl jasmonate (MeJA) was investigated by Profotova *et al*. (2006). The involvement of phospholipase C/diacylglycerol kinase (PLC/DGK)-mediated signalling in oxidative burst and hypersensitive cell death was studied by Chen *et al*. (2007) in rice suspension-cultured cells treated with BTH and infected by *Xanthomonas oryza* pv. *oryza* (*Xoo*), the causal agent of rice leaf blight disease. The results suggested that PLC/DGK-mediated signalling plays an important role in BTH-induced oxidative burst, HR, and activation of defence response in rice. Orober *et al*. (2002) concluded that the chemical SAR inducer K_2HPO_4 and the biological inducer TNV shared some common early steps in signal transduction leading to SAR in cucumber, which differed from those involved in BTH-mediated SAR.

Bostock (2005) comprehensively reviewed progress in our understanding of signalling in induced plant resistance and susceptibility to pathogens and insect herbivores, with a focus on the connections and crosstalk among phytohormone signalling networks that regulate responses to these and other stresses. Continued research in this area is predicated on the notion that effective utilization of IR in crop protection will require a functional understanding of the physiological consequences of the "induced" state of the plant, coupled with the knowledge of the specificity and compatibility of the signalling systems leading to this state. This information may guide related strategies to improve crop performance in sub-optimal environments, and define the limits of IR in certain agricultural contexts.

Reverse genetics approaches has established the functional role of the gene "NPR-1", which was found as a key regulator in plant defense against many pathogens. A number of mutant screens were performed that identified multiple alleles of a single gene, *NPR1/NIM1* to identify components involved in SA signal transduction. It was observed that the *npr1* mutant also displayed enhanced disease symptoms, when infected with virulent pathogens and is impaired in some "*R* gene"-mediated resistance, suggesting that "NPR1" is important for restricting the growth of pathogens at the site of infection (Glazebrook *et al*. 1996). Besides, NPR1 also mediates cross-talk between the SA signalling pathway, JA and ET signalling pathways that confer resistance to insects and some necrotrophic pathogens (Spoel *et al*. 2003).

Little is known about the signal transduction events that lead to the establishment of the broad-spectrum, inducible plant immunity called SAR. Characterization of a mutant non-responsive to SAR activator treatments has provided additional evidence for common signalling components between SAR and gene-for-gene resistance (Hunt *et al*. 1996). The plant hormones SA, JA and ET are major players in the regulation of signalling networks that are involved in induced defense responses against pathogens and insects. During the recent past, significant progress has been made in understanding the function of Non-expressor of PR genes1 (NPR1), a key regulator of SAR, that is essential for transducing the SA signal to activate PR gene expression. However, NPR1 and its interacting partners are not the sole regulators of SA-responsive PR gene expression and SAR. Other essential TFs and their corresponding cis-acting elements have been identified, but their role in SAR still needs to be clarified (Pieterse and Van Loon 2004). The application of genomic technologies to the study of plant defence in the recent past has similarly provided revolutionary new insights into how plants defend themselves from pathogen attack. Perhaps somewhat expectedly and largely owing to the suitability to genomic studies, the research on the model plant *Arabidopsis* has paved the way in plant genomics research. It is now clear that "IR" most likely results in a co-ordinated action of many genes with diverse functions. Although, it may be somewhat naive to assume that all genes induced or repressed in response to pathogen challenge would have direct roles in IR, pathogen responsive genes are certainly good candidates for further functional studies (Kazan and Schenzk 2007). The current knowledge on the different biotic elicitors and synthetic compounds that can stimulate disease resistance in plants is improving with the continuous flow of literature on IR.

SAR IN MONOCOTS

Conventional breeding for disease resistance cannot be relied fully as a single approach, as it needs to be complemented with other support systems. As a sequel of the continuous emergence of highly virulent races among many important pathogens *viz*. *Colletotrichum* spp., *Phytophthora* spp., etc, it is practically impossible to have a continuous flow of disease resistant varieties in pipeline. In Japan, it is recoded that for the past 20 years, not much of durable disease resistance could be achieved in a wide range of crops *viz*. tomato, tobacco, pears, banana, lettuce and other leafy vegetables, nuts and cucurbits. However, integrating SAR with other disease management strategies has been a tremendous success in rice, besides a well established list of success stories of SAR potential in many other dicot crops. Rice is an established model system for monocots in terms of many aspects by virtue of the availability of the whole genome sequence in the public domain. Similarly SAR has been a well established and demonstrated phenomenon in rice and thus could be considered as a proven model system for related works in other monocots.

The information availability on the molecular basis of IR in monocots is limited as compared to the dicots. However reports are available on the establishment of IR against relatively few monocot pathogens (Morris *et al*. 1998) using chemicals namely 2,6-dichloroisonicotinic acid (DCINA) and BTH. The relevance of the role of SA and JA as key signalling intermediaries as applicable to cereal crops *vs* pathogen interactions has been reviewed by Kogel and Langen (2005).

Arabidopsis thaliana genome characterization has opened up the gates to unravel an array of regulatory genes involved in defense in many monocots. One of the key regulatory gene for SAR is AtNPR1, for which homologues have been found in all cereals tested so far. Homologues of *AtNPR1* have been found in few cereals, the closest homologue to NPR1 in rice being OsNH1, which shares 46% identity and 60% similarity with AtNPR1. Development of genetic mutants such as *npr1-1*, *npr1-2*, and *nim1-4* facilitated to establish the crucial role of specific amino acids in the regulatory function of the gene. It was suggested that rice shares a pathway similar to the *NPR1*-mediated resistance pathway, by over-expressing *AtNPR1* and *OsNH1* in rice, which conferred resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* in rice. NH1 appears to activate defence genes more readily in rice than its orthologue from *Arabidopsis* (Chern *et al*. 2001). Similarly, Wally *et al.* (2009) established that *AtNPR1* over-expression in transgenic carrots has activated defence against both biotrophic and necrotrophic pathogens. Cao *et al*. (1998) suggested a possible difference between rice and *Arabidopsis* in the regulation of defence gene induction, due to the fact that rice contains much higher levels of endogenous SA. Literature available strongly suggests that mono- and dicotyledoneae share elements of a conserved signal transduction pathway controlling NPR1-mediated resistance.

In rice, the BTH-induced gene expression pattern is different from that primed by a bacterial pathogen *Pseudomonas syringae* pv. *syringae* against the blast fungus (Schweizer *et al*. 1999). Similarly, gene expression profiles induced by *Blumeria*. spp. in barley and wheat overlap only slightly with those induced by SA-analogous chemicals (Beber *et al.* 2000). Hence, protection of cereals by inducer fungi or bacteria may not be brought about by a typical SAR response.

Upon treatment with the functional analogues of SA *viz*. BTH and DCINA, induced barley and wheat defence responses against powdery mildew (Kogel *et al*. 1994). Similar reactions were observed in rice against *M. grisea* (Schweizer *et al*. 1997), and in maize against downy mildew fungus *Peronosclerospora sorghi* (Morris *et al*. 1998). These data suggest that in cereals, with their high SA content, SA is not an effective signal for activation of defence genes and IR. Instead, a high level of endogenous SA may exert direct and/or indirect antioxidant effects to minimize oxidative damage caused by various biotic and abiotic factors. Further development of biotechnological methods for cereal improvement requires promoters that are inducible and tissue-specific in the Monocotyledoneae.

Table 1 BTH-priming induced changes in phenolic content in sugarcane var. 'CoC 671'.

Treatments	Phenolic content ($\mu g\ g^{-1}$ fresh weight)			
	Time (h) after challenge inoculation			
	0	24	48	72
BTH (250 µg/ml): inoculated	640	785	854	935
BTH (250 µg/ml): uninoculated	596	670	712	723
Control- untreated and inoculated	645	674	767	826
Mock: untreated and uninoculated	581	662	694	710

Least significant difference to compare any two means at P=0.05 is 40. Data are mean of five replications. BTH – Benzothiadiazole

STATUS OF SAR/ ISR IN SUGARCANE

An attempt was made to study the possibility of IR in Sugarcane against the red rot pathogen – *C. falcatum* was made using biotic (fungal elicitor) and synthetic (SA and its functional analogues) inducers of resistance. The gaining importance of the disease and the nuances in successfully managing the disease has been emphasised in the very beginning introductory remarks of this chapter. Development of disease resistant varieties is the practical strategy to successfully manage the disease. However, breakdown of red rot resistance is recorded in the past, which is attributed to the possible emergence of new virulent pathotypes (Viswanathan *et al*. 2003a). Exploitation of the inherent SAR potential of highly successful commercial varieties, otherwise susceptible to red rot is a viable option to manage the disease.

The effect of a novel synthetic signal molecule, acibenzolar-S-methyl (BTH) in inducing red rot resistance in sugarcane was first reported by Ramesh Sundar *et al*. (2001). The pioneering work involved screening of several synthetic inducers for their SAR potential under glasshouse conditions, minimum efficient dosage optimization, efficient method and time of application. Among the inducers screened, BTH priming considerably reduced red rot pathogen colonization and its further penetration was arrested in sensitized stalk tissues. SAR effect was found to persist up to 30 days in the pre-treated seed canes, wherein the crop was challenged with the red rot pathogen inoculum. Similarly, a booster spray of BTH at 250 µg/ml was found to protect the canes from subsequent challenge by the pathogen (**Fig. 1**). Induction of increased phenolic content (**Table 1**) and accumulation of PRs, *viz.*, chitinase, β-1,3-glucanase and thaumatin-like protein (PR-5), were observed in the sugarcane plants primed with BTH. Similarly treatment of BTH, SA and Pf exhibited ISR in *Sorghum bicolor* (cv Rio) to *Sugarcane mosaic virus* (SCMV) isolates from sugarcane. The treatments significantly slowed down the virus titre in plants during the initial growth phase (Balamuralikrishnan *et al*. 2005). Co-ordinated induction of defense-related parameters was well observed together with onset of SAR.

A comparative study indicated that next to BTH, SA and INA were found to be promising in marginally increasing the level of red rot resistance in primed sugarcane stalk tissues. The induction of resistance was accompanied by a concomitant increase in oxidative enzymes namely peroxidases and polyphenoloxidases activities in the protected tissues. Results of the qualitative analysis indicated early induction of specific isoforms of both of these enzymes under primed state. Also a considerable decrease of pathogen titre in the pre-treated tissues as determined by ELISA was recorded, which clearly demonstrated the restriction of pathogen colonization and proliferation in the sensitized cane stalks (Ramesh Sundar *et al*. 2006). In this work, suspension cells of sugarcane responded differentially upon treatment with an elicitor molecule isolated from the myce-

Table 2 Priming effected changes in sugarcane red rot incidence due to BTH and SA treatments.

Treatments	Disease incidence (%)[a]			
	CoC 671		CoC 92061	
	Days after pre-treatment			
	30	60	30	60
Control (distilled water treated)	97 a[b]	100 a	100 a	100 a
BTH	22 c	25 c	33 c	61 b
SA	40 b	41 c	40 c	63 b

[a] Mean of three replications (10 canes per replication); DAP: Days after pre-treatment

[b] Means followed by a common letter are not significantly different at the 5% level by DMRT (Duncan's Multiple Range Test)

lial cell wall of *C. falcatum*, as compared with a non-pathogen elicitor isolated from *C. lindemuthianum.* The study opened up the possibility of inducing resistance in sugarcane against the red rot pathogen using synthetic and biotic elicitors. The observable IR in susceptible sugarcane stalk tissues pre-immunized using BTH, clearly corroborates with established reports in similar other plant-pathogen interactions (Tally *et al*. 1999) and that IR is associated with numerous plant responsive mechanisms (Hammerschmidt and Nicholson 1999).

The induction of PRs in sugarcane leaves and suspension-cultured cells in response to treatment with a glycoprotein elicitor isolated from *C. falcatum* was investigated (Ramesh Sundar *et al*. 2008). Treatment of leaves and cells with the elicitor resulted in a much marked increase in the activities of chitinase and β-1,3-glucanase in red rot resistant (BO 91) than susceptible (CoC 671) sugarcane cultivar. SDS-PAGE analysis revealed that *C. falcatum* elicitor induced the accumulation of several proteins in suspension-cultured cells of resistant cultivar (BO 91); among them the 35 kDa protein was predominant. When sugarcane leaves were treated with *C. falcatum* elicitor, two proteins with apparent molecular masses of 25 and 27 kDa were induced both in the resistant and susceptible cultivars. Immunoblot analysis revealed that the 37 kDa protein is a chitinase and the 25 kDa protein to be a thaumatin-like protein (TLP) as revealed by Western blot probed with respective antisera.

Similar results could be validated under field conditions, where BTH pre-treated susceptible cane stalks responded with enhanced resistance to subsequent challenge with the red rot pathogen (Ramesh Sundar *et al*. 2009) (**Table 2**). The overall results of the *in vitro* bioassay revealed restriction of the pathogen growth, presumably by metabolites synthesized from the primed tissues. The study clearly established that, SAR holds a promise in managing red rot in elite commercial varieties under field conditions and it can be used as an effective management strategy for control of the disease in an environment expected to favour a disease outbreak.

BIOTIC ELICITOR-MEDIATED SAR IN SUGARCANE

A high molecular weight elicitor was isolated by Ramesh Sundar *et al*. (2002a) from the mycelial walls of the red rot pathogen, and partially purified by gel filtration using Sephadex G-200. The elicitor appeared to be a glycoprotein and the activity of elicitor resided in the carbohydrate moiety. The partially purified elicitor induced the accumulation of phenolics and the activities of phenylalanine ammonia-lyase (PAL) and peroxidase (PO) in sugarcane leaves and suspension- cultured cells. Sugarcane suspension- cultured cells responded to the *C. falcatum* elicitor in a manner similar to sugarcane leaves.

The elicitor rapidly induced the generation of H_2O_2 and O_2^- within 10 min after treatment in suspension-cultured cells of both red rot resistant (BO 91) and susceptible (CoC 671) cultivars. However, the generation of active oxygen species (AOS) was more rapid in suspension-cultured cells of resistant cultivar compared to the susceptible one. In addition, an increase in the level of lipid peroxiation in resistant cultivar was recorded as early as 6 h after elicitor treatment, whereas it increased only 12 h after treatment in the cells of the susceptible cultivar. Similarly response was observed with the Lipoxygenase (LOX) activity. High constitutive expression of Superoxide dismutase (SOD) was observed in the susceptible cultivar, as compared to that of the resistant cultivar, suggesting a possible suppressor role of the antioxidant enzyme during sugarcane - *C. falcatum* interaction (Ramesh Sundar *et al*. 2002b).

Differential and specific induction of enzymes of the phenyl-propanoid pathway *viz.* Phenylalanine ammonia-lyase (PAL), Tyrosine ammonia-lyase (TAL) and 4-Cou-

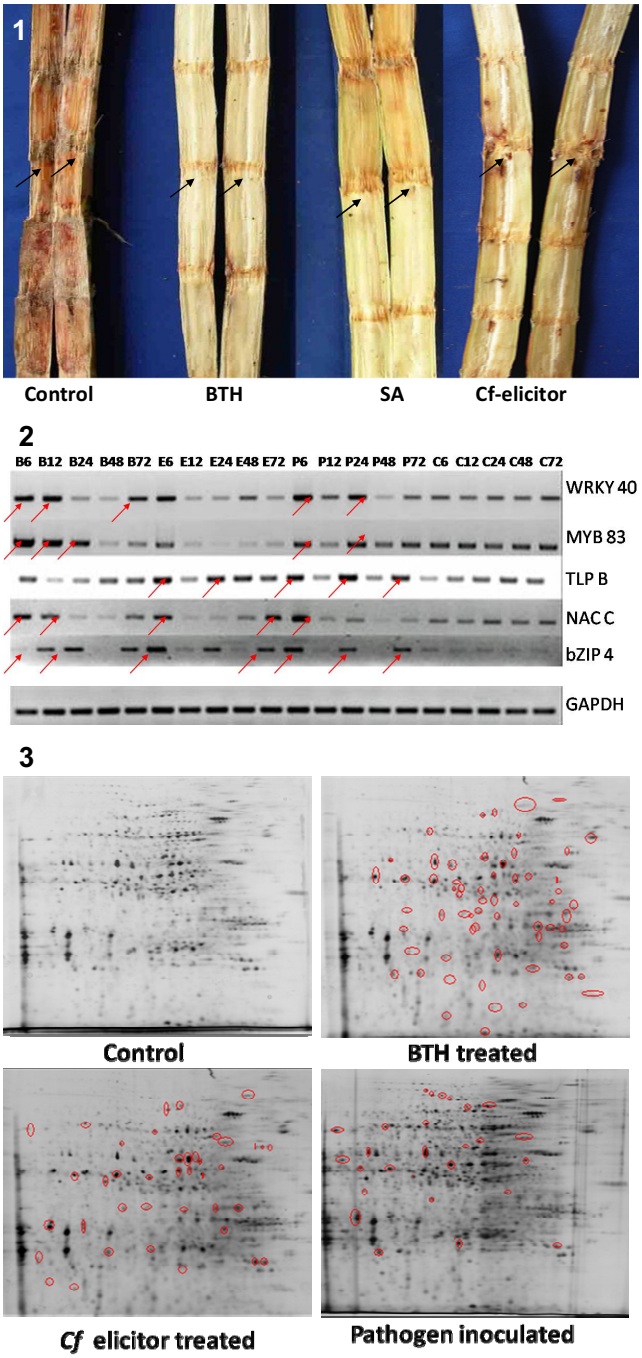

Fig. 1 BTH-induced protection in *Colletotrichum falcatum* challenged sugarcane cv. 'CoC 671'. Arrows indicate progressive lesions. **Fig. 2** RT-PCR profile indicating up-regulation of defense-related transcripts in response to SAR-priming. B – BTH-treated, E - *Cf* elicitor-treated, P - Pathogen-challenged, C - Control. 6, 12, 24, 48 and 72 indicates hours of post-inoculated samples. Arrows indicate upregulated transcripts. **Fig. 3** Representative 2D profiles from sugarcane stalk tissue in response to SAR priming and red rot pathogen challenge inoculation. Highlighted spots (differentially regulated) were analyzed by MS.

marate CoA-ligase (4-CL) was observed at cellular level, when the elicitor isolated from the red rot pathogen was used, as compared to that of a non-pathogen elicitor (*C. lindemuthianum*) (Ramesh Sundar and Vidhyasekeran 2003). Elicitor-induced browning and necrosis of cells, mimics that of a pathogen response and thus could be useful in understanding the host defense mechanism against the red rot pathogen at molecular level. Work is currently under progress to characterize the gene encoding the elicitor and for identifying the plasma membrane-bound receptor proteins mediating the defense signalling against *C. falcatum* in sugarcane.

TRANSCRIPTOME ANALYSIS OF SAR RESPONSE IN SUGARCANE

Viswanathan *et al*. (2009) established that interaction between sugarcane and *Colletotrichum falcatum* involved specific TFs contributing to host resistance. Semi-quantitative RT-PCR analyses were performed using different sets of primers designed for genes encoding targeted TFs and Resistance gene analogues (RGAs) (personal communication). Preliminary results indicated SAR-primed up-regulation of specific transcripts out of the 41 sets of primers encoding TF families – WRKY, MYB, TLP, NAC etc. Interestingly few of the TFs were differentially up-regulated in response to treatment with SAR inducers as well as with challenge with *C. falcatum* (**Fig. 2**), indicating the possible involvement of SAR-responsive transcripts in red rot pathogen defense. This study was the first attempt in sugarcane to study the TF regulated defense response against *C. falcatum* (Annual Report). The distinct temporal expression patterns of various TFs can provide guidance to the use of other experimental approaches to test the hypothesis about particular gene function. The defense-related potential transcripts identified in this study are being validated specifically using quantitative real-time PCR. Further studies will be directed towards delineating the specific role of individual TFs – functional genomics of defense and to explore the possibility of manipulating these TFs for durable red rot resistance in sugarcane.

PROTEOMIC BASIS OF SAR RESPONSE IN SUGARCANE

Proteomics of sugarcane is in its infancy, especially when dealing with the stalk tissues, where there is no study to date. A systematic proteome analysis of stalk tissue yet remains to be investigated in sugarcane, wherein the stalk tissue is well known for its rigidity, fibrous nature, and the presence of oxidative enzymes, phenolic compounds and extreme levels of carbohydrates, thus making the protein extraction complicated. Ramesh Sundar *et al*. (2010) evaluated five different protein extraction methods in sugarcane stalk tissues and optimized the best protocol for extracting soluble proteins from sugarcane stalk tissues. Both quantitative and qualitative protein analyses were performed for each method. 2-DE analysis of extracted total proteins revealed distinct differences in protein patterns among the methods, which might be due to their physicochemical limitations. Based on the 2-D gel protein profiles, TCA/acetone precipitation-LBT and phenol extraction methods showed good results. The phenol method showed a shift in pI values of proteins on 2-D gel, which was mostly overcome by the use of 2-D cleanup kit after protein extraction. Among all the methods tested, 2-D cleanup-phenol method was found to be the most suitable for producing high number of good-quality spots and reproducibility (**Fig. 3**). In total, 30 and 12 protein spots commonly present in LB, LBT and phenol methods, and LBT method were selected and subjected to eLD-IT-TOF-MS/MS (**Table 3**) and nESI-LC-MS/MS analyses respectively, and a reference map has been established for sugarcane stalk tissue proteome for the first time (**Fig. 4**). A total of 36 non-redundant proteins were identified. This is a pioneering basic study on sugarcane stalk proteome analysis and would promote further the unexplored areas of sugarcane proteome research.

ISR IN SUGARCANE

Pioneering work has been done to investigate the role of antagonistic microbes viz. *Trichoderma* sp. and *Pseudomonas* spp. in inducing systemic resistance in sugarcane against challenge with the red rot pathogen. Native rhizosphere strains of *Trichoderma* and few endophytic strains of Pseudomonads were found to be effective antagonists to *C. falcatum*. Viswanathan and Samiyappan (2001a) indicated that certain strains of fluorescent pseudomonads ISR against *C. falcatum* and has established that chitinases produced by these antagonistic strains of fluorescent pseudomonads contribute to the inhibition of the pathogen. Viswanathan *et al*. (2003b) compared mycolytic enzymes from bacterial antagonists with *T. harzianum*, and established that the latter showed more efficacy against *C. falcatum.*

Selection of efficient strains

Detailed investigations were undertaken by the Investigatory group on the possible use of *Pseudomonas* spp. for the suppression of *C. falcatum* in sugarcane. Initially the *Pseudomonas* spp. strains were isolated from the sugarcane rhizosphere and were assessed for their antagonistic and plant

Table 3 Identification of differentially expressed proteins using MALDI-TOF-MS.

Spot no.	Mw (kDa)/ pI (Exp)	Score (SwissProt / NCBInr)	Sequence coverage (SP/NCBI)	Accession No. (SP/ NCBInr)	MW/pI (Theor) (SP/NCBInr)	Description (SP/NCBInr)
A22	26 kDa / 5.9	31/0	15/0	GSTF1_ORYSJ	25022 / 5.99	Probable glutathione *S*-transferase GSTF1
A27	36 kDa / 6.7	38/0	20/0	FBL53_ARATH	36666 / 8.79	F-box/LRR-repeat protein At3g48880
A34	31 kDa / 4.6	25/0	14/0	14333_SOLLC	29401 / 4.74	14-3-3 protein 3
A47	44 kDa / 5.6	36/0	11/0	AATC_DAUCA	44375 / 6.46	Aspartate aminotransferase, cytoplasmic
A50	51 kDa / 5.9	36/0	11/0	APX8_ORYSJ	51441 / 5.36	Probable L-ascorbate peroxidase 8, chloroplastic
A54	49 kDa / 6.3	27/0	9/0	CIPK8_ORYSJ	50794 / 6.45	CBL-interacting protein kinase 8
A59	62 kDa / 6.7	40/0	30/0	PP403_ARATH	62410 / 6.71	Putative pentatricopeptide repeat-containing protein At5g37570
B2	97 kDa / 6.0	23/0	6/0	LOX2_ORYSJ	97294 / 6.16	Lipoxygenase 2
B17	45 kDa / 5.6	0/60	0/20	gi|171770699	46290 / 6.13	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Amorphophallus albus*]
B18	47 kDa / 5.1	26/0	7/0	FBD3_ARATH	46897 / 5.16	FBD-associated F-box protein At1g60410
B21	26 kDa / 6.5	25/0	9/0	CPI8_SOLTU	24963 / 7.78	Cysteine protease inhibitor 8 (Fragment)
B22	22 kDa / 6.5	66/0	30/0	RBS3_ACEAT	20899 / 8.74	Ribulose bisphosphate carboxylase small chain 3, chloroplastic
B24	21 kDa / 4.6	0/36	0/23	gi|255081366	23730 / 4.61	Mitochondrial protein translocase family [*Micromonas* sp. RCC299]
B26	19 kDa / 4.7	22/0	13/0	CML1_ORYSJ	21078 / 4.74	Calmodulin-like protein 1
B27	24 kDa / 5.2	40/0	19/0	gi|73747820	24569 / 5.52	Cold acclimation-induced protein [*Morus mongolica*]
B29	27 kDa / 6.7	38/0	26/0	RB11C_LOTJA	23859 / 6.22	Ras-related protein Rab11C
B30	26 kDa / 6.7	0/51	0/23	gi|73747820	24569 / 5.52	Cold acclimation-induced protein [*Morus mongolica*]

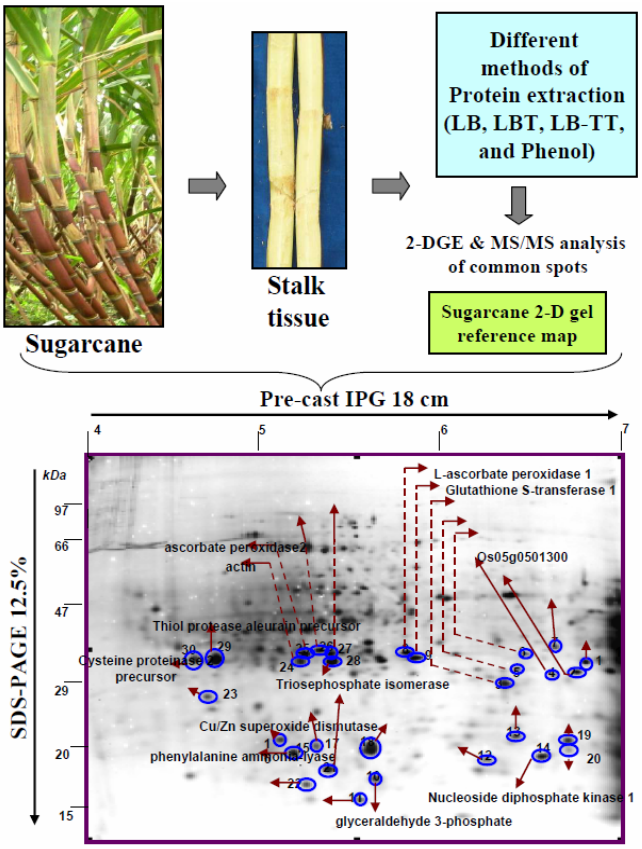

Fig. 4 A systematic proteome analyses of stalk tissue – reference map for sugarcane stalk proteome. Sample preparation was done using 5 different protocols for protein separation in 2-DGE and 30 common proteins were selected for eLD-IT-TOF-MS/MS analysis to prepare a reference map.

growth promoting abilities (Viswanathan and Samiyappan 2000). The antagonistic activity of native as well as other *Pseudomonas* strains against *C. falcatum* has been determined on different media (Viswanathan and Samiyappan 2001b). *Pseudomonas* strains CHAO, MD12, Pf1, ARR2, EP1 and EP2 suppressed the mycelial growth of the pathogen *in vitro*. Suppression of the growth of the pathogen was highest in King's B medium followed by oatmeal and potato dextrose agar. In addition, 51 bacterial strains colonizing sugarcane stalks were isolated from different genotypes/cultivars of sugarcane. These strains were characterized and the ones with antagonistic activity were identified. The strains SCEP1, SCEP3, SCEP5, etc. were highly antagonistic to *C. falcatum* (Viswanathan *et al*. 2003c). Incorporation of chitin in the medium enhanced the antagonistic activity of the strains (Viswanathan and Samiyappan 2001c). When antagonism was assessed on Fe-deficient and Fe-sufficient media, suppression of *C. falcatum* mycelial growth was significantly higher in Fe-deficient medium (Viswanathan and Samiyappan 2007) indicating involvement of many siderophores in antagonism.

Pseudomonas formulations

Since the bacterial cultures are not suitable to study their efficacy under field conditions talc formulations were prepared. The bacterial cultures were multiplied in King' B broth for 48 h to 9 × 10^8 cfu/ml. Talc formulations were prepared by mixing 400 ml of the broth with one kg of sterilized talc powder, (pH adjusted to neutral by adding $CaCo_3$ 15 g/kg and carboxy methyl cellulose 10 g/kg). After shade drying over night, the mixture was packed in polypropylene bags and sealed. The formulation had a population of about 2.5 to 3.0 × 10^8 cfu/g.

Field efficacy

Induction of systemic resistance in sugarcane has been proved by inoculating the pathogen in the stalks far away from the rhizosphere region. In the *Pseudomonas* treated canes though pathogen infection was noticed it was not able to progress beyond two to three internodes from the point of inoculation. However, in the *Pseudomonas* untreated canes, the pathogen made rapid progress up and down from the point of inoculation and caused drying of the cane stalks within 30 days. The *Pseudomonas* spp. strains were found to induce systemic resistance in two different trials in a highly susceptible cultivar. The IR has persisted up to 90 days. This information suggests that once the strains are introduced to the rhizosphere, they can offer resistance in the standing crop for at least three months (Viswanathan and Samiyappan 1999, 2002a, 2008).

Further studies have clearly proved that, *Pseudomonas* application to the crop rhizosphere significantly improved the host resistance by eliciting host defence mechanisms against the pathogen and reduced disease development under field conditions in an endemic location. The bacteria treated canes recorded higher juice parameters than the control treatments after pathogen infection and this could be due to the reduced pathogen colonization in the treated canes.

The pathogen colonization in *Pseudomonas* treated and control canes were assessed by ELISA technique at different nodal positions above the point of pathogen inoculation 30 days later. This study clearly showed reduced pathogen colonization due to the bacterial treatment in the treated canes stalks (Viswanathan and Samiyappan 2006a). Reduced pathogen colonization corroborated with reduced disease development in the treated canes.

Efficient *Pseudomonas* strains were also found to be effective against the soil borne pathogen inoculum introduced as infected cane materials in the soil (Viswanathan and Samiyappan 2000). Earlier ISR by the *Pseudomonas* spp. strains has not been established in sugarcane against different pathogens. The selected *Pseudomonas* spp. strains have reduced the disease build up in the trials where the pathogen was inoculated artificially in the upper internodes, pathogen sick plots and in disease endemic locations. Studies conducted so far indicated the potential of suppressing *C. falcatum* causing systemic infection in sugarcane stalks by *Pseudomonas* spp. strains under field conditions. These studies also proved that *Pseudomonas* spp. treatment has improved cane yield and sugar yield in the trials. However, detailed studies are to be conducted at different regions in the country to prove the efficacy of different bacterial strains (Viswanathan and Samiyappan 2006a).

Mechanism of ISR

Mechanism of IR by *Pseudomonas* strains against the disease was investigated in detail. Involvement of different PR-proteins such as β-1,3-glucanases, chitinases and thaumatin-like proteins (TLPs) was found to be associated with *Pseudomonas*-mediated IR (Viswanathan *et al*. 2003d). 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 demonstrated 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 SA, auxins, siderophores, pyocyanine, pyoluteorin and 2,4-diacetyl phloroglucinol and hydrolytic enzyme chitinase contribute to suppression of *C. falcatum*, IR and growth promotion in sugarcane (Viswanathan and Samiyappan 2004).

Lytic enzymes of fluorescent pseudomonads strains and *Trichoderma harzianum* strain T5 were inhibitory to conidial germination, germ tube elongation and hyphal growth red rot pathogen (Viswanathan *et al*. 2003b). Further characterization of *T. harzianum* extracellular chitinases was carried out by SDS-PAGE and activity gels. In this procedure, activity of chitinase isoforms viz. N-acetyl glucosaminidase, chitobiases and chitotriases were detected as discrete fluorescent bands under UV light. The partial sequences of the chitinases were cloned and characterized (Viswanathan *et al*. 2006b). Detailed studies conducted at SBI on inactivation of *C. falcatum* toxin by bacterial and fungal antagonists. The results clearly proved that *P. fluorescens* VPT4 and *T. harzianum* T5 have completely arrested biological activity of the toxin and altered spectral properties of the toxin (Malathi *et al*. 2002). Further characterization of the inactivating protein revealed it as a 97 kDa protein. Malathi *et al*. (2008) has established a correlation between disease expression and production of secondary metabolites *viz*., toxin and enzymes *viz*. cellulolytic (cellulase) and pectinolytic (exo- and endo polygalacturonases, pectin methyl esterases) enzymes by the pathogen.

SCOPE FOR SAR/ISR IN SUGARCANE

Breeding for all diseases in a crop and for specifically against a particular race is not feasible. Besides, in sugarcane regular breakdown of red rot disease resistance is an observed phenomenon regularly over a period of time. Many commercially successful varieties with agronomically superior traits like CoC 671 has been phased out of cultivation just because of a single fault of red rot susceptibility. Breakdown of red rot disease resistance has been observed in varieties, *hitherto* recorded as resistant while screening for red rot resistance. Hence a suitable strategy for inducing a race non-specific, broad-spectrum and durable red rot resistance is the need of the hour. Identification of novel signal inducers either of biotic or of synthetic origin capable of SAR-inducing potential would help in sustaining red rot resistance in proven successful varieties like CoC 671, which is much in demand by sugarcane growers and the sugar industry.

Delivery systems in sugarcane for making SAR a more practical field application strategy

In sugarcane, by virtue of the crop stand, application of plant protection measures becomes difficult under field conditions during the mid-season of the growing crop. This complicates the application of fungicide to prevent any secondary infection after 5-6 months of crop age. A suitable application mechanism need to be devised without resorting to a foliar spray and it could be achieved by integrating the application of SAR inducers along with an irrigation delivery system like surface or sub-surface drip systems. Interestingly, there is a growing awareness about the optimum use of irrigation water and hence there is a paradigm shift from conventional irrigation to drip-based irrigation systems. Large-scale installation of drip irrigation systems by corporates at subsidized cost has made this practise more attractive, which combines the fertigation process. Besides, labour and water saving, this practise has resulted in making sugarcane cultivation more remunerative. In this background, a new dimension in integrating plant protection measures through the drip irrigation network is seen as a viable delivery system for effecting the application of any plant protection component.

Priming response in sugarcane has already been established by using synthetic signal molecules like BTH and other functional analogues of SA *viz*. INA and biotic elicitor extracted from the mycelial cell wall of *C. falcatum*. Besides, induction of systemic resistance against *C. falcatum* in sugarcane by using efficient antagonistic strains of *Trichoderma harzianum* and *Pseudomonas fluorescens* has been demonstrated successfully. These priming agents are used as sett treatments before planting, which is expected to afford protection from any primary pathogen inoculum present in the soil or in the planting material. However application of these effective inducers for subsequent priming during the middle of the crop duration against any secondary infection is required. Integrating this priming along with sub-surface irrigation systems would prove beneficial by all means in successfully managing the crop disease.

In India, sugarcane breeding is undertaken with releasing location specific varieties, which has specific adaptability to the growing conditions. Classical examples are varieties 'CoC 671' and 'CoJ 64', which were ruling the sugar industry under tropical and sub-tropical conditions respectively. Once these varieties recorded epiphytotic build-up of red rot incidence, it was very difficult to replace the varieties, as they have cultivated on a large scale owing to their established popularity and suitability to the respective conditions. There is a continuous pressure for high yielding/ high sugar varieties combined with red rot resistance. However by virtue of the complex polyploidy nature of the crop and due to the rapidly evolving red rot pathogen, targeted breeding for red rot resistance is difficult, complicated and time taking. Molecular tools have come in as a handy tool to supplement conventional breeding approaches to look for red rot resistance. Also augmenting red rot resistance in commercially proven varieties would be a viable option in the successful management of the red rot disease in sugarcane. Hence it becomes pertinent to explore novel strategies like SAR/ISR, which seems to be promising for sustaining the life of such proven varieties, thus resulting in enhancing crop productivity

Pioneering work was performed by Viswanathan and Samiyappan (1999) on the possible use of biocontrol agents (BCAs) for inducing systemic resistance in sugarcane against the red rot pathogen – *C. falcatum*. The preliminary work includes enumeration of efficient strains (both rhizosphere and endophytic) from the BCAs *viz*. *Trichoderma harzianum* and *Pseudomonas fluorescens*. Highly antagonistic strains combined with plant growth promoting attributes were selected and used for bio-assay under glasshouse and field conditions. Effective strains with useful traits such as chitinase synthesis, siderophore production, phosphorous mobilization etc. were carried forward for preparing talc-based formulations for field application. Field efficacy of select strains was proven and the ISR effect persisted upto 90 days. Pathogen suppression and significant improvement of host resistance mediated by BCAs were established. Further, the mechanism of IR was partially elucidated and the involvement of antifungal PR-proteins, enzymes of the phenyl-propanoid and oxidative pathways, production of antibiotics and siderophores, etc. were found to contribute to the ISR effect in sugarcane.

Integration of biotic and synthetic elicitors was one of the key breakthroughs obtained by this investigatory group. Combining optimal doses of *P. fluorescens* along with BTH (250 µg) yielded considerable level of suppression of the pathogen growth systemically in the primed stalk tissues. Further, this kind of consortia approach would rationalise optimum use of chemical inducers and result in an eco-friendly disease management in sugarcane.

Fitness cost

Fitness costs of resistance are among the most widely discussed explanations for the evolution of IR, but studies on IR to pathogens are scarce and contradictory. Explanations are mainly based on the assumption of fitness costs. Investment in defense is thought to reduce the fitness of plants in enemy-free environments. Fitness costs often result from allocation costs, i.e. allocation of limited resources to defense, which then cannot be used for growth or other fitness-relevant processes. This theoretical concept can provide a useful tool for the interpretation of induced plant responses against pathogens, named ISR or SAR. Phenotypic plasticity, leading to induced responses, might have evolved

mainly to reduce costs, since investment in defense is restricted to situations actually requiring defense. ISR can incur allocation costs and other, indirect costs, which ultimately may lead to fitness costs (Walters and Boyle 2005).

CONCLUSION

The future stress in this line of work lies on the identification of the plant's own signal substances which mediate systemic resistance or sensitize the plants to react rapidly when challenged by pathogens. Perhaps these substances could be used directly as spray or seed dressing for protection of the plants. Research on the regulation and activation of resistance genes after induction of resistance will be certainly of interest. Artificial gene constructs of such resistance genes with suitable promoters could be used in transgenic plants in which stable and effective resistance can be induced in the field by application of inducers or signal substances. The major advantage is that induction of resistance appears to activate multiple and complex mechanisms in the plants and therefore presents a durable type of control measure. The critical factors for the establishment will be efficacy and stability of protection. Therefore, resistance inducers must be found which fulfil the criteria for practical use. This includes the application of inducing agents, existing equipment, a suitable formulation of the inducers that ensures optimal performance, persistence and a long shelf life under normal conditions and compatibility with common pesticides and fertilizers. For agronomic applications, commercially certified (agro) chemicals, including resistance inducers, are attractive and environmentally safe options under field situations. Further knowledge is also need on its efficacy under different climatic and environmental conditions and the influence of genotypes, age and on the nutritional status of the plant.

IR opens the possibility to activate latent resistance mechanisms in already existing high yielding, high quality variety of crops which otherwise are highly susceptible. The exciting applications would be the use of resistance inducers against the plant viruses for which pesticides are not available or against diseases which can be controlled with difficulty such as those caused by bacteria and soil-borne diseases. In plant protection, the use of resistance inducers appear to be realistic and a supplement to conventional methods, thus increasing the spectrum of technologies useful for disease management.

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