The effect of using uncertified citrus seeds on huanglongbing disease incidence in Karo District

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Abstract. North Sumatra Province is one of the major citrus producers for Siam cultivars in Indonesia. Recently, it experienced a significant decline in citrus production. One of the causes is the presence of huanglongbing (HLB) disease which results in decreased production and plant mortality. The research aim to obtain information on the origin of citrus seeds and to map HLB incidence in eight subdistricts within Karo District. This research was carried out through direct questionnaires to farmers, visual symptoms scoring, and molecular detection with Polymerase Chain Reaction (PCR). At least two villages were taken from each sub-district and three orchards were taken from each village as samples in January 2019. The results showed that in general, citrus plants aged 3 to 15 years. Mostly, citrus planted in Karo originated from uncertified seedlings bought from local and outside seed breeders in the province of North Sumatra. The highest symptoms severity of HLB diseases based on leaf scores and canopy scores were recorded in Barus Jahe subdistrict with 82-83% intensity. The highest HLB incidence were recorded in three subdistricts namely Kabanjahe, Munte, and Brastagi, each with 100% HLB prevalence. This shows that asymptomatic plants are not always free from CLas pathogens. Certified citrus seeds which are free from systemic diseases, followed by improve farmer’s awareness to apply good agriculture practice the initial to sustain Indonesian citrus agroindustry.

1. Introduction
Sustainable citrus agroindustry in Indonesia is highly dependent upon the use and availability of high-quality and disease-free citrus seedlings. To uphold and regulate this practice, the Ministry of Agriculture, Republic of Indonesia enshrined a policy through Keputusan Menteri Pertanian (Kepmentan) No.04/Kpts/SR.130/D/6/2019 that carefully provides systematic guidances on citrus seedling productions and regulates its supply chains. This guidance encompasses detailed information from seedlings productions, quarantine to quality control where the seedlings that embody all the criteria mentioned are labeled and certified. Moreover, this policy also aims to establish modern, advance, and high-quality national seedling productions.

In recent years, the Indonesian citrus agroindustry scene has witnessed a significant surge in the usage of uncertified citrus seedlings. These seedlings, however, despite being very cheap, are highly susceptible to multiple citrus systemic diseases. One of the most dangerous diseases found in these seedlings is huanglongbing (HLB). HLB can reduce citrus productivity, quality and in some extreme cases can cause death [1]. Young trees may never produce marketable fruit while more mature trees may gradually become unproductive. The flavour of fruits from infected trees...
can be affected and can taint extracted juice products. HLB is caused by *Candidatus liberibacter asiaticus* and can be easily transmitted from plant to plant by insect vector *Diaphorina citri* and by budding with contaminated plant materials [2]. Early detection is pivotal to control HLB, however accurate detections are hard to conduct due to the similarity of its symptoms to other disease symptoms and certain nutrient deficiencies [3].

In Indonesia, one of the provinces that are currently experiencing a significant plummet in citrus productions due to HLB outbreaks is North Sumatera. Since 2011, North Sumatra has lost its grip as the highest tangerine producer in Indonesia to East Java [4]. It mainly stems from the HLB outbreak in main citrus producers in the provinces, such as Karo District. Presumably, this outbreak resulted from the long term effect of using uncertified seedlings.

To suppress HLB incidences and transmissions in an area, early prevention and accurate detection efforts on seedling sources are critical before citrus propagation and plantation [5]. This can be done through the propagation of pathogen-free citrus seedling, elimination of inoculum sources, and prevention of secondary infection by insect vectors are important methods for integrated disease management. The establishment of a pathogen-free citrus nursery system is also fundamental to prevent disease epidemics. Other than controlling the source productions, big picture information on disease severities, intensities, and prevalences in the area is important to create a robust and accurate HLB controlling program [6]. To help address this issue, this research is conducted to obtain information on seedling sources and HLB disease intensity in Karo District, North Sumatera based on disease visual scoring and disease prevalence by Polymerase Chain Reaction (PCR) detection.

2. Materials and methods

2.1. Materials

Materials used in this research are visually infected and asymptomatic citrus leaves, chemicals such as EDTA (20 mM and 0.5 M), TAE (tris-acetate-EDTA), Tris-Cl 1.5 M, CTAB 10%, Agarose, 1 kb marker, PCR kit (Kappa), Las606 forward primer and LSS reverse primer.

2.2. Methods

The research conducted at January until April 2019. Method in this research consists of two major stages namely visual symptoms observations and molecular detections using Polymerase Chain Reaction (PCR) method. Visual observations and sample collections for PCR detection were conducted in eight sub-districts within the Karo District, namely Dolat Rayat, Barusjahe, Tiga Panah, Merek, Merdeka, Munte, Kabanjahe and Berastagi. Molecular detections with PCR were performed in the Phytopathology laboratory at Indonesian Citrus and Subtropical Fruits Research Institute, Batu, East Java, Indonesia.

This research was carried out by performing visual observations on appearing symptoms and sampling for molecular detection in the laboratory to study HLB intensities and prevalences in Karo District. Questionnaires were also employed to the farmers to acquire practical information on varieties grown, seedling sources, seedling certification statuses, and total citrus trees are grown in each field. Each sub-district studied in this research is represented by 2 orchards, from two villages. Total sample of farmer are 46 farmers. Leaf samples for molecular detection were collected from 3 visually infected citrus trees and 1 asymptomatic citrus tree.

Apparent symptoms on the leaves and canopy are scored based on the disease severity index (Table 1) to determine HLB severity in each tree [7].
Table 1. Disease scoring index for Huanglongbing symptoms in citrus leaf and tree canopy.

| Score | Leaf                                      | Canopy                              |
|-------|-------------------------------------------|-------------------------------------|
| 1     | Asymptomatic                              | Asymptomatic                        |
| 2     | Symptoms appeared in 5 or fewer leaves    | < 10% defoliation                   |
| 3     | Symptoms appeared in ≥5 leaves from the   | 10-30 % canopy defoliation          |
|       | same branch, but less than 10 % from     |                                     |
|       | overall branches                          |                                     |
| 4     | Symptoms appeared in 10-30 % branches    | 30-60 % deflorations and tree stunting |
| 5     | Symptoms appeared in 30-60% of branches   | >60 % defoliation on canopy and tree stunting |
| 6     | Symptoms appeared in >60% branches       |                                     |

Overall HLB intensity in each sub-district is determined according to the equation proposed by [8] below:

\[
\text{HLB intensity} = \left( \frac{\sum (a \times b)}{N \times Z} \right) \times 100 \%
\]

Note : \(a\) : total of symptomatic plants, \(b\) : total score, \(N\) : number of observed plants, \(Z\) : highest score.

Molecular detection of HLB in leaf samples was performed to detect asymptomatic and false positive samples, and to determine HLB disease prevalence in Karo. It is done by amplifying the 16S rDNA region of causal bacteria \(C\)Las. Chromosomal DNA of \(C\)Las were extracted according to the method proposed by [9] and amplified with Las606 forward primer (5'- GGA GAG GTG AGT GGA ATT CCG A-3) dan reserve primer LSS (5'-ACC CAA CAT CTA GGT AAA AAC C-3) [10]. The PCR mix solution consists of 2 µl DNA template, 12.5 µl PCR Master Mix, 2 µl primer forward, 2 µl primer reverse, and 6.5 µl ddH2O. The amplification of 16S rDNA was carried out under the program consisted of 1 cycle pre-denaturation at 96 ºC for 9 min, 35 cycles of denaturation at 96 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, and extension at 72 ºC for 1 min, and 72 ºC post-extension for 7 min. HLB positive samples are confirmed with the appearance of 500 bp amplicon. Disease prevalence in determined by detecting three visually infected citrus trees from each village in each sub-district. Positive results from the molecular detection in each sub-district is presented in percentage over total samples obtained in each respective sub-district.

3. Results and discussion

3.1. General condition of citrus plantation in Karo District, North Sumatera

In general, citrus trees grown in Karo are experiencing a significant increase in mortality. It is mainly due to HLB disease infection. Previously, citrus trees in this area can live up to 20 years, whereas in our findings it is only 5-13 years. This significant surge of citrus mortality and HLB prevalence in Karo is presumably related to the use of uncertified seedlings by the farmers that originated from local breeders and breeders in Bengkinang, Riau (Table 2). These uncertified seedlings usually are not grown according to the guidelines established by the government, therefore the disease status of each seedling is not thoroughly scrutinized and potentially becomes the inoculum sources of HLB.

Increasing rate of mortality due to HLB has caused farmers in Karo to change their main commodities. Recently, citrus trees and plantations have been replaced by fast-yielding, more resilient, and perennial crops such as vegetables and coffee. Apart from being free of HLB infection, cultivating these crops also requires smaller amounts of initial capitals compared to citrus. On the other hand, farmers who stick to cultivating citrus usually delay HLB symptoms manifestations by providing optimum care to each tree instead of opting for eradication of infected trees and replanting. Indeed, improving plant nutrition through fertilization might have some role in increasing HLB infected trees survival by sustaining yield for some period [11]. In particular, adding Zn fertilizers can slightly improve some metabolic processes of HLB-infected citrus [12].
Nevertheless, other alternative methods such as pulse roguing of infected citrus trees and increasing the replanting or eradication rate are still considered more reliable to control HLB [13]. However, replanting and eradication efforts of HLB infected trees in Karo are still very low and oftentimes farmer deliberately kept the infected plant, despite the overtly appearing symptoms. Infected plants can be a source of inoculum for healthy plants to become infected. To tackle this problem, once every six months of local removal of HLB-symptomatic trees is needed to curb the disease progress rate [14]. This action can be carried out with a note that the inspection of plants with HLB symptoms in the field is carried out at least once a month and vector is routinely eradicated.

Table 2. General information of citrus plantations in eight sub-districts in Karo District.

| No | Subdistricts | Village | Orchard area (m²) | Total trees in each field | Age (year) | Seedling source |
|----|---------------|---------|-------------------|--------------------------|------------|-----------------|
| 1  | Barus Jahe    | Serdang | 2.500             | 125                      | 12         | Serdang         |
|    |               | Sinaman | 3.000             | 150                      | 10         | Serdang         |
|    |               |         | 3.750             | 200                      | 14         | Sinaman         |
|    |               |         | 5.000             | 250                      | 14         | Sinaman         |
| 2  | Tiga Panah    | Tiga Panah | 4.000         | 200                      | 13         | Local           |
|    |               | Desa Singa | 2.500         | 125                      | 14         | Local           |
|    |               |         | 5.000             | 250                      | 12         | Local           |
|    |               | Kacinambun | 7.500         | 400                      | 10         | Local           |
|    |               |         | 4.000             | 200                      | 10         | Local           |
| 3  | Merdeka       | Daulu    | 3.000             | 150                      | 10         | Daulu           |
|    |               | Jaranguda | 2.500            | 125                      | 13         | Local           |
|    |               |         | 2.500             | 125                      | 12         | Local           |
|    |               | Merdeka   | 2.000             | 100                      | 9          | Local           |
|    |               |         | 3.000             | 150                      | 10         | Local           |
| 4  | Berastagi     | Raya     | 3.000             | 150                      | 12         | Riau            |
|    |               | Rumah    | 2.500             | 125                      | 10         | Riau            |
|    |               |         | 3.000             | 150                      | 12         | Local           |
|    |               | Berastagi | 2.000         | 120                      | 11         | Local           |
|    |               |         | 7.500             | 375                      | 12         | Riau            |
|    |               | Dokan    | 2.500             | 125                      | 7          | Bengkinang, Riau|
|    |               |         | 4.000             | 200                      | 10         | Bengkinang, Riau|
|    |               |         | 10.000            | 500                      | 11         | Dokan           |
| 5  | Merek         | Cingkes  | 2.500             | 125                      | 13         | Cingkes         |
|    |               |         | 2.500             | 125                      | 12         | Cingkes         |
|    |               |         | 5.000             | 312                      | 14         | Cingkes         |
|    |               |         | 5.000             | 250                      | 8          | Local           |
|    |               | Merek    | 2.500             | 125                      | 10         | Riau            |
|    |               |         | 2.500             | 100                      | 5          | Lokal           |
| 6  | Kaban Jahe    | Samura   | 5.000             | 225                      | 10         | Padang          |
|    |               | Kandibata | 4.500         | 225                      | 9          | Padang          |
|    |               | Ketaren  | 2.500             | 125                      | 11         | Riau            |
|    |               |         | 3.000             | 150                      | 14         | Padang          |
| 7  | Munte         | Barung   | 5.000             | 250                      | 13         | Local           |
|    |               | Kersap   | 4.000             | 200                      | 9          | Local           |
|    |               |         | 3.000             | 150                      | 8          | Riau            |
|    |               | Singgamanik | 5.000         | 200                      | 7          | Singgamanik     |
|    |               |         | 4.500             | 150                      | 10         | Local           |
|    |               | Kineppen | 2.000             | 100                      | 10         | Local           |
|    |               |         | 3.500             | 150                      | 8          | Local           |
| 8  | Dolat Rayat   | Kubucolia | 2.500         | 125                      | 10         | Riau            |
|    |               | Sampun   | 3.000             | 180                      | 12         | Local           |
|    |               | Dolat Rayat | 2.500         | 125                      | 9          | Local           |
|    |               |         | 4.000             | 200                      | 10         | Riau            |
3.2. Huanglongbing disease intensity in Karo District based on visual symptoms

Blotchy mottled leaves, yellow shoots, lopsided fruits with inverted color and aborted seeds are some common visual symptoms of HLB [15]. Indeed, these symptoms are pervasive in the fields especially in Singa and Katoren villages. Leaf and canopy symptoms scores in these villages reached 82-83 %, recorded as the highest disease intensity score in both categories. Contrary to the findings in the two previous villages, HLB intensities in Kacinambun and Singgamanik villages is very low. Both leaf and canopy symptoms scoring are only recorded between 8-7 % in both villages (Data are now shown).

In a subdistrict level, the highest disease intensity was recorded in Barus Jahe (Figure 1). This finding, however, is anticipated and not surprising as Barus Jahe is already known as the biggest citrus producer in North Sumatra for more than 33 years. It is also surrounded by the multiple citrus-producing sub-districts, hence the HLB local transmission level to and within Baru Jahe is expected to be very high. Apart from those factors, lack of eradication efforts and poor agricultural practice in Barus Jahe have also exacerbated HLB intensities in Barus Jahe. Severely infected trees are still very pervasive in Barus Jahe and healthy trees are still planted around them. This practice will significantly accelerate HLB infections from tree to tree as it provides a shorter flying distance for the insect vector D. citri, therefore increasing the HLB infection risks. Actually, to tackle this issue, farmers can plant windbreak plants to minimize D. citri flying distance and dispersal in citrus orchards [16]. Unfortunately, this method is relatively novel and most farmers in Karo are still not familiar with it.

![Figure 1. HLB disease intensity based on leaf score and canopy in each district in Karo District.](image)

Age and the quality of seedlings are also factors aggravating HLB transmission in Barus Jahe. Seedlings used in Barus Jahe are uncertified seedlings that originate from unregulated breeders, local markets, or self-grown seedlings. These seedlings were not grown and rigorously assessed according to the guideline set by the Ministry of Agriculture, therefore may carry HLB inoculum.

3.3 Molecular detection of huanglongbing using PCR

To investigate whether visually infected samples may harbor CLas inoculum or vice versa, we conducted molecular detection with PCR to confirm this phenomenon. During the molecular detection, we discovered multiple false-negative and false-positive results. Approximately, 6 samples tested negative for HLB but still visually manifested positive-HLB symptoms in the field. Visual manifestations of HLB symptoms in citrus are not always indicating an CLas infection. In contrast, out of 19 visually asymptomatic samples, 12 of them are tested positive for HLB (Table 3). This finding may suggests that these asymptomatic samples have just recently infected by CLas and symptoms have not fully manifested as HLB symptoms would normally emerge 4-6 month after the initial infection. Nonetheless, these plants are still very contagious. In fact, citrus may have already become infectious only after 15 days of the initial HLB infection [17].
Therefore, early detection of CLas, even before the first appearance symptom, is critical for HLB management to prevent epidemic of the disease [18].

Table 3. HLB status of asymptomatic leaf samples collected from 19 villages across Karo District based on PCR detection.

| Sample sources                | HLB status | Sample sources                | HLB status |
|-------------------------------|------------|-------------------------------|------------|
| Singa, Tiga Panah Subdistrict | +          | Merdeka, Merdeka Subdistrict  | +          |
| Kacinambun, Tiga Panah Subdistrict | +      | Singgamanik, Munte Subdistrict | -          |
| Kacinambun, Tiga Panah Subdistrict | -      | Singgamanik, Munte Subdistrict | +          |
| Raya, Berastagi Subdistrict   | +          | Kinnepen, Munte Subdistrict   | -          |
| Raya, Berastagi Subdistrict   | -          | Dokan, Merek Subdistrict      | +          |
| Samura, Kaban Jahe Subdistrict | +      | Dokan, Merek Subdistrict      | +          |
| Samura, Kaban Jahe Subdistrict | -      | Cingkes, Merek Subdistrict    | -          |
| Kandibata, Kaban Jahe Subdistrict | +    | Merek, Merek Subdistrict      | +          |
| Jaranguda, Merdeka Subdistrict | +      | Sampun, Dolat Rayat Subdistrict | -          |
| Merdeka, Merdeka Subdistrict  | +          |                               |            |

Note: + = infected by HLB, - = not infected by HLB

To also gain overall representation of HLB disease prevalence in Karo District, we analyzed 3 visually infected trees taken from each village in each sub-district with PCR. Overall, the highest prevalence of HLB are found in Kabanjahe, Munte and Brastagi sub-district, with 100% HLB prevalence (Figure 2). However, in spite of having high prevalence of HLB, symptoms found in these sub-districts are not as severe as found in Barus Jahe (Figure 1). Presumably, this may indicate that HLB infection in these sub-districts were just recently occurring and the symptoms have not yet fully manifested.

Figure 2. HLB prevalence in Karo District, North Sumatera.

As HLB has taken the center stage as one of the biggest threats to citrus agroindustry in Karo and Indonesia in general, effective agricultural policy measures need to be taken to deter poor agricultural practices can exacerbates HLB infections and dispersal. Policy on regulating seeds quality, plant maintenance and quarantine, geographic isolation, eradications of infected trees, and disease certifications for budwood sources needs to be upheld more seriously. On the other hand, policy that targets to enhances farmers awareness and understanding towards the diseases also needs to be enacted as it will allows early detection and proper handling by farmers when encountering HLB infected trees in the field.

4. Conclusions
The outbreak of HLB has significantly impacted the citrus industry. Based on visual observations and surveys, uncertified seedlings are used in all areas studied. To tackle this issue, effective agricultural policy needs to enacted and put into practice. Government should, by any means,
improve farmer’s awareness on the importance of good quality seedlings, endorse proper agricultural practices and improve breeder facilities as those are the initial capitals for preventing HLB disease.

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