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FIELD METHODS FOR RAPID AND NONINVASIVE DIAGNOSIS OF HUANGLONGBING THROUGH SYMPTOMATIC EVALUATION AND DIAGNOSTIC TESTING

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ABSTRACT

Huanglongbing (HLB) is a highly destructive disease that has inflicted significant damage to citrus orchards in major growing regions worldwide, resulting in significant reductions in production and posing a serious threat to the citrus industry. HLB is caused by a gram-negative, uncultivable bacterium, *Candidatus* Liberibacter asiaticus, which colonizes the phloem, and is naturally transmitted by vector insects, such as the Asian citrus psyllid, *Diaphorina citri*. This study will help to expand the knowledge on HLB in existing literature by focusing mainly on the incidence and diagnosis in different zones of Bahawalpur. The use of the pen test and iodine starch test to detect HLB provided a reliable and cost-efficient method for diagnosing the disease in the field. Symptoms expression was considered a fundamental criterion for HLB diagnosis, including leaf drop, stunting, mottling, and color inversion in fruits. The disease incidence ranged from 30% ± 1.15 to 44% ± 1.15 across different zones, with Bahawalpur having the highest incidence. These findings suggest that some areas may be more susceptible to the disease than others, and further investigation is necessary to determine the underlying factors responsible for this distribution.

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INTRODUCTION

Citrus (Rutaceae; Spindales) are commonly grown fruit crops that thrives in tropical and subtropical regions around the world. Citrus and closely related genera, including Fortunella, Poncirus, Eremocitrus, and Microcitrus, are commonly affected by this disease. Among these genera, citrus is the most extensively cultivated and is renowned for its production of various fruits such as oranges, mandarins, lemons, limes, and grapefruits (Scora, 1975; Khan et al., 2021). Citrus is a highly important fruit crop due to its rich contents of beneficial compounds such as carotenoids, flavonoids, terpenes, limonoids, and other bioactive components that provide both nutritional and nutraceutical benefits (Lado et al., 2018; Saini et al., 2022). Pakistan produces around 30 different varieties of fruits, with citrus fruit leading the way, accounting for over 30% of total fruit output in the country. More than 90% of citrus fruits are grown in Punjab province and distributed through various value chains in both domestic and international

markets. While the majority of citrus fruit produced in Pakistan is consumed locally with little value addition, 10-12% of total production is exported after value addition (Siddique et al., 2017).

Among all diseases of citrus described to date, citrus greening disease (syn. Huanglongbing, HLB) is considered probably the most destructive and lethal. The disease was identified in southern China for the first time (Ghosh et al., 2022). Citrus greening is a highly problematic citrus disease that is commonly referred to as the "yellow dragon disease" in Chinese. It is one of the most severe citrus diseases worldwide and poses a significant threat to the citrus processing industry. HLB affects almost all citrus varieties i.e. grapefruit, sweet oranges, certain tangelos, and mandarins being the most vulnerable, while limes, lemons, sour oranges, and trifoliate oranges are the least susceptible to the disease (Dala-Paula et al., 2019). Citrus greening was initially reported in southern China. Its discovery in India was linked to a citrus die back that occurred during the 1700s, leading to a theory that the disease was present in India before spreading to China. A comparable ailment was witnessed in South Africa in 1929 and termed "citrus greening disease" due to the inadequate color development of affected fruit. HLB was later confirmed in South America, particularly in the Brazilian state of Sao Paulo in 2004, and the state of Florida in the United States (Ghosh et al., 2022). The HLB infectious agent has been present in Pakistan for many decades, and the disease is present in all neighboring countries. The HLB pathogen is found in practically every province of Pakistan, including Sindh, KPK, Punjab, and Kashmir (Zafarullah et al., 2016).

Citrus trees are prone to a bacterial infection that can cause a significant reduction in yield and economic value of the fruits (Abbas et al., 2021). The disease is primarily caused by three species of *Candidatus* Liberibacter, which are gram-negative and restricted to the phloem. These bacteria belong to the Proteobacteria category and are identified as Africanus, Americanus, and Asiaticus (Rao et al., 2019; Widyawan et al., 2023).

Blotchy mottling patterns on the leaves are a prominent symptom of greening disease, and they appear mainly along the mid-rib, which serves as the primary axis. These patterns are highly reliable for diagnosing disease and are usually asymmetrical. Interestingly, they bear a resemblance to the yellowing of leaves that typically indicates iron deficiency, and they indicate severe infection (Inoue et al., 2020). As an infected citrus tree rapidly deteriorates, its fruits may begin to exhibit symptoms of the disease. Typically, when healthy fruits reach maturity, the stylus end turns yellow or orange, while the peduncle end remains green (Tipu et al., 2021). However, in the case of HLB disease, the peduncular end becomes yellow orange, while the stylus end remains green. Fruit dropping can begin in the early stages of development due to symptomatic fruits on diseased trees, resulting in immature fruits falling off (Dala-Paula et al., 2019). HLB infection causes the roots and fibrous roots of citrus trees to become less dense, leading to a decreased capacity to absorb water and nutrients. As a result, the tree exhibits signs of nutritional deficiency and experiences a decline in yield (Hamido et al., 2017).

Before the development of molecular techniques, HLB was primarily identified and classified based on observable symptoms. The aim of this research was to develop a rapid method for indexing HLB disease in citrus cultivar in the field. For this purpose, Mosambi cultivar is used. To achieve this objective, two tests were employed:

1. The iodine starch test, a key component of the indexing process based on the accumulation of starch.
2. The 'Pen Test' the most popular and simple approach used to distinguish between nutritional deficiencies and HLB symptoms.

These approaches have the potential not only to facilitate the rapid identification of HLB-infected plants in the field but also to aid in the development of effective management strategies.

MATERIALS AND METHODS

Incidence of HLB

To monitor the prevalence of HLB in Bahawalpur and its surrounding regions, including Yazman, Ahmadpur, Khairpur, and Hasilpur, an extensive survey was conducted. The study focused on the Musambi citrus cultivar, which was screened for HLB. Citrus samples displaying characteristic symptoms of HLB were collected from random commercial orchards. Disease incidence records were kept for 50 trees per tehsil that exhibited visible symptoms, and leaves were collected from the marked citrus trees for detection and confirmation using a pen and iodine starch test (Figure 1). The disease HLB infection causes the roots and fibrous roots of citrus trees to become less dense,

leading to a decreased capacity to absorb water and nutrients. Incidence was calculated as follows.

$$\text{Disease Incidence (\%)} = \frac{\text{No. of infected trees}}{\text{Total no. of observed trees}} \times 100$$

Pen test

A technique for distinguishing between symmetrical and asymmetrical symptoms across the mid-vein, which can indicate the presence of Huanglongbing or nutrient deficiencies, has been described in previous studies (Vashisth et al., 2016; Vashisth and Kadyampakeni, 2020). This technique involves marking the leaf with two circles on opposite halves, which should be positioned adjacent to each other along the central vein. By comparing the patterns in the circles, it becomes easier to detect the presence of greening symptoms. If the circles exhibit identical symptoms, then they are symmetrical, indicating no sign of greening. On the hand, HLB blotchy mottle patterns do not resemble the areas of the two circles, suggesting the presence of greening symptoms. A total of 20 leaves/tehsil with apparent disease symptoms from trees were collected from Bahawalpur and its surroundings (Yazman, Ahmadpur, Khairpur, and Hasilpur) for test and confirmation during the month of February.

Figure 1: (A) Citrus tree used for Huanglongbing diagnosis (B) HLB symptoms on leaf (C) Leaves collection.

Iodine starch test

During March, a sample of 20 leaves exhibiting disease symptoms were collected from Bahawalpur and its surrounding regions, namely Yazman, Ahmadpur, Khairpur, and Hasilpur, for testing and verification purposes. Iodine-starch test method of (Etxeberria et al., 2008) was used with a slight modification. When conducting the iodine starch test, it is important to use the appropriate iodine solution. Iodine tincture was chosen for this test, as other iodine solutions such as povidone. Iodine contain surfactant and other chemicals that prevent them from reacting with starch. However, undiluted iodine tincture was not used, as it reacts too strongly with even small amounts of starch, potentially leading to false positive results. To prevent this, a diluted solution was created by mixing one part iodine solution (1 ml) with nine parts distilled water (9 ml), resulting in a ten-fold dilution. This diluted iodine tincture solution was stored in a dark bottle to prevent degradation (Yaqub et al., 2020).

Procedure

To ensure a sterile procedure, a diluted solution of bleach was prepared and used to sterilize the scissors before cutting the leaf samples. The scissors were sprayed with the solution and dried with tissue paper before cutting another segment of the leaf. The bleach solution was made by mixing 20 ml of bleach with 180 ml of distilled water in a beaker. Clean and sharp scissors were used to cut sections of the selected leaves that included symptomatic tissues, from both

sides of the mid-vein. The cut sections were then immersed in the diluted iodine tincture solution for 1.5 to 2 minutes and rinsed with distilled water to remove excess dye. Finally, the stained segments were observed with a hand lens, and the presence of a dark gray to black color along the entire cut surface confirmed the presence of HLB, indicating a positive result.

Statistical analysis

The mean and SE were calculated using MS excel 2019. Difference in HLB incidence (%) among Bahawalpur zones for each test (Symptomology, Pen, and iodine-starch Test) was analyzed using ONE-Way ANOVA in SPSS (George and Mallery, 2016). The LSD test was used to comparison the % incidence among different Bahawalpur zones for each above-mentioned test.

RESULTS

Symptomology

To diagnose HLB initially, symptoms expression was considered as a fundamental criterion, and all parts of the plant were examined for symptoms. Infected trees exhibited leaf drop and stunting, along with typical symptoms like mottling and symptoms resembling zinc deficiency. The infected fruits were asymmetrical and exhibited color inversion, with thicker inside layers compared to healthy fruits. The fruit contained aborted seeds, which were visible upon cutting it in half. Additionally, infected fruits were poorly developed and failed to attain the normal orange color (Figure 2).

Figure 2: A) Leaf molting, B) Aborted seeds of infected fruit, C) Massive leaf drop and D) Poorly developed fruit and color inversion.

Disease incidence in different zones of Bahawalpur

The range of disease incidence across different zones of Bahawalpur fell between 30% and 44%, as depicted in Figure 3. The accompanying graph revealed that Bahawalpur had the highest disease incidence, reaching 44.00%, followed by Ahmedpur (38.67%), Yazman (36.67%), Hasilpur (32.67%) and Khairpur, with the lowest incidence at 30.00%.

Pen Test

The pen test is a visual diagnostic tool that involves visually inspecting the patterns on a leaf, and the results are typically reported as either symmetrical or asymmetrical, which can help identify HLB or nutritional deficiencies in plants. In the nutrient-deficient leaf on the left, the absence of certain nutrients is evident on both sides of the mid-vein in a symmetrical pattern. However, in the HLB-affected leaf on the right, the symptoms appear in an asymmetrical pattern, which is not observed in the nutrient-deficient leaf (Figure 4).

The results of the pen test conducted in various zones of Bahawalpur indicated that the detection rate of

Candidatus Liberibacter asiaticus varied across different regions. Specifically, the highest detection rate was observed in Bahawalpur at 80%, followed by Ahmedpur at 76.67%, and Yazman at 61.6%, Hasilpur at 46.67 % while the lowest detection rate was observed in Khairpur at 38.33% (Figure 5). These results suggested that *Candidatus* Liberibacter asiaticus is more prevalent in certain regions of Bahawalpur, and further investigation is necessary to determine the underlying factors responsible for this distribution.

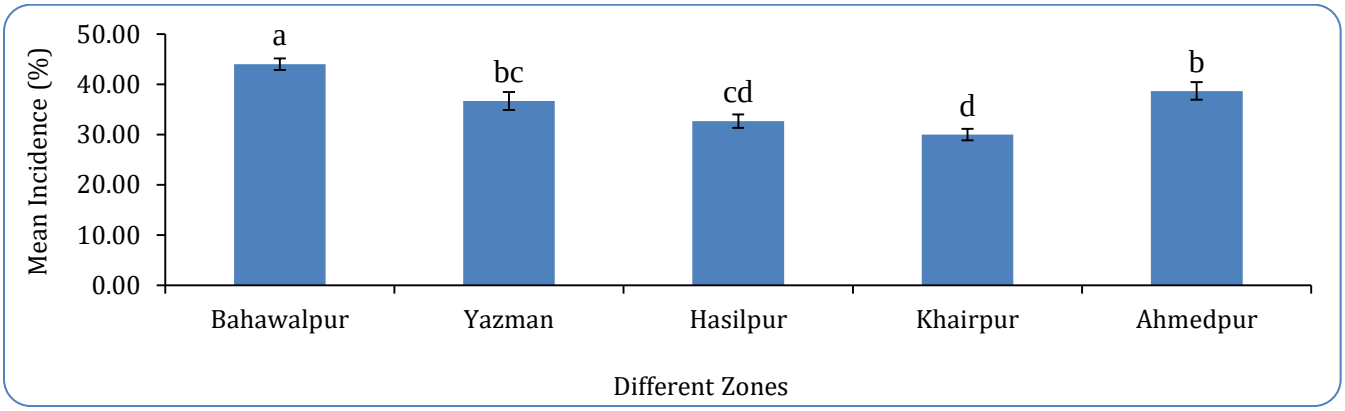

Figure 3: Mean incidence (%) via symptomology test among five different Bahawalpur zones. Means in the same bars followed by the same letter are not significantly different (P > 0.05) using LSD test.

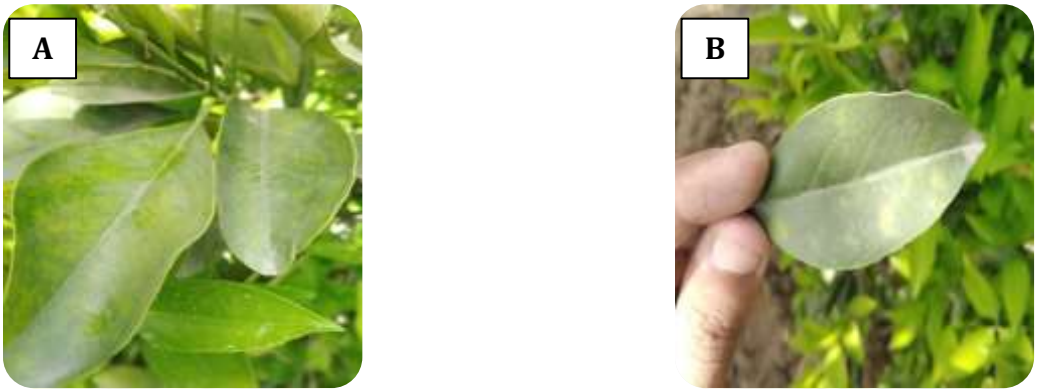

Figure 4: A) Nutrient deficient leaf in the left shows symmetrical pattern, B) HLB-affected leaf on the right, the symptoms appear in an asymmetrical pattern.

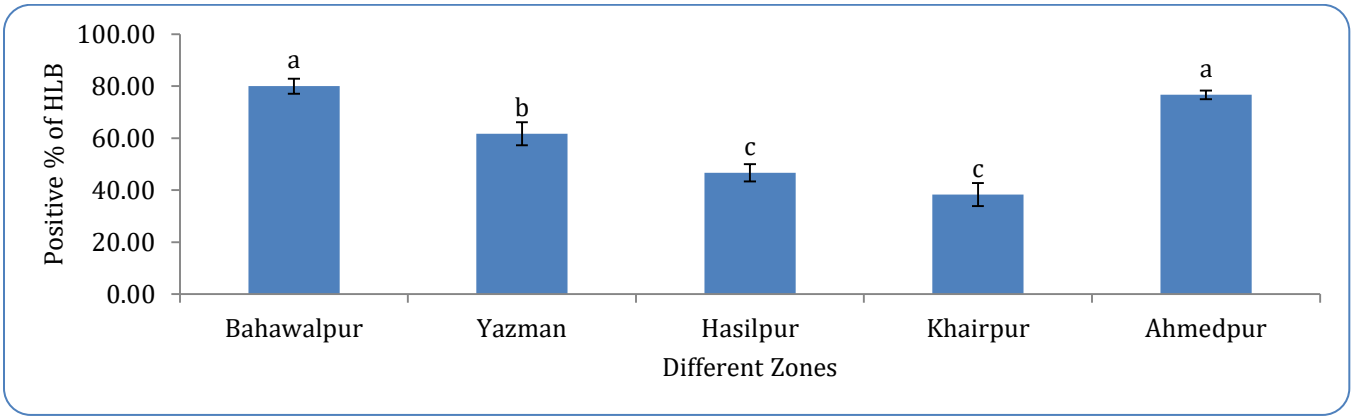

Figure 5: Detection of HLB positive (%) in five different Bahawalpur zones through Pen Test. Means in the same bars followed by the same letter is not significantly different (P > 0.05) using LSD test.

Iodine starch test

Symptomatic leaves were collected from healthy branches and subjected to iodine solution treatment, as outlined in the materials and methods section. The iodine-starch test revealed that leaves infected with HLB displayed a significantly darker gray to black color throughout the entire cut surface, whereas healthy leaves exhibited only some edges that were lightly stained. As shown in Figure 6, this test allowed for rapid indexing of citrus greening disease in the field and facilitated further characterization.

The results of the iodine test, carried out in different zones of Bahawalpur, revealed that the detection rate of *Candidatus* Liberibacter asiaticus varied across different regions. According to the data presented in Figure 7, the highest detection rate was recorded in Bahawalpur, with a rate of 71.67%. In comparison, the detection rate in Ahmedpur was 63.33%, which was lower than Bahawalpur but higher than other regions. Conversely, the lowest detection rate of 30% was observed in Khairpur. These findings indicated that the prevalence of Huanglongbing disease varied across different regions, and some areas may be more susceptible to the disease than others.

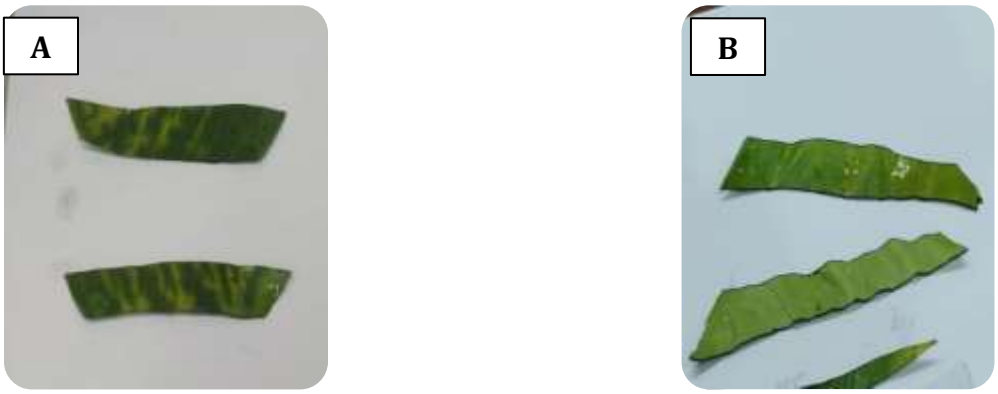

Figure 6 A): healthy leaf shows no or very little staining when immersed in iodine solution; B): HLB-positive leaves demonstrate a high intensity of dark grey to black staining throughout the entire cut surface.

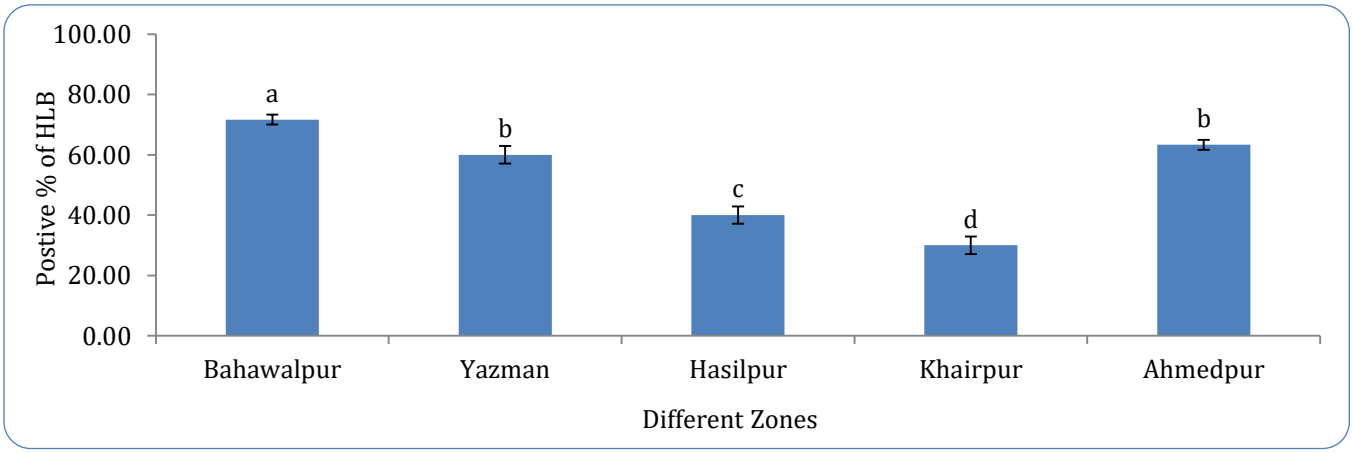

Figure 7: Detection of HLB positive (%) in five different Bahawalpur zones through Iodine Starch Test. Means in the same bars followed by the same letter is not significantly different (P > 0.05) using LSD test.

DISCUSSION

Huanglongbing is a citrus disease caused by *Candidatus* Liberibacter, which has affected citrus orchards in over 40 countries. Various symptoms have been observed as a result of citrus greening in Pakistan, leading to significant losses. The disease is characterized by symptoms such as mottling in leaves, which was observed in our study and confirmed by (Lee Abdullah et al., 2010). During our observations, we noted the presence of aborted seeds, massive leaf drop, as well as

full or half-green fruits (shown in Figure 2). Our findings regarding the symptoms of the disease were consistent with the research conducted by (McCollum and Baldwin, 2017). (Inoue et al., 2020) have also reported similar findings after reviewing symptoms such as yellowing of leaves, lopsided fruits, blotchy mottling, aborted seeds, and fruits remaining unripe (green in color). To conduct rapid indexing in the field, an iodine-starch and pen test were carried out. The accumulation of substantial amounts of starch in the palisade mesophyll tissue of infected leaves led to the suggestion that a detection method using the iodo-starch reaction could be developed. This technique offers several advantages, including reliability, simplicity, rapidity, and cost efficiency (Taba et al., 2006). The accumulation of starch was validated by treating infected leaf samples with iodine solution and observing blackening of the edges. Etxeberria et al. (2008) has also reported similar outcome. Takushi et al. (2007) found that the average amount of starch present in HLB infected leaves was found to be 514.2 mg/kg, whereas in healthy leaves, it was 85.6 mg/kg. This represents that the HLB infected leaves accumulate large amount of starch as compared to healthy leaves. Reports also suggested that this diagnostic method has shown more than 90% agreement with PCR analysis, confirming its reliability and accuracy (Paudyal, 2015). Through pen test it was observed that yellow spots on the leaves of infected trees were asymmetrical in shape, which helped distinguish them from the regular shaped spots caused by nutritional deficiencies. Similar results were also observed by Vashisth and Kadyampakeni (2020). Catara et al. (1991) and Akhtar and Ahmad (1999) initially documented the incidence of HLB in Pakistan's KPK and Punjab regions, with high rates of infection in kinnow (22%) and sweet orange (25-40%) observed in the Sargodha area. Our own research aligns with their findings, as similar symptoms were observed in our sample collections. During a survey conducted in different orchards of Punjab, Razi et al. (2014) confirmed HLB in 41% of citrus samples. Our findings align with prior studies, as we noted similar symptoms and observed a comparable trend in the incidence of HLB. Thus, the identification of symptomology plays a crucial role in detecting HLB at an early stage in the field. The consistent trend in HLB incidence further reinforces the importance of early detection. By monitoring symptomology, farmers and researchers can quickly identify and respond to the disease, preventing further spread and minimizing economic losses. Despite the significant impact of Huanglongbing on citrus production worldwide, there is a lack of data on the rapid detection of this devastating disease. In an effort to address this gap, the present study employs the pent test for the first time, thereby contributing to the advancement of knowledge about the diversity of this pathogen. The results of this study will have a strong impact on the development of effective detection and management strategies for HLB, ultimately benefiting the citrus industry and ensuring the availability of high-quality citrus products for consumers.

CONCLUSION

In conclusion, huanglongbing (HLB) is a devastating disease that poses a serious threat to the citrus industry worldwide. This study focused on diagnosing HLB disease in citrus plants and determining its incidence in different zones of Bahawalpur, Punjab, Pakistan. The findings showed a significant incidence of HLB in different regions, with Bahawalpur having the highest incidence (44%). Expression of symptoms was considered a fundamental criterion for HLB diagnosis, including leaf drop, stunting, mottling, and color inversion in fruits. The pen test and iodine starch test were also used to detect HLB, and the results showed varying detection rates across different regions. The identification of symptomology plays a crucial role in detecting HLB at an early stage in the field. By monitoring symptomology, farmers and researchers can quickly identify and respond to the disease, preventing further spread and minimizing economic losses. The consistent trend in HLB incidence further reinforces the importance of early detection. Further investigation is necessary to determine the underlying factors responsible for the distribution of the disease. The development of efficient diagnostic tools is essential for improving disease management, and continued research on HLB symptomology is crucial for the citrus industry's sustainability.

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AUTHORS' CONTRIBUTION

All authors contributed to the conception and design of the study. ANM, MSY, RA, and MI conducted material preparation, data collection, and analysis with the support of KS and AN. ANM and MBT drafted the initial manuscript, with support from BA, MFAK, and MAB. All authors provided feedback on earlier versions of the manuscript, and they all read and approved the final version. WM provided comments and suggestions for improving the manuscript.

CONFLICT Of INTEREST

The authors declare no conflict of interest.

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