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Histochemical and biochemical characterization associated with hypersensitive responses of Kerala wilt diseased coconut palm

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Abstract

As the most vexing problem of coconut growth and development in Kerala, Kerala Wilt Disease (KWD) of coconut palm has been a subject of investigation for many years. The present study was done to understand the biochemical mechanisms such as ROS production and scavenging for revealing the hypersensitive responses (HR) of the diseased palm against infection. Histochemical localization of free radicals- superoxide anion ($O_2^{\cdot-}$);derived product-lignin and the scavenging enzyme -peroxidase (POX) at sub cellular level revealed intense colour deposition displaying their level more in the leaf and root tissues of diseased palms than healthy ones/palms. The histochemical data was further substantiated by quantifying O_2, H_2O_2 and lignin. The free radical scavenging potency of KWD palms in terms of DPPH assay was found lower in them than the healthy ones, indicating low tolerance of the palm. Subsequently, the enzymes responsible for the production of ROS and its scavenging such as NADPH oxidase (NOX), superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) showed higher activities in the leaf and root samples of diseased palm than healthy palms. Thus the assay data indicate the active involvement of NOX and SOD in the production of ROS in diseased palms, as well as CAT and POX in scavenging the ROS. This rapid induction of ROS cycle in KWD palms provides a strong indication of hypersensitive response (HR) of the diseased palm against the pathogen, *Phytoplasma*.

Keywords: Coconut palm, Free radicals, Kerala wilt disease, *Phytoplasma*, Reactive oxygen species, Scavenging potency.

INTRODUCTION

In the tropical ecosystem, plants have to adjust with a plethora of potentially unfavorable conditions through biotic and abiotic means. Host plants during infection induce the expression of large array of genes encoding diverse proteins that have specific roles on defense and stress. Hence, the production and accumulation of pathogenesis related protein on plants in response to invading pathogen and or related stress situation is one of the crucial components in inducing plant's self defense mechanism. This pragmatic ideology of pathogenesis related proteins has a pertinent role in revealing the pathophysiology of diseased plants as well as in designing a remedy for restoring the health of diseased plants. In the case of plant infections, it is possible to have either a compatible interactions where the defense responses of plant succumbs to the pathogenic stress or an incompatible interaction where the pathogen is impeded form further multiplication in the system by generating an array of pathogen related proteins. These sort of interactions were related with protein at enzymic or nonenzymic levels. It is well established that virtually all biotic and abiotic stresses induce or involve in oxidative stress through reactive oxygen species (ROS). In the case of Kerala Wilt Disease of coconut palm, apart from conventional way of pathophysiological analysis, an investigation on the oxidative stress response of the host palms during the infection of *Phytoplasma* has not been attempted to date.

Adaptations during biotic stress are crucial either for the existence of the infected plants for continuing the growth orthe suppression of the plant growthwhich eventually leads to the death of plant. The role of ROS during biotic infection has become a subject of considerable interest and that have been implicated in processes leading to plant stress acclimation. It is understood that ROS are not simple products of metabolism but also function as signalling molecule at low concentrations during the biotic stress. Hence the present investigation is an attempt to reveal the ROS pathway in Kerala Wilt Diseased (KWD) palms in comparison with the healthy ones, which helps us to understand the oxidative stress response of the host palm during the infection of *Phytoplasma*. In order to detect the free radicals and other ROS metabolism in the leaf and root tissues of diseased palms, histochemical, analytical and biochemical methods were adopted.

MATERIALS AND METHOD

For the present investigation the root and leaf samples of coconut palms were collected from two identified coconut plantations. One is a Kerala Wilt diseased coconut plantation, at Ithikkara of Kollam district and other is a disease free area, at Thonnakkal, Thiruvananthapuram district.

Localization of super oxide anions $O_2^{\cdot-}$, H_2O_2, lignin and peroxidase

Super oxide anion was localized, following the procedure of Ogawa et al. (1997). Thin sections of leaf and roots were incubated in 10mM sodium phosphate buffer (pH 7.8) containing 0.25 mM Nitro Blue Tetrazolium (NBT) for 30 min. Histochemical localization of hydrogen peroxide was done by staining the tissues with Tetramethyl benzidine (TMB) reagent, as per the method of Ros Barcelo (1998b). Lignins were localized using the Wiersner test as described by Ros Barcelo (1998). Thin sections of leaf and roots were soaked in 1.0 (w/v) phloroglucinol in 25:75 (v/v) HCl: ethanol for 10 to 15 min. Red depositions in the tissue indicated the presence of lignin. Peroxidase enzyme was localized, following the procedure of Bestwick *et al.* (1998) with slight modifications. Polymerized DAB were detected as deep brown deposits, which indicated the localized enzyme.

Quantification of super oxide anions, H_2O_2, lignin

Super oxide anion was quantified, following the method of Doke (1983). An aliquot (0.1, 0.2, or 0.5ml) of the extract was taken and mixed with the assay reagent containing 0.01M potassium phosphate buffer (pH 7.8) with 0.05% nitroblue tetrazolium sodium salt (NBT) and 10 mM sodium azide (NaN_3). The assay mixture was incubated for 30 min and the initial absorbance at 580 nm was taken in a spectrophotometer. Final absorbance was taken after heating the mixture at 85°C for 15 min.

H_2O_2 concentration in the incubation medium of the treated plant tissues was estimated as per the procedure of Bellincampi et al. (2000) with some modifications. It was based on the peroxidase mediated oxidation of Fe2+, followed by the reaction of Fe^{3+} with xylenol orange. The assay mixture was incubated for 45min and the absorbance of the Fe^{3+} xylenol orange complex at 560 nm was observed. Control was performed by eliminating the H_2O_2 in the reaction mixture with catalase.

Lignin content in the leaf and roots were estimated by acetyl bromide method of Kenji Iiyama and Adrian F A Wallis (1990). Absorbance was recorded at 280 nm. A standard graph of lignin was prepared with dehydroconiferyl alcohol polymerizate (DHAP). An absorption value of 0.316/mg initial lignin was obtained.

DPPH assay

The scavenging potency of ROSs was evaluated by measuring the DPPH free radical scavenging activity. The methanolic extract of leaf sample was assayed using DPPH radical according to the procedure of Juhi Misra *et al.* (2009). The absorbance was measured at 570 nm using ascorbic acid as reference. Lower absorbance indicates high free radical scavenging activity. The capability to scavenge the DPPH radical was determined by using the following formula

The scavenging effect =

$$\frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Isolation and assay of NADPH oxidase (NOX), superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX)

NADPH oxidase assay was carried out following the procedure of Bestwick (1998). A 0.5 mL of aliquot of the sample was assayed in a freshly prepared 2.5 mL of assay reaction buffer containing 50 mM sodium acetate, 0.15 mM NADPH, 20 mM $MnCl_2$ and 2.5 mM Dichloro phenol (DCP). Absorbance was observed at 340 nm on a time scan of 10 s intervals for 5 min.

The superoxide Dismutase enzyme (SOD) was extracted and assayed following the method of Fridovich et al. (1986), with some modifications.

SOD activity was measured as the inhibition of the rate of reduction of cytochrome C by the superoxide radical observed at 550 nm.

Peroxidase was isolated and assayed as per the procedure of Goliber *et al.* (1989). POX activity was assayed using guaiacol as substrate (Ingham et al. 1998), in a reaction mixture containing 1ml of 0.1M phosphate buffer (pH 7), 1mL of 20 mM guaiacol, 50 µL of 10 mM H_2O_2 and 40 µL of the enzyme. The increase in absorbance was measured spectrophotometrically at 470 nm for 10 min at 30°C. A set of samples containing reaction mixture without guaiacol was taken as the control. One unit of POX activity is the amount of enzyme required to oxidize 1µM of guaiacol by H_2O_2 at that condition.

Catalase was extracted and assayed as per the protocol of Mullen and Clifford, (1993). The reaction mixture consisted of 2.5 mL of 30% H_2O_2 solution diluted in 0.05 M potassium phosphate buffer pH 7, and 0.5 mL enzyme. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the decomposition of 1µ mole of H_2O_2 /min at pH 7.0 at 25°C.

Statistical analysis

Analysis of variance (ANOVA) was done to analyze the variance of the above mentioned molecules and enzymes of diseased and healthy leaf and root samples. Variants were also computed along with the mean value of the treatments (Gomas and Gomas, 1984).

RESULTS AND DISCUSION

Histochemical localization

Superoxide anion ($O_2^{\cdot-}$)

As the initial phase of the study the presence of super oxide anion ($O_2^{\cdot-}$) in the root and leaf samples of KWD coconut palms was detected by NBT dye method. The blue coloured deposits localized in the vascular and cortical region indicates the presence of insoluble blue formazan produced by the reduction of NBT in the presence $O_2^{\cdot-}$ ions localized in the tissues (Fig. 1a,b,c,d). The intensity of blue colour deposits in the leaf and root tissue of diseased palm was found more than in healthy tissue indicates the higher accumulation of $O_2^{\cdot-}$ in tissues of diseased palms. Thus the efficacy in the production of $O_2^{\cdot-}$ in the tissues of diseased palms was proved by histochemical data.

Hydrogen peroxide (H_2O_2)

TMB stain was used to detect H_2O_2 in the leaf and root tissues of both diseased and healthy palms. It could be seen from the experimental samples (Fig. 2a,b,c,d), that the tissue sections incubated in the TMB medium showed deep blue deposition the cortical and vascular region of root and leaf tissue of diseased palm compared with healthy ones. The blue coloured deposits clearly indicate the oxidation of TMB in the presence of H_2O_2, suggesting the increased level of H_2O_2 production in diseased palm compared to healthy palm. Apart from the synthesis of H_2O_2 in cell system as a normal metabolic process, the over production of H_2O_2 in the symptomatic leaves of diseased palm is possibly a manifestation of oxidative stress. Moreover, it can be a part of defence mechanism contributed by the host palm against infection. It can be postulated that $O_2^{\cdot-}$ and H_2O_2 play a central role in plant host as resistance to pathogen (Musetti *et al.* 2005). ROS generating system might kill pathogens or induce a hypersensitive response (HR) for immobilizing the pathogen within the cells leading to incompatible plant pathogen reactions (Bowler and Fluhr, 2000).

Lignin

Lignin deposition in the leaf tissues of diseased and healthy palms was checked by Phloroglucinol method. As a histological barrier for defence, lignin has crucial role in regulating the adaptability of the palm and for its formation, H_2O_2 has an initiating role. In other words, lignification in plants is characterized by a burst in the production of H_2O_2. In leaf tissue of diseased plants, the xylem elements and bundle sheath expressed deep red colour throughout the region compared to the leaf tissue of healthy palm (Fig 3a & b). The data suggest the formation of more lignin in the leaf tissue of diseased palm. A similar trend of deep red colouration was noticed in the root tissue also (Fig. 3c and d). Meanwhile, no red colour deposits in control sections incubated without Phloroglucinol.

The red coloured deposits observed in the root and leaf tissues provides a clear evidence of lignin deposition in such regions. Moreover, the higher intensity of red colour deposits in the leaf and root tissues of diseased palm gives another indication of active synthesis of lignin in such tissues compared to healthy ones. Thus the histochemical data of lignin localization indirectly supports the peroxidase mediated oxidative polymerization of cinnamyl alcohol to lignin, the last step of lignin synthesis. It is also possible to interpret that the higher intensity of deep red coloured deposits in leaf and root tissues of diseased palm than healthy palms suggest the tendency of active lignin formation under diseased condition. Even

though, the high deposit of lignin in the diseased tissue looks physiologically uncommon; it is indispensable for priming the defense mechanism as a histological barrier during the pathogenesis. Moreover, the high deposition of lignin in the cell wall reduces the cell lumen, which indirectly diminishes the physiological growth rate of the diseased plant, which can also be treated as a mode of resistance.

Peroxidase (POX) and Catalse (CAT)

The active deposition of H_2O_2 and lignin in the leaf and root tissues of KWD coconut palm demands the investigation of localizing peroxidase (POX), the key enzyme responsible for lignin formation. So, an attempt has been made to localize the enzyme, POX in leaf and root tissues of diseased and healthy palm by DAB method. The tissue sections were incubated in a medium containing H_2O_2 as substrate and DAB as an oxidizing agent. As the outcome of the peroxidase reaction on H_2O_2, brownish deposits were observed as an indication of oxidized DAB in the leaf and root sections of both diseased and healthy palm (Fig. 4 a,b, c and d). The endogenous peroxidase in leaf and root tissue reacted on the H_2O_2 present in the incubation medium by getting an electron from DAB, hence giving brownish deposits in the tissues oxidized. This provides sufficient insight to the fact that the activity of POX was higher in diseased tissue than healthy. Thus, the sub-cellular study of POX localization in the leaf and root tissues of healthy and diseased palms evidently supports the histochemical data of H_2O_2 and lignin in the tissue. The incubated sections without H_2O_2 and DAB which acts as control, exhibits colour deposition in a faint way, suggesting the *in situ* oxidation of DAB.

Analytical data

As a support to the histochemical data of free radical super oxide anion ($O_2^{\cdot-}$), the reactive oxygen species H_2O_2 and lignin, were quantified in the leaf and root tissues of diseased and healthy palm.

Superoxide anion ($O_2^{\cdot-}$)

Superoxide anion was quantified spectrophotometrically by NBT method. The increased absorbance showing the reducing activity of NBT was taken as an indicator for estimating the amount of $O_2^{\cdot-}$. It is evident that, the leaf and root tissue of diseased palm showing more O.D. difference than that of healthy palm suggesting the higher accumulation of $O_2^{\cdot-}$ in the tissues of diseased palm (Table 1). The active accumulation of $O_2^{\cdot-}$ in the tissues of diseased palm provides a clear indication of biotic stress. The role of $O_2^{\cdot-}$ during the biotic stress has become a subject of considerable interest as a process leading to plant's HR against the infection (Doke *et al.* 1983; Torres *et al.* 2006). The higher concentration of $O_2^{\cdot-}$ in diseased tissues can lead to phytotoxicity, whereas relatively low level of $O_2^{\cdot-}$ can be used for signaling. These observations mean that $O_2^{\cdot-}$ is not a simple toxic product of metabolism, but also a signaling molecule. Therefore, such plants, that express a tendency to produce $O_2^{\cdot-}$ at higher level should maintain a biochemical system for reducing its content to a limited level. Even though, the reduction of molecular oxygen to water provides the energy that allows the impressive complexity of higher organism, its complete reduction to free radical, $O_2^{\cdot-}$ is an indication of internal stress stimuli. Hence the rapid accumulation of $O_2^{\cdot-}$, the free radical in the tissue system of diseased palm is an indicator of physiological event 'Oxidative burst'.

Hydrogen peroxide (H_2O_2)

H_2O_2, the ROS is derived from the $O_2^{\cdot-}$ by dismutation reaction. It is obvious from the table 1 that, the leaf and root tissues of diseased palms showed higher deposition of H_2O_2 than the normal healthy coconut palms. The accumulation of higher content of H_2O_2 in leaf and root tissues corroborates with the histochemical data of H_2O_2 localization in such tissues. Irrespective of the comparison regarding the deposition of free radical $O_2^{\cdot-}$ and ROS H_2O_2 between diseased and healthy palms, the higher accumulation of these two active oxygen species in diseased palms remains as a physiological impact of hypersensitive response of the palms against the disease.The intensity of infection indirectly reflects the amount of ROSs formation. Thus the results clearly indicates physiological impairedness of diseased coconut palms due to this oxidative burst caused by the higher level of $O_2^{\cdot-}$ and H_2O_2 production and it also highlights the HR of the palms against *Phytoplasma* infection. The relationship between biotic stress and the formation of H_2O_2 in plant tissue has been become documented by many studies (Apel and Hirt, 2004; Tsanko *et al.* 2005). Oxidative stress is induced by range of biotic and abiotic factors including uv rays, pathogenic invasion, herbicidal action and oxygen shortage (Olga Blokhinia *et al.* 2003). Thus the analytical data conclusively proved the excess generation of H_2O_2 in the root and leaf samples of diseased palm as a pathological effect of *Phytoplasma* infection. As a constitutive metabolite of cell wall for lignin formation, the presence of H_2O_2 on cell system

of plant is a common physiological feature. But the tendency of plant cell to accumulate H_2O_2 in the excessive level during pathogenesis requires further analysis at biochemical level for detecting the role of the marker enzyme involved in the.

Free radical scavenging potency

Table 1 displays the percentage of difference in the scavenging effects on healthy and diseased palm measured by DPPH radical activity. It is evident that the percentage of scavenging effect is lower in diseased palm than healthy one. In the case of healthy palm, the percentage of scavenging effects was measured to a level of 65-75 %, whereas in the diseased palms it was noted as 40-45 %. The depletion of scavenging ability in the leaf tissue of diseased palm indicates the possibility of higher accumulation of free radical in the leaf tissues of it during pathogenesis. Moreover this inefficiency of diseased palm in the *in vitro* free radical scavenging property provides sufficient evidence in the oxidative stress created by the palm during the infected phase. It is also clear from the data that the leaf tissue collected from the unaffected areas of Kerala Wilt Disease showed a powerful scavenging effect suggesting the healthy nature of the palm. Thus this data showing the scavenging potency of the diseased palm strongly supports the higher accumulation of free radical, i.e., $O_2^{\cdot-}$ in the leaf tissues of diseased palm.

Lignin

The higher accumulation of H_2O_2 in diseased palm than the healthy palm demands the study to understand the possible ways of decomposition and utilization during the growth and development. Since the source of H_2O_2 necessitates the oxidative polymerization of cinnamyl alcohol to lignin by POX action, it can be treated as one of the possible reason for the utilization of H_2O_2 in diseased palm. So the end product lignin was quantified in leaf and root samples of diseased and healthy palms for revealing the involvement of H_2O_2 in lignin formation. Table 1 reveals that the leaf and root samples of diseased palm showed higher content of the lignin than the healthy palm. In the root samples, of the diseased palm the lignin content was found extremely high from that of the healthy palm. The high deposition of lignin in the root and leaf samples of diseased palm suggests the physiological response of the palms during *Phytoplasma* infection. As a histological barrier for defense mechanism, the higher level of lignin formation in the leaf and root tissues of diseased palm limelight the response of the palm against infection. It also provides an indication to extend the longevity of the diseased palm by reducing the physiological efficacy of cell function. Moreover, the data further correlates the higher deposition of H_2O_2 with higher lignin content in the diseased palm, since the H_2O_2 act as a prerequisite for lignin synthesis.

Enzymes of ROS production

Activity of NADPH oxidase

NADPH oxidase (NOX) is an enzyme responsible for the production of $O_2^{\cdot-}$. Hence the formation of $O_2^{\cdot-}$ in the root and leaf samples of diseased and healthy coconut palm was determined by measuring NOX activity. Biochemically the enzyme is responsible for the univalent reduction of molecular oxygen to $O_2^{\cdot-}$. In the case of leaf samples, the tissue were collected from the inner, middle and outer whorls and pooled for assay. As control, tissue samples of healthy palms identified from unaffected area were used. It is evident from table 2 that NOX express a higher activity in the leaf and root tissues of diseased palm than healthy. It indicates the active involvement of NOX in the formation of $O_2^{\cdot-}$ in diseased palm from molecular oxygen compared to healthy palms. Moreover, the higher level of NOX activity in the *Phytoplasma* infected tissues of KWD coconut palms reflects the oxidative stress response of palms induced by the pathogen. The increased level of NOX activity in diseased palm compared to healthy, suggests the active conversion of molecular oxygen to $O_2^{\cdot-}$ by univalent reduction.

The increased level of NOX activity in the *Phytoplasma* infected palm was substantiated by the level of $O_2^{\cdot-}$ content. Even though the infected phase of the diseased palm favours the endogenous production of free radical $O_2^{\cdot-}$ enzymically, the plants cannot tolerate its presence in the cell system for a prolonged period due to its high reactive, toxic, penetrating and oxidative nature. Recently, it has been shown that NADPH oxidase can trigger its activity in host organism against pathogen as a part of defence during infection (Nadja Segmuller et al. 2008). Hence the higher level of NOX activity observed in the diseased palm can be interpreted as hypersensitive response of the host palm against *Phytoplasma*. Being a toxic product, its further conversion in diseased palm was investigated by assaying the enzyme Superoxide dismutase (SOD), the key enzyme responsible for dismuting of $O_2^{\cdot-}$ to H_2O_2.

Superoxide Dismutase (SOD)

Based on the enhanced level of $O_2^{\cdot-}$ in the leaf and root samples of diseased palms during infection, the key enzyme responsible for this conversion was tested for revealing its further transformation. It could be seen from table 2 that the leaf and root samples of diseased palm expressed higher level of activity than healthy samples. The data directly supports the accumulation of H_2O_2 and $O_2^{\cdot-}$, since SOD catalyze the disproportionation of $O_2^{\cdot-}$ to H_2O_2 and O_2. Thus the excessive accumulation of H_2O_2 in the leaf and root samples of diseased palm compared to healthy was confirmed by the assay data.

Enzymes of ROS scavenging

The excessive formation of the ROSs, H_2O_2 and the higher level activity of SOD in the leaf and root samples of diseased palms have provided a sound evidence towards the oxidative stress of KWD coconut palms. The specificity of POX and CAT regulating the concentration of H_2O_2 in the cell system depends on the physiological status of the plant and therefore ROS scavenging in the cell system was studied by assaying the enzymes.

Catalase (CAT)

In the leaf and root tissues of diseased palm, catalase enzyme exhibits a sound increase in the catalytic activity compared to the healthy palm (table 2). This significant increase of CAT activity in the leaf and root tissues of diseased palm directly focus on the physiological role of the enzyme for scavenging the H_2O_2 deposited in the cell system. In other words the higher concentration of H_2O_2 deposited in the leaf and root tissues of root wilt diseased palms due to infection indirectly demands more activity of scavenging enzyme CAT for the elimination of H_2O_2 from the cell system. As a pathological change noticed in the diseased palm, the extreme level of CAT activity and higher deposition of H_2O_2 in diseased palm provides an indication of liberating more oxygen and water in the cell system at the *in vivo* condition. So the excess level of molecular oxygen and water in root tissues of diseased palm has become a physiological reality to acclimatize the oxidative stress condition physiologically induced during *Phytoplasma* infection. It can also be interpreted that this evolution of oxygen in the cell system may be the possible reason for long term survival of KWD palms under diseased condition in spite of all the physiological constraints.

Peroxidase (POX)

The elimination of H_2O_2 from the cell system was further studied by assaying the activity of POX, the other scavenging enzyme of H_2O_2. As a ubiquitous enzyme, the role of POX is multidimensional physiologically. But in the present study, the activity of POX was linked with twin physiological focus. One for scavenging and other is to establish the role of H_2O_2 lignification. Since H_2O_2 is an inevitable signaling molecule for lignification in the presence of POX, the higher content of H_2O_2 and lignin in the leaf and root tissues of diseased palm demands a search for POX activity also. This establishes the correlation of H_2O_2 – POX and lignin. It is evident from the table 2 that, the root and leaf samples of diseased palm expressed a higher level activity of POX than healthy palms reveal the efficacy of the enzyme in scavenging H_2O_2. Since the lignin content estimated from the leaf and root samples found higher in the diseased palm, the assay data of POX, the key enzyme responsible for lignifications clearly supports the need of POX and H_2O_2 in lignifications. Active phase of lignifications noticed in the leaf and root tissue is a clear indication of pathological effects reflected in histologically in diseased palm.

CONCLUSION

Precisely, the results of the present investigation envisage the hypersensitive response (HR) of the Kerala Wilt Diseased (KWD) coconut palms against the *Phytoplasma* infection. Hypersensitive response is a mechanism triggered by diseased plants against infection by activating the Reactive Oxygen Species (ROS) pathway. For substantiating it, the production and scavenging of the ROS in KWD palms compared with healthy was analysed at histochemical biochemical and analytical level. So the present data remains unique and apparent in convincing the HR of KWD palms against *Phytoplasma* through the impaired action of ROS cycle. Since HR is characterized by the rapid death of cells and tissue in localized regions of the diseased plants, the visual symptoms of ribbing, depigmentation and marginal necrosis occurred on the leaf tissue and degeneration of roots of KWD palms can be considered as the hallmarks of HR of coconut palms. Further studies are planned to design a bio remedy against KWD palms commercially to effectively control this disease.

Table-1
Superoxide anion, hydrogen peroxide content, free radicals scavenging potency, lignin accumulated in the healthy and KWD diseased leaf and root of Coconut palm. Values are mean ± SD. Significant at $P < 0.0001$.

	Healthy palm	Diseased palm	*t* value
Superoxide anion (leaf) (µg/g)	0.5145±0.17	10.52±2.14	1.18
Superoxide anion (root) (µg/g)	46±12.91	109±38.15	5.5
Hydrogen peroxide (leaf) (µg/g)	23.65±1.42	154.05±25.69	22.79
Hydrogen peroxide (root) (µg/g)	3.88±1.5	15.3±2.7	19.6
Free radicals scavenging potency (%)	67.7±4.27	44.5±2.4	11.9
Lignin content (µg/g) (leaf)	29.7±3.9	440±19.2	57.09
Lignin content (root) (µg/g)	13.8±2.04	40.2±4.49	20.073

Table-2
Activity of NADPH oxidase, super oxide dismutase, catalase and peroxidase in the healthy and KWD diseased leaf and root of Coconut palm. Values are mean ± SD. Significant at $P < 0.0001$.

	Healthy palm	Diseased palm	*t* value
NADPH oxidase activity (leaf) (U/mg)	105.88±35.6	376.53±79.3	12.45
NADPH oxidase activity (U/mg) (root)	132.55± 60.5	339.39±94.3	7.5
Super oxide dismutase activity (leaf) (U/mg)	54.4±12.66	117.8±34.4	8.29
Super oxide dismutase activity (U/mg)(root)	53± 9.15	107±22.6	9.8
Catalase activity (leaf) (U/mg)	141.8±44.6	487.7±13.7	4.8220
Catalase activity (root) (U/mg)	118.64±37.2	487.7±14.3	9.19
Peroxidase activity (leaf) (U/mg)	45.8±10.4	72.42±16.2	9.27
Peroxidase activity (root) (U/mg)	21.4±6.2	57.0±1.41	15.8

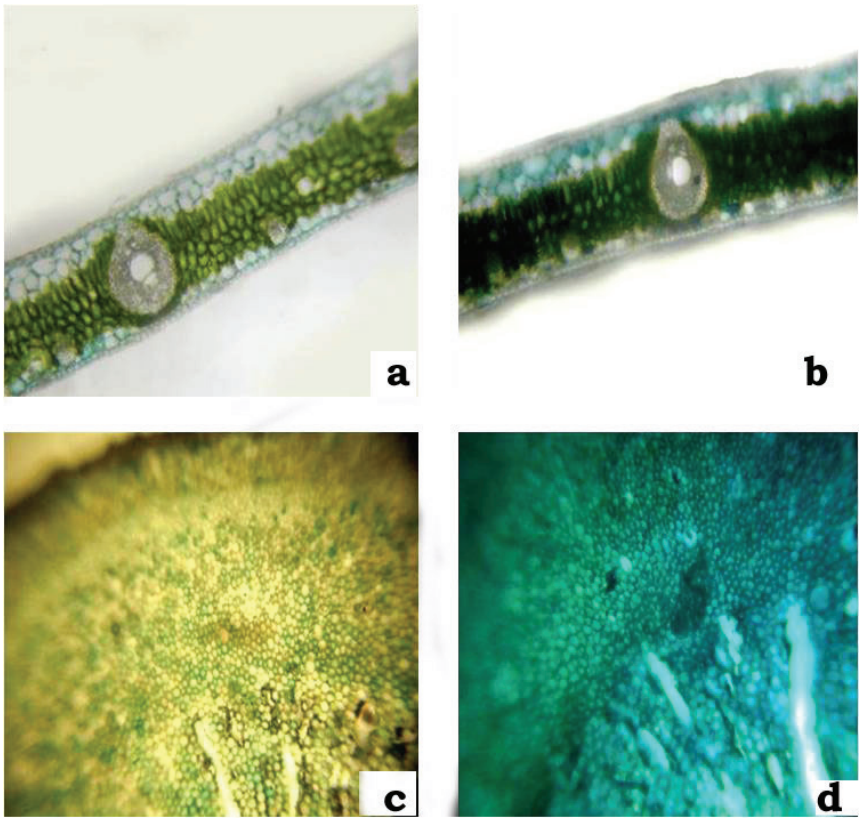

Fig. 1 a, b, c and d. Super oxide anion ($O_2^{\cdot-}$) in the leaf and root samples of KWD coconut palms detected by NBT method. a- healthy leaf, b- diseased leaf, c- healthy root d–diseased root

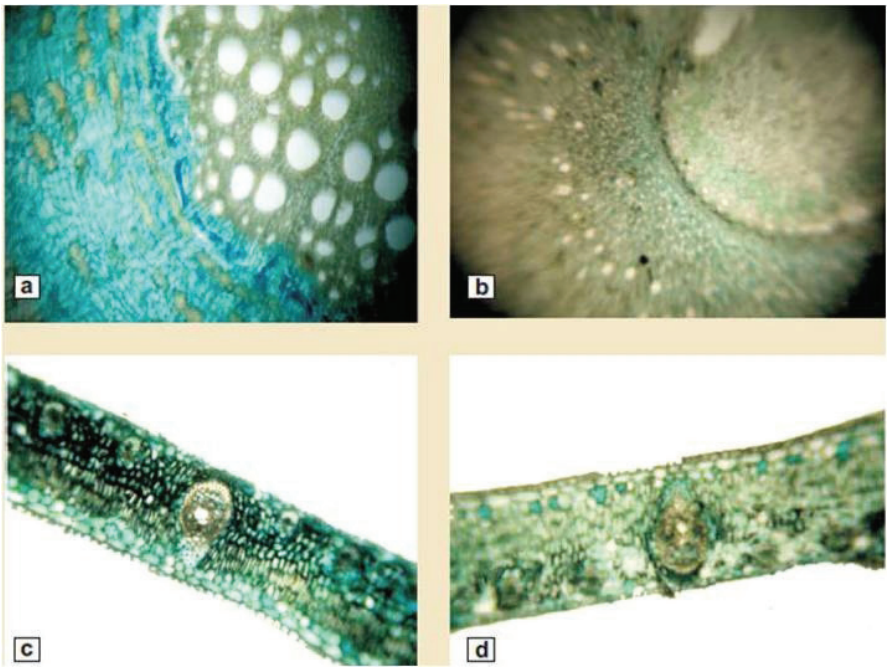

Fig. 2 a, b, c and d. Hydrogen peroxide in the leaf and root samples of KWD coconut palms. a- diseased root, b- healthy root, c- diseased leaf d- healthy leaf.

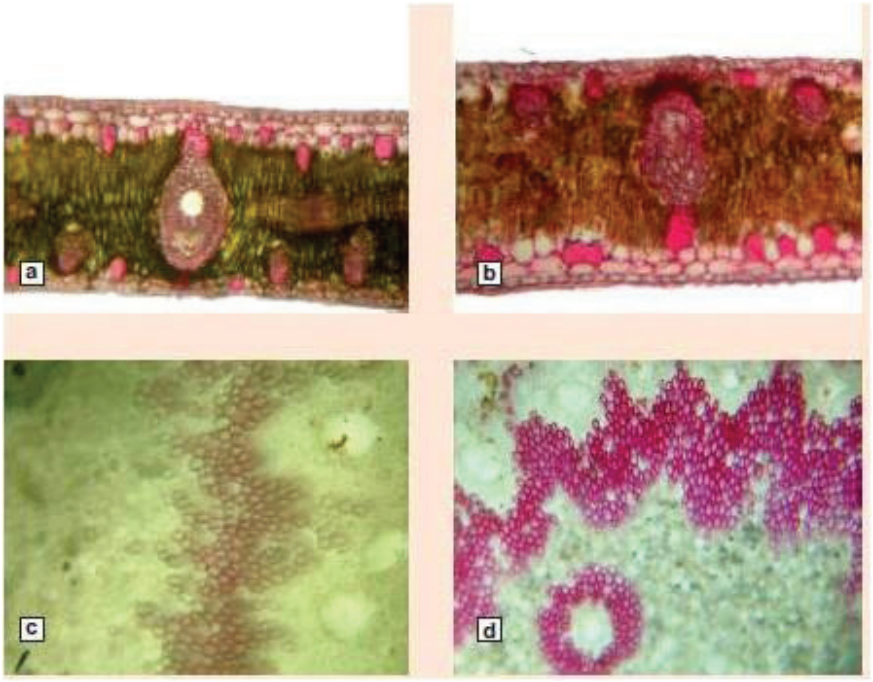

Fig. 3 a, b, c and d. Lignin accumulated in the leaf and root samples of KWD coconut palms using phloroglucinol. a- healthy leaf, b- diseased leaf, c- healthy root d–diseased root.

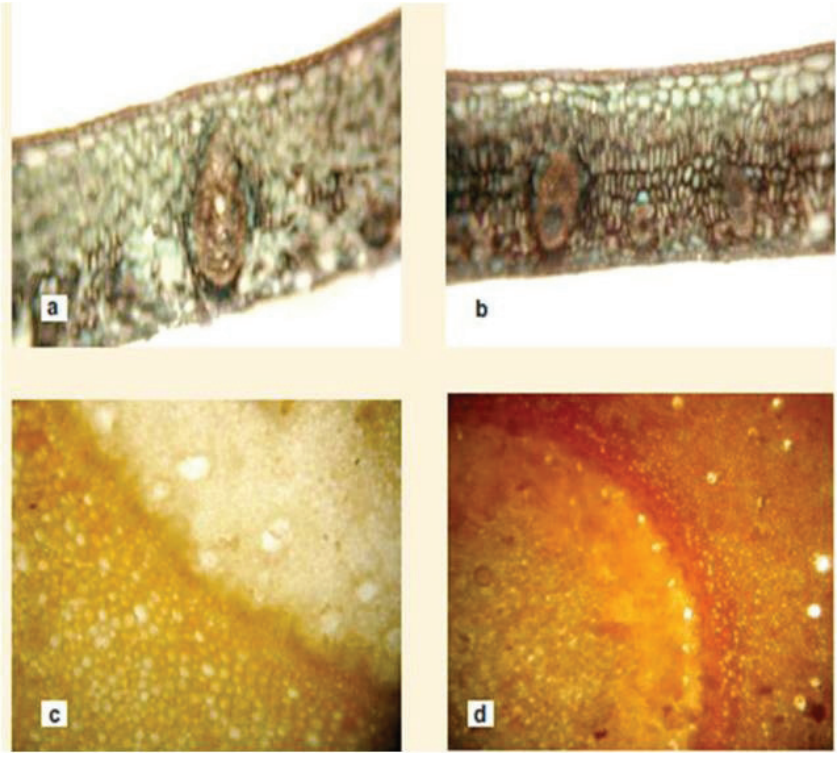

Fig. 4 a, b, c and d. Peroxidase accumulated in the leaf and root samples of KWD coconut palms. a- healthy leaf, b- diseased leaf, c- healthy root d–diseased root.

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