Physiology and Biochemistry of Lethal Yellowing in Cocos nucifera

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The coconut is the main source of oil in the tropics. These areas provide the most favorable conditions for the germination and growth of coconut trees where other oil seeds cannot succeed. Approximately 30% of the fruit can be converted to copra (Harries 1971) which, in turn, has 60–64% oil (Grimwood 1975).

The yearly world copra production in 1982 was 4.9 million tons. The main producers were The Philippines and Indonesia. Mexico supplied 2.9% of the world production (FIRA 1985).

The coconut crops in Mexico and Central America are presently threatened by a disease called lethal yellowing (LY). To date, millions of palms have died in Jamaica, Cuba, Florida and Mexico due to this disease, for which there are no adequate control measures yet.

Other reviews have focused on the etiology and epidemiology of LY (Tsai 1980, McCoy 1983). However, our present knowledge on the physiological and biochemical mechanisms involved in the host-pathogen relationship is still limited. The present paper reviews most of the physiological and biochemical studies carried out so far, and proposes an experimental model of the various alterations that take place during the disease development.

Lethal Yellowing

Since its appearance in the last century, LY has killed millions of palms in Jamaica

(Romney 1972), Florida (Fisher 1975), Tanzania (Schuiling and Mpunami 1990), and more recently, the Yucatan Peninsula (McCoy et al. 1983; Villanueva et al. 1987).

Mycoplasmalike organisms (MLO) were pointed out as causal agents of the disease based on two lines of evidence: a) cross-sections of sieve tube elements observed under the electron microscope revealed the presence of bodies with tripartite membranes and an approximate diameter of 0.3 μm (Beakbane et al. 1972, Plavsic-Banjac et al. 1972, Thomas 1979); and b) treatment of diseased palms with tetracycline, but not penicillin, stopped the development of the disease (McCoy 1973). Since MLO were first pointed out as the causal agents, other studies have documented MLO traversing cell walls (Beakbane et al. 1975) and sieve plate pores (Parthasarathy 1974) in diseased palms, suggesting how the microorganism may move to adjacent cells. A number of attempts have been made to isolate and cultivate these microorganisms from LY-affected palms. Eden-Green and Tully (1979) isolated 35 species of *Acholeplasma* from phloem sap and decaying tissue of diseased palms. These isolates were immunologically related to either *A. axanthum* or *A. oculi*. After unsuccessfully carrying out pathogenicity and transmission tests with these isolates, Eden-Green et al. (1985) concluded that these microorganisms were not the etiological agents of LY

but rather epiphytes or saprophytes of the decaying tissue. In a different study, Eden-Green and Waters (1981) isolated and characterized a *Spiroplasma* from diseased palm tissue which was different from *S. citri* or corn stunt *Spiroplasma*. Failure to repeat the obtention of these isolates in subsequent experiments, and their inability to infect susceptible palm species, suggested that these were not the causal agents of the disease (Eden-Green et al. 1983). It appears therefore, that the causal agent is very susceptible to manipulation and thus, its isolation is a difficult task that has not been accomplished. This may also be the reason why the MLO needs a vector to infect the palm.

Studies carried out in Fort Lauderdale, Florida by F. W. Howard and co-workers (1983) pointed towards the cixiid *Myndus crudus* Van Duzee as the vector for the MLO that causes LY. This insect feeds on the phloem of leaves and presumably introduces the MLO through this mechanism. The MLO can then manage to establish themselves within areas with high nutrient demand such as apical meristem, roots and inflorescences as indicated by the localization of MLO by electron microscopy in these tissues (Thomas and Norris 1980).

Lethal yellowing has been shown to require an incubation period of 4 to 9 months before visible symptoms appear (Dabek 1974, 1975). These visible symptoms have been used to grade the severity of the disease (McCoy 1973). First, the nuts fall off the tree regardless of their size, then the inflorescences become necrotic even before they open. This is followed by yellowing of the leaves from the lower to the higher fronds. At this stage the flag and spear leaves may also be yellow or dead. Finally, the whole crown falls off the tree leaving a naked trunk. This final stage is known as "telephone pole" due to its appearance. In juvenile trees, the symptoms include a yellowish discoloration from the distal ends of the fronds, followed by the total yellowing and decay

of the spear leaves and meristem (Tsai 1980). Symptoms in roots have also been reported. Eden-Green (1976, 1982) found that induced adventitious roots become increasingly necrotic as yellowing of leaves progresses. In mature palms, death occurs 3–6 months after the appearance of the first visible symptom (Grylls and Hunt 1971).

Physiological and Biochemical Alterations Caused by LY

Several physiological and biochemical modifications associated with LY have been documented. Alterations in both phloem and xylem fluxes, nutrient supply, stomatal behavior, enzyme levels, electrophoretic patterns and growth regulators, have been detected in diseased palms when compared to healthy ones.

Phloem Transport. MLO have been found confined to sieve tubes in diseased plants (Howard et al. 1983, 1984; Parthasarathy 1974; Thomas 1979). The presence of MLO in the sieve tubes has been associated with phloem malfunctions (Eden-Green and Waters 1982). Although the exudation rate increased in palms at the first stages of the disease (stage 2), in severely affected palms (stage 4–7), this rate was drastically reduced (from 15 ml/hour to less than 5 ml/hour). This phenomenon could be explained as it has been proposed by Kollar et al. (1989) for the infection of apple by MLO. When the MLO population is low, there is impairment of the sieve tube sealing mechanism. As the MLO titer increases, the tubes can become obstructed reducing phloem flux. This obstruction could be the result of necrosis, callose deposition, or even the MLO themselves occluding the sieve pores. In *Cocos nucifera*, callose deposition at early stages of the disease has been reported (Nienhaus et al. 1982). There is some electron (Beakbane et al. 1972) and fluorescence (Cardeña-López et al. 1989) microscopic evidence that MLO may be blocking the sieve

tubes. However, an actual physical blockage of the flow in the sieve tubes of LY-infected *Cocos nucifera* plants remains to be demonstrated.

Xylem Transport. As opposed to phloem, there is no evidence of the presence of MLO in the tracheids or evidence of any physical obstruction of tracheary elements on LY diseased palms (Dabek 1973). However, there is enough evidence that water transport through xylem is reduced as a result of LY (Carter 1965, Dabek 1973, Eskafi et al. 1986). Using ^{32}P, Eskafi et al. (1986), showed that xylem transport was reduced 75% in LY diseased palms when ^{32}P was applied at the base of the petiole, and 100% when applied through either stem or roots. After studying root uptake capacity and water flow in excised roots and petioles, respectively, they suggested that although malfunction of root and petiole may limit water movement in LY affected palms to a certain extent, the limiting factor is closure of stomata. However, since they did not study transport through excised stems, reduced water transport at this level cannot be ruled out.

Stomatal Behavior. Healthy palms show a typical diurnal fluctuation in xylem pressure, from −1 bar at night to −10 bars at midday. In LY diseased palms, however, these normal fluctuations did not occur showing xylem pressures of around −4 bars during most of the day (McDonough and Zimmermann 1979). This results from the fact that stomata remain closed at midday in LY affected palms (Eskafi et al. 1986, Leon et al. 1989). In fact, Leon et al. (1989) showed that stomatal diffusive conductance of LY diseased palms remained as low as 0.01 cm/s during most of the day, whereas healthy palms showed a fluctuation from 0.01 to 0.2 cm/s.

Stomatal closure has also been associated with other diseases caused by MLO in species as varied as *Catharanthus roseus*, *Ulmus americana*, *Fraxinus americana*, *Prunus virginiana* and *Zea mays* (Matteoni and Sinclair 1983). This suggests that stomatal closure may be a common mechanism of response to invasion by most MLO in plants. This stomatal closure may be explained by hormonal imbalances (see below). However, it can also be caused by other compounds like toxins (Ayres 1981), phenols (Plumbe and Willmer 1986a), and phytoalexins (Plumbe and Willmer 1986b), as found in other plant systems. Therefore, the possible role of these substances in the LY-induced stomatal closure should be studied.

In LY-affected plants of *Cocos nucifera*, stomatal closure has been detected 2 weeks before the appearance of any other LY symptoms (McDonough and Zimmermann 1979, Eskafi et al. 1986). For this reason, it has been suggested that stomatal closure might be a useful parameter for the detection of the disease at a relatively early stage.

Nutrients. Dabek and Hunt (1976) reported the effect of exogenous application of various compounds including some nutrients such as copper and zinc on diseased fronds of *Cocos nucifera*. The application of both copper and zinc to yellow leaves of LY affected palms resulted in their re-greening. This might suggest that the yellowing that LY diseased plants experience is the result of micronutrient deficiency, perhaps associated with the lack of water flow through xylem reported before (McDonough and Zimmermann 1979). However, Stemmer et al. (1982) studied the trunk phloem sap content of healthy and diseased palms and they did not find significant differences in the amounts of Cu, Zn, K, N, Mg, Ca and Fe. This discrepancy could be due to the different tissues analyzed.

Photosynthetic Rates. With stomata closure affecting CO_2 uptake in palms with LY, photosynthetic rates should be expected to be reduced. We have found that palms at early stages of the disease (partial stomatal closure) have similar photosynthetic rates to those of healthy palms,

but in severely LY affected palms (fully closed stomata) photosynthetic rates are greatly reduced (Santamaría J.M., unpublished). However, stomatal closure is not necessarily the only cause of reduction in photosynthesis. Leaf chlorophyll content is steadily reduced as the disease progresses (Sanchez et al. 1989). Additionally, we have observed a decrease in a polypeptide of Mr = 55,000, which suggests a reduction in the large subunit of ribulose bisphosphate carboxylase (RUBISCO) in leaf extracts of severely LY affected palms when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Villanueva M.A., unpublished). Certainly, more detailed studies using antibodies and studies on enzyme activity are required to define the role of RUBISCO on photosynthesis decay in LY affected palms.

Photosynthesis in diseased plants can also be altered by modifications of its end-products as occurs in various diseases caused by virus, bacteria or fungi (Buchanan et al. 1981). These modifications include the utilization of sugars produced in photosynthesis to produce amino acids. Whether this is the case in LY affected *C. nucifera* palms and whether this may contribute to the increased arginine content found in these palms (Sanchez et al. 1989) remains to be defined.

Plant Growth Regulators. Although systematic studies on plant growth regulators (PGR) in LY affected palms have not been reported so far, Dabek and Hunt (1976) made a series of observations which indirectly suggest changes in PGR availability in LY affected tissue. They studied the effect of exogenous applications of gibberellic acid (GA), kinetin and indoleacetic acid (IAA) on longevity of healthy and diseased *C. nucifera* detached pinnae. Treatment with GA caused an increase in the longevity of both healthy and diseased pinnae as well as re-greening of initially yellow areas of diseased pinnae. Kinetin and IAA did not have any effect on the longevity of either healthy or diseased pin-

nae. Regarding re-greening of yellow diseased pinnae, the effect of kinetin was only partial, whereas combined with IAA it was more effective. The authors suggested that this could be indicative of a disturbance of the hormonal balance in coconut palms affected with LY.

One of the alterations of LY is stomatal closure (Eskafi et al. 1986). Abscisic acid (ABA) has been demonstrated to control stomatal closure in several species (Zhang et al. 1987). An increase in ABA may be responsible for stomatal closure in LY affected pinnae. So far no attempts have been made to test this idea. Alternatively, a reduced supply of cytokinins might also result in stomata closure (Reid and Bradford 1984). Therefore, stomata closure in LY affected palms could also result from a reduced supply of cytokinins particularly since both phloem and xylem transport are impaired. Moreover, evidence exists that roots are damaged in LY affected palms (Eskafi et al. 1986), so that cytokinin synthesis in this tissue may be affected. It is likely that both ABA and cytokinins are involved in the stomatal closure in LY infected palms.

Some of the visible symptoms of LY include premature abscision of fruits and leaf senescence. Ethylene has been involved in both fruit abscision (Sexton et al. 1985) and leaf senescence (Roberts et al. 1985) in several species and may also be related to these processes in LY. If this was the case, an accumulation of ethylene should be expected in LY diseased palms. As far as we know, no direct measurements of ethylene have been made in LY diseased tissues. However, Dabek and Hunt (1976) tested the capacity of healthy and diseased pinnae to promote ripening of bananas. They found that healthy pinnae promoted a faster ripening than diseased pinnae which they believe may be related to a lower production of ethylene in diseased tissue. However, in the whole plant, synthesis of ethylene may also take place in the roots and the capacity for ethylene evolution of

detached pinnae may not necessarily reflect the ethylene production capacity of the whole LY affected plant.

In order to clarify whether or not changes in PGR concentrations take place in LY and if they have a causal relationship with the development of symptoms, more detailed studies on endogenous PGR concentrations of diseased tissue are required.

Enzyme Activities and Protein Electrophoretic Patterns. The response of a plant to pathogens can be varied. The most characterized responses include changes in enzyme levels such as peroxidases (Agrios 1969, Kanazawa et al. 1965, Solymosy et al. 1967). Dabek (1974) found no differences in peroxidase activity from either healthy or LY affected palms. However, in diseased palms there was a negative relationship between the polyphenolic compound content and the peroxidase activity. This relationship was not observed in healthy palms. He suggested that once well characterized and standardized, this relationship could be used as an early symptom diagnostic test for LY since the phenomenon appeared before visual symptoms occurred. Nevertheless, no further reports have been made.

In another study, catalase activity, which is involved in polyphenol metabolism, was found to increase in early stages of the disease (i.e. before the first visual symptoms) (Dabek 1974). The activity of the enzyme decreased as the disease progressed, until it was reduced to 50% of the activity found in healthy palms (Dabek and Hunt 1976). These authors suggested that this decrease was a consequence of the damage to the pathway of protein biosynthesis and was not an exclusive symptom of LY.

Other studies have focused on changes in enzyme activities from extracts analyzed by SDS-PAGE. Among the detected enzymes in both healthy and diseased palms were three peroxidases, five esterases, a ribonuclease and an alkaline phosphatase (McCoy 1983). However, no variations in

the pattern from healthy compared to diseased tissue were observed.

The determinations of protein changes in diseased palms have been analyzed by raising antibodies against diseased palms and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of extracts from phloem and petiole base tissue. Charudattan and McCoy (1975) reported that the binding of antibodies raised against phloem exudate filtrates of diseased palms was specific for LY affected palms but not for healthy palms of *Cocos nucifera* and *Veitchia merrillii*. However, no further reports of the antigenic nature of the molecule(s) recognized by the antibodies exist, nor have these antibodies been applied for diagnostic tests. In a separate study McCoy (1983) found a low molecular weight protein in extracts of bud tissue from diseased *V. merrillii* palms analyzed by SDS-PAGE, which was not present in the healthy counterpart. In contrast, in LY affected *C. nucifera* plants, this protein was not observed and the SDS-PAGE patterns were identical for both healthy and diseased palms whether bud tissue (McCoy 1983) or phloem exudate extracts (Stemmer et al. 1982) were analyzed.

It would be interesting to know if the antibodies raised against phloem exudate filtrates from diseased palms bind the low molecular weight protein found in *V. merrillii*. It would also be worthwhile to purify this low molecular weight protein for raising antibodies against it to be used in further studies or as a diagnostic test provided the antigen is present in the presymptomatic stage.

Arginine Content. If the MLO that causes LY is similar to those species of *Mycoplasma* that require arginine, it would be expected that high levels of arginine in host tissues favoured infection, whereas low levels of this amino acid would not. Barcelon and co-workers (1983) tested this idea and analyzed the arginine content in the leaves of several palm species with different degrees of susceptibility to LY.

They found that those species with higher levels of arginine were more susceptible than those with lower levels. This could be very useful as a criterion for selection of tolerant individuals.

The content of arginine in LY affected palms has been measured by Stemmer et al. (1982). The arginine content in the phloem sap of diseased leaves was about 66% greater than in that of healthy leaves. The difference was not statistically significant; however, it may be indicative of an actual difference. In fact, we have found a four- to five-fold increase in arginine content in severely affected leaves (Sanchez et al. 1989) which suggests a higher proteolysis in severely affected palms. Analysis of the proteins from the nuts of healthy palms (Hagenmaier et al. 1974, 1975) has shown arginine to be the second most abundant amino acid. If this is also true for the proteins in other plant parts (e.g. leaves), an increased availability of arginine would be expected in infected palms. This would favor the survival of arginine-utilizing MLO. In addition, preliminary results in our laboratory show a positive correlation between degradation of proteins and disease severity, as shown by the analysis of protein extracts from pinnae from intermediate leaves by SDS-PAGE (Villanueva, M.A., unpublished). Alternatively, the increased arginine content may result from an increased synthesis either as a plant response, or induced by the MLO. The latter could be brought about via transformation since MLO has been shown to contain plasmids (Sears et al. 1989, Davis et al. 1988).

Experimental Model

In an attempt to integrate the information described above, we present the following experimental model (Fig. 1).

Pre-symptomatic Stage. Once the MLO are introduced to the phloem by the vector, it may be transported by mass flow to actively-growing plant parts (e.g. young leaves and roots, developing inflorescences and vegetative meristems). There, they can be provided with an adequate supply of nutrients including arginine, which could be particularly important if MLO associated with LY requires this amino acid for its growth. In such a case, the demand of this amino acid at the beginning of the infection may be expected to be satisfied by the free arginine content within the plant.

During this stage, which takes about four to nine months (Dabek 1974, 1975), the MLO population is presumed to grow and initiate physiological and biochemical changes. Only a few of these, such as alterations in polyphenol levels and peroxidase and catalase activities, have already been documented (Dabek 1974). However, other changes can also occur in both roots and aerial parts such as production of callose, further growth of the MLO, or production of MLO substances for the alteration of the plant metabolism. These alterations may cause a general disruption of plant performance such as early root damage. This could precede the root necrosis reported in the symptomatic stage (Eskafi et al. 1986). If this is the case, root malfunction could lead to hormonal imbalance, as has been reported for flooding (Reid and Bradford 1984) and water stress (Davies et al. 1981).

Symptomatic Stage. The earliest symptom which can be detected is stomata closure (Eskafi et al. 1986). This can be reflecting changes in PGR balance. Both increases in ABA content (Davies et al. 1981) and decreases in cytokinin and gibberellin contents have been shown to promote stomata closure (Jackson and Campbell 1979). Both changes in leaf PGR contents can originate in other plant parts. Zimmermann proposed as early as 1979 that in LY affected plants a metabolite moving from the roots or stem to the leaves was responsible for stomata closure. More recently, stomata closure in water stressed plants has been shown to be mediated by
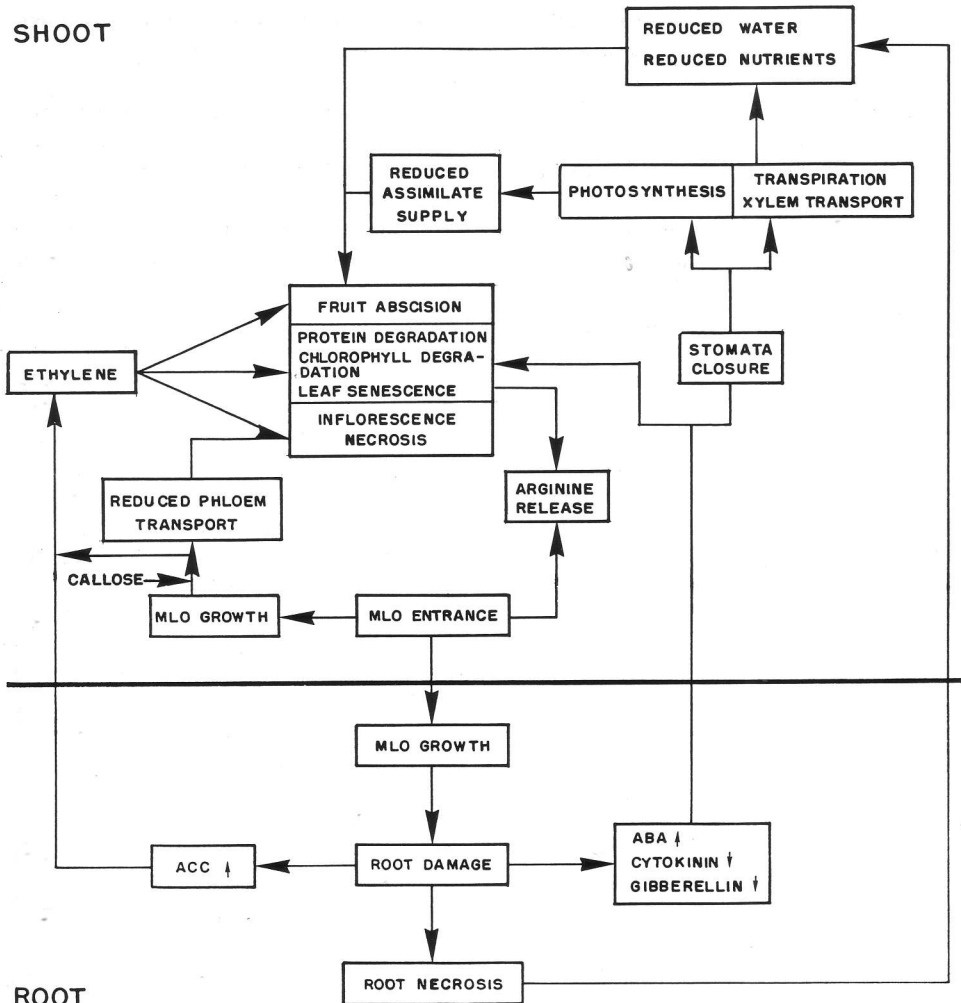
SHOOT

1. Proposed experimental model which integrates the sequence of physiological and biochemical alterations that lead to the appearance of symptoms of lethal yellowing in palms.

ABA which is apparently synthezised in the stressed roots and then mobilized to the leaves (Zhang et al. 1987). Plants under flooding also show stomata closure which has been associated with a reduced synthesis of cytokinins and gibberellins as roots are damaged (Reid and Bradford 1984). In the case of LY roots are affected; therefore stomatal closure could be the result of an altered PGR balance. This idea is consistent with the results obtained by Dabek and Hunt (1976) suggesting that

in LY infected coconut palms, cytokinin and gibberellin contents are reduced.

A sustained stomatal closure can lead to dramatic changes in the performance of the palm, for instance, reduced rates of photosynthesis due to a shortage of carbon dioxide uptake. Additionally, it could indirectly cause a reduction in the supply of water and nutrients as transpirational driving force decreases. This reduced nutrient supply may cause fruit abscision as it has been proposed by Zimmermann (1979).

Since several other types of stress have been shown to cause ethylene evolution (Jackson and Campbell 1975, Jaffe and Telewski 1984), an increased ethylene content could also be involved in promoting early fruit abscision. An increase in ethylene synthesis in aerial parts could be favored by increased 1-aminocyclopropane-1-carboxylic acid (ACC) synthesis in damaged roots as reported in the case of flooding (Reid and Bradford 1984).

In addition, the presence of MLO in the phloem vessels has been associated with callose deposition (Nienhaus et al. 1982) which, in turn, could lead to ethylene evolution. This is supported by the fact that ethylene evolution in the case of thigmomorphogenesis has been demonstrated to follow callose deposition and to keep a causal relationship with the development of symptoms (Jaffe and Telewski 1984).

As the disease develops, pronounced leaf yellowing appears. This also could be the result of a reduced nutrient supply and hormonal changes such as increases in ethylene content and decreases in cytokinins and gibberellins leading to leaf senescence. Further damage in roots could, in turn, be favored by reduced assimilate supply from senescing leaves. In this way the apical meristem and younger leaves would also suffer from reduced nutrients and assimilate supply, eventually leading to necrosis in the apical meristem and favoring senescence of younger leaves.

At later stages when the MLO population has grown, the arginine demand should be expected to be great if the microorganism depends on this amino acid. If regular levels of free arginine are not enough, then MLO would require additional sources of arginine, for instance, arginine-rich proteins from which arginine could be released by hydrolysis in senescing leaves. The possibility that the extra arginine resulted from a shift in the end products of photosynthesis, as occurs in other diseases, cannot be discarded. Alternatively, arginine *de novo* synthesis could be occurring and

maybe controlled by MLO. MLO from other diseases have been shown to contain plasmids (Davis et al. 1988, Sears et al. 1989). Therefore, the mode of action of the LY-MLO via transformation cannot be ruled out.

Conclusions

The present knowledge on physiological and biochemical aspects of lethal yellowing is in general inconclusive, since it is mostly based on indirect evidence. Therefore, it is important to extend our understanding of the alterations caused by LY, in particular during the presymptomatic stage, since several events occurring in the symptomatic stage should result from changes occurring earlier. It is difficult to study these early events since we lack both early diagnostic methods and a reliable transmission system. Some of the possible alternatives to develop the former are: a) the production of antibodies which react against antigens of both presymptomatic and symptomatic stages; b) the systematic monitoring of physiological and biochemical alterations (photosynthetic rates, enzyme activities, electrophoretic patterns, etc.) in healthy palms of infected areas until they start developing symptoms; and c) the obtention of DNA probes to identify MLO DNA in infected palms.

Other areas which require further study are: a) the culture (possibly via organ culture) and ultimate identification of the causal agent of LY, b) mechanisms of MLO dissemination, c) MLO-palm arginine relations, d) changes on the balance of growth regulators.

The integration of this knowledge might lead to the development of selection criteria for coconut palms resistant to lethal yellowing and the establishment of adequate ways to control the disease.

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