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RESEARCH

PHYSIOLOGY AND INDUCTION OF DEFENSE ENZYMES IN MERISTEM DERIVED CASSAVA (*MANIHOT ESCULENTA*. CRANTZ) PLANTS

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Abstract: Physiology and induction of defense enzymes in meristem derived and *Cassava mosaic virus* infected cassava plants (cv. MVD1 and cv. H226) were studied. Chlorophyll a, chlorophyll b, total chrolorophyll, total phenol, total sugars and total soluble protein contents were more in meristem derived healthy cassava plants as compared to *Cassava mosaic virus* infected cassava plants. SDS-PAGE analysis revealed the presence of 35 KDa protein in *Cassava mosaic virus* infected samples of cv. MVD1 and cv. H226 collected from field which was absent in meristem derived healthy plants. Induction of enzymes *viz*., PO, PPO, PAL and catalase was more in *Cassava mosaic virus* infected cassava plants.

Keywords: *Cassava mosaic virus*, Chlorophyll, Phenol, Protein, Meristem

INTRODUCTION

Plant virus infection leads to measurable changes in the physiological process of the host. Any biochemical or physiological study on infected plants may contribute to understand the virus disease (Bawden and Pirie, 1952). Porter (1959) reported that changes in metabolism of host cell induced by the virus leads to various macroscopic and microscopic symptoms. Chant and Beck (1959) reported that the chloroplasts in mosaic infected leaves are fewer in number and they have the tendency to line the cell wall. Ramakrishnan *et al*. (1969) reported that chlorophyll a and b levels were significantly lower in diseased plants than in healthy ones. But, both in diseased and healthy plants, chlorophyll a and b increased with age. Chant *et al*. (1971) reported that photosynthetic activity of immature and senescent leaves was not affected by *Cassava mosaic virus* infection but reduced by approximately 23 per cent in infected mature leaves. Chloroplasts of virus-infected mesophyll cells were irregular in shape and contained numerous swollen starch grains. Alagianagalingam and Ramakrishnan (1978) also reported that mosaic infected leaves had less chlorophyll and carotene and lower photosynthetic rate. Ayanru and Sharma (1982) observed that concentration of chlorophyll a and b of diseased samples was reduced by 32-62 per cent and 37-57 per cent respectively as compared with healthy leaves. Madhubala (2003) reported that cassava plants infected with ICMV had less chlorophyll. Chlorophyll a, b and total chlorophyll were significantly lesser in diseased plants than in healthy and regenerated ones. The per cent decrease over healthy was 9.92 with chlorophyll a, 18.83 with chlorophyll b and 12.45 with total chlorophyll in case of infected cassava leaves.

Daniel *et al*. (1977) reported that total phenols, ortho-dihydroxyphenols, flavanoids, ascorbic acid and calcium content of ICMV infected leaves were significantly reduced than healthy leaves as a result of infection. Madhubala (2003) reported that *Cassava mosaic virus* infected cassava plants showed 29.54 per cent increase in total phenol content and 22.30 per cent increase in total sugar content compared to healthy ones. Virus infection affects the host protein and the synthesis of virus protein may exert a considerable direct stress on host protein metabolism (Reddi, 1963).

Nambisan (1994) observed higher amount of soluble proteins in ICMV infected cassava plants than healthy plants and the extractability was less at low pH and increased at higher pH. But the total protein content in healthy and diseased leaves was similar. Electrophoretic protein profiles of healthy and diseased leaves revealed that new proteins were induced in diseased plants. Madhubala (2003)

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reported that the soluble protein in infected plants increased by 14.29 per cent compared to healthy plants.

Chant *et al*. (1971) found that respiration rate and peroxidase activity were increased in cassava leaves as a result of infection with *Cassava mosaic virus*. They also reported that no new virus-specific peroxidase isozymes were found as a result of infection, but one isozyme apparently occurs in greater quantity in virus infected leaves.

Alagianagalingam and Ramakrishnan (1974) reported that respiration was faster in infected cassava leaves and they observed significant difference in catalase, peroxidase and ascorbic acid oxidase activities. Vasanthi (1998) reported that there was a significant increase of peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and catalase activity in diseased plants compared with that of healthy and regenerated cassava plants. Madhubala (2003) reported that there was a significant increase in peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and catalase activity to the tune of 16.67 and 38.01 respectively in *Cassava mosaic virus* infected cassava plants compared to healthy ones.

MATERIALS AND METHODS

Physiology and biochemistry of meristem derived and Cassava mosaic virus infected cassava plants

Plant - virus interaction alters the host physiology. Hence, studies were undertaken to assess changes in physiology of healthy cassava plants (meristem derived) as well as *Cassava mosaic virus* infected cassava plants of cv. MVD1 and cv. H226

Estimation of chlorophyll

Chlorophyll a, b, and total chlorophyll were estimated using the procedure of Witham *et al*. (1971). Fresh leaf material of meristem derived and *Cassava mosaic virus* infected cassava weighing 1 gram was taken in a mortar and macerated with 20 ml of 80 per cent acetone. The homogenate was centrifuged at 5000 rpm for 5 min. The supernatant was transferred to a 50 ml volumetric flask and the residue was again extracted with acetone. The procedure was repeated till the residue became colourless. The volume of the chlorophyll extract was made up to 100 ml with 80 per cent acetone. The optical density was measured in a spectrophotometer at 663, 652 and 645 nm using 80 per cent acetone as blank. The contents of a, b and total chlorophyll were calculated using the following formulae,

$$\text{Chlorophyll b} = 22.9\ (A_{645}) - 4.68\ (A_{663}) \times V/1000 \times W$$

$$\text{Total chlorophyll} = 20.2\ (A_{645}) - 8.02\ (A_{663}) \times V/1000 \times W$$

$$\text{Chlorophyll a} = 12.7\ (A_{663}) - 2.69\ (A_{645}) \times V/1000 \times W$$

where, A= absorbance at specific wave lengths,
V = final volume of chlorophyll extract in 80 per cent acetone and
W = fresh weight of tissue extracted.

The results were expressed as mg g^{-1} on fresh weight basis.

Estimation of total phenol

Total phenol was estimated by adopting the protocol of Malik and Singh (1980). One gram of cassava leaf samples collected from meristem derived and *Cassava mosaic virus* infected plants were homogenized in 10 ml of 80 per cent ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min and the supernatant was taken. The supernatant was evaporated to dryness and the residue was dissolved in known volume of water. To 1 ml of the extract, 5 ml of distilled water and 0. 5 ml of Folin- Ciocalteau reagent were added and the solution was kept at 25ºC. After 3 min, one ml of sodium carbonate (20% solution) and 1 ml of distilled water were added and the reaction mixture was incubated for 1 h at 25ºC. The intensity of blue color was read at 650 nm in a spectrophotometer against a reagent blank. Catechol at different concentrations served as the standard. The amount of phenol was expressed as catechol equivalent in mg g^{-1} of fresh tissue.

Estimation of total sugars

Total sugar content was determined by anthrone method (Hedge and Hofreiter, 1962). One hundred milligram of cassava leaf samples collected from meristem derived and *Cassava mosaic virus* infected plants were homogenized in 5ml of 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 10 min and the supernatant was collected. Two hundred micro liters of the supernatant was taken in a boiling test tube and the alcohol was evaporated using a water bath. The residue was dissolved in 1 ml of water. To this 4 ml of anthrone reagent was added and heated for 8 min in a boiling water bath. Later, the tubes were cooled rapidly and the absorbance was read at 630nm in a spectrophotometer. D-glucose was used as a standard and the total sugar content was expressed in terms of mg of glucose equivalent per g of leaf tissue on fresh weight.

Estimation of total soluble proteins

Total soluble protein content was estimated as described by Bradford (1976).

Preparation of stock solution

One hundred mg of coomassie brilliant blue G250 was dissolved in 50 ml of 95 per cent ethanol and to that 100 ml of concentrated ortho-phosphoric acid was added. The volume was made upto 200 ml with water and kept at 4 ºC. Stock solution was

diluted four times with water to get the working solution.

Sample preparation

One gram of cassava leaf samples collected from meristem derived and *Cassava mosaic virus* infected plants were homogenized in five ml of 0.1 M phosphate buffer (pH 7.0) using chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 10 min at 4 ºC and the supernatant was used for the assay of soluble protein.

Assay

To 0.5 ml of the extract, 0.5 ml of water and 2.5 ml of the working dye solution were added and incubated for five min at room temperature. One ml of water added with 2.5 ml of dye served as the blank. The intensity of blue color was measured at 595 nm in a spectrophotometer. Bovine serum albumin at various concentrations served as the protein standard. Soluble protein content was expressed as mg albumin equivalent of soluble protein g^{-1} on fresh weight basis.

Protein profile of meristem derived and Cassava mosaic virus infected cassava plants of cv. MVD1 and cv. H226

Protein profile of infected and healthy (meristem derived) cassava samples from the varieties MVD1 and H226 were analyzed using sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to Laemmli (1970) using 12% acrylamide gel (Annexure V). Five hundred milligram of healthy and infected cassava leaf samples were homogenized separately with 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 10,000 rpm for 10 min at 4 ºC. From the supernatant, 25 µl was taken and mixed with equal quantity of sample buffer. The samples were boiled for three minutes and cooled and were loaded on to the gel. Medium range protein marker was loaded on one well. Gel running was done at a constant voltage (65V) and the gel was stained overnight. After proper staining, the gel was destained and destaining was continued till the protein bands became clear.

Enzyme activities in meristem derived and Cassava mosaic virus infected cassava plants of cv. MVD1 and cv. H226

Peroxidase (PO)

Activity of peroxidase was determined as detailed by Hammerschmidt *et al*. (1982). One gram of cassava leaf samples collected from meristem derived and *Cassava mosaic virus* infected plants were homogenized in 1 ml of 0.1 M phosphate buffer pH 7.0 in a pre-cooled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was used as enzyme source. The reaction mixture consisting of 1.5 ml of 0.05 M pyrogallol and 0.1 ml of enzyme extract was taken in a cuvette. To initiate the reaction 0.5 ml of 1.0 per cent H_2O_2 was added. The change in absorbance was recorded at 420 nm at 30 s interval for three min from zero s of incubation at room temperature. The boiled enzyme served as blank. The enzyme activity was expressed as change in absorbance $min^{-1}g^{-1}$ of fresh tissue.

Polyphenol oxidase (PPO)

One gram of fresh cassava leaf samples collected from meristem derived and *Cassava mosaic virus* infected plants were ground in 1ml of 0.1 M phosphate buffer (pH 7.0) in a pre-cooled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was used as enzyme source. The reaction mixture consisting of 1.5 ml of 0.1 M sodium phosphate (pH 7.0) and 0.1 ml of enzyme extract was taken in a cuvette. To this, 0.2 ml of catechol (0.01M) was added to initiate the reaction. The change in absorbance was recorded at 495 nm at 30 s interval for three min and the results were expressed as change in absorbance min^{-1} g^{-1} of fresh tissue (Mayer *et al*., 1965).

Phenylalanine ammonia lyase (PAL)

Five hundred mg of cassava leaf samples collected from meristem derived and *Cassava mosaic virus* infected plants were homogenized in 5 ml of cold 25 mM borate HCl buffer (pH 8.8). The homogenate was centrifuged at 10000 rpm for 15 min and the supernatant was used as enzyme source. The assay mixture consisted of 0.2 ml of enzyme extract, 1.3 ml water and 0.5 ml borate buffer. The reaction was initiated by the addition of 1ml of 12 mM L-Phenylalanine. The reaction mixture was incubated for 1 h at 32 °C. The reaction was stopped by the addition of 0.5 ml of 2 N HCl. The absorbance was measured at 290 nm. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid. The enzyme activity was expressed as n mol of trans-cinnamic acid $min^{-1}g^{-1}$ of leaf tissue (Dickerson *et al*., 1984).

Catalase

Catalase activity was assayed in the leaf samples collected from meristem derived and *Cassava mosaic virus* infected cassava plants as per the method described by Beers and Sizer (1952). Enzyme extract was obtained as described in peroxidase assay and it was used for catalase activity. Reaction mixture consisting of 0.5ml of 0.1M phosphate buffer (pH 7.0), 0.2ml of enzyme extract and 0.1ml of 1% H2O2 was incubated at 28±1°C. At the start of the enzyme reaction the absorbance of the mixture was set zero at 230nm in a spectrophotometer and changes in the absorbance was recorded at 15 sec intervals for 3 min. Catalase activity was expressed as change in the absorbance of the reaction mixture/ min/ g of leaf tissue on fresh weight basis.

Native - polyacrylamide gel electrophoresis (PAGE) analysis of PO and PPO for meristem derived and Cassava mosaic virus infected cassava plants of cv. MVD1 and cv. H226

Samples collected from meristem derived and *Cassava mosaic virus* infected plants were homogenized with 1 ml of 0.1 M sodium phosphate buffer pH 7.0 and centrifuged at 10,000 rpm for 20 min at 4°C. The protein content of the sample was determined by Bradford (1976) method. Samples (50 µg protein) were loaded onto separating gel of 8.0 per cent and stacking gel of 4.0 per cent polyacrylamide concentration for the analysis of PO and PPO isoforms.

Peroxidase (PO)

After electrophoresis, the gel was stained in 0.2 M acetate buffer at pH 4.2 containing 0.05 per cent benzidine for 30 min in dark. Then drops of H_2O_2 (30%) were added slowly with constant shaking to visualize the PO isoforms. After staining, the gel was washed with distilled water and photographed (Nadlony and Sequira, 1980).

Polyphenol oxidase (PPO)

After native gel electrophoresis, the gel was immersed for 30 min in *p*-phenylene diamine (0.1%) in 0.1 M potassium phosphate buffer (pH 7.0). Later 10 mM catechol was added and kept in a shaker with gentle shaking and observed for the appearance of dark brown bands (Jayaraman *et al*., 1987).

RESULTS AND DISCUSSION

Physiology and biochemistry of meristem derived and Cassava mosaic virus infected cassava plants of cv. MVD1 and cv. H226

Estimation of Chlorophyll

Meristem derived healthy cassava plants of cv. MVD1 and cv. H226 were having more chlorophyll content. Chlorophyll a, b and total chlorophyll were significantly more in healthy cassava plants than in *Cassava mosaic virus* infected cassava plants. Such increase in healthy plants over infected was 10.46% and 10.44% in chlorophyll a, 27.5% and 21.3% in chlorophyll b and 19.10% and 18.11% in total chlorophylls in cv. MVD1 and cv. H226 plants respectively. (Table 1) (Table 2). This is in agreement with the results of Alagianagalingam and Ramakrishnan (1978). They reported that mosaic infected cassava leaves had less chlorophyll and carotene and lower photosynthetic rate. Similar results were obtained by Vasanthi (1998). The loss in chlorophyll content might be due to the diversion of plastid protein into virus protein (Bawden and Pirie, 1952) or as a result of normal cell enzymes that attack the chlorophyll (Goodman *et al*., 1977). This has also been attributed to either the inhibition of chloroplast development or the distribution of pigments in matured chloroplasts (Matthews, 1981). In meristem derived plants because of the absence of the virus there was no degradation of chlorophyll.

Estimation of total phenols

Meristem derived healthy cassava plants of cv. MVD1 and cv. H226 exhibited 33.40% and 31.49% increase in total phenol content as compared to that in *Cassava mosaic virus* infected plants (Table 3) (Table 4). This finding is contrary with the observations of Vasanthi (1998) and Madhubala (2003). Where they reported that total phenol content was more in *Cassava mosaic virus* infected cassava plants. In general phenols are mainly responsible for resistance in plants against stresses. Because of the presence of more amount of phenols the meristem derived plants are completely free from viruses.

Estimation of total sugars

Meristem derived healthy cassava plants of cv. MVD1 and cv. H226 exhibited a significant increase in total sugar content (31.30% and 19.26% respectively) as compared to that in *Cassava mosaic virus* infected plants (Table 3). The reduced levels of total sugar content of the diseased plants may be due to higher utilization of sugars for phosphate ester formation required for virus nucleic acid synthesis (Singh and Srivastava, 1974). Conversion of sugars into amino acids and organic acids may be yet another possible reason for the reduced level of sugars in virus infected plant (Maggyaarosy *et al*., 1973). In meristem derived plants because of the absence of the virus there was no chance of conversion of sugars into amino acids and organic acids.

Estimation of total soluble proteins

Total soluble proteins in meristem derived healthy cassava plants of cv. MVD1 and cv. H226 increased to the tune of 47.49% and 31.67% respectively as compared to that in *Cassava mosaic virus* infected plants (Table 4). This is in contrary with the findings of Nambisan (1994), Vasanthi (1998) and Madhubala (2003). Where they reported that total soluble content was significantly increased in *Cassava mosaic virus* infected cassava plants.

Protein profile of meristem derived and Cassava mosaic virus infected cassava plants of cv. MVD1 and cv. H226

SDS-PAGE analysis revealed the presence of 35 KDa protein in *Cassava mosaic virus* infected samples of cv. MVD1 and cv. H226 collected from field, which was absent in meristem derived healthy plants (Picture 1).

Enzyme activities in meristem derived and Cassava mosaic virus infected cassava plants of cv. MVD1 and cv. H226

Infection of the cassava plants (cv. MVD1 and cv. H226) by *Cassava mosaic virus* led to increased Peroxidase, **Polyphenol oxidase, Phenylalanine ammonia lyase, Catalase** activity (Table 5) (Table 6). When compared to *Cassava mosaic virus* infected cassava plants Peroxidase, **Polyphenol oxidase, Phenylalanine ammonia lyase, Catalase** activity significantly decreased in meristem derived healthy cassava plants of cv. MVD1 and cv. H226

to the tune of 19.26%, 28.34%,8.43%,20.58% and 22.7%, 33.33%, 10.90%,22.63% respectively. This is in agreement with the findings of Alagianagalingam and Ramakrishnan (1974), Vasanthi (1998) and Madhubala (2003). They noticed significant differences in PO, PPO, PAL and catalase activity in the infected plants. PO is the key enzyme in the biosynthesis of lignin, It also catalyses the oxidation of phenols to quinines which are highly fungitoxic. PPO catalyses the oxidative phenolic compounds and plays major role in defense. PAL is the first enzyme in the phenyl propanoid metabolism and has a definite role in defense mechanism of plants (Friend and Threshfall, 1976). The virus-infected plant accelerates the synthesis of catalase to remove excess hydrogen peroxide produced due to increased respiration (Lodh *et al*., 1973). The abrupt rise in the enzyme activity may be due to alteration in redox potential of the host as a result of virus infection (Vidhayasekaran, 1988). Since the meristem derived plants are free from the virus infection there was no induction of the above-mentioned enzymes.

Native PAGE analysis of PO and PPO isoforms in meristem derived and Cassava mosaic virus infected cassava plants of cv. MVD1 and cv. H226

Native PAGE analysis revealed the presence of three PO and two PPO isoforms (PO1, PO2, PO3 and PPO1 and PPO2) in *Cassava mosaic virus* infected and meristem derived cassava plants of cv. MVD1 and cv. H226 whereas, two PO and one PPO isoform (PO1, PO3 and PPO2) were present in meristem derived cv. MVD1 and cv. H226 cassava plants. Two PO and PPO isoforms (PO2, PO3 and PPO1, PPO2) were found in CMD infected cv. MVD1 and cv. H226 cassava plants (Picture 2) (Picture 3).

Table 1. Estimation of chlorophyll content in meristem derived and *Cassava mosaic virus* infected cassava plants (cv. MVD1)

S. NO.	Treatments	Chlorophyll a* (mg/ g fresh weight)	% increase over infected	Chlorophyll b* (mg/ g fresh weight)	% increase over infected	Total chlorophylls* (mg/ g fresh weight)	% increase over infected
1.	Healthy (meristem derived plants)	1.498	10.46	0.612	27.5	2.114	19.10
2.	Infected	1.348	-	0.480	-	1.775	-

*Mean of eight replications

Table 2. Estimation of chlorophyll content in meristem derived and *Cassava mosaic virus* infected cassava plants (cv. H226)

S. NO.	Treatments	Chlorophyll a* (mg/ g fresh weight)	% increase over infected	Chlorophyll b* (mg/ g fresh weight)	% increase over infected	Total chlorophyll* (mg/ g fresh weight)	% increase over infected
1.	Healthy (meristem derived plants)	0.963	10.44	0.558	21.30	1.754	18.11
2.	Infected	0.872	-	0.460	-	1.485	-

*Mean of eight replications

Table 3. Estimation of total phenols, total sugars and total soluble protein contents of meristem derived and *Cassava mosaic virus* infected cassava plants (cv. MVD1)

S. NO.	Treatments	Total phenols* (mg / g fresh weight)	% increase over infected	Total sugars* (mg D-glucose equivalent / g fresh weight)	% increase over infected	Total soluble proteins* (mg / g fresh weight)	% increase over infected
1.	Healthy (meristem derived plants)	1.198	33.40	13.150	31.30	1.236	47.49
2.	Infected	0.898	-	10.015	-	0.838	-

*Mean of eight replications

Table 4. Estimation of contents total phenol, total sugars and total soluble proteins of meristem derived and *Cassava mosaic virus* infected cassava plants (cv. H226)

S. NO.	Treatments	Total phenols* (mg / g fresh weight)	% increase over infected	Total sugars* (mg D-glucose equivalent / g fresh weight)	% increase over infected	Total soluble proteins* (mg / g fresh weight)	% increase over infected
1.	Healthy (meristem derived plants)	1.023	31.49	11.125	19.26	1.105	31.67
2.	Infected	0.778	-	9.328	-	0.755	-

*Mean of eight replications

Table 5. Defense enzyme status in meristem derived and *Cassava mosaic virus* infected cassava plants (cv. MVD1)

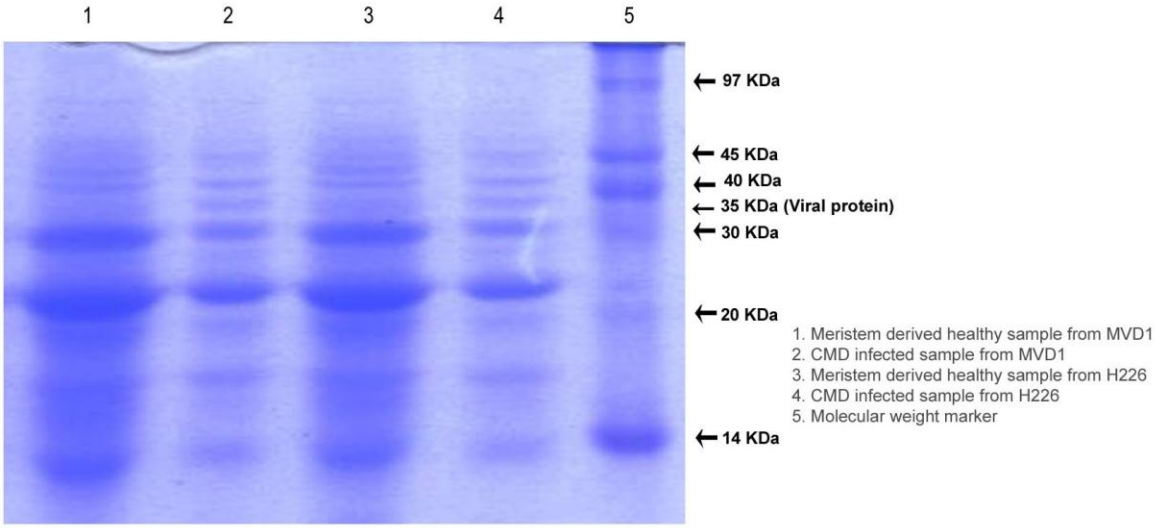
S. NO.	Treatments	PO activity * (Change in abs/ min / g)	% decrease over infected	PPO activity* (Change in abs/ min / g)	% decrease over infected	PAL activity* (n moles of transcinnamic acid / g)	% decrease over infected	Catalase	% decrease over infected
1.	Healthy (meristem derived plants)	0.826	19.26	0.526	28.34	163	8.43	0.463	20.58
2.	Infected	1.023	-	0.734	-	178	-	0.583	-

*Mean of eight replications

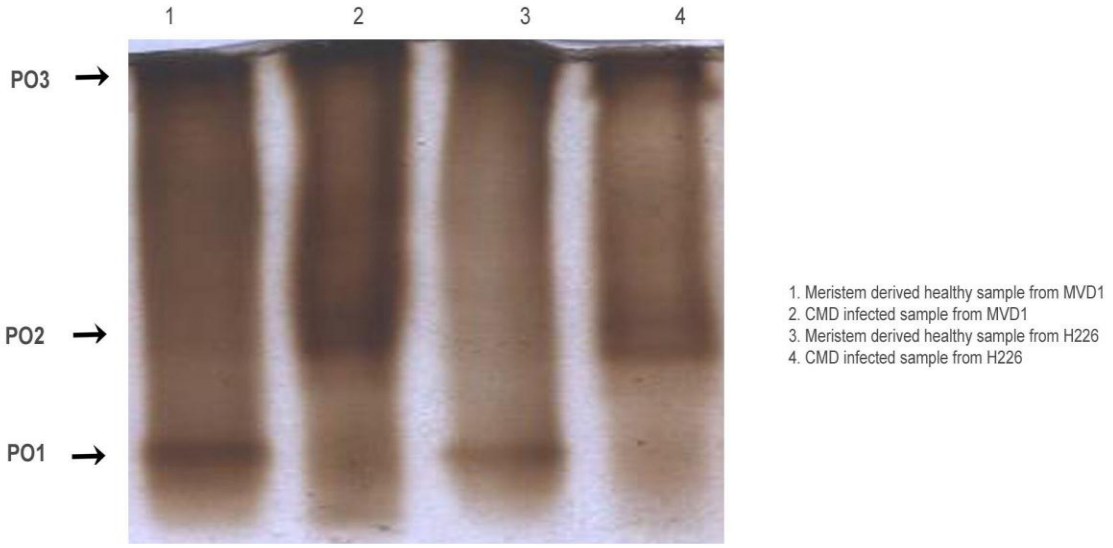
Table 6. Defense enzyme status in meristem derived and *Cassava mosaic virus* infected cassava plants (cv. H226)

S.NO.	Treatments	PO activity * (Change in abs/ min / g	% decrease over infected	PPO activity * (Change in abs/ min / g)	% decrease over infected	PAL activity* (n moles of transcinnamic acid / g	% decrease over infected	Catalase	% decrease over infected
1.	Healthy (meristem derived plants)	0.715	22.79	0.350	33.33	98	10.90	0.383	22.63
2.	Infected	0.926	-	0.525	-	110	-	0.495	-

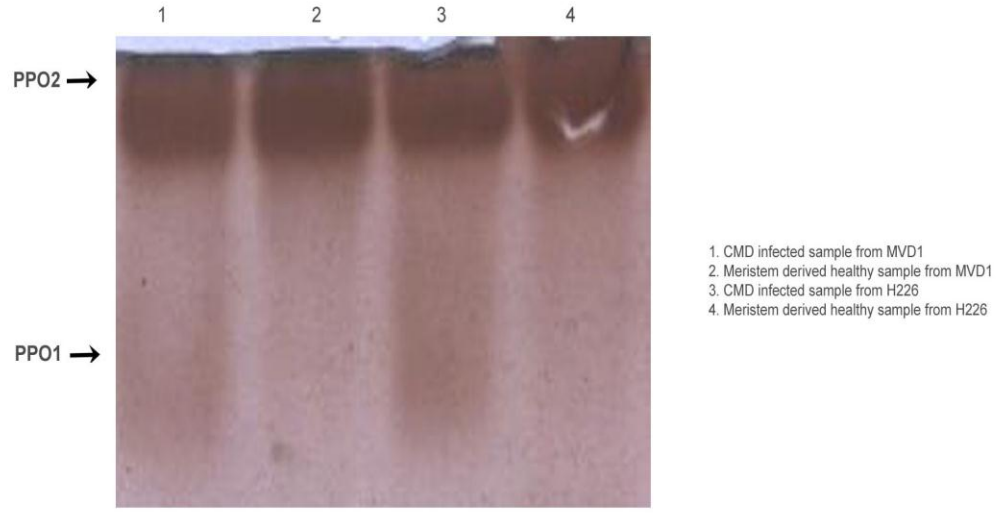
* Mean of eight replications



Picture 1. Protein profile of Cassava Mosaic Disease infected and healthy cassava plants



Picture 2. Induction of Peroxidase in Cassava Mosaic Disease infected and healthy cassava plants



Picture 3. Induction of Poly Phenol Oxidase in Cassava Mosaic Disease infected and healthy cassava plants

CONCLUSION

Chlorophyll a, Chlorophyll b, total Chlorophyll**,** total phenol, total sugars and total soluble proteins increased in healthy cassava plants.

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AUTHORS CONTRIBUTION

NR carried out the implementation and development of the work. KE and DL corrected the work and analyzed the data. All authors read and approved the final manuscript. All the authors read and approved the final manuscript.

COMPETING INTEREST

The authors declare that they have no competing interests.

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