CITRUS ROOTSTOCK SELECTION FOR ENHANCED HUANGLONGBING RESISTANCE: A STRATEGIC DISEASE MANAGEMENT PARADIGM

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Abstract. Citrus fruits, widely cultivated in tropical and subtropical regions, face considerable challenges, notably from the destructive citrus greening disease (HLB). This is caused by Candidatus *Liberibacter* spp., bacteria transmitted by psyllids, significantly reducing citrus yield and quality and posing a global threat to citrus production. Eliminating Huanglongbing (HLB) disease remains a challenge due to the lack of an effective cure, necessitating the adoption of a practical short-term management method known as the “Three-pronged” strategy. The practice of traditional plant breeding struggles to overcome two main hurdles including inter-species compatibility problems and extended juvenile phases. The use of disease-resistant rootstocks improves citrus plant tolerance against HLB which provides an effective solution to minimize damage from the disease. We examined three citrus rootstocks together with four graft combinations. The side grafting technique served to introduce the *Candidatus Liberibacter* asiaticus (*C*Las) to the citrus plants. Furthermore, the inoculation with *C*Las resulted in significant reductions of chlorophyll a, chlorophyll b, and total chlorophyll content, while photosynthetic activity and chlorophyll fluorescence intensity declined in various citrus varieties. Additionally, the inoculation led to a marked increase in reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) levels, indicating heightened oxidative stress. Interestingly, the varieties ‘Jiu Bing Le’ and ‘Hong Jiang’/‘Jiu Bing Le’ exhibited minimal damage, suggesting a high degree of resilience and potential resistance to the disease.
Keywords: *citrus, rootstock, grafting, tolerance, growth vigor*

Introduction

Citrus fruits maintain a central global agricultural position because of their nutritional and economic significance in tropical and subtropical areas (Khan et al., 2010; Hayat et al., 2022). Citrus fruits are recognized for their high nutritional contents including vitamins, minerals, dietary fibers and carbohydrates with vital compounds which offer vital health benefits to people (Hayat et al., 2017). The citrus industry faces major production obstacles because both biotic and abiotic stresses threaten its operations substantially. Among these challenges, Huanglongbing (HLB), commonly referred to as a citrus greening disease, has emerged as a profound threat, causing substantial economic losses and posing a considerable obstacle to sustainable citrus cultivation (Blaustein et al., 2018; Dala-Paula et al., 2019; Thakuria et al., 2023).

HLB, caused by the Candidatus *Liberibacter* species, is limited to the phloem and transmitted by psyllids. It stands as one of the most devastating plant diseases (Bové, 2006). *C*Las infection weakens and often leads to the death of trees, resulting in fruit loss due to premature abortion. Additionally, it negatively affects the size and quality of the remaining fruit juice. Fruits from infected trees are smaller, misshapen, and exhibit aborted seeds. Premature fruit drop is common, and the fruits that remain fail to mature properly, retaining a green color, which is why the disease is referred to as “greening disease.” Early studies in South Africa noted poor-quality, bitter-tasting juice in HLB-affected fruit (Mccleanc1 and Schwarz, 1970). The fruits exhibit improper coloring, remaining green on the shaded side, contributing to the ‘greening disease’ (Bové, 2006; Gottwald et al., 2007). Leaf symptoms range from full yellowing to asymmetric blotchy-mottling, resembling mineral deficiencies, and are often accompanied by intensive vein corking.

*C*Las infects all known Citrus species, with most commercial cultivars displaying pronounced disease symptoms post-infection (Mccleanc and Schwarz, 1970). Varied responses to HLB have been observed among different citrus cultivars, with some hybrids of Citrus and *Poncirus trifoliata*, commonly used as rootstocks, showing tolerance (Albrecht and Bowman, 2019, 2012; Folimonova et al., 2009). Commercial citrus production heavily relies on the rootstock, which plays a key role in determining its success or failure (Castle, 2010). In addition, rootstock selection not only impacts scion vigor, fruit size, quality, and yield (Hayat et al., 2021) but also depends heavily on its ability to tolerate diverse environmental conditions and resist pests and diseases (Hayat et al., 2023a).

Commercial citrus cultivars typically exhibit high susceptibility to HLB (Stover et al., 2015). However, notable exceptions lie in certain rootstock types commonly used in citrus cultivation, such as trifoliate orange and its hybrids, which demonstrate heightened tolerance to HLB (Ramadugu et al., 2016). In addressing HLB, current strategies primarily target its vectors, involve the removal of infected trees, and employ antibacterial treatments. However, a comprehensive solution to eradicate HLB remains elusive. In the face of these challenges, the exploration of disease-resistant rootstocks emerges as a promising avenue to enhance citrus resilience against HLB, offering valuable insights for sustainable management strategies. This study aims to evaluate the effectiveness of disease-resistant rootstocks in mitigating HLB-induced physiological changes and enhancing the overall resilience of citrus varieties, providing useful knowledge for the management of future citrus breeding and orchard management.

Plant material and cultivation conditions

The *C*Las-carrying budwood of ‘Hong Jiang’ was obtained from the Fruit Tree Research Institute of the Guangdong Academy of Agricultural Sciences. Three citrus seedlings: ‘Jiu Bing Le’ [*Atalantia buxifolia* (Poir.) Oliv.], ‘Carrizo citrange’ [*Citrus sinensis* Osb. × *Poncirus trifoliata* (L.) Raf.] and ‘Sour orange’ (*Citrus reticulata* Blanco). Seeds were provided by the Citrus Research Institute of the Chinese Academy of Agricultural Sciences. Four citrus grafted seedlings were produced by grafted ‘Hong Jing’ scions onto ‘Jiu Bing Le’, ‘Carrizo citrange’, ‘Sour orange’ and ‘Hong Jing’ rootstocks. Throughout the two-year cultivation period, the grafted seedlings flourished, showcasing well-formed crowns originating from the scions. During *C*Las inoculation, the affected buds of HLB were carefully grafted onto the existing branches of the scions. In September 2021, 20 plants with uniform growth were randomly selected from each variety and grafted seedlings. The grafting procedure was performed using a standard grafting method, with three buds grafted onto each plant. Among them, 10 plants were in the experimental group (grafted with buds carrying *C*Las), and the other 10 plants were in the control group (grafted with healthy buds), and both groups were managed conventionally.

Extraction of plant genomic DNA

The DNA extraction of the experimental materials was carried out using the Rapid Plant Genomic DNA Isolation Kit (Bioengineering). The concentration and quality of DNA were measured using a Nanodrop 2000 ultramicro spectrophotometer (Thermo Fisher Company, USA). DNA was stored at -20°C in a refrigerator (SANYO Company, Japan).

Routine PCR detection of CLas

To confirm whether different citrus plants were infected with Huanglongbing disease, we performed *C*Las detection on all tested plants. The method required validation and screening through PCR identification performed using specific primers OI1/OI2c (Table. 1)

Real-time quantitative fluorescence PCR (qRT-PCR) detection of CLas content

At the 2nd, 4th, 6th, 8th, 10th, and 12th month, three leaves were collected from the same position on various citrus plants infected with Huanglongbing. The collection involved selecting 3-4 mature leaves from the top to the bottom of the plant, and the total DNA of the leaves was extracted (were diluted to 50 ng/μL), and TaqMan real-time fluorescence quantitative PCR detection was performed on all DNA samples (*Table 2*).

Measurements of photosynthetic parameters

From 8:00 to 12:30 pm on September 10, 2022, three susceptible plants and three healthy plants of each variety were selected, and the branches of each plant were measured using a CIRAS-3 portable photosynthesis instrument (PP-System Company, USA). The middle mature leaves were measured to determine the net photosynthetic rate, intercellular CO_2 concentration (Ci), transpiration rate (Tr), stomatal conductance (Gs), and water use efficiency (WUE).

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Table 1. *PCR primers used in this study*

Primers	Primer sequence（5’ - 3’）
OI1	GCGCGTATGCAATACGAGCGGCA
OI2c	GCCTCGCGACTTCGCAACCCAT

Table 2. *TaqMan real-time fluorescence quantification primers*

Primer name	Primer sequence
HLBas	TCGAGCGCGTATGCAATACG
HLBr	GCGTTATCCCGTAGAAAAAGGTAG
Probe HLBp	(FAM)-AGACG GGTGAGTAACGCG(BHQ-1)

Measurements of chlorophyll fluorescence characteristics

The susceptible leaves (infected with Huanglongbing for one year) and healthy leaves were dark-treated for 30 min, and then their chlorophyll fluorescence parameters were measured using a small multifunctional plant phenotyping measurement system Plant Explorer TM (Thermo Forma Company, USA). The measurement indicators mainly include initial fluorescence (Fo) and PSII maximum photochemical efficiency (Variable fluorescence/Maximum fluorescence, Fv/Fm).

Determination of antioxidant activities

Leaf tissues (0.5 g) were finely powdered and homogenized in 5 mL of extraction buffer (phosphate buffer, pH 7.5, containing 0.1 mM EDTA, and 4% polyvinylpolypyrrolidone). Following centrifugation for 20 min at 12,000 × *g*, the supernatant was utilized for enzyme analysis (Zafar et al., 2020). Enzyme activities of antioxidants, including superoxide dismutase (SOD), polyphenol oxidase (PPO), and peroxidase (POD), were assessed using methodologies outlined by Wang et al. (2019).

Determination of soluble sugars and starch contents

Soluble sugars (glucose, fructose, and sucrose) content was extracted utilizing the method proposed by Kagan et al. (2014). Samples (0.1 g) were collected at 0, 90, 180, 270, and 360 days after inoculation (DAI) and subjected to extraction in 50 mL of deionized water for 1 h at 40°C, followed by centrifugation at 10,000 rpm for 10 min, and filtration of the supernatant through 0.45 μm syringe filters. The subsequent analysis involved high-performance liquid chromatography (HPLC; Knauer, Germany) with a Eurokat Pb column. HPLC-grade distilled water, operating at a flow rate of 1.0 mL min^{-1}, served as the mobile phase, while the column temperature was maintained at 65°C. Identification and quantification of soluble sugar concentrations were performed using a refraction index (RI) detector, with retention times of peaks guiding the determination of sugar contents based on external analytical standards. Soluble sugar concentrations were calculated through calibration curves and expressed as mg g^{-1} DW. Starch concentrations were analyzed according to the procedure outlined by Changjie et al. (1998).

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Observation of callose accumulation in the mid-vein of leaves

Citrus leaves of uniform age and size were carefully processed for histological analysis. The leaf veins, segmented into 0.5 cm sections, underwent sequential fixation in FAA solution for 24 h, dehydration in graded ethanol concentrations, and clearing in xylene: ethanol solutions. Subsequently, the samples were infiltrated with liquid paraffin, embedded to form blocks, and sectioned to the desired thickness. These wax strips were then expanded in cold water using a fine brush before being meticulously mounted on anti-drop glass slides. After a 30-min adhesion period, the slides were oven-dried at 37-40°C for approximately 1 h. De-waxing involved two treatments with pure xylene, followed by xylene: ethanol ratios of 3:1 and 1:3, and two rounds of anhydrous ethanol treatment. Rehydration ensued with sequential exposure to ethanol concentrations ranging from 95% to 35% for 2 min each. A swift staining process with aniline blue for 1 min culminated in direct observation under a fluorescence microscope (McAudi Industrial Group Co., Ltd., China).

Statistical analysis

The data was subjected to analysis of variance (ANOVA) using the Statistics 8.1 software package. Mean comparisons among treatments were performed through LSD multiple comparison tests, with statistical significance set at a P value < 0.05.

Results

Symptom development characteristics in different citrus seedlings

Phenotypic observations were carried out on a total of seven citrus plants, comprising both un-grafted and grafted plants, as illustrated in *Figure 1*. After a year of HLB infection, un-grafted citrus seedlings showed mottled yellowing symptoms in 'Carrizo citrange' and 'Sour orange'. Notably, 'Sour orange' exhibited more pronounced symptoms, including yellowing in the leaf veins. On the other hand, Jiu Bing Le' did not show any noticeable signs or symptoms. Likewise, it was noted that 12 months post-HLB infection in grafted seedlings, all four citrus plants displayed symptoms of mottled yellowing on their leaves. The severity of the mottled yellowing ranged from mild to severe, in the following order: 'Hong Jiang'/'Jiu Bing Le' < 'Hong Jiang'/Carrizo citrange < 'Hong Jiang'/Sour orange < 'Hong Jiang'/'Hong Jiang' graft combinations.

Effects of Huanglongbing on MDA and reactive oxygen species in un-grafted and grafted citrus seedlings

The MDA and reactive oxygen species (ROS) contents of healthy leaves and susceptible leaves (infected with Huanglongbing for 10 months) were measured. Both un-grafted and grafted citrus seedlings exhibited significant increases in MDA content following *C*Las infection, with the increase being most pronounced in the 'Hong Jiang'/'Sour orange' graft combination (*Fig. 2a*). In healthy plants (control), the MDA content of 'Hong Jiang'/'Jiu Bing Le' and 'Hong Jiang'/'Sour orange' is significantly greater than that of other tested varieties. As shown in *Figure 2b*, in healthy plants (control), 'Sour orange' and 'Hong Jiang'/'Jiu Bing Le' plants had the highest H_2O_2 content compared with other varieties. After *C*Las infection, un-grafted and grafted citrus varieties showed an upward trend in H_2O_2 contents. Furthermore, the H_2O_2 content of

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‘Sour orange’ and ‘Hong Jiang’/‘Hong Jiang’ plants had the highest, which was notably greater than that of the other plants. In the case of $O_2\cdot^-$ free radicals, after *C*Las infection, the $O_2\cdot^-$ production rates of different un-grafted and grafted citrus varieties were significantly increased. Moreover, after *C*Las infection, the O^{-2} production rate of ‘Jiu Bing Le’ and ‘Hong Jing’/‘Jiu Bing Le’ was significantly lower than that of the other tested varieties (*Fig. 2c*).

Effects of Huanglongbing on chlorophyll content in leaves of different citrus seedlings

The chlorophyll contents of citrus seedlings are affected by Huanglongbing. As shown in *Figure 3a*, after inoculation with *C*Las, the chlorophyll a content of un-grafted and grafted citrus seedlings was significantly reduced. The variety ‘Carrizo’ maintained the highest chlorophyll a levels under both healthy and diseased conditions, while ‘Hong Jiang/’Hong Jiang’ and ‘Hong Jiang’/‘Carrizo’ exhibited the lowest chlorophyll a content under disease conditions. As shown in *Figure 3b*, the chlorophyll b content of ‘Sour orange’ was significantly higher in healthy plants than that of the other citrus seedling varieties. After being infected with *C*Las, the chlorophyll b content of un-grafted and grafted seedlings decreased significantly. Among them, Hong Jiang’/‘Hong Jiang’ showed the largest reduction, with a decrease of 39.29%. As shown in *Figure 3c*, the total chlorophyll contents of the un-grafted and grafted citrus plants decreased significantly after inoculation with *C*Las. Among healthy plants, ‘Carrizo’ and ‘Sour orange’ showed the highest total chlorophyll levels. In contrast, diseased plants of ‘Hong Jiang’/‘Carrizo’ and ‘Hong Jiang’/‘Hong Jiang’ had the lowest levels of total chlorophyll contents.

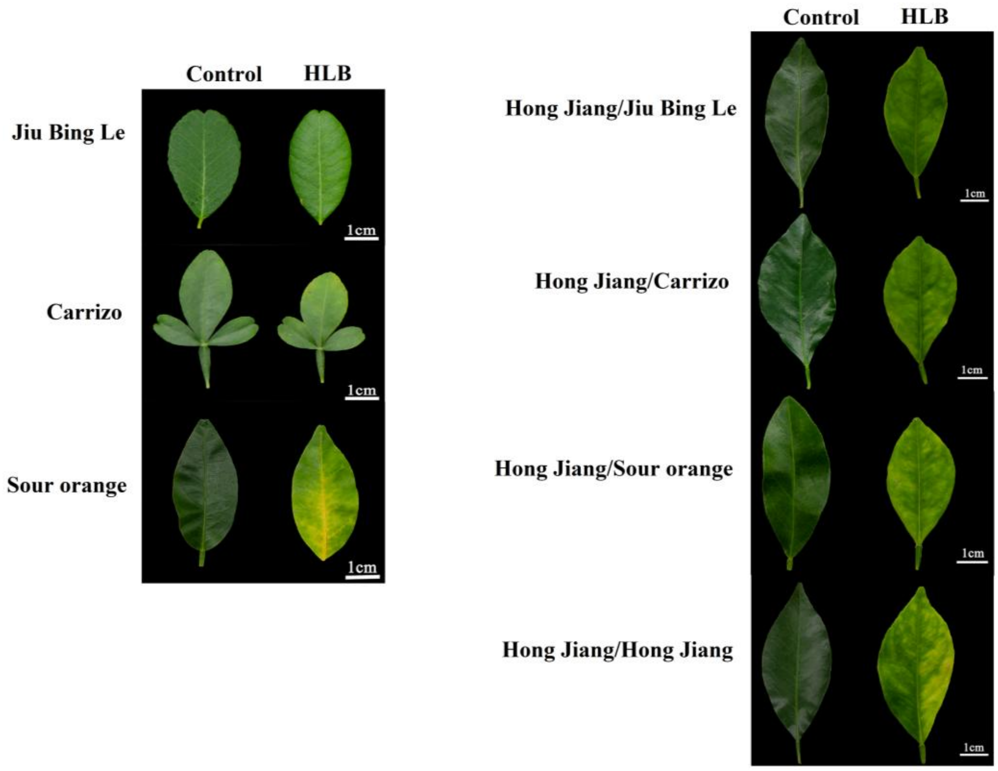

Figure 1. *Phenotypic observations of un-grafted and grafted citrus seedlings after 12 months of Huanglongbing (HLB) infection*

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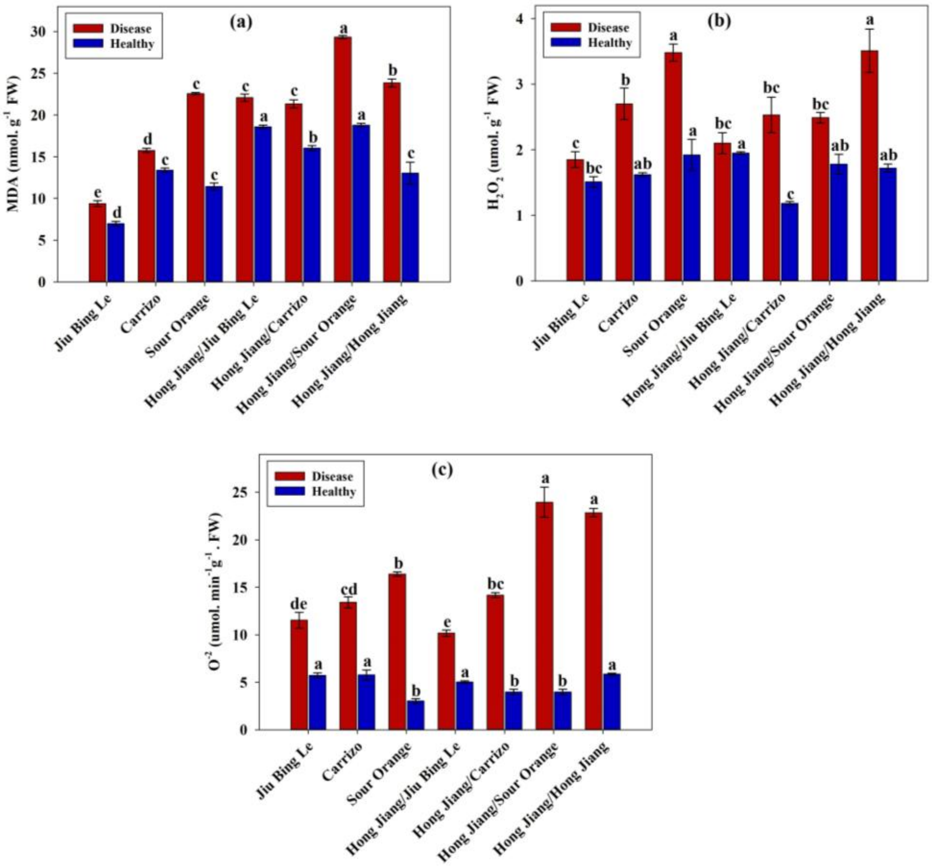

Figure 2. *The effect of Huanglongbing disease on MDA and reactive oxygen species in different citrus seedlings. (a) MDA contents; (b) H_2O_2 contents; (c) Rate of oxygen radical. Lowercase letters indicate significant differences between healthy and diseased treatments at the $p \leq 0.05$ level; n = 3*

Effects of Huanglongbing on chlorophyll fluorescence parameters of different citrus seedlings

The results showed that after inoculation with *C*Las, diseased plants consistently exhibited significantly higher F_0 values compared to healthy plants across all tested citrus seedlings with the highest values observed in ‘Hong Jiang/Sour Orange’ and ‘Hong Jiang/Hong Jiang’ (*Fig. 4a*). In contrast, ‘Jiu Bing Le’ and ‘Carrizo’ citrus seedlings exhibited significantly lower F_0 values compared to other treatments following *C*Las inoculation. As shown in *Figure 4b*, the highest Fv/Fm values in healthy plants were observed in ‘Hong Jiang’/Hong Jiang’ and ‘Sour Orange’. In contrast, diseased plants of ‘Jiu Bing Le’, ‘Hong Jiang’/‘Jiu Bing Le’ and ‘Hong Jiang’/‘Carrizo’ showed significantly greater Fv/Fm values compared to other varieties.

Effects of HLB disease on the antioxidant enzyme activity of different citrus seedlings

Antioxidant enzyme activities were measured in healthy and diseased plants (*Fig. 5*). After inoculation with *C*Las, the POD activities of the grafted and un-grafted varieties increased significantly across all citrus varieties. Among diseased plants, ‘Jiu Bing Le’ exhibited the highest POD activity, followed by ‘Carrizo’ and ‘Sour Orange’, while the lowest activity was observed in ‘Hong Jiang’/‘Carrizo’ and ‘Hong Jiang’/‘Sour Orange’

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(*Fig. 5a*). SOD activity was significantly higher in diseased plants compared to healthy plants across all citrus varieties. Among diseased plants, ‘Jiu Bing Le’ and ‘Hong Jiang’/‘Jiu Bing Le’ exhibited the largest increases in SOD activity. In contrast, healthy plants consistently displayed lower SOD activity with minimal variation between varieties, emphasizing the substantial impact of *C*Las infection on SOD levels (*Fig. 5b*). As illustrated in *Figure 5c*, PPO enzyme activity in citrus seedlings significantly increased after inoculation with *C*Las. Among the varieties, ‘Carrizo’, ‘Hong Jiang’/‘Jiu Bing Le’ and ‘Hong Jiang’/‘Sour orange’ exhibited the highest PPO activity, whereas ‘Sour Orange’ recorded the lowest activity following *C*Las inoculation. In healthy plants, the PPO activity of ‘Hong Jiang’/‘Hong Jiang’ and ‘Hong Jiang’/‘Sour orange’ was markedly greater than that of tested varieties.

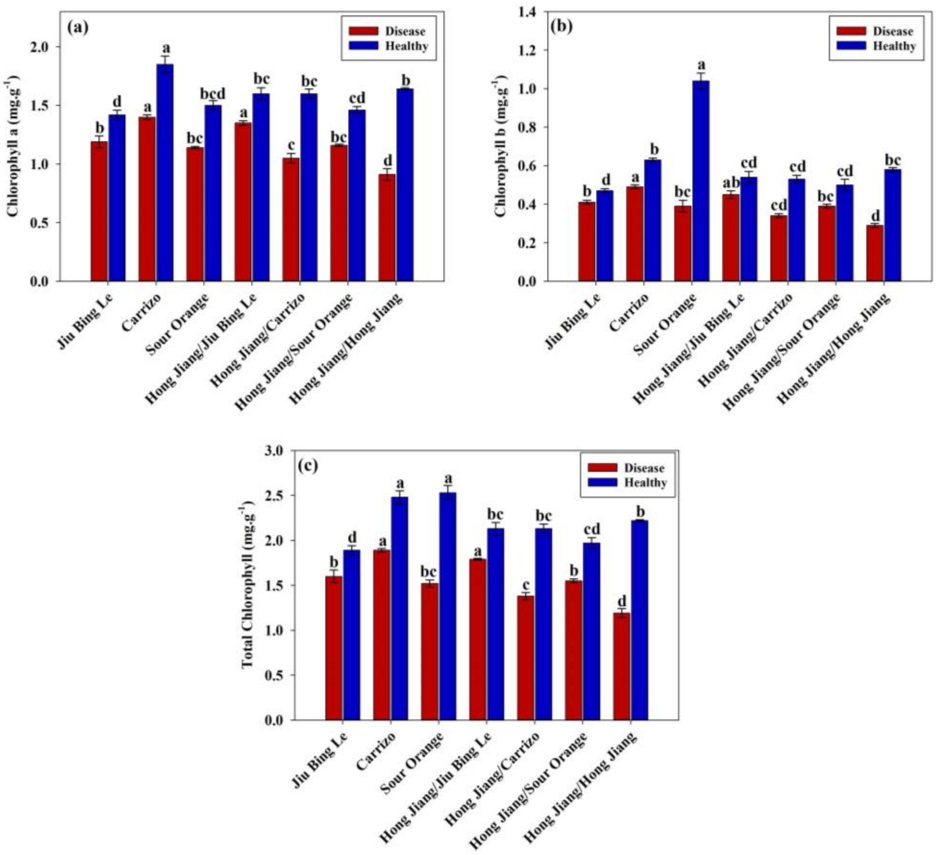

Figure 3. *Effect of Huanglongbing (citrus greening disease) on chlorophyll content in different citrus seedlings. (a) Chlorophyll A content. (b) Chlorophyll B content. (c) Total chlorophyll content. Lowercase letters indicate significant differences between healthy and diseased treatments at the p ≤ 0.05 level; n = 3*

Effects of Huanglongbing on photosynthesis of un-grafted and grafted citrus seedlings

As shown in *Figure 6a*, after inoculation with *C*Las, the net photosynthetic rate was significantly reduced in ‘Carrizo citrange’, ‘Sour orange’, ‘Hong Jiang’/‘Carrizo citrange’, ‘Hong Jiang’/‘Sour orange’ and ‘Hong Jiang’/‘Hong Jiang’ in un-grafted and grafted citrus seedlings. Furthermore, the net photosynthetic rate of ‘Jiu Bing Le’ and ‘Hong Jiang’/‘Jiu Bing Le’ plants was significantly greater than the other tested

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varieties, after *C*Las infection. To sum up, compared with the other three types of grafted seedlings, the photosynthesis of ‘Jiu Bing Le’ and ‘Hong Jiang/’Jiu Bing Le’ is less affected by Huanglongbing. It is speculated from the data that ‘Jiubianji’ and ‘Hong Jiang/’Jiu Bing Le’ has a certain tolerance to Huanglongbing.

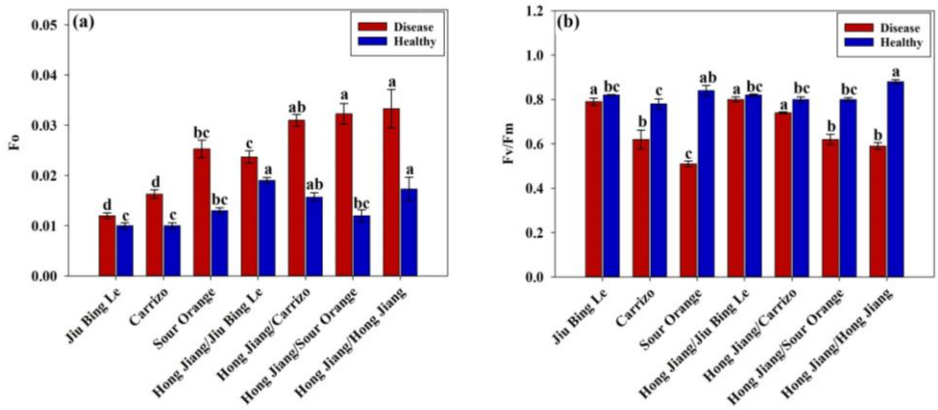

Figure 4. *Effect of Huanglongbing on Chlorophyll Fluorescence Parameters in Different Citrus Seedlings. (a) Initial fluorescence value of the leaf. (b) Maximum photochemical efficiency Fv/Fm of the leaf. Lowercase letters indicate significant differences between healthy and diseased treatments at the p ≤ 0.05 level; n = 3*

Effects of Huanglongbing on photosynthesis of un-grafted and grafted citrus seedlings

As shown in *Figure 6a*, after inoculation with *C*Las, the net photosynthetic rate was significantly reduced in ‘Carrizo citrange’, ‘Sour orange’, ‘Hong Jiang’/‘Carrizo citrange’, ‘Hong Jiang’/‘Sour orange’ and ‘Hong Jiang’/‘Hong Jiang’ in un-grafted and grafted citrus seedlings. Furthermore, the net photosynthetic rate of ‘Jiu Bing Le’ and ‘Hong Jiang’/‘Jiu Bing Le’ plants was significantly greater than the other tested varieties, after *C*Las infection. To sum up, compared with the other three types of grafted seedlings, the photosynthesis of ‘Jiu Bing Le’ and ‘Hong Jiang/’Jiu Bing Le’ is less affected by Huanglongbing. It is speculated from the data that ‘Jiubianji’ and ‘Hong Jiang/’Jiu Bing Le’ has a certain tolerance to Huanglongbing.

In the healthy control group, the intercellular CO_2 concentration of ‘Hong Jiang’/‘Jiu Bing Le’ was notably greater than that of the other tested varieties. After *C*Las infection, the intercellular CO_2 concentration of all seven un-grafted and grafted citrus seedlings increased significantly. In the disease treatment group, the intercellular CO_2 concentration of ‘Hong Jiang’/‘Jiu Bing Le’ and ‘Hong Jiang’/‘Sour orange’ was significantly greater than that of the other tested un-grafted and grafted varieties (*Fig. 6b*). Jiu Bing Le’ and ‘Hong Jiang’/‘Jiu Bing Le’ did not show any decrease in stomatal conductance (*g*s) after *C*Las infection, while all the other tested varieties experienced a reduction. Among them, ‘Sour orange’ and ‘Hong Jiang’/‘Hong Jiang’ had the largest decrease in g_s (*Fig. 6c*). As shown in *Figure 6d*, the transpiration rate (*E*) of ‘Hong Jiang’/‘Sour orange’ is significantly greater than the other tested varieties in healthy plants. In addition, the transpiration rate of ‘Hong Jiang’/‘Jiu Bing Le’ was pointedly greater than the other un-grafted and grafted citrus seedlings. As shown in *Figure 6e*, the water use efficiency of the seven citrus un-grafted and grafted seedlings decreased significantly after *C*Las infection. Moreover, in both healthy and disease

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groups, the WUE of ‘Hong Jiang’/‘Hong Jiang’ was significantly greater than that of other tested varieties.

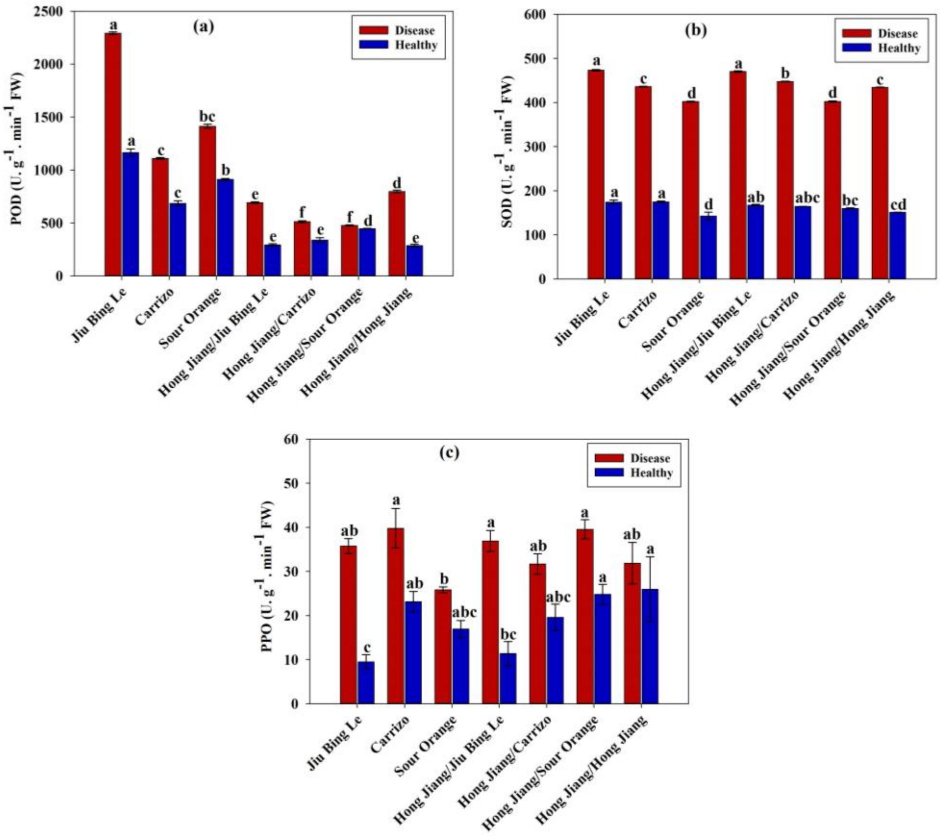

Figure 5. *Effect of Huanglongbing (Citrus Greening Disease) on Antioxidant Enzyme Activities in Different Citrus Seedlings. (a) POD enzyme activity (b) SOD enzyme activity and (c) PPO enzyme activity. Lowercase letters indicate significant differences between healthy and diseased treatments at the p ≤ 0.05 level; n = 3*

Effects of Huanglongbing on carbohydrates in different citrus seedlings and grafted seedlings

After inoculation with *C*Las, the total soluble sugar contents of different citrus grafted and un-grafted seedlings showed an overall upward trend. At 0 d, there was no significant difference in the total soluble sugar content of the tested citrus seedlings (grafted and un-grafted); from 90 d to 360 d, the total soluble sugar content of ‘Hong Jiang’/‘Hong Jiang’ and ‘Hong Jiang’/‘Sour orange’ was higher than that of other varieties (*Fig. 7a*). Similarly, the sucrose content of citrus seedlings continued to increase during the year they were infected with HLB. During the period from 0 d to 360 d, the sucrose content of ‘Jiu Bian Je’ and ‘Hong Jiang’/‘Jiu Bian Je’ was significantly lower than that of the other tested varieties. In addition, the sucrose content of these un-grafted and grafted seedlings increased rapidly from 0 d to 270 d, then increased slowly and stabilized from 270 d to 360 d (*Fig. 7b*). For both grafted and un-grafted citrus seedlings, a prolonged period of susceptibility to disease leads to an increase in fructose contents. From 90 d to 360 d, the fructose contents of ‘Hong Jiang’/‘Jiu Bing Le’ were noticeably lower than the other tested varieties. Moreover, the largest change in fructose content in one year was observed

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in ‘Sour orange’, which increased from 4.01 mg·g^{-1} on 0 d to 9.00 mg·g^{-1} on 360 d, an increase of 1.24 times (*Fig. 7c*). Moreover, the starch content of un-grafted and grafted seedlings of different citrus varieties showed an overall upward trend with the passage of HLB infection time. In addition, the starch content of ‘Hong Jiang’/‘Hong Jiang’ and ‘Hong Jiang’/‘Sour orange’ reached the maximum value on 270 d, and then decreased, with the starch content increasing by 9.39 times and 9.10 times respectively compared with 0 d (*Fig. 7d*).

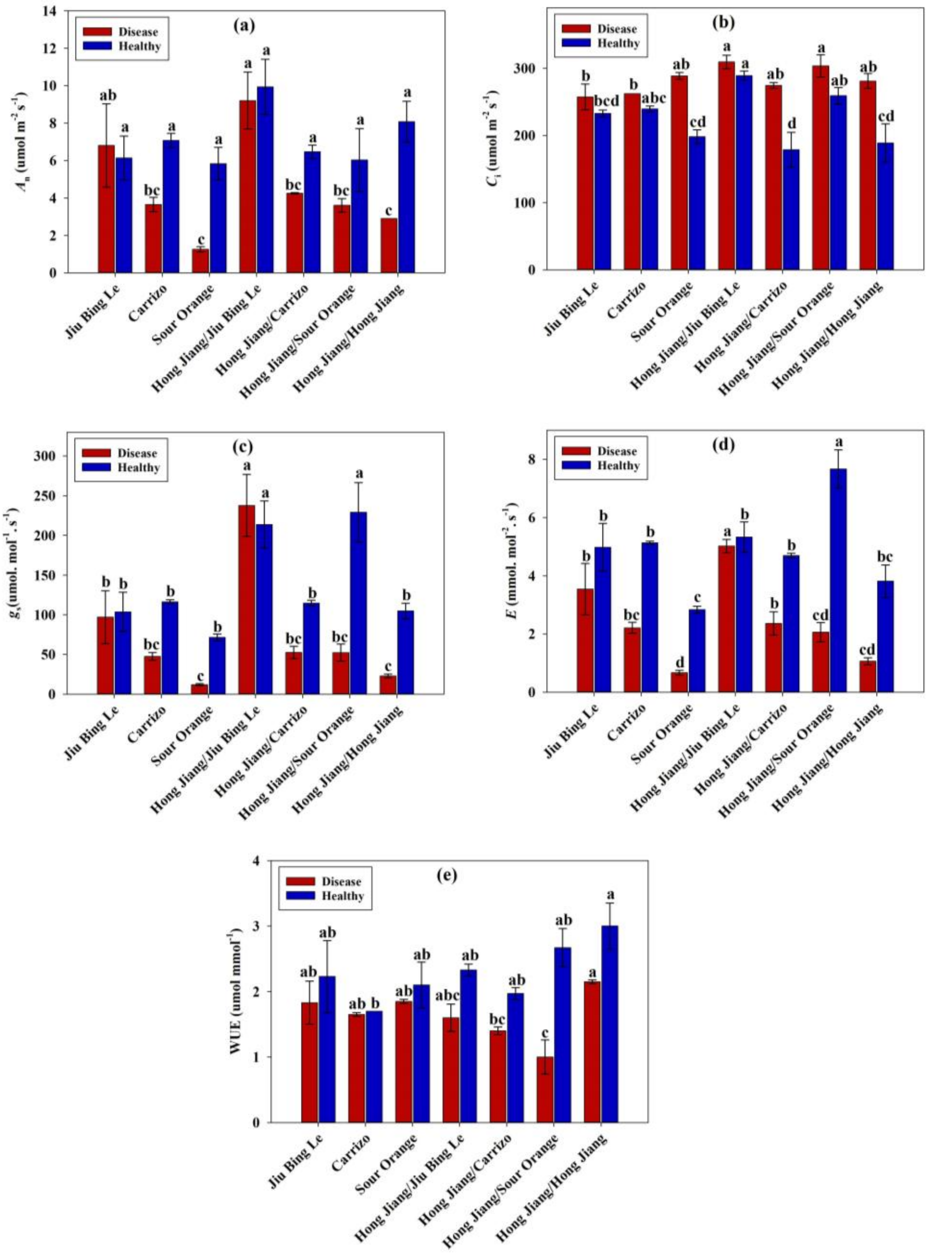

Figure 6. *The effect of Huanglongbing disease on photosynthesis of different citrus grafting seedlings. (a) Net photosynthetic rate of leaves. (b) Stomatal conductance of leaves. (c) Transpiration rate of leaves. (d) Water use efficiency of leaves. (e) Intercellular CO_2 concentration of leaves. Lowercase letters indicate significant differences between healthy and diseased treatments at the $p \leq 0.05$ level; $n = 3$*

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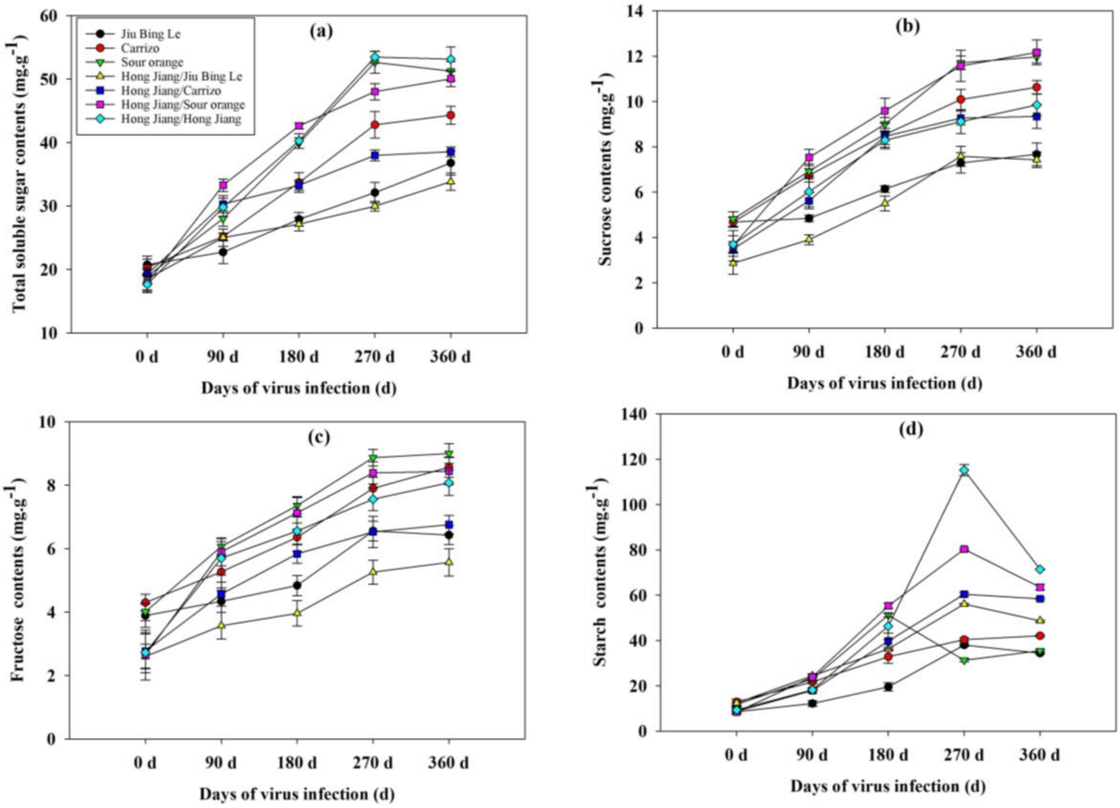

Figure 7. *Effects of Huanglongbing (Citrus Greening) disease on the carbohydrates of different citrus grafted seedlings at 0, 90, 180, 270 and 360 days of virus infection (d). (a) Soluble total sugar contents (b) Sucrose contents (c) Fructose content and (d) Starch contents.*

Effects of HLB on callose in different citrus seedlings

'Sour Orange' showed the highest number of callose deposits in un-grafted seedlings under disease conditions, compared with all other varieties. However, in grafted varieties, the highest number of callose deposits was observed in 'Hong Jiang'/'Sour Orange' under disease conditions. On the other hand, 'Jiu Bing Le' and 'Hong Jiang'/'Jiu Bing Le' had the least number of callose deposits in both ungrafted and grafted seedlings under diseased conditions after *C*las infection (*Fig. 8*). This indicates that 'Jiu Bing Le' exhibits a certain degree of tolerance to HLB.

Discussion

Currently, citrus greening is threatening the citrus industry worldwide (Hamido et al., 2019; Wang et al., 2017). So far, there is no effective cure for this destructive disease and management mainly depends on the control of *Diaphorina citri* vector using insecticides. Although the use of different rootstocks could increase citrus scions tolerance to biotic and abiotic stresses, little work has been conducted to investigate the effect of rootstocks on citrus tolerance to a citrus greening pathogen (Killiny et al., 2020). ROS concentrations triggered by *C*Las infection are above the threshold needed to induce cell death, probably resulting from the combined effect of programmed cell death induced at low ROS concentrations and necrotic cell death stimulated at high ROS concentrations. In addition, ROS positively regulate callose deposition and inhibit

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plant growth including roots (Dunand et al., 2007). MDA (malondialdehyde) is a byproduct of membrane lipid peroxidation. When plants experience adverse stress, they generate an increased amount of MDA, which in turn damages the membrane structure (Hayat et al., 2023b). Consequently, measuring the MDA content can provide insights into the extent of membrane lipid peroxidation, serving as an indirect indicator of the degree of membrane damage and the plant's resistance to disease. This current study shows that the MDA, H_2O_2 content and free radical (O^{-2}) production rate and in the leaf tissues of un-grafted and grafted varieties increased significantly after being infected with HLB. In un-grafted seedlings, 'Sour orange' accumulated the maximum amount of H_2O_2 content. Similarly, a previous study revealed that the HLB disease increased the level of MDA and H_2O_2 contents in the citrus plants as compared to the control healthy 'Kinnow' mandarin plants (Ikram et al., 2022). This study demonstrated that the MDA content in seven different citrus varieties experienced varying degrees of increase following infection with HLB, aligning with the findings of prior experiments. Consequently, it can be concluded that 'Jiu Bing Le' possesses strong resistance to HLB and has the potential to enhance the disease resistance of 'Hong Jiang' orange scion cultivar.

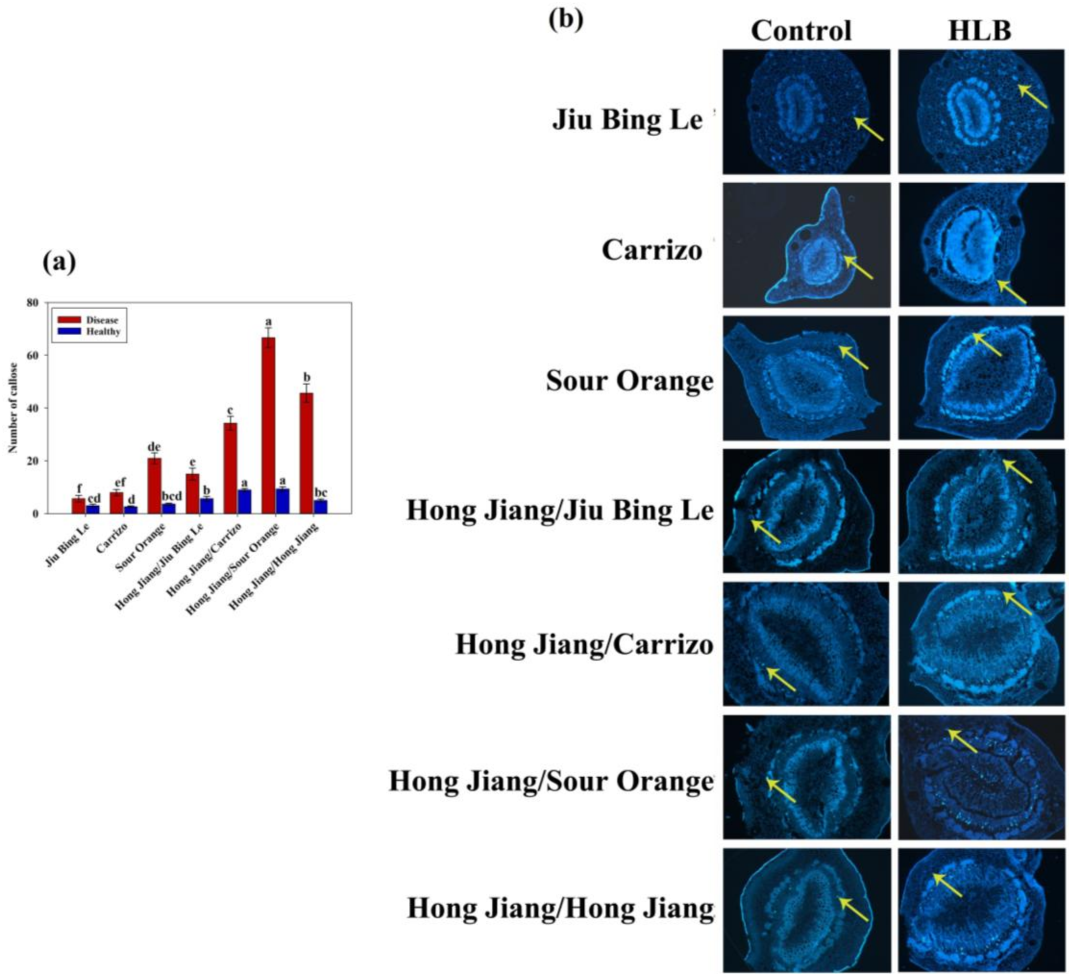

Figure 8. *Effect of Huanglongbing on callose in grafted seedlings of different citrus. (a) Quantitative picture of callose. (b) Microscopic observation picture of callose deposition. Arrow points to callose*

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HLB infection in citrus results in phloem blockage, hindering the movement of photosynthetic products, leading to a sugar metabolism disorder, and culminating in a substantial buildup of total soluble sugars in the leaves. Another study revealed that the total soluble sugar content in the leaves of 'Sweet orange' increased to 3.5 times that of healthy leaves following infection with Huanglongbing (Fan et al., 2010). Furthermore, the increase in total soluble sugar content varies among different citrus varieties after *C*Las infection, likely due to differences in their resistance to HLB. Among them, the total soluble sugar content of 'Jiu Bing Le' and 'Hong Jiang'/'Jiu Bing Le' changed relatively slowly among the other tested varieties. This shows that 'Jiu Bing Le' is less affected by HLB and has certain disease resistance. As a rootstock, it can improve the scion's resistance to HLB. Sucrose, the primary photosynthetic product, is transported from above-ground to underground parts of the plant, providing energy for growth and development. However, after HLB infection, the sucrose content in citrus leaves surges, likely due to the blockage of the plant's phloem by *C*Las, which impedes sucrose transport (Koh et al., 2012). Among the un-grafted and grafted seedlings, 'Jiu Bing Le' and 'Hong Jiang'/'Jiu Bing Le' exhibited lower values of sucrose contents compared to other tested varieties, suggesting that these varieties are less impacted by HLB. Research by Albrecht and Bowman (2019) also showed that fructose is an essential carbohydrate for the survival of *C*Las. After infection with HLB, the fructose content of resistant varieties is significantly lower than that of susceptible varieties. In the present study, the fructose content of various citrus varieties was observed to increase with the prolongation of *C*Las infection. Notably, the 'Jiu Bing Le' and 'Hong Jiang'/'Jiu Bing Le' varieties exhibited lower levels of fructose content compared to other tested varieties. It is speculated that 'Jiu Bing Le' possesses greater resistance to HLB than the other seedling varieties and has the potential to enhance the disease resistance of the scion. Additionally, it was observed that the extent of changes in starch content differed among citrus varieties. Furthermore, 'Jiu Bing Le' demonstrated only slight alterations in starch content during the *C*Las infection, and it holds the potential to enhance the scion's resistance to HLB to a certain extent.

Plants possess several reactive oxygen species (ROS) scavenging mechanisms, among which the primary ROS scavenging system includes enzymes such as peroxidase (POD), polyphenol oxidase (PPO), and superoxide dismutase (SOD) (Hayat et al., 2023). These enzymes effectively neutralize ROS, maintain a dynamic balance between ROS production and scavenging, and mitigate damage to the plant's membrane structure and function (Altaf et al., 2023). Previous research indicates a positive correlation between the increase in enzyme activities of POD, SOD, and PPO and enhanced host resistance in plants (Govind et al., 2016). HLB-tolerant cultivars such as Persian triploid lime contain higher levels of antioxidants and antioxidant enzyme activities than more susceptible cultivars such as Mexican lime (Sivager et al., 2021). This current study shows that the POD, SOD, and PPO enzyme activities of seven citrus varieties increased to varying degrees after infection with HLB, which is consistent with previous studies. Among the un-grafted seedlings, the increase in POD and SOD activities was greatest in 'Jiu Bing Le' seedlings. The variation in POD and SOD enzyme activities was less evident in the grafted 'Hong Jiang'/'Hong Jiang' seedlings. This suggests that the rootstock can influence not only the morphological traits of the scion but also enhance its stress resistance to some extent.

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Conclusions

In conclusion, this research investigated the impact of Huanglongbing (HLB) on various citrus varieties and demonstrated that ‘Jiu Bing Le’ and ‘Hong Jiang’/‘Jiu Bing Le’ were the least affected by HLB. These varieties exhibited strong resilience to HLB, as evidenced by the lower levels of reactive oxygen species, H_2O_2, and MDA contents, along with higher activities of POD, SOD, and PPO enzymes. After *C*Las inoculation, a notable decrease in chlorophyll a, chlorophyll b, and total chlorophyll content was recorded in various citrus varieties, accompanied by a decline in photosynthetic activity and chlorophyll fluorescence. This research suggests that ‘Jiu Bing Le’ plants have the potential to enhance the disease resistance of the scion to some extent, offering a valuable approach to managing HLB in citrus plants.

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