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การตรวจสอบไอโอโนมของอ้อยที่ติดเชื้อโรคใบขาวอ้อย โดยการใช้ไมโครเอ็กซ์เรย์ฟลูออเรสเซนซ์ สเปกโทรสโกปี

Ionome Visualization of Sugarcane White Leaf Disease (SCWL) Infected Sugarcane Using Micro X-ray Fluorescence Spectroscopy

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บทคัดย่อ

วัตถุประสงค์และที่มา : โรคใบขาวซึ่งเกิดจากเชื้อ sugarcane white leaf (SCWL) phytoplasma เป็นโรคที่ก่อให้เกิดความเสียหายอย่างรุนแรงต่อการผลิตอ้อยของประเทศไทย อ้อยที่ติดเชื้อ อาจแสดงอาการ หรือไม่แสดงอาการ ซึ่งอ้อยที่ไม่แสดงอาการอาจถูกใช้เป็นท่อนพันธุ์ ทำให้เกิดการแพร่กระจายของเชื้อ ในปัจจุบันข้อมูลผลกระทบของเชื้อ SCWL phytoplasma ต่ออ้อยติดเชื้อ ในระดับธาตุอาหาร โดยเฉพาะในระยะที่ไม่แสดงอาการมีน้อยมาก ทำให้การจัดการระบบปลูก และการให้ธาตุอาหารในพื้นที่ปลูกอ้อยที่เหมาะสมเป็นไปได้ยาก การศึกษานี้มีวัตถุประสงค์เพื่อ ตรวจสอบผลกระทบของ SCWL phytoplasma ต่อการกระจายธาตุอาหารในใบอ้อยและประเมินประโยชน์ของไมโครเอ็กซ์เรย์ฟลูออเรสเซนซ์ สเปกโทรสโกปี สำหรับการวิเคราะห์เปรียบเทียบธาตุอาหารในอ้อย

วิธีดำเนินการวิจัย : เก็บตัวอย่างใบจากต้นอ้อยสายพันธุ์ขอนแก่น 3 อายุ 10 เดือน ที่แสดงอาการโรคใบขาว จำนวน 15 ตัวอย่าง ซึ่งแบ่งตามความรุนแรงของอาการออกเป็น 3 ระดับ (ระดับละ 5 ต้น) และอ้อยที่ไม่แสดงอาการ จำนวน 10 ตัวอย่าง จากแหล่งปลูก อำเภอ เลาขวัญ จังหวัดกาญจนบุรี นำตัวอย่างมาตรวจหาเชื้อ sugarcane white leaf (SCWL) phytoplasma ด้วยเทคนิค polymerase chain reaction (PCR) และตรวจสอบสัดส่วนของธาตุในอ้อยที่ปลอดเชื้อไฟโตพลาสมา (ไม่แสดง

อาการ และไม่พบเชื้อ) อ้อยที่พบเชื้อแต่ไม่แสดงอาการโรค อ้อยที่แสดงอาการโรคใบขาว โดยใช้ Micro X-ray fluorescence spectroscopy (μ-XRF) และใช้การวิเคราะห์ทางเคมีแบบ Inductively Coupled Plasma Optical Emission Spectscopy (ICP-OES) เป็นวิธีการเปรียบเทียบ วิเคราะห์ข้อมูลโดยใช้การวิเคราะห์ความแปรปรวนทางเดียว (one-way analysis of variance (ANOVA) การทดสอบเปรียบเทียบของวิธี Tukey's และการวิเคราะห์ความสัมพันธ์โดยวิธี Pearson's correlation

ผลการวิจัย : อ้อยที่แสดงอาการตรวจพบเชื้อ SCWL phytoplasma ทั้ง 15 ตัวอย่าง และอ้อยที่ไม่แสดงอาการตรวจพบเชื้อ 5 ตัวอย่าง เมื่อนำอ้อยที่พบเชื้อมาวิเคราะห์พบธาตุ 6 ชนิดตามลำดับปริมาณที่พบจากมาก-น้อย ได้แก่ โพแทสเซียม ซิลิคอน แคลเซียม ซัลเฟอร์ ฟอสฟอรัส และ เหล็ก ตามลำดับ พบความสัมพันธ์อย่างมีนัยสำคัญระหว่างระดับความรุนแรงของอาการกับธาตุ 2 ธาตุ คือ ซิลิกอนและซัลเฟอร์ โดยการลดลงของซิลิกอน จะมีความสัมพันธ์ทางลบอย่างมีนัยสำคัญ (-0.824) กับความรุนแรงของอาการ ขณะที่การเพิ่มขึ้นของซัลเฟอร์จะมีความสัมพันธ์ทางบวกอย่างมีนัยสำคัญ (0.803) กับความรุนแรงของอาการ นอกจากนี้ ความสามารถในการคาดการณ์ของแบบจำลองที่สอบเทียบจากไมโครเอ็กซ์เรย์และ ICP-OES พบว่า Mean Absolute Percentage Error (MAPE) ของ ซิลิกอน โพแทสเซียม และ แคลเซียม มีค่าต่ำกว่า 20% (15.52%, 10.44% และ 9.69% ตามลำดับ) แสดงให้เห็นถึงวิธีของไมโครเอ็กซ์เรย์มีความสามารถในการตรวจวัดใกล้เคียงกับการวิเคราะห์ทางเคมีแบบ ICP-OES

สรุปผลการวิจัย : เชื้อ SCWL phytoplasma ทำให้เกิดการเปลี่ยนแปลงของธาตุอาหารในใบอ้อย โดยเฉพาะธาตุซิลิกอนและซัลเฟอร์ นอกจากนั้น ไมโครเอ็กซ์เรย์ เป็นเทคนิคที่มีประโยชน์ต่อการศึกษาการเปลี่ยนแปลงของธาตุอาหาร เป็นวิธีการที่มีความน่าเชื่อถือรวดเร็วสำหรับการแสดงการเปลี่ยนแปลงเชิงปริมาณในระดับเซลล์ ทำให้เห็นผลกระทบของไฟโตพลาสมา SCWL ต่ออ้อยในช่วงการเปลี่ยนจากระยะที่ไม่มีอาการไปเป็นระยะที่แสดงอาการ ผลการศึกษาจะเป็นประโยชน์ต่อการจัดการพื้นปลูกอ้อย และการใช้ธาตุเสริมเพื่อลดความรุนแรงของอาการโรคใบขาว

คำสำคัญ : อ้อย ; โรคใบขาว ; ไฟโตพลาสมา ; ไอโอโนมิกส์ ; ธาตุอาหาร

Abstract

Background and Objectives : Sugarcane White leaf (SCWL) is an important disease that severely affects sugarcane production in Thailand. The infected sugarcane plants could either become symptomatic or remain symptomless (asymptomatic). The infected cutting can be used for a new plantation causing disease to spread. There are very few studies on the impact of SCWL phytoplasma to infected sugarcane particularly at the elemental level making farm management and nutrient supplementation difficult. This study aims to examine the impact of SCWL phytoplasma on nutrient distribution in sugarcane leaves and to evaluate the utilization of micro x-ray fluorescence spectroscopy for the identification of nutritional elements.

Methodology : Ten-month old sugarcane plants cv. Khon Khan 3 from the plantation in Lao Khwan district, Kanchanaburi province, Thailand, were used in this study. Leaf samples were collected from 15 sugarcane plants

(one leaf per plant) with SCWL disease symptoms. These plants were divided based on the severity of the symptoms into 3 levels (5 plants per symptomatic level). Leaf samples were also collected from 10 sugarcane plants (one leaf per plant) without any symptoms of SCWL disease. The presence of SCWL phytoplasma in the samples was determined by Polymerase chain reaction (PCR). Micro x-ray fluorescence spectroscopy (μ-XRF) was used to determine element contents in healthy (free of SCWL phytoplasma), infected sugarcane without any SCWL disease symptoms and symptomatic leaf samples. Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) analysis was used as a comparative method. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests and Pearson's correlation method.

Main Results : SCWL phytoplasma was detected in all fifteen symptomatic samples and 5 asymptomatic samples. Six elements ranked based on the amount included K (the highest amount), Si, Ca, S, P, and Fe (the lowest) respectively. Correlation analysis showed a significant correlation between SCWL symptom severity and two elements; silicon (Si) and sulphur (S). Si content had a significantly strong negative correlation ($r = -0.824$) while sulphur (S) content had a significantly strong positive correlation ($r = 0.803$) with disease severity. In addition, the predictability of the calibrated model from micro x-ray and ICP-OES showed Mean Absolute Percentage Error (MAPE) of Si, K, and Ca was under 20% (15.52%, 10.44% and 9.69%, respectively). This result indicated that micro x-ray method has measurement capabilities similar to the ICP-OES method.

Conclusions : SCWL phytoplasma impacted element contents particularly Si and S in infected sugarcane. In addition, micro x-ray was applicable for the determination of element changes in sugarcane leaves. It was a reliable, rapid approach for visualizing quantitative changes at the cellular level and highlighted the impact of SCWL phytoplasma on sugarcane during the transition from asymptomatic to symptomatic stages. The outcome could be beneficial in farm management and nutrient supplementation of sugarcane plantations to delay disease progression.

Keywords : sugarcane ; sugarcane white leaf disease ; phytoplasma ; ionomics ; element

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Introduction

Macro and micro elements are essential for plant growth and development. Macro elements such as Nitrogen (N) and phosphorus (P) are required in large amounts (>1000 mg/kg DW) and the deficiency of these elements results in retardation of plant growth and development (Awasthi *et al.,* 2022; Kumar *et al.,* 2021). Though required in lesser amount than macro elements, trace elements are integral parts of catalytic enzymes and structural

roles of biological molecules, donating or accepting electrons in oxidation/ reduction reaction and facilitating biological processes (Awasthi *et al.,* 2022). In addition, macro and trace elements also play important roles in plant defense mechanism against pathogens such as modulating the activity of redox enzymes, pH fluctuation, lignin deposition, and phytoalexin biosynthesis (Tripathi *et al.,* 2022).

The term "ionome" was introduced by Lahner *et al* (2003). It was defined as the mineral nutrient and trace element composition of an organism. Ionomics includes the quantitative measurement of the elemental composition and determination of changes caused by conditions such as biotic and abiotic stresses. It has been used as a tool to understand the connection and biological significance between elemental profiles and plant genes, proteins and metabolites (Salt *et al.,* 2008). Techniques for ionomics studies include inductively coupled plasma-mass/optical emission spectroscopy (ICP-MS/OES), neutron activation analysis (NAA), X-ray crystallography and X-ray fluorescence (XRF) (Yang *et al.,* 2021). While the outcome is useful, these techniques are time consuming, expensive, require large quantity of samples and limited to laboratories. Recently, micro X-ray fluorescence (μXRF) and portable μXRF was developed allowing reduction of sample quantity and application outdoors and non-destruction of samples such as determination of heavy metal in *Noccaea caerulescens* plants (van der Zee *et al.,* 2021).

Ionomics has been used to determine elemental changes during plant pathogen infection. Oliver *et al* (2014) reported ionome changes in tobacco infected with *Xylella fastidiosa* and their correlation with pathogen virulence. Ionome and phenolic and flavonoid Compounds were used in sustainable management of *Xylella fastidiosa* infection in olive plantation in Morocco (Handi *et al.,* 2021). Nicolas *et al* (2019) reported that *Xanthomonas campestris* pv. vitaians infection affected N, S and P balance in lettuce.

Sugarcane (*Saccharum* spp.) is an important agro-industrial crop in tropical and subtropical regions such as South and Southeast Asia, South America, and Australia (Silalertruksa & Gheewala, 2020). Thailand was the 2nd global sugar exporter with the value of 1.556,35 Million EUR in 2022 (Wichakan, 2023). Among sugarcane diseases, sugarcane white leaf (SCWL) disease is one of the most severe and causing devastating losses in many sugarcane producing countries including Vietnam, India, Thailand, China and Australia (Quan *et al.,* 2020; Dayasena *et al.,* 2021; Mishra *et al.,* 2022; Roddee *et al.,* 2018; Wang *et al.,* 2022; Tran-Nguyen *et al.,* 2000). Although the disease was previously thought involving two phytoplasmas; sugarcane white leaf and sugarcane grassy shoot, a recent report confirmed that both were the same species, *Candidatus* phytoplasma sacchari (Zhang *et al.,* 2023). In this study, it was referred to as SCWL phytoplasma. SCWL infected sugarcane can be either asymptomatic or symptomatic. Moderate symptomatic sugarcane plants display white/yellow streak while severe symptomatic

sugarcane plants exhibit white leaves and stunting. While infected sugarcane with symptoms can be easily identified and confirmed with detection methods such as PCR, asymptomatic sugarcane with SCWL phytoplasma could only be identified with detection methods (Zhang *et al.,* 2020). Therefore, SCWL disease could be spread to new plantations with infected sugarcane stalks (Zhang *et al.,* 2020).

In this study, element composition and the changes during SCWL infection in sugarcane leaves were investigated using micro X-ray fluorescence (μXRF). ICP-MS/OES was used a standard method for validation of μXRF application. The involvement of elements including Si and S in plant defense was also discussed.

Methods

Plant material

Sugarcane plants cv. Khon Khan 3 (KK3) was cultivated using the furrow planting method in Lao Khwan district, Kanchanaburi province (location 14°46'45.3" N99°37'27.4" E) from February to November 2019. The soil type was Lao Khwan Series with fine-loamy, mixed, semiactive, isohyperthermic typic haplustalfs which was well-drained. The soil fertility was at a moderate level (http://oss101.ldd.go.th/web_thaisoilinf/th_reg.html). The distance between rows was 1.20 m. The plantation was irrigated using rainfed. SCWL-infected sugarcane can be divided based on disease severity into 4 groups: asymptomatic (AS), and symptomatic level 1-3 (S1-S3) (Office of The Cane and Sugar Board, 2017). The AS plants were visually indistinguishable from healthy KK3 sugarcane plants. The S1 SCWL symptomatic SCWL plants had white or yellow strips parallel to the midribs. S2 SCWL symptomatic plants had white patches on the otherwise green leaves and S3 SCWL symptomatic plants with stunted growth and white leaves (Figure 1). Five infected plants from each symptomatic level; asymptomatic, S1-S3 were selected. The third leaf from the top of the sugarcane plants based on sugarcane internode as previously reported (McCormick *et al.*, 2006; Martins *et al.,* 2016) was used in this study. The leaves were washed once with tap water, washed twice with sterilized distilled water, and superficially dried with a clean paper towel. The cleaned leaves were used for genomic DNA extraction and micro x-ray analysis.

SCWL phytoplasma detection

The mid ribs of cleaned sugarcane leaves were retrieved and cut into small pieces. Twenty-five milligrams were used for genomic DNA extraction using a DNase plant mini kit (QAIGEN, Germany). The presence and quality of the DNA was determined using a spectrophotometer (Denovix DS-11+ spectrophotometer, USA). The *SCWL* phytoplasma detection was used in a PCR machine (Biometra T-1Thermoblock, Germany). The PCR reaction

consisted of 100 ng of DNA, 1X Top *taq* PCR buffer, 0.5 μM of forwards and reverse SCWL phytoplasma specific primers (SecA-SCWL), (F 5'TGACTGGTACAGCTAAAACCGA and R 5'CCACGCCCAGCCATATTAGT), 0.2 mM dNTPs mix, 0.5 mM $MgCl_2$ and 0.5 unit Top *Taq* DNA polymerase (QAIGEN, Germany) and sterile water was added to adjust the final volume to 20 μL. The PCR cycles included initial denaturation step of 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension period at 72 °C for 1 min and followed by a final extension step of 72 °C for 10 min. The size of the expected PCR product was 361 bp that specific to *Sec*A gene. The presence of sugarcane phytoplasma was observed in 1.5% TAE agarose gel electrophoresis at 100 V for 30 min and stained with ethidium bromide, then pictured under ultraviolet light by gel documentation system (SYNGENE, UK) Water (negative control) and SCWL phytoplasma DNA (positive control) were included in all PCR experiments.

Micro X-ray fluorescence spectroscopy

A schematic overview of sugarcane leaf preparation for micro x-ray analysis was shown in Figure 2. The sugarcane leaf lamina (10 x 10 mm^2) was mounted on glass slides with an epoxy resin. It was vacuumed and scanned by a laboratory benchtop μ-XRF spectrometer (M4 Tornado, Bruker Nano GmbH, Berlin, Germany). All the analyses were performed under reduced pressure (20 mbar) by acquiring one spectrum every 50 μm step with an acquisition time of 20 msec per step giving a total pixel number of 40,000 per image. μ-XRF hyperspectral data and images were processed with the ESPRIT™ built-in software to calculate weight percentages based on the measured total fluorescence intensities for identification and selection elements against a calibration file following the manufacturer instructions. The weight percentages were calculated over the 10 x 10 mm^2 scanned region. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests pairwise were used. The data was plotted as mean ± standard deviation. Significant difference was determined by Tukey's multiple comparison tests at $p \leq 0.05$. Correlation analysis was done using Person's correlation method.

ICP-OES analysis

The element of K, S, P, Ca, Mg, Fe, Mn, Cu, Zn, Na and B contents in five healthy KK3 sugarcane leaves was determined using ICP-OES analysis (Inductively Coupled Plasma Optical Emission Spectroscopy) (Santos *et al.*, 2017). Nitrogen content was determined using Micro Kjeldahl method (Goyal *et al.*, 2022). Silicon content was determined using a method by Wei-min *et al* (2005). The mineral content from micro x-ray and ICP-OES analysis were compared using the Root Mean Square Error (RMSE) and Mean Absolute Percentage Error (MAPE) calculations. RMSE and MAPE are considered as an exceptional general-purpose error metric for numerical predictions of newly created methods or techniques (Guerra *et al.*, 2014).

Results

Sugarcane plants with SCWL symptoms (S1-S3) and those that appeared symptomless in a sugarcane plantation were collected and tested for the presence of SCWL phytoplasma and sensitivity using PCR with SecA-SCWL specific primers. The result showed that SCWL phytoplasma was detected in 5 out of 10 of asymptomatic (50%) sugarcane samples and in 15 (15/15; 100%) of SCWL symptomatic samples. Healthy and asymptomatic sugarcane samples were identified with the latter showing positive reaction with expected PCR products of 361 base pairs (Figure 3). The PCR products were sequenced and confirmed to be SCWL phytoplasma SecA gene. The sensitivity of SCWL phytoplasma detection was shown in Supplement S1 (Saengmanee *et al.,* 2023).

Using micro x-ray analysis, six elements including four macro elements (P, K, S and Ca) and 2 micro elements (Si and Fe) were identified (Table 1 and Figure 4). P content was the highest followed by Si, Ca, S, K and Fe, respectively. Compared with healthy sugarcane leaves, AS and S1 infected sugarcane leaves showed similar contents of Si, P, S and K at 67.10-69.33 g/kg, 2.27-2.63 g/kg, 3.82-5.43 g/kg and 80.75-85.73 g/kg, respectively. While Ca content was the decline from 18.91 g/kg in healthy to 12.30 g/kg in AS and 9.38 g/kg in S1 infected sugarcane leaves (Table 1). With SCWL symptomatic development, there was a significant decline in Si content compared to healthy and AS sugarcane leaves. Sugarcane epidermal cells with different degree of SCWL disease severity showed different patterns of Si accumulation with healthy sugarcane showed mild Si signal while symptomatic sugarcane displayed intense Si signal (Figure 4). On other hand, there was a significant increase in S content during SCWL symptomatic development. Although there was no statistically significant P content among healthy, AS, S1-S3 infected sugarcane leaves, P content was slightly increased during symptomatic progression corresponding with the signal pattern.

There was no significant difference in Fe content during symptomatic progression. Though there was a decline in Ca content during an early stage of infection (AS and S1), followed by an elevation of Ca content in S2 and S3 infected sugarcane leaves, the differences were not apparent in signal intensity.

Pearson correlation analysis was performed on element content from healthy, asymptomatic, and S1-S3 sugarcane and disease severity at $p \leq 0.001$ Positive correlation was indicated in shades of red, whereas negative correlation was indicated in shades of blue (Figure 5). The results revealed significant correlation between SCWL symptom severity with two elements; Si and S. Si showed significantly strong negative correlation ($r = -0.824$) while S showed significantly strong positive correlations ($r = 0.803$) with disease severity. These correlations indicate the impact of disease severity and Si and S accumulation in infected sugarcane. Furthermore, Si showed significant negative correlation with S and K at $r = -0.942$ and -0.922.

Mineral content in healthy KK3 sugarcane leaves were also determined using ICP-OES (Table 2). Using RMSE and MAPE calculation, the predictability of the calibrated model from micro x-ray and ICP-OES was determined. RMSE values of Si, P, S, K and Ca were 15.77, 9.96, 2.69, 8.76 and 1.81 g/kg, respectively. In comparison, MAPE showed percentage errors of each element. In this study, the percentage error was determined as 20% (80% accuracy). MAPE of Si, K, and Ca were under 20% (15.52%, 10.44% and 9.69%, respectively), therefore their contents were more accurate than those of P, Fe and S.

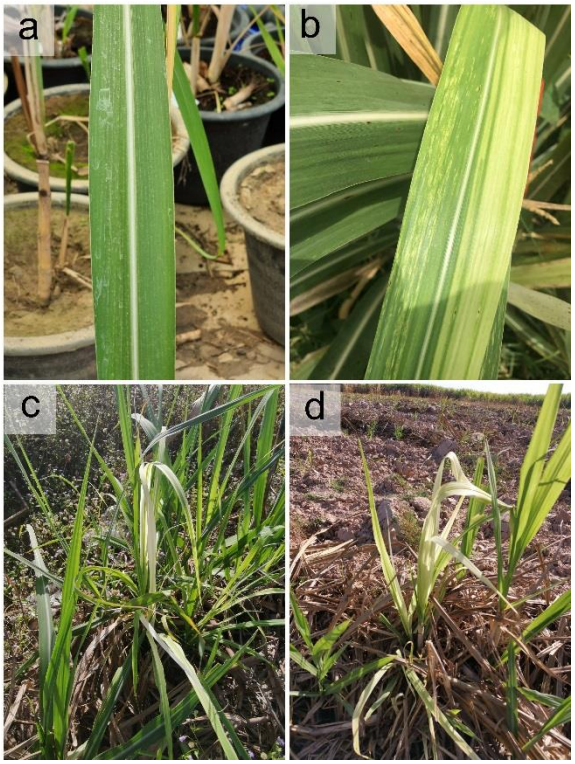

Figure 1 Symptoms of SCWL infected sugarcane; asymptomatic SCWL infected plants (a), S1 SCWL symptomatic plants (b). S2 SCWL symptomatic plants (c) and S3 SCWL symptomatic plants (d)

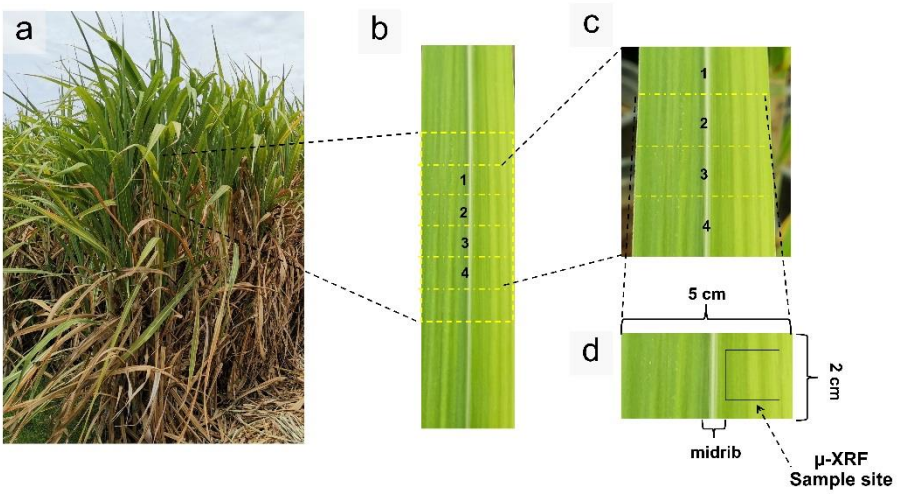

Figure 2 Schematic diagram of the preparation for micro x-ray analysis of sugarcane leaves (a). Identification of the middle sections of the leaf (b), the 2nd section was excised and used for micro x-ray analysis and SCWL phytoplasma detection via PCR (c-d)

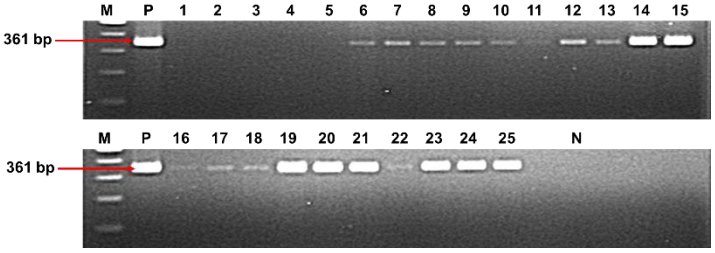

Figure 3 SCWL phytoplasma detection using SecA-SCWL specific primers. Healthy (lanes 1-5), asymptomatic (lanes 6-10), symptomatic level 1 (lanes 11-15), symptomatic level 2 (lanes 16-20) and symptomatic level 3 (lanes 21-25) samples. M = 100 bp plus marker. P = SCWL positive DNA sample N = negative PCR reaction.

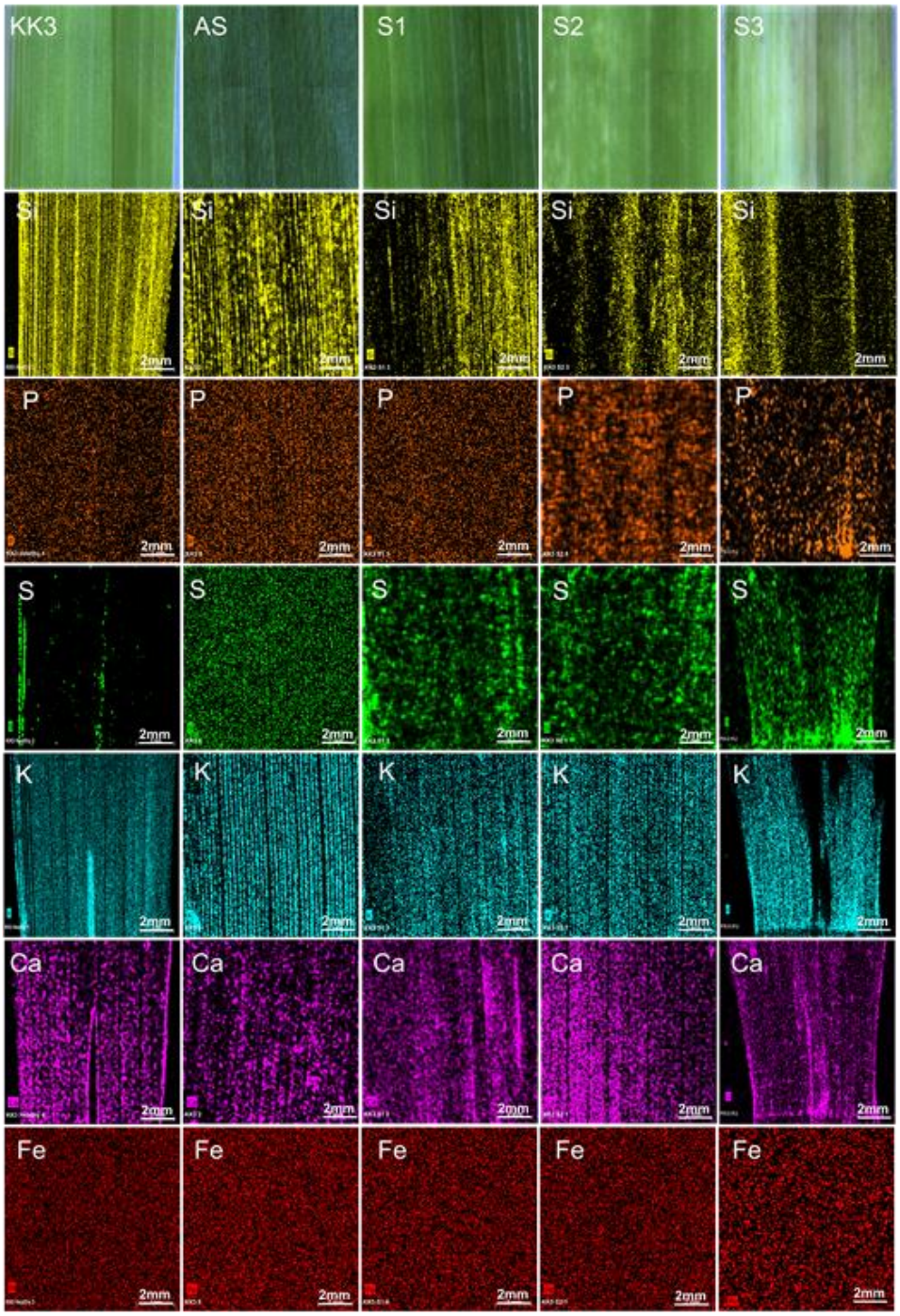

Figure 4 Micro x-ray fluorescence in Healthy (H), asymptomatic (AS) and symptomatic (S1-S3) sugarcane plants. Photos of H, AS and SCWL infected sugarcane leaves were on the top. Sample size was ten replicates

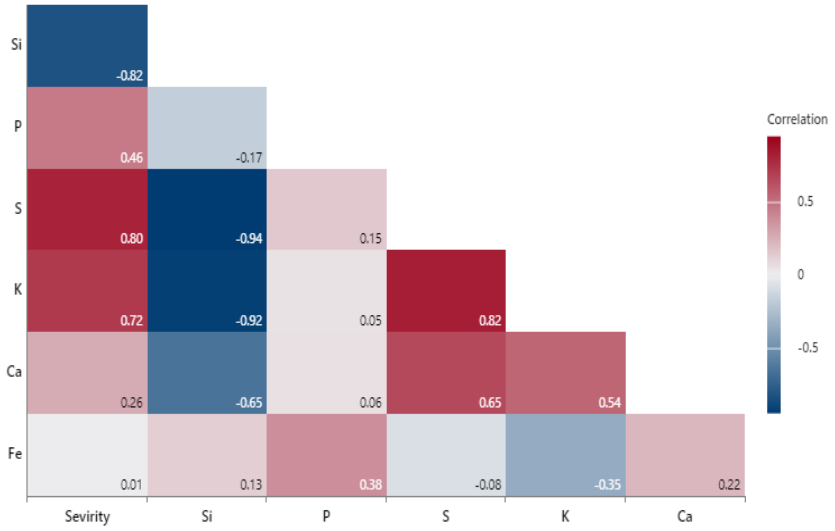

Figure 5 Correlation heatmap of element content and SCWL disease severity. The panel on the right of the plot indicated the color key and a histogram of correlation coefficients

Table 1 Quantitative analysis of elements found in sugarcane leaves using micro x-ray fluorescence. The data were analyzed using analysis of variance (ANOVA) and Tukey's multiple comparison tests. Data within the same column followed by different letters were significantly different at $p \leq 0.05$

Samples	Element (g/Kg)					
	Si	P	S	K	Ca	Fe
Healthy	68.71 ± 3.71 a	2.27 ± 0.47 a	4.68 ± 0.52 b	80.75 ± 3.03 b	18.91 ± 0.89 a	0.23 ± 0.03 a
Asymptomatic	69.33 ± 2.30 a	2.45 ± 0.62 a	3.82 ± 0.11 b	85.73 ± 2.96 ab	12.30 ± 2.25 b	0.16 ± 0.03 a
Symptomatic level1	67.10 ± 3.45 a	2.63 ± 0.86 a	5.43 ± 0.85 b	81.20 ± 6.06 b	9.38 ± 1.73 b	0.18 ± 0.10 a
Symptomatic level2	41.31 ± 10.30 b	3.03 ± 1.20 a	11.89 ± 2.89 a	96.79 ± 6.68 a	18.94 ± 1.53 a	0.13 ± 0.04 a
Symptomatic level3	38.74 ± 8.30 b	3.40 ± 0.98 a	11.89 ± 2.83 a	97.07 ± 5.77 a	18.93 ± 3.39 a	0.25 ± 0.04 a

Table 2 Absolute quantification analysis of element concentrations (g/Kg) in healthy sugarcane leaves as measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). Accuracy analysis was estimated by RMSEP: Root Mean Square Error of Prediction and MAPE: Mean Absolute Percentage Error.

Healthy	Element (g/Kg)	RMSE (g/kg)	MAPE (%)
Si	75.15 ± 14.02	15.77	15.52
P	12.20 ± 0.61	9.96	81.31
S	7.36 ± 0.55	2.70	36.48
K	73.22 ± 2.41	8.71	10.44
Ca	17.26 ± 1.03	1.81	9.69
Fe	0.40 ± 0.05	0.18	40.78

Discussion

SCWL phytoplasma impacted changes in element contents in sugarcane during symptomatic development. Micro x-ray fluorescence spectroscopy was utilized to determine element contents in fresh sugarcane leaves. It is a non-destructive technique for multielement analysis for soil and plant material that showed high measurement accuracy for Si over destructive methods (Kovács *et al.,* 2022). While 6 elements were prominent in sugarcane leaves, two elements showed correlation with SCWL disease severity. The analysis indicated the negative correlation between Si concentration and disease severity while there was positive correlation between S concentration and disease severity. The Si impact in plant defense against pathogens could be through physical, biochemical and molecular mechanism. In some cases, more than one mechanism was reported. In addition, Si supplement was reportedly enhancing disease resistance. The physical barriers were reportedly strengthened with deposition of Si in the form of SiO_2 in cell wall and root surfaces and stomata modification. Supplementation of Si content reduced blast disease incidence and severity in rice and wheat (Kim *et al.,* 2002; Chowdhury *et al.,* 2022). Si triggered the production of plant defense chemicals including callose, phytoalexins and phenolics. Si enhanced diterpenoid phytoalexin accumulation causing blast resistance in rice (Akhtar *et al.,* 2018; Rodrigues *et al.,* 2004). Si reportedly regulated expression of several genes related to antioxidant responses and signaling in tomato and rice. The Si nanoparticles activated the defense responses through reduction of ROS during *Ralstonia solanacearum* infection in tomato plants (Wang *et al.,* 2022). Si supplement increased early blight resistance by

regulating rice defense related genes and antioxidant enzymes (Gulzar *et al.,* 2021). Zhang *et al* (2022) reported the synergistic effects of Si and $Cu(OH)_2$ in Controlling Sugarcane Leaf Scald Disease. The application of Si reduced the severity of brown rust in sugarcane through physical and biochemical mechanisms (Ramouthar *et al.,* 2015). Frazão *et al* (2020) showed that Si supply improved carbon use efficiency, directly influencing yield and nutrient cycling in sugarcane. In this study, it was likely that Si was used for multiple mechanisms such as physical and biochemical during phytoplasma infection. As the severity progressed the need of Si was elevated resulting in significant reduction of Si content.

Sulphur is an essential element for plant growth and development. In addition, it also played important roles in plant defense. Four types of S containing molecules including amino acids (cysteine, methionine), glutathione, antimicrobial peptides and secondary metabolites (phytoalexin and phytoanticipins) were reportedly related to disease resistance (Künstler *et al.,* 2020). Sulphur was also important for xantham gum formation (cell adhesion) and disease virulence in *Xanthomonas citri* (Sampaio *et al.,* 2017; Tófoli de Araújo *et al.,* 2013). Cysteine riched receptor-like kinases (CRKs) interacted with bacterial flagellin resulting production of ROS and consequently cell death, a hypersensitive response to inhibit pathogen multiplication and movement. Over-expression of CRK resulted in an elevation of resistance of *Pseudomonas syringae* in Arabidopsis (Yadeta *et al.,* 2017). The elevation of S concentration was associated with *Verticillium dahliae* infection in tomato with slight increase in diseased and great elevation in resistant tomato plants (Williams *et al.,* 2002). Garcia *et al* (2023) reported that Sugarcane plants infected with high titres of *Leifsonia xyli* subsp. *xyli* (Lxx) showed high S content. However, S increase did not result in higher S assimilation confirmed by decreased methionine and glutathione levels. The decline may reflect impaired glutathione biosynthesis or a drain from oxidative stresses by pathogenesis of Lxx. A similar outcome of an elevation of S was also shown in SCWL infected sugarcane.

Brouwer *et al* (2021) reported that in the elevation of K content in Phytophthora infestans-infected potato leaves. Potassium (K) is the most abundant cation in the cytosol, and it plays a key role in the osmotic adjustment ability, biological processes such as metabolic enzymes, photosynthesis and long-distance transport (Wang *et al.,* 2013; Amtmann *et al.,* 2008). Vogel-Mikuš *et al* (2022) report that K redistribution was proposed for testing stress-induced injury of plant tissues and a criterion to evaluate the level of stress tolerance. Porfido *et al.,* 2023 reported that elevate K concentration in ash shoestring associated virus infected ash leaves. Similar results were also observed in SCWL infected sugarcane, the elevation of K in the tested sample thus indicate alleviation of osmotic stress condition in the infected cell that may cause cell damage which related to higher S and loss tissue structure as shown in Si scattering pattern.

The elemental data obtained with the benchtop μXRF method are based on samples relatively near to the leaf surface, the sample volume depends on the penetration depth of the ingoing x-ray. Moreover, it is a process that relies on the element of wavelength, and matrix parameter. In addition, a unique feature of the micro x-ray allows for non-destructive analysis of fresh plant tissues. Whereas ICP-OES method is a quantitative method that requires homogenized samples, time consuming for sample preparation and unable to visualize spatial redistributions within a sample (Brouwer *et al.*, 2021). Although both benchtop micro x-ray and ICP-OES methods can be used to determine element content in SCWL infected sugarcane, micro x-ray is more applicable to the study of ionomic changes in plant tissues. Therefore, this approach can be useful for measuring elements and concentrations in the research field. Micro x-ray provides the ability to visualize alteration of macro and micro elements at the cellular level and highlights the impact of SCWL phytoplasma on sugarcane during transition from asymptomatic to symptomatic stages. To our knowledge, this is the first report of nutrient changes in sugarcane leaves during SCWL phytoplasma infection.

Conclusion

Ionome visualization using micro-X-ray fluorescence spectroscopy is a reliable, non-destructive of flesh plant tissue and rapid approach for determining quantitative changes in element content of sugarcane plants. SCWL symptomatic progression was shown through alteration of element contents. As this micro X-ray scanned the structure of cell and able to indicate the specified element, thus the imaging implied the deposit and organizing pattern in relation to element bearing tissue. In this study, it is obvious that in healthy sample, the organization pattern of tissue is still intact and well allied, therefore, all the structural elements revealed in good scattering pattern while in the higher degree of disease severity, the imaging revealed disorganized and depletion as well as clumping of the target element implying cell or tissue destruction. This devise can thus be applied to monitor the impact or effectivity of the treatment while nutrient analysis is so far still relied in the conventional nutrient quantitation method, unless new and efficient nutrient quantitation technology has been established.

This knowledge further our understanding in plant-pathogen interaction and can be applied to nutritional supplements for delay the SCWL disease symptom.

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