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Viruliferous nature of the Sugarcane White Leaf Disease Vector; *Deltocephalus menoni* (Hemiptera: Cicadellidae)

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ABSTRACT

Sugarcane White Leaf Disease (SWLD) is one of the major threats to the cane sugar industry in Sri Lanka. *Deltocephalus menoni* (Hemiptera: Cicadellidae, Deltocephalinae) is the only locally-identified vector of this phytoplasma disease. Usually, the appearance of this disease coincides with the population dynamics of the vector. This study was conducted to determine percentage of viruliferous *D. menoni* adults in natural population and ability of the males, females and nymphs to transmit the SWLD. To determine the viruliferous percentage, the SWLDP infected insects were individually introduced to healthy plants and allowed to feed on healthy plants. Ability of *D. menoni* male adults, female adults and nymphs to transmit SWLD was studied by introducing virulified insects to healthy plants and allowing them to feed on healthy plants for one week period. The test plants were transferred into an insect proof field cage and were maintained for buildup of inoculum. Presence of Sugarcane White Leaf Disease Phytoplasma (SWLDP) was recorded by visualising disease symptoms and using Polymerase Chain Reaction (PCR) techniques. Masking ability of SWLD was confirmed by the higher number of infected clumps detected through PCR technology than the symptomatic clumps. Fifty-eight percent of adult insects in the natural vector population were found to be viruliferous and capable of transmitting SWLD after acquisition of the inoculum. It was found that male and female adults and nymphs are capable of transmitting the SWLDP. Results imply that *D. menoni* is efficient vector and management of WLD vector is vital to prevent rapid spread of this disease in sugarcane plantations.

Keywords: *Deltocephalus menoni*, Sugarcane White Leaf Disease, viruliferous insects, vector

INTRODUCTION

Sugarcane White Leaf Disease (SWLD) has become one of the major threats to sugarcane industry in several Asian countries since it causes low sugar yields due to severe reduction of cane yield, juice volume, pure obtainable cane sugar (POCS) and fiber in canes (Chandrasena *et al.,* 2003,Chanchala *et al*., 2014, Wongkaew, 2012). Sugarcane White Leaf Disease Phytoplasma (SWLDP), which is a distinct phytoplasma strain within

the 16SrXI "Phytoplasma orizea", sub group 16SrXI-B (Marcone, 2002; Ariyarathne *et al.,* 2007) is the causative agent of this disease. The disease spreads in sugarcane plantations primarily, through infected seed cane (Jayarathne, 1996) and secondarily, by an insect vector *Deltocephalus menoni* (Hemiptera: Cicadellidae, Subfamily: Deltocephalinae). True seeds do not carry phytoplasma (Cronje and Bailey, 1999; Lee *et al.,* 2000) and this pathogen does not transmit mechanically through farm equipment, by inoculation of sap (Lee *et al.,* 2000) and not air borne (Senevirathna, 2008).

Under local conditions, hot water treatment of seed cane; 54 °C for 50 minutes (Chandrasena, 2001), rogueing out of infected sugarcane plants from the field and replanting or fallowing the fields where infection level is more than 20 percent (SRI, 2015) are recommended to manage the SWLD. However, even after adopting these practices, the disease in the plantations has been spreading at an alarming rate and hence its management has become crucial. Therefore, management of SWLD vector has been identified as a strategic and integrated approach to prevent rapid spread of the disease in the plantations. The leaf hopper, *D. menoni* has only been the identified vector, which transmits SWLD in Sri Lanka. Even though, two other leafhopper species *viz., Matsumuratettix hiroglyphicus* Matsumura (Matsumuto *et al.,* 1969) and *Yamatotettix flavovitatus* Matsumura (Hanboosong *et al.,* 2006) were identified as the vectors of SWLDP in other countries, those species are not present in Sri Lanka. In Taiwan and Thailand *M. hiroglyphicus* is considered as the most active vector for SWLDP and *Y. flavovitatus* is found be less in vectoring ability.

Similar to the reported evidences regarding *M. hiroglyphicus* and *Y. flavovitatus* in other countries, *D. menoni* is also showing similar characteristics to be an active vector of SWLDP in Sri Lanka. *D. menoni* is abundant in all commercial cane growing areas *viz*., Sevanagala, Pelwatta, Hingurana and Passara in the island. There is a definite pattern of dynamics of the vector population in concurrence with the level of disease incidences recorded in the plantations (Chanchala *et al*., 2014). The vector population was recorded to be high in the 1st and 4th quarters of the year and the levels of SWLD incidences were also high in the same periods. The level of SWLD incidences in the field was significantly correlated with the level of vector population that had been recorded a month before. However, this type of relationship was appeared only in the fields with low level of disease incidences indicating that the available knowledge about relationship between the vector and the disease is not adequate to describe the pattern of spread of the disease in local sugarcane plantations (Chanchala *et al.,* 2014). This study was conducted to determine the percentage of viruliferous insect vectors in natural population and to find out the ability of males, females and nymphs of *D. menoni* in transmitting the SWLD.

MATERIALS AND METHODS

Insect-proof rearing cages in the Entomology and Biotechnology laboratories of the Sugarcane Research Institute (SRI), Uda Walawe were used for the study from 2017 to 2018. Required cultures of *D. menoni*,

SWLD-infected plants used as the phytoplasma inocula and healthy plants were maintained using the following procedures.

Maintenance of insect cultures

The adult insects of *D. menoni* were collected using sweep nets and pooters from young sugarcane plants in the research farm, SRI, Uda Walawe. The insects collected were reared in insect-rearing cages according to the protocol developed by Senevirathne (2008).

Raising SWLD-infected plants

The SWLD-infected sugarcane plants of the variety SL 96 128 were selected through external symptoms *viz.,* diffused and proliferated tillering, chlorotic leaves and yellow-colour of fully-developed leaves of the mature canes (Senevirathne, 2008). Seed setts for raising SWLD-infected plants were obtained after confirming the presence of disease in mother plants by molecular techniques (Senevirathne, 2008). Setts were established in pots filled with sterilized soil. Plants kept in an insect proof screen house were maintained under the recommended agronomic practices (SRI, 2004) to use in the experiments.

Raising healthy plants

Sugarcane plants produced through meristem culture using the *ex-plants* obtained from mother plant nursery of the variety SL 96 128, established using hot-water-treated seed cane (54 ^{0}C for 50 minutes), were used in the experiment. These plants were further tested for SWLD phytoplasma by molecular techniques (Senevirathne, 2008) and only the plants, which were confirmed free from SWLD were used to raise healthy plant population. These plants were kept in the insect proof screen house and maintained under the recommended agronomic practices (SRI, 2004).

Estimation of percentage of viruliferous insects in natural population

D. menoni adults were collected in the field using sweep nets and pooters and these insects were introduced to the SWLD-infected sugarcane plants in insect rearing cage for two days to acquire SWLDP inoculum by feeding. The infected insects were then collected individually using a pooter and they were introduced to one-month old healthy plants raised in insect proof laboratory cages as one insect per plant. Forty-five plants were used in this experiment to determine viruliferous insect percentage. Insects were allowed to feed on healthy plants in individual cages for one week. The test plants were transferred into an insect proof field cage and were maintained for eight months for development of SWLD symptoms. Disease development in healthy test plant occurs due to disease transmission of vector to particular sugarcane plant. As only one vector introduced to one plant, that vector should be virulified vector to transmit the disease. Therefore in this experiment, number of plants infected with SWLD is equal to the number of virulified insects in the population. Plants were observed regularly for appearance of the symptoms. SWLDP in test plants was confirmed using PCR techniques according to Senevirathne (2008) to determine the percentage of viruliferous insects in natural population.

Determination of the ability of *D. menoni* males, females and nymphs to transmit SWLD

Ability of *D. menoni* male adults, female adults and nymphs to transmit SWLD was studied using the insects reared in the laboratory. Both 2nd and 4th instar nymphs were considered here in confirming the ability of early and late instar nymphs to transmit the disease. One-day-old five adult males and females after ecdysis were collected separately using a pooter by considering the colour of wings and the size of the body. Males are smaller in body size and lighter in colour compared to females. Moreover, their gender was confirmed by observing genitalia with a hand-lens. Collected insects were introduced to SWLD-infected plants in insect rearing cage for 2-day period to acquire SWLDP inoculum by feeding. After feeding for two days, the insects were collected using a pooter and introduced to one-month-old healthy plants raised in the insect proof laboratory cages.

The nymphs in 2nd and 4th instars were collected using a camel hair paint brush. The nymphal growth stage was decided by considering the number of molting times as for 2nd instar, after one molting and for 4th instar, after three moltings. Five nymphs from each stage were introduced to a SWLD-infected plant in insect rearing cage and allowed to feed one day to acquire SWLDP inoculum. After feeding, the insects were collected using a camel hair paint brush and introduced to one month-old healthy plants raised in insect proof laboratory cages. Insects were allowed to feed on healthy plants for one week period. Then test plants were transferred into an insect proof field cage and maintained for eight-month period for SWLD symptom development. Presence of SWLDP in the tested-plants was confirmed by PCR techniques as per Senevirathne (2008).

Detection of SWLD in test plants

Visual detection

SWLD-infected clumps were visually-detected by considering the appearance of the test plants *viz.,* diffused and proliferated tillering, chlorotic leaves, yellow colour of fully-developed leaves of mature canes and soft texture of symptomatic leaves (Senevirathne, 2008).

Molecular detection

Molecular detection of SWLDP was done for more accurate results because relying only on disease symptoms in plants in the laboratory may lead false negatives. A twenty centimeter-long piece of the third leaf of test plants was collected individually using scissors and cleaned using distilled water. Samples of these plant leaf tissues were cut into small pieces (0.3 g) and were flash frozen in liquid nitrogen before grinding into powder in a mortar. DNA extraction was done according to Senevirathne , (2008) and quantity and the quality of the DNA were measured by Nano drop spectrophotometer. PCR amplification was performed using SPP1 forward (5′-ATTAAAGTGCCCATCATG-3′) and SPP2 reverse (5′- GTACTAAGTGTCGG GATT-3′) primer pair (senevirathne, 2008). After amplification of DNA, results were documented using gel documentation system. Size of the amplified bands was measured by referring to the 100 bp molecular weight marker and expected band size was 300pb.

RESULTS AND DISCUSSION

Appearance of initial symptoms of SWLD was observed in some test plants as two white or creamy lines with usually indistinct margin parallel to the midrib located its either sides. The pattern of symptom appearance was clearly visible on the young shoots of the clump. The symptomatic leaves were soft textured and new leaves were reduced in size. The stems of infected plants were stunted and tillers were profusely borne. Top leaves of the stalks were seen crowded due to the slow growth of the upper portion of the infected stalks. At the same time the buds at the lowest part of the stem started sprouting and at latter stages, sprouting continued on the upper part of the stem. Some infected younger clumps appeared as rosettes of grasses, whitish or yellowish in colour without stalk formation and died shortly.

Percentage viruliferous insects in natural population

White Leaf Disease symptoms appeared in the tested plants varied depending on plant age, crop cycle and method of detection (Table 1). Among the tested plants 58% plant samples produced bands when PCR was performed (Table 1 and Fig. 1). Accordingly, in the natural vector population, 58% is capable of transmitting the disease after acquisition of the inoculum.

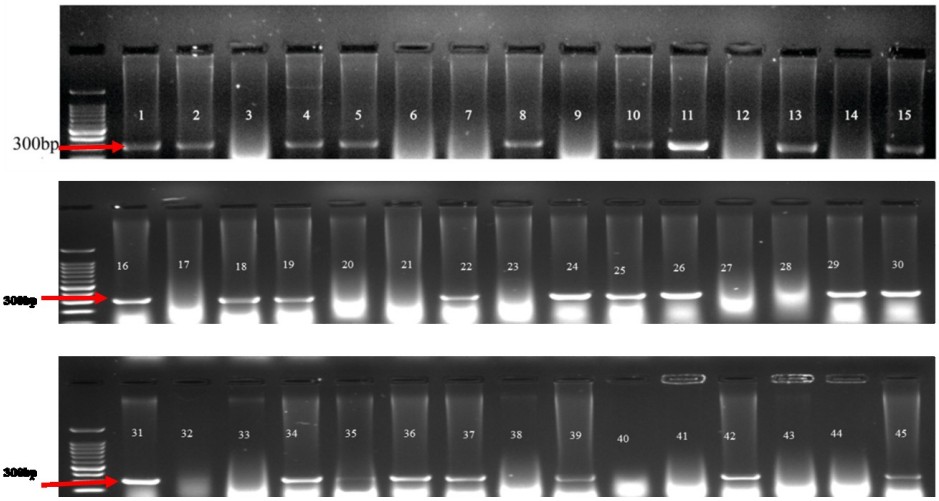

Fig. 1. PCR amplification of DNA of the tested plants used for detecting viruliferous percentage of *D. menoni* on 1.5% agarose gel

Note: 300 bp band indicates SWLDP. The numbers on the lanes represent the plants infected by individual insect vector. The samples 3, 6, 7, 9, 12, 14, 17, 20, 21, 23, 27, 28, 32, 33, 38, 40, 41, 43 and 44 are free from infection.

Table 1. The percentage of sugarcane white leaf disease (SWLD) positive plants for detecting viruliferous percentage of *D. menoni*

Method and stage of observation	Percentage SWLD positive plants
Visual observation/Plant crop at 8 month age	36
Visual observation/Ratoon I crop at 2 month age	47
Molecular detection/Plant crop	58

In the transmission tests conducted by Hanboosong *et al.* (2006) in similarly manner, percentage transmission of *M. hiroglyphicus* recorded at 55-100% and the percentage transmission of *Y. flavovitatus* has been recorded at 45%. The results of this study revealed that *D. menoni* transmitted the disease in more or less similar manner. Number of authors reported that several phytoplasma vectors had proven similar phenomena *viz., Cacopsylla pyri*; vector of pear decline (PD) (Carraro *et al*., 2008; Garcia-Chapa *et al.,* 2005), *Cacopsylla picta* and *Cacopsylla melanoneura;* vectors of Apple proliferation (AP) (Carraro *et al.,* 2008; Jarausch *et al*., 2004 and 2007), *Cacopsylla pruni* vector of European stone fruit yellows (ESFY) (Jarausch *et al*., 2004 and 2008). Jarausch and Jarausch (2010) have emphasized that acquisition of a phytoplasma by an insect does not imply that the insect is a vector since phytoplasmas may be acquired but not re-injected by feeding.

Percentage infection observed visually in the test plants in plant and ratoon I stages was comparatively lower (11% and 22% respectively), than the real infection detected by molecular techniques**.** Similar to the current study, fewer occurrences of SWLD symptoms on test plants were recorded in many transmission tests conducted in the past and PCR amplification demonstrated the presence of phytoplasma in non-symptomatic plants (Hanboosong *et al.,* 2006; Pilkington *et al.,* 2004; Tran-Nguyen *et al*., 2000). The low phytoplasma titer developed systemically in the plants during relatively shorter period of time does not allow developing visual symptoms (Hanboosong *et al.,* 2006). Jaraush *et al.* (2001) and Maixner *et al.* (2000) reported that the rate of phytoplasma disease expression in test plants was often to be low comparatively to the real infection detected by molecular techniques. Arocha *et al.* (2005) stated more or less similar transmission rates of sugarcane yellow leaf phytoplasma by plant hopper *Saccharosydne saccharivora* detected through visual symptoms and molecular techniques. Therefore, detection of phytoplasma through molecular tools always produce higher accuracy of phytoplasma transmission studies. Masking ability of SWLD in sugarcane was confirmed by the higher number of infected clumps detected through PCR technique than the number of symptomatic clumps. Leu (1983) testified that SWLD symptoms are masking in infected sugarcane during the winter and the symptoms have been developed rapidly in the same crop when approaching the warmer season and continue throughout the warmer season in Taiwan. Lue (1983) explained that the effect of temperature differences in the winter and other seasons is the major course for masking SWLD symptoms in infected sugarcane. Ouvanich *et al.* (1990) and Ouvanich and Kusalwong (1993) revealed

expression of SWLD symptoms also depends on the soil fertility and particularly on the sugarcane variety. Thus, the severity of disease expression is influenced by several factors such as seed cane quality and vigour, cultivation practices, sugarcane variety, soil type, fertilization and systemic phytoplasma titer in planting material (Wongcave, 2012). The differences in detecting SWLD infection in the test plants through disease symptoms and PCR techniques in the present study can be explained by the masking effect influenced by the environmental factors prevailed during the period of experimentation.

Ability of *D. menoni* males, females, and nymphs to transmit SWLD

All the plants subjected to study transmissivity of SWLDP by *D. menoni* males, female and nymphs of 2nd and 4th instar showed SWLD symptoms at plant crop stage and produced bands related to SWLDP when the PCR was performed (Fig. 2). Accordingly, adult males, females, 2nd instar and 4th instar nymphs are capable of transmitting SWLDP from infected plants.

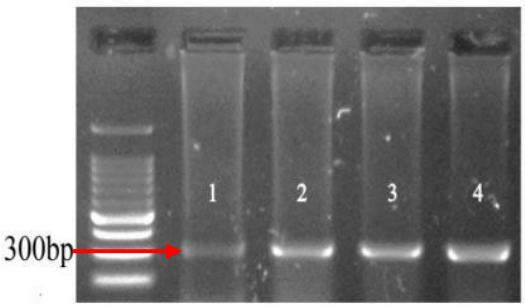

Fig. 2. PCR amplification of tested plants for determination of the ability of males, females and 2nd instar and 4th instar nymphs of *D. menoni* to transmit SWLDP on1.5% agarose gel

Note: Note: 300 bp band indicates SWLDP in the samples. The lane numbers 1-infected by adult males, 2- infected by adult females, 3- infected by 2nd instar nymphs, 4- infected by 4th instar nymphs.

Available literature demonstrated that transmission efficiency of insect vectors can vary between the sexes and among deferent growth stages of the vectors. Female vectors of Aster Yellow (AY) disease in lettuce are more efficient in transmitting the disease (Beanland *et al.,* 2000). The first instar nymphs of *Euscelidious variegatus*, the vector of Chrysanthemum Yellows disease (CY), do not transmit the disease while 5th instar nymphs of the same insect transmit the disease (Palermo *et al.,* 2001). In corn leaf hopper *Dalbulus maidis*, the vector of maize bushy stunt phytoplasma, and aster leaf hopper *Macrosteles quadrilineatus*, the vector of Ohio strains of aster yellow phytoplasma, nymphs are more efficient transmitters than the adults (Moya and Nault, 1998, Murral *et al.,* 1996). Hence, it is important to study the viruliferous nature of adult males, females and the nymphal stages of vectors separately to determine the transmission efficiency.

CONCLUSIONS

Under local conditions, fifty-eight percent of insects in the natural *D. menoni* population was found to be viruliferous and capable of transmitting SWLD after acquisition of the inoculum. Male and female adults and nymphs are capable of transmitting the SWLDP. Use of PCR technology in detection of SWLDP increases the accuracy of phytoplasma transmission studies. Masking ability of SWLD in sugarcane was

confirmed by higher number of infected clumps detected through PCR techniques than the number of symptomatic clumps. Results imply that *D. menoni* is efficient vector in secondary transmission of the SWLD. Management of SWLD vector is vital to prevent rapid spread of this disease in sugarcane plantations.

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