Mode of Transmission of Sugarcane (*Saccharum officinarum* L.) White Leaf Phytoplasma in Sri Lanka

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**ABSTRACT.** The mode of transmission of sugarcane (*Saccharum officinarum* L.) white leaf disease in Sri Lanka (SWLD-SL) was studied in a sugarcane plantation severely affected with the disease. The involvement of insects associated with the aerial parts of sugarcane in the secondary transmission of SWLD-SL was concluded other than the primary transmission by the infected seed cane. It was also revealed that the soil-inhabiting organisms or metabolites are not associated with the disease. The presence of causal phytoplasma of SWLD-SL in the affected sugarcane plants was determined by the analysis of a part of 16SrRNA gene of the phytoplasma by a direct PCR method using a pair of specific primers and also by symptomatology. The possible damage caused by the disease to a sugarcane plantation was also emphasized.

**INTRODUCTION**

Sugar industry of Sri Lanka has been threatened by a devastating phytoplasma disease locally known as grassy shoot / white leaf disease. The disease was first recorded to have appeared in Kantale in 1972 (Jayathilake, 1973) causing severe crop losses in the same area in 1991 and Siyambalanduwa in 1995, is now prevalent in all the major sugarcane (*Saccharum officinarum* L.) growing areas of the country. Most common foliar symptoms of the disease are narrowing and partially or almost chlorotic leaf lamina. Stunting, excessive tillering, formation of side shoots from the bottom to the top of the stalks are the other diagnostic symptoms of this disease. Severely infected younger plants, which appear as yellowish or whitish rosettes of grass, may die eventually. An important characteristic feature of the symptomatic plants is on and off masking of symptoms hiding the pathogen inside.

Association of a phytoplasma with this disease was first established in 1997 (Jones *et al.*, 1997) and the phytoplasma was found indistinguishable from sugarcane white leaf phytoplasma in Thailand based on leaf samples collected from Sevanagala, Uda Walawe and Siyambalanduwa (Kumarasinghe and Jones, 2001; Chandrasena *et al*., 2003). According to sugarcane pathologists, this disease however, shows both types of symptoms present in sugarcane white leaf disease (SWLD) found in the Thailand, Taiwan and Japan (Chen, 1973; Sarindu and Clark, 1993; Nakashima and Murata, 1993) and sugarcane grassy shoot disease

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(SGSD) found in the Indian sub-continent (Chona et al., 1960; Rishi and Chen, 1989). Owing to lack of studies conducted on identification of the disease in Sri Lanka, name of the disease is still ambiguous. Hence, the disease is herein referred to as sugarcane white leaf disease in Sri Lanka (SWLD-SL) and the causal phytoplasma as sugarcane white leaf phytoplasma in Sri Lanka (SWLP-SL).

It was believed that the infected seed cane was responsible for the primary transmission of SWLD-SL and many local sugarcane plantations have been affected due to the use of infected seed cane by sugar companies and farmers. The phenomenon has been observed in other closely related diseases such as SWLD and SGSD (Chona et al., 1960; Chen, 1980; Shukla et al., 1991; Harlapur et al., 1992). However, resurgence of SWLD-SL was noticed even in the rehabilitated plantations, which had been established on lands fallowed for about 6 months after completion of the program and with seed cane of well-maintained nurseries raised from heat-treated seed material (heat therapy is an accepted method to eliminate pathogen from seed cane) obtained from apparently disease-free areas. This suggests the ways of SWLD-SL spreading, other than the primary transmission in Sri Lanka. Further, it is noteworthy to mention that the seedlings in the first ground nursery in the research farm at SRI, expressed SWLD-SL symptoms after a period of five to six months. It has been emphasized that true seeds are not carriers of phytoplasma pathogens (Cronje and Bailey, 1999; Lee et al., 2000). Many diseases of phytoplasmal etiology have been reported to be secondary transmissions by different types of insect vectors around the world (Chen, 1973; Purell, 1982; Liefting et al., 1997; Hill and Sinclair, 2000). All the above evidence with the nature of rapid spread of the SWLD-SL in the local plantations suggests that the disease is secondarily transmitted. Comprehensive surveys conducted on insects associated with sugarcane in affected local plantations before the beginning of this study revealed that Matsumura Matsumuratettix hiroglyphicus, the only recorded vector of the SWLD (Chen, 1973) was not available in Sri Lanka. Spreading of SWLD-SL over three decades even in lower magnitude in a vegetatively propagated crop, absence of recorded vectors of closely related sugarcane diseases from the local plantations, nature of symptom masking and highly environment-dependent symptom expression counter-argued the concept of the secondary transmission of SWLD-SL. Farmers were of the view that the application of fertilizer and soil insecticides has direct effects on the recovery and control of the disease and that the pathogen has some connection with the soil. Further, the phenomenon of mix infection cannot easily be ruled out with the diseases of phytoplasmal etiology (McCoy, 1979; Tran-Nguyen et al., 2000; Aljanabi et al., 2001). It was in this backdrop that an investigation into the possible ways of transmission of SWLD-SL was undertaken to eliminate those ambiguities and future misinterpretations as the first step towards in-depth studies on secondary transmission of the disease.

**MATERIALS AND METHODS**

The field activities of the investigation were conducted at the Sugarcane Pathological Farm of the Sugarcane Research Institute (SRI) at Siyambalanduwa, Sri Lanka while the laboratory works were performed both in the laboratories at the College of Natural Resources and Environment, South China Agricultural University, Guangzhou, Peoples Republic of China and the Sugarcane Research Institute, Uda Walawe, Sri Lanka.
A factorial experiment was conducted to make comparisons on the development of SWLD-SL symptoms on potted test plants grown at different treatment levels, which can elucidate different avenues of transmissibility of SWLP-SL and to test possible interactions among the treatments.

**Preparation of seed cane**

'Healthy seed cane' was obtained from sugarcane (*Saccharum officinarum* L.), var Co 775 raised from apical meristem tissues of apparently healthy cane selected from a field, which had no history of SWLD-SL. Plants were grown in an insect-free environment until the stalks were ready for the experiment. The seed cane collected from matured plants of the same variety with clear SWLD-SL symptoms appeared at the latter stages of growth was referred to as 'infected seed cane'. In both cases, single-budded setts of about 5 cm in length were used for planting in polythene pots. The two seed cane types were used to confirm whether the SWLP-SL is seed cane borne or not, and as a reference treatment.

**Preparation of potting media**

Two categories of soils were used in the experiment namely 'sterilized' and 'WLD-associated'. The topsoil collected from the bases of SWLD-SL symptomatic plants up to a depth of about 20 cm and a radius of about 30-40 cm was referred to as WLD-associated soil and such soil after sterilization was called sterilized soil. Two soil types were used to check whether the soil borne organisms transmit SWLP-SL or the symptoms are produced in association with any soil inhabiting organisms. Soils were reddish brown earths with alluvial fraction having a sandy loam texture and these were sterilized in steam at 100°C for 2.0-2.5 h.

**Preparation of soil extract**

The WLD-associated soil collected from the root zone of the severely SWLD-SL affected clumps was used to prepare the soil extract with distilled water. Soils were sieved through 4 mm sieve to remove the large soil particles and the debris and samples of 250 g of soil were collected in one-liter beakers. Distilled water was added up to the volume of 1 liter level and stirred thoroughly for 3 min. with a glass rod and left for 15 min. to settle the particles. The suspension was sent through Whatman No. 01 filter paper to collect the filtrate as 'soil extract' for the experiment. The soil extract was added on to sterilized soil to test the ability of isolation of possible soil borne vectors or to determine association of any microbes or metabolites in producing SWLD-SL symptoms. The debris and other soil particles left on the sieves and the left over in the beaker were thoroughly observed by the naked eye and under the microscope for any organisms or particles.

**Maintenance of pots before and after establishment of test plants**

All plastic buckets were filled with either sterilized or WLD-associated soil up to 8 cm below the top levels after laying 6-8 cm layer of 5 cm x 5 cm metal on the bottoms of the buckets, which had four small holes (each of 1 cm diameter) for drainage. Each pot was placed on a hard polythene cover, the edge of which was extended 6 cm beyond the top surface of a concrete stand measuring 25 cm in height and 30 cm in diameter. The soil surface of each bucket was covered with a 2 cm thick rigifoam (Polyurethane rigid foam)
plate. Specially designed watering device made from pieces of a conduit tube (1.5 cm diameter) was buried into the potted soil and the water inlet of the device was set to run through the rigifoam plate. In a few pots, soil thermometers were placed at 5-7 cm depth in the potted soil to measure the soil temperature. The outer surfaces of the plastic buckets were painted in green with enamel paint and the rigifoam plate with green emulsion paint. In some pots, tensiometers were dipped in the soil to determine the moisture levels of the potted soil and soil moisture of the pots was adjusted accordingly. Thus, each plastic pot was made ready to receive young sugarcane so far grown in polythene pots.

Ten treatment combinations listed in Table 1 were replicated five times using 50 plastic pots (40 cm top diameter 30 cm bottom diameter and 45 cm height). Each pot accommodated four clumps grown from four single budded sets of seed cane, which had been sprouted and grown for two weeks in 10 cm diameter polythene pots, filled with sterilized soil in an insect proof screen house. Half of the pots were placed in an insect proof screen house, having between rows and within row spacing 1.3 m and 1.2 m, respectively, and the rest of the pots were placed at 2.0 m and 1.75 m apart accordingly in the adjoining open land, but sheltered with a canopy constructed with a green colored plastic shading material cutting about 40% sunlight on potted plants. The replicates were completely randomized within each of the two blocks, open-air and screen-house. The two environmental regimes were used to explore the possibility of the involvement of animals feeding on aerial parts of sugarcane in the secondary transmission of SWPL-SL. The first addition of soil extract was done one week after the establishment of plants in the plastic pots and continued up to three months bi-weekly. Each time, plants in each pot were treated with 100 ml of soil extract.

<table>
<thead>
<tr>
<th>Block</th>
<th>Treatment 1</th>
<th>Treatment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Open-air pots:</strong></td>
<td>Healthy seed cane</td>
<td>Sterilized soil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sterilized soil + soil extract</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WLD-associated soil</td>
</tr>
<tr>
<td>Infected seed cane</td>
<td></td>
<td>Sterilized soil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WLD-associated soil</td>
</tr>
<tr>
<td><strong>Screen-housed pots:</strong></td>
<td>Healthy seed cane</td>
<td>Sterilized soil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sterilized soil + soil extract</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WLD-associated soil</td>
</tr>
<tr>
<td>Infected seed cane</td>
<td></td>
<td>Sterilized soil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WLD-associated soil</td>
</tr>
</tbody>
</table>

With the establishment in large plastic pots, all the experimental plants were treated alike other than the differences in treatments and spraying 40% EC chlorpyrifos (20 ml in 30 l of water) on to the floor of the screen house and the bases of the screen-housed plants in 3 week interval, to ensure the environment is free of any insects. Application of fertilizer was
done according to the SRI recommendations. The plants were irrigated through the watering
device with water sterilized by boiling. The tillers of clumps were allowed to come up
through the holes made on the rigifoam plate as required by the plants after daily
observation. If the holes were too big and soil had been exposed, the spaces were covered
with green colored cotton wool. With the harvesting of the plant crop, the surface of the
potted soil was kept uncovered. However, the watering and fertilizer application continued
as in the plant crop.

Each clump was visually observed at bi-weekly intervals for the development of
SWLD-SL symptoms on plant crop, first ratoon. Further, SWLP-SL in test plants was
detected as described below at the age of 1, 6 and 9 months on the plant crop and when ever
the symptoms on test plants were not clearly distinguishable from SWLD-SL.

Detection of SWLP-SL in test plants

The DNA of the leaf samples of test plants collected from time to time was
analyzed for SWLP-SL using sugarcane phytoplasma specific primers, SPP$_1$ and SPP$_2$ by
PCR amplification as described in Seneviratne et al. (2004). Total DNA was extracted from
0.3 g samples of leaf tissues following a slightly modified method of Nakashima et al.
(1991). A 321 bp sequence of 16SrDNA of SWLP-SL was amplified using the primer pair
SPP$_1$ (5’-ATTAAAGTGCCCATCATG-3’) and SPP$_2$ (5’-GTACTAAGTGTCGGGATT-3’)
(Seneviratne et al., 2004) in PCR in a 20 µl reaction mixture containing 2 µl genomic DNA
(50-100 ng/µl) of plants, 1.6 µl dNTP mixture (each at 2.5 mM), 0.7 µl of each primer SPP,
and SPP$_2$ (10 µM), 0.1 µl Taq DNA polymerase (5U/µl) and 2 µl 10 x PCR reaction buffer
containing Mg$^{2+}$. Thirty seven PCR cycles were operated in a Progene (Techne Cambridge
Ltd, Cambridge, UK) thermocycler for the amplification of the target DNA. Each cycle
consisted of 1 min. (5 min. for the first cycle) denaturation at 94°C, 45 s annealing at 52°C
and 75 s (10 min. for the last cycle) extension at 72°C.

After amplification, each sample was added with 4 µl of loading buffer (50%
glycerol, 5 mM EDTA, 0.25% v/v bromophenol blue) and the mixture (8 µl/well) was
electrophoresed on 1.0% agarose gel containing 0.5 µg/ml ethidium bromide at 60 V for 40
min. in 0.5 x TBE (Tris Boric acid EDTA). The gels were scanned under an UV
transilluminator to analyze the bands.

The proportions of diseased clumps observed at different levels of the factors
(treatments) during the given period were compared by SAS software using CATMOD
procedure fitting the linear logistic model. The levels of three treatments, environmental
regimes, types of seed cane and soil conditions were compared after testing the interactions
among treatments on the development of SWLD-SL in potted sugarcane clumps.

RESULTS AND DISCUSSION

The convergence of higher probabilities of the maximum likelihood ratio (Table 2)
at any age of the plants indicated that the fitted model was adequate to explain the observed
variability of data, i.e. non-existence of significant interactions among treatments. The
differences in the development of symptoms between the types of seed cane have created
significant variation at any stage whereas differences between environmental regimes have
contributed to it at the latter periods under observation. No variation has been detected by any levels of soil condition confirming that no soil borne organism or metabolite is associated in producing SWLD-SL symptoms on test plants.

Table 2. Maximum likelihood analysis of variance for the fitted model in relation to the SWLD-SL symptom development on sugarcane clumps at different treatment levels and ages.

<table>
<thead>
<tr>
<th>Plant age (months)</th>
<th>Source</th>
<th>Intercept</th>
<th>Environ. regimes</th>
<th>Cane types</th>
<th>Soil types</th>
<th>Likelihood ratio**</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 (P)¹</td>
<td>DF</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Chi-square</td>
<td>0.34</td>
<td>0.24</td>
<td>50.96</td>
<td>0.16</td>
<td>0.9223</td>
</tr>
<tr>
<td></td>
<td>Probability</td>
<td>0.5605</td>
<td>0.6237</td>
<td>&lt;0.0001*</td>
<td>0.9951</td>
<td></td>
</tr>
<tr>
<td>1 (R₁)²</td>
<td>DF</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Chi-square</td>
<td>0.19</td>
<td>0.50</td>
<td>47.28</td>
<td>0.82</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>Probability</td>
<td>0.6599</td>
<td>0.4796</td>
<td>&lt;0.0001*</td>
<td>0.6643</td>
<td>0.9568</td>
</tr>
<tr>
<td>3 (R₁)</td>
<td>DF</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Chi-square</td>
<td>0.00</td>
<td>1.44</td>
<td>45.16</td>
<td>1.01</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>Probability</td>
<td>0.9707</td>
<td>0.2304</td>
<td>&lt;0.0001*</td>
<td>0.6040</td>
<td>0.8866</td>
</tr>
<tr>
<td>6 (R₁)</td>
<td>DF</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Chi-square</td>
<td>0.59</td>
<td>3.91</td>
<td>42.35</td>
<td>0.77</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>Probability</td>
<td>0.4415</td>
<td>0.0479*</td>
<td>&lt;0.0001*</td>
<td>0.6812</td>
<td>0.8748</td>
</tr>
<tr>
<td>9 (R₁)</td>
<td>DF</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Chi-square</td>
<td>1.06</td>
<td>6.30</td>
<td>36.54</td>
<td>1.17</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>Probability</td>
<td>0.3039</td>
<td>0.0121*</td>
<td>&lt;0.0001*</td>
<td>0.5558</td>
<td>0.8073</td>
</tr>
<tr>
<td>1 (R₂)³</td>
<td>DF</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Chi-square</td>
<td>1.21</td>
<td>7.87</td>
<td>34.33</td>
<td>1.33</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>Probability</td>
<td>0.2714</td>
<td>0.0050*</td>
<td>&lt;0.0001*</td>
<td>0.5131</td>
<td>0.8130</td>
</tr>
</tbody>
</table>

Note: * Significant at p = 0.05; ¹ Plant crop; ² First ratoon; ³ Second ratoon; ** The probability of likelihood ratio at any plant age is higher than 0.05.

In comparison of healthy seed cane vs. infected seed cane on the development of SWLD-SL in test plants (Tables 2 and 3), the difference between two seed cane types was significant at (p<0.05) confirming that primary transmission is caused by infected seed cane. It should be noted that the infection on the sugarcane clumps raised from infected seed cane was hundred percent irrespective of the two environmental regimes, insect-proof and open-air (Table 3). The germination percentage of healthy seed cane was approximately 82% while that of infected seed cane was approximately 48%, when both types were germinated in sterilized soil in polythene pots in an insect-proof screen house. After establishment of the plants in plastic pots, 75% of sugarcane clumps raised from the healthy seed cane survived after the period under observation whereas the survival of the clumps raised from
infected seed cane was only 30% (data not shown). All the observations show the gravity of
the damage to a sugarcane plantation caused by a possible SWLD-SL infection.

Table 3. Percentage development of SWLD-SL symptoms on sugarcane clumps in
relation to different treatment combinations.

<table>
<thead>
<tr>
<th>Treatment combination</th>
<th>Observations at different ages (months) of test plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 (P)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Open-air + heal. seed cane + st. s.&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Open-air + heal. seed cane + st. s. + s.ext.&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Open-air + heal. seed cane + wld soil&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Open-air + infec. seed cane + st. soil</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Open-air + infec. seed cane + wld soil</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Screen housed + heal. seed cane + st. soil</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Screen housed + heal. seed cane + st. soil + s.ext.</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Screen housed + heal. seed cane + wld soil</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Screen housed + infec. seed cane + st. soil</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Screen housed + infec. seed cane + wld soil</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: The figures in a column followed by the same letter are not significantly different at p = 0.05; <sup>1</sup> Plant crop; <sup>2</sup> First ratoon; <sup>3</sup> Second ratoon; <sup>4</sup> Sterilized soil; <sup>5</sup> Soil extract; <sup>6</sup> WLD associated soil.

The Tables 2 and 3 show that the difference between the symptom development of
SWLD-SL in the clumps at two environments, open-air and insect-proof, is significant at the
age of 6 months in the first ratoon and onward (p<0.05) emphasizing that animals associated
with the aerial parts of plants are involved in the development of symptoms on test plants in
open-air pots. However, it was the insects that were targeted in designing the experiment to
prevent from affecting test plants in the screen house but allure on to open plants for feeding in order to transmit the phytoplasma. It should be noted that complete prevention of insects in the screen house, which also contained many SWLD-SL affected clumps, resulted in none of the healthy plants affected by SWLP-SL (Table 3), further emphasizing that the SWLP-SL is not air-borne. Phytoplasmas are known not transmitted mechanically, by inoculation with sap containing phytoplasma (Lee et al., 2000) and thus, the air-borne ability of SWLP-SL can again be ruled out. There was slight increase of temperature and humidity in the screen house than the open land and which non-development of symptoms on healthy plants in the screen house could be counter argued on. This is not impossible to reject with the observation on 100% symptom development in the clumps raised from infected seed cane in the screen house (Table 3). Therefore, an involvement of insect(s) in the development of the disease in the open clumps could be proved beyond doubts.

The detection method used for SWLP-SL was very useful to distinguish SWLD-SL affected plants from the plants those developed yellowing symptoms close to the initial symptoms of SWLD-SL from time to time. Further, some test plants were detected positive for the SWLP-SL even two months before the expression of symptoms of the disease with the help of the PCR method (Fig. 1). This provides evidence for higher the efficacy and the reliability of the detection method.

![Amplification of template DNA extracted from leaf tissues of test plants with the primers SPP<sub>1</sub> / SPP<sub>2</sub>.](image)

**Fig. 1.** Amplification of template DNA extracted from leaf tissues of test plants with the primers SPP<sub>1</sub> / SPP<sub>2</sub>.

*Note:* M-DNA marker DL 2000 (fragment sizes-2000, 1000, 750, 500, 250 and 100 bp); 1-sugarcane (with SWLD-SL related symptoms); 2-water control; 3-sugarcane (healthy); 4-sugarcane (SWLD-SL infected, non-symptomatic); 5-sugarcane (with SWLD-SL symptoms).

**CONCLUSIONS**

The investigation on mode of transmission of SWLD-SL succeeded in concluding the involvement of an insect vector(s) in secondary transmission other than the primary transmission through the infected seed cane. The folklore that the soil related factors are
involved in spreading the disease was disproved. However, the evidence gathered from the experiment is not sufficient to conclude whether that the disease is a result of a mix infection or not. The findings are useful in narrowing down the area of research for identification of vector and finally management of a sugarcane disease of national importance.

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