Identification of Putative Vectors of Weligama Coconut Leaf Wilt Disease in Sri Lanka

A. D. N. T. Kumara, L. Perera, M. K. Meegahakumbura, N. S. Aratchige and L. C. P. Fernando

Abstract

Weligama coconut leaf wilt disease (WCLWD) is phytoplasma borne and reported in the southern part of Sri Lanka. The disease debilitates the palm, resulting in a drastic yield loss. It is well known that phytoplasma is transmitted from plant to plant by insect vectors, particularly phloem-feeders in the order Homoptera, i.e. leafhoppers and plant hoppers. A survey was conducted in the disease affected areas to collect homopterans present on the coconut palms, other plant species in the vicinity and in the environment. Insects were collected by hand, aspirator, sticky and light traps. The most abundant species were subjected to the nested polymerase chain reaction (PCR) using universal phytoplasma specific primers, P1/P7 and Pc399/P1694 to detect phytoplasma DNA present in their bodies. Thirty two homopteran and a few hemipteran species were collected from coconut plantations. Eight homopteran species, *Goniagnathus (T.) punctifer, Recilia dorsalis* Motschulsky, *Kolla ceylonica* (Melichar), *Idioscopus clypealis* (Lethierry), *Proutista moesta* (Westwood), *Proutista* sp., *Nisia nervosa* (Motschulsky) and an unknown Cixiid and a hemipteran species, *Stephanitis typica* (Distant) gave positive bands at 1280 bp. The DNA sequence of these bands was similar to WCLWD phytoplasma sequence (Gene Bank: EU635503), suggesting them as putative vector species of WCLWD.

Keywords

Coconut · Leaf Wilt Disease · Phytoplasma · Putative Vectors · Weligama

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Introduction

Coconut (*Cocos nucifera* L) is a major economically important plantation crop widely grown in Sri Lanka. Most plantations are distributed in the north western region called 'coconut triangle'. The southern region of Sri Lanka is the

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important coconut growing area found at about more than 100 km away from the coconut triangle and covering 13,498 ha. A debilitating yellowing syndrome of coconut plantation appeared in 2006, and it was identified as a phytoplasma-borne disease (Wijesekara et al. 2008). The disease was named as Weligama coconut leaf wilt disease (WCLWD) (Wijesekara et al. 2008; Perera et al. 2010). It was confirmed that WCLWD phytoplasma belong to the 16SrXI *Candidatus Phytoplasma* oryzae group and is most similar to the sugarcane white leaf phytoplasma as well as root wilt (Kerala wilt) phytoplasma in India (Perera et al. 2012). Symptoms of the disease are flattening and ribbing of the leaflets called flaccidity, marginal necrosis of leaflets and extensive yellowing of leaflets. These symptoms are more similar to the root wilt disease symptoms in India, but the symptoms of inflorescence rotting and yellowing of midworld fronds are not common in WCLWD. Both of these maladies act as predisposing factors for leaf rot disease, and palm become unproductive in a short period.

Phytoplasmas are prokaryote organisms of the class Mollicutes, affecting more than 700 plant species from tropical to temperate countries (Jones 2002). The phytoplasma cannot be cultivated invitro; therefore, molecular methods like PCR, RFLP, etc are the best approach for their detection, identification and characterization. Based on PCR-amplified ribosomal DNA (16S rgene) of phytoplasmas are classified using restriction fragment length polymorphism (RFLP) and DNA sequence analysis (IRPCM 2004). Phytoplasma diseases are transmitted through insect vectors, vegetative propagation of disease shoot to the healthy stocks, a vascular connection of healthy plant and disease plant by parasitic plants like Dodder (Dale and Kimks, 1969; Weintraub and Beanland 2006). Recently another mode of transmission of phytoplasma through seed/embryo has also been confirmed (Cordova et al., 2003; Khan et al., 2002; Oropeza, pers. comm.). However, the major mode of transmission is through insect vectors. In general, phloem-feeding insects, mainly in the suborder Auchenorrhyncha of order Homoptera, i.e. leafhoppers (Cicadellidae) and plant hoppers (Maixner 2005; Weintraub and Beanland 2006).

Most of identified phytoplasma-born disease vectors are belongs to the family Cicadellidae to date. Within Cicadellidae, subfamily Deltocephalinae contains more than 75% of all confirmed phytoplasma vector species (Nielson 1979; Weintraub and Beanland 2006). Insects in the subfamily Deltocephalinae are either monophagous or polyphagous and are able to transmit single or multiple phytoplasma species (Nielson 1979; Weintraub and Beanland 2006). Four families of plant hoppers namely Cixiidae, Delphacidae, Derbidae and Flatidae are well-known vectors of phytoplasma diseases (Weintraub and Beanland 2006). In addition, few species of Hemiptera have been reported as vectors of phytoplasma-borne diseases. *Stephanitis typica* (Hemiptera: Tingidae) has been identified as a vector of Kerala (root) wilt disease of India (Mathen et al. 1990). Brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentotomidae), responsible for transmission of the witches' broom phytoplasma to *Paulownia* trees in Asia (Hiruki 1999). Most of phytoplasma vectors are non-destructive feeders of plant and both nymphs and adult feed on same plant parts while living in the same habitat (Weintraub and Beanland 2006). In India, three insect vector species namely *S. typica* (Hemiptera: Tingidae), *Proutista moesta* (Westwood) (Homoptera: Derbidae), *Sophinia greeni* (Homoptera: Cicadellidae) have been confirmed in Kerala (root) wilt disease (Mathen et al. 1990; Solomon et al. 1998). The vector of lethal yellowing disease of coconut in the Caribbean and Central America was identified as a *Mindus crudus* Van Duzee (Homoptera: Cixidiidae) (Howard et al. 1983); whereas, in the African region, vector of lethal yellowing is not yet identified. The putative vectors of plant hoppers and leafhoppers were identified phytoplasma diseases, i.e. Kalimanthan wilt of Indonesia and lime decline phytoplasma in Saudi Arabia (Alhudaib et al. 2009; Warokka et al. 2006). This chapter reports abundance of phloem-feeding insect fauna associated with WCLWD, their seasonal variation, and PCR-based phytoplasma detection.

Material and Methods

Field Collection of Insects

Monthly surveys were conducted during 2010–2011 at ten locations in the disease-affected area in south Sri Lanka. The rainfall and maximum and minimum temperature data were obtained from the climatology department of Sri Lanka. Initially, preliminary observations and insect collections were made for the selection of suitable collection methods and insect distribution in the palms and surroundings using sticky traps, hand collection, aspirator and sweep netting. Thereafter, the survey was conducted in young palms and surrounding areas in the affected plantations based on the observations of preliminary survey. It was conducted in severely, moderately and mildly affected areas. Collected insect samples were freeze-dried and transported for further studies to the laboratory at the Coconut Research Institute. Collected insects were categorized according to the rainfall pattern as two rainy seasons and two off-rainy seasons, respectively, i.e. March–May, September–November, June–August and December–February. All the collected insects were separated and stored in the freezer for DNA extractions. Initially, insects were classified up to their genus level and sent to the Faculty of Agriculture, University of Peradeniya for further classification.

DNA Extraction and PCR Analysis

The stored insects were used for DNA extraction. The insect total nucleic acids were extracted from preserved insects using a protocol slightly modified by Mpunami (1997). CTAB extraction buffer (2% Cetyltrimethylammonium bromide); 1.4 M NaCl; 20 mM EDTA pH 8.0; 1% (wt/v) PVP−40; 0.2% (v/v) 2-mercaptoethanol) was used for DNA extraction. Pre-warmed extraction buffer—300 µl—was added into 1.5 ml sterile microcentrifuge tubes. Three insects from each species were placed in tubes and crushed using a pestle. This pestle was made from a sterile 1000 µl micropipette tip while sealing it at the distal end using a flame. Each sample was crushed and incubated at 65 °C in a water bath for 30 min for the lysis of the insect DNA. Then the tubes were allowed to cool at room temperature and were extracted by adding an equal volume of chloroform: isoamyl alcohol (24:1). The solution was gently mixed by inversion for 5 min, and was centrifuged at 12,000 rpm for 10 min to separate the phases. Then, the upper aqueous phase was transferred to a clean sterile tube. This chloroform: isoamyl alcohol (24:1) extraction method was repeated again to get pure DNA. Thereafter, separated upper aqueous phase was transfered into clean new tube and added the 0.6 volume of ice cold isopropanol into it. The mixture was inverted slowly in few minutes and was kept for few minutes at −20 °C for DNA precipitation. Tubes were then centrifuged at 12,000 rpm for 10 min for forming DNA pellet. DNA pellet was isolated by removing the liquid portion and then washing with 70% (v/v) ethanol. Washed pellet was air-dried and it was dissolved in 25 µl sterile distilled water or TE buffer and stored DNA at 4 °C for analysis.

The amplification of 16S ribosomal DNA was performed using a nested PCR and the phytoplasma universal primer pairs P1 (5′–AAG AGT TTG ATC CTG GCT CAG GAT T−3′ (Deng and Hiruki 1991) and P7 (5′–CGT CCT TCA TCG GCT CTT−3′ (Smart et al. 1996) used for the first PCR. The universal phytoplasma primers PC 399 (5′–GAA ACG ACT GCT AAG ACT GG−3′) and P1694 (5′–TGA CGG GCG GTG TGT ACA AAC CCC G−3′) (Skrzeczkowski et al. 2001) were used for the second PCR. PCR was performed in 20 µl reaction volumes in 0.25 ml micro tubes, and this reaction mixture contained 20–30 ng of template DNA, 0.5 µM of each primer, 200 µM each of the four dNTPs, 2 mM $MgCl_2$, 10 x polymerase buffer/PCR buffer, sterile water and 0.4 µl of *Taq* DNA polymerase (Go Taq polymerase, USA). Thermo-cycling parameters for primers P1/P7 and PC 399/P1694 followed the same procedure previously described by Smart et al. 1996; Heinrich et al. 2001; Skrzeczkowski et al. 2001; Perera et al. 2012. The primary PCR (first PCR) products were then diluted: 2 µl in 38 µl sterile distilled water, and 4 µl of dilution

was used for the nested PCR/2nd PCR. In nested PCR, the primers P1/P7 were used followed by universal primers Pc399/P1694, which amplify a 1280 bp DNA fragment (Lee et al. 1993; Skrzeczkowski et al. 2001; Perera et al. 2012). This PCR amplified template DNA of suspected insects collected from the diseased area, the insects collected from disease-free areas, diseased coconut leaf midribs and sugar cane white leaf disease DNA (positive control) and sterile distilled water (negative control). Amplified PCR products were subjected to electrophoresis in 1% agarose gel (using TBE buffer) by staining it with ethidium bromide (5 μg/ml). DNA bands were visualized with a UV transilluminator. The amplified DNA bands were cut and purified using Wizard ® SV Gel and PCR Cleanup System in accordance with the manufactured protocol. The purified products were sent for DNA sequencing to the University of Nottingham, UK and Gene Tech lab (Pvt.), Colombo.

Data Analysis

The mean number of each putative vector insects in each season was analyzed using ANOVA, and compared regression analysis of each insect group with rainfall and average atmospheric temperature using IBM SPSS version 19.

Result and Discussion

Relative Abundance

Preliminary observations revealed that both adult and young palms were associated with similar species of sucking insects. Lethal decline disease of coconut in Tanzania and lethal yellowing disease in the Caribbean, where disease symptoms appear in both young and old palms, and most of the insects captured were on young palms (Mpunami 1997). The insect-collection methods facilitated the trapping of a large number of airborne adult insects, and allowed collection of insects potentially moving between coconut trees or from ground-cover plants to and from coconut. Sticky traps were used to collect the insects living and flying around the coconut canopy; sweep nets caught those species living under the canopy and on grass; light traps gave an indication of species that were abundant at the vicinity of plantations as well as insects with nocturnal habit. Insects were collected from both the palms with symptoms and without symptoms in the selected plantations. In the survey, 32 Homopteran and one Heteropteran were collected. Insects belonged to seven families, i.e. Cicadellidae, Cixiidae, Delphacidae, Derbidae, Meenoplidae, Membracidae and Tingidae (Table 1). Among these, 22 species belonged to Cicadellidae. However, considering the abundance of individuals, the highest number of individuals in the affected area was from *Stephanitis typica* (Tingidae), *Proutista moesta*, *Proutista* sp. (Derbidae), *Recilia dorsalis*, *Nephotetreix virescens* (Cicadellidae) and *Nisia nervosa* (Meenoplidae) (Fig. 1). *Schwartziella typica* completes its life cycle on the coconut palm and the life stages on the coconut leaves, while adults of *P. moesta* and *Proutista* sp. were found only on the coconut palms, and their eggs and nymphal stages were found on the decaying organic material especially palm waste like decaying fronds, inflorescence etc. *N. nervosa* and *K. ceylonica* were mostly collected from surrounding vegetation, and few individuals were noticed on the coconut palm.

PCR and DNA Sequencing

According to the nested PCR test, the 1280 bp bands were given DNA samples of nine insect species collected from disease areas, four samples of the disease-affected coconut leaf midrib DNA, sugarcane white leaf disease sample. The DNA sample of insects (*P. moesta* and *S. typica*) collected from disease-free areas, sterile water samples and the three diseased coconut samples collected from diseased area were not given the required size of bands (Fig. 2a, b). The insects given to them that gave positive results among the 33 insect species were *P. moesta*, *Proutista* sp., *S. typica*, *N. nervosa*, *R. dorsalis, Goniangnathus (T.) punctifer* (Cicadelidae), *Idioscopus clypealis*

Table 1 Insect species collected in a survey from the WCLWD affected palms in Weligama

Order	Suborder	Family	Species
Homoptera	Auchenorhyncha	Cicadellidae	*Goniagnathus (T.) punctifer* *Recilia dorsalis* *Kolla ceylonica* *Kolla paulula* *Scaphoideus morosus* *Idioscopus clypealis* *Nephotetrix virescens* *Nephotetrix nigropictus* *Exitianus indicus* *Exitianus* sp. *Hishimonus* sp. *Platyrectus marginatus* *Stirellus* sp. 1 *Stirellus* sp. 11 *Balclutha* sp. *xvi. Hecalus porrectus* xvii. 5, Unknown species
Homoptera	Auchenorhyncha	Derbidae	*Proutista moesta* *Proutista sp1* Unknown sp.
Homoptera	Auchenorhyncha	Meenoplidae	*Nisia nervosa*
Homoptera	Auchenorhyncha	Cixidae	Unknown sp.
Homoptera	Auchenorhyncha	Delphacidae	Unknown sp. Unknown sp. Unknown sp. Unknown sp. Unknown sp.
Homoptera	Auchenorhyncha	Membracidae	Unknown sp.
Hemiptera		Tingidae	*Stephanitis typica*

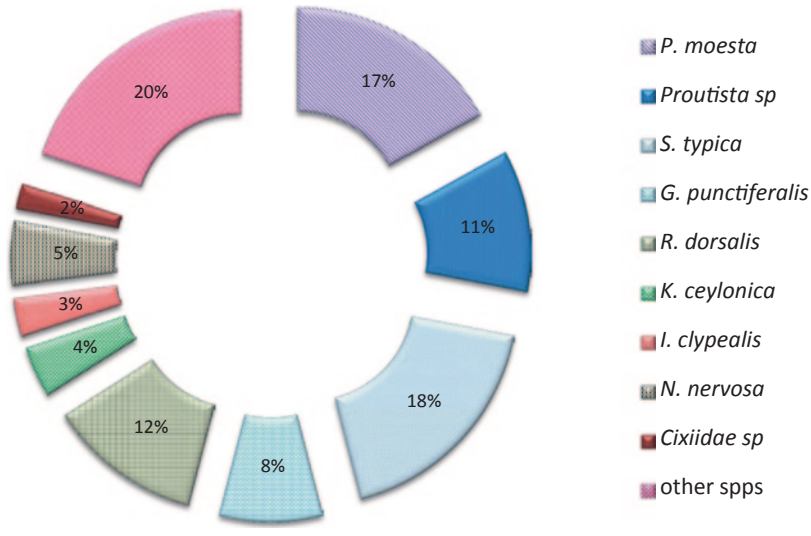

Fig. 1 Relative abundance of phloem feeding insect association in the WCLWD-affected coconut plantations

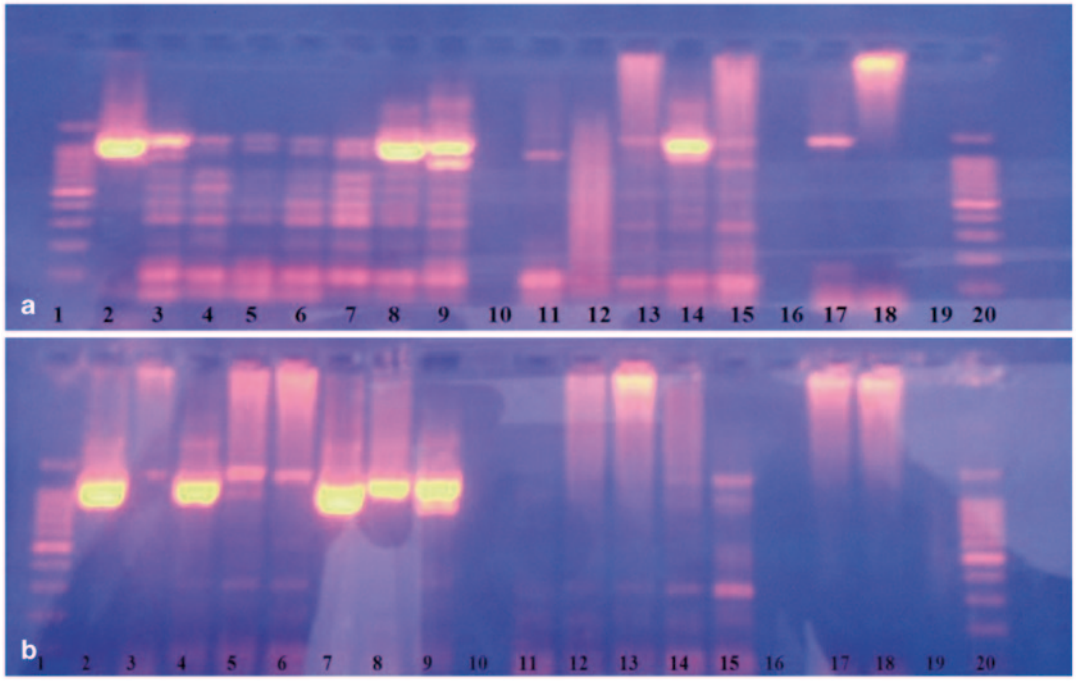

Fig. 2 Representative gels showing phytoplasma rDNA amplified from the DNA of putative insect vectors WCLWD with P1/P7 followed by nested PCR with primers PC 399/P1694; A Lane = 1 & 20, 1 kB ladder; 2–10, insect samples11 and 12 insect DNA collected from disease free area; 13, 14 and 15 infected coconut sample; 17 sugar cane white leaf sample, 18 sterile distilled water; *B lane* = 1 & 20, 1 kB ladder; 2–10, insect samples collected infected area; 10 and16 collected infected area; 10, 16 and 19 blank well, blank well, 11 and 12 insect DNA collected from disease-free area; 13, 14 and 15 infected coconut sample; 17 and 18 sterile distilled water

(Cicadelidae), *Kolla ceylonica* (Cicadelidae) and an unknown Cixiid sp. (Fig. 2 a&b). The sequencing and blasting of the positive DNA bands indicated that these sequences are matched with the gene bank deposited WCLWD sequence (Gene Bank accession number: EU635503). Among the tested insects, the highest percentage of positive results was given by *P. moesta* and *S. typica*, while other insects relatively were given low percentage of positive results. The results varied with time; due to a low titre of phytoplasma DNA present in the plant tissues especially in woody plants like coconut tree, the phytoplasma DNA was comparatively in very low concentrations in coconut tree tissues (Perera et al. 2012). However, the phytoplasma DNA concentration within the insect body was comparatively higher than in the plant due to multiplication of phytoplasma within the insect body before transmission (Weintraub and Beanland 2006). Comparatively, results showed on the DNA collected from *P. moesta*, *Proutista* sp., *S. typica* were consistent while the other six species showed a low constancy. It may be due to their feeding habit that adult insects always feed on the coconut leaf phloem sap. Their body has been filled every time with infected sap. *R. dorsalis* relatively is a more abundant insect but the percentage of positive results given was relatively low (Fig 3). The nine insect species have a wide host range and were not specific to coconut or family Palmae.

All phloem-feeding insects can acquire phytoplasma by feeding phloem sap. However, the insects are acquired the phytoplasma from plant sap through feeding, it was not translocated within the all phloem feeding insects. Within the insect digestive tract of most insects, the acquired phytoplasma was digested within short period of time before translocated. But few insects were allowed to multiple and translocation of phyto-

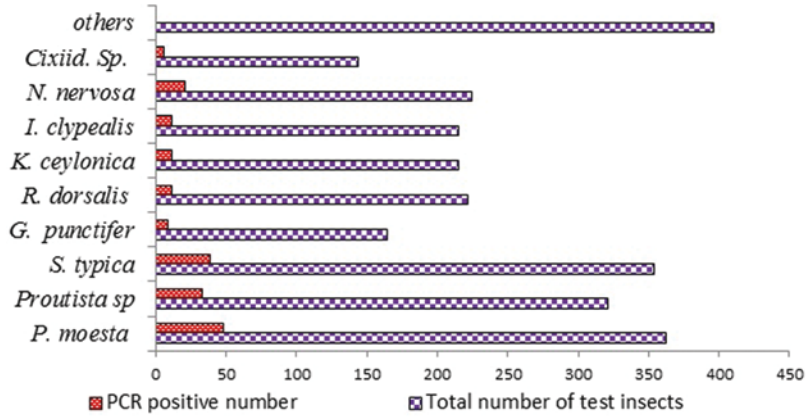

Fig. 3 Total number of each insect who were subjected to the nested PCR, and the number of those insects have given positive results

plasma in their body and they act as vectors. According to the Weintraub and Beanland (2006), vector species are found mainly in four families of Fulgorids: Cixiidae, Delphacidae, Derbidae and Flatidae. The first three families have at least one species that transmit a phytoplasma in the coconut lethal yellows group (16SrIV). Several species in these families also transmit phytoplasmas from the stolbur (Sr16XII) group. A Flatid vector, *Metcalfa pruinosa* (Say), transmits aster yellows (AY) (group Sr16I). This study was identified nine insect species as the putative vectors of WCLWD in Sri Lanka and five species of among them were recorded as vectors of several other phytoplasma diseases. The subfamily Deltocephalinae has the most highly derived lineages. More than 75% of all confirmed phytoplasma vector species are found in this subfamily. The feeding habits of the species within the Deltocephalinae range from monophagous to polyphagous, and members of this group can transmit one or more different phytoplasma taxa (Weintraub and Beanland 2006). The four species identified belong to this subfamily, viz. *R. dorsalis, K. ceylonica, I. clypealis* and *G. punctifer*. All the homopterans are non-destructive feeders (Mitchell 2004; Okuda et al. 1998), and among these nine species of insects, eight species are non-destructive feeders except *S. typical*. Hence, the feeding marks were also not visible on the coconut palms.

The presence of phytoplasma DNA within the body of these insects can be suspected as putative vectors of the WCLWD in Sri Lanka. This disease is more similar to root wilt disease of India, and they confirmed *P. moesta*, *S. typica* and *Sophonia greeni* (distant) are vectors of disease through electron microscopic studies, but they have not confirmed the disease using molecular techniques (Mathen et al. 1990). Some insects act as the dead-end host of phytoplasma and they acquire phytoplasma into body but do not transmit them. The alternate host plants also act as a source of inoculum without showing the symptoms (Weintraub and Beanland 2006). The alternative host plant present in the WCLWD-affected area surrounding the coconut plantations and insect may acquire the phytoplasma directly from that plant. This study may not clearly reflect the percentage of positive insects, as one sample contains DNA of three insects (Fig. 3).

Phytoplasma pass through insect generations via transovarian transmission, and if the infected female lays eggs, the phytoplasma goes to the next generation. Therefore, the insect, before feeding on the affected plant, may have phytoplasma in their body (Mitsuhashi et al. 2002; Alma et al. 1997). The identification and confirmation of the vector is not an easy task; however, it is important to identify the putative or possible vector before going to transmission studies.

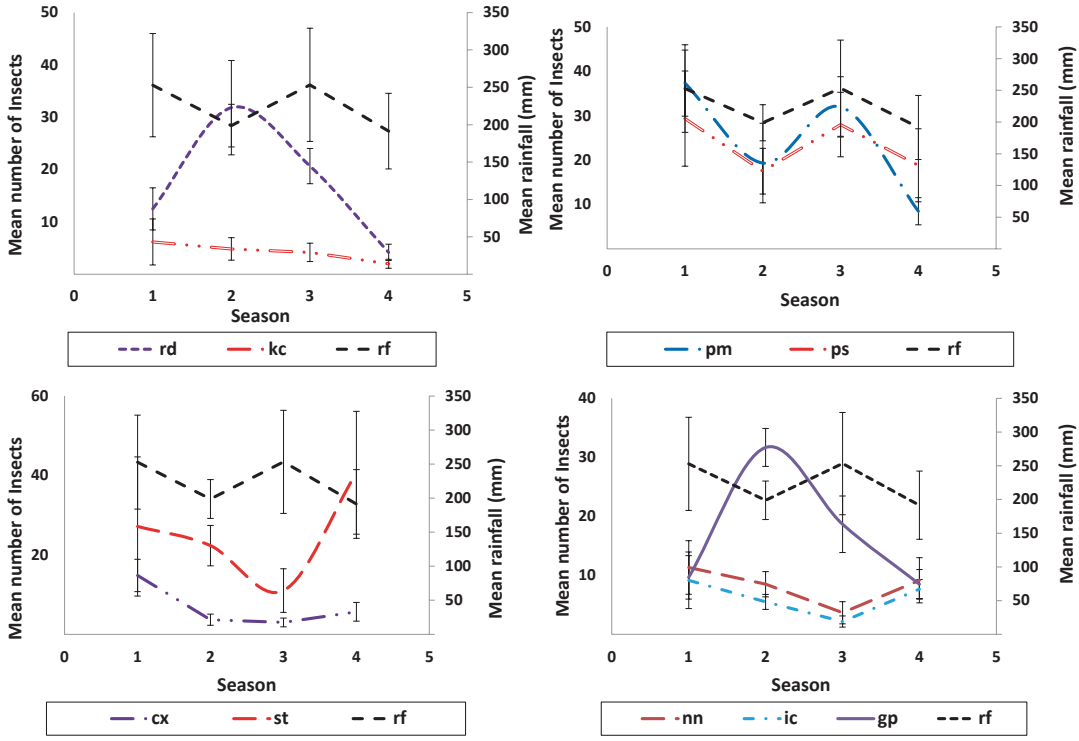

Fig. 4 Relationship insect vectors with rainfall pattern according to the season (within 2 years); season *1* December–February, *2* March–May, *3* June–August, *4* September–November. *Rf* Mean rainfall pattern, *pm P. moesta*, *ps Proutista* sp., *cx* cixiid sp., *st S. typica*, *rd R. dorsalis, kc K. ceylonica*, *nn N. nervosa*, *ic I. clypialis*, *gp G. punctifer*

Relationship with Climatic Factors

The 2-year survey indicated that the insect species and their abundance varied throughout the year. Compared to the mean number of insects with the season, *P. moesta* ($F=3.505$, $p=0.034$), *G. punctifer* ($F=8.653$, $p=0.001$), *R. dorsalis* ($F=5.015$ $p=0.009$), *Cixiid* sp. ($F=4.732$, $p=0.012$) were significantly different in the four seasons. Their abundance was generally higher in rainy seasons, and the number gradually declined with the reduction of rainfall (Fig. 3). However, only *P. moesta* and *Proutista* sp. followed the rainfall pattern (Fig. 4) (Pearson Correlation coefficient=0.460, $p=0.012$, $R^2=0.47$). The average atmospheric temperature is not significantly different in the four seasons, and there is no correlation with insect and the atmospheric average temperature ($F=0.264$, $p=0.613$). *S. typica* showed negative relationship with rainfall but this relationship is not significantly correlated (Fig. 4). In addition, there is a significant correlation of abundance pattern between insect species. The similar trends were observed in an individual species with others of their abundance in the field i.e. *R. dorsalis* and *G. punctifer* (Pearson Correlation coefficient = 0.460, $p=.012$, $R^2=0.47$) also, *K. ceylonica and Proutista* sp. (Pearson Correlation coefficient = 0.455, $p=0.025$, $R^2=0.41$), *N. nervosa* correlate with (Fig. 4) *I. clypialis* (Pearson Correlation coefficient =0.744, $p=0.001$) and Cixiid species (Pearson Correlation coefficient =0.712, $p=$ 0.001), and *Proutista* sp. also correlated with the *N. nervosa,* (Pearson Correlation coefficient = 0.368, $p=0.038$) *I. clypialis* (Pearson Correlation coefficient =0.376, $p=0.033$) as well as *K. ceylonica* (Pearson Correlation coefficient =0.455, $p=0.013$). The above data indicated that all putative vectors followed the same trend in the field (Fig. 4) and their abundance is related to the rainfall pattern, but all the data were not significantly

related due to the higher variation. It can be concluded that all the nine species are putative vectors of WCLWD in Sri Lanka, and *P. moesta, S. typica* and *Proutista* sp. are the more abundant ones.

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