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RESEARCH ARTICLE

Biochemical Profile of Transgenic Banana cv. Rasthali (*Musa spp.*) Engineered for Resistance Against Fusarium Wilt

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ABSTRACT

The biochemical and metabolic profiles of transgenic banana (*Musa spp.*) plants developed through RNAi technology for expressing resistance to *Fusarium oxysporum f.sp. cubense* race 1 were characterized by using gas chromatography coupled to electron impact ionization-time of flight-mass spectrometry (GC/EITOF-MS). A total of twenty-two metabolites were identified and annotated using the NIST data library. Several resistance-related metabolites such as 13-docosenamide, 9-octadecenamide, 9-octadecene, n-hexadecanoic acid, oleic acid, 1,2-benzenedicarboxylic acid, 2,4,6-decatrienoic acid, 1 bis (2-ethylhexyl) phthalate, methyl abietate, undecanedioic acid, 2-pentadecanone, methyl palmitate and phenols were detected in transgenic plants which were generated to silence two fungal genes *viz.,* ftf1 and/or velvet through RNAi technology. The induction of defense-related enzyme activities such as phenylalanine ammonia-lyase (PAL), superoxide dismutase (SOD), peroxidase (POD) and polyphenol oxidase (PPO) was also observed. The defense-related enzyme activities were higher in transformed plants than in untransformed plants. However, these differences did not cause unintended changes in the growth of the transgenic banana plant.

Keywords: *Transgenic banana; Ftf1 gene; Velvet gene; RNAi; Fusarium oxysporum f.sp. cubense race 1; GC/EITOF-MS*

INTRODUCTION

Fusarium wilt of banana, popularly known as Panama disease, is a severe fungal disease caused by the soil-borne fungus *Fusarium oxysporum f.sp. cubense* (Foc) that significantly affects banana production in India. The fungus enters the plant through the roots, colonizing the xylem vessels and thereby blocking water and nutrients flow. Disease progression results in the collapse of leaves at the petiole, the splitting of the pseudostem base and eventually, plant death (Ploetz, 2015; Dita *et al.,* 2018). Different races of the pathogen were identified based on the pathogenicity to reference host cultivars: race 1 (R1) affects Gros Michel (AAA) and Manzano/Apple/Latundan (Silk, AAB); race 2 (R2) affects cooking bananas of the Bluggoe (ABB) subgroup; race 3 (R3) affects Heliconia spp., and race 4 (R4) affects all cultivars in the Cavendish (AAA) subgroup in addition to those susceptible to R1 and R2 (Waite and Stover, 1960). Currently, all commercial banana cultivars in India are susceptible to Fusarium wilt, and it is difficult to manage the disease using chemical fungicides. A sustainable solution for producing bananas in infected soils is replacing susceptible cultivars with resistant ones. Due to the lack of suitable Fusarium wilt resistant resources, transgenic resistance by exploiting the concept of post-transcriptional gene silencing (PTGS) has been employed to manage the disease (Bag *et al.,* 2007; Mehta *et al.,* 2013).

Metabolomics as a biochemical tool in systems biology has the potential application not only for gene function elucidation but also as a tool to screen for disease resistance. As with genomics,

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transcriptomics, and proteomics studies, metabolomics generates large data sets of metabolites (Razzaq *et al.,* 2019). Identifying a set of metabolites could explain the functions associated with a trait or gene. Plant extracts usually comprise many secondary metabolites like terpenoids, saponins, alkaloids phenolics, flavonoids and glycosides, which possess strong antifungal effects. Several higher plants and their constituents have been reported to control a number of pathogens that cause severe plant diseases (Elzaki *et al.,* 2012; Munawwar *et al.,* 2018; Khan *et al.,* 2018; Bashir *et al.,* 2019 and Hanif *et al.,* 2017). The identification of metabolites is very important for understanding the physiology of plants, including plant growth, development, and defense mechanisms which have been elucidated clearly in transgenic Nicotiana tabacum (Lucas *et al.,* 2003, 2006) and transgenic groundnut (Shomo *et al.,* 2016) against viral and fungal diseases. Research evidence showed that the level of expression of phenylalanine ammonia-lyase (PAL3) and phenol oxidising enzymes increased in cavendish Brazilian banana when infected with Foc TR4 strain compared with non-virulent Foc strain TR1 (Chittor *et al.,* 1999; De Ascensao and Dubery, 2000 and Li *et al.,* 2013). Employing RNAi technology, transgenic banana lines (cv. Rasthali) harboring two different RNAi constructs targeting fusarium transcription factor1 (ftf1) and velvet gene of Foc were generated (unpublished). Analysis of these transgenic lines for Foc 1 resistance revealed that transgenic lines expressed better resistance than the untransformed control lines. In the present study, the metabolic profiling of transgenic banana plants was compared with that of un-transformed control by using gas chromatography coupled to electron impact ionization time of flight mass spectrometry (GC/EI-TOF-MS). Besides, the activity of stress-related enzymes was also determined in transgenic banana to understand if there were any significant differences in the biochemical profile of transgenic banana compared to its non-transgenic counterpart.

MATERIAL AND METHODS

Plant material

Ten transgenic Rasthali plants (harboring RNAi construct of ftf1 or ftf1+velvet RNAi), developed in our lab were used in this study. These plants were generated through Agrobacterium mediated transformation using RNAi constructs with a view to silence fungal genes, fusarium transcription factor 1 (ftf1) or ftf1 and velvet genes. These plants were found to exhibit resistance against *Fusarium oxysporum f.sp. cubense* (Foc1) race 1 (6 months post-inoculation) in an earlier study (results not published). Five plants in each of these constructs were selected based on disease severity score and PCR assays. These plants were grown in the transgenic greenhouse under controlled conditions along with untransformed plants.

Fungal strain and virulence assay

Highly virulent Foc race 1 (strain KP) was used in this study. Mycelial disc of Foc race 1 was multiplied in PDA medium. A nine mm disc of Foc race 1 was inoculated into sterile Potato Dextrose Broth and incubated in an orbital rotary shaker at 28±2 ^{o}C (150 rpm) for five days. Subsequently, the suspension was homogenized, and the conidial load was adjusted to 106 colony-forming unit (cfu) mL^{-1}. This suspension was used to challenge both transgenic and non-transgenic plants. Six months old Fusarium resistant transgenic Rasthali plants together with untransformed plants were used for challenge inoculation with Foc 1. Prior to inoculation, plants were removed from pots; roots were trimmed to a length of ~40 cm and were individually inoculated by dipping the roots for 30 min in the conidial suspension comprising of 106 conidia mL^{-1}. The plants were incubated for 28 days in a transgenic greenhouse maintained at 28 °C. The disease symptoms were recorded at 0 h, 24 h, 48 h, 72 h and 28 days of post-inoculation. For resistance evaluation, the standard evaluation method of Perez-Vicente et al. (2014) was adopted.

Sample preparation for metabolite assay using GC/EITOF-MS

Root samples (young roots or newly emerged roots) from the resistant transgenic plants and untransformed plants (cv. Rasthali) infected with Foc race 1 were collected after 72 h of inoculation and stored at -80 °C until metabolite extraction. Polar primary metabolites were extracted from the root tissues and then subjected to derivatization by following the procedure described by Erban et al. (2007). Root samples were ground to a fine powder using a pre-chilled mortar and pestle. The root powder (120 mg) was transferred to 2 mL round bottom microcentrifuge tubes, and 1.4 mL of 100 % methanol and 60 µL of internal standard ribitol were added and mixed well by vortexing. The mixtures were incubated at 70 °C for 10 min on a rotary orbital shaker at 150 rpm and then centrifuged at 11,000 x g for 10 min. To the supernatant, 750 µL of chloroform and 1.4 mL of double distilled water were added and vortexed for uniform mixing. The mixture was centrifuged at 2,200 x g for 15 min and 1 mL of the supernatant was transferred to 1.5 mL of microcentrifuge tube and concentrated in a vacuum concentrator. The dried samples were then stored at -70 °C until derivatization. For derivatization, 300 µL of methoxy amination reagent (5 mg of 4- dimethyl aminopyridine and 40 mg of methoxy amine hydrochloride dissolved in 1 mL of pyridine) was added to the micro centrifuge tube and shaken at 37 °C for 2 h. The sample was vortexed every 30 min

during 2 h of shaking to dissolve the sample completely in the reagent. After methoxy-amination, the samples were centrifuged at 10,000 x g for 1 min and 40 µL of the supernatant was transferred to 1.5 mL microcentrifuge tube and 70 µL of N, O-Bis (trimethylsilyl) trifluoro acetamide (BSTFA) and 10 µL of retention time standards (n-alkanes) were added to the sample and shaken at 37 °C for 30 min. The n-alkane mixture was prepared by using pyridine at a final concentration of 0.22 mg mL^{-1} each. The samples were then centrifuged at 10,000 x g for 1 min and 95 µL of the supernatant was transferred to GC-MS glass vial for analysis.

Gas chromatography and mass spectrometry (GC-MS) analysis

The sample was injected into column Elite 5MS (100% Dimethyl poly siloxane), 30 x 0.25 mm x 0.25 µm equipped with GC Clarus 500 (Perkin Elmer). The turbo Mass-Gold-Perkin-Elmer detector was used. The carrier gas flow rate was 1 mL min^{-1}, split 10:1, and injected volumes were 3 µL. The column temperature was maintained initially at 110 °C at the rate of 10 °C min-1 no hold followed by an increase up to 280 °C at the rate of 5 °C/min to 9 min (hold). The injector temperature was 250 °C, which was held constant for 36 min. The electron impact energy was 70 eV, julet line temperature was set at 2000 °C and the source temperature was set at 200 °C. Electron impact (EI) mass scan (m/z) was recorded in the 45-450 amu (atomic mass unit) range. An ion mass spectrometer and OMA detector were used to monitor the eluted compounds from 10 to 30 min (total analysis time 35min).

Data processing and identification of metabolites

The unprocessed raw data from the GC/MS was converted to a readable format by AMDIS (Automated Mass Spectral Deconvolution and Identification System Version 2.64). The AMDIS software was linked to the NIST (National Institute of Standards and Technology) MS library (Version 06) (Kopka *et al.,* 2005; Erban *et al.,* 2007) to determine the best mass spectral matches and the poor matches (<60%) were considered unidentified. The output data from GC/MS scans, the mass ions and their areas were processed by running the AMDIS software. Each metabolite across the replicates was manually verified for consistency using retention time as a reference. Compound structures were putatively identified and evaluated by comparing the molecular masses (m/z values) of the eluted compounds with literature data and standards.

Assessment of defense related enzymes activity

The samples from the most recent completely expanded leaf of the infected plants were used for biochemical analysis. The phenylalanine ammonia lyase enzyme activity was carried out at 24 h intervals after Foc1 inoculation. Meanwhile, peroxidase, polyphenol oxidase and superoxide dismutase activities were measured at 0 h and 28 days after inoculation. All biochemical parameters were measured using a spectrophotometer (Jasco V-730 BIO spectrophotometer, USA).

Phenylalanine ammonia lyase assay (PAL)

PAL was assayed according to the method of Robert and Helmut (1992). The absorbances of the reaction mixture of the samples were measured at 290 nm with its corresponding control at every 30 min intervals. PAL activity was expressed as changes in absorbance at 290 nm h^{-1} mg^{-1} protein.

Peroxidase assay

Peroxidase activity was assessed following the oxidation of pyrogallol according to the method given by Hammerschmidt et al. (1982). The absorbance was recorded at 430nm at 30 sec intervals up to 3 min. Peroxidase activity was expressed in unit mg-1 protein. A change in 0.1 absorbance min^{-1} mg^{-1} protein refers as one unit.

Polyphenol oxidase assay

Polyphenol oxidase activity was determined according to the method described by Mozzetti et al. (1995). The activity was measured by monitoring the increase in absorbance for 3 min at 410 nm. The specific activity was expressed in unit mg^{-1} protein. Like peroxidase, a change in 0.1 absorbance min^{-1} mg^{-1} protein refers to one unit.

Superoxide Dismutase (SOD)

The SOD activity was estimated by following the method described by Du and Bramlage (1994). The inhibition of photochemical reduction of nitro blue tetrazolium (NBT) was measured at 560 nm and expressed in units g^{-1} fresh weight. The amount of enzyme inhibiting the NBT photo reduction by 50% was one unit of SOD activity.

Statistical analysis

Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple-range test. All statistical analyses were performed at 5% using SPSS 10.0 (SPSS Inc.,).

Results and Discussion

Bioassay of transgenic banana expressing ftf1 and ftf1 + velvet gene sequences

The selected Foc 1 resistant transgenic banana plants derived from the two RNAi constructs were subjected to Foc race 1 infection. Fusarium wilt symptoms were observed in untransformed control banana plants only after 28 days of inoculation. Infected plants were characterized with yellowing of older leaves coupled with splitting of the pseudostem. Subsequently, infected plants wilted completely after 6 weeks.

However, there was no visible symptom on non-inoculated transgenic plants after 28 days of post-inoculation. Subsequently, few transgenic plants (FTF-289, 335; FTF+VELVET (F+V) - 51, 116) exhibited yellowing of older leaves and pseudostem splitting, whereas the plants *viz.,* FTF-215; FTF+VELVET-93, 97 were free of Foc symptoms at 28 days post inoculation (DPI) (Figure 1).

Figure 1. Bioassay of the transgenic banana plants for Foc resistance at 28 days post inoculation

a) UC 1- Rasthali control (non-inoculated), UC 2- Rasthali control (inoculated), UC-3 Grand naine control (inoculated), ftf1 plants and b) UC 1- Rasthali control (non-inoculated), UC 2- Rasthali control (inoculated), UC-3 Grand naine control (inoculated), ftf1+velvet plants (UC-untransformed control)

As shown in Figure 2, results from the disease incidence among transgenic plants varied between 10 and 80% from both the construct-derived plants. Ghag et al. (2014) reported that the differences in disease resistance of Rasthali transgenic plants with wild type against Foc in banana could depend on genotype, environmental conditions and virulence of the fungal strain.

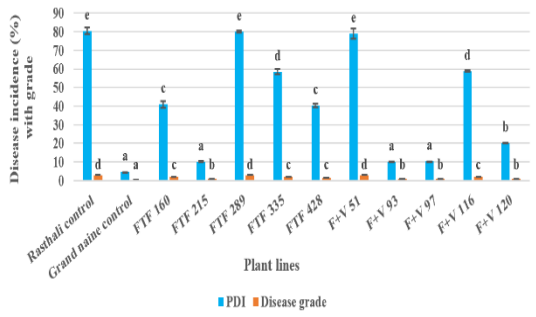


Figure 2. Disease incidence and disease grade of transformed Rasthali plants against Foc race 1 (28 DPI). Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Values indicated in superscripts (a-e) are significantly different ($p<0.05$)

Metabolite compounds in transgenic banana

Fungal resistance in various crop plants has been successfully achieved by transforming the host plant with genes derived from fungus. The entire or partial gene sequences of the pathogen (both structural and functional) are used to transform host plants to generate dsRNA in order to protect them against the pathogen or a range of closely related pathogens (Baulcombe, 1996). This strategy has been employed to protect crop plants against several genera of fungus in various plant species (Galvez *et al.,* 2014). Detailed analysis of transgenic plants revealed that resistance in plants is primarily due to transgene-induced post-transcriptional gene silencing (PTGS) via the formation of dsRNA, termed as "RNA silencing" (Baulcombe, 2002). In the present study, the metabolic profile of transgenic Rasthali banana plants transformed with gene construct encoding dsRNA ftf1 and/or velvet gene sequence were compared with untransformed counterparts against Foc race 1.

The samples of resistant transgenic plans (FTF-215, FTF1+VELVET-97) and susceptible wild type plant after 72 h of inoculation with Foc race 1 were chosen for metabolite analysis based on the disease severity score obtained 28 DPI. The parameters such as retention time, molecular formula, area percentage, and biological activity of different compounds were identified by the comparison of their mass spectra against the NIST library database (Table 1). The peak area represented a quantitative proportion of the predicted compound to the total of crude extracts. These compounds with antifungal actions were related to various chemical classes, including esters, fatty acids, aldehydes, tertiary amines, alkaloids, and ketones. Among these bioactive compounds obtained, transgenic plants FTF-215 and F+V-97 had significant area (per cent) in comparison with control plants, indicating that they played a substantial role in antifungal activities. Results obtained on the GCMS analysis of the transgenic and untransformed banana plants are presented in Figure 3 a, b, c.

Several resistance-related metabolites were consistently detected in both transgenic and untransformed plants. The differentially expressed bioactive metabolites during the interaction of transgenic plants with Foc1 KP revealed that transgenic plants produced seven unique bioactive metabolites such as 1,2-benzenedicarboxylic acid, undecanedioic acid, 2-pentadecanone, bis (2-ethylhexyl) phthalate, methyl palmitate, methyl abietate, and 2,4,6-decatrienoic acid exclusively in the transgenic plant (Table 1).

Likewise, Rasthali wildtype with Foc KP infection induced secretion of secondary metabolites *viz.,* octadecanoic acid methyl ester, eicosane, heptadecane, 2,6-dimethyl, 4,6-dimethyldodecane, trioxsalen, hexanoic acid and butanamide. Comparison

of bioactive metabolites between Rasthali wild type with Foc KP and ftf1 or ftf1+velvet with Foc KP revealed the production of 15 bioactive metabolites in common *viz.,* 9-octadecenoic acid, octadecane, 2-ethyl-3-methylpyrazine, 2-methyl-5,6 diethyl pyrazine, phenol 2,4-bis (1,1-dimethlethyl)-4-methyl, octadecanoic acid, 1-ascorbic acid, n-hexadecanoic acid, 1-butanone, 9-octadecenamide, ethyl iso-allocholate, 9-octadecene, lauric acid and oleic acid (Figure 3 a, b, c). In this study, a volatile resin, 2-pentadecanone detected exclusively in transgenic plants exhibiting a higher peak area percentage of 8.92, thereby indicating that this metabolite plays a potential role in plant defense by showing antifungal activity similar to other fungal pathogens such as *Aspergillus sp., Fusarium oxysporum, Pythium sp*., and *R. solani* in beans (Hanif *et al.,* 2022).

The metabolite, 13-docosenamide or erucamide was detected at a retention time of 25.45 min with 2.66 % area increase in transgenic plants. Generally, it acts as anti-inflammatory, dermatigenic, hypocholesterolemic and anaemiagenic (Li *et al.,* 2017). The antifungal mechanism of erucamide, destroy cell structure and inhibit the germination and growth of fungal spores (Makarieva *et al.,* 2002). Enhanced disease resistance in plants induced by erucamide has been demonstrated against Foc TR4 in banana (Qi *et al.,* 2019). The metabolite, 9-octadecenamide, expressed in transgenic Rasthali expressing siRNA specific to ftf1 and velvet has been shown to have antifungal activity against *Botrytis cineria* and *Macrophomina Phaseolina* (Khan *et al.,* 2022) in soybean. Dodecanoic acid (Lauric acid), a saturated fatty acid was detected at a retention time of 28.52 min in transgenic plants (2.25 %), and is known to exhibit antifungal activity against Foc. It also exhibits antimicrobial activity against various bacteria and antifungal activity against *Pythium ultimum, Rhizoctonia solani, Blumeria, Aspergillus* and *Fusarium spp*. (Walters *et al.,* 2003; Liu *et al.,* 2008).

The metabolite, 2-methyl-5,6 diethyl pyrazine, which occurs in much larger amounts in bacteria and in some plants was detected at a retention time of 9.25 in transgenic plants with an area percentage of 6.05 (ftf1) and 6.25 (ftf1+velvet). The antifungal activity of the metabolite was reported earlier against *Fusarium solani, Fusarium sp.,* and *Colletotrichum gloeosporioides* (Edgar *et al.,* 2019). Rahman and Anwar (2006) reported the broad-spectrum antifungal activity of 1,2-benzenedicarboxylic acid against six phytopathogenic fungi *viz., Alternaria alternata, Botryodiplodia theobromae, Curvularia lunata, Fusarium equiseti, Macrophomina phaseolina* and *Colletotrichum corchori*. Similarly, the metabolite bis (2-ethylhexyl) phthalate exhibited antimicrobial, antioxidant and antifungal activities against unicellular and filamentous fungi like *Fusarium oxysporum* (Zishan *et al.,* 2020). The antifungal activity of 1-ascorbic acid which transgenic plants produce was reported by Bokhari et al. (2013) against *Fusarium oxysporum, Alternaria alternata, Macrophomina phaseolina* and *Colletotrichum* in *Citrullus sp.* Shobier et al. (2016) reported the antibacterial and antifungal activities (Aspergillus spp., Fusarium sp.,) of methyl palmitate that damages microbial cellular membranes in various crops. Likewise, methyl abietate possessing high antioxidant activity, exhibited antibacterial activity against *E. coli, B. cereus* and antifungal activity against *Puccinia sp., Fusarium oxysporum, Alternaria alternata* and *Fusarium graminum* in several crops.

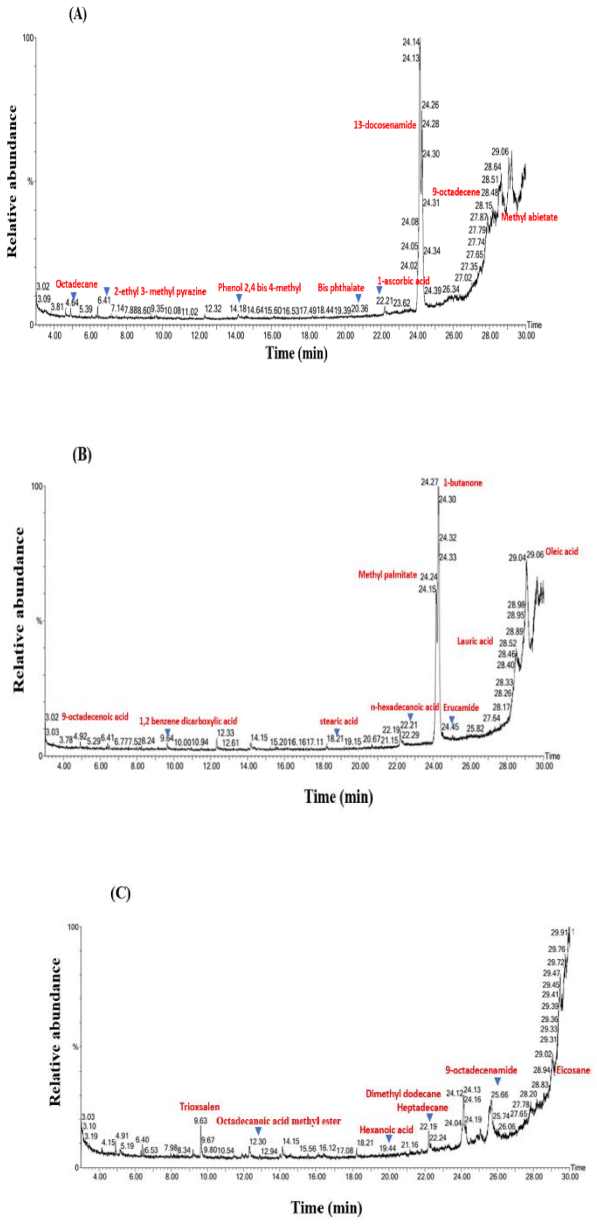


Figure 3. Total ion chromatogram of transgenic plants at 72 h post-inoculation (A) FTF-215 (B) FTF+VELVET-97 and (C) wild-type banana plants

Table 1. Metabolite profiles of transgenic and wild-type banana plants assessed by GC-MS upon inoculation with Foc race 1

S. No.	Retention time (min)	Name of the compound with molecular formula	Peak area (%) in wild type plant	Peak area (%) in transgenic plant		Inhibitory role / Biological activity	Reference
				FTF1	F+V		
1	3.02	9-Octadecenoic acid (Ricinoleic acid), $C_{21}H_{40}O_4$	7.56	9.74	9.84	Antibacterial activity against *E. coli, Salmonella* sp. and antifungal activity against *Aspergillus flavus* and *Fusarium* spp.	Krishnaveni *et al*. (2014)
2	4.64	Octadecane, $C_{42}H_{86}$	0.76	1.12	1.15	Antifungal activity against *Fusarium* sp. and *C. gloeosporioides*	Edgar *et al*. (2019)
3	6.41	2-Ethyl-3-methylpyrazine, $C_7H_{10}N_2$	2.12	4.02	3.99	Inhibits the growth of *Fusarium oxysporum*, *Botryosphaeria* sp., *Trichoderma atroviride*, *Colletotrichum gloeosporioides* and *Penicillium expansum*	He *et al*. (2020)
4	9.25	2-Methyl-5,6 diethyl pyrazine, $C_9H_{14}N_2$	1.97	6.05	6.25	Antifungal activity against *Fusarium solani, Fusarium* sp. and *Colletotrichum gloeosporioides*	Edgar *et al.* (2019)
5	9.64	1,2-Benzenedicarboxylic acid, $C_8H_6O_4$	-	1.67	1.71	Antifungal activity against six phytopathogenic fungi *viz*., *Alternaria alternata, Botryodiplodia theobromae, Curvularia lunata, Fusarium equiseti, Macrophomina phaseolina* and *Colletotrichum corchori.*	Rahman and Anwar (2006)
6	10.95	Undecanedioic acid, $C_{11}H_{22}O_2$	-	5.74	5.87	Antifungal activity against *Fusarium oxysporum* f. sp. *cubense*	Yang *et al.* (2021)
7	12.85	2-Pentadecanone, $C_{15}H_{30}O$	-	8.96	8.88	Antifungal activity against *Aspergillus* sp., *Fusarium oxysporum*, *Pythium* sp. and *Rhizoctonia solani*	Hanif *et al*. (2022)
8	14.18	Phenol 2,4-bis (1,1-dimethlethyl)-4-methyl, $C_{14}H_{22}O_2$	0.33	3.92	3.99	Completely inhibited the growth of *Fusarium oxysporum* f.sp. *cubense*	Yuan *et al.* (2012)
9	18.21	Octadecanoic acid (Stearic acid),$C_{18}H_{36}O_2$	0.36	1.47	1.45	Exhibit antifungal property against *F. oxysporum* and *M. phaseolina*	Aftab *et al*. (2019)
10	20.36	Bis (2-ethylhexyl) phthalate, $C_{24}H_{38}O_4$	-	1.8	1.9	Exhibited antimicrobial, antioxidant and antifungal activities against unicellular and filamentous fungi like *Fusarium oxysporum*	khadim *et al.* (2016)
11	22.21	l-Ascorbic acid, $C_{38}H_{68}O_8$	1.69	5.77	5.89	Antifungal activity against *Fusarium oxysporum*, *Alternaria alternata*, *Macrophomina phaseolina* and *Colletotrichum musae*	Bokhari *et al.* (2013)

12	22.21	n-Hexadecanoic acid (Palmitic acid), $C_{16}H_{32}O_2$	1.77	9.20	9.34	Exhibited antifungal properties against a number of fungal species namely *Fusarium sporotrichioides, F. subglutinans, F. verticillioides, F. cerealis, F. proliferatum, F. oxysporum, Aspergillus carbonarius, A. tubingensis, Penicillium* sp. and *Alternaria alternata*	Morcia *et al*. (2012)
13	24.24	Methyl palmitate, $C_{17}H_{34}O_2$	-	2.21	2.30	Exhibited antibacterial and antifungal activities (*Aspergillus* spp., *Fusarium* sp.) by damaging microbial cellular membranes	Shobier *et al*. (2016)
14	24.27	1-Butanone, C_4H_8O	0.56	0.89	0.97	Inhibits the growth of *Fusarium* spp.	Khan and Javaid (2022)
15	25.45	13-Docosenamide (Erucamide), $C_{22}H_{43}NO$	0.57	3.19	3.23	Antibacterial and antifungal activity by destroying the cell structure and inhibit the germination and growth of fungal spores in *Foc* TR4	Qi *et al*. (2019)
16	25.57	9-Octadecenamide, $C_{18}H_{35}NO$	1.07	3.19	3.17	Exhibits antifungal activity against *Colletotrichum acutatum, C. coccodes, C. gloeosporioides, F. oxysporum,* and *T. roseum.*	Kim *et al*. (2019)
17	27.21	Ethyl iso-allocholate, $C_{26}H_{44}O_5$	0.53	1.96	1.99	High inhibitory activity against different strains of gram-negative bacteria, anti-diabetic activity and resistance property against *Fusarium oxysporum* f.sp. *lycopersici*	Rodriguez *et al.* (2015)
18	27.85	Methyl abietate, $C_{21}H_{32}O_2$	-	1.95	1.96	Antibacterial against *E. coli, B. cereus*, antifungal activity against *Puccinia* sp., *Fusarium oxysporum, Alternaria alternata, Fusarium graminum* and high antioxidant activity	Benlembarek *et al*. (2021)
19	28.17	2,4,6-Decatrienoic acid, $C_{10}H_{14}O_2$	-	2.23	2.20	Antimicrobial activity against five bacteria and fourteen fungi (includes *Fusarium* spp.) and yeast.	Kadhim *et al*. (2016)
20	28.48	9-Octadecene, $C_{38}H_{76}O_2$	0.34	2.27	2.28	Antifungal, antioxidant, anticarcinogenic, and antimicrobial activity against yeast fungus, *S. rolfsii*	Akpuaka *et al*. (2013)
21	28.52	Dodecanoic acid (Lauric acid), $C_{12}H_{24}O_2$	1.22	2.27	2.26	Antimicrobial activity against various bacteria and antifungal activity against *Pythium ultimum*, *R. solani*, *Blumeria*, *Aspergillus* and *Fusarium* spp.	Liu *et al*. (2008)
22	29.06	Oleic acid, $C_{18}H_{34}O_2$	0.32	2.27	2.35	Antifungal activities against *Aspergillus niger*, *A. flavus*, *Penicillium parasiticus*, *Candida utilis, Fusarium solani* and antibacterial activity against gram-positive bacteria.	Mohy El-Din and Mohyeldin (2018)

The metabolite, undecanedioic acid was detected at a retention time of 10.95 min in transgenic plants alone with an area percentage of 5.74 (ftf1) and 5.87 (ftf1+velvet). Antifungal activity of undecanedioic acid in plants has been reported earlier against *Fusarium oxysporum f. sp. cubense* in banana by Yang et al. (2021). A phenolic compound, phenol, 2,6-bis (1,1-dimethylethyl)-4-methyl, commonly known as butylated hydroxytoluene is an antioxidant. This metabolite which has been demonstrated as an antimicrobial agent due to its ability to remove free radicals was also detected in transgenics (Pintac *et al.,* 2019). Yuan et al. (2012) reported that the presence of phenol completely inhibited the growth of *Fusarium oxysporum f. sp. cubense* in transgenic banana.

The metabolite, 9-octadecenoic acid or ricinoleic acid, was detected in the transgenic and wild-type plants at a retention time of 3.02. The ricinoleic acid area percentage in wild type was 7.56 %, whereas 9.74 % and 9.84 % were recorded in ftf1 or ftf1+velvet plants, respectively. The concentration of ricinoleic acid increased as infection progressed and was implicated in the activation of defense responses against injury/infection of Aspergillus flavus and Fusarium spp. (Krishnaveni *et al.,* 2014). Octadecanoic acid or stearic acid was detected at a retention time of 18.21 min with an increase in 1.11 % area in transgenic plants compared to wild type. Stearic acid is known to exhibit antifungal property against soil-borne diseases such as *F. oxysporum* and *M. Phaseolina* in black cumin (Aftab *et al.,* 2019).

In this study, the differences in peak area percentage of various metabolites among transgenics and wild type were compared. However, these differences did not cause any changes in the growth and development of transgenic plants. Besides, similar observations on the production of metabolites in Tobacco streak virus TSV) resistant transgenic groundnut plants engineered to express dsRNA of coat protein gene of TSV (Shomo *et al.,* 2016) and transgenic cotton plants engineered for down-regulation of tryptophan synthesis-related gene (GbTRP1) for controlling *Verticillium dahliae* and *Botrytis cinerea* (Miao *et al.,* 2019).

Phenylalanine ammonia lyase activity in transgenic plants

L-Phenylalanine ammonia lyase, in the phenylpropanoid pathway, acts as an inducible physical barrier against pathogen ingress and has shown to be strongly induced with the fungal pathogen infection, *Cercospora nicotianae* in transgenic tobacco (Gail *et al.,* 2003). In this study, PAL activity was monitored in Rasthali (AAB) transformed and untransformed plants following inoculation with Foc race 1 up to 72 h. PAL activity increased to maximum level by 24 h in transgenic banana plants (F+V 97; FTF 215) that correlate to the disease incidence obtained at 28 DPI with Foc1 (Table2; Figure 2). Similar observation was reported by Aguilar et al. (1999) in gold finger cultivar (AAAA), which is known to be more resistant to Fusarium wilt disease with the accumulation of lignin (De Ascensao and Dubery 2000) when compared with banana cultivar susceptible to Fusarium wilt such as Williams (AAA) and Gros Michel (AAA). The observation of the present study is also supported by the findings of Subramaniam et al. (2006), who showed that the transgenic banana Rasthali-pisang cultivar (AAB) expressing two genes (chitinase and β-1,3 glucanase) had higher PAL activity against Foc after 24 h of inoculation.

Peroxidase, superoxide dismutase and polyphenol oxidase activity in transgenic banana plants during pre and post inoculation with Foc

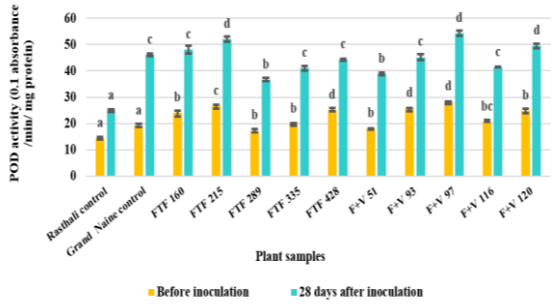
In stress-related environment, ROS accumulation appears to be at a higher level and found to be a common phenomenon. The activity of ROS scavenging enzymes *viz.,* SOD, POD and PPO were studied in transgenic plants before and in response to pathogen infection. SOD detoxifies the O_2^- radicals to H_2O_2 and acts as the first line of defense mechanism, POD detoxifies the peroxides into H_2O and serves as the next line of defense by the formation of lignin or suberin, a physical barrier to limit pathogen invasion and enhance cell wall rigidity. Further, PPO is responsible for the oxidation of phenolic compounds into antimicrobial quinones in plant cells attacked by phytopathogens (Malolepsza and Urbanek, 2000). In the present experiment, the activity of these ROS enzymes substantially increased after 28 days of Foc inoculation in the transgenic plants compared to the untransformed ones. The results obtained also correlates to the disease severity score.

Table 2. Time course of changes in Phenylalanine ammonia-lyase (PAL) activity (Foc 1 post-inoculation) in control and transgenic Rasthali (AAB) plants.

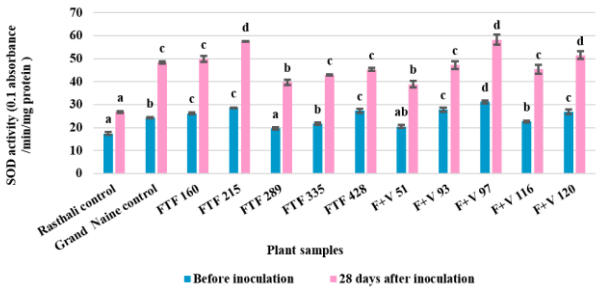
	Hours			
Plant Id	0	24	48	72
Rasthali control	3.7^{a}	12.1^{b}	22.5^{cd}	6.4^{a}
Grand Naine control	4^{a}	16.9^{c}	25.3^{d}	13.6^{b}
FTF 160	4.3^{a}	16.5^{c}	25.1^{d}	14.5^{b}
FTF 215	4.9^{a}	20.6^{c}	26.9^{d}	15.8^{c}
FTF 289	3.6^{a}	14.5^{b}	21.2^{c}	11.9^{b}
FTF 335	3.9^{a}	15.3^{c}	23.4^{d}	13.7^{b}
FTF 428	4.1^{a}	16.4^{c}	24.5^{d}	14.3^{b}
F+V 51	4.6^{a}	15.7^{c}	19.6^{c}	11.4^{b}
F+V 93	5.2^{a}	19.3^{c}	25.6^{d}	15.1^{c}
F+V 97	6.2^{a}	21.1^{c}	27.8^{d}	16.9^{c}
F+V 116	5.9^{a}	17.2^{c}	22.3^{cd}	13.1^{b}
F+V 120	5.4^{a}	19.1^{c}	24.7^{d}	14.9^{b}

The specific activity of peroxidase was higher (46%) in the transgenic plant F+V-97 (54.3-unit mg^{-1} protein) without disease symptom expression when compared to the untransformed plant (24.8-unit mg^{-1} protein) (Figure 4a) suggesting that peroxidase enzyme could be involved in defense response against pathogens. Morpurgo et al. (1994) reported that the activity of peroxidase was five times higher in the leaves, roots, and corm tissues of the *F. oxysporum* resistant banana cultivar, Gold Finger (AAAA) than in the susceptible cultivar, Pisang Mas (AA). Subsequently, the increase in superoxide dismutase activity was recorded as 47 and 46.5 per cent higher in transgenic plants F+V-97, FTF-215 (58.3, 57.5 unit mg^{-1} protein) compared to untransformed plant (26.8 unit mg^{-1} protein) (Figure 4b). Similar observations were made by Sunisha et al. (2020) after stacking of antimicrobial genes in banana against Fusarium wilt and Karmakar (2017) in the leaf sheath of transgenic lines of rice to *R. solani* infection and reported increased peroxidase, superoxide dismutase and ascorbate peroxidase activities.

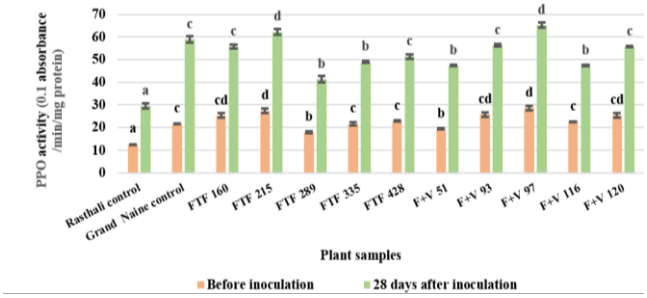
Besides, an increase in polyphenol oxidase activity in the transformed Rasthali (AAB) plant was also observed (Figure 4c). The highest PPO activity in F+V 97 (65.09-unit mg^{-1} protein) transgenic plant was recorded, which was 44% higher than that recorded from untransformed plants. Subsequently, other transformed plants, F+V-93 and FTF-215, vary significantly from each other, where the PPO activity ranged between 56.3 and 62.3 unit mg^{-1} protein. Similar findings were observed by Mohammadi and Kazemi (2002) and Subramaniam et al. (2006) with increased PPO activity in transgenic wheat heads and pisang-rasthali cultivars, respectively, following inoculation with respective Fusarium conidia.



a) Peroxidase (POD) activity



b) Superoxide dismutase (SOD) activity



c) Polyphenol oxidase (PPO) activity

Figure 4. Changes in enzymatic activities in wild type and transgenic Rasthali (AAB) plants before and 28 DPI of Foc. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Values indicated in superscripts (a-d) are significantly different ($p<0.05$)

The plant defense mechanism is a complex one with the emergence of new pathogenic strains that makes it difficult to study. Considering the pathogenic nature, various biochemical parameters against Foc transformed and untransformed banana cv. Rasthali indicated a significant increase in defense enzyme activities in the transformed plants compared to untransformed ones. An increase in the amount of hydrogen peroxide, PAL activities after 24 h, and phenol oxidizing enzymes after 28 days of Foc inoculation was found higher in transformed Rasthali (AAB) plants with combined ftf1+velvet gene than the single inserted ftf1 gene.

CONCLUSION

The present study revealed the resistance activity of transgenic banana plants against Foc race 1 by understanding their biochemical profile. The bioactive metabolite compounds identified using GC-MS analysis support and signify the importance of the resistant action of transgenic plants against Foc. However, a detailed characterization of these antifungal compounds needs to be carried out to know the exact mode of action.

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