***Original Research Article***

# Differential biochemical response among Banana (*Musa* spp.) genotypes against Banana Bunchy Top Virus (BBTV)


## ABSTRACT

The present study was carried out to explore the adaptive mechanism and biochemical responses in two banana cultivars viz., Rasthali and Grand Naine against the banana bunchy top virus (BBTV). In the leaf samples of BBT-~~ ~~infected Rasthali and Grand Naine, estimated the total chlorophyll, carbohydrates, phenols and enzyme activities such as peroxidase (POX), polyphenol oxidase (PPO), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and superoxide dismutase (SOD). The infected samples of both ~~the~~ cultivars revealed a significant increase in the defense enzymes ~~such as~~ PPO, POX, APX, GPX and CAT over the healthy sample. Higher total phenols in healthy Rasthali ~~healthy~~ plants which further significantly increased after BBTV infection was observed in comparison to Grand Naine. In contrast to Grand Naine, Rasthali showed higher polyphenol oxidase (PPO) activity contributing to increased polyphenol formation. ~~The~~ H~~h~~igher superoxide dismutase (SOD) activity in virus-~~ ~~infected Rasthali was observed in comparison to Grand Naine. The increased amount of total phenols, polyphenols and SOD activity in Rasthali might have contributed to less susceptibility to bunchy top virus. However, total protein and chlorophyll content were reduced after BBTV infection in both the banana cultivars.



*Keywords: [Banana Bunchy top virus, Rasthali, Grand Naine, Biochemical changes and Defense enzymes]*


## 1. INTRODUCTION

Plants are frequently exposed to infection by a wide array of pathogens that ~~show~~ lead to different responses in the host plant. During compatible plant-pathogen interaction, along

with the development of visible symptoms, the pathogen also adversely affects the growth and development, physiological status and yield of a plant [35]. Plants have evolved various pre-existing physical and chemical barriers, as well as inducible defense responses that restrict pathogen colonization [18,37,38]. Globally, bananas and plantains are the fourth most important agricultural produce which is attacked by various pests and pathogens causing major production problems. Grand Naine (AAA subgroup Cavendish) is one of the most commonly cultivated commercial Cavendish cultivar in the world. Rasthali (AAB subgroup Silk) is cultivated mostly in India and is popular in the local and world market as a premium dessert variety similar to Cavendish bananas. Plant viral diseases cause significant losses by reducing plant growth and yield. Among the banana viral diseases, Banana bunchy top disease (BBTD) is one of the most destructive viral diseases affecting various banana cultivars. There is no resistance germplasm available in bananas and plantains; however, the level of susceptibility varies among the banana cultivars.

Banana bunchy top disease affects the fruit and foliage and is caused by a single-strand DNA virus, the banana bunchy top virus (BBTV; Genus *Babusvirus*; Family *Nanoviridae*). The BBTV virus colonizes in the phloem tissue, which ~~is~~ damages~~ing~~ the host cells. The name of the disease comes from the symptom which occurs in banana plants, in which the emerging new leaves are narrower than usual, yellow and flat, which causes a "bunchy" appearance at the top. In addition, few distinctive symptoms are 'morse code streaking,' 'green J hooks' and 'keikis'. Viruses depend on the host for their replication and other metabolic processes, which instigate significant changes in their usual physiological processes such as loss of pigment contents, increasing respiration rates, soluble sugar~~,~~ and starch accumulation and production ~~level~~ of higher levels of enzymatic antioxidants. Due to ~~the~~ viral infection, various changes occur in the host plants at the molecular level, thereby leading to various biological and physiological changes. Hence, it is ~~necessary~~ of value to estimate the physiological and biochemical changes in banana cultivars Rasthali and Grand Naine ~~to known the~~and to measure biochemical changes occurring due to BBTV infection. The present investigation will lead to better understanding of the defense mechanism in two banana cultivars, which will be useful for adopting suitable control strategy against bunchy top disease in banana.

## 2. MATERIAL AND METHODS

### 2.1 Plant material and source of infection

Banana cultivars Rasthali and Grand Naine were used in the present investigation. Leaf samples were collected from BBTV infected plants in Orchard of Tamil Nadu Agricultural

University, Coimbatore district, Tamil Nadu, India. ~~The~~ Lleaf samples from ~~the~~ healthy plants of each cultivar were taken as control.

### 2.2 PCR confirmation of BBTV presence in the infected banana samples

The plant genomic DNA was extracted ~~from~~ from 100 mg leaf samples of healthy and infected (showing characteristic symptoms of BBTV) samples of Rasthali and Grand Naine ~~by~~ using the cetyl trimethyl ammonium bromide (CTAB) method with some modification as described by [10] ~~from 100 mg leaf samples~~ and subjected to PCR using the BBTV specific primers designed for Replicase gene F-**5'** ACGACAGAATGGCGCGA**3'** and R-**5'**TCAGCAAGAAACCAACTTTATTC**3'.** The PCR products were resolved on 1 % Agarose gel, electrophoresed at 70 V for one h and the amplicons were assessed with 1.0 kb DNA ladder.

### 2.3. Leaf samples for biochemical analyses

The most recent fully expanded leaves of BBTV infected samples were collected for various analyses. All biochemical parameters were measured using a spectrophotometer (Jasco V-730 BIO spectrophotometer, USA).

### 2.4 Estimation of photosynthetic pigments:

The photosynthetic pigments such as total chlorophyll, chlorophyll 'a' and chlorophyll 'b' contents of healthy and infected leaves were estimated according to the non-destructive DMSO method [16]. The absorbance was recorded at 663 and 645 nm, respectively in a spectrophotometer, taking full concentration of the DMSO ~~h~~as blank. Chlorophyll a, b and total chlorophyll were calculated by the following formulas:

$$\text{Chlorophyll a (mg g}^{-1}\text{ tissue)} = \frac{[12.7(OD663) - 2.69(OD645)]/ x\ V}{1000} \times W$$

$$\text{Chlorophyll b (mg g}^{-1}\text{ tissue)} = \frac{[22.9(OD645) - 4.68(OD663)]/ x\ V}{1000} \times W$$

$$\text{Total Chlorophyll (mg g}^{-1}\text{ tissue)} = \frac{[8.02(OD663) + 20.20(OD645)]/ x\ V}{1000} \times W$$

Where OD, Optical density at respective nm, V, Final volume of chlorophyll extract, W, Fresh weight of the tissue extracted.

### 2.5 Total sugars and starch content:

Total reducing sugars were calculated according to the method described by [11] and the total starch content method explained by [26]. About 0.5 gm of healthy and infected leaves were ~~taken and~~ homogenised with 80 % ethanol and centrifuged at 5000 rpm for 15 min. The supernatants were pooled and heated in a water bath at 85 °C until the ethanol was evaporated entirely from the samples.

For total reducing sugar determination, healthy and virus-infected pooled supernatants were taken and cold anthrone reagent was rapidly added to each tube and incubated for 10 min on ice and cooled at room temperature. The absorbance of the samples was recorded at 625 nm in a spectrophotometer along with the blank sample. The amount of total sugars was estimated by using a standard curve prepared for D-glucose. The content of reducing sugar was expressed as mg $g^{-1}$ fresh weight.

For starch determination, the extract for total sugar was solubilized in five ml of 52 % perchloric acid (PCA) and boiled at 80 °C for 10 min in a water bath. Three ml of distilled water and five ml of anthrone reagent were~~as~~ addednd~~,~~ the sam;ples incubated for 10 min on ice. The absorbance of the samples was recorded at 625 nm in a spectrophotometer. The amount of starch was determined by using D-glucose standard curve. The content of total starch was expressed as mg $g^{-1}$ fresh weight.

**2.6 Phenolic content:**

Phenol content was measured using ~~the~~ Folin-Ciocalteau reagent. One-gram of fresh plant material was homogenized using 80 % ethanol. The extract was subjected to centrifugation at 3000 rpm for 15 min and the supernatant was separated. Then, 0.1 ml of ethanol extract was evaporated on a water bath, to which six ml water was added and shaken well before the addition of 0.5 ml Folin-Ciocalteau reagent. Two ml of 20 % sodium carbonate solution was added to each test tube and after 30-45 min of incubation, the absorbance was recorded at wavelength 660 nm against a reagent blank. Using pyrocatechol as standard, a standard curve was generated to determine the concentration of total phenols in the leaf extract [12].

**2.7 Measurement of total protein content**

Total protein was estimated by using the Bradford method [3] and absorbance was recorded at 595 nm. Bovine serum albumin was used as a standard. Protein contents in leaf samples were recorded as µg of protein per gram of leaf tissue.

**2.8 Preparation of enzyme extract:**

To obtain the total enzyme extract, a one-gram leaf sample was homogenized at 4°C in 1 ml of extraction buffer [50 mM potassium phosphate buffer (pH 7.0), 1 % Triton X-100 and 7 mM 2-mercaptoethanol]. The obtained homogenate was then centrifuged at 12000 rpm for 20 min at 4°C. The resulting supernatant was used for analysis of enzymes.

### *2.8.1* Peroxidase activity

POX activity was assessed following the oxidation of pyrogallol [23]. For the assay, 3.5 ml of phosphate buffer (pH-6.5) was taken in a clean and dry cuvette. To it, 0.2 ml of enzyme extract and 0.1 ml of freshly prepared pyrogallol solution were added. Then 0.2 ml of 0.2 M $H_2O_2$ was added and instantly the absorbance of the reaction mixture was recorded at 430 nm at every 30-sec intervals up to 3 min. The specific activity of the enzyme was expressed as micromoles pyrogallol oxidized per minute per milligram protein.

### *2.8.2* Polyphenol oxidase activity

PPO activity was determined according to the method described by [28]. For the assay, 0.2 ml of the enzyme extract was taken, to which 1 ml of catechol and 3.5 ml phosphate buffer were added. The extract was incubated at 30 °C for 30 min. The activity was measured by monitoring the increase in absorbance for 3 min at 410 nm. The specific activity of the enzyme was expressed as micromoles catechol oxidized per minute per milligram protein.

### *2.8.3* Catalase activity

Catalase activity was calculated by measuring the rate of disappearance of $H_2O_2$ using the method followed by [22]. The reaction mixture containing 2.5 ml of 50 mM phosphate buffer (pH 7.4), 0.1 ml of 1 % $H_2O_2$ and 50 µl of enzyme extract was diluted to keep measurements within the linear range of the analysis. The decrease in $H_2O_2$ was followed as a decline in absorbance at 240 nm. Catalase activity was expressed as micromoles of $H_2O_2$ oxidized per minute per milligram protein.

### *2.8.4* Ascorbate peroxidase activity

APX activity was determined using the method described by [6]. The one ml reaction mixture consisteds of 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.5 mM ascorbate, 1.54 mM $H_2O_2$ and 50 µl of enzyme extract. The oxidation of ascorbate was followed by a decrease in the absorbance at 240 nm. The enzyme-specific activity is expressed as micromoles ascorbate oxidized per minute per milligram protein.

### *2.8.5* Guaiacol peroxidase activity

GPX activity was calculated using the method described by Upadhyaya et al. [36]. The reaction mixture contained 2.5 ml of 50 mM phosphate buffer (pH 6.1), one ml of 1 % $H_2O_2$, one ml of 1 % guaiacol and 20 µl of enzyme extract. The increase in absorbance at 420 nm was recorded for 1 min. The enzyme-specific activity is expressed as micromoles guaiacol oxidized per minute per milligram protein.

158

159 ***2.8.6*** **Superoxide dismutase activity**

160 SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue
161 tetrazolium (NBT) using the method described by [8]. The reaction mixture consists of 50
162 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 M NBT, 2 M riboflavin, 0.1 mM EDTA
163 and 50 µL of enzyme extract. Riboflavin was added last and the tubes were subjected to
164 intermittent shaking. The absorbance of the reaction mixture was recorded
165 spectrophotometrically at 560 nm.

166

167 **2.9 Statistical analysis:**

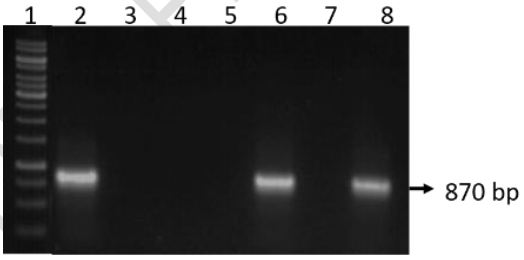
168 All the experiments were performed in two duplicates (n=4). The significance of differences
169 between healthy and infected samples was determined by using one-way analysis of
170 variance (*ANOVA)* and means and standard errors were calculated. Differences in means
171 were considered significant when the *P*-value was <0.05.

172

173 **3. RESULTS AND DISCUSSION**

174 **3.1 PCR based confirmation of BBTV**

175 The presence of BBTV in symptomatic leaves of Rasthali and Grand Naine was confirmed
176 by PCR amplification of 870 bp BBTV Rep gene using designed gene specific primers
177 (Fig.1.)




178

179 **Fig. 1. PCR amplification of BBTV Rep gene in symptomatic Rasthali and Grand Naine**
180 **plants**

181 *Lane 1, 1 kb ladder; 2, positive control; 3, negative control; 4, water control; 5-6, Rasthali healthy and*
182 *infected sample; 7-8, Grand Naine healthy and infected sample.*
183

184 **3.2 Effect of BBTV incidence on the photosynthetic pigment**

185 The BBTV infected cultivars exhibited a two-–fold significant reduction in photosynthetic
186 pigment contents (chlorophyll a, chlorophyll b and total chlorophyll) compared to healthy
187 cultivars (Fig. 2). The reduction in the chlorophyll content in the infected plant reduces the

photosynthetic capacity and plant growth, leaving the plant stunted and chlorotic resulting in the symptom expression. This difference in the chlorophyll content was attributed to the stimulation of cell enzymes like chlorophyllase that degrades chlorophyll [14], or it may be the effect of the virus on pigment synthesis [2, 33] which disturbs ~~the~~ physiological processes like photosynthesis. A recent study suggests the possibility of BBTV utilizing the chloroplast for the synthesis of viral proteins [39]. They found during BBTV infection, outer membranes of chloroplasts are disrup~~pu~~ted and crystalline aggregation of virus-~~-~~like particles accumulate~~s~~ in it.

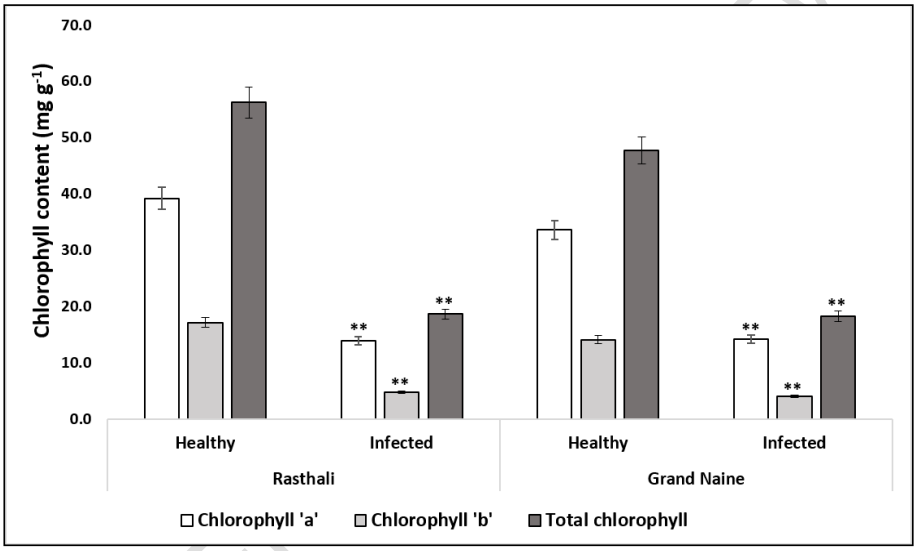
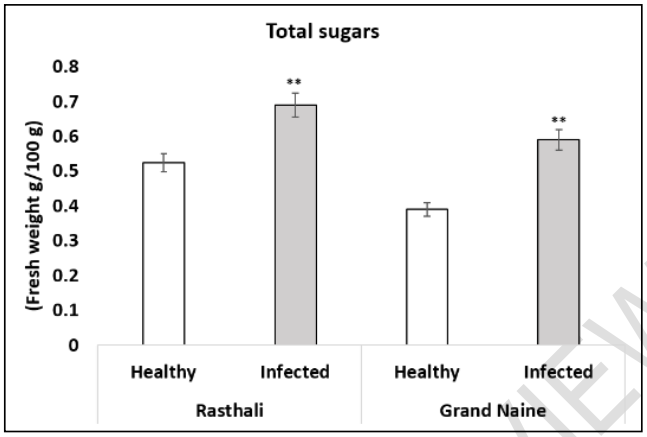



**Fig. 2. Chlorophyll content of healthy and infected Rasthali and Grand Naine plants.**

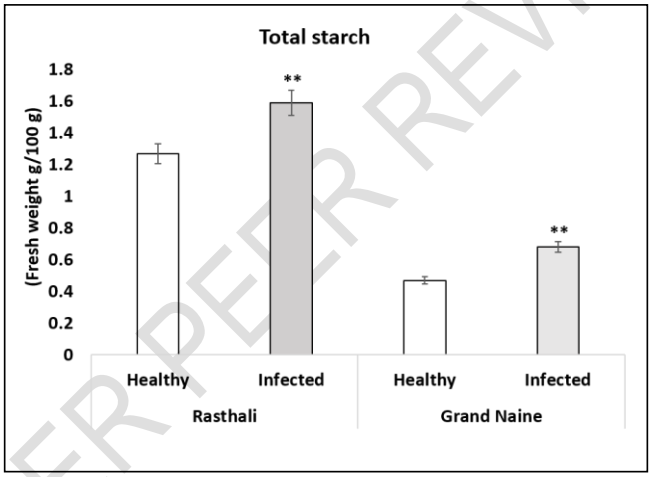
*Data represent the mean ± standard error of mean of four independent replications. Significant differences in healthy and infected from each cultivar analysed by Student's t test (*P<0.05, **P<0.01) are shown.*

### 3.3 Carbohydrates

The total sugars and starch were significantly high~~er~~ in infected plants compared to healthy in both the cultivars of banana (Fig. 3 a-b). Our study suggests that sugar increases during BBTV infection may control photo~~-~~-inhibitory processes and produce symptoms. The carbohydrate content reported by [1] was similar to the findings of our study where changes in the sugar and starch content were the same in all the banana cultivars viz., Virupakshi, Grand naine and Rasthali. Some viruses appear to have little effect on carbohydrates in the leaves, while others may alter both their rate of synthesis and rate of translocation which affects the overall growth of the plant [13].




(a)




(b)

**Fig. 3. Carbohydrate content (a) Total sugars and (b) Starch of healthy and infected Rasthali and Grand Naine plants.**

*Data represents the mean ± standard error of mean of four independent replications. Significant differences in healthy and infected from each cultivar analyzed by Student's t test (*P<0.05, **P<0.01) are shown.*

### 3.4 Total Phenol

In the present investigation showed a significant variation in the total phenolic compound of banana cultivars in response to infection with BBTV (Fig. 4a) was apparent. The amount of total phenol was significantly higher in virus-infected leaves in both the cultivars of banana. and the increased quantities of phenols might be attributed to a defense mechanism where plant polyphenols act as secondary metabolites. It was has been reported that the resistance to disease caused by pathogens was can be attributed to the presence of a high amount of

phenol [17,21,23,29,33]. It has been reported that ~~the~~ Rasthali ~~takes~~ viral ~~infection~~ infection occurs later than in the Grand Naine cultivar because of the difference in the genomes [27]. Although no Musa genotype is known to be resistant to BBTV, cultivars in the AA and AAA genomic groups are highly susceptible, whereas cultivars containing the B genome are regarded as less susceptible. The less BBTV susceptible Rasthali had higher total phenol content in healthy plants which further increased >2 fold after BBTV infection. This is in contrast to Grand Naine displaying lower total phenol content in healthy plants which increased, but to a lower level after BBTV infection. Hence, the increased quantity of phenolics in the infected plant of the banana may be contributing to the resistance against the infection of viral pathogens [24].

### 3.5 Total Protein

Protein content was found to **decrease** significantly in the BBTV infected plants of both ~~the~~ cultivars (Fig. 4b). ~~Results in our study showed that the total protein content changes regarding viral infection in both the cultivars thereby confirming the accumulation~~??~~ of protein as a response to viral infections~~. The involvement of host protein components in plant disease resistance has been documented in various plant pathogenic interactions [4, 36]. This result is in accordance with the results of Tobacco mosaic virus-infected tobacco plants [19], Tomato yellow leaf curl virus-infected tomato plants [9], Banana bunchy top virus-infected cultivars of banana [7], geminivirus infected *Capsicum annum* [25] and cotton with CLCuBuV [34].

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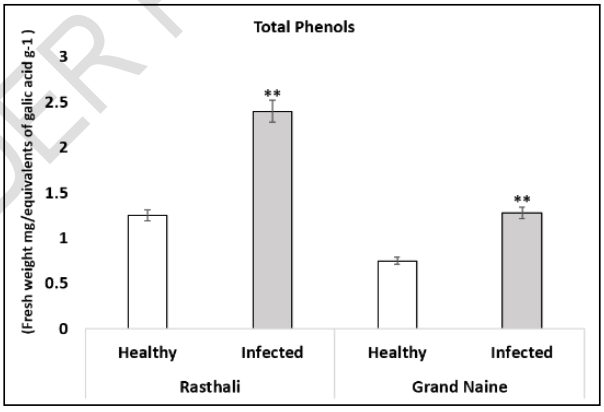
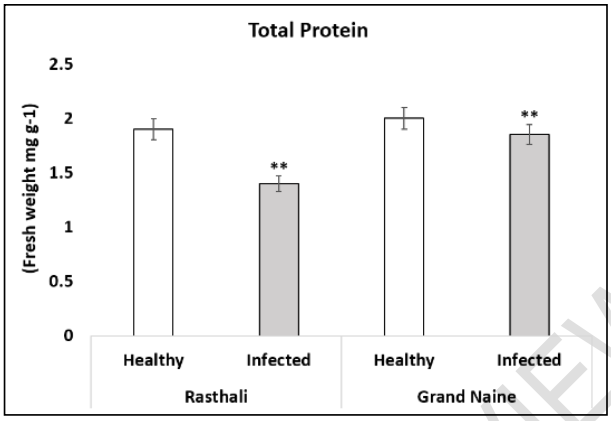



**(a)**




**(b)**

**Fig. 4. (a) Total phenol content and (b) Total protein of healthy and infected Rasthali and Grand Naine plants.**

*Data represents the mean ± standard error of mean of four independent replications. Significant differences in healthy and infected from each cultivar analysed by Student's t test (*P<0.05, **P<0.01) are shown.*

### 3.6 Enzyme activities

#### 3.6.1 Peroxidase

The POX activity was increased significantly in BBTV infected plants of both cultivars, in comparison to healthy (Fig. 6). ). 5 should come before 6! In an earlier report, a similar increase in the activity of POX was observed in Virupakshi and Grand Naine cultivar [1]. The peroxidases are enzymes whose primary function is to oxidize hydrogen donors at the expense of peroxides which is known to be involved in oxidative damage in response to stress to the plant. POX activity was found to be increased in chilli against chilli leaf curl virus as reported by [30].

#### 3.6.2 Poly Phenol Oxidase

The Ppolyphenol oxidase is involved in the formation of insoluble polyphenols in plants by the oxidation of the soluble phenols. The Hhigher poly phenoloxidase activity was observed in Rasthali in healthy plants which marginally reduced after BBTV infection, in contrast, Grand Naine showed lower activity in healthy plants which increased during BBTV infection (Fig. 6). Higher total soluble phenols, together with higher PPO have been demonstrated? to play a role in resistance to viral pathogens [1, 28].

#### 3.6.3 Catalase

A significant elevation was observed in the CAT activity of BBTV-infected samples in both the banana cultivars tested (Fig. 5). Changes in catalase activity have been found to be a significant monitoring index for~~of~~ plant responses under abiotic or biotic conditions. An increase in foliar CAT activity was observed in leaves of *Arachis hypogaea* infected with Peanut mottle virus [20] and cotton plants infected with the Cotton leaf curl burewala virus [34].

**3.6.4 Ascorbate Peroxidase**

The activity of ascorbate peroxidase was significantly higher in BBTV infected plants of both cultivars when compared to the healthy (Fig. 5). APX acts as an antioxidant response triggered by the increasing presence of $H_2O_2$ within cells. One of the major peroxide detoxifying system in plant cells is the ascorbate-glutathione cycle, in which ascorbate peroxidase (APX) enzyme has a key role catalyzing the conversion of $H_2O_2$ into $H_2O$, using ascorbate as a specific electron donor. The increase in APX activity in BBTD infected banana was similar to that reported for *Hibiscus cannabinus* infected with begomovirus *Nicotiana benthamiana* infected with Pepper mild mottle virus [15] and sunflower infected with sunflower chlorotic mottle virus [31].

**3.6.5 Guaiacol Peroxidase**

Guaiacol peroxidase (GPX) activity was found to be significantly higher in BBTV infected cultivars when compared with healthy (Fig. 5 and 6). GPX is an essential group from peroxidase enzymes, which oxidize guaiacol and are?~~is~~ found in cellular cytoplasm and apoplasm fractions. ~~,~~ It is involved in a range of processes related to plant growth and development. GPX activity was found be higher in mesta plants infected with yellow vein mosaic virus as reported by [5]

**3.6.6 Superoxide dismutase**

S~~The s~~uperoxide dismutase (SOD) is an enzyme that breaks down superoxide radical generated during stress into molecular oxygen or hydrogen peroxide, thereby preventing cell damage. ~~The~~ SOD activity was significantly higher in the leaves of healthy plants of Rasthali in contrast to healthy plants of Grand Naine (Fig. 6). Upon BBTV infection, Rasthali showed increased SOD activity, whereas, Grand Naine showed decrease in SOD activity. In contrast, early reports show increase in SOD activity upon BBTV infection in Grand Naine [1]. SOD constitutes the front-line of defense against ROS and oxidative stress in plant cells and it is one of the most important scavenging enzymes. It is also reported that the induction of antioxidant enzymes, including SOD, is vital for the development of plant stress tolerance. Based on the present result, it can be concluded that higher SOD activity in Rasthali compared to Grand Naine might contribute to increased level of tolerance to BBTV infection

in Rasthali and Grand Naine. But aren't the peroxides made to inhibit pathogen growth? If so, removing them could favor pathogen growth

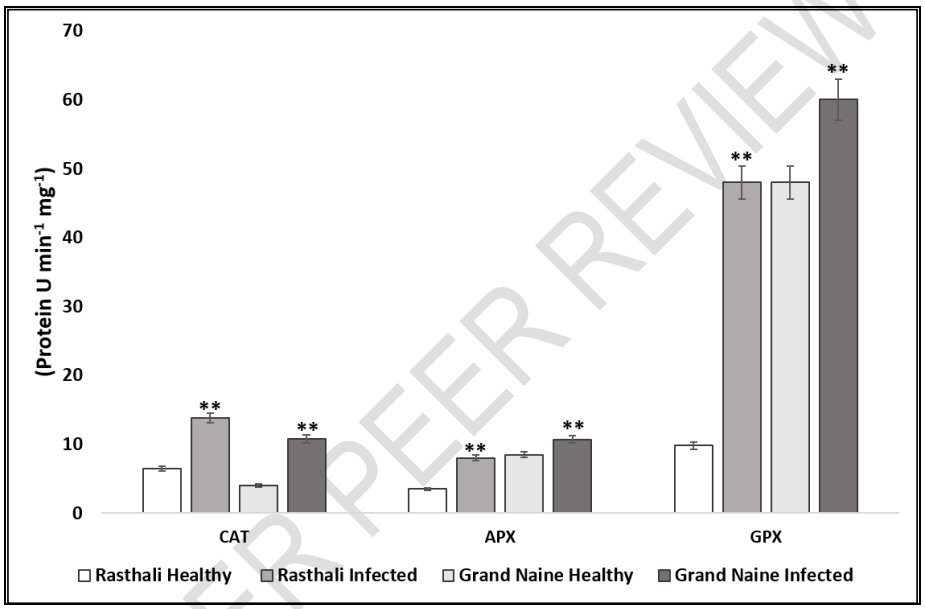



**Fig. 5. Changes in enzyme activities of CAT, APX and GPX in healthy and BBTV infected Rasthali and Grand naine banana plants**.

*Data represents the mean ±–standard error of mean of four independent replications. Significant differences in healthy and infected from each cultivar analysed by Student's t test (*P<0.05, **P<0.01) are shown.*

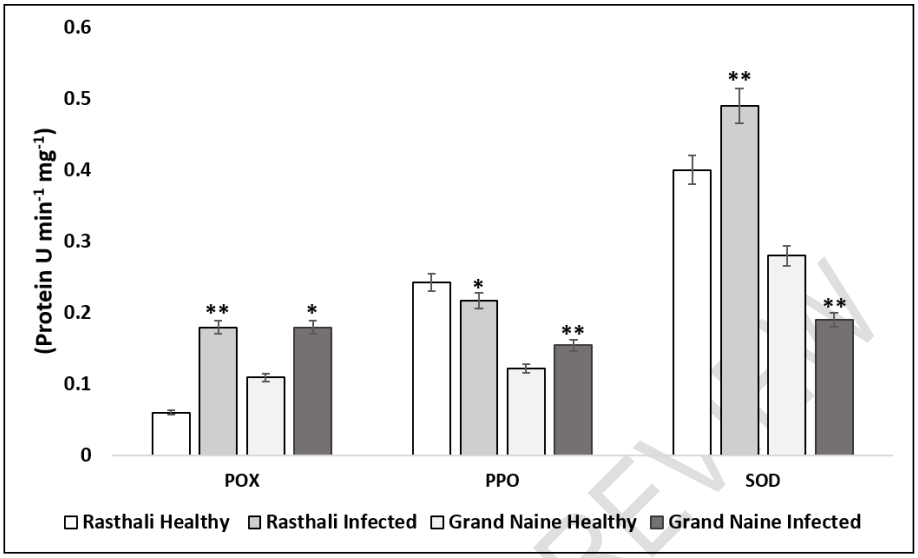



**Fig.6. Changes in enzyme activities of POX, PPO and SOD in healthy and BBTV infected Rasthali and Grand Naine banana plants.**

*Data represents the mean ± standard error of mean of four independent replications. Significant differences in healthy and infected from each cultivar analysed by Student's t test (*P<0.05, **P<0.01) are shown.*

## 4. CONCLUSION

It is well known that plant defense mechanism is complex, and the evolution of new strains of pathogens makes it a very difficult task to study. Various physiological and biochemical parameters were analyzed in BBTV infected and healthy banana cultivars Grand Naine and Rasthali. Our results indicated significant increase in defense enzyme activities in the BBTV infected cultivars compared to the healthy. There was a significant increase in amount of phenol and polyphenols in Rasthali in comparison to Grand Naine. The level of difference of biochemical constituents between the genotypes reverberates the variation of genotypes in defense against the BBTV. The findings of this study will help in better understanding of various physiological changes that occur in banana species against the BBTV and will contribute to plant resistance mechanisms which in turn will provide new tools for crop improvement.

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448 **ABBREVIATIONS**

449 APX Ascorbate Peroxidase
450 CAT Catalase
451 GPX Guaiacol peroxidase
452 PPO Poly Phenol Oxidase
453 POX Peroxidase
454 SOD Superoxide dismutase

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