NUTRIO-PHYSIOLOGICAL AND MOLECULAR ANALYSES AND CARBON DIOXIDE ENRICHMENT STUDIES OF COCONUT PALMS (*Cocos nucifera* L.) WITH FOLIAR YELLOWING

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DECLARATION

I hereby declare that this thesis entitled **"Nutrio-Physiological and Molecular Analyses and Carbon Dioxide Enrichment Studies of Coconut Palms (*Cocos nucifera* L.) with Foliar 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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Certified that this thesis, entitled **"Nutrio-Physiological and Molecular Analyses and Carbon Dioxide Enrichment Studies of Coconut Palms (*Cocos nucifera* L.) with Foliar Yellowing"** is a record of research work done independently by **Deepa, S. (2012-21-119)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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'Now, this is not the end, it is not even the beginning of the end. But it is perhaps, the end of the beginning'- Sir Winston Churchill.

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Dedicated to

My husband

and

My dear son

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LIST OF ABBREVIATIONS

MWY	Mid whorl yellowing
RW	Root (wilt)
OTC	Open top chamber
cm	centimeter
Fig.	Figure
g	gram
h	hour
ha	hectare
kg	kilogram
l	litre
M	Molar
m	meter
mg	milligram
ml	millilitre
mM	milli molar
mm	millimeter
mm^2	square millimeter
N	Normal
nm	nanometer
^oC	degree Celcius
ppm	Parts per million
PR protein	Pathogenesis related protein
var.	Variety
µm	micrometer
µg	microgram

Introduction

1. INTRODUCTION

Coconut palm (*Cocos nucifera* L.) is a benevolent provider of the basic needs of millions of people in the tropics and subtropics. The palm is intimately associated with social, cultural and economic lives of people around the world. It is the most useful crop under the class Monocotyledonae. It is popularly known as the 'Kalpavriksha' which translates as "tree that gives all that is necessary for living." The diversity of products offered by the palms ranging from the coconut fruit- tender juice, nira, coconut meat, shell, fibre, copra, trunk and edible heart of the palm provides the rationale for bestowing the title 'tree of life', 'tree of heaven', 'tree of plenty' to the palm. The coconut palm not only provides sustainable income to millions of people who are directly and indirectly dependant on it, but also provides 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. The different parts of the palm possess potential medicinal properties also (DebMandal and Mandal, 2011).

India is the leading country in coconut production and productivity with an area of 1.98 mha and production of 20439 million nuts and a productivity of 10345 nuts/ha (Press Information Bureau, 2016). Coconut is grown in 17 states and 3 union territories in India. Kerala 'the Land of Coconut' occupies the first position in area (33%) under coconut cultivation in India. Kerala has an area of 0.65 mha, production of 4896 million nuts and a productivity of 7535 nuts/ ha (CDB, 2015). However the productivity is behind the national average and far behind the neighbouring state Tamil Nadu (14873 nuts/ha) where the area under coconut cultivation is only 23.5%.

The diminution in production is because of the several constraints faced in the coconut cultivation in Kerala. Coconut is a perennial crop and mostly grown as a rainfed crop in Kerala. Scientific management practices are not being followed by many coconut growers. There is prevalence of old, senile and unproductive palms, dearth of good quality seedlings, high cost of inputs, labour

problems in cultivation and harvesting and lack of reasonable price for coconut which all lead to the dwindling coconut production in Kerala. To add to the woes there is serious incidence of pests and diseases of which the root (wilt) disease is the most prominent one which results in a yield loss of 35% and in severe cases may lead upto 80% reduction in yield (Ramjegathesh *et al.*, 2012; Rajesh *et al.*, 2015).

Root (wilt) disease is a complex debilitating malady of coconut caused by phytoplasma which causes maximum damage to the palms. The annual loss due to the disease is estimated to be about 968 million nuts (CPCRI, 1985, Koshy, 1999; Ramjegathesh *et al.*, 2012; Manimekalai *et al.*, 2014; Thomas *et al.*, 2015).

The most obvious and diagnostic symptom of the root (wilt) disease is the abnormal inward bending of the leaflets termed ribbing or flaccidity. Wilting and drooping of leaves, yellowing and necrosis of leaflets are the other typical symptoms (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 a devastating phytoplasma disease affecting coconut palms mostly reported in African and American countries causing tremendous destruction of palm. The most conspicuous symptom is premature nut fall. The affected palms die within 3-6 months after acquisition of pathogen and once the disease appears in an area, it can spread rapidly (Aguilar *et al.*, 2009). The disease spread is very fast and ultimately the entire crown of the palm becomes withered and toppled, leaving a bare and barren trunk (Harrison *et al.*, 2008).

Mid whorl yellowing (MWY) is another severe malady of recent origin in the coconut growing tracts especially in the southern tracts of Kerala causing serious reduction in the nut yield. It is characterised by sudden appearance of yellowing in two or three leaves in the middle whorl. When the symptom advances, the entire middle whorl turns yellow. Other symptoms of palms with MWY are drying and necrosis of inflorescence and shedding of buttons and

immature nuts, ultimately leading to a substantial yield loss. In severe cases it may cause a yield loss up to 70% (Deepa, 2011). The most distinguishing feature of the palms with mid whorl yellowing which differentiates it from the root (wilt) disease is the absence of flaccidity. In the case of lethal yellowing the palms die within a span of 6 months which does not happen in MWY.

There are reports suggesting that the yellowing in the middle whorls can be an associated symptom of the root (wilt) disease (Koshy, 1999). No elaborate studies have been reported in the case of palms with MWY to confirm this. A preliminary study was carried out in the Department of Plant Physiology to analyse the physiological, biochemical, nutrient and anatomical changes associated with foliar yellowing (Deepa, 2011). Significant reduction in the chlorophyll, carbohydrate and protein contents and accumulation of phenol were found in palms affected with yellowing in the study. There is no literature available reporting any detailed study on this fast spreading and serious problem. A comparative study between the mid whorl yellowing and the root (wilt) has also not been carried out. At this juncture, a programme was formulated to address this issue by including detailed analyses on the nutrio-physiological and biochemical changes occurring in palms with mid whorl yellowing. The study was also extended to root (wilt) affected palms in order to make a comparative analysis.

Climate change has a varying impact on plant pathogens. Whilst the life cycle of some pathogens will be limited by increasing temperatures, other climatic factors, such as increasing atmospheric carbon dioxide, may provide more favourable conditions for some other pathogens. Very few studies have been done on the impact of climate change on phytoplasmal response. Effect of altered levels of oxygen and carbon dioxide on phytoplasma abundance in *Oenothera* leaf tip cultures has been studied by Sears *et al.* (1997). Galetto *et al.*, 2011 has reported that changes in climatic conditions can have an influence on phytoplasma diseases.

Association of phytoplasma was already evident from the preliminary study conducted in the Department of Plant Physiology (Deepa *et al.*, 2012). In this context, a study on phytoplasmal response to enriched carbon dioxide

conditions was also formulated for understanding the phytoplasmal epidemiology under the changing climate scenario.

The main objectives of the present programme are

1. To analyse the nutrio-physiological changes associated with foliar yellowing
2. To assess the impact of enhanced carbon dioxide on phytoplasmal response.

The programme will bring to light any association between yellowing and root (wilt) disease. The information generated will also pave way for the development of better management strategies for this problem. Phytoplasmal response studies to enriched carbon dioxide conditions will help to address the phytoplasma epidemiology under the changing climatic scenario.

Review of literature

2. REVIEW OF LITERATURE

Cocos nucifera L. (2n=32) is a perennial tropical species of the *Arecaceae* family, a monocotyledon belonging to the monospecific genus Cocos and the order Palmae. The coconut is cultivated in the warm damp regions of the tropics and is particularly distributed on all tropical shores. The palm sustains the livelihood of millions of people in coastal regions of the tropics and subtropics.

Coconut grows well in warm humid climate with an ideal temperature of 27^0C with 5-7^0C diurnal variation. Coconut prefers an annual precipitation between 1300 and 2500 mm or above. The palm can be grown in different soil types and it tolerates salinity and a wide range of pH (5.0-8.0).

The coconut palm grows up to a height of about 25 m or more depending on ecological conditions and age. The trunk is single, stout, annulate, light grey, smooth and erect or slightly curved and rises from a swollen base, the bole. The trunk is surmounted by a graceful crown of giant, featherlike leaves. The leaves are long, pinnatisect, leaflets equidistant, narrow, tapering and petioles stout. The coconut inflorescence is called spadix which is stout, erect, androgynous and simply branched. The palm is monecious with male flowers numerous and female flowers relatively low. Mature fruits are botanically known as drupe which are large obovoid or ellipsoid in shape. The pericarp is thick and fibrous and is a hard shell encloses the embryo with its abundant endosperm, composed of both meat and liquid. The coconut palm has an adventitious root system which produce numerous uniformly thick roots from the base of the stem.

Kerala is the 'land of coconut' having a productivity of 7535 nuts/ha (Coconut Development Board, 2015). But the productivity is much less when compared to that of other coconut growing states. The coconut cultivation in Kerala is constrained by many factors. This includes unscientific cultivation practices, old, senile and unproductive palms, dearth of good quality seedlings, high cost of inputs, labour problems in cultivation and harvesting and lack of reasonable price for coconut. To add to the woes there is serious incidence of pests and diseases of which the root (wilt) disease is the most prominent one

which results in an average annual loss of 968 million nuts in Kerala (CPCRI, 1985, Koshy, 1999; Ramjegathesh *et al*, 2012; Manimekalai *et al*., 2014; Thomas, *et al*., 2015).

Mid whorl yellowing (MWY), a type of yellowing of recent origin, is a severe problem in the coconut growing tracts in Kerala. The characteristic symptom of root (wilt) disease "flaccidity" is absent in palms with mid whorl yellowing. There is rampant spreading of yellowing to other coconut cultivated areas. Since it is causing serious economic consequence, it is necessary to have an understanding on the physiological changes occurring in palms with mid whorl yellowing in order to adopt a better management strategy. In the present investigation, an attempt has been made to analyze the nutrio - physiological changes occurring in the palms affected with foliar yellowing, bringing to light any association between yellowing and root (wilt) disease. Phytoplasmal response studies to enriched carbon dioxide conditions will help to address the phytoplasma epidemiology under the changing climatic scenario.

2.1 Root (wilt) disease

Root (wilt) disease, the age old disease is the most devastating disease affecting coconut in Kerala. It was first reported in 1874 in Erattupetta of Meenachil Taluk in Kottayam district (Butler, 1908; Menon and Pandalai, 1958; Solomon *et al*., 1999, Koshy, 1999; Sasikala *et al*., 2005). Since then it has spread in all directions from the original sites of infection and now the disease is reported to be present in all districts of Kerala and many of the adjoining districts of Tamil Nadu and Karnataka, and also from Goa and Orissa (Mishra *et al*., 1989; Koshy, 1999; Thomas, 2009).

Detailed surveys were conducted at different times to estimate extend of spread of the disease. Pillai *et al*., 1973 reported a contiguous distribution of the disease in six districts viz. Thiruvananthapuram, Kollam, Alappuzha, Kottayam, Ernakulam and Thrissur. According to them more than 30 percent of the 0.75 million ha under coconut in Kerala was affected by the disease. A comprehensive survey was undertaken by the Central Plantation Crops Research Institute (CPCRI) in collaboration with the Department of Agriculture, Kerala and other

agencies in 1984-85 to estimate the spread and intensity of disease. According to the survey the disease was prevalent in the eight southern districts of Kerala and the intensity of the disease was highest in Kottayam district (75.6%) followed by Alappuzha (70.7%), Pathanamathitta (38.2%), Ernakulam (34.5%), Idukki (34.2%) and Kollam (28.6%). The districts of Thrissur and Thiruvananthapuram recorded a disease intensity of 2.6% and 1.5% respectively. The survey also brought out the annual loss of 968 million nuts due to the disease. In 1996, Department of Agriculture, Government of Kerala had undertaken a detailed survey in the eight southern districts and revealed that the overall disease incidence came down to 24.05% from 32.37% in 1984. This decline was mainly due to the cutting and removal of diseased palms and replanting with quality seedlings.

However, one of the recent investigations of Coconut Development Board, Kochi indicated that the incidence and severity of the disease has greatly increased from that of 1996 survey in certain districts of Kerala such as Thiruvananthapuram (2.09 to 26.89 %), Kollam (25.97 to 60 %) and Thrissur (6.19 to 43.95 %) (Anon., 2009).

The recently observed yellowing has not been studied properly. The appearance of intervenal yellowing in the leaves of middle whorl was described as important symptom for the diagnosis of the root (wilt) disease (Koshy, 1999; Anithakumari *et al.*, 2003). Palms with healthy outer leaves but yellowing in some of the inner whorls also were noticed during the early investigations of root (wilt) disease (Menon, 1937). But elaborate studies were not conducted to explicate the etiology of yellowing and to compare it with the root (wilt) disease. Deepa (2011) and Anju (2011) made an initial attempt to throw light on predisposing factors, causative agents, symptamatology and various alterations brought at the cellular level.

Similar phytoplasmal diseases are reported in coconut in North America, Africa and few other Asian countries also. Coconut Lethal Yellowing Disease was first reported from Grand Cayman Islands in the Caribbean region in 1834

(Myrie *et al.*, 2006). Recently a 'Kerala wilt' like disease has been reported on coconut from Srilanka which is described as Weligama Coconut Leaf Wilt disease. It is fast spreading from Weligama area in the South to northwards (Pathiraja *et al.*, 2010).

2.2 Symptamatology

Root (wilt) disease is a complex debilitating malady of coconut. The diseased palms are mainly identified by the visual symptoms. The symptoms appear on the leaves, reproductive parts and roots. Wilting and drooping of the leaves, flaccidity, ribbing, paling/ yellowing and necrosis of the leaflets are typical symptoms of the disease (Menon and Nair, 1951; Menon and Pandalai, 1958; Pillai and Rawther, 1999). When the disease progresses, there is a reduction in the number of leaves and successive leaves become smaller, shorter and narrower finally resulting in the stunting of the crown (Menon and Pandalai, 1958). Radha and Lal (1972) recorded flaccidity as the earliest consistent visual symptom. This appears mainly in the central and outer whorls. Dwivedi *et al.* (1979) opinioned softening and whitening of spindle leaves and interveinal yellowing in the leaves of middle whorl as the initial symptoms of root (wilt) disease.

The expression of the foliar symptom varies with the soil type and ecological conditions (Pillai and Rawther, 1999). In general flaccidity occurs in 67-91% of palms; yellowing in 38-67% of palms and necrosis in 28-48% of palms. Out of the three main symptoms viz- flaccidity, yellowing and necrosis, flaccidity is considered as the most frequent and common symptom. In the case of young palms (palms below the age of 10 years) flaccidity is the main symptom; yellowing and necrosis are more or less absent (Solomon *et al.*, 1999).

The disease has an effect on the reproductive system also. Inflorescence necrosis, lack of ability to produce female flowers and pollen sterility makes the palm unproductive (Varghese, 1934; Varkey and Davis, 1964). The spathes of the affected palms become smaller in size and do not open normally (Maramorosch, 1964). Shedding of immature nuts and poor quality of nuts are other characteristic symptoms (Pillai and Rawther, 1999). The husk becomes thinner, shell turns black and kernel exhibits uneven thickness and remains flexible (Menon and Pandalai,

1958; Varghese, 1934). Shedding of immature nuts even before the appearance of other visual symptoms or after is another important characteristic of the disease (Koshy, 1999). All these result in a huge decline in the yield of palms (Radha and Lal, 1972).

Many of the earlier workers considered rotting of roots to be another characteristic symptom of the disease (Butler, 1908; Menon and Nair, 1949; Menon and Pandalai, 1958; Michael, 1964). The percentage of root decay varied with the intensity of disease (Michael, 1964). According to Menon and Nair (1949) rotting of roots and rootlets start from the tip backwards.

In certain cases the expression of symptom in palms with root (wilt) is greatly varied. Sudden appearance of bright yellowing of 3-4 leaves in the middle whorl, followed by the appearance of large number of brown spots of various shapes with a halo around on all leaflets of yellowed leaves was the first symptom of RWD. In the beginning, yellowing was sometimes restricted from the leaf tip to the middle of the leaf. Shedding of buttons/ immature nuts and inflorescence necrosis were the other prominent symptoms in these palms. These yellowed leaves dried up faster and shed while leaves of lower whorls remained green. Very bright yellowing and more incidence of mid whorl yellowing were noticed in the variety Chowghat Orange Dwarfs, their seggregants and their hybrids (Koshy, 1999).

The root (wilt) disease affected palms are also superimposed with leaf rot disease (Radha and Lal, 1968; George and Radha, 1973; Pillai, 1975; Mathai, 1980; Srinivasan, 1991). Srinivasan *et al.* (2000) reported a close relationship between the occurance of root (wilt) disease and leaf rot disease. About 65% of the root (wilt) diseased palms are affected by leaf rot disease caused by fungi (Srinivasan, 1991, Rajan, 2011) and contributed to the rapid decline and reduction in the yield of the affected palm.

In the case of lethal yellowing, the most conspicuous symptom is premature nut fall. As the disease progresses there will be inflorescence necrosis, leaf chlorosis and senescence and spear leaf death. Plant death usually ensues within 3-6 months. The disease spread is very fast and ultimately the entire crown

of the palm becomes withered and toppled, leaving a bare and barren trunk (Plavsic-Banjac *et al.*, 1972; Mpunami, 1997; Harrison *et al.*, 2002; Myrie *et al.*, 2006; Roca *et al.*, 2006; Howard and Harrison, 2007; Nipah *et al.*, 2007; Harrison *et al.*, 2008).

However, in the recent type of yellowing spreading in the southern parts of Kerala the most prominent symptom is the appearance of yellowing in the middle whorl. The yellowing appears either in the 3- 4 leaves in the middle whorl or the whole middle whorl turn yellow. In severe conditions the yellowing spreads to the outer whorl and this results in the yellowing of the 2/3 rd portion of the crown. As time progresses leaves turn necrotic and in some cases the leaves are seen drooping around the crown. A distinctive character of palms with MWY is the absence of flaccidity which is the prominent and diagnostic symptom of root (wilt) disease. Premature nut fall, button shedding and inflorescence necrosis are other characteristic symptoms of palms with yellowing. With the advance of disease, reduction in spathe size with few female flowers, drying up of spathes and necrosis of spikelets were also observed. Necrosis started from the tips of yellowed leaflets and progressed inwards. Root rotting was also observed in the case of palms with yellowing. The rotting of roots started from the tip backwards (Deepa, 2011).

2.3 Physiological and biochemical variations

Coconut root (wilt) disease results in various physiological and biochemical changes (Rajagopal *et al.*, 1986; CPCRI, 2007; Ramjegathesh *et al.*, 2012). Phytoplasmal infection severely damages the physiological and biochemical processes in plants. Maust *et al.* (2003) reported that coconut trees with lethal yellowing exhibit numerous physiological and biochemical changes before or with the first appearance of the visual symptoms.

2.3.1 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. 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). 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. 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. The mid whorl yellowing affected palms showed lower carbohydrate content and higher reducing sugars and starch compared to the control palms (Deepa *et al.*, 2012).

2.3.2 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) and Anjum *et al.* (2011) reported that water stress resulted in a reduction in the chlorophyll content in plants.

Foliar yellowing is the most conspicuous symptom in root (wilt) and lethal yellowing disease. Deepa *et al.* (2012) reported 72%, 70% 79% and a 60% reduction in the total chlorophyll, chlorophyll a chlorophyll b and carotenoid contents in palms with middle whorl yellowing compared to the control palms. Koshy (1999) reported a reduction in the chlorophyll content in the root (wilt) affected coconut palms. A similar reduction in the chlorophyll and carotenoid contents of coconut affected with lethal yellowing has been observed 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).

2.3.3 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; Gomes and Prado, 2007). Shivashankar *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. Deepa *et al.*, 2012 reported that the palms with mid whorl yellowing exhibited lower relative water content of 73.95% compared to the control palms. 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 has been reported by Leon *et al.* (1996) also.

2.3.4 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 and Duduk (2009) have reported an increase in phenolic compounds in host plants due to infection by phytoplasma. Musetti

(2010) 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. CPCRI, 2007 reported a reduction in the phenol content of coconut palms infected with root (wilt) disease. An accelerated phenol metabolism has been reported in roots of root (wilt) affected coconut palms (Joseph and Jayasankar, 1979). A 3 times increase in the phenolic compounds was observed in palms with yellowing compared to the control palms (Deepa, 2011).

2.3.5 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 and Duduk (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). Similarly a 3% reduction in the protein content was observed in palms with mid whorl yellowing (Deepa *et al.*, 2012)

2.1.6 Antioxidant 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 (2007) has reported that the peroxidase activity did not differ in root (wilt) affected and healthy palms but there was a significant increase in the polyphenols oxidase activity in palms with root(wilt). 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. A higher peroxidise activity and a lower poly phenol oxidase activity was observed in palms with mid whorl yellowing (Deepa *et al.*, 2012).

2.4 Soil and Plant Nutrient Analyses

Organic carbon content, pH and electrical conductivity are considered as the soil quality indicators (Reeves, 1997). These factors affect the availability of nutrients to crop plants. Soil organic carbon is an index of sustainable land management and is critical in determining response to N and P fertilization (Bationo *et al.*, 2007). Soil pH influences solubility, concentration in soil solution and mobility of nutrients in soil and consequently acquisition of these elements by plants (Fageria *et al.*, 1990).

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)

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, 1999a)

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

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 leaves may 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 midrib 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.

A deficiency in the case of nitrogen, phosphorus, magnesium and the micronutrient manganese and accumulation in the case of potassium, calcium iron and copper were reported in palms with mid whorl yellowing (Deepa *et al.*, 2012).

2.5 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). Phytoplasmas belong to the class Mollicutes, phylum Tenericutes along with mycoplasmas, spiroplsama and acholeplasmas. Although spiroplasma and mycoplasma species have been cultured in artificial medium outside their host, phytoplasmas have not been cultured and hence were assigned the genus name *Candidatus Phytoplasma.* The phytoplasma have small genomes, a low G+C content, a few rRNA operones, a few tRNA genes and limited metabolic activities. The genome size of phytoplasma ranges from 680 - 1600 kbp (Bertaccini *et al.*, 2014).

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 phytoplasmas are predominantly restricted to the cytoplasm of phloem sieve cells. Phytoplasmas

systemically infect their host plants by moving through the sieve plates, thereby spreading throughout the plant's vascular system. 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).

The major symptoms induced by phytoplasma in plants include stunting, yellowing, virescence and phyllody. The phytoplasmas produce effectors that modulate cellular processes in plant development. The effectors are proteins that are secreted by microbial pathogens into a host cell to enhance colonisation and facilitate multiplication of the pathogen. These effectors induce various physiological and morphological changes during infection of their hosts, and phytoplasmas gain fitness advantages from inducing such changes.

The effectors interact with plant TCP transcription factors thereby inducing cell proliferation and morphological alterations. They may also interfere with auxin biosynthetic and signalling pathways. The effectors also interfere with floral meristem establishment/maintenance, specification of organ identity, and termination of floral meristem (Sugio *et al.*, 2011).

2.5.1 Transmission

Phytoplasmal diseases are spread primarily by sap-sucking insect vectors belonging to the families Cicadellidea (leafhoppers) and Fulgoridea (planthoppers) (Lee *et al.*, 2000). Insects feed on phloem tissues, where they acquire phytoplasmas and transmit them from plant to plant. Insect vectors acquire phytoplasmas by feeding on the nutrient rich sieve cells containing phytoplasmas by a process called acquisition feeding. The phytoplasmas which enter the insects replicate inside the intestinal epithelial cells. They are released into the hemolymph from which they infect various other organs including the vacuoles of the salivary gland. The phytoplasmas are released into the plant sieve cells from these vacuoles during feeding. This process is called inoculation feeding. The period between acquisition and inoculation is called latency period. The spread of the root (wilt) disease occurs in leaps and jumps which suggested a possible involvement of aerial vectors in the transmission of the disease (Pillai et

al., 1980, Rajan, 2011). *Proutista moesta* a plant hopper and *Stephanitis typica* a lace bug were established as the vectors for the transmission of phytoplasma in the case of coconut root (wilt) disease (Solomon *et al.*, 1999, Rajan, 2011). But studies by Edwin and Mohankumar (2007) recorded that *Stephanitis typica* is a non vector of Kerala (wilt) disease.

Other mechanisms which introduce phytoplasmas into the vulnerable tissue of host plants: (*a*) vegetative propagation or grafting of infected plant material, (*b*) vascular connections made between infected and non infected host plants by parasitic plants such as dodder c) seed transmission (Weintraub and Beanland, 2006).

2.5.2 Detection of phytoplasma

Detection and identification of phytoplasma is of paramount importance for accurate disease diagnosis. The localisation of phytoplasmas in the plant is also vital for understanding the resistance mechanism and for disease management (Saracco *et al.*, 2006). 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.

2.5.3 DNA extraction

The phytoplasma titer varies greatly from plant to plant. Phytoplasma DNA may constitute less than 0.1 % of total DNA in extracts from woody hosts exhibiting disease symptoms (Kirkpatrick *et al.*, 1987). 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 contains exceptionally high amounts of polysaccharides, polyphenols, tannins which interfere with the DNA isolation procedure (Angeles *et al.*, 2005). It is difficult to obtain a pure quality DNA from plants with high amounts of polysaccharides and polyphenols (Lebrun *et al.*, 1998; Maltas *et al.*, 2011). 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 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 *et al.*, 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).

2.5.4 PCR

Polymerase Chain Reaction is the most versatile tool for detecting phytoplasmas in their plant and insect hosts (Nejat and Vadamalai, 2010). Phytoplasma detection has been based on the phytoplasma specific universal primers or phytoplasma group specific primers PCR primers designed on the basis of highly conserved 16S rRNA gene sequences.

2.5.5 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 groupspecific 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.

2.5.6 Real Time PCR

Standard nested PCR are labour intensive and time consuming. The introduction of a second round of amplification and the simultaneous manipulation of the previously amplified products in the nested PCR vastly increases the risks of cross contamination in routine analysis (Torres *et al.*, 2005; Klemsdal and Elen, 2006). Real-time PCR is the most recent technique in phytoplasma detection. It is really advantageous over conventional PCR methods due to its accuracy, specificity, less analysis time and absence of post- PCR manipulations that prevent carry-over contamination (Manimekalai *et al.*, 2011). It also eliminates the need to analyze the reaction products by gel electrophoresis (Crosslin *et al.*, 2006). Now real time PCR has been employed in the detection of various phytoplasma diseases including the coconut root (wilt) disease (Manimekalai *et al.*, 2011). Real-time PCR assays employ TaqMan probes for a wide range of phytoplasmas using both SYBR Green (Nejat and Vadamalai,

2010) and TaqMan minor grove binding (MGB) probe technology. SYBR Green chemistry is the most economic alternative and the precision of the PCR amplification can be checked by melting curves analysis (Martini *et al.*, 2007).

2.6 Anatomical analyses

External symptoms are the manifestations of biochemical and histological alterations caused by or as an effect of pathogenic infection. The symptoms can only be identified by the occurrence of visible indications when the phytoplasma is well established. The use of microscopic techniques can help in studying the anatomical alterations and detecting the presence of phytoplasma.

Phytoplasmas were originally detected by electron microscopy of ultra-thin sections from symptomatic plants (Doi *et al.* 1967). Electron microscopy of phloem tissue was reported tobe the most reliable method for demonstrating the presence of phytoplasma in diseased plants (Navratil *et al.* 2009). Direct detection of phytoplasmas is difficult, because they are not visible through a normal light microscope. Two different methods can be used to localize and identify phytoplasmas in the infected tissues by means of light microscopy. Dienes-reagent which eventually gives a blue colour (Dienes *et al.* 1948), and DNA-binding fluorochrome, 4'-6-diamidino- 2-phenylindole (DAPI) is the stain most used for phytoplasma diagnosis (Seemuller, 1976) both in herbaceous (Hiruki & Da Rocha 1986) and woody host plants such as coconut palms, ash, pear and sandal trees (Schuiling & Forstel-Neuhaus 1992, Sinclair *et al.* 1992, Malinowski *et al.* 1996, Thomas & Balasundaran 1998, Musetti, 2009, 2010).

These procedures are simple, quick and relatively cheap, and can be used as a preliminary method to detect phytoplasmas in infected plants with high concentrations of the pathogen but not specific for the different phytoplasmas (Das and Mitra, 2004). However, it is limited when the phytoplasma population is very low in the phloem tissue of a diseased host plant.

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 coconut 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. Three to five layers of closely packed, chloroplast- filled palisade cells lie below the two layered adaxial hypodermis. 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. Five or more small vascular bundles in the abaxial mesophyll lie between two large veins, each of which cover the entire palisade area and are attached only to the adaxial and abaxial hypodermal layers with a few sclerotic cells. Fibers are regularly interspersed in the second adaxial hypodermal layer and in the abaxial hypodermis. Each vascular bundle consists of a few tracheids, three to four phloem elements, and fiber cells. Each large vein has the xylem vessels on the upper side, a large vessel in the centre, and the phloem elements on the lower side towards the center of the cross -section. Narrow rings of fibres encircle the vascular bundles of large veins. Very few spongy parenchyma cells can be noticed in the abaxial hypodermis. Stomata are regularly distributed in the abaxial epidermis. Also in the abaxial epidermis there occur at regular intervals in shallow depressions dark brown, short stalked scales containing tannins. Spherical to oval chloroplasts, slightly smaller than the cell nucleus, are abundant in healthy palisade cells.

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 revealed a more or less compactly arranged , elongated palisade cells extending from the upper hypoderm to the lower epidermis with very little spongy tissues and air spaces in between. The chloroplasts evenly distributed along the periphery of cells, were uniformly spherical to oval in shape with compact yellowish green chlorophyll. While no variation was seen in the mesophyll of disease affected palms or palms showing physiological yellowing. However there was a great variation in the distribution, size and the chlorophyll contents. The chloroplasts were 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.

Ultra structural changes were observed by Solomon et al., 1999 in palms affected with the root wilt by the use of electron microscopy. The sieve tubes that containing phytoplasma and the bordering cells had fibrilar tubules. The tubules had electron dense granules which were identified as P proteins. The P –proteins which are similar to microfilaments are supposed to provide the motive force to assimilate movement in sieve elements. Although P- proteins are normal host components, their massive accumulation in sieve cells of diseased palms may be part of the host-parasite interaction.

The protophloem elements in roots and rachilla of diseased palms were compressed and had electron dense contents indicating necrotic obliteration. Deposition of callose a response to injury was often observed in sieve pores of

phytoplasma infected cells. Sieve plate pores of infected sieve elements were lined with callose.

Another prominent ultrastructural change was the elaboration of the membrane system. Plasma lemma which lined the cell wall makes deep intrusion into cell lumen forming invagination. The invaginations often enclosed vesicular or membraneous structures lacking contents and were described as paramural bodies. The sieve tube plastids contained crystalline inclusions with well defined crystalline lattice were identified as Cuneate crystalline inclusions. Clusters of electron dense osmiophilic membrane bound crystals rhombic or hexagonal were observed in rachilla of diseased palms. Wildman and Hunt (1976) interpreted these as phytoferritin, a normal host component that accumulated in plants infected with the biological agents.

2.7 Climate change and Phytoplasma

Climate change is one of the major challenges of our time and adds considerable stress to our societies and to the environment. The catasrophical impacts of climate change include erratic climate and weather extremes, altered ecosystems and habitats and risks to human health and society. Apart from its effect on the food supply the climate change will also determine how much land people choose to farm, and the number of crops they plant (Science daily, 2016).

Climate change drives changes in the timing of seasonal biological activities. Living things are intimately connected to their physical surroundings. Even small changes in the temperature of the air, the moisture in the soil, or the salinity of the water can have significant effects.

The importance of the environment on the development of plant diseases has been known for over two thousand years. Plant diseases are one of the important factors which have a direct impact on global agricultural productivity and climate change will further aggravate the situation (Gautam *et al.*, 2013). Climate change and its impact on plant diseases). Plant pathogens are the first to demonstrate the effects of climate change due to the numerous populations, ease of reproduction and dispersal, and short time between generations (Ghini *et al.*,

2008). The plant pathogens will have varying responses to the climate change. Changes in the climatic factors may influence the distribution and biology of plant pathogens with positive, negative or neutral effects (Luck *et al.*, 2011). Climatic variability can affect not only the pathogen, but also plant host and insect vector, as well as the interactions between or among these organisms (Galetto *et al.*, 2011).

There are only few studies on the effect of climate change on the phytoplasma diseases and insect vectors associated with the diseases (Foissac and Wilson 2010). Boudon-Padieu and Maixner (2009) reported the potential effects of climate change on the distribution and activity of insect vectors related to grapevine phytoplasma and Polak, 2009 studied the influence of climate changes on the distribution of phytoplasmas in the Mediterranean sub tropical region. Galetto *et al.*, 2011 conducted an experiment in order to investigate the effects of global warming on phytoplasma epidemiology and found out that phytoplasma multiplication was faster under cooler conditions in insects and under warmer conditions in plants. He also suggested that the effect of environmental conditions on phytoplasma multiplication was host-dependent.

Materials and methods

3. MATERIALS AND METHODS

The objectives of the present study entitled "Nutrio - physiological and molecular analyses and carbon dioxide enrichment studies of coconut palms (*Cocos nucifera* L.) with foliar yellowing" was to conduct nutrio physiological, anatomical and molecular analyses of coconut palms affected with yellowing and to assess the impact of enhanced carbon dioxide on phytoplasmal response. The study was conducted in the Department of Plant Physiology, College of Agriculture, Vellayani during the period 2012-2017 as two experiments.

Experiment I

Nutrio - physiological and molecular analyses

3.1 Experimental material

3.1.1 Identification of location and selection of palms

As a first step for identifying the locations for selection of palms, a preliminary data with regard to the presence and spread of types of yellowing in coconut was collected from the Agriculture Officers in the Thiruvananthapuram district. Based on the information collected, field visits were carried out in the different locations of Thiruvananthapuram district like the Instructional Farm, College of Agriculture, Vellayani, Thiruvallam, Venganoor, Kuravankonam and Varkkala. The first part of the study included a detailed analysis of mid whorl yellowing along with a comparative analysis of mid whorl yellowing and root (wilt) with healthy palms as control. To achieve this, locations where all the three categories of palms were available were to be identified in order to avoid any discrepancies in the soil factors and agronomic practises. Such areas were identified in two locations.

a) Instructional Farm, College of Agriculture, Vellayani
b) Venganoor region

Palms of similar age (15- 20 years) showing the following symptoms from these two locations were selected for the study (Plate 1-Plate 7).

1. Palms showing mid whorl yellowing with no flaccidity symptom

2. Palms with root (wilt) disease
3. Healthy palms

3.1.1.1 Location I

Palms of similar age group showing different patterns of yellowing were selected from D 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).

3.1.1.2 Location II

The farm is located at $8^0$4' N latitude, 77^0 01'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.

3.1.2 Collection of sample

Leaves were selected from the inner (3^{rd} fully opened) and middle whorls of the selected palms for physiological and biochemical analyses. Spindle leaves and roots were selected for anatomical analyses. Nutrient analyses were carried out in the index leaves (14^{th} leaves) and soil. Molecular analyses were carried out in the spindle leaves

3.2 METHODS

3.2.1Physiological parameters

3.2.1.1Total 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

a) MWY* in one or two leaves in the middle whorl

b) MWY* in the entire middle whorl

Plate 1. Palms with MWY

*MWY- Mid Whorl Yellowing

Plate 2. Palm with MWY* with lower half of the crown turned yellow

*MWY- Mid Whorl Yellowing

a) Necrosis of the leaves

b) Palms with MWY* showing necrosis and severe blighting

Plate 3. Palms with MWY* showing necrosis symptom

*MWY- Mid Whorl Yellowing

4 a)

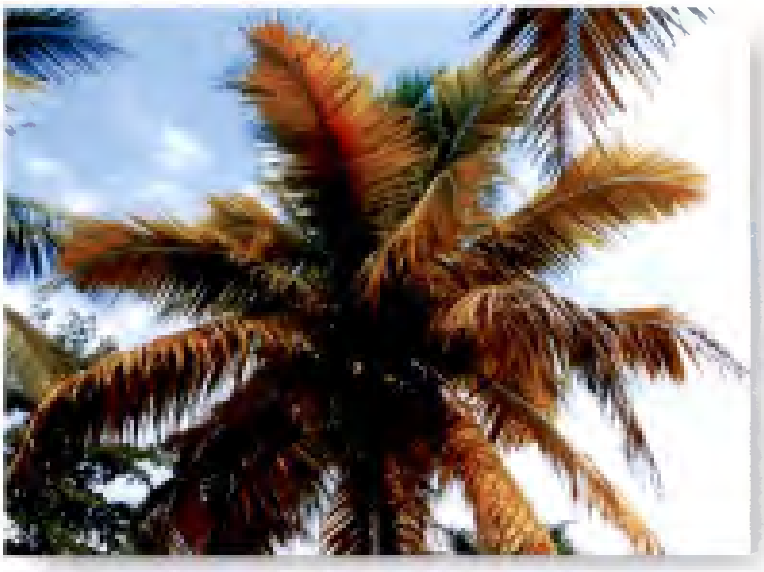
4 b)

Plate 4. Palms with MWY* showing inflorescence necrosis and button shedding

*MWY- Mid Whorl Yellowing

Plate 5. Palm with Root (wilt) disease

6 a) Flaccidity symptom



6 b) Yellowing with flaccidity

Plate 6. Root (wilt) diseased palms showing flaccidity symptoms

7 a)

7 b)

Plate 7. Healthy palm

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.

3.2.1.2 Estimation of reducing sugar

Reducing sugar was estimated following the Dinitro salicylic acid (DNS) method (Somogyi, 1952). The sample was weighed (100 mg each) and the sugars were extracted with hot 80% ethanol, twice. The supernatant was collected and evaporated by keeping it on a boiling water bath at 80^0 C. The residue was dissolved in 5 ml distilled water.1 ml aliquot was taken into test tubes and equalized to 3 ml with distilled water. To this 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, 1 ml of 40% Rochelle salt solution was added. Then the test tubes were cooled and the intensity of dark red colour was read at 510 nm. A series of the standard, Glucose (0- 500 micro g) was run and a standard curve was plotted. The amount of reducing sugars was calculated from the standard graph.

3.2.1.3 Estimation of Starch

The estimation of starch in plants was done following the Anthrone method (Mc Cready et al., 1950). A known quantity of plant sample (0.1 g) was homogenized in hot 80% ethanol to remove sugars. The homogenate was centrifuged and residue was retained. The residue was washed repeatedly with hot 80 % ethanol till the washing does not give any colour with anthrone reagent. Then the residue was dried well over a water bath. The dried residue was mixed with 5 ml water and 6.5 ml 52% perchloric acid and was extraxted at 0^0 C for 20 min. This solution was centrifuged and the supernatant was saved. The extraction was repeated using fresh perchloric acid. The supernatants after centrifugation was pooled and made up to 100 ml.

An aliquot of 0.1 ml of the supernatant was taken and again made up to 1 ml using distilled water. The standard was prepared by taking 0.2, 0.4, 0.6, 0.8

and 1 ml of the working standard solution and made up the volume to 1 ml in each tube using distilled water. Anthrone reagent (4 ml) was added to both the sample and standard test tubes. These test tubes were heated for eight minutes in a boiling water bath and cooled rapidly. The intensity of colour change from green to dark green was measured at 630 nm. The glucose content in the sample was calculated using the standard curve. This value was multiplied by a factor of 0.9 to arrive at the starch content.

3.2.1.4 Chlorophyll estimation

Chlorophyll content (chlorophyll a, b and total 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.

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)

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

Where V= volume of the solution made up

W= fresh weight of leaves

3.2.1.5 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

3.2.1.6 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 273 nm. 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

3.2.1.7 Total soluble protein

Total soluble protein of leaf was estimated using simple protein dye binding assay of Bradford (1976); Sadasivam and Manickam (1996) 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 (40 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) (*EC.1.11.1.7*)

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 phosphate buffer (pH 6.5) to which a pinch of polyvinyl pyrrolidone (PVP) was added. The homogenisation was done at 40^0C using a mortar and pestle. The supernatant was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 minutes at 40^0C. 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) (*EC. 1.14.18.1*)

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

3.2.2.1. Soil analysis

Soil samples were collected from all the four sides of basins of the selected palms (1 m away from the tree trunk at a depth of 0-30 cm and 30-60 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

Properties	Method	Reference
Chemical Properties		
pH	1:2.5 Soil: water, pH meter	Jackson (1973)
EC	Conductivity meter	Jackson (1973)
Organic Carbon	Walkley and Black rapid titration method	Jackson (1973)
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	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^{0}C 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 0-30 cm and 30-60 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 Anatomical Analyses

Anatomical analysis was carried out in spindle leaves and fresh roots. Scanning Electron Microscopy (SEM) technique was used to detect phytoplasmas in the leaf tissues of coconut. Samples were incised with razor, attached to SEM stubs and coated with palladium in an ion sputter (DESK II, Denton Vacuum), for 40 seconds. Prepared specimens were examined in a SEM (S-3000N Hitachi) at 5-20 kV (Lebsky and Poghosyan, 2014).

Fresh roots of pencil thickness were traced out and tip portion of the root of 10cm length were collected from the selected palms. Root tip morphology and transverse section of root tips were done using Stereo zoom microscope Leica EZ4D. Sections of roots were done and viewed under microscope (45X) (Motic BA 310 DIGITAL) and photographed.

3.2.5 Molecular analyses

3.2.5.1 Sample collection

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

3.2.5.2 Procedure for DNA Isolation

Phytoplasma enrichment protocol was standardised for isolating DNA.

3.2.5.2.1 Phytoplasma Enrichment protocol (Manimekalai et al., 2010)

3g of tender leaf samples with PVPP were powdered in liquid nitrogen. The powdered leaf samples were transferred to 10 ml Phytoplasma extraction buffer at 4^0 C for 5 minutes. The mixture was centrifuged at 5000 rpm for 5 minutes. The supernatant was transferred to fresh tube through muslin cloth. The samples were centrifuged at 13000 rpm for 25 minutes. The supernatant was discarded and the pellet was dissolved in 1 ml SDS buffer. The tubes were incubated at 60^0 C in a water bath for 1 hour. 500 µl each of sample were transferred to two centrifuge tubes and equal volumes of chloroform: isoamyl alcohol 24:1 mixture added to each tube. The tubes were centrifuged at 13000 rpm for 15 minutes. The supernatant was taken and equal volumes of isopropanol was added and incubated at -20^0 C for 1 hr / overnight. The tubes were then centrifuged at 13000 rpm for 15 minutes. The supernatant was discarded and the pellet dried and dissolved in 400µl TE buffer. 40 µl Sodium acetate and 900 µl absolute alcohol was added to the tubes and incubated at -20^0 C for overnight. The samples were centrifuged at 13000 rpm for 15 minutes. The pellet was washed with 80% ethanol twice then dried and dissolved in 50 µl TE.

The above procedure did not yield sufficient quantity of DNA for PCR analysis. So the protocol was slightly modified which yielded a pure quality DNA. Instead of SDS buffer, CTAB buffer was used. One time DNA precipitation was followed instead of two times precipitation. The modified phytoplasma enrichment procedure is given below.

3g of tender leaf samples with PVPP were powdered in liquid nitrogen. The powdered leaf samples were transferred to 10 ml Phytoplasma extraction buffer at 4^0 C for 5 minutes. The mixture was centrifuged at 5000 rpm for 5 minutes. The supernatant was transferred to fresh tube through muslin cloth. The samples were centrifuged at 13000 rpm for 25 minutes. The supernatant was discarded and the pellet was dissolved in 1 ml SDS buffer. The tubes were incubated at 60^0 C in a water bath for 1 hour. 500 µl each of sample were transferred to two centrifuge tubes and equal volumes of chloroform: isoamyl alcohol 24:1 mixture added to each tube. The tubes were centrifuged at 13000 rpm

for 15 minutes. The supernatant was taken and equal volumes of isopropanol was added and incubated at -20^0 C for 1 hr / overnight. The tubes were then centrifuged at 13000 rpm for 15 minutes. The supernatant was discarded and the pellet dried and dissolved in 400µl TE buffer. 40 µl Sodium acetate and 900 µl absolute alcohol was added to the tubes and incubated at -20^0 C for overnight. The samples were centrifuged at 13000 rpm for 15 minutes. The pellet was washed with 80% ethanol twice. The pellet then dried and dissolved in 50 µl TE.

3.2.5.3 Spectrophotometric analysis

Spectrophotometric analysis of the extracted DNA was made for determining the quality and quantity. Spectrophotometer (Elico) 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. 5 µl of DNA was dissolved in 3ml of sterile distilled water and optical density was measured at 260nm and 280 nm.

3.2.5.4 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) = A_{260} x 50 x dilution factor

(where A_{260} is absorbance value at 260nm)

3.2.5.5 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.

3.2.5.6 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 (Syngene) and the data was recorded using the Quantity One Software.

3.2.5.7 PCR analysis

3.2.5.7.1 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.

3.2.5.7.2 Semi nested PCR

Semi nested PCR is the one in which in the second PCR run, one of the primers used in the first run is used again and the other primer is within the target sequence.

3.2.5.7.3 Real Time PCR

Real-time PCR is the most recent technique in phytoplasma detection. It is really advantageous over conventional PCR methods due to its accuracy, specificity, less analysis time and absence of post- PCR manipulations that prevent carry-over contamination (Manimekalai et al., 2011). Real Time PCR facility at CPCRI Kasargode was utilised. Real Time PCR was done using double-stranded DNA intercalating dye, SYBR Green Real-time PCR was performed with the Stratagene Real Time PCR system and the results were analysed using MxPro software.

Table 5. Primer sequence and PCR conditions

Primer pair	Sequence	Annealing Condition	Reference
Nested PCR			
P1[a]	5'AAGAGTTTGATCCTGGCTCAGGATT 3'	63^0 c for 1 min	(Deng and Hiruki, 1991 Schneider et al,1997, Smart et al., 1996)
P7[a]	5' CGTCCTTCATCGGCTCTT 3'		
R16F2n[a]	5'-GAAACGACTGCTAAGACTGG-3'	56^0C for 1 min	Gundersen and Lee, 1996
R16R2[a]	5' GACGGGCGGTGTGTACAAACCCCG-3'		
Semi-nested PCR			
1F7[b]	5'- AGTGCTTAACACTGTCCTGCTA-3'	57^0 C for 1 min	Manimekalai et al., 2011
7R3[b]	5'- TTGTAGCCCAGATCATAAGGGGCA-3'		
1F7[b]	5'- AGTGCTTAACACTGTCCTGCTA-3'	57^0 C for 1 min	
7R2[b]	5'- GACAAGGGTTGCGCTCGTTTT-3'		
Real Time Primers			
QPF2[c]	5'-ACGCTTAACGTTGTCCTGCT-3'	60 ^{0}C for 30 sec	Manimekalai et al.,2011
QPR2[c]	5'-TGCCTCAGCGTCAGTAAAGA-3'		

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3.2.5.8 PCR Conditions

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

Nested and Semi- nested reactions

DNA (25ng/µl) -5µl

dNTPs (2.5mM) -1µl

Forward primer (10pmol) -1.50µl

Reverse primer (10pmol) -1.50µl

10x assay buffer -2.5µl

Taq polymerase (3units/µl) -0.33µl

Sterile distilled water -13.17µl

Real time PCR (15 µl reaction)

DNA (50 ng/µl) -2µl

Forward primer (10pmol) -1.2µl

Reverse primer (10pmol) -1.2µl

Master mix -7.5 µl

Sterile distilled water -3.1 µl

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

1. P1/ P7 and nested primers R16F2n/ R16R2;
2. 1F7/7R3 and semi nested primer pair 1F7/7R2 (root (wilt) specific pimers)
3. Real time primers QPF2/R2

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 1.2% agarose with ethidium bromide (0.5µg/ml) using TAE buffer (Tris buffer, Glacial acetic acid and water) as the running buffer. DNA fragments of 490 bp from the semi nested PCR were sequenced.

Other temperature conditions followed for nested PCR

a) 94^0C for 5 minutes and followed by 35 cycles of 94^0 C for 45 seconds appropriate annealing condition and 72^0 C for 2minute with final extension at 72^0 C for 10 minute

b) 94^0 C for 2 minutes and followed by 35 cycles of 94^0 C for 1 minute appropriate annealing condition and 72^0 C for 1 minute 30 seconds with final extension at 72^0 C for 10 minute

c) 95°C for 10 min (segment 1), followed by 40 cycles of amplification at 95°C for 10 s and 55°C for 30 s (segment 2), and finally the melting curve analysis at 95°C for 10 s, 55°C for 30 s and 95°C for 30 s (segment 3)

Experiment II Effect of enhanced carbon dioxide on phytoplasmal responses

3.3 Location

The experiment was conducted in Open Top Chamber (OTC) system established by Department of Plant Physiology, College of Agriculture, Vellayani for carrying out simulation studies on elevated carbon dioxide environment.

3.3.1Experimental material

a) Coconut seedlings

Six month old seedlings showing yellowing were selected from the Instructional Farm, College of Agriculture, Vellayani.

b) Other plant hosts

Other plant host – phytoplasma pathosystem like Catharanthus and Brinjal were used for the study. Catharanthus and brinjal plants infected with phytoplasma maintained at the Department of Plant Pathology were utilised for grafting to the healthy catharanthus and brinjal plants maintained at the Department of Plant Physiology.

3.3.2 Graft Transmission

The infected shoots having the symptoms of phytoplasma were selected as the source or scion. The base of the scion was trimmed into a wedge shaped structure of approximately 4 cm and then it was inserted into the cleft of about 4 to 5 cm made on the healthy plant which was selected as the root stock. The graft was tied firmly using a high density polythene strip and covered using a polypropylene cover to keep the graft union moist. The grafted plants were kept for the expression of symptoms on the new sprouts. After the grafts were

established, the plants were transferred into the OTC and kept inside OTC for a period of one month.

3.3.3Lay out of the experiment

The experiment was laid out in CRD with four treatments and four replications.

3.3.4 Open top chamber

Open Top Chambers (OTC) are square type chambers constructed to maintain near natural conditions and elevated CO_2 conditions for experimental purposes. The basic structure of OTC was built of metal frame and installed in the experimental field. OTCs were covered with a 200 micron UV poly sheet. The chamber is 3 X 3 X 3 dimension, 45^0 slope and 1 m^2 opening at the top. Two such chambers were built in the experimental field; one serves to impose CO_2 enrichment and the other serves as the control chamber to study the chamber effects. Elevated CO_2 was released into the chamber from a CO_2 cylinder in a controlled manner. Measurements of micro climatic parameters (temperature, humidity and light) were done within and outside the OTCs with the help of sensors on a real time basis. Potted plants were kept within these chambers at 500 ppm CO_2 concentration for a period of one month and observations were taken.

3.4.1Biochemical parameters

The biochemical parameters reducing sugar content, total soluble protein content, phenol content, antioxidant enzymes Peroxidase, polyphenol oxidase and phenyl alanine ammonia lyase (PAL) were estimated. The procedures for the biochemical analyses except PAL were followed as per 3.2.1.

3.4.1.1 Phenyl alanine Ammonia Lyase (PAL)

PAL activity was assayed spectrophotometrically by assaying the ratio of conversion of L-Phenyl alanine to trans-cinnamic acid at 290 nm as described by Dickerson et al., 1984. The enzyme extract was prepared by homogenising one gram leaf samples in 5 ml of 0.1 M borate buffer (pH 8.8) containing a pinch of PVP using chilled mortar and pestle. The homogenate was centrifuged at 10000 rpm for 10 minutes at 4^0C. The supernatant was used for th assy of PAL activity.

The reaction mixture contained 3 ml of 0.1 M sodium borate buffer (pH 8.8) and 0.1 ml of 12 mM L-Phenyl alanine. The reaction mixture and blank were incubated at 40 ^{0}C for 30 minutes and reaction was stopped by adding 0.2 ml f 3N HCl. The absorbance was read at 290 nm in a spectrophotometer (Elico). PAL activity was expressed as micrograms of cinnamic acid produced per minute per gram on fresh weight basis.

3.4.2 Molecular Analysis

3.4.2.1 DNA isolation

Total DNA was extracted from the infected leaves using the DNeasy Plant Mini Kits (Qiagen) following the manufacture's protocol. The DNA isolation were done after 15th day of exposure and 30th day of exposure. 50 ng DNA was used for the real time PCR.

3.4.2.2 Real Time PCR

Real time PCR was done using Power SYBR Green master mix with the real time primers QPF2/QPR2 (Table 5). Real Time PCR facility at RGCB, Thiruvananthapuram was utilised for the study. Real-time PCR was performed with the Stratagene Real Time PCR system and the results were analysed using MxPro software.

Results

4. RESULTS

Coconut cultivation in Kerala faces severe constraints. Mid whorl yellowing (MWY) is a serious problem in the coconut growing areas which causes a yield loss up to 70% under severe conditions. The major symptoms of MWY are sudden appearance of yellowing in the middle whorls, shedding of immature nuts and inflorescence necrosis without the presence of flaccidity. No elaborate studies have been done in this rampantly spreading malady.

The present study "Nutrio - physiological and molecular analyses and carbon dioxide enrichment studies of coconut palms (*Cocos nucifera* L.) with foliar yellowing" was implemented in two experiments. In the first experiment efforts were made to generate informations on the nutrio-physiological and biochemical changes occurring in palms with MWY. Molecular analyses of palms with MWY were also a part of the first experiment. For understanding the phytoplasmal epidemiology under the changing climate scenario, a study on phytoplasmal response to enriched carbon dioxide condition was formulated as the second experiment. The results based on statistically analysed data pertaining to the experiment conducted during the course of investigation are presented below.

4.1 Experiment 1 Nutrio - physiological and molecular analyses

Field visits were conducted in the Thiruvananthapuram district to select the three categories of palms- palms with MWY, palms with RW disease and healthy palms. Six regions were visited after consultation with the agricultural officers. Two areas –Instructional Farm, College of Agriculture, Vellayani (location 1) and Venganoor area (location 2) were selected out of the six locations. Observations on the total carbohydrate content, chlorophyll a, chlorophyll b and total chlorophyll content, membrane integrity, relative water content, total phenol content, total soluble protein content, anti oxidant enzymes, and soil and plant nutrient analyses and molecular analyses were carried out in order to achieve the first objective. The observations were taken from the inner and middle whorl of the selected palms and compared with the healthy palms.

Significant variations were observed in all these parameters among the different categories of the selected palms.

4.1.1 Physiological and Biochemical parameters

4.1.1.1 Estimation of carbohydrate fractions

The total carbohydrate content in the leaves of coconut palms with MWY and root (wilt) disease (RW) was studied and the data is presented in table 6. Increased carbohydrate content was shown by the palms with MWY and RW compared to the healthy palms in location 1. The highest carbohydrate content was shown by the inner whorl of the RW affected palms in location 1 (69.52 mg g^{-1}). Significant increase in the carbohydrate content was observed in the inner whorls (64.97 mg g^{-1} and 56.50 mg g^{-1}) in location 1 and 2 and the middle whorls (65.45 mg g^{-1}) in location 1 of palms with MWY. The carbohydrate content of the middle whorl of the mid whorl yellowing affected palms in location 2 was on par with the healthy palms. Across the whorls in the case of MWY palms, the total carbohydrate content slightly increased from the inner whorl to the middle whorl (64.97 mg g^{-1} to 65.45 mg g^{-1} in location 1 and 56.50 mg g^{-1} to 59.86 mg g^{-1} in location 2). In the case of RW palms the total carbohydrate content in the middle whorls in location 2 was significantly lower than the MWY and healthy palms.

The palms with MWY showed an increasing trend in the total carbohydrate content in the inner and middle whorls in location 1 (18.30% and 25.15%) and location 2 (19.99% and 11.54%) respectively compared to the healthy palms. Compared to the RW affected palms, the inner whorl of the MWY palms showed 6.55% and 5.32% decrease and the middle whorl showed 0.62% and 31.21% increase in location 1 and 2 respectively (table 6).

4.1.1.2 Estimation of reducing sugars

The reducing sugar status of the infected palms was estimated and the data is presented in table 7. The palms with MWY and RW showed increased reducing sugar content compared to the healthy palms. The maximum reducing sugar content was shown by the middle whorl of the RW palm in location 2 (33.60 mg g-1). Significant increase was shown by inner whorl (26.32 mg g-1) of the palms

Table 6. Carbohydrate content (mg g^{-1}) of coconut leaves affected with MWY*

Treatments	Location 1		Location 2		Mean	
	IW	MW	IW	MW	Locn 1	Locn 2
MWY	64.97	65.45	56.50	59.86	65.21	58.18
RW	69.52	65.05	59.68	41.18	67.29	50.43
Healthy	54.92	52.30	47.09	53.67	53.61	50.38
Mean	64.97	60.93	54.42	51.57		
SE m (±)	2.92		2.92			
CD (PXW)	8.54		8.51			
SE m (±)	1.69		1.68			
CD (W)	4.93		4.91			

Percent change in the carbohydrate content of palms with MWY

	Palms with MWY over healthy palms (%)		Palms with MWY over RW affected palms (%)	
	IW	MW	IW	MW
Location 1	(+) 18.30	(+) 25.15	(-) 6.55	(+) 0.62
Location 2	(+) 19.99	(+) 11.54	(-) 5.32	(+) 31.21

* Mean of 5 replications

MWY- Mid whorl yellowing RW – Root (wilt) IW- Inner whorl MW- Middle whorl

Table 6. Reducing sugar content (mg g^{-1}) of coconut leaves affected with MWY*

Treatments	Location 1		Location 2		Mean	
	IW	MW	IW	MW	Locn 1	Locn 2
MWY	26.32	24.43	16.64	26.7	25.38	21.67
RW	28.57	29.94	17.82	33.6	29.26	25.71
Healthy	20.56	23.68	14.29	23.50	22.12	18.90
Mean	25.15	26.02	16.26	27.93		
SE m (±)	1.66		1.75			
CD (PXW)	4.86		5.10			
SE m (±)	0.96		1.01			
CD (W)	2.80		2.94			

Percent change in the reducing sugar content of palms with MWY

	Palms with MWY over healthy palms (%)		Palms with MWY over RW affected palms (%)	
	IW	MW	IW	MW
Location 1	(+) 28.02	(+) 3.18	(-) 7.88	(-)18.39
Location 2	(+) 16.37	(+) 13.60	(-) 6.66	(-) 20.53

* Mean of 5 replications

MWY- Mid whorl yellowing RW – Root (wilt) IW- Inner whorl MW- Middle whorl

with MWY in location 1. Although the reducing sugar content increased in all cases, the values were on par with the healthy palms. In the case of RW affected palms all the values except the inner whorl in location 2 was significant. Across the whorls, there was no trend in the case of MWY palms. The reducing sugar content of the inner whorl (26.32 mg g^{-1}) of the MWY palms was greater than the middle whorl (24.43 mg g^{-1}) in location 1. But in location 2, the middle whorl (26.70 mg g^{-1}) expressed increased reducing sugar content compared to the inner whorl (16.64 mg g^{-1}) of MWY palms. In the case of RW palms; there was an increasing trend in the reducing sugar content from the inner to the middle whorl in both locations. The inner whorl of the root wilt palms showed lesser reducing sugar content (28.57 mg g^{-1}) than the middle whorl (29.94 mg g^{-1}) in location 1. But the difference was not significant. In location 2, the reducing sugar content in the middle whorl (33.60 mg g^{-1}) was more than that of the inner whorl (17.82 mg g^{-1}).

The palms with MWY recorded an increasing trend in the reducing sugar content in the inner and middle whorls in location 1 (28.02% and 3.18%) and location 2 (16.37% and 13.6%) respectively compared to the healthy palms. Compared to the RW affected palms, the palms with MWY showed 7.88% and 18.39% (location 1) and 6.66% and 20.53% (location 2) decrease in the inner and middle whorls in the reducing sugar content (Table 7).

4.1.1.3 Estimation of starch

The starch content of the palms with MWY and RW was estimated and the data is presented in table 8. Significant increase in the starch content was shown by the palms with yellowing and RW compared to the healthy palms. The highest starch content was shown by the middle whorl of the MWY affected palms in location 2 (41.28 mg g^{-1}). The lowest value was given by the inner whorl of the healthy palms (24.41 mg g^{-1}) in location 1. The inner and middle whorl of the palms with MWY in both the locations showed a significantly increased starch content except for the inner whorl in location 2. The RW affected palms showed significantly increased starch content in all cases except in location 2 where the

Table 8. Starch content (mg g^{-1}) of coconut leaves affected with MWY *

Treatments	Location 1		Location 2		Mean	
	IW	MW	IW	MW	Locn 1	Locn 2
MWY	31.32	34.20	30.07	41.28	32.76	35.67
RW	32.80	37.89	34.17	33.85	35.34	34.01
Healthy	24.41	27.53	26.50	30.04	25.99	28.27
Mean	29.51	33.20	30.25	35.06		
SE m (±)	1.32		1.55			
CD (PXW)	3.86		4.54			
SE m (±)	0.76		0.90			
CD (W)	2.23		2.62			

Percent change in the starch content of palms with MWY

	Palms with MWY over healthy palms (%)		Palms with MWY over RW affected palms (%)	
	IW	MW	IW	MW
Location 1	(+) 28.33	(+) 24.22	(-) 4.50	(-) 9.74
Location 2	(+)13.44	(+) 37.40	(-) 5.17	(+) 21.95

* Mean of 5 replications

MWY- Mid whorl yellowing RW – Root (wilt) IW- Inner whorl MW- Middle whorl

starch content (33.85 mg g^{-1}) was on par with the healthy palms. Across the whorls, starch content increased from the inner whorl to the middle whorl in MWY. The starch content in the inner whorl (31.32 and 30.07 mg g^{-1}) of the mid whorl yellowing palm was lower than that in the middle whorl (34.20 and 41.28 mg g^{-1}) in location 1 and 2.

The palms with MWY showed increased starch content in the inner and middle whorls in location 1 (28.33% and 24.22%) and location 2 (13.44% and 37.40%) compared to the healthy palms. The palms with RW disease expressed 4.5% and 9.74% and 5.17% increase in the starch content in the inner and middle whorls in location 1 and 2 respectively compared to the palms with MWY. But the middle whorl of the MWY palms in location 2 showed a 21.95% increase in the starch content compared to the root (wilt) palms (table 8).

4.1.2 Chlorophyll a content

The results of the study on chlorophyll a content is presented in table 9. The highest chlorophyll a content was shown by the healthy palms (1.41 and 1.15 mg g^{-1} in location 1 and 1.74 and 1.06 mg g^{-1} in location 2 in the inner whorl and middle whorl respectively). The palms with MWY showed a chlorophyll a content of 0.63 and 0.39 mg g^{-1} in location 1 and 0.88 and 0.25 mg g^{-1} in location 2 in the inner whorl and middle whorl respectively. The root (wilt) affected palms showed a chlorophyll a content of 0.56 and 0.33 mg g^{-1} in location 1 and 0.89 and 0.74 mg g^{-1} in location 2 in the inner whorl and middle whorls respectively. Across the whorls, the chlorophyll a content significantly decreased from the inner to the middle whorls in all palms in both the locations. The inner whorls (0.63 & 0.88 mg g^{-1}) of the mid whorl yellowing palms showed a significantly higher chlorophyll a content compared to the middle whorls (0.39 & 0.25 mg g^{-1}) in both the locations.

4.1.3 Cholorophyll b content

Chlorophyll b content was estimated in infected and healthy palms and the results are presented in table 10. The palms with MWY showed a reduction in the chlorophyll b content 0.34 and 0.18 mg g^{-1} in location 1 and 0.47 and 0.15 mg g^{-1}

Table 9. Chlorophyll a content (mg g^{-1}) of coconut leaves affected with MWY*

Treatments	Location 1		Location 2		Mean	
	IW	MW	IW	MW	Locn 1	Locn 2
MWY	0.63	0.39	0.88	0.25	0.51	0.56
RW	0.56	0.33	0.89	0.74	0.45	0.82
Healthy	1.41	1.15	1.74	1.06	1.28	1.40
Mean	0.87	0.63	1.17	0.69		
SE m (±)	0.07		0.07			
CD (PXW)	0.19		0.19			
SE m (±)	0.04		0.04			
CD (W)	0.11		0.11			

* Mean of 5 replications

MWY- Mid whorl yellowing RW – Root (wilt) IW- Inner whorl MW- Middle whorl

Table 10. Chlorophyll b content (mg g^{-1}) of coconut leaves affected with MWY*

Treatments	Location 1		Location 2		Mean	
	IW	MW	IW	MW	Locn 1	Locn 2
MWY	0.34	0.18	0.47	0.15	0.26	0.31
RW	0.21	0.13	0.36	0.35	0.17	0.36
Healthy	0.81	0.75	0.55	0.73	0.78	0.64
Mean	0.45	0.35	0.46	0.41		
SE m (±)	0.03		0.06			
CD (PXW)	0.09		0.18			
SE m (±)	0.02		0.03			
CD (W)	0.05		0.10			

* Mean of 5 replications

MWY- Mid whorl yellowing RW – Root (wilt) IW- Inner whorl MW- Middle whorl

in location 2 in the inner and middle whorls respectively. The healthy palms maintained the highest chlorophyll b content both in the inner and middle whorls in both the locations. Chlorophyll b content was also reduced in the case of root (wilt) infected palms, but it maintained higher chlorophyll b content compared to the mid whorl yellowing palms. The least chlorophyll b content was shown by the middle whorl of the palms with MWY in location 2 (0.15 mg g^{-1}). Across the whorls, the chlorophyll b content showed similar pattern of that of chlorophyll a content. The inner whorls (0.34 & 0.47 mg g^{-1}) of MWY affected palms showed more chlorophyll b content than that of the middle whorls (0.18 & 0.15 mg g^{-1}) in both the locations.

4.1.4. Total Chlorophyll content

The total chlorophyll content was estimated in infected and healthy palms and the data is presented in table 11. It was observed that the total chlorophyll content in the MWY affected palms and RW affected palms showed significant reduction in both the whorls compared to the healthy palms. The palms with MWY showed a total chlorophyll content of 0.96 and 0.57 mg g^{-1} in location 1 and 1.35 and 0.40 mg g^{-1} in the location 2 in the inner whorl and middle whorl respectively. The highest total chlorophyll content was shown by the healthy palms 2.21 and 1.90 mg g^{-1} in location 1 and 2.29 and 1.79 mg g^{-1} in the location 2 in the inner whorl and middle whorl respectively. Across the whorls, there was a declining trend in the total chlorophyll content from the inner to the middle whorls in all palms in both the locations. The inner whorl (0.96 & 1.35 mg g^{-1}) of the MWY palms showed a higher total chlorophyll content than that in the middle whorl (0.57 & 0.40 mg g^{-1}) in both the locations.

Compared to the healthy palms there was 56.44% and 70% reduction in the total chlorophyll content in location 1 and 41.37% and 77.5% reduction in location 2 in the inner whorl and middle whorl respectively of the palms with MWY. The MWY affected palms showed a 24.81% and 24.26% increase in the total chlorophyll content in location 1 and 7.26 % increase in inner whorl in location 2 compared to the root (wilt) palms. But there was 63.14% reduction in

Table 11. Total chlorophyll content (mg g^{-1}) of coconut leaves affected with MWY*

Treatments	Location 1		Location 2		Mean	
	IW	MW	IW	MW	Locn 1	Locn 2
MWY	0.96	0.57	1.35	0.40	0.77	0.87
RW	0.77	0.46	1.25	1.10	0.62	1.17
Healthy	2.21	1.90	2.29	1.79	2.06	2.04
Mean	1.32	0.98	1.63	1.10		
SE m (±)	2.92		2.91			
CD (PXW)	8.54		8.51			
SE m (±)	1.69		1.68			
CD (W)	4.93		4.91			

Percent change in the total chlorophyll content of palms with MWY

	Palms with MWY over healthy palms (%)		Palms with MWY over RW affected palms (%)	
	IW	MW	IW	MW
Location 1	(-) 56.44	(-) 70.00	(+) 24.81	(+) 24.26
Location 2	(-) 41.37	(-) 77.50	(+) 7.26	(-) 63.14

* Mean of 5 replications

MWY- Mid whorl yellowing RW – Root (wilt) IW- Inner whorl MW- Middle whorl

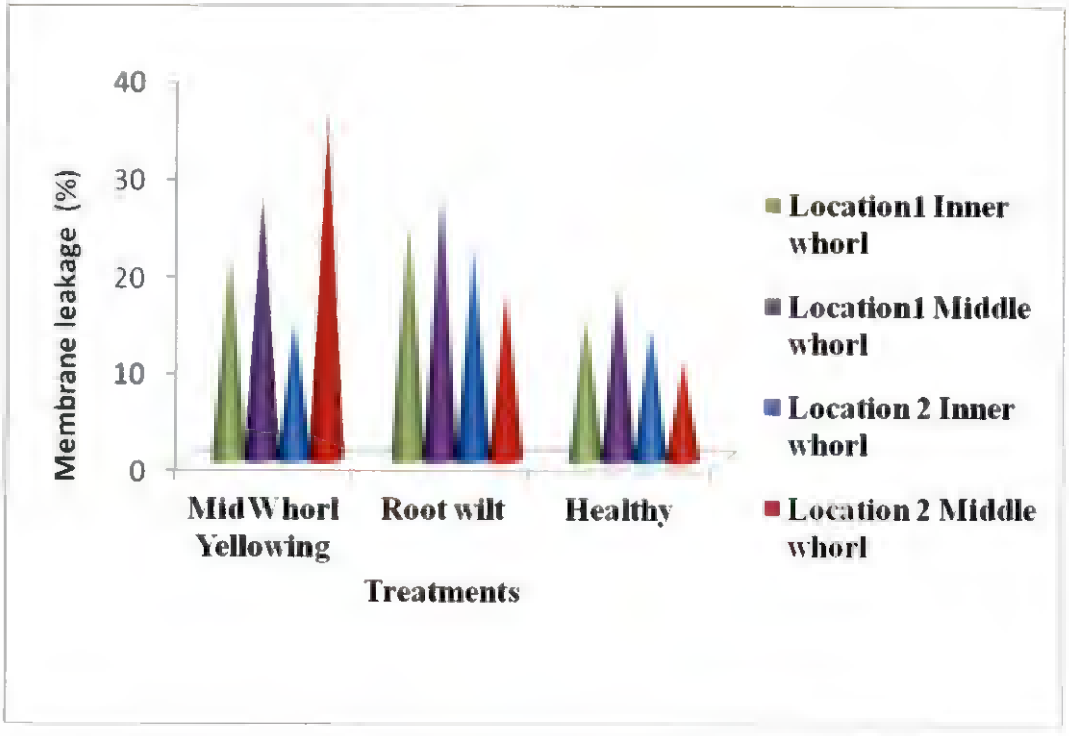
the total chlorophyll content in the middle whorl of palms with MWY in location 2 compared to the palms with RW disease.

4.1.5 Membrane integrity

There was considerable reduction in the membrane integrity in palms with MWY and RW compared to the healthy palms (Fig 1). The MWY affected palm showed a membrane leakage of 21.21% and 28.55% in location 1 and 14.39% and 36.71% in location 2 in the inner and middle whorls respectively. The reduction in the membrane integrity was significant only in the case of the middle whorl of the mid whorl yellowing affected palms. The RW affected palms showed a membrane leakage of 24.88% and 28.09% in location 1 and 22.13% and 17.49% in location 2 in the inner and middle whorls respectively. In root (wilt) palms the reduction in the membrane integrity was significant in all cases except in the middle whorl of palms in location 2. A declining trend was observed in the membrane integrity in the middle whorls of palms with MWY in both the locations. But the trend was significant only in location 2. The same trend was not observed in the case of root (wilt) palms.

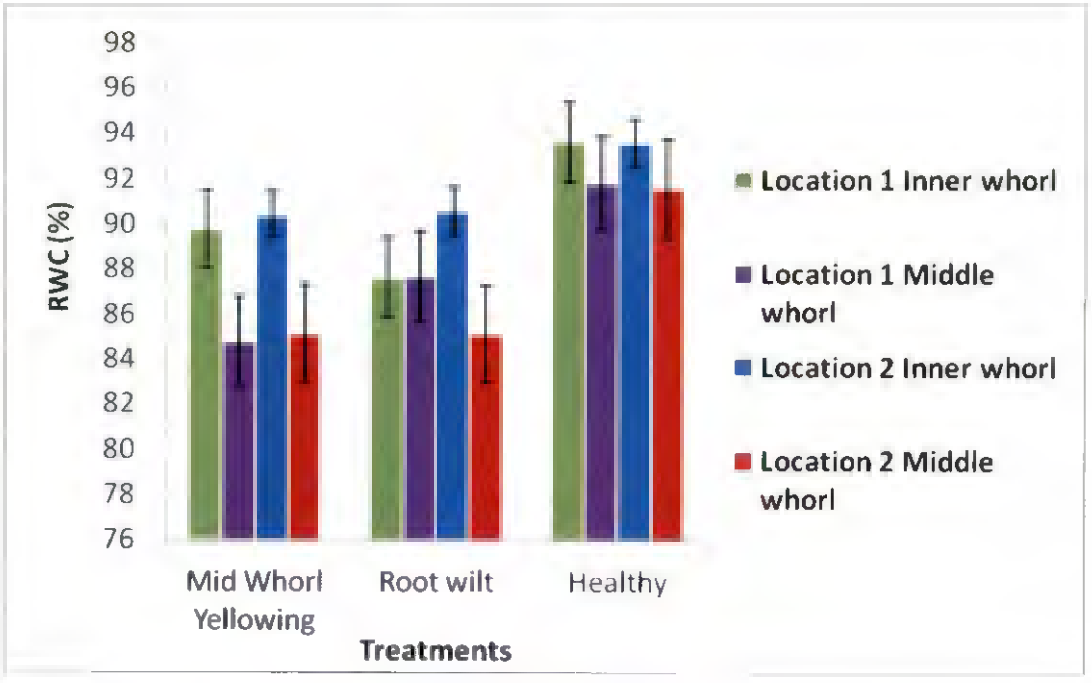
4.1.6. Relative Water Content

Data on the relative water content (RWC) of infected and healthy palms are depicted in (Fig 2). The palms with MWY and RW showed a reduction in the RWC compared to the healthy palms. The palms with mid whorl yellowing showed RWC of 89.77% and 84.79% in location 1 and 90.42% and 85.16% in location 2 in the inner and middle whorls respectively. Significant reduction was seen in the case of middle whorls of palms with MWY in both the locations and in the inner whorls in location 1 and middle whorls in location 2 of the root (wilt) affected palms. When the inner whorl and middle whorls of MWY affected palms were compared, a declining trend in the RWC of middle whorl was observed. The healthy palms showed RWC of 93.61% and 91.80% in location 1 and 93.61% and 91.59% in location 2 in the inner and middle whorls respectively. The declining trend was significant only in the case of the mid whorl yellowing affected palms in location 2.



MWY- Mid Whorl Yellowing

Fig 1. Loss of membrane integrity (membrane leakage) of coconut leaves affected with MWY



MWY- Mid Whorl Yellowing

Fig 2. Relative water content (RWC) of coconut leaves affected with MWY

Table 12. Phenol content (mg g^{-1}) of coconut leaves affected with MWY*

Treatments	Location 1		Location 2		Mean	
	IW	MW	IW	MW	Locn 1	Locn 2
MWY	19.70	16.75	16.00	17.75	18.22	16.87
RW	13.16	17.98	14.58	17.77	15.57	16.18
Healthy	19.84	23.94	19.70	20.16	21.89	19.93
Mean	17.57	19.56	16.76	18.56		
SE m (±)	2.10		1.65			
CD (PXW)	6.14		4.83			
SE m (±)	1.21		0.96			
CD (W)	3.54		2.79			

Percent change in the phenol content of palms with MWY

	Palms with MWY over healthy palms (%)		Palms with MWY over RW affected palms (%)	
	IW	MW	IW	MW
Location 1	(-) 0.73	(-) 30.03	(+) 49.62	(-) 6.85
Location 2	(-) 18.79	(-) 11.94	(+) 9.69	(-) 0.11

* Mean of 5 replications

MWY- Mid whorl yellowing RW – Root (wilt) IW- Inner whorl MW- Middle whorl

There was 4% and 7.6% reduction in the RWC in location 1 and 3.4% and 7.02% reduction in location 2 in the inner and middle whorls of palms with MWY compared to the healthy palms. The percent change in the RWC of mid whorl yellowing palms compared to the root wilt affected palms was highly varied. The inner whorl of palms with MWY were showing 2.4% higher RWC in location 1 and 0.03% higher RWC in middle whorl in location 2 compared to the root (wilt) affected palms. The middle whorl and inner whorl of the palms with mid whorl yellowing in location 1 and location 2 respectively showed a reduction in RWC compared to palms with RW disease.

4.1.7 Estimation of phenol

The healthy palms maintained higher phenol content compared to the infected palms (Table 12). The highest phenol content was observed in the middle whorl of the healthy palms in location 1 (23.94 mg g^{-1}). The palms with MWY showed a slight reduction in the phenol content, but the value was significantly lower only in the case of the middle whorl of the palms with MWY in location 1(16.75 mg g^{-1}). In the case of root (wilt) affected palms, the inner whorls showed a significantly lower phenol content (13.16 mg g^{-1}). While the middle whorl recorded values which were on par with that of the healthy palms.

The phenol content in the MWY affected palms showed 0.73% and 30.03% decrease in location 1 and 18.79% and 11.94 % in location 2 in the inner and middle whorls respectively when compared with the healthy palms. Compared to the root wilt palms, the palms with MWY showed increased phenol content (49.63% and 9.69% in inner whorls in location 1 and 2) and decreased phenol content (6.85% and 0.11 % in middle whorl in location I and 2).

4.1.8 Estimation of total soluble protein

The total soluble protein content of palms was estimated and the data is presented in table 13. The infected palms showed significantly higher protein content compared to the healthy palms. The peak protein content was shown by the middle whorl of the RW affected palms in location 2 (6.46 mg/g). The palms

Table 13. Total soluble protein content (mg g^{-1}) of coconut leaves affected with MWY*

Treatments	Location 1		Location 2		Mean	
	IW	MW	IW	MW	Locn 1	Locn 2
MWY	3.36	4.14	3.94	5.57	3.75	4.75
RW	3.70	5.81	4.09	6.46	4.76	5.27
Healthy	2.72	3.20	2.74	2.95	2.96	2.85
Mean	3.26	4.39	3.59	4.99		
SE m (±)	0.30		0.33			
CD (PXW)	0.88		0.95			
SE m (±)	0.17		0.19			
CD (W)	0.51		0.55			

Percent change in the total soluble protein content of palms with MWY

	Palms with MWY over healthy palms (%)		Palms with MWY over RW affected palms (%)	
	IW	MW	IW	MW
Location 1	(+) 23.77	(+) 29.17	(-) 9.03	(-) 28.81
Location 2	(+) 43.75	(+) 88.61	(-) 3.58	(-) 13.78

* Mean of 5 replications

MWY- Mid whorl yellowing RW – Root (wilt) IW- Inner whorl MW- Middle whorl

with MWY exhibited protein content of 3.36 mg g^{-1} and 4.14 mg g^{-1} in location 1 and 3.94 mg g^{-1} and 5.57 mg g^{-1} in location 2 in the inner and middle whorls respectively. The increased protein content was significant in the palms with MWY in all cases except in the inner whorl of palms in location1.The least protein content was recorded in the inner whorl of the healthy palm in location 1.

The protein content in the palms with MWY showed 23.77% and 29.17% increase in location 1 and 43.75% and 88.61 % in location 2 in the inner and middle whorls respectively when compared with the healthy palms. The palms with MWY recorded a decreased protein content (9.03% and 28.81% decrease in location 1 and 3.58% and 13.78 % in location 2 in the inner and middle whorls respectively) compared to the root (wilt) infected palms (Table 13).

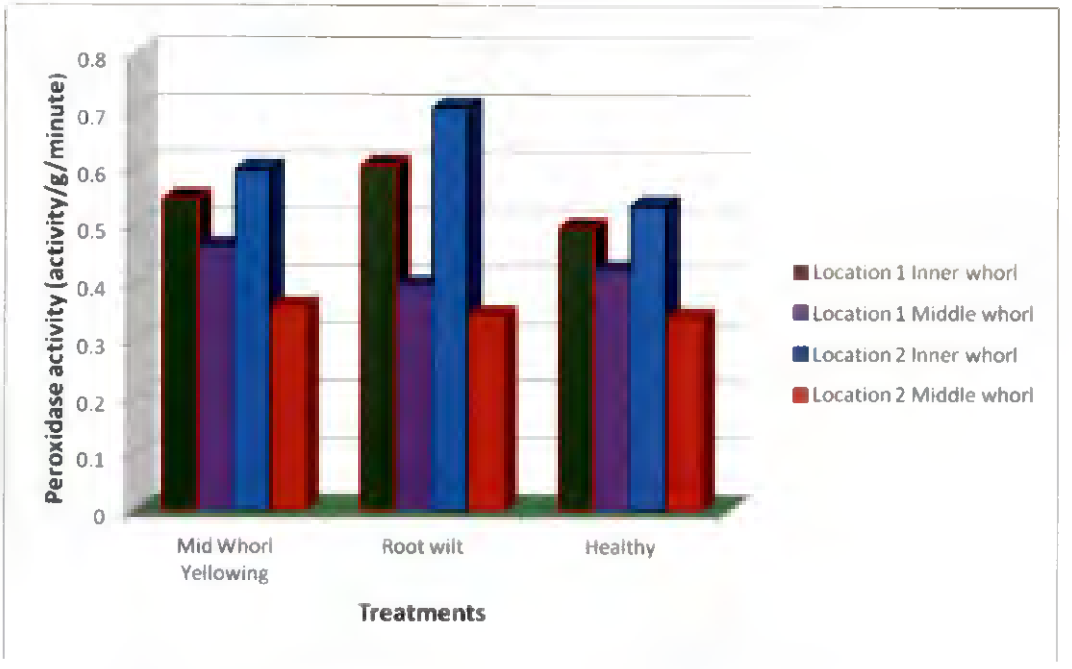
4.1.9 Antioxidant enzymes

4.1.9.1 Estimation of Peroxidase (POX)

All the palms except the middle whorl of the RW affected palms in location 1 exhibited an increased peroxidase activity compared to the healthy palms (Fig 3). But the increase in the POX activity was non significant in all cases. The highest activity was shown by the inner whorl of the palms with root (wilt) in location 2 (0.70 activity/ g/ min). The palms with MWY showed POX activity of 0.55 activity/ g/ min and 0.46 activity/ g/ min in location 1 and 0.60 activity/ g/ min and 0.36 activity/ g/ min in location 2 in the inner and middle whorls respectively. Across the whorls, a decrease in the peroxidase activity was observed in the middle whorls of all the selected categories of palms in location 1 and 2.

4.1.9.2. Estimation of Polyphenol oxidase (PPO)

The PPO activity of the infected and healthy palms was studied and the data are presented in table 14. There was a significant increase in the polyphenol oxidase activity in palms with MWY and RW compared to healthy palms. The healthy palms maintained lower activity in both the whorls and locations. In the case of palms with MWY, the middle whorl of palms in location 2 showed highest polyphenol oxidase activity (4.97 activity/g/min) than the inner whorl. The



MWY- Mid Whorl Yellowing

Fig 3. Peroxidase activity (activity/g/minute) of coconut leaves affected with MWY

Table 14. Polyphenol oxidase activity (activity/g/min) of coconut leaves affected with MWY*

Treatments	Location 1		Location 2		Mean	
	IW	MW	IW	MW	Locn 1	Locn 2
MWY	2.99	3.18	3.28	4.97	3.13	4.08
RW	3.71	4.50	4.46	5.45	4.08	4.98
Healthy	1.35	1.36	1.54	2.02	1.45	1.69
Mean	2.68	3.01	3.09	4.15		
SE m (±)	0.20		0.14			
CD (PXW)	0.57		0.42			
SE m (±)	0.11		0.08			
CD (W)	0.33		0.24			

Percent change in the polyphenol oxidase activity of palms with MWY

	Palms with MWY over healthy palms (%)		Palms with MWY over RW affected palms (%)	
	IW	MW	IW	MW
Location 1	(+) 121.57	(+) 112.62	(-) 19.28	(-) 26.50
Location 2	(+) 134.22	(+) 145.38	(-) 29.34	(-) 8.79

* Mean of 5 replications

MWY- Mid whorl yellowing RW – Root (wilt) IW- Inner whorl MW- Middle whorl

maximum activity was observed in the middle whorls of the palms with root (wilt) disease (5.45 activity/g/min). Among the palms also there was a significant increase in the activity from the inner whorl to the middle whorl (2.99 activity/g/min and 3.18 activity/g/min in the inner and middle whorl in location 1 and 3.28 activity/g/min and 4.97 activity/g/min in location 2 of the MWY affected palms).

The study revealed that the middle whorl of the palms with mid whorl yellowing in location 2 showed a maximum increase in the polyphenol oxidase activity of 145.38%. compared to the healthy palms the palms with mid whorl yellowing showed 121.57% and 112.62% increase in the polyphenols oxidase activity in location 1 and 134.22% and 145.38% in location 2 in the inner and middle whorl respectively. When the palms with MWY and RW were compared the palms with MWY showed 19.28% and 26.50% reduction in polyphenol oxidase activity in location 1 and 29.34% and 8.79% in location 2 in the inner and middle whorls respectively (table 14).

4.2. Soil analyses

The soil chemical properties like pH, EC, organic carbon, available macro and micronutrient status were analysed at two different depths (0-30 cm and 30-60cm) in two locations. The results did not show any significant variations indicative of any particular role for any of these soil characters in the development of yellowing symptom. The results are illustrated in table 15, 16, 17, 18, 19, 20 and 21.

4.3 Plant Nutrient Analyses

The index leaves (14^{th} leaf) were selected for various plant nutrient analyses. Analyses on plant nutrients indicated a wide variation in the values compared to the healthy palms.

Significant reduction in the nitrogen content was observed in the palms with MWY and RW affected palms in both the locations (Table 22). MWY affected palms in location 1 expressed the least nitrogen (1.62%) content. The root wilt palms in location 1 also showed reduction in nitrogen content (2.28%). The highest nitrogen content in location 1 was shown by the healthy palms (3.10%). In

Table 15. Soil characters in location 1

Treatments	pH		EC (dSm-1)		Organic carbon (%)	
	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm
MWY	5.00	4.80	0.05	0.05	1.20	1.30
RW	5.50	5.30	0.10	0.08	1.75	1.10
Healthy	5.65	5.20	0.09	0.09	1.40	0.84
Mean	5.38	5.10	0.08	0.08	1.45	1.08
SE m (±)	0.30	0.19	0.05	0.03	0.23	0.09
CD (0.05)	NS		NS		NS	

Table 16. Soil characters in location 2

Treatments	pH		EC (dSm-1)		Organic carbon (%)	
	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm
MWY	5.08	4.68	0.19	0.14	0.48	0.14
RW	5.22	5.12	0.07	0.07	0.74	0.24
Healthy	4.96	5.08	0.06	0.08	0.56	0.32
Mean	5.09	4.96	0.11	0.09	0.59	0.23
SE m (±)	0.29	0.18	0.07	0.05	0.10	0.07
CD (0.05)	NS		NS		NS	

MWY- Mid whorl yellowing RW- Root (wilt)

Table 17. Available nutrient status of soils in location 1

Treatment	Nitrogen (kg/ha)		Phosphorus (kg/ha)		Potassium (kg/ha)		Calcium (ppm)		Magnesium (ppm)	
	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm
MWY	385.70	374.20	42.70	18.80	433.40	468.60	260.00	178.00	81.60	68.40
RW	364.60	352.20	77.80	55.20	378.40	404.80	166.00	140.00	32.40	37.20
Healthy	380.70	371.30	46.90	22.70	444.40	332.20	154.00	102.00	42.00	55.20
Mean	359.70	348.60	55.80	32.20	276.70	401.90	193.30	140.00	52.00	53.60
SE m (±)	8.85	6.12	18.01	10.75	129.56	141.02	84.00	38.80	21.19	9.38
CD(0.05)	NS		NS		NS		NS		NS	

MWY- Mid whorl yellowing RW- Root (wilt)

Table 18. Available nutrient status of soils in location 2

Treatments	Nitrogen (kg/ha)		Phosphorus (kg/ha)		Potassium (kg/ha)		Calcium (ppm)		Magnesium (ppm)	
	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm
MWY	285.38	275.62	80.95	34.60	104.50	94.70	57.31	64.38	129.63	108.00
RW	290.64	230.15	128.05	79.20	143.00	138.20	86.83	87.01	159.25	186.25
Healthy	300.15	290.23	73.80	50.60	82.50	74.60	79.54	52.04	121.25	81.50
Mean	292.06	795.97	84.27	54.80	110.00	102.50	74.56	67.81	136.71	125.25
SE m(±)	8.34	14.32	20.01	24.32	28.02	24.78	38.80	21.19	33.41	46.12
CD(0.05)	NS		NS		NS		NS		NS	

MWY- Mid whorl yellowing RW- Root (wilt)

Table 19. Available micronutrient status of soils in location 1

Treatments	Iron (ppm)		Manganese (ppm)		Copper (ppm)		Zinc (ppm)	
	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm
MWY	240.56	237.94	34.94	40.82	0.60	0.44	3.60	4.08
RW	198.40	171.46	29.12	48.02	1.50	0.78	2.70	2.80
Healthy	266.92	258.62	31.68	27.68	1.36	1.26	2.72	1.96
Mean	235.29	222.67	31.91	38.84	1.15	0.83	3.01	2.95
SE m(±)	74.94	54.84	10.92	13.83	0.45	0.46	0.90	1.70
CD(0.05)	**NS**		**NS**		**NS**		**NS**	

MWY- Mid whorl yellowing RW- Root (wilt)

Table 20. Available micronutrient status of soils in location 2

Treatments	Iron (ppm)		Manganese (ppm)		Copper (ppm)		Zinc (ppm)	
	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm
MWY	582.64	437.94	63.2	53.61	1.32	0.98	11.33	9.23
RW	642.75	471.46	65.77	58.04	1.98	0.56	8.50	7.50
Healthy	584.74	558.62	70.62	67.21	2.03	0.85	25.13	20.96
Mean	603.38	489.34	66.53	59.62	1.78	0.80	14.99	12.56
SE m(±)	152.35	163.45	12.36	15.26	0.54	0.47	1.59	5.64
CD(0.05)	NS		NS		NS		NS	

MWY- Mid whorl yellowing RW- Root (wilt)

Table 21. Available boron (ppm) content in soils of location 1 and 2

	Location 1		Location 2	
Treatments	**30 cm**	**60 cm**	**30 cm**	**60 cm**
MWY	0.20	0.26	0.21	0.13
RW	0.12	0.18	0.23	0.22
Healthy	0.16	0.22	0.18	0.16
SE m(±)	0.44	0.10	0.56	0.11
CD(0.05)	NS		NS	

MWY- Mid whorl yellowing RW – Root (wilt)

location 2 the nitrogen content of the mid whorl yellowing palms and root wilt palms were on par (1.73% and 2.69% respectively) but were significantly reduced compared to the healthy palms (2.89%).

The palms with MWY exhibited 47.74% and 40.14% reduction in the nitrogen content in location 1 and location 2 respectively compared to the healthy palms. When the root (wilt) affected palms were compared the mid whorl yellowing palms showed 28.95% and 35.69% reduction in the nitrogen content in location 1 and 2 respectively.

Data on the phosphorus content of coconut leaves are depicted in (Fig 4). There was no significant variation in the phosphorus content in the palms with MWY and RW affected palms compared to the healthy palms in both the locations. But the phosphorus content was slightly reduced in the MWY affected palms compared to the healthy palms.

The results on the potassium content are shown in table 23. Significant accumulation in potassium content was observed in both the MWY and RW affected palms compared to the healthy palms. The palms with MWY showed 3.78% and 3.66% potassium content in location 1 and 2 respectively which were on par with the root wilt palms (3.66% and 3.68%). The control palms showed the least potassium content (2.74% and 2.84%).

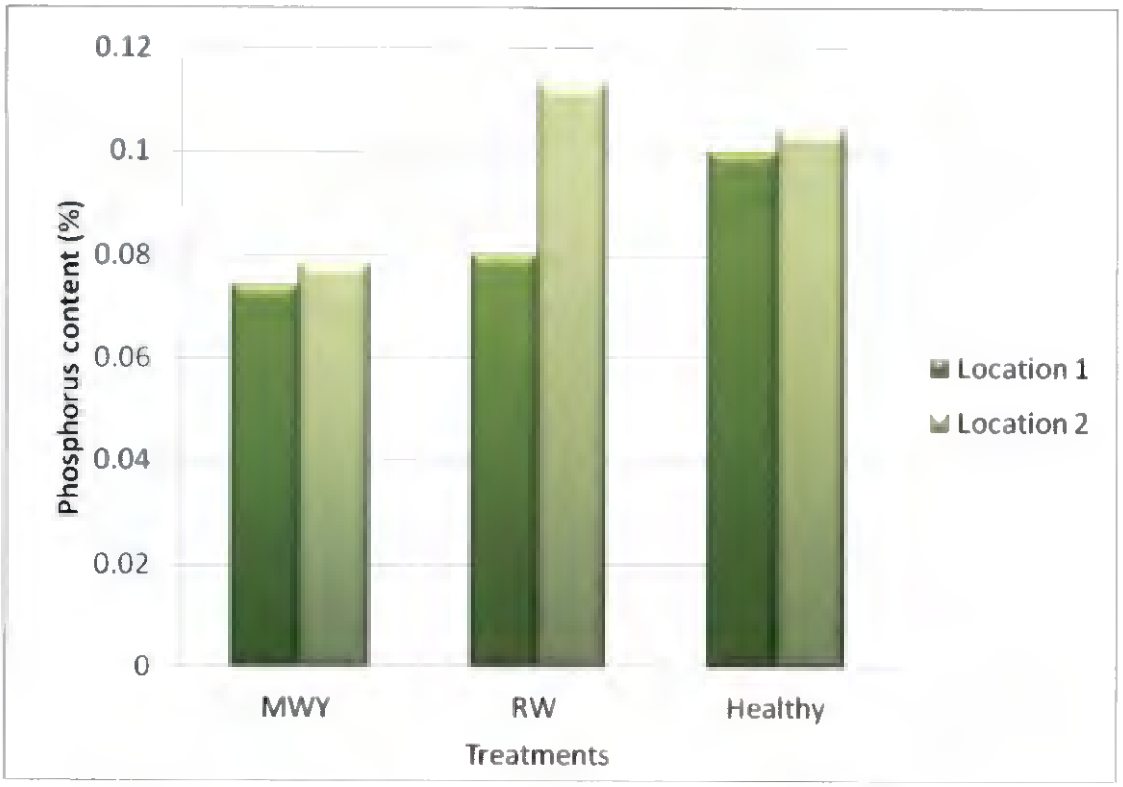
The palms with MWY showed 37.96 and 28.87 % increase in the potassium content compared to the healthy palms in location 1 and 2 respectively. The change in the potassium content compared to the RW affected palms was insignificant.

Data on the calcium content of the coconut leaves is shown in table 24. No significant variation was observed in the case of calcium content in the selected palms. The palms with mid whorl yellowing showed calcium content of 5.00% and 5.66% in location 1and 2 respectively which is slightly more than the healthy palms (4.66% and 4.02%). The root (wilt) affected palms also recorded slightly increased calcium content (4.76% and 6.42%).

Table 22. Nitrogen content (%) of coconut leaves affected with MWY*

Treatments	Location 1	Location 2
MWY	1.62	1.73
RW	2.28	2.69
Healthy	3.10	2.89
Mean	2.33	2.44
SE m (±)	0.18	0.16
CD (0.05)	0.58	0.51
Percent change in nitrogen content of palms with yellowing		
	Palms with MWY over healthy palms (%)	**Palms with MWY over RW affected palms (%)**
Location 1	(-) 47.74	(-) 28.95
Location 2	(-) 40.14	(-) 35.69

MWY- Mid whorl yellowing RW – Root (wilt)



MWY- Mid Whorl Yellowing RW- Root (wilt)

Fig 4. Phosphorus content (%) of coconut leaves affected with MWY

Table 23. Potassium content (%) of coconut leaves affected with MWY

Treatments	Location 1	Location 2
MWY	3.78	3.66
RW	3.66	3.68
Healthy	2.74	2.84
Mean	3.39	3.39
SE m (±)	0.15	0.19
CD (0.05)	0.49	0.62
Percentage change in potassium content of palms with mid whorl yellowing		
	Palms with MWY over healthy palms (%)	**Palms with MWY over RW affected palms (%)**
Location 1	(+) 37.96	(+) 3.28
Location 2	(+) 28.87	(-) 0.54

Table 24. Calcium content (%) of affected coconut leaves

Treatments	Location 1	Location 2
MWY	5.00	5.66
RW	4.76	6.42
Healthy	4.66	4.02
Mean	4.81	5.37
SE m(±)	0.50	0.45
CD(0.05)	NS	NS

MWY- Mid whorl yellowing RW – Root (wilt)

Table 25.Magnesium content (%) of coconut leaves affected with MWY

Treatments	Location 1	Location 2
MWY	0.17	0.21
RW	0.21	0.20
Healthy	0.22	0.25
Mean	0.20	0.22
SE m (±)	0.01	0.01
CD (0.05)	0.04	0.04
Percentage change in magnesium content of coconut palms with yellowing		
	Palms with MWY over healthy palms (%)	**Palms with MWY over RW affected palms (%)**
Location 1	(-) 22.72	(-) 19.05
Location 2	(-) 16.00	(+) 5.00

MWY- Mid whorl yellowing RW – Root (wilt)

Table 26. Iron content (ppm) of coconut leaves affected with MWY

Treatments	Location 1	Location 2
MWY	426.36	428.80
RW	398.50	416.80
Healthy	440.70	278.44
Mean	421.85	374.68
SE m (±)	50.61	41.58
CD (0.05)	NS	135.58
Percentage change in Iron content of coconut palms with yellowing		
	Palms with MWY over healthy palms (%)	**Palms with MWY over RW affected palms (%)**
Location 1	(-) 14.34	(+) 6.99
Location 2	(+) 54.00	(+) 2.90

Table 27. Manganese content (ppm) of coconut leaves affected with MWY

Treatments	Location 1	Location 2
MWY	98.36	199.28
RW	123.95	197.12
Healthy	156.70	86.68
Mean	126.34	161.03
SE m(±)	35.88	47.51
CD (0.05)	NS	NS

MWY- Mid whorl yellowing RW – Root (wilt)

Table 28. Copper content (ppm) of coconut leaves affected with MWY

Treatments	Location 1	Location 2
MWY	13.56	19.44
RW	10.80	23.88
Healthy	11.40	14.32
Mean	11.92	19.21
SE m(±)	2.87	5.29
CD (0.05)	NS	NS
Percentage change in Copper content of coconut palms with yellowing		
	Palms with MWY over healthy palms (%)	**Palms with MWY over RW affected palms (%)**
Location 1	(+) 18.95	(+) 25.55
Location 2	(+) 35.75	(-) 18.66

MWY- Mid whorl yellowing RW – Root (wilt)

Table 29. Zinc content (ppm) of affected coconut leaves

Treatments	Location 1	Location 2
MWY	50.50	46.70
RW	55.75	48.30
Healthy	61.75	61.60
Mean	56.00	52.20
SE m (±)	2.74	1.59
CD (0.05)	NS	4.90
Percentage change in Zinc content of coconut palms with yellowing		
	Palms with MWY over healthy palms (%)	**Palms with MWY over RW affected palms (%)**
Location 1	(-) 18.22	(-) 9.41
Location 2	(-) 24.19	(-) 3.31

MWY- Mid whorl yellowing RW – Root (wilt)

Table 30. Mean cycle threshold (Ct) value and melting temperature (Tm) of the product

Sample	Mean Ct	Tm
M1	34.05	82.50
M2	32.40	82.00
M3	No Ct	57.23
M4	32.92	82.53
M5	No Ct	57.74
R	32.19	82.53
C1	37.07	82.50
C2	No Ct	74.75
C3	32.69	82.50
C4	No Ct	58.77
C5	No Ct	57.24
NTC	No Ct	56.74
Sugarcane Plasmid	24.33	82.00

Magnesium content in the 14th leaf of coconut was estimated and the data is presented in the table 25. Significant reduction in the magnesium content was observed in the case of palms with MWY compared to the healthy palms. The palms with MWY showed magnesium content of 0.17% and 0.21% in location 1 and 2 respectively. The magnesium content of the RW affected palms (0.21%) in location 1 was on par with the healthy palms, but in location 2 it was significantly lower than the healthy palms.

Compared to the healthy palms, the palms with MWY showed 22.72% and 16% reduction in the magnesium content in location 1 and 2 respectively. In location 1, there was 19.05% reduction and in location 2, 5.00% increase in the magnesium content in the MWY affected palms compared to the root wilt palms.

Iron content of the selected palms was analysed and the data is presented in table 26. No significant variation was observed in the iron content in palms with MWY and root (wilt) affected palms compared to the healthy palms in location 1. Slight reduction was observed in the iron content of MWY and RW affected palms in location 1. Mean while in location 2 there was significant accumulation of iron content in both the MWY and RW affected palms. The palms with MWY recorded iron content of 426.36 ppm and 428.8 ppm in location1 and 2 respectively. The least iron content in location1 was observed in the case of RW affected palms 398.5 ppm and in location 2 in the case of healthy palms 278.44 ppm.

Palms with MWY showed 14.34 % reduction in location 1 and 54.00% increase in location 2 in the iron content compared to the healthy palms. Compared to the root wilt palms, the mid whorl yellowing palms showed 6.99% and 2.90% increase in the iron content in location 1 and 2 respectively.

Data on the manganese content of coconut leaves is presented in table 27. The manganese content of the palms with MWY and RW did not maintain the same trend in the two locations. But in both the cases the value was not significant.

Copper content was analysed in the selected palms and the data is presented in table 28. No significant variation was observed in the MWY affected

palms and root (wilt) palms in both the locations. But the mid whorl yellowing palms maintained a slightly higher copper content compared to the healthy palms in both the locations. The mid whorl yellowing palms showed copper content of 13.56 ppm and 19.44 ppm in location 1 and 2 respectively. In the case of root wilt palms, the copper content was 10.8 ppm in location 1 which was lower than the healthy palms (11.40 ppm). In location 2 the copper content of the root wilt palms was 23.88 ppm which was slightly higher than the healthy palms (14.32 ppm).

The zinc content of coconut leaves was estimated and the data is illustrated in table 29. Both the MWY and RW palms exhibited reduced zinc content, but the reduction was significant only in location 2. The mid whorl yellowing palms showed zinc content of 50.5 ppm and 46.7 ppm in location 1 and 2. The root (wilt) palms showed zinc content of 55.75 ppm and 48.3 ppm.

The mid whorl yellowing palms exhibited 18.22% and 24.19% reduction in the zinc content compared to the healthy palms in location 1 and 2 respectively. Compared to the root wilt palms, the mid whorl yellowing palms showed 9.41% and 3.31% reduction in the zinc content.

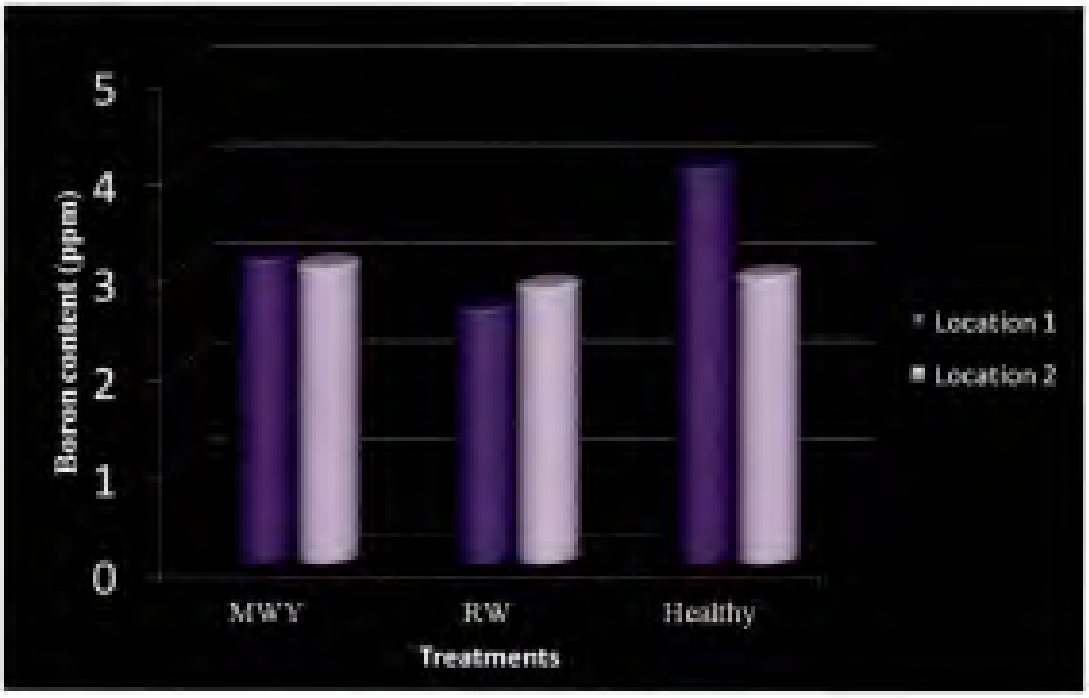
Boron content in the selected palms did not show any significant variation compared to the healthy palms (Fig 5). A slight decrease in the boron content was observed in palms with MWY (3.12 ppm) in location 1, while a slight increase (3.04 ppm) was observed in location 2 compared to the healthy palms. The RW affected palms in both the cases (2.62 ppm and 2.83 ppm) maintained lower boron content compared to the healthy palms.

4.4 Soil moisture status

The soil moisture content was estimated by gravimetric method and the result obtained is expressed in the (Fig 6). There was no significant variation in the soil moisture status between healthy and affected palms.

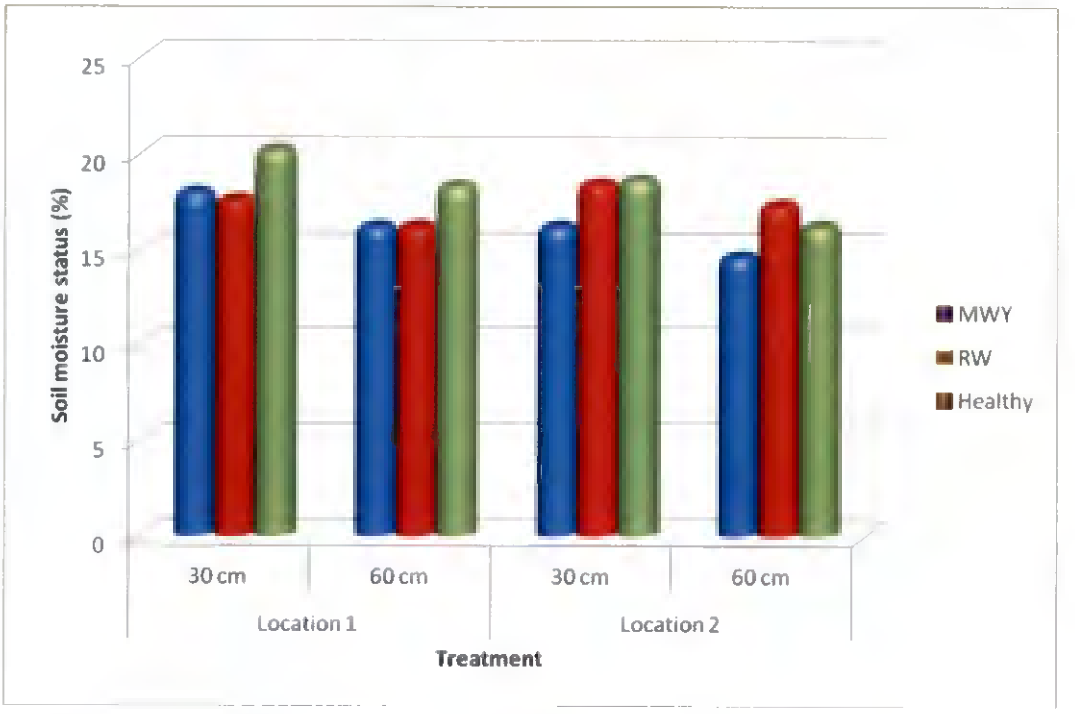
4.5 Anatomical Analyses

Scanning Electron Microscopy (SEM) technique was used to detect phytoplasmas in the leaf tissues of coconut. Spindle leaves were selected for the study. Samples were incised with razor, attached to SEM stubs and coated with



MWY- Mid Whorl Yellowing

Fig 5. Boron content of coconut leaves affected with MWY



MWY- Mid Whorl Yellowing RW- Root (wilt)

Fig 6. Soil moisture status at two locations

palladium in an ion sputter (DESK II, Denton Vacuum), for 40 seconds. Prepared specimens were examined in a SEM (S-3000N Hitachi) at 5-20 kV.

Phytoplasma cells were detected in the phloem tissue of both MWY and RW samples analysed. They were observed as spherical bodies. They appeared as separate cells or clustered particles in phloem tissue (Plate 8).SEM images of infected brinjal plants was also taken (Plate 9).

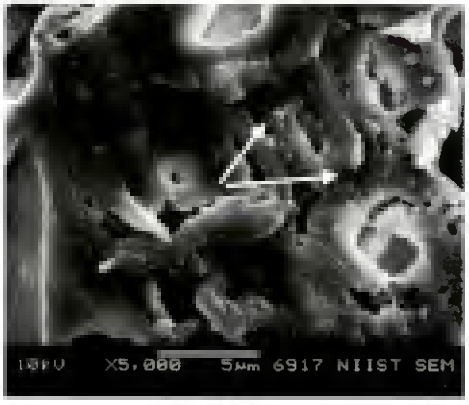
Morphological and anatomical analyses of fresh roots were also carried out in the selected palms. Pencil thick root of uniform size and maturity were selected for the study. In the control set of palms, roots were found to be healthy (Plate 10). Both the categories of the affected palms showed browning and necrotic symptoms, but the chance of getting deteriorating roots was more in the case of the root (wilt) affected palms. The palms with different types of yellowing had roots exhibiting browning and tissue damage (Plate 11). The anatomical analyses showed that there was internal browning of vascular elements extending into the cortex (Plate 12). Disintegration of vascular elements was also seen in palms with MWY and RW.

4.5 Molecular analyses

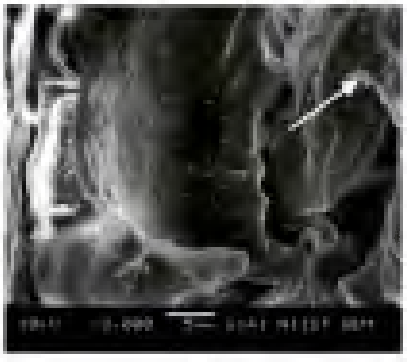
The DNA was extracted from 3 g of spindle leaf (two to three leaflets) midrib tissues (Plate 13) using a modified phytoplasma enrichment protocol (Ahrens and Seemuller, 1992). Grassy shoot diseased sugarcane samples collected from Sugarcane Breeding Institute (Coimbatore, India), and infected catharanthus samples obtained from Department of Plant Pathology, College of Agriculture, Vellayani were used as positive control.

The presence of phytoplasma in the selected samples were checked by nested PCR analysis using phytoplasma specific universal primers-P1/P7-R16F2n/ R16R2, Phytoplasma 16S rDNA specific semi nested primers 1F7/7R3 - 1F7/7R2 and by real time PCR technique using real time primers QPF2/R2. Real-time PCR was performed with Stratagene MX3005P machine (Agilent Technologies) and the results were analyzed using MxPro software.

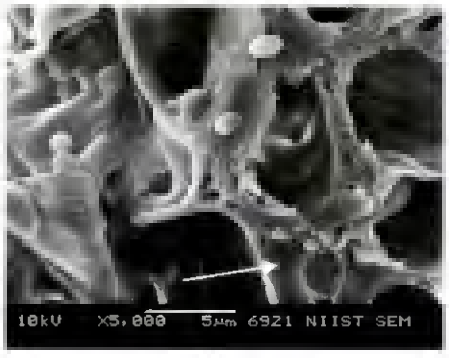
a)



b)



c)



d)

e)

f)

Plate 8 . Scanning electron micrographs of phytoplasmas in phloem tissues of coconut leaves a) leaves of MWY under 8500X magnification b) leaves of MWY under 5000X magnification c) leaves of RW under 3000X magnification d) leaves of RW under 5000X magnification (e) & (f) healthy palms no phytoplasma. Arrows indicate: phytoplasma cells

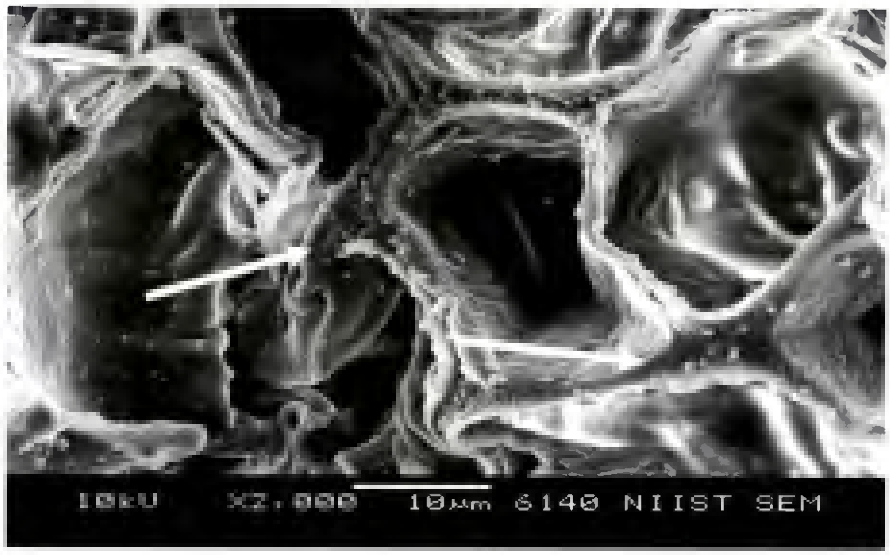


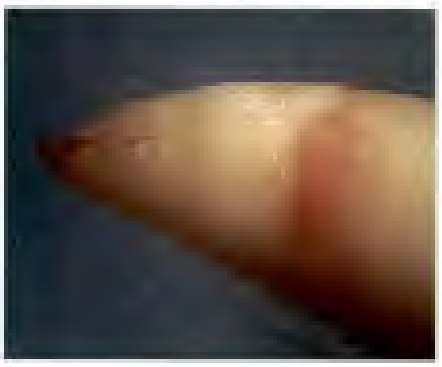
Plate 9. Scanning electron micrographs of phytoplasmas in phloem tissues of infected brinjal leaves

Leaves of brinjal under 2000X magnification Arrows indicate: phytoplasma cells

Plate 10. Roots of palms with yellowing in comparison with healthy palms

a)

b)



c)

Plate 11. Root tips showing necrosis

a) Root tips of palms with MWY b) Root tips of palms with RW

c) Root tips of healthy palms

a)

b)

c)

Plate 12. Transverse sections of root tips showing necrosis

a) Root tips of palms with MWY b) Root tips of palms with RW

c) Root tips of healthy palms

a)

b)

c)

Plate 13. Cross section of roots showing browning and vascular disintegration

a) Root section of palms with MWY b) Root section of palms with RW

b) Root section of healthy palms

Plate 14. Spindle leaves of the selected palms

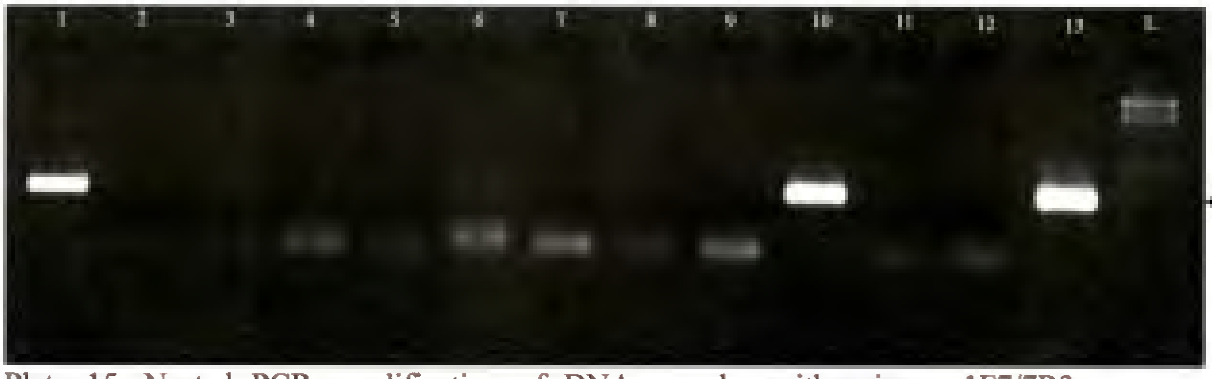


Plate 15. Nested PCR amplification of DNA samples with primers 1F7/7R3 - 1F7/7R2 (amplicon size - 490 bp). Lanes 1– Root wilt palms. Lane 2-6 – Mid whorl yellowing palms. Lane 7-11 Healthy palms, Lane 12- NTC, Lane 13- Sugarcane grassy shoot sample. Lane 14– 1Kb DNA ladder (band size from bottom – 250 bp, 500 bp, 750 bp, 1, 000 bp etc.)

4.5.1 Nested PCR (Location 1)

Nested PCR with the phytoplasma 16S rDNA specific semi nested primers 1F7/7R3 - 1F7/7R2 yielded an amplicon of 493 bp in the mid whorl yellowing palms, root (wilt) palms and the sugarcane positive control (sugarcane grassy shoot) in location 1 (Plate 14). 2 out of 5 healthy palms also showed amplification indicating latent infection before symptom expression. Nested PCR with semi nested primers 1F7/7R3 - 1F7/7R2 was carried out in another 5 sets of mid whorl yellowing palms in which all 5 showed amplification (Plate 15). No amplification was seen in the NTC.

Location 2

Nested PCR with the phytoplasma 16S rDNA specific semi nested primers 1F7/7R3 - 1F7/7R2 was also carried out in the samples collected from location 2. An amplicon of 490 bp was obtained in mid whorl yellowing palms, root (wilt) palms and catharanthus positive sample. No amplicon was obtained for the healthy palms (Plate 16).

Nested PCR with phytoplasma specific universal primer pairs P1/P7-R16F2n/ R16R2 yielded an amplicon of 1.2 kb in mid whorl yellowing palms and root (wilt) palm selected and the catharanthus positive sample. No amplification was observed in the healthy palms and NTC (Plate 17).

4.5.2 Real Time PCR

Real time PCR using real time primers QPF2/R2 yielded an amplicon of 140 bp in the root wilt palms, 3 mid whorl yellowing palms and 2 healthy palms. In the amplification plot, the mean Ct value of coconut samples ranged between 32 and 37. One sample showed a higher Ct value of 37 which indicates the low titer of phytoplasma in the host. The Ct value of sugarcane positive control was 24.33 and NTC did not produce fluorescence signal and hence no Ct (Fig 7 & table 30)

4.5.3 Sequence Analysis

The PCR product obtained from the semi-nested PCR were sequenced and analysed. The sequence showed 89% similarity with the root (wilt) phytoplasma (Table 31.)

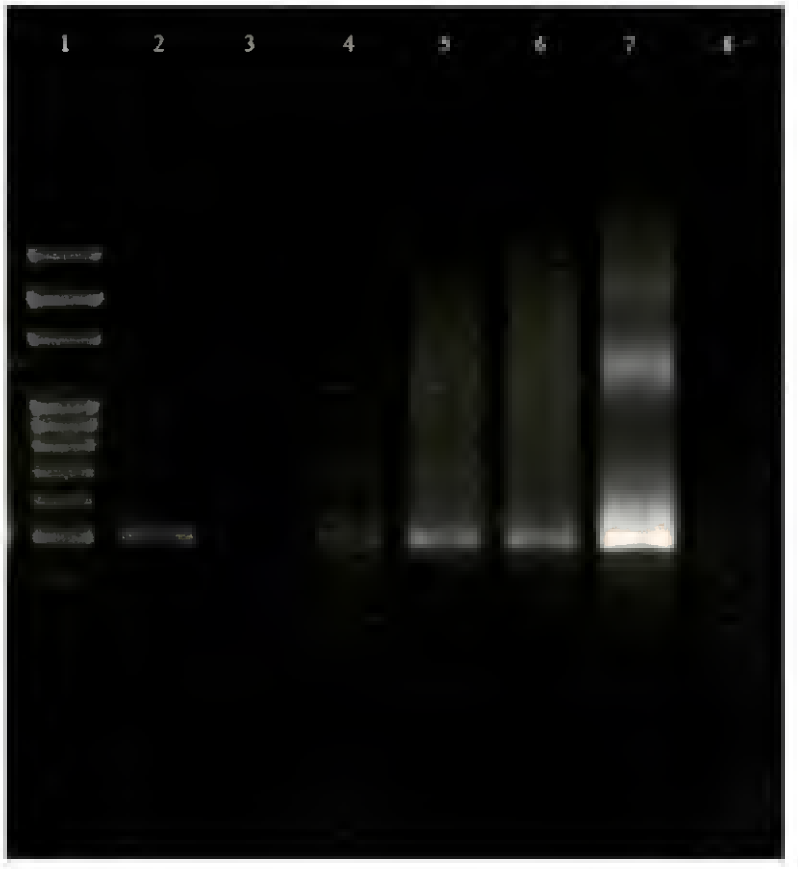


Plate 16. Nested PCR amplification of DNA samples with primers 1F7/7R3 - 1F7/7R2 (amplicon size - 490 bp). Lane 1 100 bp Lanes 2– 6 Mid whorl yellowing palms. Lane 7 Catharanthus positive sample Lane 8- NTC (ladder band size from bottom – 100 bp, 200 bp, 300 bp, 400 bp, 500 bp etc.)

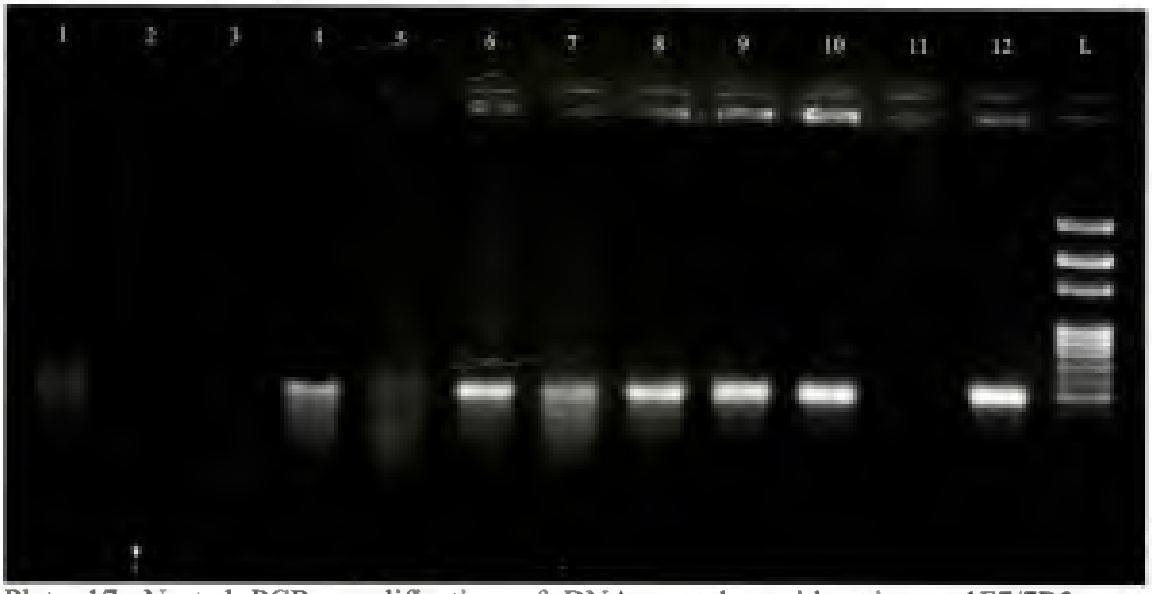


Plate 17. Nested PCR amplification of DNA samples with primers 1F7/7R3 - 1F7/7R2 (amplicon size - 490 bp). Lane 1-5 Mid whorl yellowing palms. Lanes 6– 10 Root wilt palms. Lane 11-Healthy palms. Lane 12- Catharanthus positive sample Lane 13- 100 bp (ladder band size from bottom – 100 bp, 200 bp, 300 bp, 400 bp, 500 bp etc.)

Plate 18. Nested PCR amplification of DNA samples with primers P1/P7-R16F2n/ R16R2 (amplicon size – 1.2 kbp). Lane 1-5 Mid whorl yellowing palms. Lanes 6 Root wilt palms. Lane 7 Healthy palms. Lane 8- Catharanthus positive sample. Lane 9- NTC Lane 10- 100 bp (ladder band size from Top – 3000 bp, 2000 bp, 1000 bp etc.)

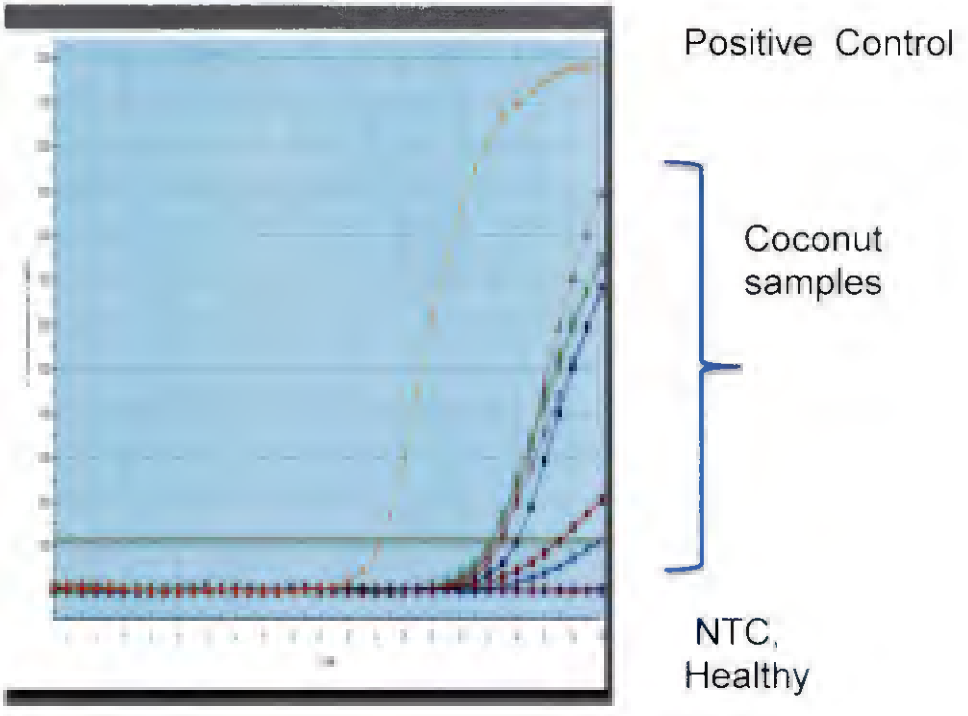


Fig 6. Amplification plot for diseased coconut samples. The Ct value is calculated by plotting the normalized fluorescence against the number of cycles. NTC have no Ct

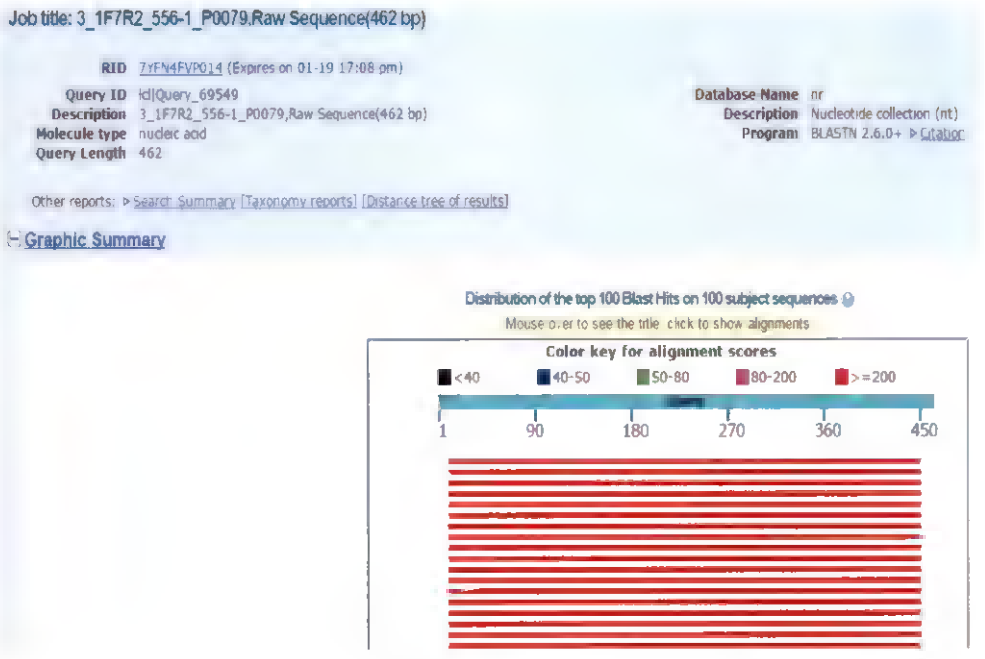


Fig 7. Blast analysis of the sequence amplified by the primer 1F7R2

Table 31. Percent sequence identity between the nucleotide sequences of the MWY phytoplasma isolates with selected phytoplasma

Sequences used for comparison	**Sequence identity (%)**
Coconut palm root (wilt) phytoplasma isolate Kottayam 16S ribosomal RNA gene, partial sequence (KP739433.1)	89
Coconut palm root (wilt) phytoplasma isolate Sullia 16S ribosomal RNA gene, partial sequence (KP739429.1)	87
Sugarcane grassy shoot phytoplasma clone TH04 16S ribosomal RNA gene, partial sequence (KX871707.1)	89
Coconut lethal yellowing phytoplasma strain LYC347 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer and tRNA-Ile, complete sequence; and 23S ribosomal RNA gene, partial sequence (DQ645635.1)	87

4.6 Experiment II Effect of enhanced carbon dioxide on phytoplasmal responses

The second experiment was formulated to understand the effect of changing climate on phytoplasmal response. The systems selected for this study are six months old coconut seedlings showing yellowing and little leaf infected catharanthus and brinjal plants (Plate 19 & 20).

Coconut seedlings showing yellowing were selected from the Instructional Farm, College of Agriculture, Vellayani. Nested PCR analysis was done with phytoplasma specific universal primer pairs P1/P7-R16F2n/ R16R2. No amplification was observed in any of the seedlings except for the catharanthus positive control (Plate 21). Since no amplification was obtained for the phytoplasma, the seedlings were not placed inside the OTC.

Catharanthus and brinjal plants infected with phytoplasma maintained at the Department of Plant Pathology were utilised for grafting to the healthy catharanthus and brinjal plants maintained at the Department of Plant Physiology. After the grafts were established, the plants were transferred into the Open Top Chamber (A chamber) and exposed to CO_2 at 500 ppm for a period of one month (Plate 31-34). Molecular analyses were carried out in the 15^{th} and 30^{th} day of exposure. Various physiological analyses were carried out in plants after one month of exposure to enriched carbon dioxide condition.

4.6.1 Graft Transmission

Graft transmission was carried out by wedge grafting of phytoplasma infected scion on to healthy root stock and observed for the symptom development. The initial symptoms were noticed in the new sprouts of the rootstock 7-10 days after the graft transmission (plate 22-30).

After exposure period, the infected plants showed improved growth and development (Plate 35).

4.6.2 Molecular analyses

The DNA isolation was done using DNeasy Plant Mini Kits (Qiagen) after 15^{th} day of exposure and 30^{th} day of exposure. 50 ng DNA was used for the real time PCR.

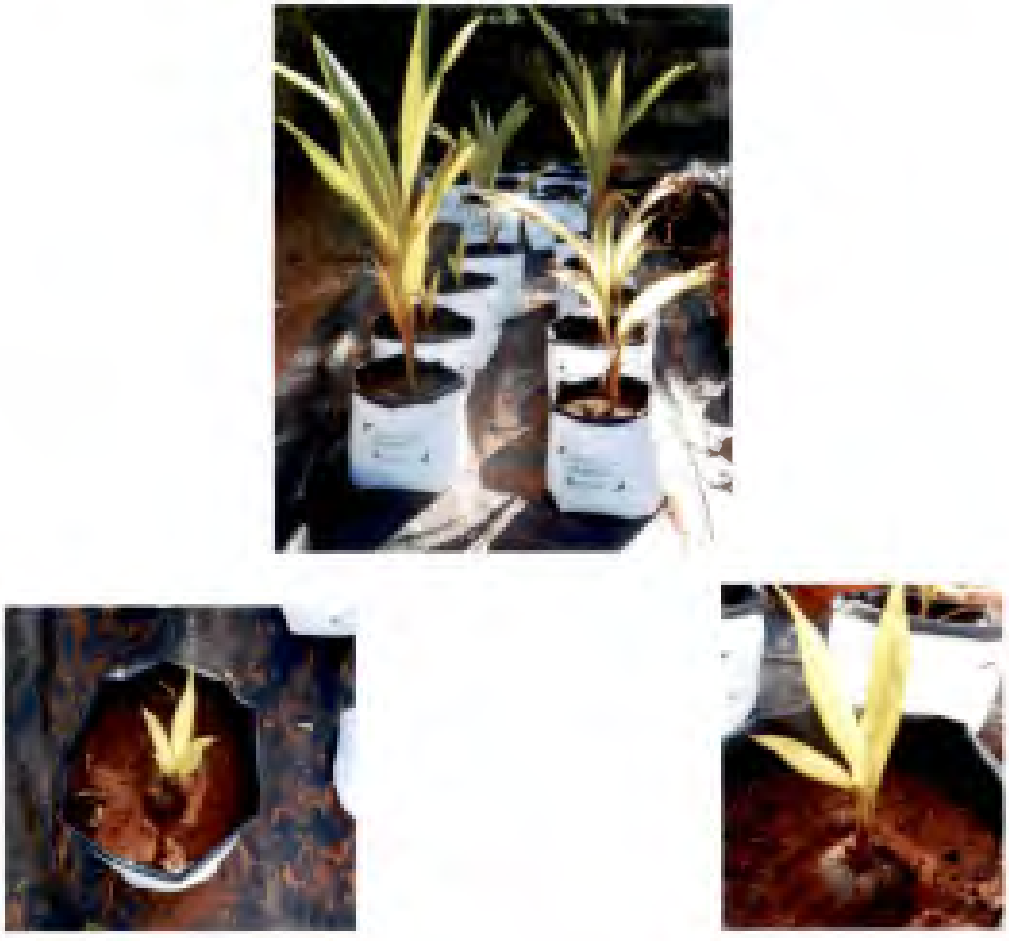


Plate 19. Coconut seedlings showing yellowing symptoms

Plate 20. Comparison of healthy and infected catharanthus plants

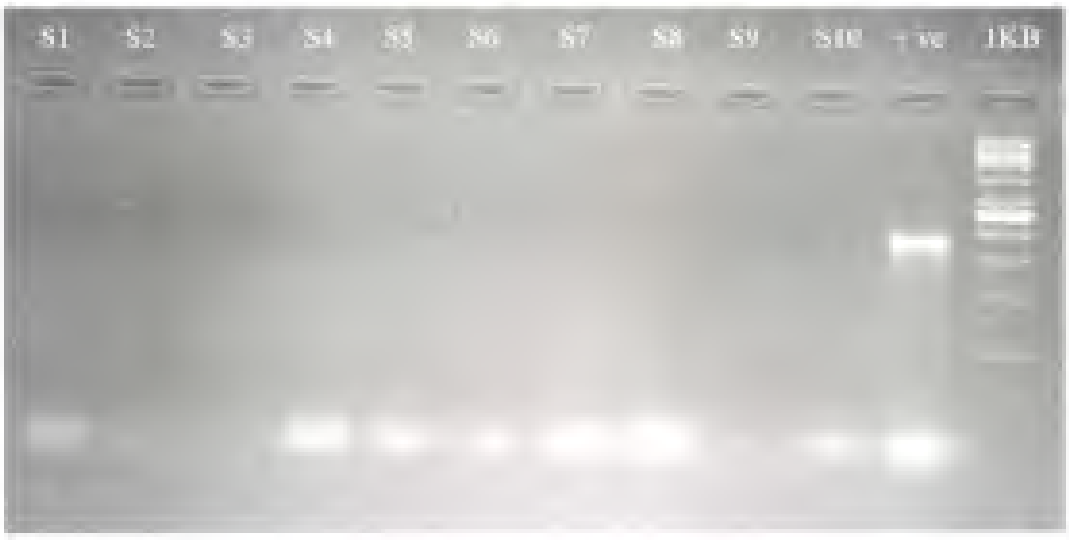


Plate 21. Nested PCR amplification of DNA samples with primers P1/P7-R16F2n/ R16R2 (amplicon size – 1.2 kbp). Lane 1-10 Seedlings showing yellowing, Lane 11- Catharanthus positive control Lane 12- 1 kbp (ladder band size from bottom – 250 bp, 500 bp, 1000 bp etc.)

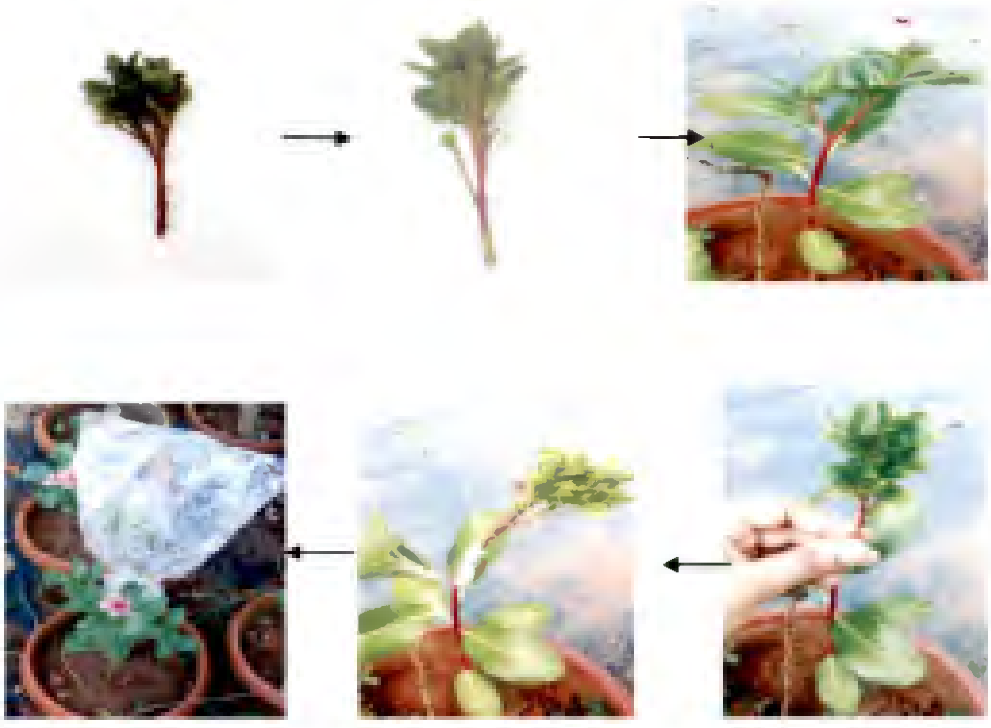


Plate 22. Different stages of graft inoculation in catharanthus

Plate 23. Grafts established after inoculation

a) Catharanthus b) Brinjal

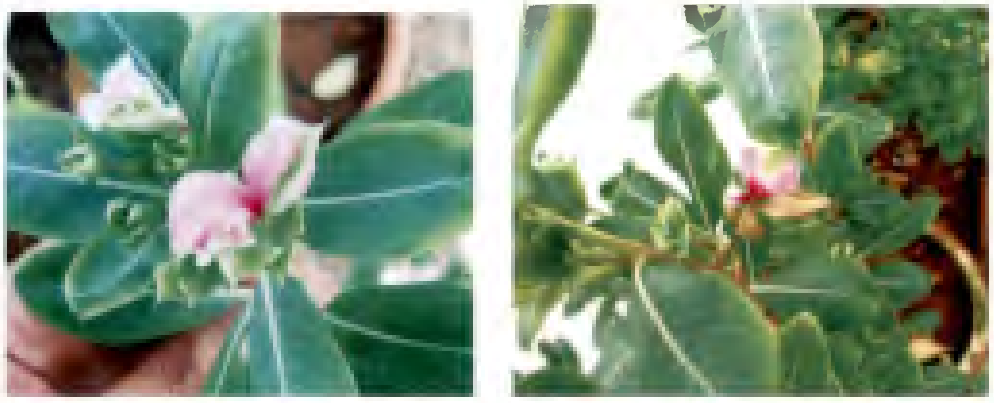
Plate 24. Symptom expression on new sprouts after graft inoculation

a) Catharanthus b) Brinjal

Plate 25. Symptom expression: Little leaf symptom

Plate 26. Floral symptom: green phylloid flowers in catharanthus

Plate 27. Floral symptom: green phylloid flowers in brinjal



a) b)

Plate 28. a) Floral symptom : Colour degradation at early stage b) Colour degradation at advanced stage

Plate 29. Normal flowers of healthy catharanthus plants

Catharanthus

Brinjal

Plate 30. Symptom expression: Yellowing symptom

Plate 31. Open Top Chamber (OTC) general view and sensors

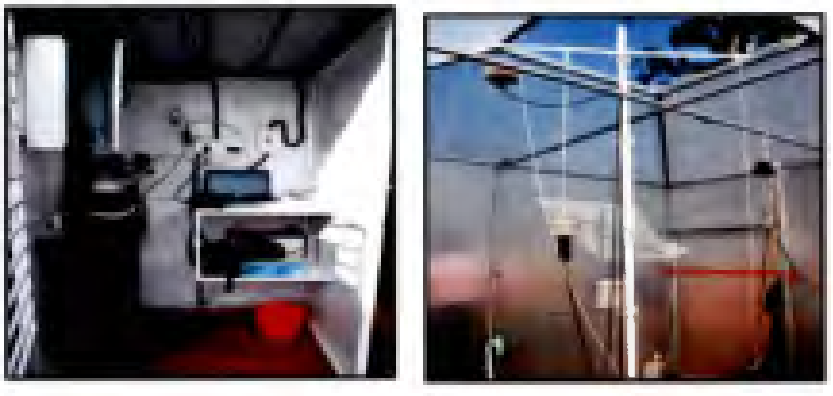


Plate 32. Sensors in Open Top Chamber

Plate 33. Plants kept in Open Top Chamber

Plate 34. Plants kept in open condition

a) OTC b) Open condition

A

a) OTC b) Open condition

B

Plate 35.Infected plants after exposure period A. Catharanthus B. Brinjal

4.6.2.1Real Time PCR

Real Time PCR was carried out with real time primers QPF2/R2 (Fig 7). Real-time PCR was performed with Applied Biosystems and the results were analyzed. It did not reveal any significant changes in the phytoplasmal load.

4.6.2.1 PCR

Normal PCR was carried out with the real time primers BCF2/R2 (AATGGAGGAGGAAACTCTGAC/TACCAGGGTATCTAATCCTG). An amplicon of 238 bp was obtained for all the samples. But no significant change was observed in the phytoplasmal load in our study (Plate 36).

4.6.3 Biochemical Parameters

Various biochemical parameters like reducing sugar content, total soluble protein content, phenol content, antioxidant enzyme activity- Peroxidase, Polyphenol oxidase and Phenyl Ammonia Lyase activity were carried out in both the catharanthus and brinjal plants kept inside OTC and in open condition after one month of exposure to enriched CO_2 condition.

4.6.3.1 Reducing sugar content

The infected plants both catharanthus and brinjal exposed to enhanced CO_2 concentration showed an accumulation of reducing sugar (table 32) compared to the plants kept in open condition. The infected catharanthus plants kept inside OTC showed a reducing sugar content of 15.95 mg g^{-1}. The least reducing sugar content was shown by healthy plants in open condition.

The infected brinjal plants kept inside OTC showed a reducing sugar content of 13.99 mg g^{-1}. The brinjal healthy plants kept in open condition showed least protein content (8.36 mg g^{-1}).

The infected catharanthus and brinjal plants kept in OTC exhibited 63.92% and 31.48% increase in the reducing sugar content compared to the infected plants kept in open condition.

4.6.3.1 Total soluble protein content

The total soluble protein content in catharanthus and brinjal samples kept in OTC and Open condition was estimated and the results are presented in table 33. Significant increase in the protein content was observed in the infected

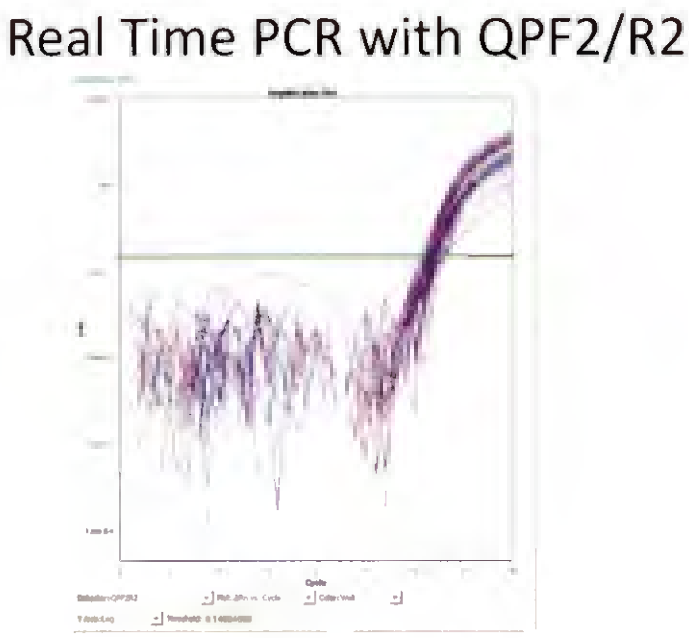


Fig 7. Amplification plot for diseased samples. The Ct value is calculated by plotting the normalized fluorescence against the number of cycles.

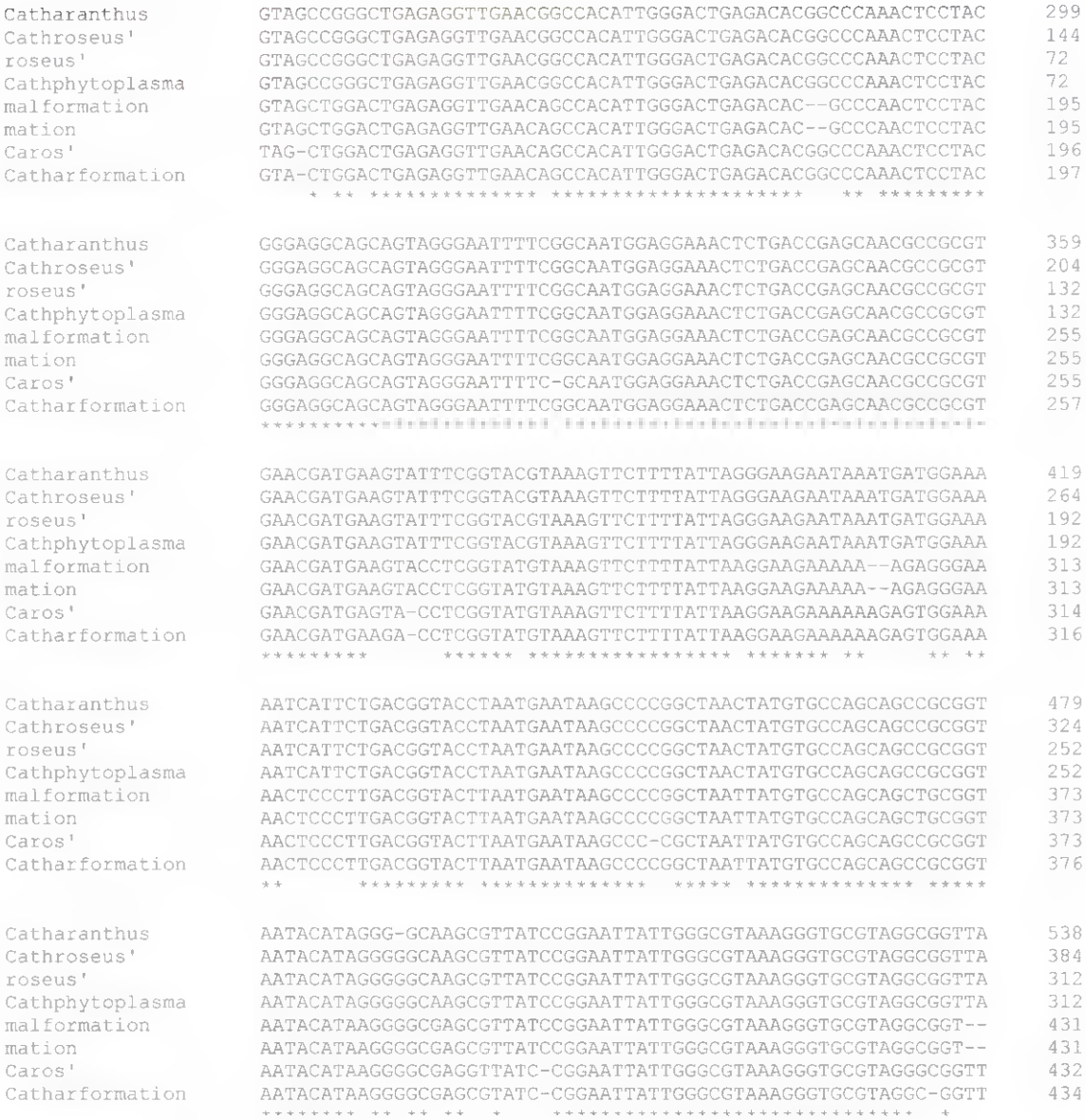


Fig 8. Multiple sequence alignment of catharanthus phytoplasma using Clustal Omega

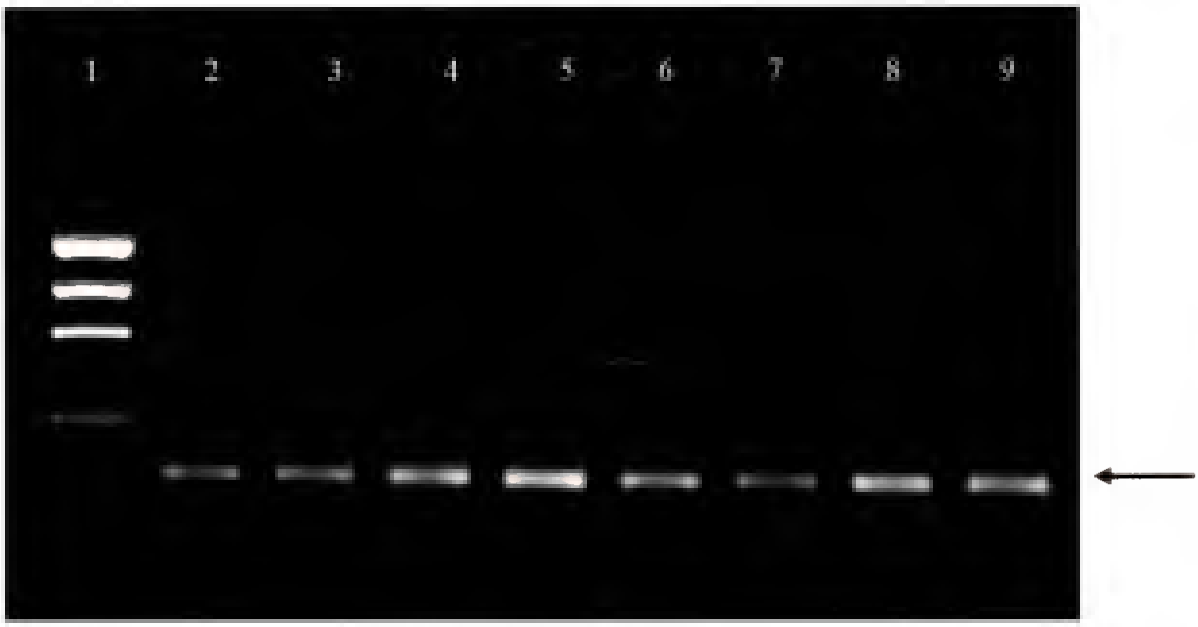


Plate 36. Real Time PCR with real time primers BCF1/BCR1

1-low mass DNA ladder

2- OC 15- Infected catharanthus kept in open condition at 15th day

3-AC 15- Infected catharanthus kept in OTC at 15th day of exposure

4- OC 30- Infected catharanthus kept in open condition at 30th day

5- AC 30- Infected catharanthus kept in OTC at 30th day

6- OB 15- - Infected brinjal kept in open condition at 15th day

7- AB15- - Infected brinjal kept in OTC at 15th day

8- OB30- - Infected brinjal kept in open condition at 30th day

9- AB30- - Infected brinjal kept in OTC at 30th day

Table 32. Effect of enhanced CO_2 on reducing sugar content (mg g^{-1}) in infected catharanthus and brinjal plants

Treatments	Catharanthus Diseased	Catharanthus Healthy	Mean	Brinjal Diseased	Brinjal Healthy	Mean
OTC	15.95	10.85	13.40	13.99	12.38	13.19
Open	9.73	7.79	8.76	10.64	8.36	9.50
Mean	12.84	9.32		12.32	10.37	
SE m (±)	0.73			0.39		
CD (0.05)	2.24			1.22		
Percent change over the infected plants kept in open condition						
Catharanthus	(+) 63.92					
Brinjal	(+) 31.48					

Table 33. Effect of enhanced CO2 on total soluble protein content (mg g^{-1}) in infected catharanthus and brinjal plants

Treatments	Catharanthus Diseased	Catharanthus Healthy	Mean	Brinjal Diseased	Brinjal Healthy	Mean
OTC	7.11	8.47	7.80	3.67	7.84	5.75
Open	5.63	7.01	6.32	1.48	7.44	4.45
Mean	6.36	7.75		2.58	7.65	
SE m (±)	0.52			0.50		
CD (0.05)	1.60			1.52		
Percent change over the infected plants kept in open condition						
Catharanthus	(+) 26.29					
Brinjal	(+) 147.97					

Table 34. Effect of enhanced CO_2 on total phenol content (mg g^{-1}) in infected cathranthus and brinjal plants.

Treatments	Catharanthus Diseased	Catharanthus Healthy	Mean	Brinjal Diseased	Brinjal Healthy	Mean
OTC	1.92	3.60	1.26	0.90	2.59	2.22
Open	2.80	4.87	3.84	1.44	3.55	2.01
Mean	2.36	2.74		1.17	3.07	
SE m (±)	0.19			0.11		
CD (0.05)	0.57			0.35		
Percent change over the infected plants kept in open condition						
Catharanthus	(-) 31.28					
Brinjal	(-) 37.80					

Table 35. Effect of enhanced CO_2 on peroxidase activity (acitivity/g/min) in infected cathranthus and brinjal plants

Treatments	Catharanthus Diseased	Catharanthus Healthy	Mean	Brinjal Diseased	Brinjal Healthy	Mean
OTC	7.38	6.37	6.87	2.11	2.68	2.39
Open	9.95	5.99	7.97	2.29	2.34	2.31
Mean	8.66	6.18		2.20	2.51	
SE m (±)	0.69			0.20		
CD (0.05)	2.12			0.63		

catharanthus plants kept in OTC (7.11 mg g^{-1}) compared to the plants kept in open condition. The catharanthus diseased samples kept in open condition exhibited the least amount of protein (5.63 mg/g).

The total soluble protein content significantly increased in the diseased brinjal plants kept inside OTC (3.67 mg/g) compared to the plants kept at open condition (5.63 mg/g). The brinjal diseased plants kept in open condition showed least protein content (1.48 mg g^{-1}).

The infected catharanthus and brinjal plants kept in OTC exhibited 26.29% and 147.97% increase in the total soluble protein content compared to the infected plants kept in open condition.

4.6.3.2 Total phenol content

The total phenol content of brinjal and catharanthus plants was estimated and the data is depicted in table 34. Significant reduction in the phenol content was observed in both the infected catharanthus and brinjal plants kept in OTC compared to the plants kept in open conditions. In catharanthus, the least phenol content was shown by infected plants kept inside OTC (1.92 mg/g).

A similar trend was seen in brinjal plants also. The least phenol content was shown by the diseased brinjal plants kept inside OTC (0.90 mg/g).

The infected catharanthus and brinjal plants kept in OTC exhibited 31.28% and 37.8% reduction in the total phenol content compared to the infected plants kept in open condition.

4.6.3.3 Peroxidase activity

The peroxidase activity of brinjal and catharanthus plants was estimated and the data is depicted in table 35. The peroxidase activity in the infected plants kept in OTC was seen to be significantly reduced (7.38 activity/g/min) compared to the plants kept in open condition. There was significant difference in the peroxidase activity in the infected brinjal plants.

4.6.3.4 Polyphenol oxidase activity

The polyphenol oxidase activity of catharanthus and brinjal plants was estimated and the data is depicted in table 36. Significant reduction in the

Table 36. Effect of enhanced CO_2 on polyphenol oxidase activity acitivity/g/min) in infected cathranthus and brinjal plants

Treatments	Catharanthus Diseased	Catharanthus Healthy	Mean	Brinjal Diseased	Brinjal Healthy	Mean
OTC	1.37	3.26	2.31	3.74	7.26	5.50
Open	3.61	4.53	3.97	4.39	10.07	7.23
Mean	4.98	7.58		4.06	8.66	
SE m (±)	0.25			0.80		
CD (0.05)	0.77			2.45		
Percent change over the infected plants kept in open condition						
Catharanthus				(-) 31.28		
Brinjal				(-) 37.80		

Table 37. Effect of enhanced CO_2 on PAL activity (acitivity/g/min) in infected cathranthus and brinjal plants

Treatments	Catharanthus Diseased	Catharanthus Healthy	Mean	Brinjal Diseased	Brinjal Healthy	Mean
OTC	10.51	17.99	14.25	7.04	8.93	7.98
Open	8.91	24.06	16.48	5.33	12.38	8.85
Mean	9.21	21.02		6.18	10.65	
SE m (±)	0.40			0.35		
CD (0.05)	1.22			1.06		
Percent change over the infected plants kept in open condition						
Catharanthus				(+) 18.05		
Brinjal				(+) 32.15		

polyphenol oxidase content was observed in both the infected catharanthus and brinjal plants kept in OTC compared to the plants in open condition. In cathranthus, the least polyphenol oxidase content was shown by infected plants kept inside OTC (1.37 activity/g/minute).

A similar trend was seen in brinjal plants also. The least polyphenol oxidase activity was shown by the diseased brinjal plants kept inside OTC (3.74 activity/g/minute). The diseased brinjal plants in open condition showed PPO activity of 4.39 activity/g/minute.

4.6.3.5 Phenyl Ammonia Lyase content

The Phenyl Ammonia Lyase activity of catharanthus and brinjal plants was estimated and the data is depicted in table 37. Significant increase in the PAL activity was observed in both the infected catharanthus and brinjal plants kept in OTC compared to the infected plants kept in open condition. In cathranthus, the least PAL content was shown by infected plants kept in open condition (8.91 activity/g/minute). The infected plants in OTC showed PAL activity of 10.51 activity/g/minute.

A similar trend was seen in brinjal plants also. The least PAL content was shown by the diseased brinjal plants kept in open condition (5.33 activity/g/minute). The diseased brinjal plants in OTC (7.04 activity/g/minute) showed significantly higher PAL activity. The infected catharanthus and brinjal plants kept in OTC exhibited 18.05% and 32.15% increase in the PAL activity compared to the infected plants kept in open condition.

Discussion

5. DISCUSSION

Mid whorl yellowing (MWY) is a serious malady in the coconut growing tracts in Kerala which can cause yield loss up to 70% under severe conditions. The major symptoms of MWY are sudden appearance of yellowing in the middle whorls of the palms, shedding of immature nuts and buttons and inflorescence necrosis. Absence of flaccidity is a distinguishing feature of MWY from the root (wilt) disease. No detailed study has been carried out on this malady yet. Hence no specific management strategy to contain the loss caused by the problem has been formulated.

The present study entitled "Nutrio-physiological and molecular analyses and carbon dioxide enrichment studies of coconut palms (*Cocos nucifera* L.) with foliar yellowing" was taken up at Department of Plant Physiology, College of Agriculture, Vellayani to address this issue. The main objective was to generate information on the nutrio-physiological and biochemical changes occurring in palms with mid whorl yellowing which will help to design a better management strategy. The second objective was formulated to have an understanding on the phytoplasmal responses under enhanced carbon dioxide conditions. The results of various experiments conducted to address the above objectives are discussed with sufficient supports from previous studies.

5.1 Experiment I Nutrio- physiological and molecular analyses

Field visits were conducted in the Thiruvananthapuram district to select locations with all the three categories of palms namely palms with MWY, palms with RW disease and healthy palms. Six regions were visited after consultation with the Agricultural Officers of respective Krishibhavans. The idea was to select all the three categories of the palms from the same location in order to avoid any discrepancies in the soil factors and agronomic practises. Such areas were identified in two locations. -Instructional Farm, College of Agriculture, Vellayani and Venganoor area. So the palms were selected from these areas.

5.1.1 Physiological and Biochemical Variations

Observations on the carbohydrate fractions, photosynthetic pigments, membrane integrity, relative water content, total phenol, total soluble protein content and antioxidant enzymes revealed significant variations in the selected categories of the palms.

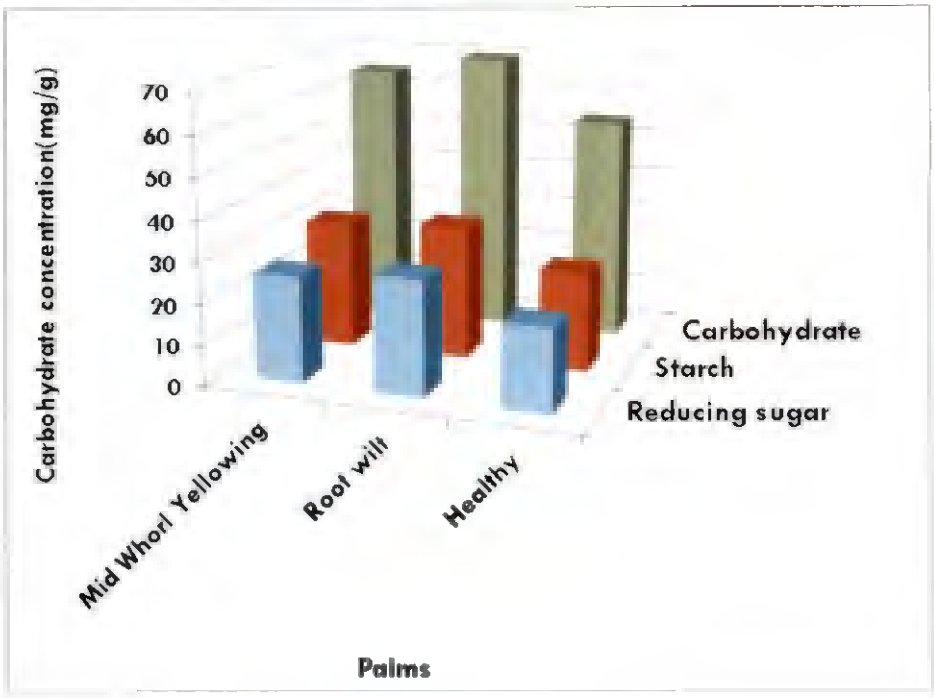
Carbohydrates in plants act as vital sources of energy and carbon skeletons for organic compounds and storage components. They play a pivotal role as signalling molecules. Sugars are considered to be the key components of an integrated cellular redox network (Trouvelot *et al.*, 2014). In plant microbe interactions, sugars are essential to fuel the energy required for defenses and serve as signals for the regulation of defense genes (Ehness *et al.*, 1997; Roitsch *et al.*, 2003; Bolton, 2009).

In this context, the carbohydrate fractions-total carbohydrates, reducing sugars and starch were analysed under our study which revealed significant accumulation of all the three componenets in the MWY affected palms (Fig 8). An increase of 19% and 25% in total carbohydrates, 28% and 13% in reducing sugar and 28% and 33% in starch content was observed in palms with MWY in the inner and middle whorls respectively compared to the healthy palms. Similar increasing trend was observed in the RW diseased palms also except in one location where there was significant reduction in the carbohydrate content. Across the whorls, there was an increase in the carbohydrate fractions in the middle whorl compared to the inner whorls in the palms with MWY. Such trend was absent in RW affected palms. Mathew (1977) analysed the carbohydrate content of the RWD affected palms and showed that total, reducing and non-reducing sugars were significantly higher in the leaves of infected palms. He inferred this as due to impaired translocation to the roots in affected palms. 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 newly 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

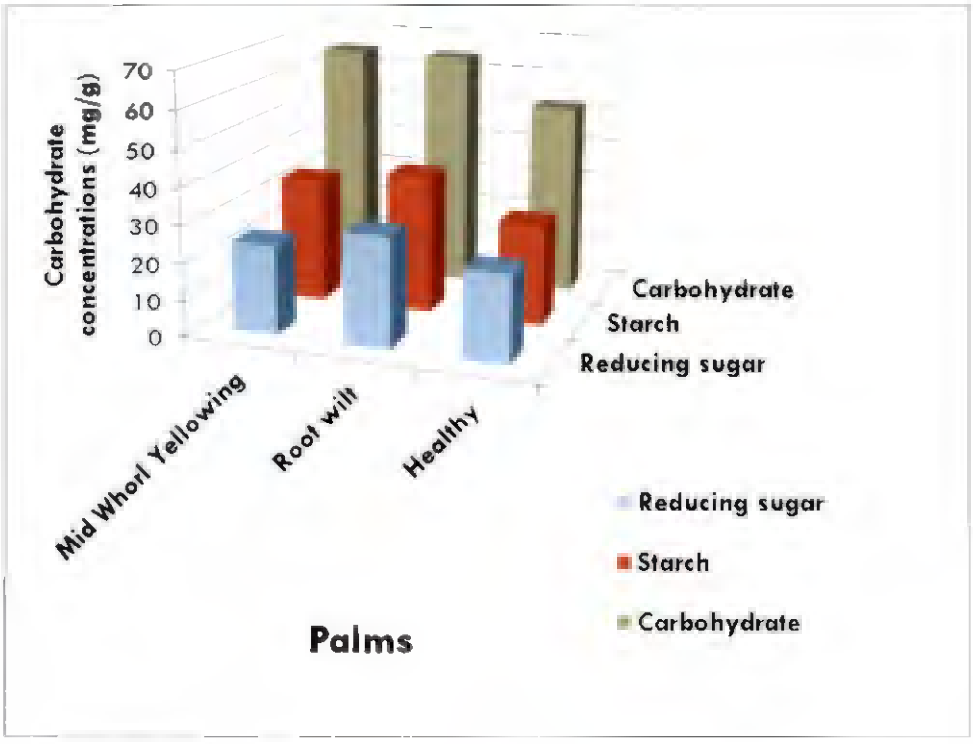
the disease before decreasing in later stages. Accumulation of sugar and starch in the leaves in pear decline affected pear has also been reported (Catlin *et al.*, 1975). Guthrie *et al.* (2001) reported higher carbohydrate content in diseased leaf tissue in papaya infected with phytoplasma. Higher reducing sugar content has been reported in corn plants infected by the maize bushy stunt phytoplasma (Junqueira *et al.*, 2004).

Hren *et al.*, 2009 reported that phytoplasmas could impair the phloem function thereby altering the carbohydrate metabolism of the plant and thus affecting sugar transport which subsequently results in the accumulation of sugars in the source leaves. Reduced sugar transport could be hindered by physical blockage of sieve tubes by phytoplasma, deposition of callose or other material in response to phytoplasma infection (Maust *et al.*, 2003). These accumulated sugars act as a food source for the phytoplasma. Phytoplasmas contain a minimal genome and lack genes coding for ATP synthases and sugar uptake and use, making them dependent on their host (Christensen *et al.*, 2005). Impaired photosynthesis, accumulation of carbohydrates in mature leaves and decreased starch content in sink tissues such as roots, often described for phytoplasma infection, seem to be secondary effects and can easily be related to inhibition of phloem transport (Lepka *et al.*, 1999; Tan and Whitlow, 2001; Bertamini *et al.*, 2003; Maust *et al.*, 2003; Choi *et al.*, 2004; Junqueira *et al.*, 2004). Accumulation of sugars lead to feed back inhibitions resulting in stomatal closure, reduced photosynthetic rates, and leaf yellowing or chlorosis (Maust *et al.*, 2003). Deepa *et al.*, (2012) and Anju (2011) have also reported accumulation of carbohydrate fractions in palms with yellowing. The reduction in the carbohydrate content in the RW affected palms of the present study may be because these palms were more severe in symptom expression.

Plant productivity is a unique process that depends greatly on the amount of chlorophyll present in the chloroplast. The chlorophyll plays an important role in the physiology, productivity and economy of the green plants (Taiz and Zeiger, 2006). Leaf chlorophyll content is a good indicator of photosynthesis activity, stress condition and nutritional status of plants (Ghasemi *et al.*, 2011).



Inner Whorl



b) Middle whorl

Fig 9. Carbohydrate concentrations of different whorls of coconut palms affected with MWY

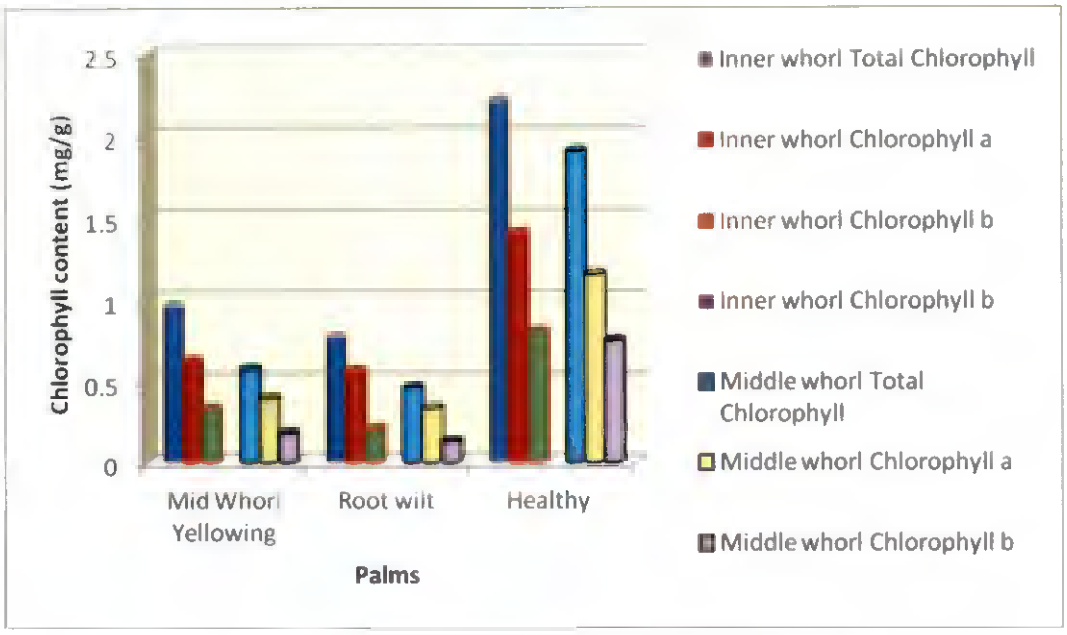


Fig 10. Chlorophyll content of coconut leaves affected with MWY

The palms with MWY showed a reduction in the photosynthetic pigments-chlorophyll a, chlorophyll b and total chlorophyll contents. A 77% and 50% reduction in the total chlorophyll content was exhibited by the inner and middle whorls of the palms with MWY. Compared to the inner whorl, the middle whorl showed significant reduction in the chlorophyll content. Palms with RW disease also showed significant reduction in the chlorophyll content (Fig 9).

Reduction in the chlorophyll content have been reported in coconut palms affected with root (wilt) 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. 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). Maust *et al.*, (2003) has reported that accumulation of sugars can lead to feed back inhibitions resulting in leaf yellowing or chlorosis. 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.

The plant cell wall and plasma membrane are involved in different biological processes like cell morphogenesis and biotic/abiotic stress responses. As one of the first points of contact for environmental signals upon the cell, they play important roles in stress responses. This is of particular relevance in plants, which cannot move or take shelter from potentially damaging environmental conditions. Maintaining functional integrity of these components is essential under stress conditions (Eckardt, 2008; Hamann and Denness, 2011).

The present study revealed a massive reduction in the membrane integrity of palms with MWY. There was 2.5 times reduction in the membrane integrity of palms with MWY compared to the healthy palms. The middle whorl showed increased membrane leakage compared to the inner whorl. Root (wilt) diseased palms also expressed an increased membrane leakage. The result was in accordance with the report of Deepa (2011) in palms with yellowing.

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

In this study, the palms with MWY showed a significant reduction in the RWC compared to the healthy palms. There was 4% and 7% reduction in the RWC in the inner and middle whorls of palms with MWY. The root (wilt) affected palms also showed reduction in the RWC. 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). Deepa, 2011 reported that there was 20% reduction in the RWC of palms with yellowing.

Phenolics play important roles in plant development, particularly in lignin and pigment biosynthesis. Rivero *et al.*, 2001 and Michalak, 2006 reported that an enhancement of phenylpropanoid 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) have 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 current study, the palms with MWY were found to maintain a lower level of phenol content. Significant reduction was observed only in the middle whorls (30.03%) of the palms affected with MWY. The healthy palms maintained maximum phenol content. The study was in accordance with the report of Sasikala *et al.*, (2005) in the palms with root (wilt) where the phenol content in diseased samples decreased compared to the healthy palms.

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) disease (Padmaja *et al.*, 1981). In the present work, 43% and 88% increase in the total soluble protein content was observed in palms with MWY compared to the healthy palms. The RW affected palms also recorded increased protein content. Across the whorls, the middle whorl recorded highest protein content compared to the inner whorl. A decrease in protein fractions were observed in the leaf tissues of root (wilt) affected coconut palms (Pillai, 1975). The leaf protein content showed no significant difference in the early stages of Lethal Yellowing affected palms and reduced from the foliar discolouration phase. At this stage the protein content dropped to 45 % of its original level (Leon *et al.*, 1996). An increase in the total amount of proteins has been found in maize bushy stunt phytoplasma-infected maize plants (Junqueira *et al.*, 2004). Contradictory results have been obtained in different host plants infected with different Mollicutes. A possible explanation for this difference could be due to the fact that extremely susceptible plants were used in those experiments. In particular, resistant host plants accumulate higher protein content than susceptible ones, supporting the hypothesis that accumulation of PR-proteins contributes to the increase of total proteins in infected tissues (Musetti, 2010).

Peroxidase (PO) and Polyphenol oxidase (PPO) have 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 have a 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.

No significant variation was observed in the peroxidase activity in palms with MWY. But a slight increase was observed in MWY affected palms compared to the control palms. The polyphenol oxidase activity significantly increased in palms with MWY and RW compared to the healthy palms. There was 121% and 145% increase in the PPO activity in palms with MWY. Compared to the inner whorls, middle whorls recorded maximum PPO activity. The study was in accordance with the report of Deepa, 2011 in palms with yellowing where there was significant increase in the PPO activity in the diseased samples. An increased peroxidase and polyphenol oxidase activity has been reported in the roots of palms affected with root (wilt) (Joseph and Jayasankar, 1979, Sasikala *et al.*, 2005).

Overall, the physiological and biochemical variations in the palms with MWY indicated that the health status of the affected palms was highly deteriorated. The carbohydrate metabolism was highly altered. There was accumulation of carbohydrate in the source leaves indicating that the sugar translocation was affected. These sugars act as a food source of phytoplasma. Reduced sugar transport could be caused by the physical blockage of the sieve tubes caused by phytoplasma. The accumulation of sugars lead to feed back inhibitions resulting in stomatal closure, reduced photosynthetic rates, and leaf yellowing or chlorosis. Loss of membrane integrity and maintainance of low relative water content was another major physiological change observed in palms with MWY. The palms with MWY recoreded a low phenol content. All these

contributed to the worsening of the health status of the palms and making it more susceptible.

5.1.2 Soil analysis

Organic carbon content, pH and electrical conductivity are considered as the soil quality indicators (Reeves, 1997). These factors affect the availability of nutrients to crop plants. Soil organic carbon is an index of sustainable land management and is critical in determining response to N and P fertilization (Bationo *et al.*, 2007). Soil pH influences solubility, concentration in soil solution and mobility of nutrients in soil and consequently acquisition of these elements by plants (Fageria *et al.*, 1990).

The present study did not indicate any significant variation in the organic carbon, pH and electrical conductivity in the soils taken at two different depths. But low organic carbon was maintained by the soils in location 2.

The mineral nutrients need to be at appropriate concentrations in the soil for the plants to obtain them for proper physiological and biochemical functioning. The major and minor nutrient availability in soil was estimated in the experiment and it revealed that the soils in both the locations maintained a medium nutrient status. The results did not show any significant variations indicative of any particular role for any of these soil characters in the development of yellowing symptom.

5.1.3 Plant analysis

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).

Plant nutrition has always been an important component of disease control. Mineral nutrients in plant tissue increase resistance by maximising the inherent resistance of plants, facilitating disease escape through increased nutrient availability or stimulated plant growth and altering external environments to influence survival, germination and penetration of pathogens. Nutrient concentrations in plants are important in host ability to resist or tolerate infectious pathogens (Fageria *et al.*, 2002).

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 14^{th} leaf data from the healthy palms and palms with MWY and RW were analysed. The data showed that there were significant reductions in the levels of major nutrient nitrogen (47%), phosphorus (26%) and also in the levels of magnesium (22.72%) Zinc (24%) in case of palms showing mid whorl yellowing compared to the control palm. The level of nitrogen and phosphorus in palms with MWY was critically lower. Magnesium deficiency is often associated with high leaf potassium level (Wahid, 1984). Higher levels of accumulation were found in case of potassium (37.96%), calcium (40.79%), iron (54%) and copper (35.75%). 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. 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 and 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.

Foliar analysis of the RW palms by several workers (Pillai *et al.*, 1975; Cecil *et al.*, 1991) indicated a higher concentration of N, P and K and micronutrients Cu, Mn and Fe in the leaf tissues of diseased palms. The accumulation of the nutrients in the crown of the diseased palms can be due to reduced rate of dry matter content of the foliage and partly due to the reduced rate of nut yield with increased rate of disease intensity. It was observed that the palms with MWY recorded low yield. When the nut yield in diseased palms is restricted the excess nutrients are likely to get accumulated in the crown. Pandalai, 1959 suggested that the absence or non- availability of nutrients was not the cause of tissue abnormalities, but was actually the inability of the palms to transact the normal processes at the appropriate site.

5.1.4 Soil moisture status

No significant variation was observed in the soil moisture status in both the locations.

5.1.5 Anatomical variations in roots and leaves

Phytoplasmas were originally detected by electron microscopy of ultra-thin sections from symptomatic plants (Doi *et al.* 1967). Electron microscopy of phloem tissue was reported to be the most reliable method for demonstrating the presence of phytoplasma in diseased plants (Navratil *et al.* 2009). Direct detection of phytoplasmas is difficult, because they are not visible through a normal light microscope. In our experiment, phytoplasma cells were observed by Scanning Electron Microscopy (SEM) in the spindle leaves of coconut palms with MWY and RW. They appeared as spherical bodies either singly or in cluster. It was absent in the healthy palms.

The external symptoms in the roots of coconut palms affected with yellowing showed correlation with various anatomical changes. Roots of both the palms with MWY and RW showed necrosis, while the control palms maintained healthy roots. 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 in palms with RW disease.

5.1.6 Detection of phytoplasma by PCR

Phytoplasma enrichment protocol for isolating DNA was standardised. The modified method resulted in extracting high quality, low polysaccharide genomic DNA which was suitable for PCR analysis.

Phytoplasma was detected by the nested, semi-nested and real time PCR. The nested PCR analysis with the phytoplasma specific universal primers P1/P7-R16F2n/R16R2 (Nejat and Vadamalai, 2010)) provided an amplicon of 1240 bp (1.2 kb) in the MWY affected palms. The semi nested primer pairs IF7/7R3-IF7/7R2 (Manimekalai *et al.*, 2010) provided an amplicon of 493 bp in the MWY affected palms and in the RW affected palms. Two healthy palms also showed amplification indicating latent infection before symptom expression. Real time PCR using real time primers QPF2/R2 yielded an amplicon of 140 bp in the root (wilt) palms and mid whorl yellowing affected palms.

Nested PCR with universal primers often result in inconsistent results (Manimekalai, *et al.*, 2010). The semi- nested PCR with the semi- nested primer pairs IF7/7R3- IF7/7R2 could detect phytoplasma in symptomless carriers, even 6 months before the appearance of the symptom proving its specificity and accuracy in phytoplasmal detection. So this technique can be utilised for phytoplasmal indexing and disease free mother palm and seedling selection.

The PCR product obtained from the semi-nested PCR were sequenced and analysed. The blast analysis showed 89% sequence similarity with the root (wilt) phytoplasma. This indicates that the MWY phytoplasma can be a variant of root (wilt) phytoplasma.

5.2 Effect of enhanced carbon dioxide on phytoplasmal responses

The second experiment was formulated to understand the effect of changing climate on phytoplasmal response. The systems selected for this study were six months old coconut seedlings showing yellowing and little leaf infected catharanthus and brinjal plants.

Nested PCR analyses was done with phytoplasma specific universal primer pairs P1/P7-R16F2n/ R16R2 in coconut seedlings to detect phytoplasma. No amplification was observed in any of the seedlings except for the catharanthus positive control. Since no amplification was obtained for the phytoplasma, the seedlings were not placed inside the OTC.

The infected cathranthus and brinjal plants maintained by repeated grafting were kept in the Open Top Chamber (OTC) at an enriched CO2 concentration of 500 ppm for a period of one month.

After the exposure period improved growth and development was observed in both the infected catharanthus and brinjal plants kept inside OTC. The infected plants showed more number of leaves and branches, but symptom remission was not there. Real time PCR analyses was carried out in the 15^{th} and 30^{th} day of exposure. It did not reveal any significant variation in the phytoplasmal load between the infected plants kept inside OTC and in the open condition.

The major biochemical compounds studied in the current experiment are reducing sugar content, total soluble protein content, phenols and antioxidant enzymes- peroxidase, polyphenol oxidase and phenyl alanine ammonia lyase.

The biochemical analyses carried out in the experimental plants revealed a significantly higher reducing sugar content (63% and 31%), protein content (26% and 147%) and phenyl alanine ammonia lyase activity (18% and 32 %) and significantly lower phenol content (31% and 37%) and poly phenol oxidase activity (62% and 14%) in the infected catharanthus and brinjal plants kept in OTC compared to the infected plants kept in open condition.

Accumulation of carbohydrates in leaves is one of the most important responses observed in plants to elevated atmospheric CO_2 (Long *et al.*, 2004). Elevated CO_2 increases the accumulation of starch, total soluble sugars and reducing sugars in black gram (Sathish *et al.*, 2014). The present study also recorded increased reducing sugar content in the infected plants exposed to elevated CO_2 condition.

Elevated CO_2 usually results in reduced protein content in plants exposed (Tezra *et al.*, 2002; Robredo *et al.*, 2011). The infected catharanthus and brinjal plants exposed to elevated CO_2 showed increased protein content compared to the plants in open condition. This may be due to the enhanced production of PR proteins under elevated CO_2.

Various stresses can lead to the overproduction of Reactive Oxygen Species (ROS) in plants which are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates which results in oxidative stress mechanisms against ROS (Matsuura and Fett-Neto, 2013). The antioxidant enzymes studied in this experiment were peroxidase, polyphenol oxidase and phenyl alanine ammonia lyase. Increased PAL activity can result in contributing towards activated defense mechanism in plants.

Plant growth is nearly always stimulated by elevation of CO_2. Since CO_2 is one of the substrates for the process of photosynthesis, this influences the growth rates and development of plant species (Geissler *et al.*, 2009). Carbon dioxide enrichment causes stimulation of photosynthesis, inhibition of photorespitration

and increase in water use efficiency resulting in higher biomass production (Bowes, 1991 and Drake *et al.*, 2008). Elevated CO_2 increases plant biomass, root mass and total leaf area (Rogers *et al.*, 1994; Curtis and Wang, 1998; Minu et al., 2015 and Dheeraj 2016). The present experiment showed an improved growth and development in infected plants exposed to elevated CO_2. The soluble sugar content in the infected plants was found to be significantly higher. Under elevated carbon dioxide condition the defense system was activated in plants kept inside OTC, even though no variation in the phytoplasmal load was detected. Thus elevated carbon dioxide condition can have an impact on the physiological alterations induced by phytoplasma.

Summary

6. SUMMARY

Coconut palm the 'Kalpavriksha' is intimately associated with the economic, social and cultural life of millions of people in the tropics and subtropics. Coconut is a perennial tropical species usually grown as a rainfed crop and its cultivation is exposed to severe constraints. Kerala is the 'land of coconut' having an average productivity of 7535 nuts/ha. But the productivity is much less when compared to that of other coconut growing states owing to various biotic and abiotic stresses. The coconut palms are prone to several diseases and disorders which cause a substantial yield loss. Among the diseases, root (wilt) disease (RWD) caused by phytoplasma is the most important one. It causes 35% yield reduction and the losses may extend up to 80% in severe cases.

Mid whorl yellowing (MWY) is another severe malady of recent origin in the coconut growing tracts especially in the southern tracts of Kerala causing serious reduction in the nut yield. It is characterised by sudden appearance of yellowing in two or three leaves in the middle whorl. When the symptom advances, the entire middle whorl turns yellow. Other symptoms of palms with MWY are drying and necrosis of inflorescence and shedding of buttons and immature nuts ultimately leading to a substantial yield loss. In severe cases it may cause a yield loss up to 70%. The most distinguishing feature of the palms with mid whorl yellowing which differentiates it from the root (wilt) disease is the absence of flaccidity.

A detailed study on this fast spreading and serious problem has not yet been reported. There are reports suggesting that the yellowing in the middle whorls can be an associated symptom of the root (wilt) disease. A comparative study between the mid whorl yellowing and the root (wilt) has also not been carried out. Since it is causing serious economic consequence, it is necessary to have an understanding on the nutrio-physiological changes occurring in palms with MWY in order to adopt a better management strategy.

Climate change has varying impact on plant pathogens. Very few studies have been done on the impact of climate change on plant phytoplasmal response. Association of phytoplasma in palms with MWY have already been established.

How CO_2-induced changes in plant growth will influence the complex interactions with diseases especially phytoplasmal disease is a priority area of research.

In this context, the current programme entitled "Nutrio-physiological and molecular analyses and carbon dioxide enrichment studies of coconut palms (*Cocos nucifera* L.) with foliar yellowing" was undertaken to analyze the nutrio - physiological changes occurring in the palms affected with foliar yellowing and to assess the impact of enhanced carbon dioxide on phytoplasmal response.

Palms showing mid whorl yellowing, root wilt affected palms and healthy palms were selected from two different locations viz the Instructional Farm, College of Agriculture, Vellayani (location 1) and Venganoor region (location 2) in the Thiruvananthapuram district. Comparative analyses on physiological and biochemical parameters were carried out in the leaves of upper and middle whorls of the selected palms. Nutrient analyses were done in both leaves and soil. Anatomical and molecular analyses were carried out in the selected categories of palms. In order to study the effect of enhanced carbon dioxide on phytoplasmal response, coconut seedlings showing yellowing were selected from the Instructional Farm, College of Agriculture, Vellayani. Catharanthus and brinjal plants with 'little leaf symptom' maintained at the Department of Plant Pathology were utilised for grafting into healthy catharanthus and brinjal plants and these were also taken for studying the phytoplasmal response under elevated carbon dioxide condition.

The study revealed significant variations in the physiological and biochemical parameters analysed. Accumulation in the total carbohydrate fraction was observed in palms with MWY. An increase of 19% and 25% in total carbohydrates, 28% and 13% in reducing sugar and 28% and 33% in starch content was observed in palms with MWY in the inner and middle whorls respectively compared to the healthy palms. This accumulation in the carbohydrate fractions can be due to the damage in the phloem function caused by phytoplasma which alters the carbohydrate metabolism and results in the reduced sugar transport. Reduced sugar transport could be caused by physical blockage of

sieve tubes by phytoplasma and deposition of callose in response to phytoplasma infection.

The palms with MWY exhibited significant reduction in the physiological parameters- photosynthetic pigment composition, membrane integrity and relative water content. A 77% and 50% reduction in the total chlorophyll content was exhibited by the inner and middle whorls of the palms with MWY. Loss of membrane integrity or membrane damage is the major physiological change in coconut due to MWY. There was 42% and 257% reduction in the membrane integrity of palms with MWY compared to the healthy palms. Palms with MWY showed 4% and 7% reduction in the RWC in the inner and middle whorls respectively in comparison with the healthy palms.

The current study observed a slight reduction in the phenol content in the palms with MWY. Significant reduction was observed only in the middle whorls (30.03%) of the palms affected with MWY. The healthy palms maintained maximum phenol content.

In the present work, 43% and 88% increase in the total soluble protein content was observed in palms with MWY compared to the healthy palms. This can be due to the accumulation of PR proteins in response to phytoplasmal infection.

The polyphenol oxidase activity significantly increased in palms with MWY and RW compared to the healthy palms. There was 121% and 145% increase in the PPO activity in palms with MWY. Compared to the inner whorls, middle whorls recorded maximum PPO activity.

Soil factors-pH, organic carbon and electrical conductivity were analysed for the root-zone soils of all the experimental palms. The major and minor nutrient availability in soil was estimated in the experiment and it revealed that the soils in both the locations maintained a medium nutrient status. The results did not show any significant variations indicative of any particular role for any of these soil characters in the development of yellowing symptom.

Plant nutrient analyses were carried out in the 14th leaf of the healthy palms and palms with MWY and RW. The data showed that there were significant

reductions in the levels of major nutrient nitrogen (47%), phosphorus (26%) and also in the levels of magnesium (22.72%) and Zinc (24%) in case of palms showing mid whorl yellowing compared to the control palm. The level of nitrogen and phosphorus in palms with MWY was critically lower. Higher levels of accumulation were found in case of potassium (37.96%), calcium (40.79%), iron (54%) and copper (35.75%). These variations in the nutrient levels can have an influence on symptom development in coconut palms.

No significant variation was observed in the soil moisture status.

Phytoplasma cells were observed by Scanning Electron Microscopy (SEM) in the spindle leaves of coconut palms with MWY. The external symptoms in the roots of coconut palms affected with yellowing showed correlation with various anatomical changes. Roots of both the palms with MWY and RW showed necrosis, while the control palms maintained healthy roots. Vascular browning which extended to the cortex and the vascular disintegration in the roots of affected palms was observed.

Phytoplasma enrichment protocol for isolating DNA was standardised. Phytoplasma was detected by the nested, semi-nested and real time PCR. 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 MWY palms. The semi nested primer pairs IF7/7R3- IF7/7R2 provided an amplicon of 493 bp in palms with MWY and RW affected palms. Two out of five healthy palms also showed amplification indicating latent infection before symptom expression. Real time PCR using real time primers QPF2/R2 also yielded an amplicon of 140 bp palms with MWY and RW affected palms.

The PCR product obtained from the semi-nested PCR were sequenced and analysed. The blast analysis showed 89% sequence similarity with the root (wilt) phytoplasma.

The second experiment was formulated to understand the effect of changing climate on phytoplasmal response. The systems selected for this study were six months old coconut seedlings showing yellowing and little leaf infected catharanthus and brinjal plants. Nested PCR with universal primer pairs showed

no amplification in the coconut seedlings and hence only catharanthus and brinjal grafts with phytoplasmal infection were kept in Open Top Chamber (OTC) with 500 ppm Carbon dioxide concentration for a period of one month.

After the exposure period, the plants kept in OTC showed an increased rate of growth and development with phytoplasmal symptoms. Physiological and biochemical analyses showed a significant increase in the protein content (147%) and PAL activity (32%) and significant reduction in the phenol content (37%), and polyphenol oxidase activity (62%) in the infected plants kept in OTC compared to the infected plants kept in open condition. Molecular analyses of the infected samples kept in OTC and open condition with the DNA samples isolated at the 15^{th} and 30^{th} day of exposure was done using real time PCR. No significant variation was detected in the phytoplasmal load after elevated carbon dioxide exposure in our study.

The results of the study is summarised below:

The role of phytoplasma in causing MWY was established by molecular and anatomical studies. Overall, the physiological and biochemical variations in the palms with MWY indicated that the health status of the affected palms was highly deteriorated. The carbohydrate metabolism was highly altered. There was accumulation of carbohydrate in the source leaves indicating that the sugar translocation was affected. These sugars act as a food source of phytoplasma. Reduced sugar transport could be caused by the physical blockage of the sieve tubes caused by phytoplasma. The accumulation of sugars lead to feed back inhibitions resulting in stomatal closure, reduced photosynthetic rates, and leaf yellowing or chlorosis. Loss of membrane integrity and maintainance of low relative water content was another major physiological change observed in palms with MWY. Significant alterartions in the nutrient profile was observed. Maintainance of critically low nitrogen and phosphorus content was observed. The phosphorus is essential for maintaining the structural integrity of the cell membrane as it forms an important component of the phospholipids in the cell membrane. The root tips of palms with MWY showed necrosis in root tips. Hence there can be an effect on the elements absorbed via root tips. Accumulation of

elements like potassium and calcium reveals that the assimilation of these nutrients was highly affected. The accumulation of the nutrients in the crown of the diseased palms can be due to reduced rate of dry matter content of the foliage and partly due to the reduced rate of nut yield with increased rate of disease intensity. It was observed that the palms with MWY recorded low yield. When the nut yield in diseased palms is restricted the excess nutrients are likely to get accumulated in the crown. The palms with MWY recorded low phenol content. All these contributed to the worsening of the health status of the palms and making it more susceptible.

Elevated carbon dioxide showed an improved growth and development and activated defense response to phytoplasma in the infected plants but the CO_2 induced modifications in phytoplasmal load was not detected. The results indicated a better tolerance strategy to phytoplasma under changing environmental conditions

The salient findings of the present study are

- Role of phytoplasma in causing MWY was established by molecular and anatomical analysis
- Physiological and biochemical analyses indicate
    - Altered carbohydrate metabolism
    - Maintainance of low water content
    - Low membrane integrity

Overall health status of the palms with MWY is deteriorated

- Significant variation in nutrient profile
    - accumulation of potassium, calcium, iron and copper
    - reduction in nitrogen, phosphorus, magnesium and zinc
- Anatomical studies indicate
    - Deformity and necrosis of roots
    - SEM analysis establish the presence of phytoplasma
- Molecular analysis
    - Phytoplasma enrichment protocol for DNA isolation standardised
    - Semi nested PCR found to be a better technique compared to the nested PCR
    - Sequence analysis revealed MWY phytoplasma can be a variant of RW phytoplasma

- Carbon dioxide enrichment
  - Improved growth and development of plants exposed to elevated carbon dioxide condition irrespective of phytoplasmal infection
  - Activated defense mechanism
  - No variation in the phytoplasmal load

6.1 Future line of work

The role of phytoplasma in causing MWY was established by molecular and anatomical studies. Sequencing of the PCR product revealed that MWY phytoplasma can be a variant of root (wilt) phytoplasma. The present study indicated that the overall health status of the palms with MWY was highly deteriorated. Significant variation in the nutrient profile was noted which emphasise the need for proper nutrient management. Semi- nested PCR was found to be more accurate and specific in phytoplasmal detection which can be utilised for phytoplasmal indexing and mother palm and disease free seedling selection. Elevated carbon dioxide showed an improved growth and development and activated defense response to phytoplasma in the infected plants but the CO_2 induced modifications in phytoplasmal load was not detected. The results indicated a better tolerance strategy to phytoplasma under changing environ - mental conditions.

The information generated from the present programme with regard to the modifications in the nutrient profile in palms with MWY in comparison with the healthy palms in conjunction with information on the physiological parameters like hormonal profile will help to develop management strategy for ameliorating field performance and productivity of coconut palms in with MWY. Molecular characterisation of phytoplasmal variants involved in MWY is necessary. Information generation on carbondioxide induced phytoplasmal dynamics and its exploitation in managing phytoplasmal diseases.

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Abstract

NUTRIO-PHYSIOLOGICAL AND MOLECULAR ANALYSES AND CARBON DIOXIDE ENRICHMENT STUDIES OF COCONUT PALMS (*Cocos nucifera* L.) WITH FOLIAR YELLOWING

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ABSTRACT

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8. ABSTRACT

A study entitled "Nutrio-physiological and molecular analyses and carbon dioxide enrichment studies of coconut palms (*Cocos nucifera* L.) with foliar yellowing" was carried out with the objective to analyze the nutrio - physiological changes occurring in the palms affected with foliar yellowing and to assess the impact of enhanced carbon dioxide on phytoplasmal response. In this study, palms showing mid whorl yellowing (MWY), root (wilt) affected palms (RW) and healthy palms were selected from two different locations viz the Instructional Farm, College of Agriculture, Vellayani (location 1) and Venganoor region (location 2) in the Thiruvananthapuram district. Coconut seedlings showing yellowing were selected from the Instructional Farm, College of Agriculture, Vellayani to study the effect of enhanced carbon dioxide on phytoplasmal response. Catharanthus and brinjal plants with 'little leaf symptom' maintained at the Department of Plant Pathology were utilised for grafting into healthy catharanthus and brinjal plants and these were also taken for studying the phytoplasmal response under elevated carbon dioxide condition.

Physiological and biochemical analyses revealed significant variations in all the parameters studied. Palms with mid whorl yellowing (MWY) showed a significant reduction in the chlorophyll a, b and total chlorophyll contents, relative water content, membrane integrity and phenol content compared to the healthy palms. An increase in the protein (88%), carbohydrate (25.14%), reducing sugar (28%) and starch content (28.33%) was noted in palms with MWY. In the case of antioxidant enzymes there was build up of polyphenol oxidase (145.38%) and peroxidase activities in palms with MWY compared to the healthy palms. A similar trend was observed in almost all cases of RW affected palms.

Nutrient analyses revealed significant alterations in the nutrient content of the selected palms. Significant reduction in the nitrogen (47.74%), magnesium (22.72%) and zinc content (24.19%) was observed in palms with yellowing compared to the healthy palms. Accumulation of elements like potassium

(37.96%) calcium (40.79%), iron (54%) and copper (35.75%) was observed in palms with mid whorl yellowing compared to the healthy palms. These variations in the nutrient levels can have an influence on symptom development in coconut palms. The results on the soil nutrient analyses did not show any particular role in development of yellowing symptoms.

Phytoplasma cells were detected in the phloem tissues of both mid whorl yellowing and root wilt affected palms under Scanning Electron Microscopy (SEM) study. Morphological and anatomical analyses of fresh roots indicated browning and necrosis of roots and vascular disintegration in MWY and RW affected palms.

The presence of phytoplasma in the selected samples were checked by nested PCR analysis using phytoplasma specific universal primers-P1/P7-R16F2n/ R16R2, Phytoplasma 16S rDNA specific semi nested primers 1F7/7R3 - 1F7/7R2 and by real time PCR technique using real time primers QPF2/R2. Semi-nested PCR yielded an amplicon of 493 bp in all the MWY and RW palms in both the locations. Real time PCR yielded an amplicon of 140 bp in the RW palms, 3 MWY palms in location 1. Nested PCR with phytoplasma specific universal primer pairs P1/P7-R16F2n/ R16R2 yielded an amplicon of 1.2 kb in MWY palm and RW palm. Sequence analysis of the mid whorl yellowing phytoplasma revealed 89% similarity to the root wilt phytoplasma.

In experiment II, nested PCR with universal primer pairs showed no amplification in the coconut seedlings and hence only catharanthus and brinjal grafts with phytoplasmal infection were kept in Open Top Chamber (OTC) with 500 ppm Carbon dioxide concentration for a period of one month. After the exposure period, the plants kept in OTC showed an increased rate of growth and development with phytoplasmal symptoms. Physiological and biochemical analyses showed a significant increase in the reducing sugar content (63%), protein content (147%) and PAL activity (32%) and significant reduction in the phenol content (37%), and polyphenol oxidase activity (62%) in the infected plants kept in OTC compared to the infected plants kept in open condition. Molecular analyses of the infected samples using real time PCR kept in OTC and

open condition with the DNA samples isolated at the 15^{th} and 30^{th} day of exposure was done. No significant variation was observed in the phytoplasmal load after elevated carbon dioxide exposure.

The role of phytoplasma in causing MWY was established by molecular and anatomical studies. Sequencing of the PCR product revealed that MWY phytoplasma can be a variant of root (wilt) phytoplasma. The present study indicated that the overall health status of the palms with MWY was highly deteriorated. Significant variation in the nutrient profile was noted which emphasise the need for proper nutrient management. Semi- nested PCR was found to be more accurate and specific in phytoplasmal detection which can be utilised for phytoplasmal indexing and mother palm and disease free seedling selection. Elevated carbon dioxide showed an improved growth and development and activated defense response to phytoplasma in the infected plants but the CO_2 induced modifications in phytoplasmal load was not detected. The results indicated a better tolerance strategy to phytoplasma under changing environmental conditions.

Appendices

APPENDIX - I

Anthrone reagent

Anthrone reagent made by dissolving 200 mg of Anthrone in 100 ml ice cold 95 per cent concentrated Sulphuric acid.

APPENDIX – II

Buffers for biochemical analysis

1. Acetate buffer (0.1 M)

A: 0.2 M solution of acetic acid = 11.55 ml in 1000 ml

B: 0.2 M solution of sodium acetate = 16.4 g $C_2H_3O_2Na$ in 1000 ml

22.7 ml of A was mixed with 27 ml of B and the volume made upto 100 ml.

2. Borate buffer (0.1 M)

A: 0.2 M solution of boric acid = 12.4 g in 1000 ml

B: 0.05 M solution of borax = 19.06 g in 1000 ml

50 ml of A was mixed with 30 ml of B and diluted to a total of 200 ml. 0.05 g PVP was added.

APPENDIX – III

Stock dye solution for estimation of protein

100 mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 95 per cent ethanol and 100 ml of concentrated orthophosphoric acid was added. The volume was made upto 200 ml with water and kept at 4°C. The working dye was prepared just before use by diluting the stock solution to five times with water.

APPENDIX – IV

Buffers for DNA isolation

1. Phytoplasma Extraction Buffer

Tris	100mM (pH 8)
Sucrose	10%
Ascorbic acid	25 mM
BSA	0.15%

2. SDS Buffer

SDS	2%
Tris	100mM
NaCl	1.4 M
EDTA	20mM

3. TE buffer

Tris buffer	0.01 M
EDTA	0.001 M
pH	8.0