Cord 2009, **25** (1)

Toxins Produced by *Fusarium* Species in Leaf Scorch Decline Affected Coconut Palms (*Cocos nucifera* L.): Quantitative Analysis of Fusaric Acid, Zearalenone and T-2

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Abstract

Leaf Scorch Decline (LSD) is a disorder of coconut (*Cocos nucifera* L.) prevalent in Sri Lanka, for which the etiology is yet unknown and hence a study was conducted to identify the toxins produced by *Fusarium* spp. associated with LSD-affected palms. Root, stem and leaf tissues of LSD-affected and healthy palms were used to analyze the toxin content. Gas Chromatographic (GC) analysis carried out for Fusaric acid (FA), Zearalenone (ZEA) and T-2 (a Trichothecene) revealed the presence of FA in stem or leaves of many affected palms, but not in any of the roots. Total FA in stem and leaves was significantly higher ($p>0.01$) in affected palms than in healthy palms. Healthy palms were free of FA. Although ZEA and T-2 were present in both healthy and affected palms, the only significant difference between the two groups was ZEA in stem tissues. A strong association between FA and the presence of ZEA suggest they influence the production of LSD symptoms. The similarity of symptoms of *Fusarium* wilt diseases in other plants and LSD are discussed.

Keywords: Coconut palm, Fusaric acid, *Fusarium* toxins, Leaf Scorch Decline, T-2, Zearalenone.

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Introduction

Leaf Scorch Decline (LSD), Premature Decline (PD) and Rapid Decline are debilitating disorders prevalent in coconut plantations in Sri Lanka. LSD is the most prevalent disorder and it was first reported in the Southern Province of the country in 1955 (Kirthisinghe, 1966. unpublished) and now occurs in all coconut growing areas. A recent survey in the North-western Province revealed a mean incidence of 3.2% (Ranasinghe *et al.*, 2006).

Palms affected by LSD show several external and internal symptoms. The most noticeable visual symptom of the disorder is necrosis of the tips of leaflets, which progresses towards the midrib of the leaf. In a palm, leaf scorching progresses from the lower to the upper leaves and when leaves fall, prominent leaf scars remain on the stem. Gradually, a reduction in the number and size of leaves occur leading to a reduction in the size of the crown, a reduced number and size of nuts, and a reduced rate of inflorescence emergence, while a progressive reduction of stem girth results in a tapering of the trunk. The formation of fibrous roots and root decay has also been observed. Finally, the crown becomes a tuft of small leaves, without inflorescences and falls off (Ekanayake, 1963). LSD is evident in all ages of trees over 20 years (Kirthisinghe, 1966. unpublished). Even though the disorder is said to run its full course over a period of 2-6 years (Ekanayaka, 1963), on some occasions, palms take a much longer time to die or remain without change the severity spending their natural ageing (Humphries, 1970). Further, fluctuation, or reversal of symptoms or even total recovery have been observed.

Numerous studies have found that LSD-affected palms were associated with several histological, physiological and biochemical changes. Cortical and vascular browning, vascular damages, clogging of xylem vessels with tyloses have been observed (Peries, 1968). Reduced transpiration, leaf water potential, leaf chlorophyll level, functional canopy area, photosynthetic rate, and volume of inflorescence sap; increased stomatal diffusive resistance and water deficit; and a significantly low number of active roots were also found to occur in LSD-affected palms. Increased level of total polyphenolic compounds, higher polyphenols in withered leaves, abscisic acid and reduction of sugar and starch in leaf and various micro- and macronutrient imbalances have also been observed (CRI, 1988; Ranasinghe, 2005). However, no relationship between LSD and nutrient deficiency has been established. A recent study found an improvement of greenness of the canopy of LSD-affected palms after application of Zn (Maddurapperume, 2004).

Mycological studies have not revealed any relationship of LSD with leaf pathogens (Peries, 1968), virus (Hamphries, 1970), bacteria (Gunasekara and Kannangara, 1973) or phytoplasma (CRI, 1987). However, higher incidence of *Fusarium* was found to be associated with LSD-affected palms. Approximately 50% and 60% of fungi isolated from decaying root tips and rhizosphere of affected palms respectively belonged to few species of *Fusarium;* F. *oxysporium, F. moniliform, F. sambacinum, F. soloni,* and *F. javanicum.* In healthy plants, although *Fusarium* spp. were present, they were at low levels, 15% in decayed root tips and 14% in rhizosphere (Ekanayake, 1963; Peries, 1968). Therefore, it is thought that *Fusarium* spp. could possibly play a significant role in development of LSD symptoms. It has been suggested that factors such as reduced vigor of palms due to lack of nutrients and adverse soil conditions could predispose palms for *Fusarium* invasion (Peries, 1968). Failure of inoculum tests with *Fusarium* may be due to the fact that predisposing conditions has not been met in those studies. It is thought that root burrowing nematodes also provide access to *Fusarium* as in the case of *Radopholus similis* and *Fusarium spp* association in grapefruit seedlings (Feder and Feldmesser, 1961) and *F. solanai* and *F. moniliform* in banana root

lesions (Pinochet and Stover, 1980). In LSD affected palms, the parasitic nematode, *R. similis* is abundantly found in lesions of decayed roots (Koshy, 2000).

Plants infected by *Fusarium* spp. exhibit symptoms, which are very similar to those of LSD. They are yellowing, tip scorching, marginal desiccation, epinasty, withering, wilting, and death of leaves and remains of an abscission layer after leaves fall off, stunting, inability of leaves to expand to their normal size and browning of xylem (Dimond, 1972). These symptoms have been reported in vascular wilt in oil palm (Ho and Varghese, 1988), Canary Island date palm wilt (*Phoenix cararіensis)* (Pfalzgraf, 2002), collar rot disease in rattan (Zakaria *et. al.*, 1994), Panama disease in banana and wilt diseases in tomato, cotton, pea, cabbage (cabbage yellows) caused by *Fusarium* spp. (Kern, 1972). Many investigators consider that symptoms of fusarial diseases are primarily due to toxins produced by fungi. *Fusarium* spp simultaneously produce more than one toxin and of those zearalenone (ZEA), fusaric acid (FA) and trichothecenes are commonly found in plants (Gilbert, 1984; Kern, 1972). As *Fusarium* spp. are prevalent in roots of LSD affected palms and symptoms of LSD are mostly similar to that of fusarial wilt diseases, this study was conducted to examine whether these three toxins produced by *Fusarium* spp are found in LSD-affected coconut palms and determine their levels in different tissues.

Materials and method

The study was conducted at the Coconut Research Institute of Sri Lanka during February-November, 2006 following preliminary studies on Thin Layer Chromatography (TLC) for pooled and single samples and Gas Chromatography (GC) for qualitative analysis from 2003. Tissues were collected from roots, stems and leaves of LSD-affected and healthy palms in the field for screening.

Sample collection

Samples of LSD-affected palms were collected from Bandirippuwa estate (Kurunegala District, North-Western Province). Samples of healthy palms were obtained from a site at Navagaththegama (Puttalam District, North-Western Province). This site was isolated by paddy fields and no LSD-affected palms were found in the vicinity. For sampling, eight LSD-affected and five healthy palms of 20-25 years of age range in the same variety were selected.

From each palm, approximately 2 kg of roots were collected within 50 cm of the stem base and down to a depth of 30 cm. Stem tissues were extracted by boring 3 holes into the stem to a 10-15 cm depth at 80-90 cm of height above the ground level using a power drill with 13mm of drill bit. These holes were resealed with sand and cement mixture to prevent from the secondary attacks or infections. For leaf tissues, twenty leaflets were collected from five leaves of the middle whorl of healthy palms and from five uppermost affected leaves of affected palms.

Sample preparation:

Root and leaf samples were thoroughly washed in tap water followed with distilled water and cut into 50 mm pieces. For affected leaves, the pieces were obtained from the marginal scorched regions; stem tissues were used directly. From each sample, 40 g was put into glass bottles and 60 ml of methanol added. Leaf and root preparations were further crushed in a homogenizer to obtain fine particles. All root, stem and leaf preparations were centrifuged at 8000 rpm for 30 minutes at 5°C. and the extracts were collected in 100ml glass volumetric flasks. Samples were purified using C_{18} Cep-pack (Waters, U.K.) cartridges to remove substances that could interfere in the subsequent analysis. Each sample was concentrated using a rotary evaporator at 65°C to get a dry extract. That was mixed with 2-3 ml of BSTFA (Bis (trimethylsilyl) trifluoro-acetamide) and dissolved in 2-3 ml of hexane

(Gilbert, 1984). Standard solutions of 100mg/l of FA, 20mg/l of ZEA and 20mg/l of T-2 were prepared to determine the retention times of each toxin.

Sample analysis and data analysis

From each preparation 1□l was used for GC analysis. Analysis was carried out with a 25 m x 3 mm capillary silica fused column at 90°C for 2 min programmed at 30°C / min to 300°C, splitless injector at 200°C with Flame Ionization Detection (Gilbert, 1984). Data were analyzed by the general linear model procedure in SAS comparing least squares means to consider the probability difference for significance.

Results and discussion

All three toxins produced by *Fusarium* species were found in LSD-affected palms; FA was found in stem or leaf tissues of many affected palms, but was absent from the roots [of any]. In healthy palms only ZEA and T-2 were found; tissues of healthy palms were totally free from FA (Table 1). Presence of FA in the stem of affected palms was, statistically, significantly higher than in healthy palms whereas in leaves it was not (Table 1).

Table 1. Mean (±SE) amount (ηg/μl) of FA, ZEA and T-2 in different tissues of healthy (H) (n=5) and Leaf Scorch Decline-affected (A) (n=8) coconut palms

Tissue	FA	ZEA	T-2
Leaf H	0±0	872±471	114±41
A	1469±750	2277±398	76±35
P value	**0.23**	**0.04***	**0.50**
Stem H	0±0	2326±1959	64±346
A	1457±407	5249±1655	677±293
P value	**0.04***	**0.28**	**0.20**
Root H	0±0	933±823	155±53
P value	-	**0.55**	**0.22**

* Significant at p=0.05

ZEA was present at significantly higher amounts in the leaf tissues of affected palms ($P<0.05$) compared to healthy palms. Although not significant, mean levels of ZEA were considerably higher in stem and roots of affected palms than in healthy palms (Table 1).

T-2 was present in all tissues of healthy and affected palms in varying quantities, but the levels were much lower than the other two toxins. Both ZEA and T-2 levels in stem tissues of affected palms were relatively higher than levels found in roots and leaf tissues (Table 1).

The total amounts of ZEA and T-2 in above ground parts (stem+leaf) did not show any significant difference between healthy and affected palms. The FA of LSD-affected palms was, statistically, highly significant ($p<0.01$) because FA is not present in healthy palms (Table 2).

Table 2. Mean (±SE) amount (ηg/μl) of FA, ZEA and T-2 in above ground parts (stem+leaf) of healthy (n=5) and Leaf Scorch Decline affected (n=8) coconut palms

Palms	FA	ZEA	T2
Heathy	0±0	3197±1429	178±32
Affected	2927±706	7526±1973	753±666
P value	0.006	0.10	0.22

In fusarial wilt diseases several chemical compounds are produced by both the fungus and the host plant inflicting many anatomical and physiological changes in the plant (Dimond, 1972). However, the symptoms expressed by the host plant are mainly due to the toxins released by *Fusarium* spp. The host plant produces chemical compounds in response to the initial infection by *Fusarium,* which stimulate defenses such as browning, tyloses, vascular damage, gels, gums, foliar abscission and adventitious roots (Dimond, 1972). Stomatal closure, reduced

photosynthetic rate, reduced transpiration and leaf water potential are also plant defense responses to invasion of *Fusarium* spp (Dimond and Waggoner, 1953). Most of these changes reported in LSD-affected palms have been discussed. However, later in the colonization process the toxins produced by *Fusarium* spp overrides the defense mechanisms and cause severe disease symptoms in the host plants (Turner and Granili, 1969; Drysdale, 1984).

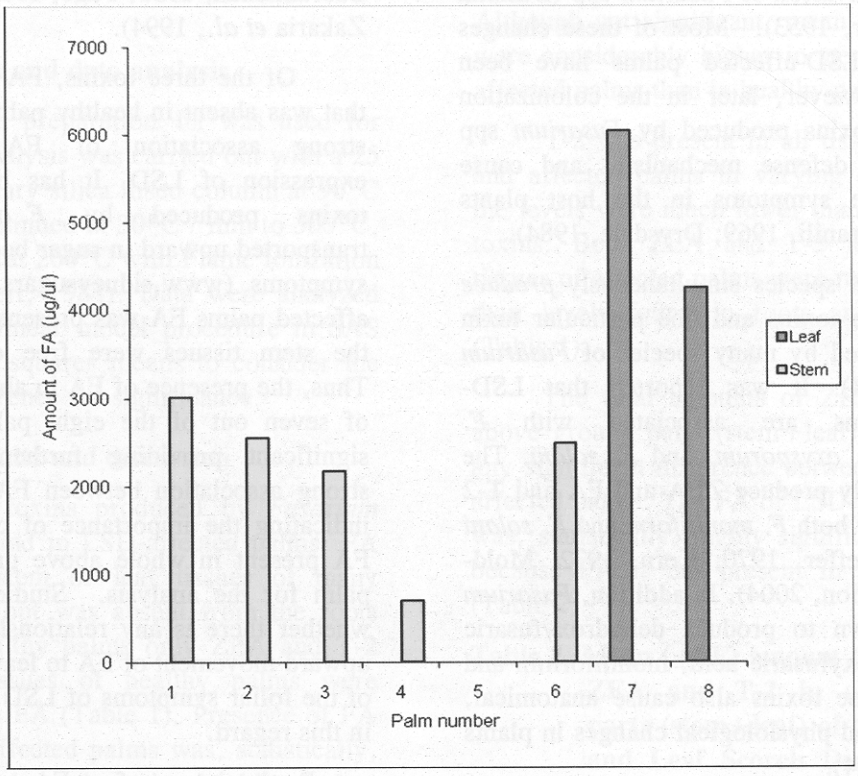
Fusarium species simultaneously *produce* more than one toxins and one particular toxin can be produced by many species of *Fusarium* (Gilbert, 1984). It was reported that LSD-affected palms are associated with *F. moniliform, F. oxysporum,* and *F. solani*. The first two mainly produce ZEA and FA and T-2 is produced by both *F. moniliform* and *F. solani* (Kuo and Scheffer, 1970; Kern, 1972; Mold-Help organization, 2004). In addition, *Fusarium* spp. are known to produce dehydroxyfusaric acid, 10-hydroxyfusaric acid, moniliformin and enniatins. These toxins also cause anatomical, biochemical and physiological changes in plants (Drysdale, 1984).

Inhibition of polyphenol oxidase activity may enhance the accumulation of polyphenols and auxins (IAA) and FA inhibits polyphenol oxidase and feroxidase activity in plants leading to low vascular browning (Wood, 1972) and a decrease in the respiration rate (Noef-Roth, 1957). In tomatoes, FA causes excessive water losses by damaging cell membranes, leading to leakage of water and other compounds, and it also chelates metal ions, causing scorching of leaf tips (Drysdale, 1984). High polyphenol levels have also been observed in LSD-affected palms. IAA promotes stomatal opening (Snaith & Mansfield, 1982) causing excessive water losses that lead to the scorching of leaves. In LSD-affected palms, characteristic scorching symptom that starts from leaf tips, low volume of inflorescence sap production and ionic imbalances could be possible effects of FA. [However, it appears that LSD is mainly associated with excessive water losses caused by fusaric acid]. The role of FA in cotton, pea, banana, flax, water melon and rattan has also been established (Lakshminarayanan & Subramanian, 1955; Page, 1959; Davis, 1969; Zakaria *et al.*, 1994).

Of the three toxins, FA is the only one that was absent in healthy palms, suggesting a strong association of FA in symptom expression of LSD. It has been found that toxins produced by *F. oxysporum* are transported upward in sugar beet causing foliar symptoms (www.sidneysugars.com). In LSD affected palms FA was present in leaves when the stem tissues were free of FA (Fig.1). Thus, the presence of FA in above ground part of seven out of the eight palms was highly significant providing further evidence for strong association between FA and LSD and indicating the importance of consideration of FA present in whole above ground part of a palm for the analysis. Studies to determine whether there is any relationship between the upward movement of FA to leaves and severity of the foliar symptoms of LSD are worthwhile in this regard.

Production of FA depends on environmental and nutritional factors as well as its rapid metabolisation and derivatization in a plant. Low oxygen levels (Kern, 1972), low potassium levels, for example in cotton infected with *F. oxysporum* (Ramassamy and Prasad, 1975b) and low iron levels enhance production of FA (Kern, 1972). It was also reported that weak pathogenic strains of *Fusarium* form large amounts of FA in media without Zn+ (Egli, 1969). Stomatal closure at initial infection by *Fusarium*, low levels of K, Fe and Zn may have contributed to high levels of FA in LSD-affected palms. Accumulation of Zn in roots without translocation to trunk and leaves (Maddurapperume, 2004) and presence of FA only in above ground parts of affected palms provides further evidence for the relationship. Metabolisation of FA to CO_2, small fragments and water soluble compounds (Braun, 1960: Raffner, 1974) and derivatization of FA to less toxic compounds such as N-methylated FA

Figure 1. Amounts of FA (ηg/μl) in above ground part (leaf and stem) of each LSD-affected palms



amide is common in resistant plants i.e. pea, cabbage, tomato, cotton (Heiatfuss et. al., Kluepfel, 1957). Absence of FA and presence of ZEA and T-2 produced by the same species of *Fusarium* in healthy palms would be due to such factors. Further, due to this nature of FA, even though the palms are infected with *Fusarium* they may remain as apparently healthy palms, since presence of FA is the most important factor in symptom expression.

ZEA interacts with membrane permeability and may inhibit H^+ and K^+ transport, K^+ stimulated ATPase activity, depolarize membrane potential and increase electrolyte leakage, for example ionic imbalance in red beet, potato, corn and cotton (Vianello and Macri, 1978). In addition, ZEA reduces synthesis of protein nucleic acid and chlorophyll, retards growth of plants, inhibits root elongation, and induces necrosis (Sadasivan and Saraswawathi-devi, 1957; Brodnik, 1975: Bilgrami, 1995). Observed effects of ZEA on LSD-affected palms may involve K and other ionic imbalances, reduced chlorophyll a and b, photosynthesis, functional canopy area, growth of crown and stem, growth of roots and their necrosis. In addition, inhibited K^+ transportation by ZEA may create a low K+ environment in the plant that is required for the production of FA.

The effects on other plants of T-2 involve chlorophyll content and synthesis of protein and nucleic acid (Bilgrimi, 1991). Even though the presence of T-2 is not statistically significant, the somewhat higher amount in stem tissues of affected palms suggests an influence on disease development. Low amounts of T-2 and ZEA present even in healthy palms would be due to low populations of *Fusarium*.

The close resemblance of symptoms produced by *Fusarium* wilts and LSD strongly suggests that FA produced by *Fusarium* spp. plays a major role in development of LSD symptoms. Further studies are needed to confirm the relationship.

Acknowledgements

The Sri Lanka Council for Agricultural Research Policy (CARP) and Dr. (Mrs.) C. S. Ranasinghe, Coordinator of the Multi-disciplinary Project, Head, Plant Physiology Division, CRI are gratefully acknowledged for the financial support given for part of the study. We are highly thankful to Mr. W.W. Noel Fernando, Senior Field Assistant and Mrs. Sweneetha Peries, Crop Protection Division of CRI for their hard work during the study. Thanks are also due to Mrs. P. Waidyaratna, Research Officer, Biometry Division, CRI for the Statistical Analysis. We also express our sincere gratitude to Dr. K.A.S. Hemachandra, Lecturer, Faculty of Agriculture, University of Peradeniya, for his valuable comments on the manuscript. We also thank rest of the staff of CPD, Mrs. W.P.K.K Fernando, Senior Technical Officer, Plant Physiology Division and Staff of the CRI Library, for their assistance in various ways.

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