PHYSIOLOGICAL, ANATOMICAL AND MOLECULAR ANALYSES OF COCONUT PALMS (*Cocos nucifera* L.) AFFECTED WITH YELLOWING

By

DEEPA S.

(2009-11-107)

THESIS

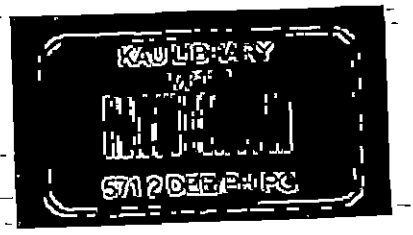
Submitted in partial fulfillment of the

requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University



DEPARTMENT OF PLANT PHYSIOLOGY

COLLEGE OF AGRICULTURE

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KERALA, INDIA

2011

DECLARATION

I, hereby declare that this thesis entitled **"Physiological, anatomical and molecular analyses of coconut palms (*Cocos nucifera* L.) affected with yellowing"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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ACKNOWLEDGEMENT

First and foremost I bow my head before the Almighty God whose grace has endowed me the strength and confidence to complete the thesis work successfully on time.

With immense pleasure I avail this opportunity to express my deep sense of gratitude and indebtness to my major advisor Dr. R. V. Manju, Associate Professor and Head, Department of Plant Physiology, for her expert guidance, constructive suggestions, motherly approach, constant encouragement, affectionate advice and above all, the extreme patience, understanding and wholehearted co-operation rendered throughout the course of my study. I consider myself very fortunate in having the privilege of being guided by her.

I thankfully acknowledge Dr. Roy Stephen, Associate Professor, Department of Plant Physiology for his meticulous help, unwavering encouragement, forbearance, well timed support and critical scrutiny of the manuscript which has helped a lot for the improvement and preparation of the thesis.

I am greatly thankful to Dr. M. M. Viji, Associate Professor, Department of Plant Physiology for her friendly approach, constructive criticisms and help rendered during the course of study.

I avail this opportunity to express my deep felt reverence and gratitude to Dr. Umamaheswaran, K, Professor, Department of Plant Pathology, for his expert advice, constant inspiration, precious suggestions, generous support and constructive criticisms during my entire study which helped in successful completion of this work.

I extent my heartiest gratitude to Dr. K. B. Soni, Associate Professor, Department of Plant Biotechnology, for her ever willing help , constructive comments , critical discussions and creative suggestions throughout the period of my research.

I sincerely thank Dr. Anoop E. V. Associate Professor and Head, Department of Wood Science, College of Forestry, Vellanikkara for his valuable guidance, friendly approach, support and kind co-operation during my research work.

I wish to extend my sincere gratitude to **Dr. B. R. Reghunath**, *Professor and Head, Department of Plant Biotechnology for providing the lab facilities for the molecular work and* **Dr. Swapna Alex**, *Associate Professor, Department of Plant Biotechnology for her valuable suggestions during the work.*

I am greatly thankful to **Dr. Abdul Salam**, *Professor and Head, Department of Agronomy, and* **Dr. N. Saifudeen**, *Professor and Head, Department of Soil Science and Agricultural Chemistry, for providing the facilities for chemical analysis during the course of study.*

I sincerely thank **Dr. Shalini Pillai**, *Associate Professor, Department of Agronomy for her timely help and sustained interest during my course of study.*

I duly acknowledge the valuable help and service rendered during plant analysis by **Dr. Sunitha**, *Scientist, Central Soil Analytical Lab, Parottukonam.*

My profound sense of gratitude to **Mrs. Liji, Mrs. Shiji, Mrs. Aruna, Miss Akhila, Mr. Saji, Mr. Aravind** *for their help and service during my analysis work.*

I also express my sincere thanks to **Dr. Jeena Mathew, and Mrs. Nihad**, *Scientists, CPCRI, Kayamkulam for their timely help in literature collection.*

I wish to place on record the help rendered to me by all my friends especially **Rekha, Anisa, Dhanya, Divya, Shimi, Mariya, Kavitha, Hajara, Anulekshmi, Anju, Sheeba, Adrika, Shameena Beegam, Shameena S., Priya, and all my PG classmates.**

My heart never forgets **Mrs. Nikitha, Mrs. Lekshmi, Mrs. Nya, Mrs. Lali,** *of my department whose helping hands, love and affection helped me in my difficult times.*

I thank **Kerala Agricultural University** *for granting me the KAU Junior Research Fellowship.*

I am forever beholden to my **Parents and family members** *for their moral support and prayers, which helped me to undertake the strenuous toil successfully.*

Deepa S.

DEDICATED

TO

MY FAMILY

&

TEACHERS

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LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrophotometer
BSA	Bovine Serum Albumine
CBB	Coomassie brilliant blue
DMSO	Di methyl sulfoxide
DNA	De oxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
et al.	And others
FW	Fresh weight
PCR	Polymerase chain reaction
PO	Peroxidase
PPO	Poly phenol oxidase
RWC	Relative water content
N	Nitrogen
P	Phosphorus
K	Potassium
Ca	Calcium
Mg	Magnesium
Fe	Iron
Mn	Manganese
Cu	Copper
Zn	Zinc
µ	micro

Introduction

1. INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is a perennial tropical tree of the family Arecaceae, the most important one under the class Monocotyledoneae. The sole species of the genus *Cocos* is coconut. It includes more than 300 cultivars or varieties (Lebrun *et al.,* 1998). According to Walther *et al.* (2007), species of the Arecaceae family have been quoted as important indicators for obtaining information of the past climate in the Earth's history, and have become significant global bioindicators across continents for present-day climate change and the projected global warming of the near future. The coconut palm sustains the livelihood of millions of people in coastal regions of the tropics and subtropics (Ohler *et al.*, 1999a). Philippines, Indonesia, India, Sri Lanka, Thailand, Tanzania, Brazil and Malaysia are the main coconut producing countries (Gomes andPrado, 2007). Coconut palm is a versatile tree, popularly known as 'Tree of Life', the Consols of the East', Tree of Abundance', 'Tree of Plenty', and 'King of Palms'. The Vedas describe coconut tree as 'Kalpavriksha', which translates as "tree that gives all that is necessary for living." Considered the most useful tree in the world, the coconut palm not only provides sustainable income to millions who are directly and indirectly dependant on it, but also provides highly nutritious food, drink, clothing, shelter, heirloom history, financial security, alleviate poverty and export earnings (Nejat *et al.*, 2009). The coconut is known as a wonder food. It is a near perfect diet as it contains almost all the essential nutrients needed by the human body.

India is the largest producer of coconut and it is grown in 18 states and 3 union territories with an area of 1.89mha and production of 15730 million nuts and a productivity of 8303 nuts/ha (Balakrishnanan, 2011). Fossil records indicate that the coconut palms existed as many as 15 million years ago and older fossils were found in Kerala, Tamil Nadu Rajasthan and on the banks of the river Palar (Tania, 2011). Scholars opine that the word Kerala is derived from 'Keram' meaning 'land of coconut tree'. Kerala is the largest producer of coconut in India with an area of 0.79 mha and production of 3992 million nuts (NHB, 2010).

However the productivity of Kerala (5066 nuts/ha) is behind the national average (8303 nuts/ha) and far behind the neighbouring state Tamil Nadu (9467 nuts/ha) where the area under coconut cultivation is only half that of Kerala (NHB, 2010). This is because of unproductive and senile palms, lack of adaptation of recommended cultivation practises and serious incidence of pests and diseases.

Root (wilt) is the major endemic disease of coconut in Kerala, and is of great concern to the coconut growers because of the quantum of economic loss incurred and the non- availability of an immediate control measure (Solomon *et al.*, 1999). Root (wilt) disease was first noticed after the great floods of 1882 in three isolated pockets 50 km away from each other in the erstwhile princely state of Travancore, presently included in the Kerala state. Since then it has spread in all directions from the original foci of infection and according to a survey of 1984-85 the disease occurred in a contiguous manner in 0.41 million ha in eight southern districts and in isolated pockets in the northern districts of Kerala, and also in districts of Tamil Nadu bordering Kerala state (CPCRI, 1985). The annual loss due to the disease is estimated to be around 968 million nuts (Balakrishnan, 2011).

Root (wilt) disease (RWD) is a debilitating disease caused by phytoplasma which causes a gradual decline in the yield over time. The most obvious and diagnostic symptom of the disease is the abnormal inward bending of the leaflets termed ribbing or flaccidity. Yellowing and marginal necrosis of leaflets are other associated foliar symptoms. Rotting of roots increases with the progress of the disease. Shedding of immature nuts, drying up of spathes and necrosis of spikelets in unopened inflorescence is noticed in certain cases (Pillai and Rawther, 1999; Chandramohanan and Peter, 2008). The symptom expressions vary with the age, nutritional status management practises, variety and the time lag after disease incidence. In general, 67 -97% palms show flaccidity, 38- 67% develop yellowing and 28- 48% show marginal necrosis (Koshy, 1999).

Lethal yellowing (LY) is another devastating phytoplasma disease affecting coconut palms in several African and American countries causing tremendous destruction of palm. The affected palms die within 3-6 months after

acquisition of pathogen and once the disease appears in a certain area, it can spread rapidly (Aguilar *et al.*, 2009). The palms affected with lethal yellowing exhibit a variety of symptoms including premature nut drop, inflorescence necrosis, leaf chlorosis and senescence and spear leaf death (Leon *et al.*, 1996; Maust *et al.*, 2003).

Phytoplasmas are plant pathogenic mycoplasmas that are non-helical, non culturable, pleomorphic and transmitted by arthropod insect vectors. They are minute cell wall less prokaryotes with a diameter less than 1μm, generally confined to the phloem (Bertamini, 2003; Hogenhout *et al.*, 2008; Aguilar *et al.*, 2009 and Nejat and Vadamalai, 2010). Constant association of phytoplasma with the root (wilt) and lethal yellowing has been established by several workers. Molecular diagnostic techniques developed like nested PCR have proven to be more accurate, reliable and sensitive for the detection of phytoplasma. The presence of phytoplasma in root (wilt) disease has been detected by Edwin and Mohankumar (2007) and Manimekalai *et al.*, 2010 and in lethal yellowing by Mpunami (1999) and Maust *et al.*, (2003).

Recently another type of yellowing characterised by mid whorl yellowing shedding of immature nuts, drying of inflorescence without showing characteristic ribbing symptom is rapidly spreading in many parts of Kerala. Although sudden appearance of bright yellowing of 3-4 leaves in the middle whorl, followed by the appearance of large number of brown spots with a halo around on all leaflets of yellowed leaves has been reported to be an associated symptom of root (wilt) (Koshy, 1999). No studies has yet cited specifically on mid whorl yellowing of coconut. Since there is rampant spreading of this type of yellowing to other coconut cultivated areas causing a serious reduction in the nut yield, this problem needs immediate attention.

At this juncture, the present study was taken up as an initial attempt to throw light on the predisposing factors, causative agents and the various alterations brought about at the cellular and tissue level by mid whorl yellowing of coconut with the following specific objectives.

1. To analyse the physiological, biochemical and anatomical changes associated with mid whorl yellowing of coconut palm.
2. To detect the presence of phytoplasma in the affected palms.

Review of Literature

2. REVIEW OF LITERATURE

Cocos nucifera L. is a perennial tropical species of the *Arecaceae* family, one of the most important in the Monocotyledoneae class. The coconut palm sustains the livelihood of millions of people in coastal regions of the tropics and subtropics. Though Kerala is known as the land of coconut, the per palm production is much less when compared to that of other coconut growing states. This is because the coconut is of which the root (wilt) disease is the most prominent one which results in an average annual loss of 968 million nuts in Kerala. Recently another type of yellowing of coconut characterized by mid whorl yellowing is fast spreading in many parts of Kerala. Since there is rampant spreading of yellowing to other coconut cultivated areas, this problem demands immediate attention.

In the present investigation, an attempt has been made to analyze the physiological, biochemical and anatomical changes associated with foliar yellowing in coconut and to detect the presence of phytoplasma in affected palms

2.1 Physiological parameters

2.1.1 Chlorophyll content

Plant productivity is a unique process that depends greatly on the amount of chlorophyll present in the chloroplast. Chlorophyll is the pigment that gives plant their characteristic green colour; it plays a unique role in the physiology, productivity and economy of green plants (Taiz and Zeiger, 2006). The quantity of chlorophyll per unit area is an indication of photosynthetic capacity and productivity of a plant. Therefore, the amount of chlorophyll in the leaf tissues may be influenced by nutrient availability and environmental stresses (Palta, 1990; Otitoju and Onwurah, 2010). Mukherjee and Kumar (2005); Anjum *et al.* (2011) reported that water stress resulted in a reduction in the chlorophyll content in plants.

Phytoplasmal infection severely damages the physiological and biochemical processes in plants. Foliar yellowing is the most conspicuous symptom in root (wilt) and lethal yellowing disease. A reduction in the chlorophyll content in the root (wilt) affected coconut palms has been reported (Koshy, 1999). A similar reduction in the chlorophyll and carotenoid contents of coconut affected with lethal yellowing has been studied by Leon *et al.* (1996). In maize plants infected by Maize bushy stunt phytoplasma a significant reduction in chlorophyll content has been reported (Junqueira *et al.*, 2004). The carotenoid content in aster yellows phytoplasma-infected leaves was diminished (Choi *et al.*, 2004).

2.1.2 Relative Water Content (RWC)

Leaf water status is intimately related to several leaf physiological variables, such as leaf turgor, growth, stomatal conductance, transpiration, photosynthesis and respiration. RWC is a useful indicator of the state of water balance of a plant (Yamasaki and Dilenberg, 1999).

Shivshankar *et al.* (1993) reported that under soil moisture depletion, the coconut showed a gradual reduction in leaf water status. Changes in leaf water status were reflected in changes in the levels of chlorophylls, free amino nitrogen, epicuticular wax and total soluble sugars. Rajagopal (1991) has reported a lower leaf water potential in root (wilt) affected coconut palms compared to healthy ones. A reduction in leaf water potential in coconut affected with lethal yellowing also has been reported (Leon *et al.*, 1996).

2.1.3 Total carbohydrate

Lepka *et al.* (1999) described the effect of phytoplasma infection on concentration and translocation of carbohydrates in periwinkle and tobacco plants. They found higher levels of reducing sugars and sucrose in source leaves of infected plants than in healthy ones. Maust *et al.* (2003) reported that in coconut palms

affected with lethal yellowing leaf carbohydrate concentration increased in infected leaves. Sugar and starch concentrations increased slowly in recently-expanded leaves with the development of the disease before decreasing in later stages of lethal yellowing. Sugar and starch concentrations increased more rapidly in intermediate leaves with the advance of the disease before decreasing in later stages. In coconut palms affected with root (wilt) also a significantly higher total reducing and non-reducing sugars and a reduction in the total carbohydrate and starch have been reported (Mathew, 1977). Accumulation of sugar and starch in the leaves in pear decline affected pear has also been reported (Catlin *et al.*, 1975). Guthrie *et al.* (2001) reported higher carbohydrate content in diseased leaf tissue in papaya infected with phytoplasma. Higher reducing sugar content has been reported in corn plants infected by the maize bushy stunt phytoplasma (Junqueira *et al.*, 2004)

Lakmini *et al.* (2006) has reported increased levels of sucrose or reducing sugars in coconut palms under moisture stress. According to Magat (1993) shortage of proteins as a result of N deficiency increases the C/N ratio, resulting in excessive carbohydrates.

2.1.4 Total soluble proteins

Usually plants infected by pathogens show a high protein content which could be due to both the activation of the host defense mechanisms and the pathogen attack mechanisms (Agrios, 1997). Bertaccini (2009) has reported that phytoplasma infection can lead to the production of defense proteins in host plants. An increase in the protein content of leaves was reported in coconut affected with root wilt (Padmaja *et al.*, 1981). But in lethal yellowing affected coconut palms there was a reduction in the leaf protein content(Leon *et al.*, 1996).In maize bushy stunt phytoplasma infected maize plants, an increase in the total amount of proteins has been found (Junqueira *et al.*, 2004). Contradictory results have been obtained for plants affected with different mollicutes- a decrease in total soluble protein has been reported in tomato plants

affected by STOL, in grape wine affected by bois noir and in apple trees affected by apple proliferation (Bertamini *et al.*, 2002).

2.1.5 Phenols

It has been observed that certain common phenolic substances are toxic to pathogens and accumulate in plants after infection, especially in resistant varieties (Agrios, 1997). Bertaccini (2009) has reported an increase in phenolic compounds in host plants due to infection by phytoplasma. Musetti *et al.* (2000) has determined the total polyphenol content in phytoplasma – infected apples and plums and three- fold higher phenol content in the infected tissues as compared with healthy ones have been reported. Accumulation of phenolics in phytoplasma infected *Zea mays* has also been reported (Junqueira *et al.*, 2004). Choi *et al.* (2004) has reported higher amounts of polyphenols in phytoplasma infected *Catharanthus roseus* leaf tissues. An accelerated phenol metabolism has been reported in roots of root (wilt) affected coconut palms (Joseph and Jayasankar, 1979).

2.1.6 Defence related enzymes

Peroxidase (PO) and Polyphenol oxidase (PPO) has been extensively studied and implicated in plant resistance to various diseases. Peroxidase catalyses dehydrogenation of a large array of compounds like hydroxy cinnamyl alcohols, phenolics, and aromatic amines. Physiological roles of peroxidase include lignin synthesis, cross linking cell wall polysaccharide and wound healing. Peroxidase and polyphenol oxidase mediate the oxidation of phenols and oxidized phenols are highly toxic to the pathogen (Sequeira, 1983). PO and PPO catalyse the oxidation of phenolic compounds through a PPO-PO-H2O2 system (Srivastava, 1987). The role of phenol oxidases in resistance is based on the observations that the activity of these enzymes is increased in infected tissues and that the oxidized phenols i.e., quinones are more reactive and more toxic to microorganisms compared to their non-oxidized form (Khatun, *et al.*, 2009).

Khatun *et al.* (2009) has reported an increased activity of peroxidase and polyphenol oxidase in plants in response to pathogen infection. Mayilvaganan and Gupta (1999) detected two isoforms of peroxidase (1and 2) in root (wilt) affected coconut palms. The activity of peroxidase 2 was greatly reduced in early stage of disease and totally absent in other stages. CPCRI (2006) has reported that the peroxidase activity did not differ in root (wilt) affected and healthy palms. But Joseph and Jayasankar (1979) reported an increase in the activity of peroxidase and poly phenol oxidase in roots of the root (wilt) affected coconut palms.

2.2 Nutrients

The importance of different plant nutrient factors in the soil for the optimum growth and productivity of coconut palm has been emphasized by several workers. Coconut is a perennial crop and is unique among the plantation crops in that it flowers and fruits throughout the year. Hence maintenance of adequate water and nutrient during the entire period is of paramount importance. Palms suffer quickly and conspicuously from improper mineral nutrition, whether due to insufficient or incorrect fertilization. They also may exhibit certain nutritional disorders in unique ways. Some nutritional problems in palms are difficult to diagnose accurately because symptoms of several different mineral deficiencies may overlap (Broschat, 1992). Nutrient removal by coconut palm has been computed by several workers (Nelliat, 1972; Thampan, 1982; Wahid, 1984). Although wide variations were observed in the reported values, there was a resemblance in the relative proportion of various nutrients removed by the palm. Wahid (1984) reported the absolute quantities of nutrients removed by a hybrid for an annual production of 6.7 t copra/ ha (Table 1).

Table 1. Nutrient removal by a hybrid coconut palm

Nutrient	Annual removal(kg/ha)
N	174
P	20
K	249
Ca	70
Mg	39

An investigation conducted in some districts in Kerala State, India revealed that the majority of farmers did not apply fertilizer at all, whereas about 30% applied fertilizers only at a low level (Ohler, 1999b).

The soil and nutritional factors are believed to exert considerable influence on the development, spread and intensity of the plant disease (Cecil *et al.*, 1991).Macro- and micronutrients have long been recognized as being associated with changes in plant susceptibility or tolerance and resistance to diseases and pests. (Fageria *et al.*, 2002).

Many of the coconut disease cannot be primarily caused by unfavourable soil conditions or nutrient imbalances but such factors provide an environment conducive to infection by biological agents (Cecil *et al.*, 1991). Soil sickness characterized by nutrient imbalance with mineral deficiencies especially of K, Ca and Mg were reported to have a decisive role on the incidence of the root (wilt) disease (Lal, 1964). Pillai *et al.*, (1975) has reported that the total nitrogen content of healthy soils was lower than that of root (wilt) affected area; available P did not differ but exchangeable K was lower in the diseased tract. No difference in Ca and Mg levels in the soils between healthy and diseased zones is reported.

Among the soil micronutrients Fe, Mn, Cu and Zn were significantly higher in the healthy zones compared to diseased tracts (Pillai *et al.*, 1975).

Leaf analysis is an accurate system to determine the nutritional status of the plant (Wahid, 1984 and Ohler, 1999b). The 14th leaf starting from the first fully opened one is the most widely used leaf for chemical analysis as recommended by the IRHO (Fremond, 1966). Kamaladevi *et al.* (1983); Ohler (1999b) also reported the suitability of 14th frond as index leaf in coconut. The critical level of nutrients in the 14th frond is presented in the Table 2 (Wahid, 1984; Ohler, 1999b)

Table 2 Foliar critical nutrient levels in coconut palm

Nutrients	Nutrient level in 14th frond
N	1.8-2.0 %
P	0.12 %
K	0.8-1.0 %
Ca	0.3-0.4 %
Mg	0.24 %
Fe	50 ppm
Mn	60 ppm

The following is an account of the visual characteristic symptoms of nutrient deficiencies reported in coconut (Menon and Pandalai, 1958; Manicot *et al.*, 1980, Wahid, 1984; Ohler, 1999b)

Nitrogen deficiency reduces the chlorophyll content of leaves. In the early stage of the deficiency, the crown of the palm loses its glossy appearance and turns pale green, followed by yellowing of the leaves. In advanced stages young leaves also turn pale green giving the leaflets a dull appearance. Phosphorus deficiency in coconut palms is rare and very difficult to recognize as it shows hardly any visible symptoms. Only in severe cases may leaves turn yellow before dying prematurely. It plays an important role in the efficient functioning of nitrogen. The first symptoms of potassium deficiency begin in the older functional leaves. They are characterized by

yellowing of the leaflets, followed by necrosis. Yellowing starts at the tip of the leaflets, progressing along the margins towards the base. This characteristic distinguishes it from nitrogen deficiency, where the yellowing is more pronounced along the mid-rib. In the case of potassium deficiency, the yellow colour usually has an orange tinge, in this respect also differing from nitrogen deficiency. Yellowing of the middle leaves and drying up of the older leaves are common in K deficient palms. Magnesium plays a vital role as a constituent of the chlorophyll, and deficiency leads to a loss of chlorophylls. The first deficiency symptom is the intervascular yellowing of older leaves. Yellowing starts at the tip and spreads to the base. In Ca deficiency the petioles turn a deep yellow or orange colour, orange blotches occur along the mid-rib frequently.

Among the micronutrients Fe, Cu, Mn and Zn, Fe is closely connected with chlorophyll formation but is not a constituent of chlorophyll. Manicot *et al.* (1980) described the symptoms as a general chlorosis, with all leaves discolouring to pale green or dark yellow. In Cu deficiency, there occurs a severe bending of the rachis of the youngest leaves, accompanied by yellowing and desiccation of the leaf tip, which appears to be rimmed with brown and yellow, whilst the central part remains green. In Mn deficiency symptoms occur only on new leaves which emerge chlorotic, weak, reduced in size, and with extensive necrotic streaking in the leaves. No descriptions are available on zinc deficiency in the case of coconut.

Mineral deficiencies may also enhance disease or pest attacks, which may blur the deficiency symptoms. On the other hand, diseases may also cause nutritional disturbances, showing unbalanced mineral relations. Extensive studies have been conducted to investigate on the role of nutrients in relation to the incidence of root (wilt) disease.

Pandalai (1959) showed that there was a tendency for N, P, and K to get accumulated in the leaf tissues of diseased palms and the accumulation increased with the advancement of the disease. Cecil (1975) suggested that the nutrient accumulation

was only apparent, possibly the result of a low dry matter content of leaf tissues consequent on disease incidence. He also reported that the N, P, and K contents did not differ between healthy and diseased palms in the early stage of infection. But Ca and Mg contents of healthy palms in disease free areas were significantly higher than those of apparently healthy or diseased palms in the affected tracts. Pillai *et al.* (1975) indicated that the palms in the disease affected areas whether apparently healthy or visibly diseased, were in a state of imbalanced nutrition, possibly the result of a relatively higher content of N, P, and K on the one hand and a lower content of Ca and Mg on the other.

Lal (1964) reported a reduction in the foliar yellowing and increase in yield of diseased palms by applying NPK, lime and farmyard manure and spraying with Bordeaux mixture, micronutrients and magnesium. Davis and Pillai (1966) reported that the application of micronutrients and Mg did not prevent the fresh incidence of disease. However, Mg application had decidedly a favourable response on the yield of diseased palms. Lal (1964) reported that the yellowing associated with the disease might be largely due to Mg deficiency. Cecil *et al.* (1982) based on fertility trials concluded that the disease was not caused by deficiency of any major nutrients. The soil and nutritional aspect of the disease were recently reviewed by Cecil and Amma (1998) indicating no direct involvement of major and micro nutrients in the disease. While discussing the nutritional disturbances in relation to root (wilt) disease, Pandalai (1959) suggested that absence or non- availability of nutrients was not the cause of tissue abnormalities, but was actually the inability of the palm to transact the normal processes at the appropriate site.

Khan *et al.* (1981) observed that the micronutrients viz., Cu, Mn, and Fe were higher in the crown of root (wilt) affected palms compared to healthy ones. A systematic micro nutrients manurial experiment consisting of all combinations of two levels each of Fe, Mn, Cu, Zn, B and Mo since field planting had shown that the disease was not related to micronutrient nutrition of the palm (Cecil and Amma, 1998).Zn both as soil application and foliar spray had no effect on incidence or

intensity of the disease even though tissue levels of Zn increased considerably (Mathew *et al.*, 1986). According to Cecil and Amma (1998) the diseased tract and disease free tract are geochemically different and perhaps it is due to this that the disease is confined to a particular region.

2.3 Yield

Though Kerala is known as the land of coconut the per palm production is much less when compared to that of other coconut growing states (Balakrishnan, 2011). This is because the coconut is prone to several maladies of which the root (wilt) disease is the most prominent one. It has been reported that root (wilt) results in an annual loss of 968 million nuts (Balakrishnan, 2011).

2.3 Anatomy

External symptoms are the manifestations of biochemical and histological alterations caused by or as an effect of pathogenic infection. Anatomical studies on root (wilt) affected coconut palms have been attempted by Shanta *et al.* (1959); Joseph and Shanta (1963), Indira and Ramadasan (1968); Kutty and Vellaichamy (1976); Dwivedi *et al.* (1977).

The normal leaf is protected on both abaxial and adaxial surface by a layer of cuticle. The upper epidermis is highly cuticularised than the lower one. There is a thin layer of wax on the outer surface of the cuticle. Below the epidermis there is a two-layered tissue of hypodermis on the upper surface and a single broken layer of storage tissue on the lower surface. There are about 20-25 vascular bundles running lengthwise on each side of the leaflet. Each vascular bundle is surrounded by a highly lignified 2-3 layered bundle sheath.

Joseph and Shanta (1963) reported considerable changes in the epidermal, mechanical and conducting tissues of coconut affected with root (wilt). The thickness

of cuticle on the upper surface was considerably reduced in the diseased compared to healthy. A considerable change in the number of the epidermal cell per unit area was reported where transverse divisions were accelerated and longitudinal divisions were curtailed in the upper epidermis of leaves of the diseased palm. The stomata on the lower surface of leaf are slightly narrower and there is increased percentage distribution of stomata per unit area in the diseased compared to that of healthy leaves. Failure of development of all types of schlerenchymatous tissues and phloem proliferation are the other important changes.

Shanta *et al.* (1959) reported that the mesophyll of a healthy leaf sample reveals a more or less compactly arranged , elongated palisade cells extending from the upper hypoderm to the lower epidermis with very little spongy tissues and and air spaces in between. The chloroplasts evenly distributed along the periphery of cells, are uniformly spherical to oval in shape with compact yellowish green chlorophyll. While no variation is seen in the mesophyll of disease affected palms or palms showing physiological yellowing. However there is a great variation in the distribution, size and the chlorophyll contents. The chloroplasts are more or less disintegrated.

Anatomical changes in roots of coconut palm affected with root (wilt) also have been reported (Kutty and Vellaichamy, 1976). Root damage is observed in disease prevalent areas. Walls of metaxylem elements of diseased roots were disorganized wall thickness much reduced. Tyloses were seen in roots. Phloem tissues showed degeneration in the roots of affected palms. The cells of protophloem and a few members of metaphloem showed necrotic effects. The phloem walls were distorted and collapsed. Phloem degeneration was noted. Callose accumulations were found in sieve plates.

2.4 Molecular analyses

2.4.1 Phytoplasmas

Phytoplasmas are plant pathogenic mycoplasmas that are non-helical, non culturable, pleomorphic and transmitted by arthropod insect vectors (Solomon, *et al.*, 1998).They are minute cell wall less prokaryotes with a diameter less than 1μm, generally confined to the phloem (Bertamini *et al.*, 2003; Hogenhout *et al.*, 2008; Aguilar *et al.*, 2009; Nejat and Vadamalai, 2010).The genome size of phytoplasma ranges from 530 to 1350 kbp (Edwin and Mohankumar, 2007).

Phytoplasmas are associated with hundreds of plant diseases globally affecting several plant species including many food vegetable and fruit crops, ornamental plants, timber and shade trees. Coconut cultivation faces severe phytopathological constraints caused by phytoplasma which includes root (wilt), lethal yellowing etc.(Nejat and Vadamalai, 2010).The presence of phytoplasma was identified in sieve tubes of roots, tender stem, petiole and developing leaf bases of root (wilt) diseased palms (Solomon *et al.*, 1998).

2.4.2. Detection of phytoplasma

Detection and identification of phytoplasma is of paramount importance for accurate disease diagnosis. Serological and DNA hybridization methods were used in the past for this purpose. Molecular diagnostic techniques developed in the last decade have proven to be more accurate, reliable and sensitive for the detection of phytoplasma. Phytoplasma diagnostics based on phytoplasma specific universal (Generic) or phytoplasma group specific polymerase chain reaction (PCR) primers designed on the basis of highly conserved 16S ribosomal RNA (rRNA) is now widely used. (Mpunami, 1997; Edwin and Mohankumar, 2007;Nejat and Vadamalai, 2010). It is particularly useful for detection of phytoplasmas because the major obstacles to diagnosis of phytoplasma-induced diseases have been the relatively low titres and

uneven distribution of these organisms in plant hosts. By use of PCR to amplify 16S ribosomal DNA (rDNA) sequences, detection of different phytoplasma strains from low titre plant hosts like chrysanthemum (Bertaccini *et al.,* 1992) and coconut palms (Harrison *et al.,* 1994) has been enhanced.

Diagnosis of phytoplasma can be divided into three phases-total DNA extraction from symptomatic tissues; PCR amplification of phytoplasmic DNA; nested PCR with group specific primers.

2.4.3. DNA extraction

The phytoplasma titer varies greatly from plant to plant. The coconut phytoplasma is confined to the phloem and is found primarily in sink tissues such as the apical meristem, immature leaves, root meristems, and inflorescences that are actively importing sugars, but usually is undetectable in mature source leaves (Maust *et al.,* 2003). The success of PCR in detecting phytoplasma in field-collected samples largely depends on obtaining total nucleic acid preparations of good quality and enriched with phytoplasma DNA, but this has always been difficult (Firrao *et al.,* 2007). The amount of phytoplasma DNA is lower than 1% of total DNA extracted from tissue (Bertaccini and Duduk, 2009).

Coconut contain exceptionally high amounts of polysaccharides, polyphenols, tannins which interferes with the DNA isolation procedure (Angeles *et al.*, 2005). It is diificult to obtain a pure quality DNA from plants with high amounts of polysaccharides and polyphenols (Maltas *et al.,* 2011). The problems encountered in the isolation and purification of DNA include degradation of DNA due to endonucleases, coisolation of highly viscous polysaccharides, inhibitor compounds like polyphenols which directly or indirectly interfere with the enzymatic reactions. Moreover the contaminating RNA that precipitates along with the DNA causes many problems including suppression of PCR amplification, improper priming of DNA templates during thermal cycling sequence etc.

Different protocols for total DNA extraction have been reported for the detection of the phytoplasma. The main goal of each protocol has been to concentrate

phytoplasma DNA while reducing enzyme-inhibitory plant polyphenolic and polysaccharide molecules. This is generally obtained by including a phytoplasma enrichment step in the nucleic acid extraction procedure (Bertacini and Duduk, 2009). The Cetyl Trimethyl Ammonium Bromide (CTAB) method of Doyle and Doyle (1990), phytoplasma enrichment method of Kirkpatrick *et al.* (1987) have been used to extract total nucleic acids from infected coconut tissues (Porebski, 1997; Sharmila *et al.*, 2004; Edwin and Mohankumar, 2007; Nejat and Vadamalai, 2010). DNA extraction methods by modification of the CTAB method of Doyle and Doyle (1990), phytoplasma enrichment method of Kirkpatrick *et al.* (1987) and Ahrens and Seemuller (1992) provided a good template for DNA amplification for phytoplasma (Mpunami, 1997; Nejat *et al.*, 2009).

2.4.4. Nested PCR

Nested-PCR assay designed to increase both sensitivity and specificity, is necessary for the amplification of phytoplasma DNA from samples having unusually low titers or inhibitors that may interfere with PCR efficacy (Gundersen *et al.*, 1994). Nested-PCR is performed by preliminary amplification using a universal primer pair followed by a second amplification using a second universal primer pair. By using a universal primer pair followed by PCR using group-specific primer pairs, nested-PCR can detect phytoplasmas present in mixed infections (Lee *et al.*, 1994).

The design of primers based on conserved sequences such as the 16S rRNA gene, has been a major breakthrough in the detection, identification, and classification of phytoplasmas (Schneider *et al.*, 1997; Bertacini and Duduk, 2009). The choice of the primer sets for phytoplasma diagnosis by nested PCR mostly depends on the phytoplasma we are looking for. Nested PCR with a combination of different universal primers can improve the diagnosis of unknown phytoplasma present with low titre in the plant. Generally the 16S rDNA is amplified by universal primer pair P1/P7 followed by nested PCR with R16F2n/R16R2 primer pair which gives a 1.2 kb amplicon (Deng and Hiruki, 1991; Gundersen and Lee, 1996; Nejat *et al.*, 2009).

Primers fU5/ rU3 used in nested PCR were reported to be more reliable to detect phytoplasma from the spear leaves and inflorescence of palms (Nejat and Vadamalai, 2010). The use of two universal primer pairs R16mF2/R16mR1 and R16F2n/R16R2 designed for amplification of phytoplasma 16S rDNA has also been reported which yielded a 1.3 kb amplicon (Gundersen and Lee, 1996). Edwin and Mohankumar (2007) obtained a 650 bp fragment from the palms affected with root (wilt) using the primers P4/P7, while no amplification was seen using the primers P1/P7 and P1/P6. According to them, the Kerala Wilt Disease phytoplasma belong to 16SrIV- C. Manimekalai *et al.* (2010) has reported that the Root (Wilt) Disease phytoplasma belong to a new group-16SrXI group with the use of two primer sets designed-1F7/7R3, 3Fwd/3 Rev and semi nested primer pairs 1F7/7R2, 3Fwd/5Rev from sequencing of a 1.8 kb fragment amplified by primers P1/P7 from a diseased sample. They obtained a 1.3kb amplicon using the semi nested primer pair-3Fwd/3 Rev-3Fwd/5Rev and a 493 bp amplicon for 1F7/7R3-1F7/7R2.

Materials and Methods

3. MATERIALS AND METHODS

The objective of the present study was to conduct physiological, biochemical, anatomical and molecular analysis of coconut affected with yellowing. To achieve this objective, palms with different types of yellowing were selected from Instructional farm, College of Agriculture, Vellayani and the study was conducted in the Department of Plant Physiology College of Agriculture, Vellayani (2009-11). Since elucidation of possible factors contributing towards mid whorl yellowing was a part of the objective, PCR based detection of phytoplasma was also carried out during the programme utilizing the facilities available at the Department of Plant Biotechnology, College of Agriculture, Vellayani.

3.1 MATERIALS

3.1.1 Experimental material

3.1.1.1 Selection of palms

Palms of similar age group showing different patterns of yellowing were selected from B block of Instructional farm, College of Agriculture, Vellayani. The farm is located at 8^0 30' N latitude, 76^0 9 'E longitude at an altitude of 29 m above mean sea level. The soil of the experimental site is red sandy loam belonging to the order oxisol and taxonomic class loamy kaolinite rhodic haplustox (Vellayani Series). The soil was acidic, with pH of 5.2 and an EC of 0.002dSm.

Palms exhibiting the following types of symptoms were selected for the study (Plate I).

1. IWY-Palms with inner whorl of leaves showing yellowing.
2. MWY-Palms with middle whorl of leaves showing yellowing.
3. OWY-Palms with outer whorl of leaves showing yellowing.

IWY

MWY

OWY

GY

Control

Plate 1 Palm showing yellowing in comparison with control

IWY = Inner whorl yellowing MWY = Middle whorl yellowing OWY = Outer whorl yellowing
GY = General yellowing C = Control

4. GY-Palms showing general yellowing.
5. C-Healthy palms.

3.1.1.2 Collection of leaf sample

Leaves were selected from the inner, middle and outer whorls of the selected palms. Leaflets from both the sides of the middle portion of the selected leaves were collected for various type of analysis.

3.2 METHODS

3.2.1 Physiological parameters

3.2.1.1Chlorophyll estimation

Chlorophyll content of leaf samples were estimated as per the procedure described by Arnon (1949). Hundred mg each of leaf sample were taken and were chopped into pieces. 5 ml of DMSO (Dimethyl sulfoxide): Acetone (80%) (1:1) mixture was added to these samples and incubated overnight. The supernatant was collected and absorbance was measured at 645 and 663 nm. The chlorophyll a, chlorophyll b, and total chlorophyll contents were calculated using the formulae given below and expressed in mg g^{-1} of fresh leaf weight.

Total Chlorophyll= {[20.2(OD at 645) + 8.01(OD at 663)] x V}/ (Wx1000)

Chlorophyll a= {[12.7(OD at 663)-2.69(OD at 645)] x V}/Wx1000)

Chlorophyll b= {[22.9(OD at 645)-4.68(OD at 663)] x V}/Wx1000)

Where V= volume of the solution made up

W= fresh weight of leaves

3.2.1.2 Relative Water Content (RWC)

Relative Water Content was estimated as per Barr and Weatherly (1962) by measuring the fresh weight, dry weight and turgid weight of known number of leaf discs of the treatments taken. After measuring the fresh weight of the sample,

it was submerged in distilled water under darkness for three hours and taken the turgid weight. The dry weight of the sample was measured after keeping the samples in oven at 70°C for three days. The relative water content calculated using the following formula.

RWC= [(Fresh weight-Dry weight)/ (Turgid weight-Dry weight)] x100

3.2.1.3 Membrane integrity

Measurement of loss of membrane integrity was estimated as per the procedure described by Leopold *et al.* (1981). Leaves were allowed to gain turgidity by incubating in distilled water for 45 minutes. After noting the turgid weight, leaves were allowed to live wilt under shade. When leaves lost 60% of their fresh weight, leaf punches of 1cm diameter taken. Leaf punches were washed for 1 to 2 minutes to leach out the solutes from the cut ends and blotted on clean filter paper. Ten leaf punches were incubated in a beaker containing 20 ml distilled water for 3 hours. The leakage of the solutes in the bathing medium was estimated by recording its absorbance at 273nm. This is the initial leakage of solutes.

After this, the beakers were incubated in hot water bath (100^0 C) for 15 minutes. After suitable dilution, the absorbance was again read at 273nm to indicate the final absorbance due to the leakage of total solutes contained in the tissue. The percentage leakage of solutes which is a direct reflection of the extent of loss of membrane integrity is calculated as

Percentage leakage= (Initial absorbance of bathing medium/ Final absorbance of bathing medium) x100

3.2.1.4 Total carbohydrate

Total carbohydrate content was estimated by Anthrone method (Hedge and Hofreiter, 1962). Leaf samples of 100 mg each were weighed out from all the selected palms and hydrolysed with 5ml of 2.5 N hydrochloric acid (HCl) in a boiling water bath. The hydrolyzate was neutralised with solid sodium carbonate

until the effervescence ceased. The volume was made up to 100ml and centrifuged at 5000 rpm for 15 minutes. From the supernatant 0.5 ml aliquot was taken and made up to one ml by adding distilled water. To this 4 ml anthrone reagent was added and heated for eight minutes in a boiling water bath. This was cooled rapidly and absorbance was measured at 630 nm in a spectrophotometer. Amount of carbohydrate present was calculated from standard graph prepared using glucose and expressed in terms of milligrams of glucose equivalent per gram of leaf tissue on fresh weight basis.

3.2.1.5 Estimation of reducing sugar

Reducing sugar was estimated as per the procedure described by Sadasivam and Manickam (1996) 100 mg of each of leaf samples were collected and homogenised in 80% ethanol. The supernatant was collected and evaporated. The residue was dissolved in 5 ml distilled water.1 ml aliquot was taken and made upto 3 ml with distilled water.3 ml dinitrosalicylic acid(DNS) reagent was added and boiled the contents in a water bath for 5 minutes. When the contents were still warm, added 1 ml of 40% Rochelle salt solution. This was cooled and absorbance was read at 510 nm. Sugar content was calculated using glucose as the standard.

3.2.1.6 Estimation of starch

Starch was estimated by the method of Sadasivam and Manickam (1996). One gram of leaf was homogenised in 80% ethanol. The residue was dried and to this 5 ml of water and 6.5 ml of 52% perchloric acid was added. The supernatant was extracted at 0°C for 20 minutes. The extraction was repeated using fresh perchloric acid and the supernatants were pooled and made up to 100 ml.0.2 ml aliquot of supernatant was taken and made up to 1 ml with distilled water. . To this 4 ml anthrone reagent was added and heated for eight minutes in a boiling water bath. This was cooled rapidly and the absorbance at 630 nm was read. The starch content was calculated using glucose standard and multiplied with a factor of 0.9 to arrive at the starch content. The starch content was expressed as mg per g fresh weight of the leaf.

3.2.1.7 Total soluble protein

Total soluble protein of leaf was estimated using simple protein dye binding assay of Bradford (1976) using bovine serum albumin as the standard. One hundred milligram of CBB 250 was dissolved in 50 ml of 95% ethanol. To this solution 100 ml of 85% (w/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 200 ml with distilled water.

One gram of leaf material was ground to a thin paste and soluble protein was extracted with 10 ml of phosphate buffer (pH 7.8) containing 1 mM EDTA, 2% (w/w) PVP. The extract was centrifuged in cold (4^0 C) at 10,000 rpm for 10 minutes. To the 50µl of the supernatant 4 ml of Bradford reagent was added and mixed well. The absorbance of the solution was recorded after two minutes and within 30 minutes using spectrophotometer at 595 nm.The protein content was calculated using the BSA standard in the range of (10-100µg). The protein content was expressed as mg/g FW.

3.2.1.8 Phenol

The phenol content was estimated following the procedure described by Bray and Thorpe (1954). One gram each of leaf samples were ground in 10 ml of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes, supernatant was collected and residues were extracted with five times the volume of 80% ethanol and centrifuged. The supernatant was collected, pooled and evaporated to dryness in a boiling water bath. The residue was dissolved in 5 ml distilled water. An aliquot of 0.3ml was pipetted out and made up to 3 ml with distilled water. Folin-Ciocalteau reagent (0.5 ml) was added and 2 ml of 20% sodium carbonate solution was added to each tube after three minutes. This was mixed thoroughly and kept in boiling water for one minute. The reaction mixture was cooled and absorbance was measured at 650nm against reagent blank. Standard curve was prepared using different concentrations of catechol and expressed in catechol equivalents as microgram per gram leaf tissue on fresh weight basis.

3. 2.1.9 Estimation of defence related enzymes

3.2.1.9.1 Estimation of peroxidase (PO)

Peroxidase activity was determined according to the procedure described by Srivastava (1987). Leaf sample of 200 mg was homogenised in 1 ml of 0.1 M sodium pho0sphate buffer (pH 6.5) to which a pinch of polyvinyl pyrrolidone (PVP) was added. The homogenisation was done at 4^0 C using a mortar and pestle. The supernatant was filtered through a muslin cloth and centrifuged at 5000rpm for 15 minutes at 4^0 C. The supernatant was used as the enzyme extract for the assay of PO activity.

The reaction mixture consisting of 1 ml of 0.05 M pyrogallol and 50µl of enzyme extract was taken in both reference and sample cuvettes, mixed and kept. in a spectrophotometer and the reading was adjusted to zero at 420 nm. The enzyme reaction was started by adding 1 ml of one percent hydrogen peroxide (H_2O_2) into sample cuvettes and change in absorbance was measured at 30 seconds interval.

3.2.1.9.2 Estimation of polyphenol oxidase (PPO)

Polyphenol oxidase activity was determined as per the procedure given by Mayer *et al.* (1965).The enzyme extract was prepared as per the procedure given for the estimation of peroxidase.

The reaction mixture consisting of one ml of 0.1 M sodium phosphate buffer (pH 6.5) and 50µl of enzyme extract was taken in both reference and sample cuvettes, mixed and kept in a spectrophotometer and the reading was adjusted to zero at 420 nm. The reaction was initiated after adding one ml of 0.01 M catechol. The observations were recorded in a spectrophotometer. The change in absorbance was recorded at 495nm and PPO activity was expressed as change in the absorbance of the reaction mixture per minute per gram on fresh weight basis.

3.2.2 Nutrient analysis

To understand the nutrient dynamics due to yellowing in coconut both soil and plant samples were analysed for both major and minor nutrients.

3.2.2.1. Soil analysis

Soil samples were collected from four sides of the selected palms (1 m away from the tree trunk at a depth of 30 cm). Samples from each treatment site were pooled, air dried and passed through 2 mm sieve for nutrient analysis.

Table 3 Analytical methods followed in soil analysis

Element	Method	Reference
Available N	Microkjeldhal digestion and distillation	Jackson(1973)
Available P	Bray extraction and photoelectric colorimetry	Jackson(1973)
Available K	Flame Photometry	Pratt(1965)
Exchangeable Ca and Mg	Neutral Normal Ammonium Acetate extraction and titration with EDTA (Versenate titration)	Hesse (1971)
Fe, Mn, Cu, Zn	Extraction using DTPA and read in AAS	Lindsay and Norvell (1978)

3.2.2.2 Plant analysis

Samples from index leaf (14th leaf from the top) was taken for nutrient analysis. Four leaflets from both the sides of the middle portion of index leaf were collected, midrib was removed and was oven dried at 70^0C for three days and analysed for major and minor nutrients as per the following procedures.

Table 4 Analytical methods followed in plant analysis

Element	Method	Reference
N	Microkjeldhal digestion in sulphuric acid and distillation	Jackson(1973)
P	Nitric-perchloric acid(9:3) digestion and colorimetry making use of vanado molybdo phosphoric yellow colour method	Jackson (1973)
K	Nitric-perchloric acid(9:3) digestion and flame photometry	Jackson (1973)
Ca	Nitric-perchloric acid (9:3)digestion and versenate titration	Tandon(1993)
Mg	Nitric-perchloric acid (9:3)digestion and versenate titration with standard EDTA	Tandon(1993)
Fe, Mn, Cu, Zn	Nitric-perchloric acid (9:3) digestion and AAS	Lindsay and Norvell (1978)

3.2.3 Soil moisture content

The soil moisture content was estimated by the gravimetric method. Soil samples were collected 1 m away from the tree trunk at a depth of 30 cm. The samples were pooled. Fresh weight of the samples were taken immediately and

dry weight of samples were taken after keeping the samples in the oven at 104±5°C until constant weight was obtained. The moisture content was estimated by the formula

Soil moisture content = [(Fresh weight-dry weight) x100]/dry weight

3.2.4 Yield

Yield per four harvest of each coconut palm was taken.

3.2.5 Anatomical studies

Anatomical studies were initiated in the Department of Wood Science, College of Forestry, Vellanikkara, Thrissur

Fresh roots were traced out and tip portion of the root of 10cm length were collected from the selected palms.

3.2.5.1 Killing, Fixing and Aspiration

Root bits were fixed in FAA solution for 24 hours aspirated in an aspirator. The remaining procedure of tissue processing was carried out in a Leica tissue processor (Model-Jung Histokinette 2000) (Johansen, 1940).

Composition of FAA solution

Formalin - 10 ml

Glacial acetic acid - 5 ml

Ethyl alcochol - 50 ml

Distilled water - 35 ml

3.2.5.2 Dehydration in alcohol series

The samples were gradually dehydrated in the following alcohol series

a) 30 minutes in 30% alcohol

b) 30 minutes in 50% alcohol

c) 30 minutes in 70% alcohol

d) 60 minutes in 95% alcohol

3.2.5.3 Infiltration of paraffin wax

Paraffin was infiltrated into the dehydrated samples in ethanol paraffin solvent mixture (Tertiary butyl alcohol) in the following proportion.

a) 30 minutes in ethanol+ TBA (3:1)

b) 30 minutes in ethanol+ TBA (1:1)

c) 30 minutes in ethanol+ TBA (1:3)

d) 30 minutes in pure TBA

3.2.5.4 Embedding

Paraffin infiltrated root tips were embedded in a Leica histoembedder

3.2.5.5 Microtomy

The wax embedded root samples were sectioned in a Leica Jung Multicut Rotary Microtome.

3.2.5.6 Dewaxing, staining washing dehydration and mounting

Sections placed on slides with adhesive, were dewaxed using the following solutions in the given order.

1. Xylene
2. Mixture of absolute alcohol and xylene
3. Absolute alcohol
4. Water.

Dewaxed sections were stained in Toluidine Blue (0.5% ,pH<4.4) in a coupling jar, washed, dehydrated and mounted in DPX, left undisturbed for 24 hours and photographed in an image analyser.

3.2.5.7 Hand sections

Hand sections of leaves and roots were done and viewed under microscope (10X) (Labomed) and photographed.

3.2.6 Molecular analysis

3.2.6.1 Sample collection

Spindle leaves from all the selected palm were collected for molecular analysis.

3.2.6.2 Standardisation of extraction of DNA

3.2.6.2.1. Isolation of genomic DNA

Coconut contains exceptionally high amounts of polysaccharides, polyphenols, tannins which interferes with the DNA isolation procedure (Angeles et al., 2005). The problems encountered in the isolation and purification of DNA include degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols which directly or indirectly interfere with the enzymatic reactions. Moreover the contaminating RNA that precipitates along with the DNA causes many problems including suppression of PCR amplification, improper priming of DNA templates during thermal cycling sequence. So suitable modification was adopted to obtain a pure quality DNA.

Genomic DNA was isolated from fresh leaves using modified CTAB method (Porebski et al., 1997).

1 g of tender leaf sample was taken in a clean autoclaved mortar and frozen samples in liquid nitrogen were powdered in liquid nitrogen along with 0.1 g of PVP.

↓

The pulverized tissues were quickly transferred to 2 ml of freshly prepared pre-warmed ($65^{O}C$) extraction buffer and shaken vigorously by inversion to form slurry.

↓

The tubes were incubated at $65^{0}C$ in water bath for 60 minutes with intermittent shaking and swirling for every 30 min.

↓

The mixture was then centrifuged at 10,000rpm at $4^{0}C$ for 10 minutes and to the supernatant **20 µg/ ml RNase** was added and incubated at $37^{0}C$ for half an hour.

↓

To the aqueous phase an equal volume of **phenol: chloroform: isoamyl alcohol** (25:24:1) was added and centrifuged at 10,000rpm for 10 minutes at room temperature.

↓

An equal volume of chloroform isoamyl alcohol (24:1) was added to the supernatant.

↓

The aqueous phase was collected and equal volume of chloroform isoamyl alcohol (24:1) was added and centrifuged at 10,000rpm for 10 minutes at room temperature.

↓

The supernatant was collected and one tenth volume of sodium acetate and two third volume of ice cold isopropanol was added and kept overnight at -20^{0}C for precipitation.

The solution was centrifuged at 12000rpm for 10 minutes and the supernatant was discarded without dislodging the pellet.

The precipitate was then washed twice using 70% ethanol and dried.

After drying the precipitate was dissolved in 100 µl 0.1XTAE buffer (Tris buffer 10mM, EDTA 1mM) and stored at – 20°C

Composition of reagants used for DNA extraction

1. CTAB buffer

Tris HCl	-100mM
NaCl	-1.4mM
EDTA	-20mM
CTAB	-3%,
PVP	-2%,
β-mercaptoethanol	-1%

2. TAE buffer (50x, 100 ml)

Tris buffer	- 24.2 g
Glacial acetic acid	- 5.71 ml

EDTA - 1.861g

pH - 8.0

3. TE buffer

Tris buffer -0.01 M

EDTA - 0.001 M

pH - 8.0

3.2.6.3 Spectrophotometric analysis

Spectrophotometric analysis of the extracted DNA was made for determining the quality and quantity of DNA. Spectrophotometer (Spectronic Genesis 5) with deuterium lamp as a UV source was used for measuring the absorbance measurements. Spectrophotometer was calibrated for sterile distilled water which was used as a blank further. Optical density of 5 µl of DNA dissolved in 3ml of sterile distilled water was measured at 260nm and 280 nm.

3.2.6.4 Quantification of DNA

An optical density value of 1.0 at 260 nm indicates the presence of 50 µg of double stranded DNA in an ml of solution. So the quantity of DNA present in the extracted sample was estimated by employing the formula

Amount of DNA (µg/g) = A260 x 50 x dilution factor

(where A260 is absorbance value at 260nm)

3.2.6.5 Purity of DNA

The quality of DNA was judged from ratio of absorbance values at 260nm and 280nm. A ratio of 1.8-2.0 indicates best quality of DNA.

3.2.6.6 Agarose gel electrophoresis

The genomic DNA was confirmed by horizontal gel electrophoresis unit (Genei). Gel was run at 5 V/cm. Electrophoresis of genomic DNA was done at 0.8% agarose made of 1x TAE buffer. The DNA samples were mixed with required volumes of gel loading buffer comprising 0.25% w/v of bromophenol blue :30% glycerol: 70% sterile water. Each well was loaded with 12µl of sample and run till the loading dye moved about ¾ th of the gel. After electrophoresis, gel was visualised using gel documentation unit (BIORAD) and the data was recorded using the Quantity One Software.

3.2.6.7 PCR analysis

3.2.6.7.1 Nested PCR

Nested polymerase chain reaction is a modification of polymerase chain reaction intended to reduce the contamination in products due to the amplification of unexpected primer binding sites. It is performed by preliminary amplification using a universal primer pair followed by a second amplification using a second universal primer pair which amplifies a secondary target within the first run product which is shorter than the first one. The advantage of nested PCR is that if the wrong PCR fragment was amplified, the probability is quite low that the region would be amplified a second time by the second set of primers (Fig 1).

3.2.6.7.2 PCR conditions

PCR was done in 0.2 ml PCR tubes in a 25µl reaction mixture

DNA (25ng/µl)	-5µl
dNTPs (2.5mM)	-2µl

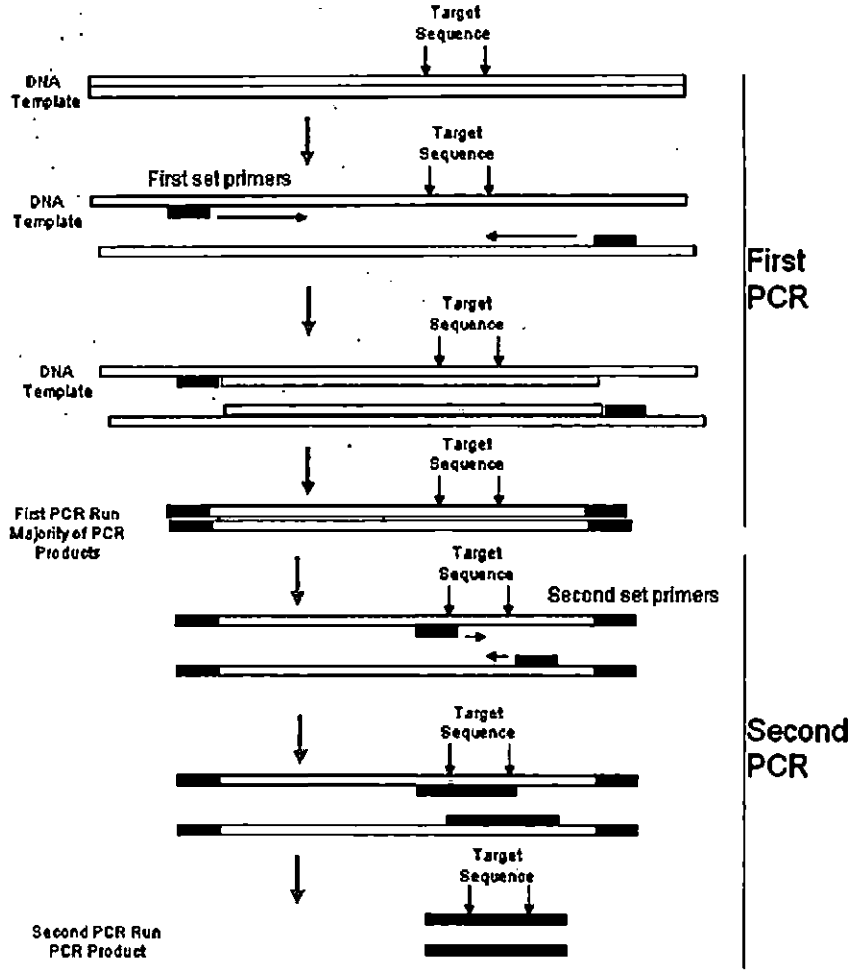


Fig 1: Nested PCR diagram

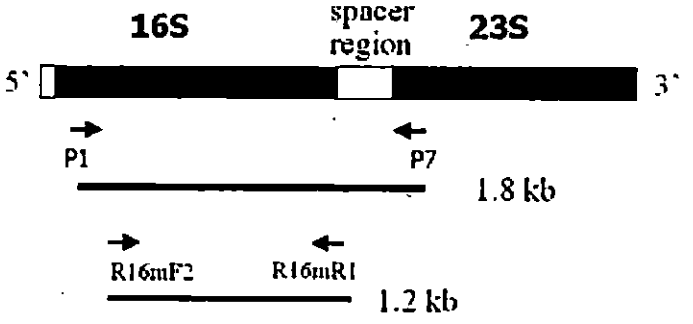


Fig 2: Diagrammatic representation of phytoplasma rRNA operon including the16S and 23 S rRNA genes and the intergeneric spacer region

Forward primer(10pmol) -1.00µl

Reverse primer(10pmol) -1.00µl

10x assay buffer -2.5µl

Taq polymerase3units/µl) -0.33µl

Sterile distilled water -13.17µl

PCR amplification was carried out using three sets of primers designed from Sigma (Table 5). -

1. P1/ P7 and nested primers R16F2n/ R16R2; (fig 2).
2. R16mF2 /R16mR1 and nested primer primers R16F2n/ R16R2
3. 1F7/7R3 and semi nested primer pair 1F7/7R2 (root (wilt) specific pimers)

All PCR assays were performed in a thermal cycler (Eppendorf Gradient Master Cycler). An aliquot of 10µl of PCR product was analysed by horizontal electrophoresis unit through 1.4% agarose with Ethidium Bromide (0.5µg/ml) using TAE buffer (Tris buffer, Glacial acetic acid and water) as the running buffer.

Table 5 Primer sequence and PCR conditions adapted for nested PCR

Sl no.	Primer pair	Design	Primer sequence	Location	Annealing condition	Reference
1	Nested PCR I [a] I[st] round PCR	P1 P7	5[1]-AGAGTTTGATCCTGGCTCAGGATT-3[1] 5[1]-GTCCTTCATCGGCTCTT-3[1]	6-30 68-51	48[0] C for 1 minutes	Deng and Hiruki, 1991 Schneider et al,1997
2	II[nd] round PCR	R16F2n R16R2	5[1]-GAAACGACTGCTAAGACTGG-3[1] 5[1]-GACGGGCGGTGTGTACAAACCCCG-3[1]	149-168 1397-1373	60[0] C for 2 minutes	Alhudaib et al,2007
4.	Nested PCR II [b] I[st] round PCR	R16mF2 R16mR1	5[1]-CATGCAAGTCGAACG-3[1] 5[1]-CTTAACCCCAATCATCGA-3[1]		55[0] C for 2 minutes	Gundersen and Lee,1996
5	II[nd] round PCR	R16F2N R16R2	5[1]-GAAACGACTGCTAAGACTGG-3[1] 5[1]-GACGGGCGGTGTGTACAAACCCCG-3[1]	149-168 1397-1373	60[0] C for 2 minutes	Alhudaib et al,2007
6.	Nested PCR III[c] I[st] round PCR	3Fwd 3Rev	5[1]-ACCTGCCTTTAAGACGAGGA-3[1] 5[1]-AAAGGAGGTGATCCATCCCCACCT-3[1]		63[0] C for 2 minutes	Manimekalai et al., 2010
7.	II[nd] round PCR	3Fwd/5Rev	5[1]-ACCCCGAGAACGTATTCACCGCGA-3[1]		63[0] C for 2 minutes	Manimekalai et al., 2010

Other temperature conditions followed for nested PCR

a) 95^0 C for 3 minutes and followed by 30 cycles of 95^0 C for 1 minute appropriate annealing condition and 72^0 C for 1 minute with final extension at 72^0 C for 10 minute

b) 95^0 C for 10 minutes and followed by 35 cycles of 95^0 C for 1 minute appropriate annealing condition and 72^0 C for 1 minute with final extension at 72^0 C for 10 minute

c) 95^0 C for 10 minutes and followed by 35 cycles of 95^0 C for 1 minute appropriate annealing condition and 72^0 C for 1 minute with final extension at 72^0 C for 10 minute

Results

4. RESULTS

Kerala ranks first in area and production of coconut in India (NHB, 2010). But the productivity is below the national average and that of neighbouring Tamil Nadu. Kerala faces serious problems in coconut cultivation. The debilitating root (wilt) disease is the most serious disease affecting coconut which is widely prevalent in Kerala since 1882. Recently there is rampant spreading of another malady-the yellowing of coconut characterized by mid whorl yellowing, shedding of immature nuts and drying of inflorescence that is prevalent in many parts of Kerala.

In the present study efforts were made to generate basic information on the changes of physiological, anatomical and molecular realms of coconut palm affected by mid whorl yellowing. There is no research report available in this emerging problem in the coconut growing tracts of Kerala and this is the first attempt made to disclose the predisposing factors and causative agents leading to the mid whorl yellowing of coconut palm. The results obtained are presented in this chapter.

4.1 PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS

The various physiological and biochemical parameters analysed include different types of pigments (total chlorophyll content, chlorophyll a, chlorophyll b and carotenoid content), relative water content, membrane integrity, total carbohydrates, total soluble protein, total phenol content, anti oxidants, soil and plant nutrients. Significant variations were observed in all these parameters between different palms and across the whorls of the palm.

4.1.1 Total chlorophyll content

The total chlorophyll content showed significant variation between different palms (Fig 3). A reduction in the chlorophyll content was observed in the palms with yellowing. The control palm showed highest total chlorophyll content (1.83 mg/g of fresh weight) compared to other palms. The palms with outer whorl

yellowing had more chlorophyll content than the palms with middle whorl and inner whorl yellowing, which were on par. The palms with general yellowing showed least total chlorophyll content (0.62 mg/g of fresh weight).

On comparing the middle whorls of all the selected palms, highest total chlorophyll content was recorded for the control palm (1.208 mg/g of fresh weight). The middle whorl of the palm with outer whorl yellowing had more chlorophyll content than other palms. The middle whorl of the palms with general yellowing and middle whorl (0.52 mg/g of fresh weight each) affected palms showed least chlorophyll content(Table 6).

When all the whorls of the selected palms were considered, the inner whorl of the control palm showed maximum total chlorophyll content (2.18 mg/g of fresh weight) and the least in the outer whorl of palms with outer whorl yellowing(0.34 mg/g of fresh weight).

4.1.2 Chlorophyll a

Data on chlorophyll a content showed significant variation. The highest chlorophyll a content was recorded for the control palm (1.21 mg/g of fresh weight). The next best was palm with outer whorl yellowing. The palms with inner whorl and middle whorl yellowing showed similar chlorophyll a content but were significantly different from palms with general yellowing which showed the least value (0.44 mg/g of fresh weight) (Table 7).

Among the middle whorls of all the selected palms, the control palm showed highest chlorophyll a (1.27 mg/g of fresh weight) content followed by palms with outer whorl and inner whorl yellowing. The middle whorl of palms with middle (0.39 mg/g of fresh weight) and general yellowing (0.35 mg/g of fresh weight) showed the least chlorophyll a content.

The maximum chlorophyll a content was recorded for the inner whorl of the control palm (1.36 mg/g of fresh weight) and the least by the outer whorl of outer whorl yellowing affected palm (0.24 mg/g of fresh weight).

Table 6: Total Chlorophyll content (mg/g FW) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	1.151	0.875	1.159	1.062
MWY	1.978	0.517	0.704	1.066
OWY	1.987	1.547	0.340	1.291
GY	0.490	0.517	0.842	0.616
C	2.180	1.898	1.401	1.826
Mean	1.557	1.071	0.889	
CD(Palm)	0.181			
CD(Whorl)	0.126			
CD(PxW)	0.281			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 7 : Chlorophyll a content (mg/g FW) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	0.837	0.629	0.792	0.752
MWY	1.310	0.350	0.490	0.716
OWY	1.323	1.088	0.236	0.882
GY	0.341	0.388	0.591	0.440
C	1.356	1.270	0.998	1.208
Mean	1.033	0.745	0.621	
CD(Palm)	0.105			
CD(Whorl)	0.073			
CD(PxW)	0.162			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

4.1.3 Chlorophyll b

There was a significant variation in chlorophyll b content between healthy and yellowing affected palms (Table 8). The control palm showed highest chlorophyll b content (0.62 mg/g of fresh weight. There was no significant variation between palms with outer and middle whorl yellowing. The palm with general yellowing showed the least chlorophyll b content (0.18 mg/g of fresh weight).

The middle whorl of the control palm (1.27mg/g of fresh weight) had more chlorophyll b content than other palms followed by palms with outer, inner and middle whorl yellowing. The least chlorophyll b content was shown by the palms with general yellowing (0.13 mg/g of fresh weight).

The maximum chlorophyll b content was exhibited by the inner whorl of the control palm (0.83 mg/g of fresh weight) and the least by the outer whorl of outer whorl yellowing affected palm (0.10 mg/g of fresh weight).

4.1.4 Carotenoid content

The carotenoid content varied between the selected palms. The control palm showed highest carotenoid content (0.67 mg/g of fresh weight). The palms with outer, middle and inner whorl yellowing were on par but significantly differed from the palms with general yellowing (0.35 mg/g of fresh weight) (Table 9).

When the middle whorls of the selected palms were considered, the carotenoid content was highest in the control palm (0.63 mg/g of fresh weight). The middle whorl of the palms affected with general yellowing showed least carotenoid content (0.28 mg/g of fresh weight).

When all the whorls were considered, the maximum carotenoid content was shown by the inner whorl of the control palm (0.72 mg/g of fresh weight) and the least shown by the outer whorl of the palms with outer whorl yellowing (0.29 mg/g of fresh weight).

Table 8 : Chlorophyll b content (mg/g FW) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	0.315	0.247	0.368	0.310
MWY	0.668	0.167	0.215	0.350
OWY	0.665	0.459	0.104	0.409
GY	0.149	0.129	0.251	0.176
C	0.824	0.629	0.403	0.619
Mean	0.524	0.326	0.268	
CD(Palm)	0.089			
CD(Whorl)	0.060			
CD(PxW)	0.135			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 9 : Carotenoid content (mg/g FW) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	0.413	0.433	0.512	0.453
MWY	0.656	0.389	0.430	0.492
OWY	0.626	0.611	0.291	0.509
GY	0.330	0.283	0.424	0.346
C	0.717	0.713	0.576	0.669
Mean	0.549	0.486	0.447	
CD(Palm)	0.061			
CD(Whorl)	0.041			
CD(PxW)	0.093			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

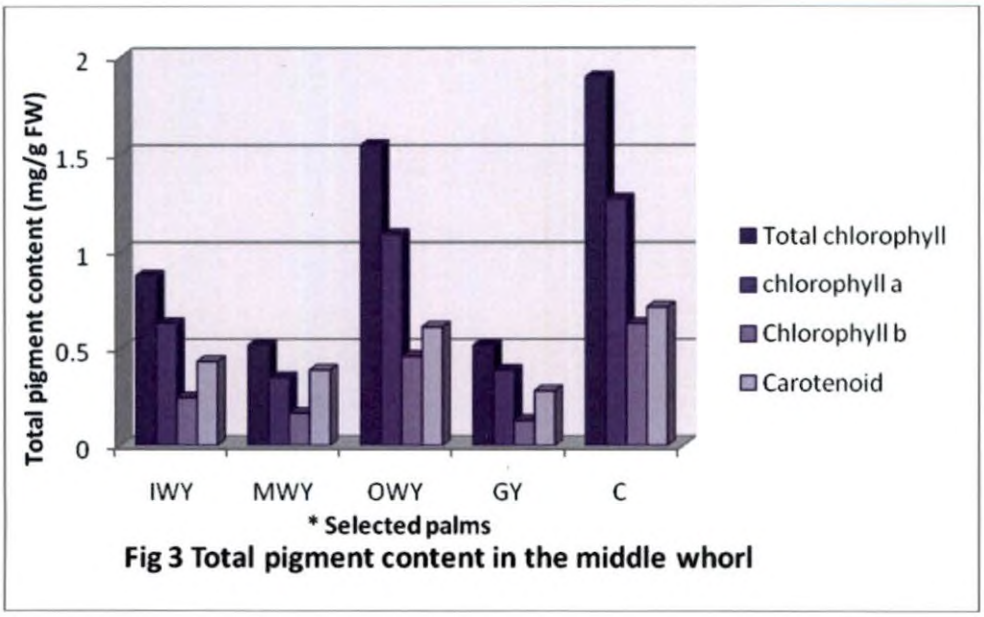


Fig 3 Total pigment content in the middle whorl

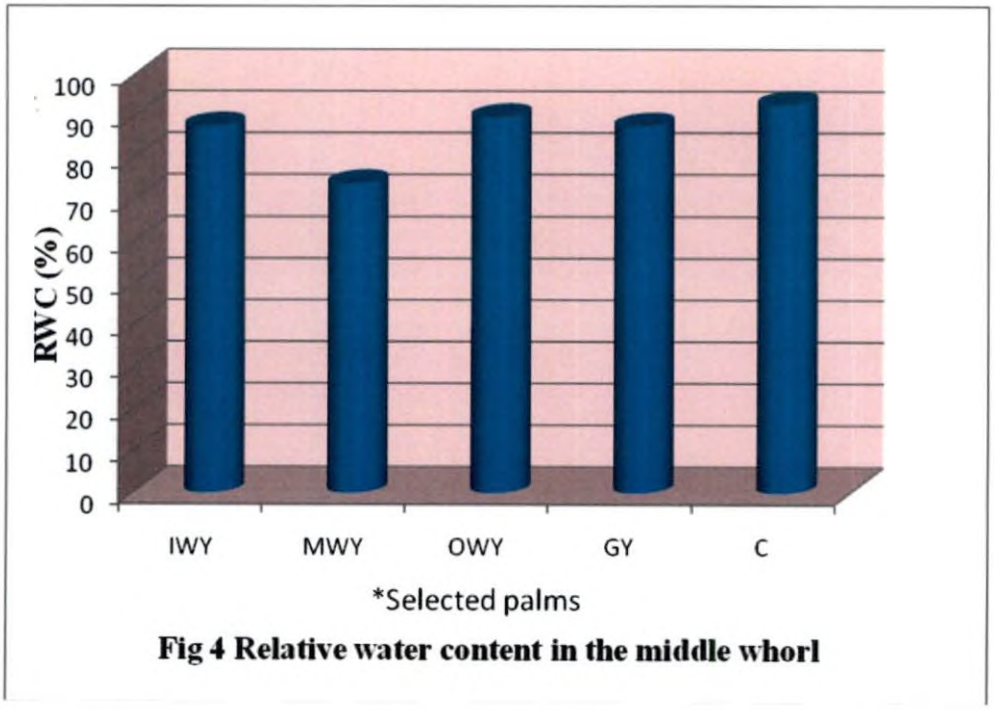


Fig 4 Relative water content in the middle whorl

* IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

4.1.5 Relative water content (RWC)

The relative water content showed significant variation between the palms and the whorls of the palm (fig 4). The control palm showed the highest RWC (93.02%). Palms with inner whorl and outer whorl yellowing were on par but was significantly different from palms with general (84.62%) and middle whorl yellowing (82.22%) (Table 10).

When the RWC of middle whorl across all palms were considered, it showed that the RWC in the middle whorl of the control (92.93%), inner (89.89%), outer (87.74%) and middle whorl (87.67%) yellowing were on par but significantly different from the palms with general yellowing (73.95%).

Among all the whorls of the selected palms, the highest RWC was recorded for the inner whorl (94.08%) of the control palm and the lowest for the middle whorl (73.95%) of the middle whorl yellowing affected palm.

4.1.6 Membrane integrity

The effect of yellowing on the loss of membrane integrity is shown in Table 11. There was no significant variation in loss of membrane integrity in palms with inner and outer whorl yellowing. The control palm (4.17) and palm with general yellowing (4.93) showed maximum membrane integrity.

No significant variation was observed in the loss of membrane integrity in the middle whorls of the outer, middle and inner whorl yellowing affected palms. The control palm (2.93) and palm with general yellowing (5.38) showed maximum membrane integrity (fig 5).

4.1.7 Total carbohydrate

The total carbohydrate content showed significant variation between the selected palms (Table 12). The palm with outer whorl yellowing showed maximum carbohydrate content (67.84 mg/g of fresh weight) followed by palms with middle whorl yellowing. The control palm had a carbohydrate content of

Table 10 : Relative water content (%) of coconut leaves affected with yellowing

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	91.66	87.74	89.73	89.71
MWY	94.64	73.95	78.08	82.22
OWY	93.56	89.89	79.04	87.50
GY	78.51	87.68	87.68	84.62
C	94.08	92.93	92.04	93.02
Mean	90.49	86.44	85.31	
CD(Palm)	2.656			
CD(Whorl)	3.181			
CD(PxW)	7.111			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 11 : Loss of membrane integrity(%) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	5.74	5.64	18.25	9.87
MWY	3.42	7.43	12.16	7.67
OWY	3.65	8.34	21.51	11.17
GY	2.74	5.38	6.66	4.93
C	1.47	2.92	8.10	4.17
Mean	3.41	5.94	13.33	
CD(Palm)	2.21			
CD(Whorl)	1.26			
CD(PxW)	2.82			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

51.29 mg/g of fresh weight which significantly differed from palms with general yellowing. The palms with inner whorl yellowing showed least carbohydrate content (37.09 mg/g of fresh weight).

Among the middle whorls of all selected palms, the palms with outer whorl yellowing (82.43 mg/g of fresh weight) and the control palm (77.6 mg/g of fresh weight) showed maximum carbohydrate content which significantly differed from the palms with middle whorl yellowing (fig 6). The total carbohydrate content of middle whorl of palms with middle whorl yellowing was more than the palms with general yellowing. The middle whorl of palms with inner whorl yellowing showed least carbohydrate content (31.55 mg/g of fresh weight).

The maximum total carbohydrate content was recorded in the outer whorl of the palms with outer whorl yellowing (83.44 mg/g of fresh weight).

4.1.8 Reducing sugar

Data on reducing sugar content showed significant variation among the selected palms. The palms with general yellowing showed maximum reducing sugar content (28.9 mg/g of fresh weight). The reducing sugar content of palms with inner and middle whorl yellowing were on par but significantly differed from with outer whorl yellowing (Table 13). The control palm exhibited least reducing sugar content (17.11 mg/g of fresh weight).

When the middle whorls of the selected palms were considered, the palms with middle whorl yellowing showed maximum reducing sugar content (43.79 mg/g of fresh weight)which is the maximum reducing sugar content when all the whorls were considered, followed by palms with general and outer whorl yellowing. The reducing sugar content of palms with outer and inner whorl yellowing was on par. The control palm exhibited least reducing sugar content (10.1 mg/g of fresh weight).

Table 12 : Carbohydrate content (mg/g FW) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	39.33	37.46	41.68	39.49
MWY	35.02	62.88	63.67	53.86
OWY	37.66	82.43	83.44	67.84
GY	39.37	52.69	49.85	47.30
C	36.57	77.6	39.70	51.29
Mean	37.59	62.61	55.67	
CD(Palm)	2.4			
CD(Whorl)	2.65			
CD(PxW)	5.92			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 13 : Reducing sugar(mg/g FW) content of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	22.15	28.01	23.39	24.52
MWY	12.37	43.79	17.07	24.41
OWY	13.32	24.21	26.82	21.45
GY	25.17	28.68	32.86	28.90
C	18.38	12.68	20.28	17.11
Mean	18.28	27.47	24.08	
CD(Palm)	1.78			
CD(Whorl)	1.16			
CD(PxW)	2.58			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

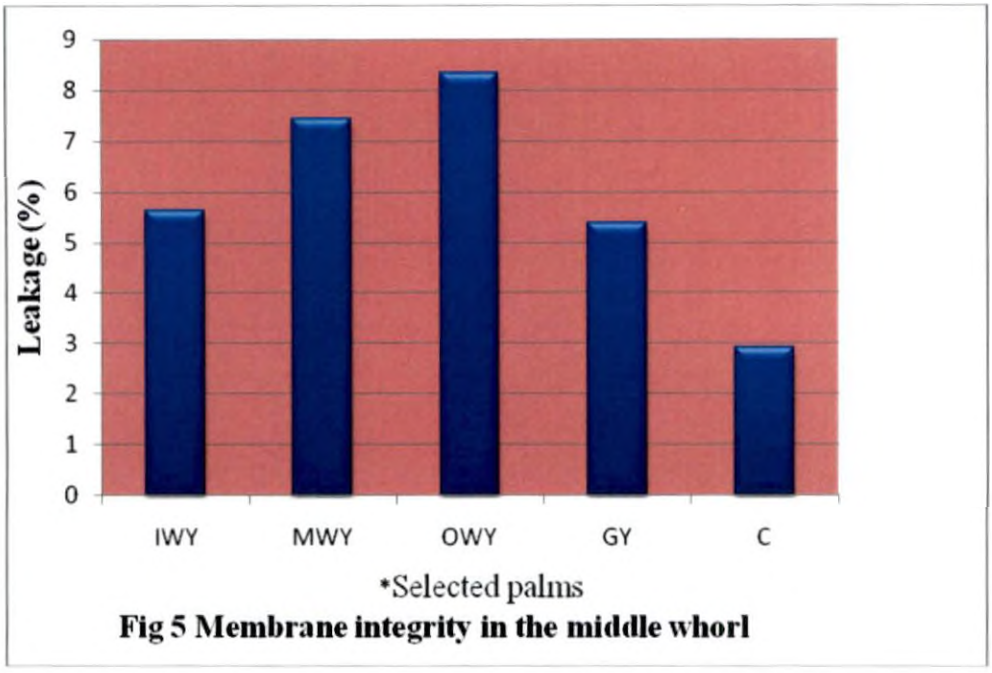


Fig 5 Membrane integrity in the middle whorl

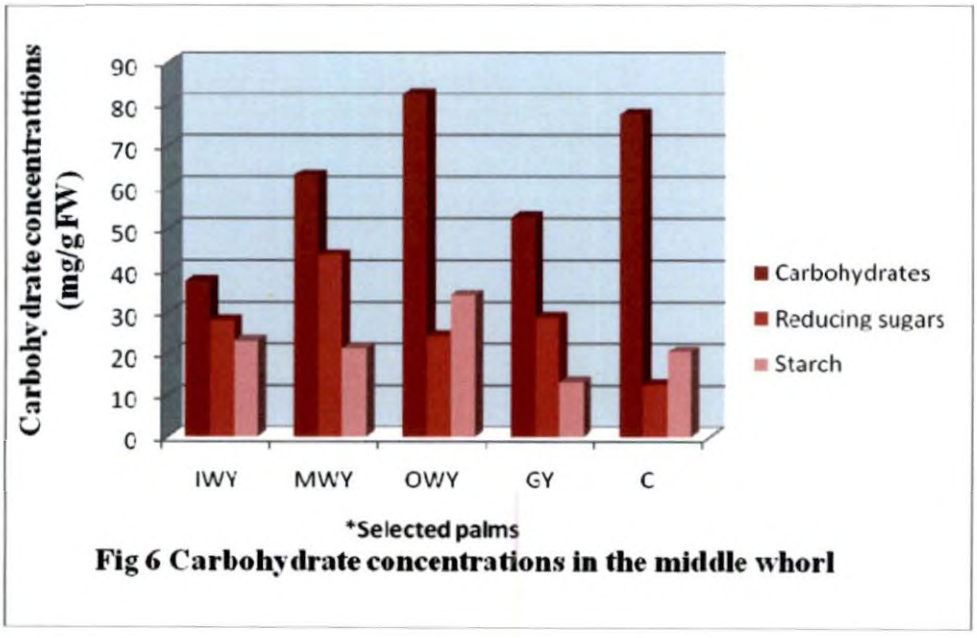


Fig 6 Carbohydrate concentrations in the middle whorl

* IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Across the whorls, the least reducing sugar content was shown by the inner whorl (12.37 mg/g of fresh weight) of middle whorl yellowing affected palm and the middle whorl (12.68 mg/g of fresh weight) of the control palm.

4.1.9 Starch content

A significant variation was observed in the starch content of all the selected palms. The palms with inner whorl yellowing showed maximum starch content (24.27 mg/g of fresh weight) which significantly differed from the palms with outer whorl and general yellowing. The palms with middle whorl yellowing showed next best starch content. The control palm showed least amount of starch content (18.46 mg/g of fresh weight).

When the starch content of middle whorl of all the selected palms was considered, the palms with outer whorl yellowing (34.11mg/g of fresh weight) showed maximum starch content followed by palms with inner and middle whorl yellowing. The control palm (20.5 mg/g of fresh weight) showed similar starch content with that of middle whorl yellowing palm. The palm with general yellowing showed lowest starch content (13.25 mg/g of fresh weight), which is least among all the whorls considered (Table 14).

4.1.10 Total soluble protein

No significant variation was observed in the total soluble protein content in the control palms (2.98 mg/g of fresh weight) and palms with outer whorl yellowing (2.86 mg/g of fresh weight) (Table 15) but was significantly different from the palms affected with inner whorl (2.57 mg/g of fresh weight) and general yellowing (2.51mg/g of fresh weight) which showed least total soluble protein content.

While the middle whorls of all the selected palms, where considered, the control palm showed maximum total soluble protein content (3.22 mg/g of fresh weight) followed by palms with middle and inner whorl yellowing. The palms

Table 14 : Starch content (mg/g FW) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	24.80	23.10	24.89	24.27
MWY	15.22	21.46	25.43	20.70
OWY	15.35	34.11	22.80	24.09
GY	24.52	13.25	31.95	23.24
C	18.73	20.5	16.15	18.46
Mean	19.72	22.49	24.25	
CD(Palm)	0.90			
CD(Whorl)	0.57			
CD(PxW)	1.28			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 15 : Total soluble protein content(mg/g FW) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	3.27	2.06	2.39	2.57
MWY	2.30	2.90	2.39	2.76
OWY	2.75	3.10	2.75	2.86
GY	2.48	2.04	3.02	2.51
C	2.86	3.22	2.87	2.98
Mean	2.87	2.66	2.68	
CD(Palm)	0.22			
CD(Whorl)	4.86			
CD(PxW)	0.11			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

with inner whorl and general yellowing showed similar total soluble protein content (fig7).

4.1.11 Total phenol content

The phenol content showed significant difference between the palms and across the whorls of the palm (Table 16).The palms with general yellowing expressed maximum phenol content (27.47 mg/g fresh weight). A significant reduction in the phenol content was shown by the palms affected with middle whorl yellowing followed by inner whorl yellowing, and outer whorl yellowing The control palm showed the least phenol content (9.11 mg/g fresh weight).

Similar trend was seen in the middle whorls, inner whorls and outer whorls of the selected palms. Across the outer whorls of all the selected palm showed maximum phenol content (fig 8).

4.1.12 Peroxidase activity

Data on peroxidase activity of coconut leaves affected with yellowing is presented in Table 17. The palms with middle whorl yellowing showed maximum peroxidase activity (15.59 per minute per gram on fresh weight basis).The peroxidase content of control and palms with outer whorl yellowing were on par. The palms with outer whorl yellowing showed more peroxidase activity than the palms with inner whorl yellowing. The palm with general yellowing showed least peroxidase activity (6.82 per minute per gram on fresh weight basis).

Among the middle whorls of the selected palms, the middle whorl showed maximum peroxidase activity (10.25 per minute per gram on fresh weight basis) followed by palms with inner whorl, general yellowing, control palm and outer whorl yellowing. The least peroxidase activity was shown by the middle whorl (3.74 per minute per gram on fresh weight basis) of palms with middle whorl yellowing (fig 9).

When all the whorls of the selected palms were considered, the inner whorls (15.93 per minute per gram on fresh weight basis) of the palms with outer

Table 16 : Phenol content (mg/g FW)of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	15.23	14.36	16.50	15.63
MWY	19.37	21.50	21.81	20.89
OWY	11.30	10.50	14.38	12.06
GY	25.90	26.57	29.93	27.47
C	8.52	7.60	11.20	9.11
Mean	16.06	16.11	18.77	
CD(Palm)	0.858			
CD(Whorl)	0.352			
CD(PxW)	0.788			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 17: Peroxidase activity(activity/g/min) in coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	6.05	7.23	11.8	8.36
MWY	10.36	10.25	26.17	15.59
OWY	15.93	3.74	6.13	8.6
GY	9.16	6.63	5.25	7.01
C	9.55	5.46	11.21	8.74
Mean	10.21	6.66	12.11	
CD(Palm)	0.19			
CD(Whorl)	0.14			
CD(PxW)	0.31			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

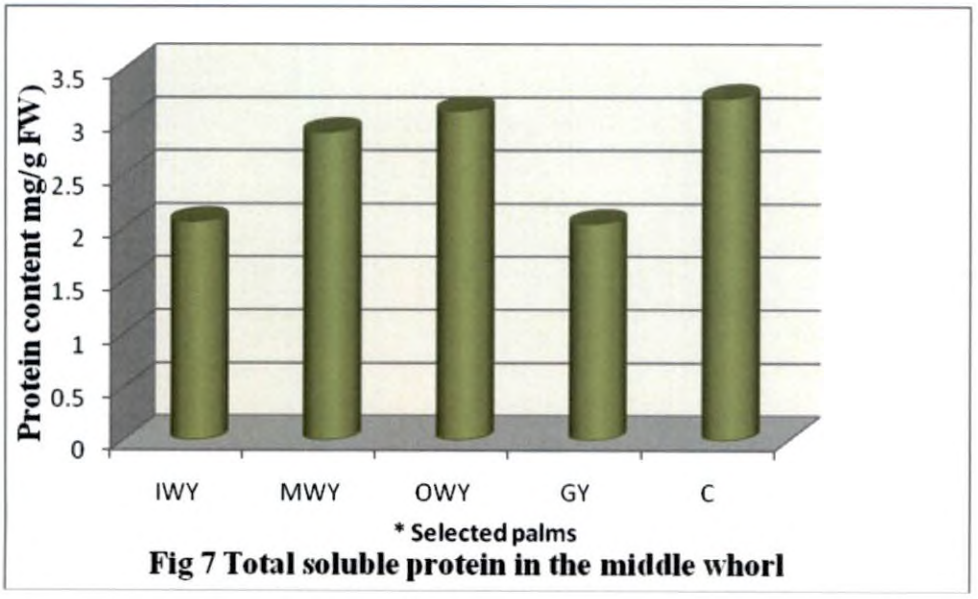


Fig 7 Total soluble protein in the middle whorl

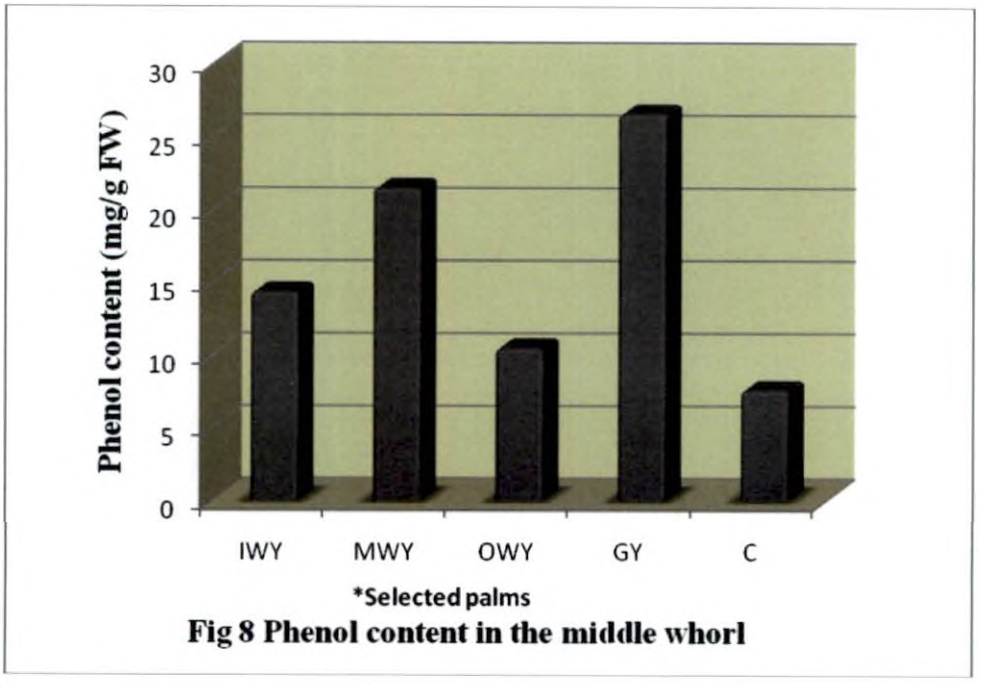


Fig 8 Phenol content in the middle whorl

* IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

whorl yellowing and the outer whorl (26.17 per minute per gram on fresh weight basis) of the palms with middle whorl yellowing showed maximum peroxidase content and the least value was exhibited by the middle whorl (3.74 per minute per gram on fresh weight basis) of the middle whorl affected palm.

4.1.13 Polyphenol oxidase activity

The polyphenol oxidase activity showed significant variation among the selected palms (Table 18). The palms with inner whorl yellowing showed maximum polyphenol oxidase activity (4.72 per minute per gram on fresh weight basis) followed by palms with middle whorl, general and outer whorl yellowing. The control palm exhibited least polyphenol oxidase activity (2.04 per minute per gram on fresh weight basis).

Considering the middle whorls of the selected palms, the palms with inner whorl yellowing showed maximum polyphenol oxidase activity (5.83 per minute per gram on fresh weight basis). The polyphenol oxidase activity of palms with general, middle whorl and outer whorl yellowing were on par. The control palm exhibited least polyphenol oxidase activity (1.29 per minute per gram on fresh weight basis).

4.1.14 Nutrient analysis

4.1.14.1 Plant nutrient analysis

There was a significant variation in the nitrogen content in all the selected palms. The control palm showed maximum nitrogen content (2.06%) followed by palms with outer and inner whorl yellowing. There was no significant variation in the nitrogen content in the palms with inner whorl middle whorl and general yellowing (Table 19).

Among the middle whorl of all the selected palms, the control palm showed maximum nitrogen content (2.35%). The nitrogen content in the middle whorl of the palm with outer and middle whorl yellowing were on par but

Table 18 : Polyphenol oxidase activity (activity/g/min) in coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	5.85	5.83	2.48	4.72
MWY	3.63	3.25	1.80	2.89
OWY	2.72	3.20	2.03	2.65
GY	3.12	3.26	1.81	2.73
C	2.76	1.29	2.14	2.07
Mean	3.61	3.37	2.05	
CD(Palm)	2.98			
CD(Whorl)	3.12			
CD(PxW)	6.97			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 19 : Nitrogen content (%) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	1.87	1.38	1.62	1.62
MWY	1.43	1.71	1.43	1.53
OWY	2.29	1.73	1.34	1.78
GY	1.72	1.39	1.38	1.5
C	1.68	2.35	2.15	2.06
Mean	1.8	1.71	1.58	
CD(Palm)	0.11			
CD(Whorl)	0.05			
CD(PxW)	0.12			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

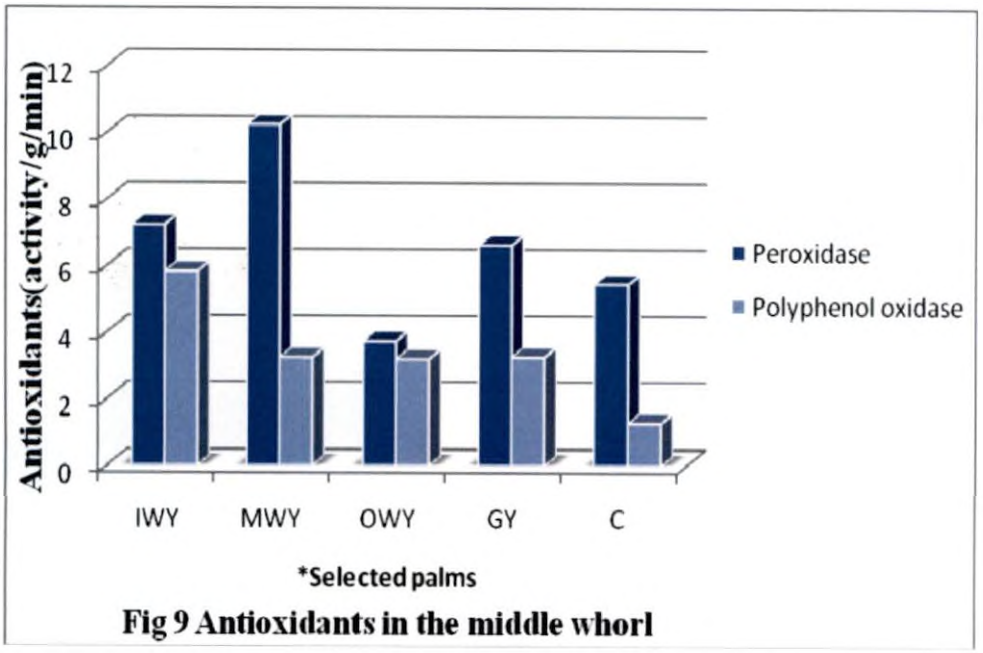


Fig 9 Antioxidants in the middle whorl

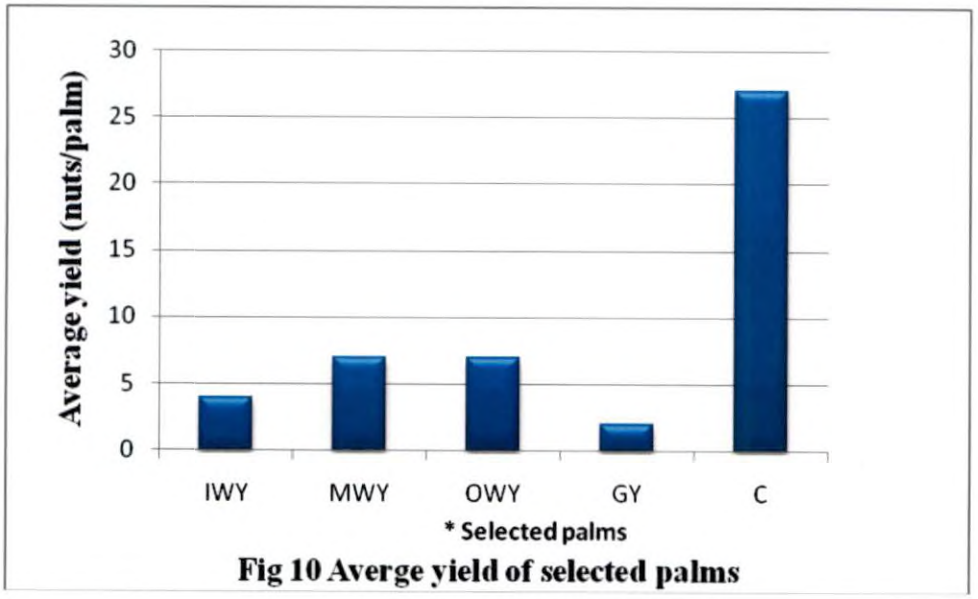


Fig 10 Averge yield of selected palms

* IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

significantly different from palm with general yellowing. The palms with inner whorl yellowing showed least nitrogen content (1.38%).

Data on the phosphorus content of palms affected with yellowing is presented in Table 20. The palms with inner whorl yellowing showed maximum phosphorus content (0.23%) followed by control palm then palms with outer whorl and middle whorl yellowing. The palms with general yellowing showed least amount of phosphorus (0.14%).

The middle whorl of the control palms and palms with inner whorl yellowing showed maximum phosphorus content. The middle whorl of the palm with outer whorl yellowing showed significantly higher phosphorus content than palms with middle whorl yellowing. The palms with general yellowing showed least phosphorus content (0.13%).

The potassium content in the control palm and palms affected with yellowing differed significantly (Table 21). The palms with inner whorl yellowing had maximum potassium content (1.62%) followed by palms with general and outer whorl yellowing. The control palm showed next best potassium content. The least potassium content is shown by palms with middle whorl yellowing.

The potassium content in the middle whorl of the palms with inner whorl and general yellowing were on par. The potassium content in the palm with general yellowing showed more potassium content than palms with mid whorl and outer whorl yellowing. The control palm showed least potassium content (0.93%).

Data on the calcium content showed significant variation between control and yellowing affected palms (Table 22). The palms with inner whorl yellowing showed maximum calcium content followed by palms with general and outer whorl yellowing. The control palm showed a calcium content of 172.78 ppm. The palms with middle whorl yellowing showed least calcium content (167.11 ppm).

Table 20 : Phosphorus content (%) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	0.26	0.28	0.14	0.23
MWY	0.21	0.14	0.12	0.16
OWY	0.20	0.22	0.14	0.18
GY	0.15	0.13	0.12	0.14
C	0.198	0.28	0.14	0.206
Mean	0.20	0.21	0.13	
CD(Palm)	0.008			
CD(Whorl)	0.005			
CD(PxW)	0.01			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 21 :Potassium content (%)of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	2.4	1.74	0.75	1.62
MWY	1.54	1.25	0.25	.01
OWY	1.44	1.16	0.74	1.12
GY	1.64	1.74	1.24	1.54
C	1.35	0.97	0.93	1.08
Mean	1.67	1.37	0.78	
CD(Palm)	2.72			
CD(Whorl)	1.92			
CD(PxW)	4.29			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

When the middle whorl of all the selected palms were considered, the palms with inner whorl yellowing showed maximum calcium content(238 ppm) followed by palms with general, middle whorl, and outer whorl yellowing.The control palm exhibited least calcium content (169 ppm).

The magnesium content of control palm and palms affected with yellowing differed significantly (Table 23). The control palm showed maximum magnesium content (64.67 ppm). The magnesium content of palms with middle and inner whorl yellowing was on par. There was no significant variation in the magnesium content of palms with general and outer whorl yellowing.

The magnesium content in the middle whorl of the control palm was maximum (64.67 ppm) followed by palms with outer, inner and general yellowing. The palms with middle whorl yellowing exhibited least amount of magnesium (13.33ppm).

Data on the iron content of coconut leaves affected with yellowing has been depicted in Table 24. The palms with general yellowing showed maximum iron content (5.01 ppm) followed by palms with middle whorl yellowing. The control palm showed more iron content than palms with outer whorl yellowing. The least iron content was exhibited by palms with inner whorl yellowing (1.86 ppm).

Among the middle whorls of all the selected palms, maximum iron content was shown by palms with middle whorl yellowing(6.64 ppm) followed by palms with general yellowing. The control palm exhibited an iron content of 2.85 ppm. The palms with outer whorl yellowing showed least amount of iron content (0.87 ppm).

Across the whorls, the iron content of the inner whorl of the palms with general yellowing (7.76 ppm) and the middle whorl (6.64 ppm) of the middle whorl yellowing palm was maximum. The least being shown by the middle whorl (0.95 ppm) of the outer whorl yellowing affected palm.

Table 22 : Calcium content (ppm) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	26800	23800	13250	21283
MWY	22400	19433	8300	16711
OWY	22383	19083	13367	18278
GY	19223	22100	21300	20874
C	22000	16900	12933	17278
Mean	22561	20263	13830	
CD(Palm)	155			
CD(Whorl)	101			
CD(PxW)	226			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 23: Magnesium content (ppm) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	2267	2567	2500	2444
MWY	2367	1333	3900	2533
OWY	2033	2800	1100	1978
GY	2167	1600	2400	2056
C	1600	6467	1300	3122
Mean	2087	2953	2240	
CD(Palm)	142			
CD(Whorl)	60			
CD(PxW)	1.34			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

The manganese content differed significantly between the control and yellowing affected palms (Table 25). The control palm showed more manganese content (1.34 ppm). The manganese content of palms with inner whorl, outer whorl and general yellowing were similar. The least manganese content was exhibited by palms with middle whorl yellowing (0.61 ppm).

While in the case of middle whorls of selected palms the maximum manganese content was shown by palms with outer whorl yellowing, followed by control palm and then palms with inner whorl, general and middle whorl yellowing.

The inner whorl (1.32ppm) of the general yellowing affected palm showed a slightly greater value of manganese content compared to the inner whorl of other selected palms.

The observation on the copper content of palms affected with yellowing is illustrated in Table 26. The palms with general yellowing showed maximum copper content (0.21 ppm) followed by palms with inner whorl middle whorl yellowing, control palm and palms with outer whorl yellowing.

When the middle whorls of all the selected palms were considered, the palms with inner and middle whorl yellowing showed similar copper content which significantly differed from the rest of the palms.

The maximum copper content was recorded for the inner whorl (0.35 ppm) of the general yellowing affected palm.

The result of the estimation of zinc content in coconut palms affected with yellowing is presented in table 27.There was no significant variation in the zinc content between the control palm and palm affected with outer whorl yellowing, but significantly differed from the rest of the palms. The greatest reduction in the zinc content was shown by the palms affected with inner whorl yellowing (0.19 ppm).

Table 24 : Iron content (ppm) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	238	154	165	186
MWY	142	664	215	340
OWY	225	95	371	231
GY	776	324	404	501
C	337	226	294	285
Mean	343	293	290	
CD(Palm)	4			
CD(Whorl)	3			
CD(PxW)	7			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 25 : Manganese content (ppm) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	59	88	176	108
MWY	35	43	104	61
OWY	52	148	124	108
GY	132	70	68	90
C	74	144	84	134
Mean	71	99	31	
CD(Palm)	0.8			
CD(Whorl)	0.6			
CD(PxW)	1			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 26 : Copper content (ppm) of coconut leaves

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	11	14	10	12
MWY	8	14	5	9
OWY	9	7	6	7
GY	35	12	15	21
C	12	8	4	8
Mean	15	11	8	
CD(Palm)	0.2			
CD(Whorl)	0.4			
CD(PxW)	0.7			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 27 : Zinc content (ppm) of coconut leaves affected with yellowing

Palms	Inner whorl	Middle whorl	Outer whorl	Mean
IWY	16	0.19	0.22	0.19
MWY	22	0.30	0.20	0.24
OWY	19	0.18	0.38	0.25
GY	27	0.17	0.17	0.21
C	21	0.31	0.31	0.28
Mean	21	0.23	0.26	
CD(Palm)	0.9			
CD(Whorl)	0.8			
CD(PxW)	1			

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Considering the middle whorls of the selected palms, the control palm (0.31 ppm) and palms affected with middle whorl yellowing (0.304 ppm) showed similar zinc content. It was followed by the palm with inner whorl yellowing (0.135 ppm) which was on par with the palms with outer whorl yellowing. The least zinc content was exhibited by palms with general yellowing (0.17 ppm).

The 14th leaf starting from the first fully opened one is the most widely used leaf for nutrient analysis as recommended by the IRHO. (Fremond et al., 1966). The 14th leaf data from the control palms and palms with different types of yellowing were analysed. The data showed that there were significant reductions in the levels of major nutrient nitrogen, phosphorus and also in the levels of magnesium and the micronutrient manganese in case of palms showing mid whorl yellowing compared to the control palm. But significantly higher levels of accumulation were found in case of potassium, calcium, iron and copper. These variations in the nutrient levels can have an influence on symptom development in coconut palms.

4.1.14.2 Soil nutrient analysis

Though the soils collected from the root zones of palms exhibiting varying types of yellowing showed significant variations in the nutrient levels, they were not following any particular pattern indicative of any particular roles in development of yellowing.(Table 28 and 29).

4.2 Soil moisture content

The soil moisture content was estimated by gravimetric method and the result obtained is expressed in the table30. The soils of palms affected with outer whorl yellowing showed a maximum moisture content of 21.27% followed by palms affected with inner whorl (18.82%), control (18.4%), and general yellowing (17.59%). The least amount of soil moisture was shown by the palms affected with middle whorl yellowing (12.16%).

Table 28 : Major nutrients (kg/ha) in the soil

Palms	Nitrogen	Phosphorus	Potassium	Calcium	Magnesium
IWY	385.73	15.37	182.56	229.63	24.75
MWY	213.25	44.56	58.24	205.01	25.00
OWY	316.74	52.30	62.72	209.83	23.75
GY	285.38	41.87	91.28	206.45	21.5
C	316.74	44.80	105.84	205.63	15.5
Mean	303.56	39.78	100.13	211.31	22.1
CD(Palm)	39.95	10.43	14.07	33.82	3.84

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 29 : Micronutrients (ppm) in the soil

Palms	Iron	Manganese	Copper	Zinc
IWY	6.72	9.42	0.43	1.24
MWY	6.43	8.07	0.24	1.27
OWY	7.29	9.43	0.35	2.65
GY	8.33	9.85	0.54	1.93
C	8.69	7.19	0.26	0.75
Mean	7.49	8.79	0.36	1.57
CD(Palm)	0.183	0.16	0.028	0.08

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 30 : Soil moisture (%) content

Palms	Moisture content
IWY	18.82
MWY	12.16
OWY	21.27
GY	17.59
C	18.40
Mean	17.65
CD(palm)	0.723

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

Table 31: Average yield (per harvest)

Palms	Average yield (nuts)
IWY	4
MWY	7
OWY	7
GY	2
C	27
Mean	9
CD(palm)	2.04

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

4.3 Average yield

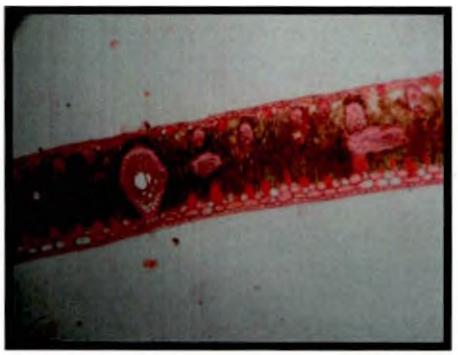
The average yield per four harvest was estimated and presented in table 31. The control palm showed a maximum yield of 27 nuts per harvest followed by pals affected with middle whorl and outer whorl yellowing (7 nuts each per harvest), then comes the palms affected with inner whorl yellowing (4 nuts per harvest). The greatest reduction in yield was observed for palms affected with general yellowing (2 nuts per harvest).

4.4 Anatomical analyses

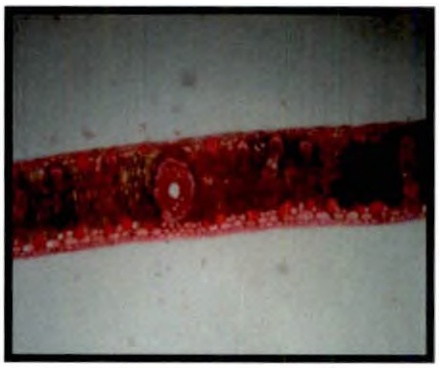
The middle leaflets of the middle whorls of all the selected palms were hand sectioned and stained in safranin. In all the yellowing affected palms, chlorophyll degradation was seen. Structural integrity of the yellowing affected leaves was lower than that of control palm (Plate 2).

Microtome sections of roots could not be taken properly as the fresh roots got shrunk when tissue processing was done and the internal structures were difficult to be viewed. However the fully matured roots of palms showing inner whorl yellowing palms provided good microtome sections. Since the idea was to view the anatomical changes in the fresh roots, hand sections of fresh roots were taken.

The morphological and anatomical analyses of fresh roots showed maintenance of healthy roots by control palms (Plate 3). The palms with different types of yellowing had roots exhibiting browning and tissue damage along with healthy roots (Plate 4). The anatomical analyses showed that there was internal browning of vascular elements extending into the cortex (Plate 5). Disintegration of vascular elements was also seen in palms with middle whorl and general yellowing.



IWY



MWY

OWY

GY

Control

Plate 2 Cross sections of leaves of selected palms

IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

IWY

MWY

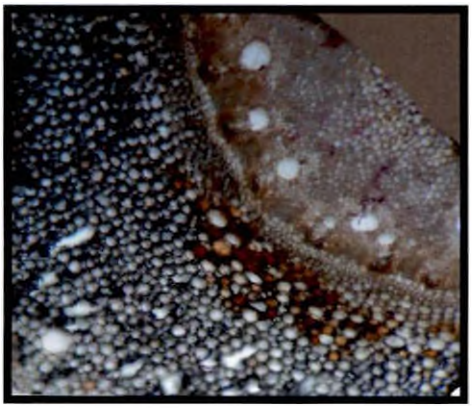
OWY

GY

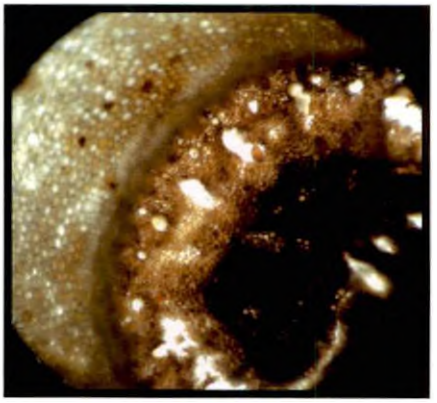
Control

Plate 3 Roots of palms with yellowing in comparison with control

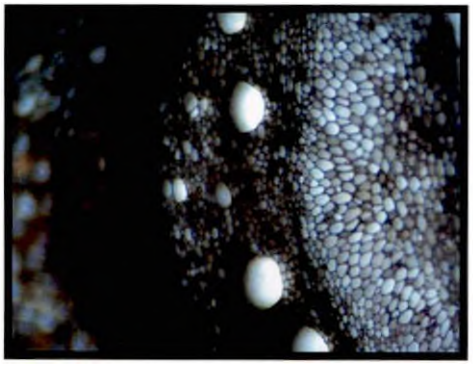
IWY = Inner whorl yellowing MWY = Middle whorl yellowing OWY = Outer whorl yellowing
GY = General yellowing C = Control



IWY



MWY



OWY

GY

Control

Plate 4 Cross sections of roots of palms with yellowing in comparison with control

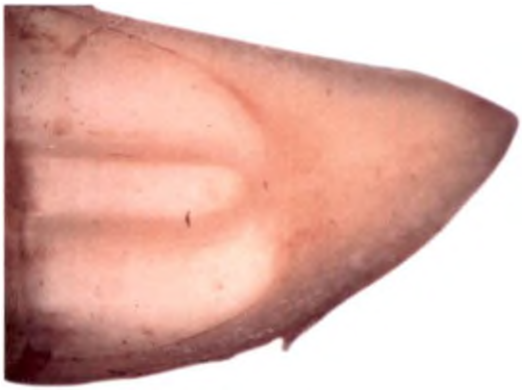
IWY- Inner whorl yellowing, MWY-Middle whorl yellowing, OWY- outer whorl yellowing, GY- general yellowing, C- Control

IWY

MWY

OWY

GY



Control

Plate 5 Transverse sections of roots of palms with yellowing comparison with control

IWY = Inner whorl yellowing MWY = Middle whorl yellowing OWY = Outer whorl yellowing
GY = General yellowing C = Control

4.4 Molecular analyses

4.4.1 DNA Isolation

Coconut contains higher amount of polysaccharides and polyphenols which interfere with the process of DNA isolation. So the total genomic DNA extraction was standardised for obtaining a better quality DNA. The DNA extraction was improved by bringing out modifications in the original CTAB DNA isolation protocol (Doyle and Doyle, 1990) and DNA isolation protocol by Porebski et al., 1997. The DNA isolation by original CTAB protocol provided DNA of purity between 1.2-1.4 and a concentration of 1.2µg/ µl.

The genomic DNA was isolated from the phytoplasma affected Vinca plants (*Catharanthus roseus)* which were maintained as a positive control and from the spindle leaves of selected coconut palms. The leaves selected for DNA isolation were fresh and young. The modifications adopted were- addition of PVP along with grinding of the tissues to remove impurities, incubation with RNase for the removal of RNA, and phenol: chloroform: isoamyl alcohol purification step to remove the proteins. The DNA degradation and precipitation were avoided to some extent by carrying out all the steps at room temperature. An agarose gel (0.8%) was run to confirm the DNA isolation. This modified CTAB method provided DNA of quality 1.7-1.8 and a concentration of 5.4 µg/ µl.

4.4.2 Nested PCR amplification

The presence of phytoplasma in the selected samples were checked by nested PCR analysis using phytoplasma specific universal primers (P1/P7-R16F2n/ R16R2 and R16mF2 /R16mR1 -R16F2n/ R16R2 and root (wilt) phytoplasma specific primers (IF7/7R3and semi nested primer pair IF7/7R2) designed from the sequencing of a 1.8 kb fragment amplified by primers P1/P7.

Nested PCR with primers P1/P7 -R16F2n/ R16R2 yielded an amplicon of 1.2 kb fragment in the positive control (*Catharanthus roseus*) and the palms showing inner whorl and middle whorl yellowing (Plate 6). A 1.3 kb fragment

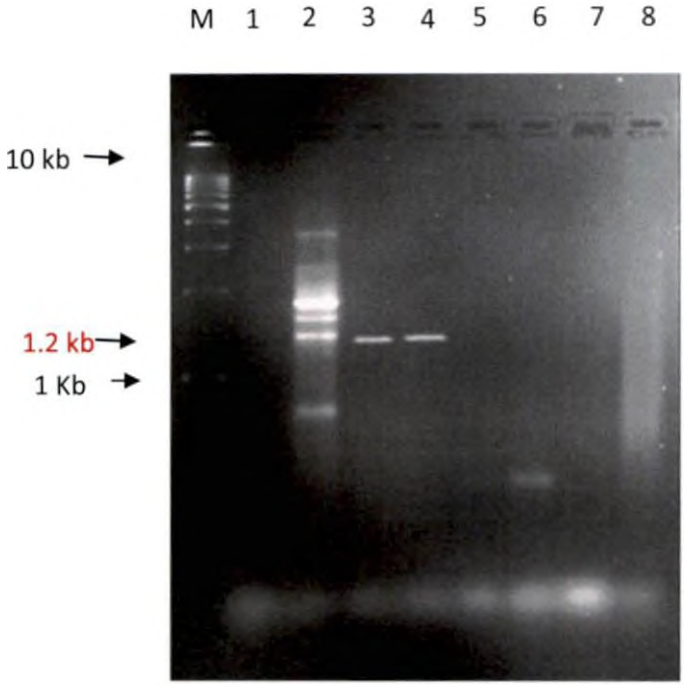


Plate 6 .PCR amplification with primers P1/P7/ R16F2n/ R16R2

M: DNA ladder, lane 1: negative control lane 2: positive control

Lane 3: IWY, lane 4: MWY Lane 5: OWY, Lane 6: GY

Lane 7: C1, Lane 8: C2

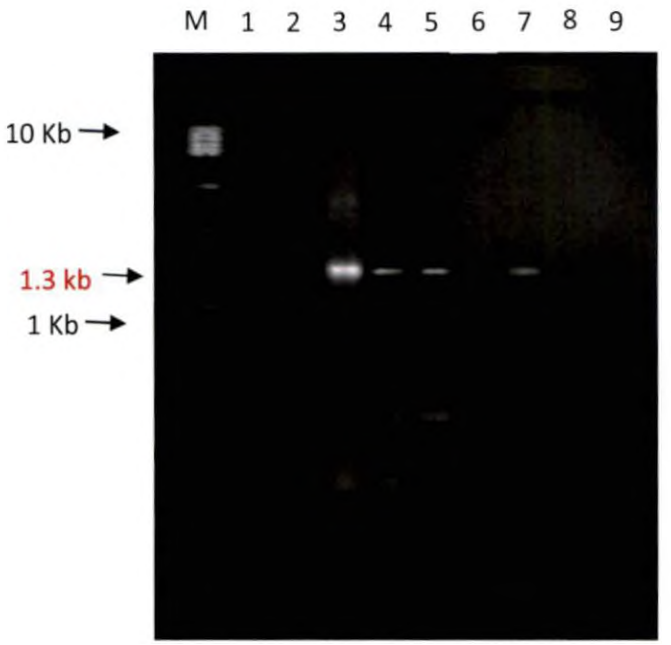


Plate 7 : PCR amplification with primers R16MF2/R16MR1- R16F2N/R16R2

M: DNA ladder, lane 1: negative control, lane2: water control

lane 3: positive control, lane 4: IWY, lane 5: MWY, lane 6: OWY,

lane 7: GY, lane 8:C1, Lane 9: C2

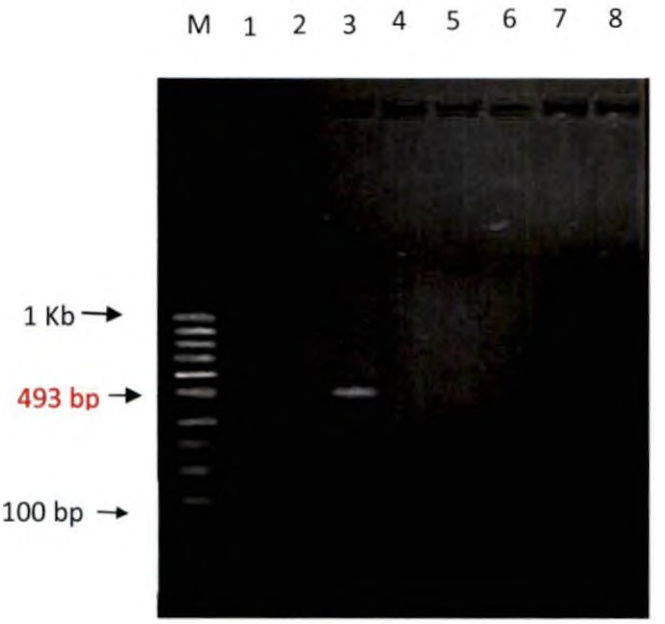


Plate 8 : PCR amplification with semi-nested primer pairs IF7/7R3- IF7/7R2

M: DNA ladder, lane 1: negative control, lane2: water control

lane 3: positive control, lane 4: IWY, lane 5: MWY, lane 6: OWY,

lane 7: GY, lane 8:C1, Lane 9: C2

was obtained for the positive control and for tissues from the palms with inner, middle and general yellowing with the primers R16mF2 /R16mR1 -R16F2n/ R16R2 (Plate 7).

With the root (wilt) phytoplasma specific semi-nested primer pairs IF7/7R3-IF7/7R2 a 493 bp fragment (Plate 8) was obtained only for the positive control and no amplification was seen for any of the selected palms.

The control palms and palms with outer whorl yellowing did not show any amplification for any of the primers

Discussion

5. DISCUSSION

The coconut (*Cocos nucifera* L.) palm refered to as the 'Tree of Life' due to its multifaceted uses is a source of livelihood to several millions of people in 93 countries particularly in the humid tropics in the Asian and Pacific regions, where the crop has several cultural and socio-economic significance. Kerala known as the land of coconut occupies first rank in area and production in India (NHB, 2010), but the productivity is below the national average. This is because coconut is prone to several maladies of which the root (wilt) is the most serious disease which is widely prevalent in Kerala since 1882. Recently there is rampant spreading of another type of yellowing of coconut characterized by mid whorl yellowing, shedding of immature nuts, drying of inflorescence etc in many parts of Kerala.

There is no research report available on this emerging problem. Hence an experiment was proposed to generate the basic information on the changes of physiological, molecular and anatomical realms of coconut affected by yellowing. This is the first attempt to disclose the predisposing factors and causative agents leading to mid whorl yellowing of coconut palm. The results of various experiments conducted to address the above objectives are discussed with sufficient supports from previous studies.

5.1 Physiological parameters

A wide variation has been observed in the physiological parameters like total pigments, relative water content, carbohydrate fractions, proteins, phenols and antioxidants between yellowing affected and control palms.

Plant productivity is a unique process that depends greatly on the amount of chlorophyll present in the chloroplast. The green colour giving pigment chlorophyll plays an important role in the physiology, productivity and economy of the green plants. Leaf chlorophyll content is a good indicator of photosynthesis

activity, mutations, stress condition and nutritional status of plants (Ghasemi *et al.*, 2011).

Mineral nutrients like nitrogen and magnesium are constituents of chlorophyll and micronutrient iron is closely associated with chlorophyll biosynthesis. The deficiency of these elements will result in reduced chlorophyll b and chlorosis of leaves (Ohler, 1999b and Broschat, 1992). A reduction in the chlorophyll content in coconut affected with root (wilt) has been reported by Koshy (1999). He has reported a 16 and 70% increase in the chlorophyll content in the first fully opened leaves and middle leaves of healthy palms over that in diseased palms. Leon *et al.* (1996) has reported a similar reduction in the chlorophyll and carotenoid contents in coconut palms affected by lethal yellowing. According to his study a 60% reduction in the chlorophyll and carotenoid content was observed in palms affected with lethal yellowing. In maize plants infected by Maize bushy stunt phytoplasma, a significant reduction in chlorophyll content has been reported (Junqueira *et al.*, 2004). The carotenoid content in phytoplasma-infected leaves of aster yellows was diminished (Choi *et al.*, 2004).

In the present study, the yellowing affected palms have shown a significant reduction in the total chlorophyll, chlorophyll a, chlorophyll b and carotenoid content when compared to the healthy palm. Across the middle whorls, a 19% reduction in the total chlorophyll content was observed in palms with outer whorl yellowing followed by, 54% reduction in the inner whorl yellowing and, 72% reduction in palms with middle whorl and general yellowing over the control palm. Similarly a 14%, 39%, 45%, and 60% reduction in the carotenoid content was observed in palms with outer whorl, inner whorl, middle whorl and general yellowing respectively. Also the nitrogen and magnesium content in the middle whorls of yellowing affected palms were significantly lower than that of the control palm. This suggests a possible role for these nutrients also in the reduction of the chlorophyll content.

Leaf water status influences several leaf physiological variables, such as leaf turgor, growth, stomatal conductance, transpiration, photosynthesis and

respiration. RWC is a useful indicator of the state of water balance of a plant (Yamasaki and Dilenberg, 1999).

Rajagopal *et al.*, (1986) found abnormal stomatal opening in the palms affected with root (wilt) with impaired regulation which lead to excessive water loss. He has reported that the root (wilt) affected palms had consistently lower water potential than the healthy palms. A significant reduction in the water potential under severe yellowing has been reported in the palms affected with lethal yellowing (Leon *et al.*, 1996).

In this experiment it was found that across the middle whorls, the entire yellowing affected palms have recorded lower water content than the control palm. A significant reduction (20%) was shown by the palms affected with middle whorl yellowing compared to the control palm. The soil moisture content in palms affected with middle whorl yellowing was very low (12.16%). This can also be a reason for the low relative water content in the middle whorl yellowing palms. The inner whorl of the general yellowing affected palm showed a significant reduction in the relative water content compared to other yellowing affected palms and control palms.

The plasma membrane is the selectively permeable lipid bilayer that surrounds living cells. As one of the first points of contact for environmental signals upon the cell, the plasma membrane plays an important role in stress responses. This is of particular relevance in plants, which cannot move or take shelter from potentially damaging environmental conditions. So the maintenance of membrane integrity is very important to thrive under stress conditions (Eckardt, 2008).

Significant variations were observed in the loss of membrane integrity between palms affected with yellowing and control palms. The maximum membrane integrity was shown by the control palm, but it did not significantly vary with palms affected with general yellowing. The other yellowing affected palms showed a significant reduction in the membrane integrity.

A wide variation was observed in the total carbohydrate fractions between the yellowing affected palms and the control palms. The carbohydrate content was maximum for palms with outer whorl yellowing and control palm. The other yellowing affected palms showed a lower carbohydrate content.

Maust *et al.*, (2003) reported that in coconut palms affected with lethal yellowing leaf carbohydrate concentration increased in infected leaves. Sugar and starch concentrations increased slowly in recently expanded leaves with the development of the disease before decreasing in later stages of lethal yellowing. Sugar and starch concentrations increased more rapidly in intermediate leaves with the advance of the disease before decreasing in later stages.

A reduction in the total carbohydrate content and starch content has been reported in palms affected with root (wilt) (Mathew, 1977). Several reports on increased carbohydrate fractions in infected plants have been reported in other plants by several workers (Catlin *et al.*, 1975). Guthrie *et al.* (2001), reported higher carbohydrate content in diseased leaf tissue in papaya infected with phytoplasma. Higher reducing sugar content has been reported in corn plants infected by the maize bushy stunt phytoplasma (Junqueira *et al.*, 2004). In accordance with these reports in the present study also, the control palm exhibited least amount of reducing sugars compared to other palms with yellowing. A 48% increase in outer whorl yellowing 55% increase in both inner whorl and general yellowing affected palm and 64% increase in the middle whorl yellowing affected palm in the reducing sugar content has been recorded. A similar increase in the reducing sugar has been reported in palms affected with root (wilt) (Mathew, 1977) and in palms affected with lethal yellowing (Maust *et al.*, 2003).

All the yellowing affected palms except the general yellowing showed an increased starch content in the middle whorls.

The lower carbohydrate in leaves can be due to retarded rate of net photosynthesis and the higher accumulation of reducing sugar and starch indicate a possible block in the translocation of photosynthates. Lakmini *et al.* (2006) have

reported increased levels of sucrose or reducing sugars in coconut palms under moisture stress. The soil moisture content in palms affected with middle whorl yellowing was very low (12.16%) and there was 20 % reduction in RWC also. This can also be a reason for the significant increase in the reducing sugar content in the coconut palm.

Usually plants infected by pathogens show a high protein content which could be due to both the activation of the host defense mechanisms and the pathogen attack mechanisms (Agrios, 1997). An increase in the protein content of leaves was reported in healthy palms compared to coconut affected with root wilt (Padmaja *et al.*, 1981).

In the present experiment, the control palm recorded maximum protein content compared to other yellowing affected palms. Across the middle whorls, a 36% reduction in the protein content in the palms affected with inner whorl yellowing, and a 37% reduction in the palms affected with general yellowing compared to the control palms have been observed. This was in accordance with the palms affected with root (wilt) where 19.9% increase in alkali extractable protein in the middle whorls and also with palms affected with lethal yellowing, where 45 % reduction in the leaf protein content was recorded in affected palms. This might be due to the decelerated protein synthesis and accelerated protein break down or decrease in the polypeptide Rubisco, a key enzyme in the photosynthetic reduction cycle which constitutes 50% or more of the total soluble protein.

Several contradictory results have been reported in plants affected with different mollicutes a decrease in total soluble protein in tomato plants affected by STOL. in grape wine affected by bois noir and in apple trees affected by apple proliferation (Bertamini *et al.*, 2002).

Phenolics have various functions in plants. Rivero, 2001 and Michalak, 2006 reported that an enhancement of phenylopropanoid metabolism and the amount of phenolic compounds can be observed under different environmental

factors and stress conditions. It has been observed that certain common phenolic substances are toxic to pathogens and accumulate in plants after infection, especially in resistant varieties (Agrios, 1997). Bertaccini and Duduk (2009) has reported an increase in phenolic compounds in host plants due to infection by phytoplasma. An accelerated phenol metabolism has been reported in roots of root (wilt) affected coconut palms (Joseph and Jayasankar 1979)

In the present study a significant increase in the phenolic compounds was observed in palms with yellowing compared to the control palms. Across the middle whorls palms with general yellowing showed maximum phenol content followed by palms with middle whorl, inner whorl and outer whorl yellowing. 88.9% increase was observed in the phenol content of palms with inner yellowing. A 3 times increase was shown by the palms with middle whorl yellowing.

Peroxidase (PO) and Polyphenol oxidase(PPO) has been extensively studied and implicated in plant resistance to various diseases. Khatun *et al.*, (2009) has reported an increased activity of peroxidase and polyphenol oxidase in plants in response to pathogen infection. Recent studies have indicated that phenol oxidizing enzymes may participate in the response to various abiotic stresses including drought (Zivkovic *et al.*, 2010). PPO is a copper-containing protein widely distributed in the plant kingdom that catalyzes the oxygen-dependent oxidation of monophenols or *o*- diphenols to *o*-quinones. The *o*-quinones are highly reactive substances that can react with amino acids, peptides and proteins, thus altering the structural and functional properties of the cell. This enzyme has been implicated to the function in tissue browning. PO is a widely distributed plant enzyme with various physiological functions in plant cells, including auxin metabolism and defense against numerous abiotic stresses.

In the present study, across the middle whorls, all palms with yellowing except the one with outer whorl yellowing, recorded a significantly higher peroxidase activity than the control palm, while in the case of polyphenol oxidase, the control palm showed the least activity. An increased peroxidase and

polyphenol oxidase activity has been reported in the roots of palms affected with root (wilt) (Joseph and Jayasankar, 1979).

Coconut is a perennial crop and is unique among the plantation crops in that it flowers and fruits throughout the year. The coconut palm removes large quantities of nutrients from the soil continuously. Hence maintenance of adequate water and nutrient during the entire crop growth period is of paramount importance. Palms suffer quickly and conspicuously from improper mineral nutrition, which result from insufficient or incorrect fertilization. Some nutritional problems in palms are difficult to diagnose accurately because symptoms of several different mineral deficiencies overlap (Broschat, 1992).

The nutrient analyses have revealed that the yellowing affected palms maintained lower nitrogen content than the critical level in the 14th leaf. While the inner whorls were considered, the nitrogen content was more in the entire yellowing affected palm than that of the control palm. In the case of phosphorus both the control and yellowing affected palm maintained higher phosphorus content than the critical level in the 14th leaf. The inner whorl of the inner whorl and middle whorl yellowing affected palm maintained a higher concentration of phosphorus than the control palm. Higher potassium content was recorded in the inner whorl and the 14th leaf than the critical level in yellowing affected palms when compared to the control palm. When all the secondary nutrients- calcium and magnesium and the micronutrients- iron, manganese, copper and zinc were considered, it revealed that these nutrients were present in a very low level than that of the critical level in both control and yellowing affected palms.

The 14th leaf starting from the first fully opened one is the most widely used leaf for nutrient analysis as recommended by the IRHO. (Fremond *et al.*, 1966).The 14th leaf data from the control palms and palms with different types of yellowing were analysed. The data showed that there were significant reductions in the levels of major nutrient nitrogen, phosphorus and also in the levels of magnesium and the micronutrient manganese in case of palms showing mid whorl

yellowing compared to the control palm. But significantly higher levels of accumulation were found in case of potassium, calcium, iron and copper. These variations in the nutrient levels can have an influence on symptom development in coconut palms.

The nutrient deficiency symptoms have been studied by several workers (Menon and Pandalai, 1958; Manicot *et al.*, 1980; Wahid, 1984; Ohler, 1999b, Broschat, 1992; and Justin *et al.*, 2005).Nitrogen and magnesium are the constituents of chlorophyll and iron is closely connected with chlorophyll formation. The deficiencies of these nutrients will result in chlorosis of leaves. In the early stage of nitrogen deficiency, the crown of the palm loses its glossy appearance and turns pale green, followed by yellowing of the leaves. In advanced stages young leaves also turn pale green giving the leaflets a dull appearance. The first deficiency symptom of magnesium is the intervascular yellowing of older leaves. Yellowing starts at the tip and spreads to the base. Gradually the leaflet becomes almost devoid of green pigmentation except on the portion nearer to the rachis. The symptoms of iron deficiency results in general chlorosis, with all leaves discolouring to pale green or dark yellow. When potassium is present in excess it interferes with magnesium utilisation and thus inducing chlorosis of older leaves. Phosphorus deficiency in coconut palms is rare and very difficult to recognize as it shows hardly any visible symptoms. Only in severe cases leaves may turn yellow before dying prematurely. Calcium is mainly concerned with proper growth and functioning of stem and leaves rather than with the palm's productivity of nuts .In Ca deficiency the petioles turn a deep yellow or orange colour, orange blotches occur along the mid-rib frequently. In Mn deficiency symptoms occur only on new leaves which emerge chlorotic, weak, reduced in size, and with extensive necrotic streaking in the leaves. The Cu deficiency, results in a severe bending of the rachis of the youngest leaves, accompanied by yellowing and desiccation of the leaf tip, which appears to be rimmed with brown and yellow, whilst the central part remains green. Zinc is directly involved in the synthesis of auxin precursors and also involved in numerous enzyme systems. No

descriptions are available on zinc deficiency in the case of coconut. But generally zinc deficiencies result in shortened internodes, small narrow leaves that form rosette like whorls.

5.2 Yield

The control palm recorded the maximum yield of 27 nuts per harvest. All the yellowing affected palms showed a tremendous decline in yield. The maximum reduction was shown by palms affected with general yellowing (93 % reduction). Similar reductions in yield have been reported for coconut affected with root (wilt) (Balakrishnan, 2011) and lethal yellowing. The major elements like nitrogen, phosphorus potassium and magnesium have a foremost role in the productivity of the palms. A deficiency of these elements especially nitrogen, phosphorus and magnesium in all the yellowing affected palm in the present study might also have a role in the declined yield in the affected palms.

5.3 Anatomical changes associated with yellowing in coconut

The external symptoms in the leaves and roots of coconut palms affected with yellowing showed correlation with various anatomical changes. Chlorophyll degradation and loss of structural integrity was seen in the leaves of yellowing affected palms. Vascular browning which extended to the cortex and the vascular disintegration in the roots of affected palms was in accordance with the studies of Indira and Ramadasan, 1968.

5.4 Molecular analysis

The DNA extraction was improved by bringing out modifications in the original CTAB DNA isolation protocol (Doyle and Doyle, 1990) and DNA isolation protocol by Porebski *et al.*, 1997 .The modified CTAB method resulted in extracting high quality, low polysaccharide genomic DNA which was suitable for PCR analysis.

The nested PCR analysis with the phytoplasma specific universal primers P1/P7-R16F2n/R16R2 provided an amplicon of 1240 bp (1.2 kb) in the positive control and palms affected with inner and middle whorl yellowing as reported by

Nejat and Vadamalai (2010) and the primers R16mF2 /R16mR1 -R16F2n/ R16R2(Rojas-Martinez *et al.*, 2003) provided an amplicon of 1.3 kb for the positive control and palms affected with inner, middle and general yellowing. The root (wilt) phytoplasma specific semi- nested primers IF7/7R3- IF7/7R2 (Manimekalai *et al.*, 2010) provided an amplicon of 493 bp only for the positive control and no amplification was seen in any of the selected palms. The control palm and the palms affected with outer whorl yellowing provided no amplification at all for any of the primers tested.

The absence of amplification for the root (wilt) phytoplasma specific semi-nested primers indicate the absence of the 16SrXI root (wilt) phytoplasmal group in any of the selected palms, while amplifications of universal primers in palms with the inner whorl, middle whorl and general yellowing indicate that some phytoplasmal groups are present in these palms which need to be characterised. Lack of amplifications of any of the phytoplasma specific primers in the control palm and the palms with outer whorl yellowing can be an indication of absence of phytoplasma in these palms. The results of molecular studies points to the fact that the yellowing of outer whorls of the selected palms can just be a developmental phenomenon associated with the senescing phase of the leaves.

Summary

6. SUMMARY

Coconut known as the 'tree of life 'is a benevolent provider of the basic needs of millions of people in 93 countries particularly in the humid tropics in the Asian and Pacific regions where in the crop has cultural and socioeconomic significance. The importance of the palm lies in the fact that not only does it supply food, drink and shelter, but it also provides raw material for a number of important industries. Kerala well known as the 'Land of coconut' occupies first position in the area and production of the coconut in India. But the productivity in Kerala is below the national average. This is because of unproductive and senile palms, lack of adaptation of recommended cultivation practises and serious incidence of pests and diseases.

Coconut root (wilt) an endemic disease in Kerala causes an annual loss of 968 millions of nuts. Lethal yellowing (LY) is another devastating phytoplasma disease affecting coconut palms in several African and American countries causing tremendous destruction of palms. Recently another type of yellowing characterised by mid whorl yellowing, shedding of immature nuts, drying of inflorescence without showing characteristic ribbing symptom is rapidly spreading in many parts of Kerala. There is no research reports and published reports available in this emerging problem in the coconut growing tracts of Kerala Since there is rampant spreading of yellowing to other coconut cultivated areas causing a serious reduction in nut yield, this problem demands immediate attention. This study is the first attempt to disclose the predisposing factors and causative agents leading to mid whorl yellowing of coconut palm. In the present programme, efforts were made to generate basic informations on the changes of physiological, anatomical and molecular realms of coconut palm affected by mid whorl yellowing. The salient findings of the study are summarised below.

The palms with yellowing showed a significant reduction in the total chlorophyll, chlorophyll a, chlorophyll b and carotenoid content when compared to the healthy palm. A 19% and 54% reduction in total chlorophyll content was

shown by palms with outer whorl yellowing, inner whorl yellowing and a 72% reduction each in middle whorl and general yellowing respectively.

Relative water content was lower for all the palms with yellowing compared to the control palm. A significant reduction (20%) was shown by the palms affected with middle whorl yellowing compared to the control palm.

Significant variations were observed in the loss of membrane integrity between palms affected with yellowing and control palms. The maximum membrane integrity was shown by the control palm, but it did not significantly vary with palms with general yellowing. The other palms with yellowing showed a significant reduction in the membrane integrity.

All the palms with yellowing recorded an accumulation of reducing sugars compared to the healthy palms. All the palms with yellowing except general yellowing showed an increased accumulation of starch content in the middle whorls.

In the present experiment, the control palms recorded maximum protein content compared to other palms with yellowing. A significant increase in the phenolic compounds was observed in palms with yellowing compared to the control palms. Across the middle whorls, palms with general yellowing showed maximum phenol content followed by palms with middle whorl, inner whorl and outer whorl yellowing. Across the middle whorls, all palms with yellowing except the one with outer whorl yellowing, recorded a significantly higher peroxidase activity than the control palm, while in the case of polyphenol oxidase, palms with all types of yellowing recorded significantly higher activities.

The 14^{th} leaf starting from the first fully opened one is the most widely used leaf for nutrient analysis as recommended by the IRHO. The 14^{th} leaf data from the control palms and palms with different types of yellowing were analysed. The data showed that there were significant reductions in the levels of major nutrient nitrogen, phosphorus and also in the levels of magnesium and the

micronutrient manganese in case of palms showing mid whorl yellowing compared to the control palm. But significantly higher levels of accumulation were found in case of potassium, calcium, iron and copper. These variations in the nutrient levels can have an influence on symptom development in coconut palms.

Though the soils collected from the root zones of palms exhibiting varying types of yellowing showed significant variations in the nutrient levels, they were not following any particular pattern indicative of any particular roles in development of yellowing.

The maximum yield of 27 nuts per harvest was recorded for the control palm. All the yellowing affected palms showed a tremendous decline in yield.

The external symptoms in the leaves and roots of coconut palms affected with yellowing showed correlation with various anatomical changes. Chlorophyll degradation and loss of structural integrity was seen in the leaves of yellowing affected palms. Vascular browning which extended to the cortex and the vascular disintegration in the roots of affected palms were seen.

The DNA extraction was improved by bringing out modifications in the original CTAB DNA isolation protocol. The modified CTAB method resulted in extracting high quality, low polysaccharide genomic DNA which was suitable for PCR analysis.

The nested PCR analysis with the phytoplasma specific universal primers P1/P7-R16F2n/R16R2 provided an amplicon of 1240 bp (1.2 kb) in the positive control and palms with inner and middle whorl yellowing and the primers R16mF2 /R16mR1 -R16F2n/ R16R2 provided an amplicon of 1.3 kb for the positive control and palms with inner, middle and general yellowing. The root (wilt) phytoplasma specific semi- nested primers IF7/7R3- IF7/7R2 provided an amplicon of 493 bp only for the positive control and no amplification was seen in any of the selected palms. The control palm and the palms affected with outer whorl yellowing provided no amplification at all for any of the primers tested.

Future line of work

Amplification of phytoplasma specific universal primers in palms with mid whorl yellowing indicates that phytoplasma has got a role in development of the mid whorl yellowing symptom. This necessitates further studies on the specificity of phytoplasmal strains associated with mid whorl yellowing in coconut. But in case of the root (wilt) phytoplasma specific primers, there was no amplification in palms with mid whorl yellowing. This suggests that the mid whorl yellowing of coconut palm which is an emerging problem in Kerala may not be associated with the root (wilt) disease.

The results of biochemical and physiological analyses of the selected palms clearly indicate an altered primary metabolism, source-sink relation and a complexity in the nutriophysiology which can be explained by phytoplasmal influence of the palm. These altered conditions can act as predisposing factors for the development of specific symptoms.

The outcome of the present programme in conjunction with informations on the physiological parameters like hormonal profile will help to develop management strategy for ameliorating field performance and productivity of coconut palm in Kerala.

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Appendices

Table 1.Weather Parameters during the experimental period (4/10 to 4/11)

Months	Maximum temperature (^{0}C)	Rainfall (mm)	Relative Humidity (%)	Evaporation (mm)
April	34.50	61.00	85.50	4.60
May	32.60	278.90	86.80	3.80
June	30.10	245.20	89.40	3.10
July	30.29	199.99	84.63	3.36
August	30.37	91.63	84.96	3.46
September	30.62	134.40	82.79	3.38
October	30.33	504.98	83.74	3.17
November	30.33	289.80	83.71	3.02
December	29.75	105.98	85.77	2.89
January	30.48	7.35	86.09	3.18
February	31.59	35.21	82.21	3.50
March	33.00	0.00	80.00	4.14

Abstract of the thesis

PHYSIOLOGICAL, ANATOMICAL AND MOLECULAR ANALYSES OF COCONUT PALMS (*Cocos nucifera* L.) AFFECTED WITH YELLOWING

By

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(2009-11-107)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the

requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT PHYSIOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM - 695522

KERALA, INDIA

2011

ABSTRACT

Kerala the 'Land of coconut' occupies first position in area and production of coconut in India but the productivity is below the national average. This is because of unproductive and senile palms, lack of adaptation of recommended cultivation practises and serious incidence of pests and diseases. The root (wilt), an endemic disease of Kerala causes an annual loss of 968 million nuts. Recently another type of yellowing characterised by mid whorl yellowing shedding of immature nuts and drying of inflorescence without showing characteristic ribbing symptom is rapidly spreading in many parts of Kerala. There is no research report available in this emerging problem in the coconut growing tracts of Kerala. Since there is rampant spreading of yellowing to other coconut cultivated areas causing a serious reduction in nut yield, this problem demands immediate attention. Hence an experiment was conducted in the Department of Plant Physiology, College of Agriculture, Vellyani to generate the basic information on the causative agents and on the changes of physiological, molecular and anatomical realms of coconut affected by mid whorl yellowing.

A wide variation was observed in the physiological parameters like total pigments, relative water content, carbohydrate fractions, proteins, phenols and antioxidants betweenpalms with yellowing and control palms. The palms with yellowing showed a significant reduction in the total chlorophyll, chlorophyll a, chlorophyll b and carotenoid content when compared to the healthy palm. Relative water content was lower for all the palms with yellowing compared to the control palm and the healthy palms were maintaining a better water status and membrane integrity. All the selected palms with yellowing recorded an accumulation of reducing sugars compared to the healthy palms. The trend was similar for starch content except for palms with general yellowing. The control palms recorded maximum protein content compared to other palms with yellowing. A significant increase in the phenolic compounds was observed in palms with yellowing compared to the healthy palms. This results point to an

activated defense system. The 14th leaf starting from the first fully opened one is the most widely used leaf for nutrient analysis as recommended by the IRHO. (Fremond et al., 1966).The 14th leaf data from the control palms and palms with different types of yellowing were analysed. The data showed that there were significant reductions in the levels of major nutrient nitrogen, phosphorus and also in the levels of magnesium and the micronutrient manganese in case of palms showing mid whorl yellowing compared to the control palm. But significantly higher levels of accumulation were found in case of potassium, calcium, iron and copper. These variations in the nutrient levels can have an influence on symptom development in coconut palms. The maximum yield was recorded for the control palm. Chlorophyll degradation and loss of structural integrity were evident from the anatomical studies of leaf tissues in case of palms with yellowing. Vascular browning which extended to the cortex and the vascular disintegration in the roots of affected palms were seen on anatomical analyses.

A pure quality DNA was obtained by modified CTAB DNA isolation protocol. Molecular analyses using nested PCR showed that with the phytoplasma specific universal primers P1/P7-R16F2n/R16R2 provided an amplicon) in the positive control and palms with inner and middle whorl yellowing and the primers R16mF2 /R16mR1 -R16F2n/ R16R2 provided an amplicon for the positive control and palms with inner, middle and general yellowing. The root (wilt) phytoplasma specific semi- nested primers IF7/7R3- IF7/7R2 provided an amplicon of 493 bp only for the positive control and no amplification was seen in any of the selected palms. The control palm and the palms affected with outer whorl yellowing provided no amplification at all for any of the primers tested. Amplification of phytoplasma specific universal primers in palms with mid whorl yellowing indicates that phytoplasma has got a role in development of the specific symptom. This demands immediate attention and extensive studies on the etiology, changes in biochemical profile, anatomy etc.

Salient Findings

Amplification of phytoplasma specific universal primers in palms with mid whorl yellowing indicates that phytoplasma has got a role in development of the mid whorl yellowing symptom. But in case of the root (wilt) phytoplasma specific primers, there was no amplification in palms with mid whorl yellowing. This suggests that the mid whorl yellowing of coconut palm which is an emerging problem in Kerala may not be associated with the root (wilt) disease.

The results of biochemical and physiological analyses of the selected palms clearly indicate an altered primary metabolism, source-sink relation and a complexity in the nutriophysiology. These altered conditions can act as predisposing factors for the development of specific symptoms.

The anatomical analyses showed damages of roots in the palms with mid whorl yellowing. They will have internal browning of vascular elements, extending into the cortex and also disintegration of vascular elements.

Extension of molecular studies into identification of specific phytoplasmal strains associated with mid whorl yellowing of coconut palms.

Future line of work

Detailed analysis of nutriophysiology, source-sink relation and hormonal profile.

Studies on soil factors like nutrient level and water status.

Extension of anatomical studies to different type of tissues.

Development of management strategy for the improvement of the field performance and productivity of coconut palms with mid whorl yellowing.