RESEARCH ARTICLE

Alterations in biochemical components in mesta plants infected with yellow vein mosaic disease

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ALTERATIONS IN BIOCHEMICAL COMPONENTS IN MESTA PLANTS: Yellow vein mosaic disease of mesta (kenaf, *Hibiscus cannabinus* L.; and roselle, *H. sabdariffa* L.) is a new entrant to the disease scenario and it is associated with a novel monopartite *Begomovirus.* Changes in different biochemical parameters in diseased mesta plants were observed as compared to healthy ones. Isozyme pattern and assays of different enzymes, namely catalase, acid phosphatase, peroxidase, esterase, polyphenol oxidase and superoxide dismutase, revealed lower activities of catalase, acid phosphatase and peroxidase enzymes and enhanced activities of esterase, polyphenol oxidase and superoxide dismutase in diseased plants as compared to healthy ones. Due to the infection, chlorophyll content, phenolics and total soluble protein decreased whereas free amino acid, proline and disease-related proteins increased in the host plants. Differential responses of polyacetylene and isoflavone content as well as SDS-PAGE band profiling of total soluble proteins were also observed in plants due to the infection.

Key Words: *Begomovirus,* chlorophyll, *Hibiscus,* isozyme pattern, phenolics, proline.

INTRODUCTION

Plants in nature are constantly challenged by a diverse array of pathogenic microorganisms. In many cases, their protective mechanisms involve an inducible defense system. The ability of plants to invoke such defense reactions is presumed to be mediated by an initial recognition process that involves detection of certain unique signal molecules of incompatible pathogens by receptor-like molecules in plants, resulting in a cascade of biochemical events that leads to the expression of resistance and susceptibility to a disease (Ryals et al., 1994). Host-pathogen interactions are presumed to generate signals that activate nuclear genes involved in plant defense responses leading to the induction of stress-related enzymes, differential expression of proteins and release of free amino acids and the associated accumulation of high levels of phenolic compounds. Antimicrobial phytoalexins such as sesquiterpenoids, isoflavanoids, coumarins, acetylenic and phenolic compounds also contribute to multilayered plant defense systems (Keen, 1992).

The occurrence of yellow vein mosaic disease of mesta (kenaf, *Hibiscus cannabinus* L.; and roselle, *H. sabdariffa* L.; Malvaceae) is a new entrant to the disease scenario in India (Chatterjee and Ghosh, 2007a,b). It was found in endemic form in different parts of India during the last few years and the disease has spread fastly causing strong reductions in yield and thus becoming a major threat to production. The association of a novel *Begomovirus*, namely *Mesta yellow vein mosaic virus*, with this disease was confirmed by electron microscopy and molecular techniques using PCR, sequence information and southern hybridization (Chatterjee et al., 2006; Chatterjee and Ghosh, 2007a,b). However the alterations in the host physiology and its associated biochemical components induced by the infection with this recently known *Begomovirus* pathogen in mesta plants

remain unknown. Hence, the present investigation was undertaken with the diseased plants in order to determine the patho-physiological changes that take place.

MATERIALS AND METHODS

Mesta plants (*H. cannabinus* cv. HC-583 and *H. sabdariffa* cv. HS-4288) were raised using seeds in healthy conditions in a glasshouse. Leaves from infected mesta plants showing the typical yellow vein mosaic disease symptom were used as a source of inoculum. Artificial inoculation of healthy plants was carried out using viruliferous whiteflies, the natural vector of this disease. The inoculated plants, along with their respective healthy controls, were then maintained in insect-proof wooden cages kept at 30°C in a temperature controlled glasshouse under a photoperiod of 18/6 h (light/dark) and 60% RH. After the development of symptoms in infected plants the experiment was terminated and the plants harvested for analysis.

Concentration of chlorophyll in leaves from diseased mesta plants and the respective healthy plant controls was determined at 30-d intervals using a standard procedure (Sadasivam and Manickam, 1992). Phenolic compounds (bound phenols, ortho-dihydric phenols and total phenols) (Malick and Singh, 1980), total free amino acids (Misra et al., 1975), proline (Bates et al., 1973), and total protein (Lowry et al., 1951) were estimated using standard protocols after day 110 of inoculation. For protein, a standard curve was prepared from a stock standard solution of BSA (200 μg protein mL^{-1}). Polyacrylamide gel electrophoresis (SDS-PAGE) of total soluble protein was conducted using a 12% resolving gel and 5% stacking gel in tris-glycine-SDS buffer following the protocol of Laemmli (1970). Analysis of disease-related proteins (Mitra et al., 1990), and extraction and identification of polyacetylenes and isoflavones by thin-layer chromatography (TLC) (Harborne, 1973) were performed from healthy and diseased mesta plants. Activity assays of catalase (CAT, EC. 1.11.1.6) (Braber, 1980), esterase (EST, EC 3.1.1.8 (Thimmaiah, 1999), acid phosphatase (ACP, EC. 3.1.3.2) and peroxidase (POD, EC. 1.11.1.7) (Malik and Singh, 1980), polyphenol oxidase (PPO, EC 1.14.18.1) (Sarvesh and Reddy, 1988) and superoxide dismutase (SOD, EC 1.15.1.1) (Oberley and Spitz, 1985) were performed as described for diseased and healthy mesta plants after day 110 of inoculation. In the enzyme assays, H_2O_2 was used as the substrate for CAT (assay pH 7.0), indophenyl acetate for EST (assay pH 5.5), p-nitrophenyl phosphate for ACP (assay pH 5.2), orthodianisidine for POD (assay pH 6.0), *o*-catechol for PPO (assay pH 6.8), and diethylenetriamine pentaacetic acid, nitroblue tetrazolium and xanthine for SOD (pH 7.8). The isoenzyme profiles of CAT (Woodbury et al., 1971), EST (Brewbaker et al., 1968), ACP (Murray and Collier, 1977), POD (Sheen and Calvert, 1969), PPO (De Ascensao and Dubery, 2000) and SOD (Chen and Pan, 1996) were examined by native PAGE. Each experiment was replicated five times. Data are depicted as a histogram and values represent the means of five observations (n = 5). The vertical bar above the mean represents SD.

RESULTS AND DISCUSSION

The present investigation revealed enormous changes in biochemical components in mesta plants due to the infection with yellow vein mosaic virus. A gradual reduction in green pigments like chlorophyll (*a*, *b* and total) at different stages of pathogenesis in both species was observed (Figure 1). The disease development in mesta also altered the ratio between chlorophyll a and b, probably affecting the photosynthetic efficiency (Endo et al., 2000). Lower amounts of phenolics (total phenols, ortho-dihydric phenols and bound phenols) in diseased plants after 110 days of inoculation in both species were also observed as compared with their respective controls (Figure 2). Defense responses are characterized by the early accumulation of phenolic compounds at the infection site and the slowing down of pathogen development through rapid cell death (Fernandez and Heath, 1989). A role for phenolics and phenol oxidizing enzymes like PPO and POD in plant resistance against viral diseases has been implicated by several investigators (e.g., Rathi et al., 1986). With symptom development, phenols decrease in susceptible varieties whereas in resistant varieties they accumulate (Thimmaiah, 1999). Hence, reduced levels of phenolics, as evident in the present study, would appear to be a possible factor for disease development in the two species investigated.

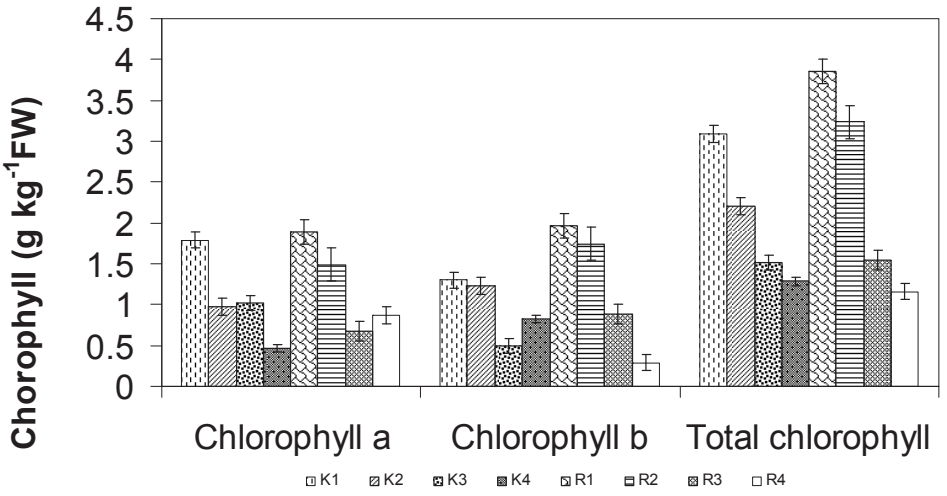

Figure 1. Chlorophyll concentration of healthy and diseased *Hibiscus cannabinus* (kenaf) and *H. sabdariffa* (roselle) at different ages. K1 = healthy kenaf before inoculation, K2 = diseased kenaf after one month of inoculation, K3 = diseased kenaf after two months of inoculation, K4 = diseased kenaf after three months of inoculation; R1 = healthy roselle before inoculation, R2 = diseased roselle after one month of inoculation, R3 = diseased roselle after two months of inoculation, R4 = diseased roselle after three months of inoculation. Each bar represents the mean (n = 5) ± SD.

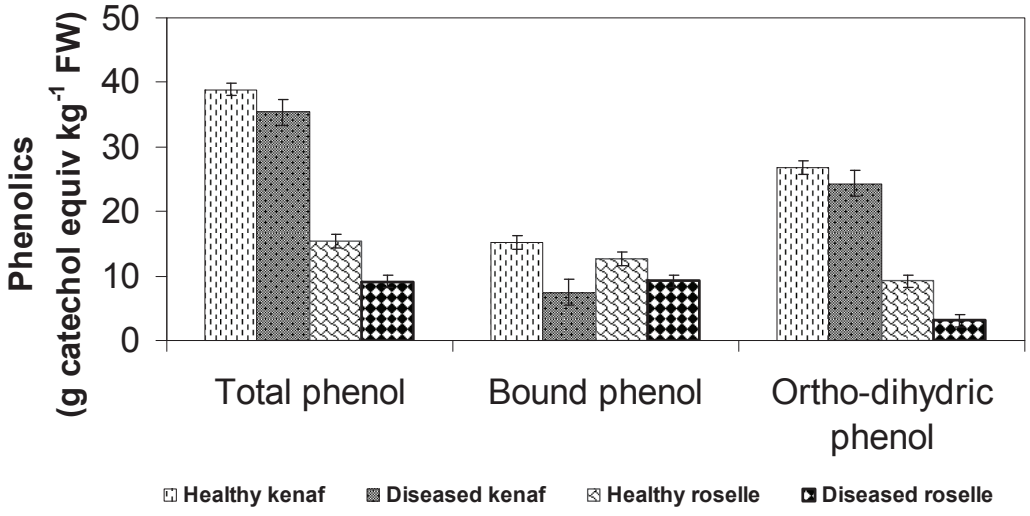

Figure 2. Phenolic (bound phenols, ortho-dihydric phenols and total phenols) concentrations from healthy and diseased Hibiscus cannabinus (kenaf) and H. sabdariffa (roselle) plants (after 110 d of inoculation). Each bar represents the mean (n = 5) ± SD.

The protein concentration was low in diseased plants of both species compared to controls (Figure 3A). In diseased leaves after 110 d of inoculation the free amino acid concentration was greater than in the control (Figure 3B). A higher amount of proline in diseased material was also observed compared to the respective controls (Figure 3C). Low protein content and higher free amino acid content in diseased samples indicate that the disease might have caused denaturation or breakdown of proteins, as well as polypeptide chains and bound amino acids, resulting in enhanced free amino acid content of the host tissues. Proline is also a major component of structural proteins in animals and plants and a known osmo-protectant capable of mitigating the impacts of drought, salt, temperature and pathogenic stress in plants. When plants are exposed to microbial pathogens, they produce reactive oxygen

species (ROS) that induce programmed cell death in the plant cells surrounding the infection site to effectively wall off the pathogen and terminate the disease process (Apel and Hirt, 2004). The amino acid proline may act as a potent scavenger of ROS and this property of proline might prevent the induction of programmed cell death by ROS (Chen and Dickman, 2005). Proline may also function as a protein-compatible hydrotrope (Srinivas and Balasubramanian, 1995), and as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989). In any case, the higher proline accumulation in diseased tissue as noted in the present study might be related to pathological disorder (Stewart, 1980).

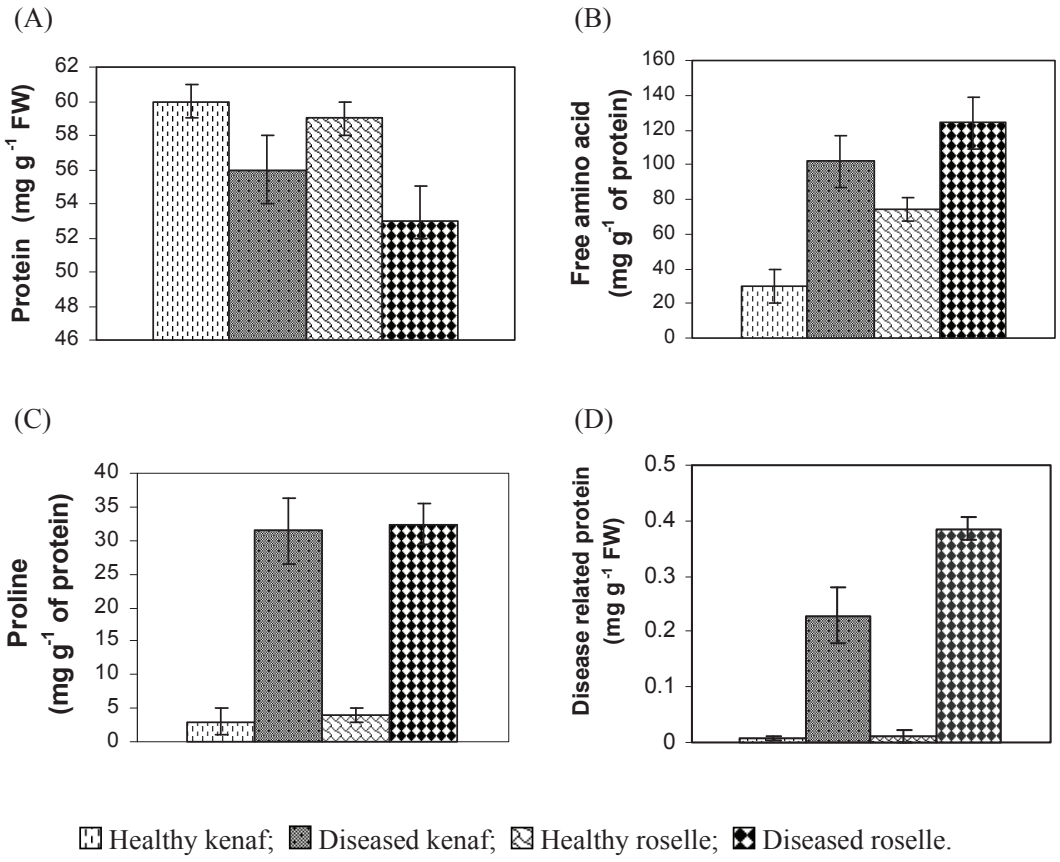

Figure 3. Concentrations of protein (**A**), free amino acid (**B**), proline (**C**) and disease-related protein (**D**) from healthy and diseased *Hibiscus cannabinus* (kenaf) and *H. sabdariffa* (roselle) plants (after 110 d of inoculation). Each bar represents the mean ($n = 5$) ± SD.

The SDS-PAGE protein profile of total soluble proteins from diseased leaves of both *H. cannabinus* and *H. sabdariffa* showed differences in band patterns when compared with their respective healthy plants (Figure 4). In *H. cannabinus* the virus infection caused the disappearance of protein bands at 27 kDa and near 85 kDa which were present in the healthy plant, while some new protein bands at 49 kDa, 170 kDa and 175 kDa were observed in diseased samples that were absent in the healthy sample. Additionally, one hypersensitive 20 kDa protein band was present in healthy *H. cannabinus* that was absent in the virus-infected plant. In the case of *H. sabdariffa*, 22 kDa and 26 kDa protein bands, pronounced in healthy plants, appeared to be absent in diseased material. Moreover, two protein bands of 20 kDa and 28 kDa appeared to be hypersensitive in healthy plants as compared to diseased *H. sabdariffa*. Resistance-associated proteins are reported in several virus-host interactions (Sela, 1981). Plant pathogens such as viruses, bacteria, fungi and nematodes elicit the synthesis of host proteins which help in restricting the multiplication and spread of pathogens in the healthy tissue (Datta et al., 1999).

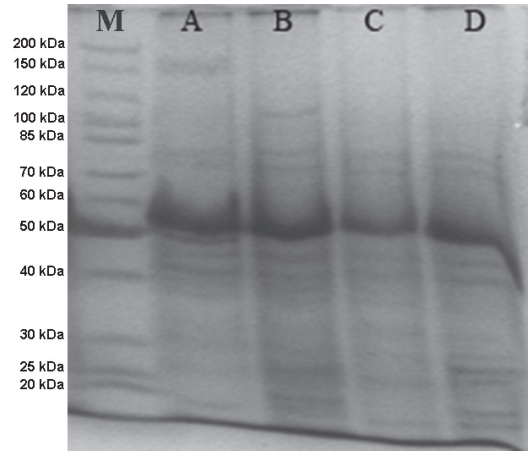

Figure 4. SDS-PAGE profile of total soluble proteins from healthy and diseased leaves of *Hibiscus cannabinus* (kenaf) and *H. sabdariffa* (roselle) plants. A = diseased *H. cannabinus,* B = healthy *H. cannabinus,* C = diseased *H. sabdariffa,* D = healthy *H. sabdariffa.* M = Protein Ladder (10-200 kDa).

Analysis of disease-related proteins revealed that the content of such proteins was greater in diseased *H. cannabinus* and *H. sabdariffa* than in the respective controls (Figure 3D). In the present investigation, TLC separation and UV-spectrum analysis revealed the presence of a higher amount of polyacetylenes in healthy than in diseased plants, whereas lower concentrations of isoflavones were found in the healthy plants compared to the diseased ones (Figure 5). Plants have flexible detection systems and probably employ several recognition and signal transduction pathways to activate their defense (Johal et al., 1995). Overall, precise temporal and spatial coordination of induced defense responses are required to successfully kill or restrict the invading microbe while simultaneously minimizing the damage to host tissue (Hammond-Kosack and Jones, 1996).

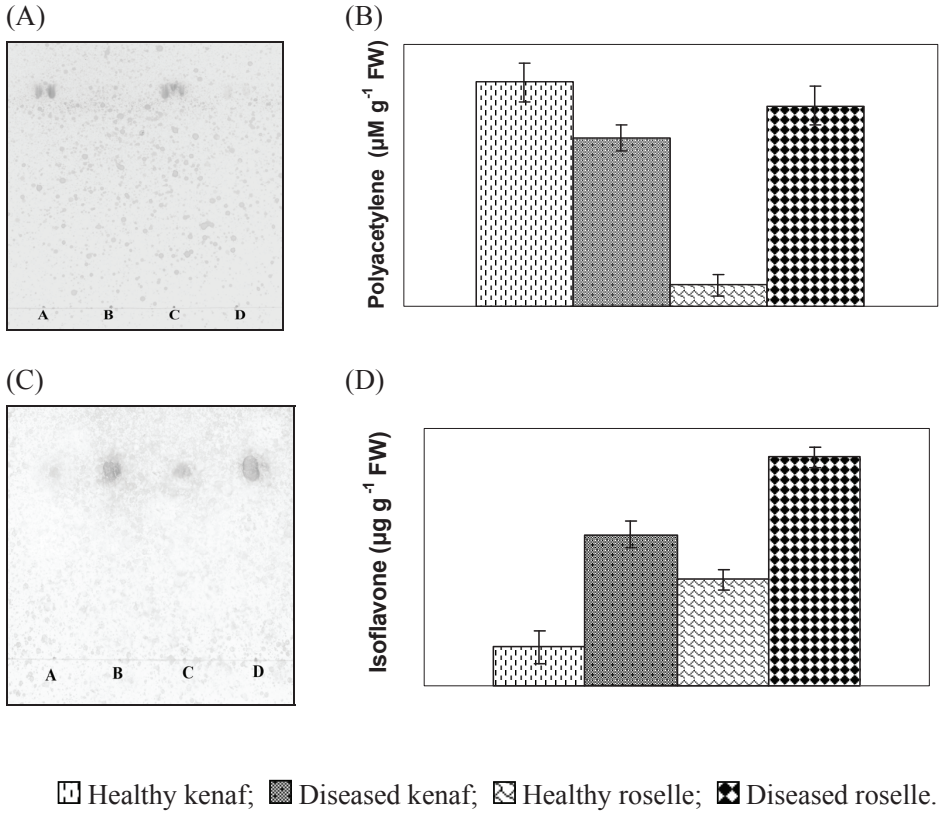

Figure 5. Estimation of polyacetylenes and isoflavones from healthy and diseased *Hibiscus cannabinus* (kenaf) and *H. sabdariffa* (roselle) plants (after 110 d of inoculation); thin-layer chromatographic separation (**A**) and UV-spectrum analysis of polyacetylenes (**B**), thin-layer chromatographic separation (**C**) and UV-spectrum analysis of isoflavones (**D**). Each bar represents the mean (n = 5) ± SD.

Incompatible host-pathogen interaction results in the synthesis of inhibitors, known as phytoalexins, which have different structures according to the plant source, such as sesquiterpenoid, isoflavanoid, acetylenic or phenolic. The results thus indicate that the possible variation in balance of such defense-related components like polyacetylenes and isoflavones might be one of the factors for establishment of this disease in host plants.

Analyses of isozyme patterns and activities of CAT, EST, ACP, POD, PPO and SOD in both the species, as shown in Figure 6, indicated alteration in activities of different enzymes due to the infection. Enzyme assays revealed lower activity of CAT, ACP and POD enzymes in diseased plants in comparison with healthy ones; in contrast, a marked increase in activities of EST, PPO and SOD was found in diseased plants as compared with the respective healthy plants. The isozyme patterns of these enzymes from diseased and healthy mesta plants produced similar types of band pattern in the case of ACP, PPO and SOD (Figure 7). For PPO and SOD the bands were found to be hyperactive in diseased plants in comparison with control plants, whereas in case of ACP the reverse was true. In the case of EST band profiling a clear extra band was found in diseased plants, and the other hyperactive bands observed in diseased plants indicate higher enzyme activity compared with their respective healthy plants. The isozyme profile of POD revealed the disappearance of some bands in diseased material which were present in their respective controls. In the case of CAT the isozyme pattern of diseased mesta was different from the healthy plant; a new band was noted in diseased material and some other bands were pronounced in healthy material but missing in the diseased plants.

Since enzymes control biochemical reactions, and their syntheses are under the control of specific gene(s), any change in the activity of an enzyme would reflect the pattern of gene expression and corresponding metabolic events in the cell. Hence, enzymes can be used as tools to study the induced responses of plants showing disease symptoms at the biochemical level (Neog et al., 2004). In addition, phenol-oxidizing enzymes such as POD and PPO are associated with many diseases (Pegg, 1985). In the present investigation, changes in the activities of CAT, EST, ACP, POD, SOD and PPO along with total amount of protein have been studied in mesta plants to understand the fate of existing biochemical components in these plants upon infection by yellow vein mosaic disease. Altered zymogram patterns of isocatalases suggest inactivation of existing isocatalases, activation of an inactive form and/or synthesis of new isocatalases. The appearance of new isozymes of CAT in infected tissue might play a unique role in disease development. ACP catalyzes the hydrolysis of phosphate esters with consequent release of inorganic phosphate and plays an important role in phosphorus metabolism (Thimmaiah, 1999). Thus our investigation revealed that this normal metabolism of ACP was found to be hampered in mesta plants due to virus infection. The higher EST and SOD activity in diseased leaves indicates a probable mechanism of overcoming the stress situation developed due to virus infection. The lower activity of POD enzyme, a key enzyme of lignin biosynthesis, in diseased plants probably resulted in the slowing down of the metabolic pathway for ligno-cellulosic bast fibre formation, thereby providing a possible clue for the reduction in fibre yield due to virus infection. Based on the differential ability of PPO and POD to drive the oxidation and condensation of lignin precursors, it has been suggested that PPO might be primarily responsible for the initial polymerization of monolignols into olignols (Sterjiades et al., 1993), whereas POD would be more likely to catalyze the reactions leading from olignols to highly condensed macromolecular lignin. PPO activity is ubiquitous in higher plants, and functions attributed to the enzyme include phenol metabolism and a defense mechanism against pathogens (Mace and Wilson, 1964; Lax and Cary, 1995). Several observations have identified a role for PPO in the polymerization of monolignols into olignols, the precursor molecules of lignin. The hyperactive profiling of PPO in diseased plants is normally associated with an improved host defense mechanism (Lax and Cary, 1995), but in the present investigation the host defense system in mesta plants appeared to have totally failed despite the enhanced PPO activity observed in diseased leaves. The reason behind such a situation is still unknown and warrants further study.

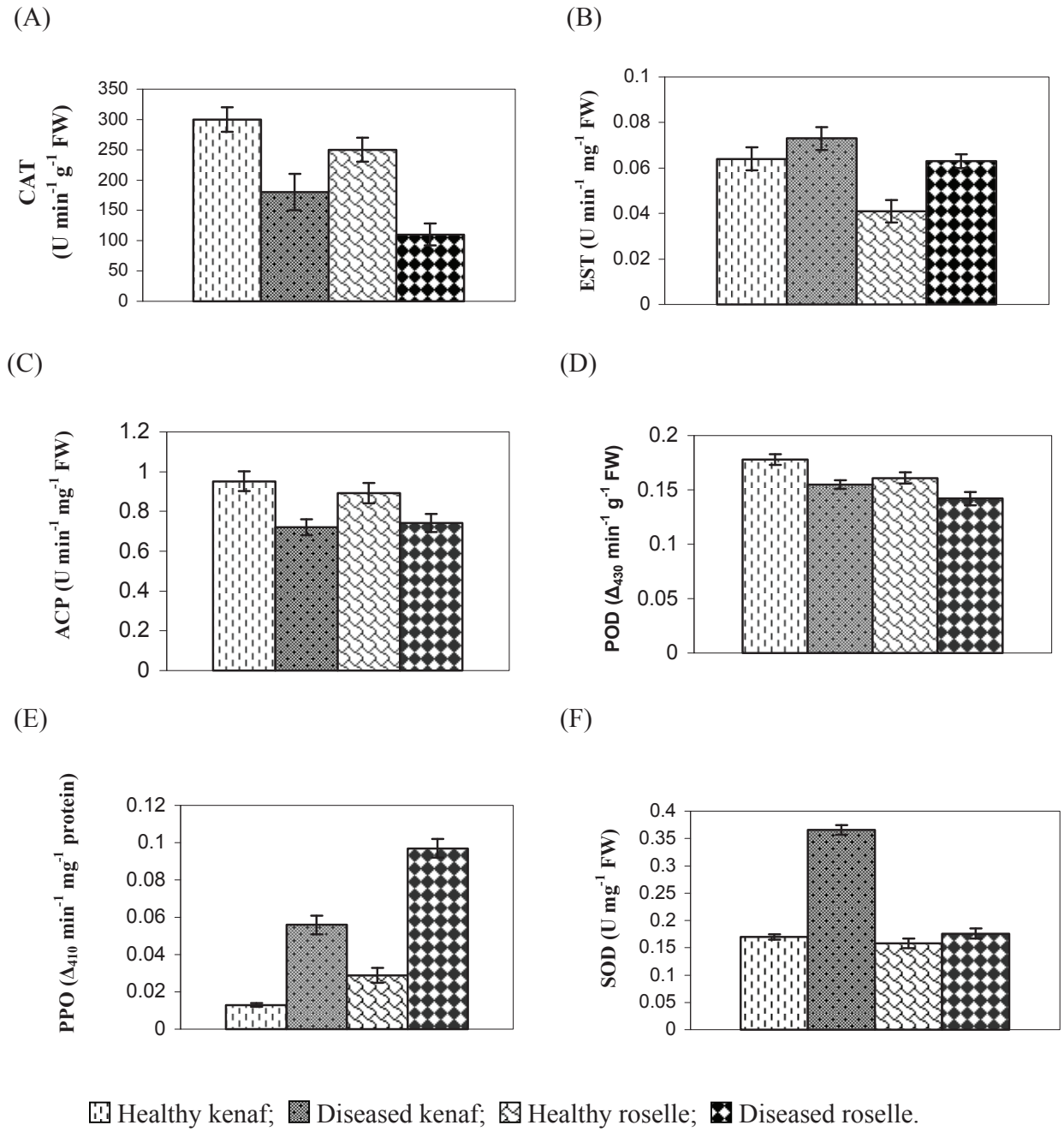

Figure 6. Activities of different enzymes from healthy and diseased leaves of *Hibiscus cannabinus* (kenaf) and *H. sabdariffa* (roselle) plants (after 110 d of inoculation). Catalase (**A**), esterase (**B**), acid phosphatase (**C**), peroxidase (**D**), polyphenol oxidase (**E**) and superoxide dismutase (**F**). Each bar represents the mean ($n = 5$) ± SD.

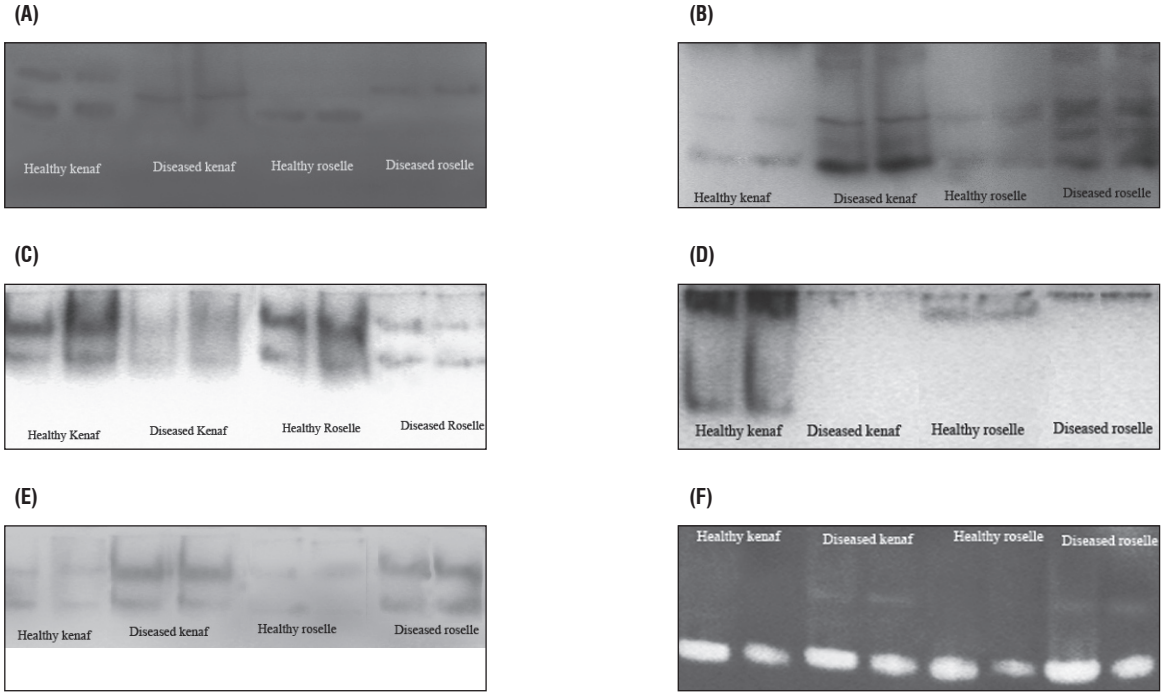

Figure 7. Isozyme polymorphism profiles from healthy and diseased leaves of *Hibiscus cannabinus* (kenaf) and *H. sabdariffa* (roselle) plants. Catalase (**A**), esterase (**B**), acid phosphatase (**C**), peroxidase (**D**), polyphenol oxidase (**E**) and superoxide dismutase (**F**).

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