Research Article

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Comparison of enzyme activities with diagnostic potential in healthy and root (wilt) diseased coconut palms, differing in cultivars and growth stages

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Abstract

Activities of superoxide dismutase (SOD), succinic dehydrogenase (SucDH), exochitinase (Cht) and 1, 4 β-glucanase (Glucn) were quantified to assess their diagnostic potential for detection of, or their role in imparting tolerance against, root (wilt) disease (RWD). The enzymes were determined in healthy and infected palms of contrasting coconut cultivars - Chowghat Green Dwarf (CGD) and/or Malayan Green Dwarf (MGD) tolerant and West Coast Tall (WCT) - susceptible, in the adult palms and in some at the seedling growth stage. Healthy and RWD plants differed little and that too inconsistently with respect to the activities, specific activities and soluble protein content of all the 4 enzymes, offering no diagnostic potential, in the three cultivars. However, cultivars and growth stage differences for enzyme activities were found to be significant for some enzymes. The RWD tolerant cultivar CGD (10-year old palms) had higher SOD and SucDH activity, higher soluble protein in the SOD and SucDH extract and lesser SOD specific activity than the RWD susceptible cultivar WCT (40-year old palms). The RWD tolerant cultivar MGD had lesser SOD activity than WCT in the seedling stage, lesser SOD specific activity in the adult stage (15-year old palms), higher SucDH activity and specific activity, higher soluble protein in the SOD extract, but lesser soluble protein in the Cht extract than 40-year old WCT adult palms. Seedlings of WCT and MGD had higher SOD activity than their adult plants. Seedlings of WCT and CGD had higher soluble protein in SOD extract than their adult plants. Seedlings of CGD had higher soluble protein in SucDH extract than its adult plants. Metabolic differences in enzyme activities indicated that the cultivars CGD followed by MGD and the seedling stage or younger plants seem to have a better capacity in resisting the effects of root (wilt) disease.

Keywords: Coconut, enzyme activities, cultivars, growth stage, soluble protein, root (wilt) disease

Introduction

Root (wilt) disease of coconut is widely prevalent, in southern districts of Kerala and also in isolated tracts in northern districts of the State, border districts of Tamil Nadu, Goa and Karnataka. The RWD incidence is very high (about 36%) in these districts of Kerala situated between Thiruvananthapuram and Thrissur and these areas are categorized as root (wilt) diseased tracts of Kerala. In other districts including Thiruvananthapuram and all districts which are north of Thrissur the RWD incidence is low and less than 6%, and these areas are categorized as healthy tracts. The disease is considered to be caused by phytoplasma (Solomon *et al*., 1983). The main symptoms are flaccidity of leaflets which droop downwards forming the shape of human ribs in the leaves of outer and middle whorls of coconut palms. Later, yellowing of leaves and marginal necrosis gradually develops. There is a progressive debilitation of the plant and reduction in nut yield, husk, copra, oil and leaf over a period of 10 years or more. The disease is non-curable but manageable by removing low yielding advanced diseased palms and planting seedlings from disease free mother palms/disease tolerant varieties. Presently disease

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free mother palms are being selected using serology test (Sasikala *et al.*, 2010).

By the serology test we can screen about 15 palms in four days. With a view to search for a method which could be faster or easier than serology, enzyme activity differences between healthy and RWD coconut palms were taken up in this study to asses their diagnostic potential. Superoxide dismutase (SOD) activity has been reported to be enhanced on infection by pathogen (Moghaddam *et al*., 2006). Succinate is reported to accumulate in phytoplasma infected *Catharanthus roseus* leaves (Choi *et al*., 2004). Succinate accumulation could occur due to decrease in the activity of succinic dehydrogenase (SucDH) enzyme which converts succinate to fumarate in the plant. Chitinase (Cht) and glucanase (Glucn) enzymes have been reported to be enhanced on infection by various pathogens in different plants (El-Khallal, 2007; Prasada *et al*., 1987). But, changes in activities of these four enzymes in coconut palms in relation to root (wilt) disease has not been studied. Hence the present study was conducted to assess the changes, if any, in the activities of these four enzymes in root (wilt) diseased coconut palms, compared to healthy ones and whether these enzymes had any potential for detection of the disease or in imparting resistance to the host plant.

Materials and Methods

The enzymes were studied in healthy and RWD palms of tolerant - Chowghat Green Dwarf (CGD) and /or Malayan Green Dwarf (MGD) - and susceptible - West Coast Tall (WCT) - coconut cultivars. For the study, the healthy and diseased palms of CGD (10-year old) and WCT (40-years old) were selected from Central Plantation Crops Research Institute Regional Station farm at Kayangulam, State Nursery farm at Karunagapally and homestead gardens of farmers at Kottayam. The MGD (15-year old) palms were selected from Coconut Development Board Farm at Neriamangalam. These farms and gardens were selected for the study because they had palms of known uniform age of each cultivar, although different for different cultivar, where different stages of root (wilt) disease could be identified. Seedlings (1-year old) of all the three cultivars were selected from nursery beds located at CPCRI farm at Kayamkulam. Selection of healthy and RWD adult palms were done based on the presence or absence of disease symptoms and were confirmed by serological test (Sasikala *et al*., 2010). The seedlings were differentiated as healthy or RWD based on serology test.

Six to twelve leaflets of spindle leaves were cut from each of the adult palms of the three cultivars for serological confirmation of disease and estimation of enzyme activities. In seedlings, one half or the entire spindle leaves were cut and used for analysis of enzyme activities. Spindle leaves collected from the adult palms or seedlings were immediately stored between frozen dry ice packs and then kept in refrigerator at -20°C for enzyme analysis and at 8-10°C for serology test. At least 7 healthy palms and 7 RWD palms (consisting of a mixture of early, middle and advanced stages of the disease) or 7 plants in each of the four category *viz*., healthy, early, middle and advanced stages of the disease, were individually analyzed for enzymes. SOD and SucDH, Cht and Glucn activities, as well as their soluble protein contents were determined in WCT cultivar in the adult palm and in the seedlings. All the four enzymes were determined in the adult palms of CGD and only SucDH was determined in the seedling stage. Three enzymes were analyzed in MGD in the adult palms only.

SOD extraction and assay: The enzyme was extracted and assayed by the method of Nishikimi *et al*., 1972. Leaf lamina (500 mg) was finely shredded with scissors and homogenized in 5 ml of 0.01M sodium phosphate buffer pH 7.6 using 1.5 g acid washed sand and liquid nitrogen. The homogenate was centrifuged at 14,000 g for 15 minutes. The supernatant was collected and 1 ml of supernatant was diluted with 9 ml of the phosphate buffer (pH 7.6) before enzyme assay. The assay mixture consisted of one ml of 0.03 M sodium phosphate buffer (pH 7.8), 0.2 ml of 750 µM nitroblue tetrazolium, 0.2 ml of 1.17 mM NADH, an aliquot (0.2 ml and 0.8 ml) of 1 to 10 diluted enzyme and aliquot of water to bring the total volume to 2.8 ml. Then 0.2 ml of 150µM phenazine methosulfate was added and mixed and

spectrophotometer reading taken at 560 nm at exactly 4 minutes after mixing. The blank contained 1.4 ml water and all other reagents and was without enzyme. Log of absorbance values obtained for the 0.2 and 0.8 ml of diluted enzyme extract was plotted against the volume of the diluted enzyme. From this plot, the volume of diluted enzyme corresponding to log of (half the absorbance of blank) was determined for each sample which represented one enzyme unit.

SucDH extraction and assay: The enzyme was extracted and then assayed by the method of Kun and Abood, (1949). Leaf lamina (1g) was finely shredded with scissors and homogenized in 10 ml of 0.1 M sodium phosphate buffer pH 7.2 using 1.5 g acid washed sand and liquid nitrogen. The homogenate was centrifuged at 1500 g for 20 minutes. The supernatant was collected and used for assay. The assay mixture contained 1 ml of 0.2 M sodium succinate, 0.5 ml of 0.1M sodium phosphate buffer pH 7.2, 0.5 ml of 0.1% 2, 3, 5 triphenyl tetrazolium chloride (TTC) and 1 ml of undiluted enzyme. The reaction was allowed for 4 and 6 minutes at 30°C. Thereafter it was stopped by adding 3.5 ml of acetone. In the blank (carried out in duplicates) 3.5 ml of acetone was added and mixed before adding the enzyme. After assay the contents were centrifuged at 2000 g at 4°C for 30 minutes. A set of standards was prepared by taking 1ml of 0.2M sodium succinate solution, 0.5 ml of 0.1 M sodium phosphate buffer pH 7.2, aliquots of 25 to 100 µl of 0.1% TTC and then distilled water (400 to 475 µl) added to make total volume to 2 ml. Thereafter 1 ml of freshly prepared sodium dithionite solution (15 mg in 10 ml distilled water) was added to the standards. Blank was prepared similarly except that 0.1 ml of 0.1% TTC and 1.4 ml of distilled water was added and sodium dithionite was not added to it. Finally 3.5 ml of acetone was added to all the tubes, mixed and absorbance taken at 460 nm in a spectrophotometer. Enzyme activity was calculated for the assay durations of 4 and 6 minutes and their mean value are reported.

Cht. extraction and assay: The enzyme was extracted and assayed by the method of Abeles *et al*., 1970. Leaf lamina (1 g) was finely shredded and homogenized in 5 ml of 0.1M sodium citrate buffer (pH 5.0) along with 40 mg of polyvinylpyrrolidone using 1.5 g acid washed sand and liquid nitrogen. The homogenate was centrifuged at 10,000 g for 10 minutes. The supernatant was collected as the enzyme. The enzyme samples as well as enzyme blanks were assayed in duplicates. The reaction mixture in a total volume of 0.5 ml contained, 0.06 ml of sodium acetate buffer (233.3 mM, pH 5.0), 0.04 ml sodium azide solution (7.5 mM), 0.1 ml of chitin suspension (10 mg chitin/ml) and 0.3 ml of the crude undiluted enzyme extract. In the enzyme blank, instead of crude enzyme extract, 0.3 ml of heat killed enzyme was added. For preparing the heat killed enzyme 1.0 ml of crude enzyme extract was heated on a burner and as soon as it reached boiling and frothing, it was immediately kept aside to cool to room temperature. The reaction mixture was incubated at 37°C in a shaking water bath for four hours. Then the reaction was stopped by adding 0.1 ml sodium borate buffer (0.8M, pH 9.1) to all the microcentrifuge tubes and then centrifuged at 1000 g for 5 minutes. An aliquot of 0.3 ml of the supernatant was used for the colorimetric determination of N-acetylglucosamine by the method of Reissig *et al.,* 1955.

Glucn. extraction and assay: The enzyme was extracted and then assayed by a modified method of Hinton and Pressey, 1974. Leaf lamina (1 g) was finely shredded and homogenized in 5 ml of 0.1M sodium acetate buffer (pH 5.2) along with 40 mg polyvinylpyrrolidone using 1.5 g acid washed sand and liquid nitrogen. The homogenate was centrifuged at 12,000 g for 10 minutes. The supernatant was the crude enzyme extract. The enzyme samples as well as enzyme blanks were assayed in duplicates. The reaction mixture consisted of 4 ml of 0.5 % carboxy methyl cellulose, 1 ml of 0.1 M acetate buffer (pH 5.2) and 0.5 ml of crude enzyme extract. In the enzyme blank, 0.5 ml of heat killed enzyme instead of crude enzyme extract was added. For preparing the heat killed enzymes 1.5 ml of crude enzyme extract was heated on a burner and as soon as it reached boiling and frothing, it was immediately kept aside to cool to room temperature. The reaction mixtures in all the test tubes were mixed thoroughly and incubated at 37°C

for 60 minutes in a water bath incubator. After incubation an aliquot of 1 ml of the reaction mixture were taken from all the tubes and glucose estimated by the method of Somogyi (1952).

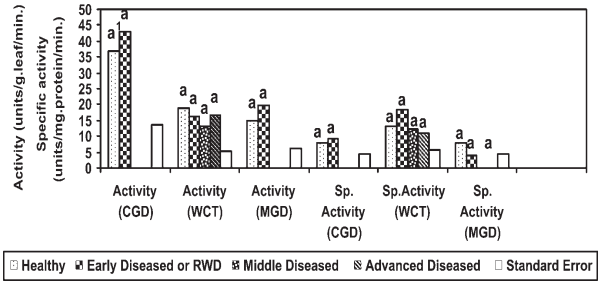
Soluble protein content in enzyme extracts: For protein estimation, 0.5 ml of crude enzyme was mixed with 0.5 ml of 20% TCA in a 1.5 ml microcentrifuge tube and left for 15 minutes and votexed again and left for another 15 minutes. The solution was then centrifuged at 4°C at 3000 rpm for 10 minutes in a biofuge. The supernatant was discarded and the precipitate dissolved in 1 ml of 2 N sodium hydroxide solution. From this solution 0.1 and 0.2 ml aliquots were taken and protein estimated by the method of Lowry *et al*. (1951).

The data was statistically analyzed as for a completely randomized design (Bailey, 1997).

Results and Discussion

Enzyme activities in healthy and root (wilt) diseased coconut palms

SOD activity as well as its specific activity in spindle leaf of healthy and root (wilt) diseased CGD, WCT and MGD adult palms and seedlings are presented in Figs. 1 & 2. No significant difference in the activity or specific activity of SOD was observed between healthy and root (wilt) diseased coconut palms in the three cultivars. Only in MGD cultivar, RWD seedlings had higher SOD specific activity than the healthy ones (Fig. 2). Sunukumar *et al*, (2010) reported significantly higher SOD activity in RWD adult coconut palms (from diseased tract) compared to healthy ones (from disease free tract - Thiruvananthapuram district) in the WCT cultivar. In the present study both healthy



[1]Bars, within each cluster, having any common alphabet above them are not significantly different at P(<0.05).

Fig. 1. Superoxide dismutase activity and specific activity in spindle leaf of healthy and root (wilt) diseased coconut palms

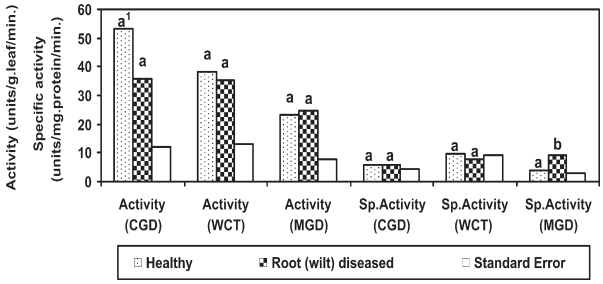


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Fig. 2. Superoxide dismutase activity and specific activity in spindle leaf of healthy and root (wilt) diseased coconut seedlings

and diseased palms were selected from the same diseased tract and no difference in SOD activity was found in all the three cultivars indicating no hypersensitive response in the plants due to the disease, except in the case of MGD seedlings.

Similarly, succinic dehydrogenase activities as well as its specific activity was unaltered due to phytoplasma infection in the three cultivars (Figs. 3 & 4). Succinate is reported to accumulate in phytoplasma infected *Catharanthus roseus* leaves

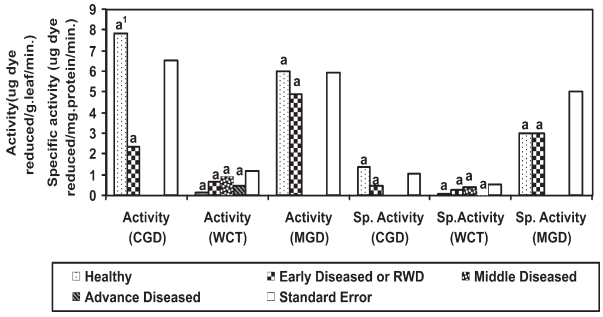

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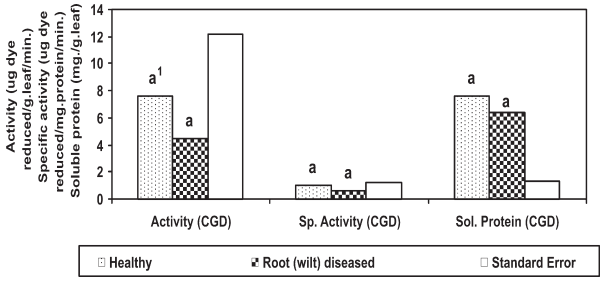
Fig. 3. Succinic dehydrogenase activity and specific activity in spindle leaf of healthy and root (wilt) diseased coconut palms

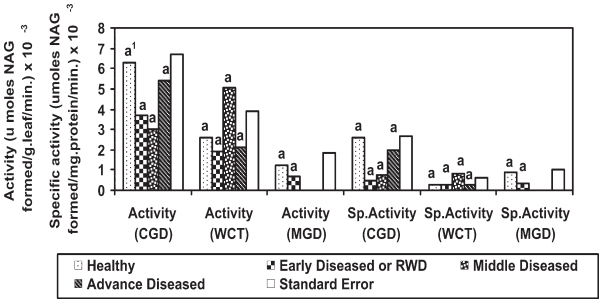


[1]Bars, within each cluster, having any common alphabet above them are not significantly different at P(<0.05).

Fig. 4. Succinic dehydrogenase activity, specific activity and soluble protein content in spindle leaf of healthy and root (wilt) diseased coconut seedlings of CGD cultivar

(Choi *et al*., 2004). Based on this it was considered that succinic dehydrogenase activity, which converts succinate to fumarate in the plant, may be altered in the plant system due to phytoplasma infection. But the current results indicated that succinic dehydrogenase activity is not altered.

Cht activities and its specific activities were unaltered due to root (wilt) disease in the three cultivars (Figs. 5). El-Khallal (2007) found increased chitinase activity in tomato leaves on infection with *Fusarium oxysporium* which causes wilt disease of tomato. This response is generally attributed to the presence of chitin in cell walls of fungi which is the substrate for chitinase enzyme. As phytoplasma lack cell wall and therefore possibly chitin, similar response of chitinase activity in root (wilt) affected

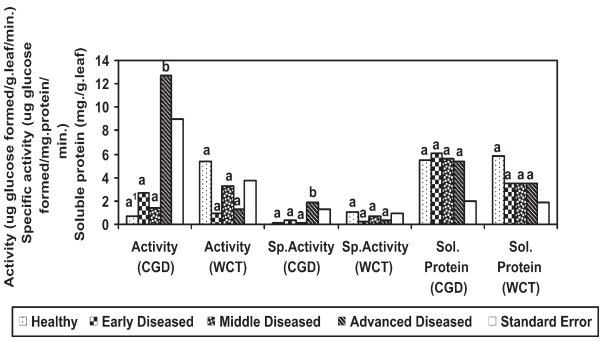


[1]Bars, within each cluster, having any common alphabet above them are not significantly different at P(<0.05).

Fig. 5. Chitinase activity and specific activity in spindle leaf of healthy and root (wilt) diseased coconut palms

coconut palms as that reported in wilt disease of tomato was not observed.

Glucn activity as well as its specific activity were significantly higher in advanced stage of RWD in CGD cultivar but no such differences were seen



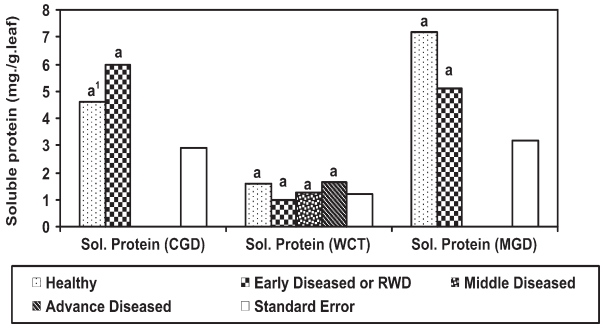
[1]Bars, within each cluster, having any common alphabet above them are not significantly different at P(<0.05).

Fig. 6. Glucanase activity, specific activity and soluble protein in spindle leaf of healthy and root (wilt) diseased coconut palms

in the WCT cultivar (Fig. 6). Leaf rot disease was in the advanced RWD stage palms in the CGD cultivar. Generally at the advanced stage of RWD, leaf rot disease may occur, due to which the tips of leaflets start necrotting and at this stage Glucn is produced to digest the plant cellwall. Probably, the presence of leaf rot disease in the advanced stage of RWD, in the CGD cultivar only, may be the cause for the higher Glucn activity observed in this cultivar but not in the WCT cultivar. Padmaja and Amma (1979) also found that the cellulase (glucanase) specific activity was significantly higher in decayed roots of root (wilt) diseased palms as compared to those from healthy palms.

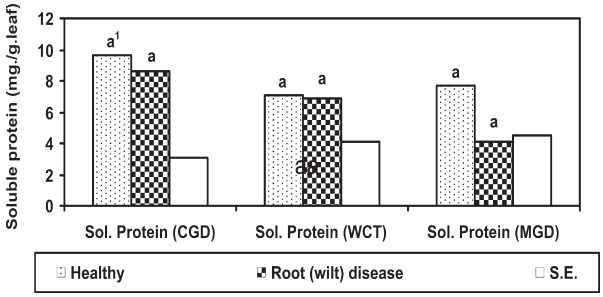
Soluble protein content in healthy and root (wilt) diseased coconut palms

The soluble protein content of the SOD enzyme extracts were similar in both healthy and diseased palms selected from the diseased tract, irrespective of cultivars (Figs. 7 & 8). Padmaja *et al*., 1981, also working with WCT cultivar, found no difference in soluble protein content between



[1]Bars, within each cluster, having any common alphabet above them are not significantly different at P(<0.05).

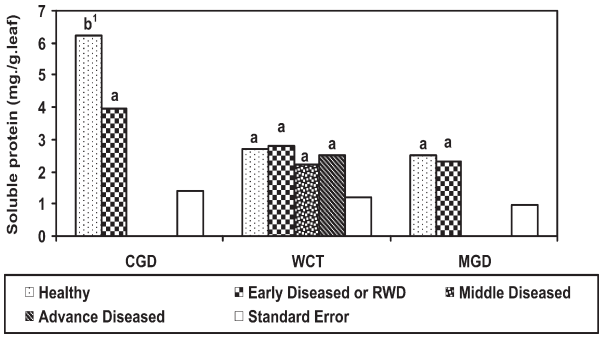
Fig. 7. Soluble protein in superoxide dismutase extract from spindle leaf of healthy and root (wilt) diseased coconut palms



[1]Bars, within each cluster, having any common alphabet above them are not significantly different at P(<0.05).

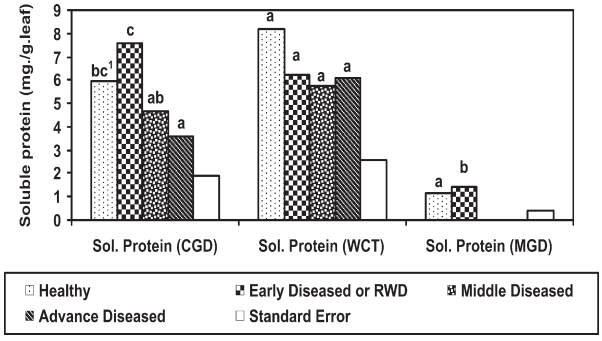
Fig. 8. Soluble protein in superoxide dismutase extract from spindle leaf of healthy and root (wilt) diseased coconut seedlings

healthy and diseased palms from the same diseased tract. But they found significantly higher soluble protein content in leaves of healthy palms (from disease free tract - Kasaragod district) as compared to RWD palms (from diseased tract). In contrast to this, in the CGD cultivar, even though both healthy and diseased palm samples were obtained from the same diseased tract, significantly higher soluble protein content in the SucDH and Cht extract was found in the healthy palms as compared to the RWD palms (Figs. 9 & 10). However, in the MGD cultivar, higher soluble protein in the Cht extract was found in the RWD adult palms than in healthy ones (Fig. 10).



[1]Bars, within each cluster, having any common alphabet above them are not significantly different at P(<0.05).

Fig. 9. Soluble protein in succinic dehydrogenase extract from spindle leaf of healthy and root (wilt) diseased coconut palms



[1]Bars, within each cluster, having any common alphabet above them are not significantly different at P(<0.05).

Fig. 10. Soluble protein in chitinase extract from spindle leaf of healthy and root (wilt) diseased coconut palms

The results indicate differences in response of cultivars to root (wilt) disease in the soluble protein content.

Enzyme activity and soluble protein in cultivars and growth stages

CGD had higher SOD activity than WCT and MGD in the adult plants (Fig. 1). CGD and WCT had higher SOD activity than MGD in the seedlings (Fig. 2) and higher soluble protein in the Cht extract in the adult plants (Fig. 10). WCT had higher SOD specific activity than CGD and MGD in the adult palms (Fig. 1) while the reverse was true with respect to soluble protein content in the SOD extract (Fig. 7). The RWD tolerant cultivars *viz*., CGD and MGD had higher SucDH activity and soluble protein in the SOD extract (Fig. 7) than the RWD susceptible WCT (Fig. 3). MGD had higher SucDH specific activity than CGD and WCT cultivar (Fig.3). CGD and WCT had higher soluble protein in Cht extract than the MGD cultivar (Fig. 10).

The higher SOD and SucDH activities found in CGD and higher SucDH activity found in MGD than in WCT indicate a higher potential in clearing superoxide radical and succinate and may be contributing towards better tolerance of CGD and MGD to RWD than WCT. Moghaddam *et al*. (2006) found no difference in SOD activity in leaves of strawberry cultivars that were resistant and tolerant to *Mycosphaerella fragariae*. They found higher SOD activity, only for a short period, in the resistant cultivar as compared to the tolerant one, after infection by pathogen.

WCT and MGD seedlings had higher SOD activity than their adult palms (Figs. 1 & 2). Seedlings of WCT and CGD had higher soluble protein in the SOD extract than their adult palms. In the CGD cultivar, seedlings had higher soluble protein in the SucDH extract than their adult palms (Fig. 4 & 9).

The results of the present study, therefore, indicated that changes in the activities of the four enzymes due to root (wilt) disease were mostly non-significant in the cultivars and offered no potential for use in detection of RWD. However cultivar differences in some enzyme activities and soluble protein of enzyme extracts were significant as also between the seedlings and adult stage of growth of palms. Enzyme activity differences between cultivars indicated that cultivars as well seedlings or younger plants appear to have a better potential in resisting the effects of root (wilt) disease.

Lack of differences in these enzyme activities between healthy and RWD plants supports the very

slow nature of deterioration of health of RWD plants. Hence controlling other diseases which cause rapid deterioration in plant health such as leaf rot which make its appearance in RWD plants could maintain the yield of root (wilt) diseased palms. Enzyme activity differences between cultivars indicate that planting relatively tolerant cultivars could help in the reduction of root (wilt) disease incidence. Perhaps studying the activities of enzymes involved in those metabolic processes that are effected by RWD, such as respiration, photosynthesis and transpiration, may prove useful in identifying the enzymes having diagnostic potential.

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