TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Note

Turk J Bot
(2016) 40: 461-471

doi:10.3906/bot-1506-27

Preliminary analysis on the transcripts involved in resistance responses to eumusae leaf spot disease of banana caused by *Mycosphaerella eumusae*, a recent add-on of the sigatoka disease complex

Asoor Santhanam SARAVANAKUMAR, Subbaraya UMA*, Raman THANGAVELU, Suthanthiram BACKIYARANI, Marimuthu Somasundram SARASWATHI, Viswanathan SRIRAM
ICAR-National Research Centre for Banana, Trichy, Tamil Nadu, India

Received: 16.06.2015 • **Accepted/Published Online:** 09.02.2016 • **Final Version:** 19.07.2016

Abstract: Eumusae leaf spot disease, caused by *Mycosphaerella eumusae*, is widely distributed in different banana growing countries in Asia and Africa, causing severe losses in yield and quality of banana. The aim of this study was to identify the differentially expressed defense responsive genes through subtractive suppression hybridization (SSH) in the *Musa–M. eumusae* interaction system. In this method, leaf samples collected at different intervals between 0 and 72 h postinoculation (hpi) of *M. eumusae* from resistant (Manoranjitham-AAA) and susceptible (Grand Naine-AAA) cultivars were subjected to cDNA-SSH library construction. From the SSH library, 832 clones having the insert were selected and sequenced. All these sequences were assembled using CAP3, which resulted in 498 unigenes (59.85%), consisting of 78 contigs and 420 singletons. Furthermore, the BLAST2GO analysis performed showed that, out of the 498 unigenes, only 161 (32.32%) were involved in molecular functions, cellular component and biological processes. However, among these 161 unigenes, 57 were found to have top BLAST hits. Six genes were selected based on the function and subjected to validation through qPCR. Four genes, namely ethylene responsive factor, flavin containing monoxygenase, serine glyoxylate, and metallothionein, were found to have the highest-fold regulation in the resistant cultivar at 48 hpi, followed by lipoxygenase at 36 hpi. The main findings are that resistant plants deploy HR activity as a strategy to evade the fungus followed by the activation of various ROS scavengers to maintain homeostasis and the role of phytohormones may be induced as a part of plant defense against *M. eumusae*.

Key words: Eumusae leaf spot disease, gene expression, *Musa*, subtractive suppression hybridization

1. Introduction

Eumusae leaf spot disease (ELSD) of banana is caused by *Mycosphaerella eumusae* (Carlier) (anamorph *Pseudocercospora eumusae*), which belongs to the largest genera of plant-pathogenic fungi, *Mycosphaerella* (Carlier et al., 2000). *M. fijiensis* (Morelet) and *M. musicola* (Leach) are two other *Mycosphaerella* spp. that also cause serious leaf spot diseases of banana such as black leaf streak disease (commonly known as black sigatoka) and sigatoka leaf spot disease (commonly known as yellow sigatoka), respectively. The chronology of disease records around the world suggests that Southeast Asia is the center of origin for all three species (Jones, 2002; Rivas et al., 2004). Among these three species of *Mycosphaerella*, *M. eumusae* is recognized as a new constituent of the sigatoka complex of banana (Carlier et al., 2000; Crous and Mourichon, 2002) only in the mid-1990s. These leaf spot diseases cause necrotic leaf lesions, resulting in reduced photosynthetic activity eventually leading to reduced crop yield and fruit quality and pose serious threats for major banana producing zones (Arzanlou et al., 2007). Information on ELSD is limited to symptoms and distribution only. Presently, *M. eumusae* is known from Southeast Asia and parts of Africa. In India, so far only *M. eumusae* was recorded, causing severe economic losses particularly in the Cavendish cultivar in the major banana growing states like Maharashtra, Tamil Nadu, Andhra Pradesh, and Karnataka (Thangavelu et al., 2013), altogether contributing about 15.3% of the world's total banana production (FAOSTAT, 2013; NHB, 2013). Owing to spontaneous spread and serious economic threat caused by the disease an "exotic pest alert" warning has been called for in many countries (Exotic Pest Alert, 2014). ELSD typically begins with the development of thin light brown streaks on the adaxial surface. As the disease progresses the streaks develop into a dark brown margin and enlarge into an elliptical spot with grayish white

* Correspondence: umabinit@yahoo.co.in

centers. At the advanced stages the spots merge together, resulting in necrotic leaf areas, and the dead leaf appears as grey spots (Figure 1). The mature spots of *M. eumusae* are larger than the leaf spots caused in black sigatoka and yellow sigatoka. Moreover, a *M. eumusae* strain has been reported to infect cultivars that have shown resistance to both *M. fijiensis* and *M. musicola* (Jones, 2002). All three banana leaf spot disease causing species affect the banana plant differently by the production of various mycotoxins (Stierle et al., 1991) and their management varies. Hence understanding the interaction of *Musa* with this particular species is necessary. At present, the chemical control strategies available may cause development of races resistant to fungicides and pollute the environment (Aguilar-Barragan et al., 2014). To overcome these disadvantages, the development of resistant lines would be very useful and for this identification of resistant genes is essential. Evidence from previous studies has shown that the potential mode of *Musa* resistance against fungal pathogens would be through detoxifying phytoallexins like anigorufones and phenylphenalanone-type compounds that are biosynthesized via the phenylpropanoid pathway, shikimate pathway, and diarylheptanoid pathway (Luis et al., 1996, 1997; Binks et al., 1997; Hölscher et al., 2014; Hidalgo et al., 2015). Hence, in the present study, efforts were made to identify the defense response/resistant genes in the eumusae resistant cultivar. Subtractive suppression hybridization (SSH) has been successfully employed in banana to isolate differentially expressed genes during *Musa*–pathogen interaction such as Fusarium wilt (Van Den Berg et al., 2007; Swarupa et al., 2013), *M. fijiensis* (Portal et al., 2011), and nematode–*Pratylenchus coffeae* (Backiyarani et al., 2014). Here, we identified pathogen-induced genes and particularly focused on a series of genes to assemble the molecular basis of the *Musa*–*M. eumusae* resistance mechanism.

2. Materials and methods

2.1. Preparation of *M. eumusae* inoculums

Leaf samples having mature *eumusae* leaf spot (*M. eumusae*), which were collected from a susceptible banana cultivar, were cut into 2 cm^2 pieces and dipped in sterile water for 10 min. Then leaf pieces were stapled onto sterile filter paper and placed in the lid of a petri dish containing 3% water agar. The plates were incubated at room temperature for a day and the release of ascospores was observed under the microscope. The ascospores were picked from the plain agar media and transferred to potato dextrose agar (PDA) and incubated for 4 weeks to grow colonies to a diameter of 0.5 cm. The colonies raised in the center were with light gray to green and the other side of the colony was light brown to black. The fungal spores were diluted (10^8/mL) using sterile distilled water and used for challenging the banana cultivars.

2.2. Fungal inoculation and sample collection

ELSD resistant cv. Manoranjitham and susceptible cv. Grand Naine, belonging to the same triploid acuminata group (AAA), were used as contrasting cultivars for this study. A total of twelve tissue culture plants of each cultivar with three replications were planted in a pot containing potting mixture of soil, sand, and farm yard manure (2:1:1) and acclimatized under controlled conditions (95% RH, 22 °C, 16 h light and 8 h dark). The prepared *M. eumusae* spores were inoculated by spraying on both surfaces of leaves. Inoculated leaf samples were collected from 0 to 72 hours postinoculation (hpi) at an interval of 6 h and leaf samples were frozen with liquid nitrogen and stored at –80 °C.

2.3. RNA isolation from M. eumusae challenged leaf samples

Total RNA was isolated from stored leaf samples using the modified protocol from Gasic et al. (2004) and analyzed

Figure 1. Symptom of banana leaf upon *M. eumusae* infection.

for its concentration and quality using a bioanalyzer 2100 (Agilent Technologies). The high quality RNA was considered for mRNA separation and SSH library construction.

2.4. SSH library construction

Total RNA isolated from *M. eumusae* inoculated leaf samples (0 to 72 hpi at an interval of 6 h) of resistant cultivar were pooled and used as 'tester'. Similarly, RNA samples from challenged susceptible cultivar were pooled and used as 'driver'. The messenger RNA was extracted from the tester and driver RNA pools independently using a Genelute mRNA Miniprep kit (Sigma, USA) following the manufacturer's instructions. Then 2 µg of mRNA from each cultivar was taken and SSH was performed using the commercial PCR-Select cDNA Subtraction Kit (BD Biosciences Clontech, Heidelberg, Germany). The forward library was constructed by subtracting driver RNA from tester RNA and during the experiment two rounds of hybridization and PCR were performed to enrich differentially expressed sequences. The subtracted PCR products generated by SSH were subjected to PCR cleanup using a QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's protocol and purified PCR products were inserted into pGEM-T easy vector (Promega, Madison, WI, USA). It was then transformed into *Escherichia coli* DH5α and plated onto LB agar with 100 µg/mL ampicillin, 0.5 mM IPTG, and 80 µg/mL X-gal. Individual white clones were picked and grown overnight at 37 °C. Well grown cultures were added to 15% glycerol and stored at –80 °C.

2.5. Sequence submission and bioinformatics analysis

The recombinant white colonies were grown individually overnight in liquid broth (LB) containing ampicillin (100 µg/mL) and plasmids were isolated using a GenElute HP Plasmid Miniprep Kit (Sigma, USA) according to the manufacturer's protocol. Plasmids were confirmed using *Eco*RI digestion to remove false positive clones and sequenced unidirectionally using universal primer M13. Sequences were subjected to vector sequence removal by Vecscreen analysis tool at NCBI (http://www.ncbi.nlm.nih.gov/VecScreen) and submitted to NCBI GenBank. Sequences were assembled using CAP3 (http://pbil.univ-lyon1.fr/cap3.php) to generate accurate contigs. Resulting contigs and singletons were annotated using the BLAST2GO tool (Conesa et al., 2005). Annotation was also carried out using the banana genome hub (http://banana-genome.cirad.fr).

2.6. Validation using gene expression studies

Total RNA of both resistant and susceptible cultivar was isolated independently from *M. eumusae* inoculated leaf samples. qPCR analysis was performed for five time points (12, 24, 36, 48, and 72 hpi) along with controls (water sprayed). Messenger RNA was purified from each total RNA using the Genelute mRNA Miniprep kit (Sigma, USA) following the manufacturer's instructions. First strand cDNA was synthesized using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) with oligo dT primers according to the manufacturer's protocol. Synthesized cDNA was diluted (1:10) with nuclease-free water and used as template for gene expression studies. A total of six genes were selected based on their function from the forward library and primers were designed using the online Primer3 tool (Table 1). A SYBR green assay was performed using a LightCycler 480 instrument (Roche Diagnostics, Germany). A reaction volume of 20 µL was set up in triplicate for each primer set with 5 µM of forward and reverse primers, 1X SYBR Green PCR Master Mix (Roche, Germany), and the final reaction volume was made up with nuclease-free water. The RPS2 gene was used as endogenous gene (Chen et al., 2011). Cycling conditions were one cycle of 95 °C for 10 min followed by 40 cycles of initial denaturation at 94 °C for 20 s, annealing at 50–60 °C for 20 s, and extension at 72 °C for 25 s. Finally, the expression level of each gene was relatively quantified based on calculated normalized ratio using advanced relative quantification using Roche LightCycler software version 1.5.

Table 1. Primer details used for gene expression studies using real time PCR.

Gene name	Forward primer	Reverse primer	Product size (bp)	Annealing temperature
CO	GGTGGCCAAAGAATCAGAAA	CCTATTCGTCTGAGCCGTTC	180	58
ERF	TCATGTTCCTGCTGCTATCG	GGCCGAGGTACGAGTCATAA	161	58
FMO	GTCGGACCGCTCGAGTATAA	AACAGTGCAGCGAAAAGTGA	235	55
SG	CTGTCACTGCTGTGGTAGTTCC	CCGAGGTACCATTTCCACAC	200	55
MT	CGGCCGAGGTATAATTTGTT	TGGAGTGGTCTACCTCGCTAA	400	54
LOX	TGCGAGTCATAGCGGTTGTA	GACCATGATTACGCCAAGCTA	190	51

3. Results

3.1. Construction of the cDNA-SSH library

The forward cDNA-SSH library was constructed from the double-stranded cDNAs of *M. eumusae* challenged leaf samples of a resistant (tester) and susceptible cultivar (driver). The efficiency of subtraction was confirmed through the differential expression of RPS2 among the subtracted and unsubtracted cDNAs of both the tester and driver. The amplification of the RPS2 gene was detected using agarose gel and found that amplicons were observed in subtracted cDNAs only after 32 cycles, whereas in unsubtracted cDNAs they were detected within 17 cycles. The reduction in expression level observed in the subtracted cDNAs confirmed the successful subtraction. A total of 1100 clones were obtained from the *M. eumusae* challenged cDNA-SSH library. To confirm the positive clones, all the clones were digested with *EcoRI* and resulted in identification of 832 clones with insert. These clones were sequenced and submitted in the NCBI Genbank (Accession numbers: GO0248764 – 248776; GT067848 – 067888; GT086299 – 086405; GT153720 – 153761; GT153911 – 153943; HS032618 – 032711; HS091928 – 092099; HS107192 – 107521). Sequences were assembled using CAP3 analysis, which resulted in 498 unigenes (59.85%), containing 78 contigs and 420 singletons.

3.2. Annotation of SSH library

The analysis of the 498 unigenes using BLAST2GO revealed 161 unigenes (32.32%) hit with putative function. The BLAST result showed that 44 and 56 unigenes hit with E value ≥1.00E–13 with reference to *Musa* and *Viridiplantae*, respectively (Table 2), which included genes responsible for photosynthesis and defense/resistance related genes like cytochrome oxidase (*CO*), lipoxygenase (*LOX*), ethylene response factor (*ERF*), serine-glyoxylate (*SG*), flavin-containing monooxygenase (*FMO*), and metallothionein (*MT*).

Species distribution of top BLAST hits with reference to *Viridiplantae* exhibited more similarity with *Zea mays*, *Oryza sativa*, *Elaeis guineensis* etc., whereas BLAST hits with reference to *Viridiplantae* had higher similarity with *Oryza sativa* followed by *Vitis vinifera* and *Medicago truncatula* (Figure 2).

Gene ontology (GO) was performed subsequent to BLAST results. GOs were categorized into sequence distribution of molecular function, cellular component, and biological processes. Plotting of GOs using the WEGO chart subcategorized the molecular functions into binding (90%), catalytic (60%), transmembrane transporter (5%), antioxidant (3%), and peroxidase (2%); biological processes into cellular and metabolic processes (85%), response to stimulus (7%), response to stress (4%), and cellular response to stimulus (4%); and cellular component into membrane bound organelle, organelle (75%), and cell and cell part (70%) (Figure 3). In total 337 ESTs (67.67%) did not have any GO terms and were considered genes with unknown function.

After GO annotation, sequences were analyzed for identification of protein regions using InterPro scan. It resulted in 159 hits in IPR (InterPro region), which were categorized into family (74), domain (73), active site (4), binding site (6), conserved site (2), and 680 hits with no IPR. Catalase was found to have high hits with IPR in many categories like domain, family, and binding site followed by photosystem. Some of the ESTs hits with IPR categorized families such as fructose-bisphosphate aldolase, chlorophyll binding protein, carbonic anhydrase, and glycine cleavage H protein. Similarly, some were found to hit with domains like zinc finger CCCH-type, chlorophyll a/b binding protein, nucleotide binding, peptidase, ATPase, aldolase type TIM barrel, pyridoxal, and phosphate dependent transferase. ESTs hit with active sites are cysteine peptidase, histidine active site, and also with binding sites like catalase heme and 2-oxoacid dehydrogenase lipoyl binding site.

Finally, sequences were subjected to KEGG pathway analysis, which resulted in tryptophan metabolism, one carbon pool by folate, pentose phosphate pathway, oxidative phosphorylation, drug metabolism–cytochrome P450, carbon fixation in photosynthetic organisms, fructose and mannose metabolism, glyoxylate and dicarboxylate metabolism, glycine, serine and threonine metabolism, purine metabolism, thiamine metabolism, methane metabolism, nitrogen metabolism, and glycolysis/gluconeogenesis (Table 3).

3.3. Gene expression studies

A total of six genes were selected based on their function to study their expression in the *M. eumusae* challenged leaf samples of two contrasting cultivars against five time points (12, 24, 36, 48, and 72 hpi) along with a control. The expression of each gene was normalized based on the expression of a banana housekeeping gene RPS2 and analyzed using advanced relative quantitative software (Roche LightCycler software version 1.5). Except *SG*, all genes recorded high-fold expression in the unchallenged resistant cultivar than in the susceptible one, whereas in the resistant cultivar, all genes except *CO* were observed to be overexpressed under challenged condition compared to unchallenged one. Out of the five upregulated genes in the resistant cultivar, the highest-fold expression at 48 hpi was observed for four genes, namely *ERF*, *FMO*, *SG*, and *MT*, and at 36 hpi for *LOX*. Interestingly, three genes, namely *MT*, *LOX*, and *FMO*, were not overexpressed in the susceptible cultivar even under *M. eumusae* challenged condition (Figure 4).

Table 2. BLAST details of *Musa* cDNA – SSH library with reference to *Musa* (*) and *Viridiplantae*.

Singleton / contig	Protein name	Sequence length (bp)	Hit accession/	E value	Similarity (%)
GT154768	serine-glyoxylate aminotransferase	263	AEW69795	1.00E–35	94
HS092079	ribulose-bisphosphate carboxylase oxygenase large subunit	866	AET25092	1.04E–133	100
GT086386	photosystem ii 32 kda protein	243	ABL86528	1.11E–20	98
Contig29	microfilarial sheath protein	477	AAA99147	1.11E–51	96
GT153926	snf1-related protein kinase regulatory subunit beta-2*	245	GSMUA_AchrUn_randomT07990_001	1E–13	46
|GT153761	senescence-associated protein	321	ACA30301	1.34E–54	95
GT086359	photosystem i reaction center subunit iv a*	290	GSMUA_AchrUn_randomP10600_001	7E–20	98
GT086363	photosystem i subunit b	360	XP_002535619	1.54E–61	100
Contig64	ethylene response factor	446	ADG58085	1.55E–14	55
GT086329	metallothionein-like protein	441	ACB10219	1.59E–18	76
Contig59	Xylem cysteine proteinase 1*	402	ABR19829	1.73E–43	98
GT086364	photosystem i p700 apoprotein a2	654	YP_004769713	1.75E–15	70
Contig11	chlorophyll a-b binding protein	466	BAG96979	1.89E–56	98
GT153923	zinc finger ccch domain-containing protein 14-like*	753	GSMUA_Achr7P20530_001	1E–116	100
GT086353	ubiquitin-conjugating enzyme-like protein	403	ACF06534	2.00E–31	94
Contig43	metallothionein-like protein	824	CAB52585	2.03E–32	89
GT153728	ribulose-bisphosphate carboxylase oxygenase large partial	263	BAJ08767	2.04E–42	100
GT153938	photosystem ii protein m	631	XP_002318750	2.10E–18	83
GT086377	ribulose bisphosphate carboxylase oxygenase activase*	458	GSMUA_Achr11P24320_001	2E–66	92
GT067862	catalase 2	654	ABG33767	2.12E–63	97
GT086358	photosystem ipsah protein*	338	GSMUA_Achr2P09140_001	2E–48	96
GT153727	allatotropin neuropeptide precursor	444	CAD98809	2.26E–07	75
Contig68	fructose-bisphosphate chloroplast	1414	ADD52195	8E–13	82
GT086380	carbonic anhydrase*	428	GSMUA_Achr3P31010_001	3E–34	81
GT067866	predicted protein [*Populus trichocarpa*]	290	XP_002338056	2.71E–23	100
GT067886	acrs-like protein	1084	XP_002863304	2.81E–27	91
Contig61	lipoxygenase [*Capsicum annuum*]	468	CAQ58078	2.90E–07	71
GT067860	Fructose-bisphosphate aldolase, chloroplastic*	661	GSMUA_Achr6P17900_001	1E–106	96
Contig78	retrotransposon ty1-copia subclass	463	AAC26250	2.99E–44	73
Contig19	pentatricopeptide repeat-containing protein	773	NP_001170726	2.99E–67	90
Contig31	cytochrome oxidase subunit i	723	XP_002804370	3.04E–64	84
GT086324	retrotransposon unclassified	337	CAD40278	3.13E–10	75

Table 2. (Continued).

Contig17	spinach plastocyanin	330	P35476	3.24E–11	88
GT086403	alanine glyoxylate aminotransferase	187	AEW69795	3.52E–25	92
GT086321	major cab protein*	255	GSMUA_ Achr4P07890_001	3E–24	92
GT153737	auxin response factor 8*	437	GSMUA_ Achr8P13630_001	2E–16	84
Contig33	pinustaeda anonymous locus 0_7614_01 genomic sequence	714	NP_001170726	4.59E–51	92
GT086303	h-protein	380	CAA85767	4.88E–63	93
Contig76	ribulose- -bisphosphate carboxylase oxygenase activase*	563	GSMUA_ Achr11P24320_001	1E–83	100
GT086369	cytochrome b6-f complex iron-sulfur subunit 2*	357	GSMUA_ Achr9P24590_001	5E–50	98
GT086375	fructose-bisphosphate aldolase	307	AEO33212	5.43E–55	95
GT153933	flavin-containing monooxygenase yucca	854	XP_002468464	6E–27	60
GT086305	tetracycline efflux protein*	434	GSMUA_AchrUn_ randomP25860_001	5.87E–30	75
GT153912	copper ion binding	354	XP_002321953	6.19E–07	71
GT153738	retrotransposon opie-2	668	CAN80699	6.30E–67	80
GT153758	chlorophyll a b-binding protein cp29	152	GSMUA_AchrUn_ randomP28100_001	2E–13	72
Contig35	plastocyanin a a	395	P35476	6.91E–10	81
GT086352	Actin	324	ADZ73581	7.42E–53	100
GT153934	apr protease precursor	1048	ZP_04979063	7.43E–12	97
GT086343	atp synthase beta subunit*	142	GSMUA_ Achr3P29100_001	3E–12	86
Contig50	dimethylaniline monooxygenase	845	XP_002468464	7.83E–11	85
GT086330	light-harvesting complex	180	EIE23074	7.83E–16	79
Contig69	Fructose-bisphosphate aldolase, chloroplastic*	1294	GSMUA_ Achr6P17900_001	1E–106	98
HS032641	chloroplast pigment-binding protein cp26	615	ABG73417	9.11E–04	59
GT067857	photosystem ii 32 kda protein	613	AAQ91580	9.11E–34	81
GT154760	Oryzain alpha chain*	402	GSMUA_ Achr3P03740_001	5E–11	40

4. Discussion

To compare the gene expression during *M. eumusae* interaction in ELSD resistant banana cv. Manoranjitham (AAA) and susceptible cv. Grand Naine (AAA), SSH was performed and a total of 498 differentially expressed unigenes were obtained. Contrasting cultivars were chosen with the same genetic composition (AAA) to reduce genome variations. Annotation of SSH derived ESTs revealed that nearly 32.32% of the genes hit with known biological functions. Our aim was to mainly focus on the defense genes and to characterize the resistance mechanism for ELSD. Within 72 hpi, in the resistant cultivar, we found that few transcripts encoded defense-related proteases and resistance-related transcription factors, while EST clones responsible for metabolism, photosynthesis, cell growth, and signal transduction were high. These six genes were selected based on their function for qPCR validation in which the expression of defense-related genes like *LOX,*

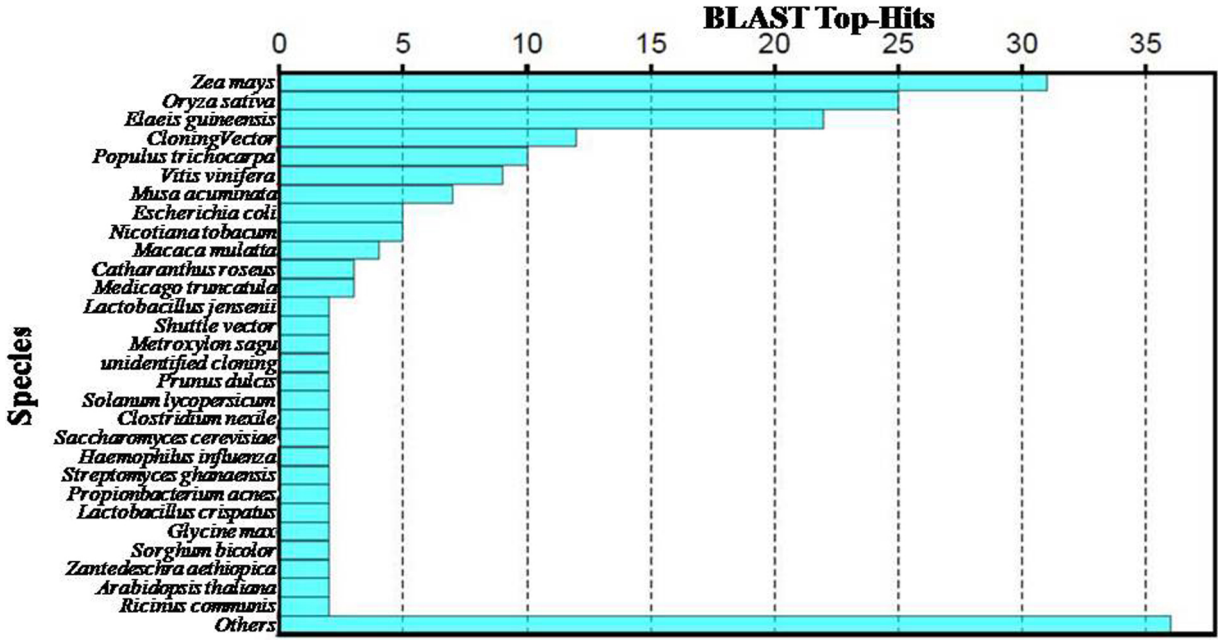

Figure 2. Species distribution of top-hit sequences of *Musa* cDNA-SSH library derived from *M. eumusae* infected banana cultivars.

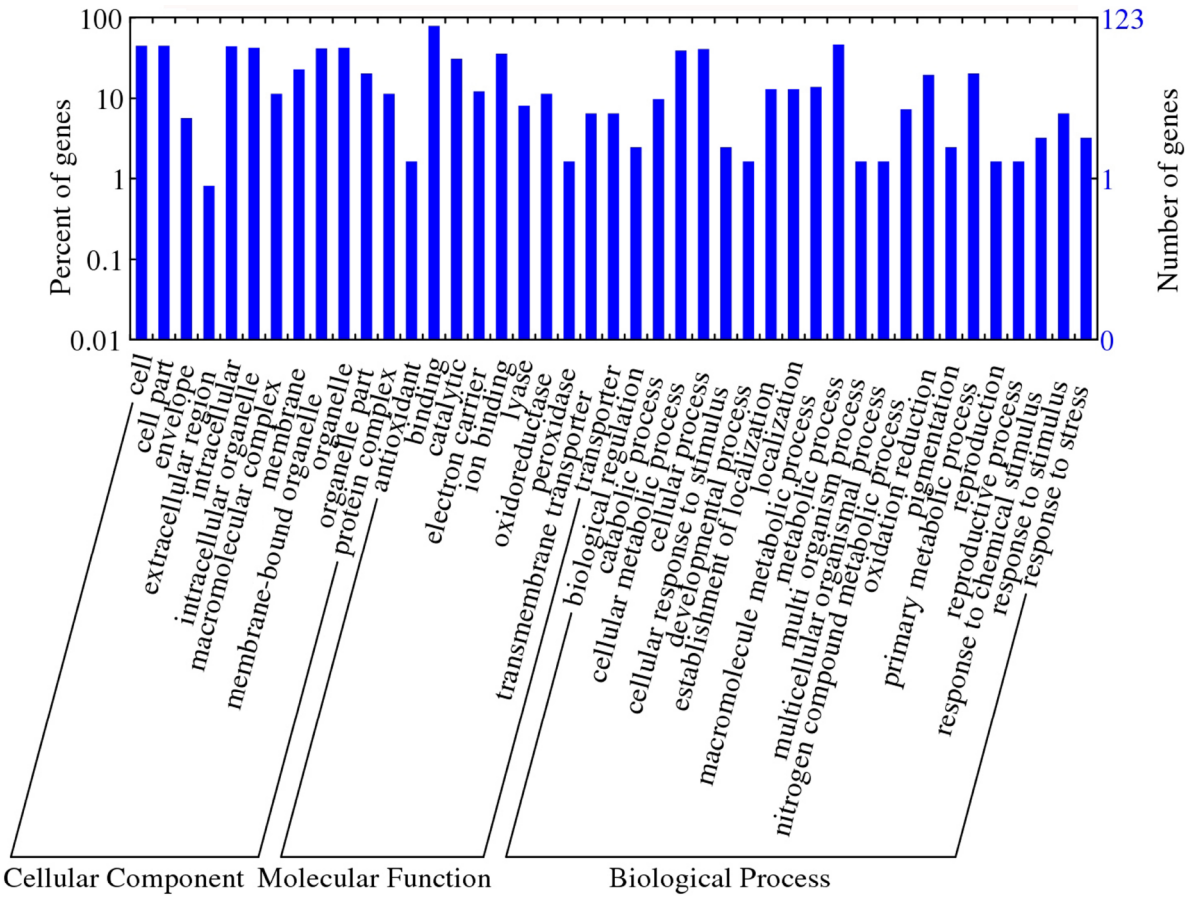

Figure 3. Gene ontology classification of *Musa* cDNA-SSH library derived from *M. eumusae* infected banana cultivars.

Table 3. Pathway of the genes derived from *Musa* cDNA-SSH library.

Sl no.	Pathway	Enzyme	Ezyme ID	Nonredundant sequences
1.	One carbon pool by folate	aminomethyltransferase	ec:2.1.2.10	1
2.	Tryptophan metabolism	catalase	ec:1.11.1.6	2
3.	Pentose phosphate pathway	fructose-bisphosphate aldolase	ec:4.1.2.13	4
4a.	Oxidative phosphorylation	ubiquinol-cytochrome-c reductase	ec:1.10.2.2	1
4b.		cytochrome-c oxidase	ec:1.9.3.1	5
5.	Drug metabolism cytochrome P450	flavin-containing monooxygenase	ec:1.14.13.8	2
6a.	Carbon fixation in photosynthetic organisms	fructose-bisphosphate aldolase	ec:4.1.2.13	4
6b.		ribulose-bisphosphate carboxylase	ec:4.1.1.39	3
7.	Fructose and mannose metabolism	fructose-bisphosphate aldolase	ec:4.1.2.13	4
8a.	Glyoxylate and dicarboxylate metabolism	serine-glyoxylate transaminase	ec:2.6.1.45	2
8b.		catalase	ec:1.11.1.6	2
8c.		ribulose-bisphosphate carboxylase	ec:4.1.1.39	3
9a.	Glycine, serine and threonine metabolism	serine-glyoxylate transaminase	ec:2.6.1.45	2
9b.		aminomethyltransferase	ec:2.1.2.10	1
10.	Purine metabolism	nucleoside-triphosphatase	ec:3.6.1.15	2
11.	Thiamine metabolism	nucleoside-triphosphatase	ec:3.6.1.15	2
12a.	Methane metabolism	flavin-containing monooxygenase	ec:1.14.13.8	2
12b.		serine-glyoxylate transaminase	ec:2.6.1.45	2
12c.		catalase	ec:1.11.1.6	2
12d.		fructose-bisphosphate aldolase	ec:4.1.2.13	4
13a.	Nitrogen metabolism	ubiquinol-cytochrome-c reductase	ec:1.10.2.2	1
13b.		carbonate dehydratase	ec:4.2.1.1	1
13c		cytochrome-c oxidase	ec:1.9.3.1	5
13d.		aminomethyltransferase	ec:2.1.2.10	1
14.	Glycolysis / Gluconeogenesis	fructose-bisphosphate aldolase	ec:4.1.2.13	4

ERF, and *MT* was maximal between 36 hpi and 48 hpi in the resistant cultivar.

Expression of hypersensitive response (HR)-related genes such as carbonic anhydrase (*CA*), *MT*, and catalases (*CAT*) was found to be induced during the resistance process. Hypersensitive response (HR) leads to the production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$) in the region surrounding the infection to limit the growth and spread of pathogens. HR has been reported as one of the factors in banana plant resistance against *M. musicola* and was followed by ROS detoxification (Passos et al., 2013). Additionally, the role of H_2O_2 has been shown to be an important defense mechanism by the *M. fijiensis* resistant cv. Calcutta 4 (Maria de Jesus et al., 2011). ROS homeostasis in cells is regained by the production of ROS scavenging antioxidant compounds and enzymes. The expression of oxidation-related genes such as *MT*-like protein type 3 that function as scavengers of ROS is involved in blocking oxidant-mediated programmed cell death by converting H_2O_2 into hypochlorite (HOCl) (Barcelo and Laura, 2009). Metallothionein also protects against ROS-induced DNA degradation (Zinoveva et al., 2001). Upregulation of *MT* in the resistant cultivar is suggestive of its involvement in a complex balance of oxide concentration. Activation of genes like *CA* was previously shown to exhibit antioxidant activity in the HR as a part of plant defense and interestingly in tobacco *CA* is a salicylic acid binding protein that can activate salicylic acid-based systemic acquired resistance (Slaymaker et al., 2002). Moreover, ROS scavenging enzyme *CAT* was found to be induced in resistant plants that can convert H_2O_2 into H_2O and $1/2O_2$ (Das and Roychoudhury, 2014). A similar kind of *CAT*-dependent resistance mechanism was reported in a *M. fijiensis* resistant banana cultivar (Beltran-Garcia et al.,

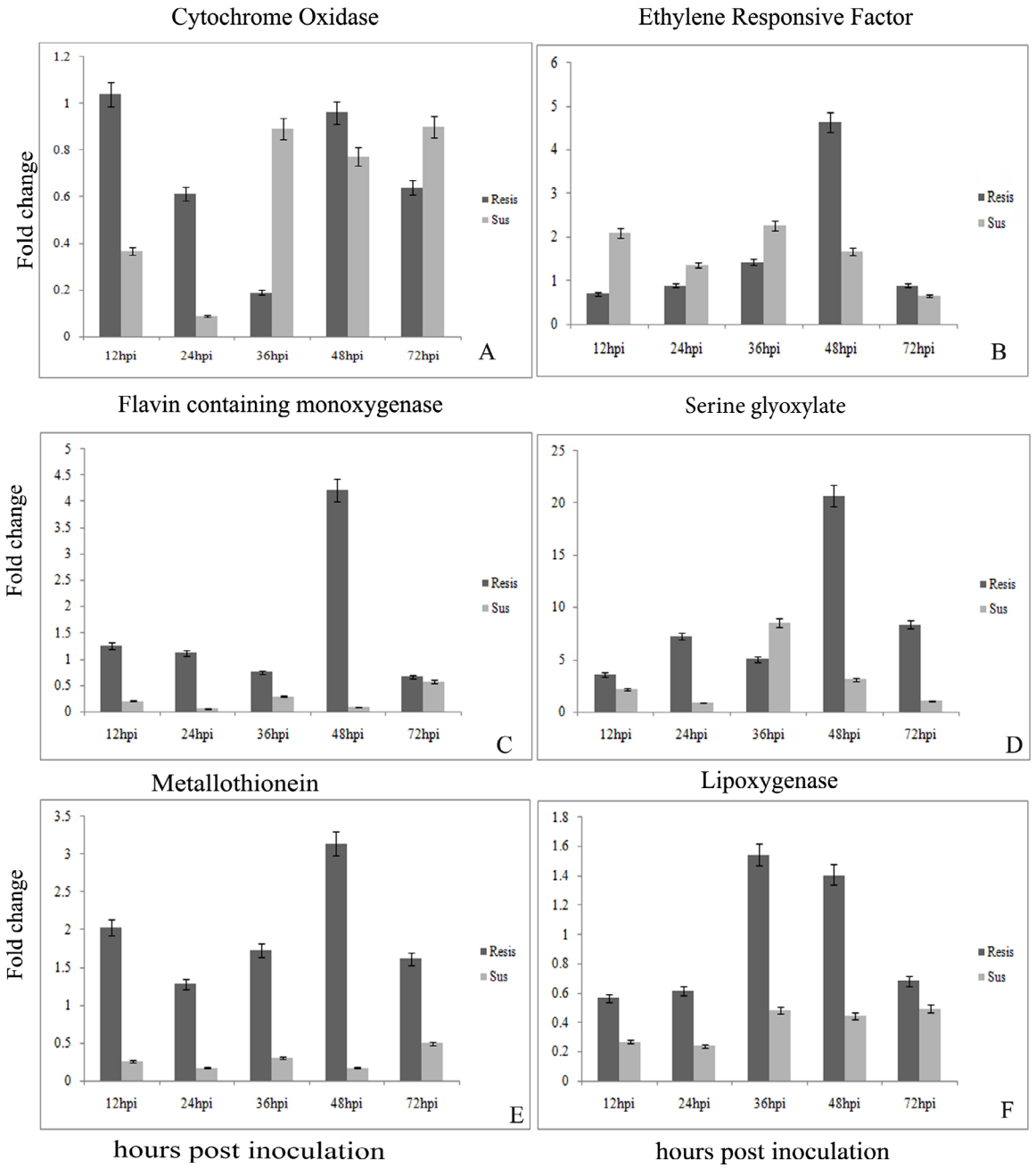

Figure 4. qPCR analysis of banana defense genes in *M. eumusae* challenged and unchallenged resistant and susceptible cultivars (at different time points 12, 24, 36, 48, and 72 hpi). Cytrochrome oxidase (A), Ethylene responsive factor (B), Flavin containing oxygenase (C), Serine glyoxylate (D), Metallothionein (E), and Lipoxygenase (F). RPS2 was used as internal reference.

2009). An early stimulation of ROS scavengers had been associated with pathogen resistance in *Musa* by assisting host cells to recover from the stress condition caused by ROS release against mycotoxins like juglone (5-hydroxy-1,4-naphthoquinone), a pathogenicity factor secreted by *M. fijiensis* (Hadrami et al., 2005). Thus, it appears that *M. eumusae* is attacked by ROS and the recovery of plant cells from ROS damaging effects includes balancing of intracellular redox potential and oxygen detoxification. Proteases have been implicated at the level of perception of the pathogen and defense activation signaling (Van der Hoorn and Jones, 2004). We also found expression of proteases such as Protease Do-Like 1 (serine-protease) and cysteine proteases like Vignain, Oryzain, and

cysteine protease 1 in the resistant plants upon infection. Interactions between ROS and phytohormones like auxin, salicylic acid, gibberellin, and ethylene had been previously reported. Induction of YUCCA *FMO*, an enzyme that catalyzes the rate-limiting step in auxin biosynthesis, indicates the involvement of auxin in the resistance process. Auxin biosynthesis is required for cell viability, cell cycle progression, and growth. This phytohormone may assist in the inhibition of programmed cell death caused by HR and also to recover from the adaptive modulation in plant development during pathogen stress (Xia et al., 2005). Overexpression of *ERF* in the resistant cultivar under challenged condition revealed that it plays a major role in the *Musa* defense mechanism. Involvement of *ERF* in the resistant mechanism against *Botrytis cinerea* and *Plectosphaerella cucumerina* has been reported in *Arabidopsis* (Berrocal-Lobo et al., 2002). Lorenzo et al. (2003) also have shown that *ERF* is a key factor in the expression of ethylene/jasmonate responsive genes and it is a downstream component of ethylene signaling (Solano et al., 1998). Similarly, transcriptome studies in plants resistant to *M. musicola* and *M. fijiensis* have shown induction of transcription factors of the *ERF* families and upregulation of genes involved in biosynthesis of ethylene after pathogen challenge. However, the actual role of ethylene in *M. eumusae* resistance is not known. Induction of *LOX* genes during plant–pathogen interactions has been reported in several plant species and high expression of *LOX* was observed in the resistant cultivar at 36 hpi. *LOX*s are well known for their role in the biosynthesis of many oxilipins and jasmonic acid, a defense signaling phytohormone.

Our SSH results were similar to those of a study conducted on *M. fijiensis* resistant banana cv. DH Pahang with very few upregulated genes (D'Hont et al., 2012) and most of them accounting for carbohydrate metabolism. In summary, the plants recognized the fungal phytotoxins and triggered a ROS burst and induced defense signaling pathways. The main findings are that resistant plants deploy HR activity as a strategy to evade the fungus followed by the activation of various ROS scavengers to maintain homeostasis. We have also shown that phytohormones may be induced as a part of plant defense against *M. eumusae*. However, this study lacked information on the pathogen suppression of host immunity. A detailed analysis of expression of various defense genes will help us to understand the defense signaling network and currently work is in progress for in-depth transcriptional profiling using the next generation sequencing method.

Acknowledgment

We would like to thank the Indian Council of Agricultural Research (ICAR) under the Network Project on Transgenics in Crops (NPTC), for the financial support, and Director, ICAR - National Research Centre for Banana (NRCB), for the facilities provided to conduct this study.

References

Arzanlou M, Abeln EC, Kema GH, Waalwijk C, Carlier J, Vries I, Guzmán M, Crous PW (2007). Molecular diagnostics for the sigatoka disease complex of banana. Phytopathology 97: 1112-1118.

Aguilar-Barragan A, García-Torres AE, Odriozola-Casas O, Macedo-Raygoza G, Ogura T, Manzo-Sánchez G, James AC, Islas-Flores I, Beltrán-García MJ (2014). Chemical management in fungicide sensitivity of *Mycosphaerella fijiensis* collected from banana fields in México. Braz J Microbiol 45**:** 359-364.

Backiyarani S, Uma S, Arunkumar G, Saraswathi MS, Sundararaju P (2014). Differentially expressed genes in incompatible interactions of *Pratylenchus coffeae* with *Musa* using suppression subtractive hybridization. Physiol Mol Plant P 86: 11-18.

Barcelo AR, Laura VGR (2009). Reactive oxygen species in plant cell walls. In: Rio LA, Puppo A, editors. Reactive Oxygen Species in Plant Signaling. Berlin, Germany: Springer, pp. 73-93.

Beltran-Garcia MJ, Manzo-Sanchez G, Guzman-Gonzalez S, Arias-Castro C, Rodriguez-Mendiola M, Avila-Miranda M, Ogura T (2009). Oxidative stress response of *Mycosphaerella fijiensis*, the causal agent of black leaf streak disease in banana plants, to hydrogen peroxide and paraquat. Can J Microbiol 55: 887-894.

Berrocal-Lobo M, Molina A, Solano R (2002). Constitutive expression of ethylene-response-factor 1 in *Arabidopsis* confers resistance to several necrotrophic fungi. Plant J 29: 23-32.

Binks RH, Greenham JR, Luis JG, Gowen SR (1997). A phytoalexin from roots of *Musa acuminata* var. Pisang sipulu. Phytochemistry 45: 47-49.

Carlier J, Zapater MF, Lapeyre F, Jones DR, Mourichon X (2000). Septoria leaf spot of banana: a newly discovered disease caused by *Mycosphaerella eumusae* (anamorph *Septoria eumusae*). Phytopathology 90: 884-890.

Chen L, Zhong HY, Kuang JF, Li JG, Lu WJ, Chen JY (2011). Validation of reference genes for RT-qPCR studies of gene expression in banana fruit under different experimental conditions. Planta 234: 377-390.

Conesa A, Götz S, Garcia-Gomez JM, Terol J, Talon M, Robles M (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21: 3674-3676.

Crous PW, Mourichon X (2002). *Mycosphaerella eumusae* and its anamorph *Pseudocercospora eumusae* spp. nov.: causal agent of eumusae leaf spot disease of banana. Sydowia 54: 35-43.

D'Hont A, Denoeud F, Aury JM, Baurens FC, Carreel F, Garsmeur O, Noel B, Bocs S, Droc G, Rouard M et al. (2012). The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. Nature 488: 213-217.

Das K, Roychoudhury A (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front Environ Sci 2: 1-13.

Exotic Pest Alert: Eumusae leaf spot (2014). Primefact 1329, 1st edition Plant Biosecurity & Product Integrity, Orange.

Food and Agriculture Organization of the United Nations, FAOSTAT database (2013), available at http://faostat3.fao.org/browse/Q/*/E.

Gasic K, Hernandez A, Korban SS (2004). RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. Plant Mol Biol Rep 22: 437a-437g.

Hadrami AE, Kone D, Lepoivre P (2005). Effect of juglone on active oxygen species and antioxidant enzymes in susceptible and partially resistant banana cultivars to Black Leaf Streak Disease. Eur J Plant Pathol 113: 241-254.

Hidalgo W, Jima NC, Riya CM, Felipe O, Bernd S (2015). Phenylphenalenones protect banana plants from infection by *Mycosphaerella fijiensis* and are deactivated by metabolic conversion. Plant Cell Environ. doi: 10.1111/pce.12630.

Hölscher D, Dhakshinamoorthy S, Alexandrov T, Becker M, Bretschneider T, Buerkert A, Crecelius AC, De Waele D, Elsen A, Heckel DG et al. (2014). Phenalenone-type phytoalexins mediate resistance of banana plants (*Musa* spp.) to the burrowing nematode *Radopholus similis.* P Natl Acad Sci USA 111: 105-110.

Jones RD (2002). The distribution and importance of the Mycosphaerella leaf spot diseases of banana. Proceeding. 2nd International. Workshop on Mycosphaerella Leaf Spot Disease of Bananas. INIBAP, Montpellier, France: 25-42.

Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell 15: 165-178.

Luis JG, Lahlou EH, Andrés LS, Echeverri F, Fletcher WQ (1997). Phenylphenalenonic phytoanticipins. New acenaphtylene and dimeric phenylphenalenones from the resistant *Musa* selected hybrid SH-3481. Tetrahedron 53.

Luis JG, Quinones W, Echeverri F, Grillo TA, Kishi MP, Garcia-Garcia F, Torres F, Cardona G (1996). Musanolones: Four 9-phenylphenalenones from rhizomes of Musa acuminata. Photochemistry 41: 753-757.

Maria de Jesus BC, Jacques E, Jean PM, Rocio ER, Michel RN, Luis CO, Chantal H, Marc L, Jean LV (2011). Reactive oxygen species and cellular interactions between *Mycosphaerella fijiensis* and banana. Trop Plant Biol 4: 134-143.

National Horticulture Board – NHB (2013), available at http://www.nhb.gov.in.

Passos MA, de Cruz VO, Emediato FL, de Teixeira CC, Azevedo VC, Brasileiro AC, Amorim EP, Ferreira CF, Martins NF, Togawa RC et al. (2013). Analysis of the leaf transcriptome of *Musa acuminata* during interaction with *Mycosphaerella musicola*: gene assembly, annotation and marker development. BMC Genomics 14: 78.

Portal O, Izquierdo Y, De VlD, Sánchez RA, Mendoza RM, Acosta SM, Ocaña B, Jiménez E, Höfte M (2011). Analysis of expressed sequence tags derived from a compatible *Mycosphaerella fijiensis*-banana interaction. Plant Cell Rep 30: 913-28.

Rivas GG, Zapater MF, Abadie C, Carlier J (2004). Founder effects and stochastic dispersal at the continental scale of the fungal pathogen of bananas *Mycosphaerella fijiensis*. Mol Ecol 13: 471-482.

Slaymaker DH, Navarre DA, Clark D, Del Pozo O, Martin GB, Klessig DF (2002).The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defense response. P Natl Acad Sci 99: 11640-11645.

Solano R, Stepanova A, Chao Q, Ecker JR (1998). Nuclear events in ethylene signaling: A transcriptional cascade mediated by ethylene-insensitive3 and ethylene response- factor1. Gene Dev 12: 3703-3714.

Stierle AA, Upadhyay R, Hershenhorn J, Strobel GA, Molina G (1991). The phytotoxins of *Mycosphaerella fijiensis*, the causative agent of Black Sigatoka disease of bananas and plantains. Experientia 47: 853-859.

Swarupa V, Ravishankar KV, Rekha A (2013).Characterization of tolerance to *Fusarium oxysporum* f.sp., cubense infection in banana using suppression subtractive hybridization and gene expression analysis. Physiol Mol Plant P 83: 1-7.

Thangavelu R, Ganga Devi P, Gopi M, Mustaffa MM (2013). Management of Eumusae leaf spot disease of banana caused by *Mycosphaerella eumusae* with Zimmu *Allium Sativum x Allium cepa*) leaf extract. Crop Prot 46: 100-105.

Van Den Berg N, Berger DK, Hein I, Birch PR, Wingfield MJ, Viljoen A (2007). Tolerance in banana to Fusarium wilt is associated with early up-regulation of cell wall-strengthening genes in the roots. Mol Plant Pathol 8:333-41.

Van der Hoorn RA, Jones JD (2004).The plant proteolytic machinery and its role in defense. Curr Opin Plant Biol 7: 400-407.

Xia QZ, Zhang XL, Nie YC, Guo XP (2005). Withdrawal of exogenous auxin induces programmed cell death of cotton embryonic suspension cultures. Journal of Plant Physiology and Molecular Biology 31: 78-84.

Zinoveva SV, Udalova ZV, Vasileva IS, Vanyushkin SA, Paseshnichenko VA (2001). Role of isoprenoids in plant adaptation to biogenic stress induced by parasitic nematodes. Appl Biochem Micro 37: 456-463.