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RESEARCH

Morpho-chemical characterization of Huanglongbing in mandarin (*Citrus reticulata*) and orange (*Citrus sinensis*) varieties from Pakistan

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ABSTRACT

Huanglongbing (HLB) is one of the world's worst diseases of citrus trees. The research was conducted to characterize positive citrus samples of two local varieties, 'Kinnow' mandarin (*Citrus reticulata* Blanco) and 'Mosambi' orange (*C. sinensis* (L.) Osbeck), based on morphological, physical and biochemical characters. Physical and biochemical parameters of greening infected and healthy samples of both the cultivars were significantly different. Citrus fruits were lopsided and half to full green in the HLB infected trees compared to healthy. The HLB infected leaves samples were small and narrow. Fruit diameter, leaf area, and juice weight of HLB infected samples were significantly less than healthy samples. The rind thickness of infected fruits (70% and 80%) was significantly higher than healthy ones (20% and 30%). Starch content of leaves from mandarin (5.54 μg mL^{-1}) and orange (6.12 μg mL^{-1}) were significantly higher in HLB infected plants, while juice percentage of HLB infected fruit samples was lesser in mandarin and orange (22.73%, and 14.70%, respectively) than healthy fruits (38.22% and 31.30%, respectively). Acidity was significantly increased in juice of HLB infected mandarin and orange fruits (0.57%, and 0.53%, respectively). Similarly, there was significant reduction in biochemical parameters; total sugars in mandarin and orange (5.16% and 4.79%), total soluble solids (8.54% and 8.28%), total chlorophyll (13.50 and 14.92 mg mL^{-1}) and ascorbic acid (34.28% and 34.12%) in HLB infected samples of both cultivars. This study determined differences of physical and biochemical parameters of two local citrus varieties, commonly cultivated in the Asian region.

Key words: Citrus, HLB, juice quality, physical parameters, physiology.

INTRODUCTION

Citrus in terms of related products and by-products have a potential role in generation of employment that lead to raise the income for Pakistan citrus industry. The export of citrus is an important source of foreign exchange (Iftikhar et al., 2021), because citrus industry in Pakistan represents 2.89 million metric tons in production. From the Pakistan citrus growing areas, Punjab province has vital role in contribution of more than 95% this production (Sajid et al., 2021). Although Pakistan is among the top 16 leading citrus producing countries, but its production has been affected by or citrus greening disease (CGD) (syn. Huanglongbing, HLB) since late 80's (Catara et al., 1988), which is a real threat to the citrus industry not only in Pakistan but also in the world (Bove, 2006; Gottwald et al., 2007; Sajid et al., 2021). This disease debilitates the tree and has a bad impact on the flowering, tree vigor, nutrient uptake, fruit quality, and yield of infected plants (Gottwald et al., 2007). The influence of HLB on horticultural traits of scion variety is due to phloem limited causative bacterium and its transmission through citrus psyllid (Gottwald et al., 2007). Once the disease was established in citrus orchards, it showed different types of symptoms leading to the plant's death. Leaf chlorosis of different intensity, stunting of tree, low fruit, and juice quality were the common and characteristic effects of HLB in citrus (Bove, 2006; Iftikhar et al., 2016). The nutritional status of citrus plants also compromised due to HLB (Razi et al., 2011). Different types of symptoms and physiological changes have been observed and found effective in disease characterization on a biochemical basis. Some biochemical changes like starch accumulation, high acidity, lower soluble solids, and secondary metabolites have also been attributed to this disease (Albrecht et al., 2016; McCollum and Baldwin, 2017; Dala-Paula et al., 2019). However, it is not reported how these biochemical and physiological changes affect Pakistani citrus varieties which are also being cultivated in the Asian region.

Bacterium infection produced different symptoms on citrus depending on the degree of tolerance to HLB of citrus varieties from sweet orange, mandarin, grapefruit, lemon, and lime (Hijaz et al., 2020). Biochemical and physical parameters of HLB infected citrus plants have been studied to understand mechanism of infection and response of plants (Baldwin et al., 2010; Shokrollah et al., 2011). Starch accumulation was one of the characters most commonly studied (Etxeberria et al., 2007). Extreme environmental conditions such as high and low temperatures, rainfall and relative humidity had a role in masking of symptoms (Whitaker et al., 2014). Health of tree, fruit development, ripening and quality of fruits, juice quality, high acidity, soluble solids and solid/acid ratio, total sugars, and other metabolites produced in HLB infected plants have been also studied (Massenti et al., 2016; Baldwin et al., 2018; Dala-Paula et al., 2019).

This devastating disease infects all the important citrus commercial varieties at different level of susceptibility. These negative impact of HLB can be minimized by using the tolerant rootstock. In a previous study, PCR detected the *Candidatus* Liberibacter asiaticus in HLB-infected citrus samples and showed genetic variation in HLB-infected samples of highly planted citrus varieties, 'Kinnow' mandarin (*Citrus reticulata* Blanco) and 'Mosambi' orange (*C. sinensis* (L.) Osbeck), upon sequencing (Sajid et al., 2021) from citrus orchards of Punjab, Pakistan. These two cultivars, mandarin and orange, shared the greatest amount of citrus production in Pakistan (Nawaz et al., 2020). Few research papers have been published on biochemical activities in HLB-infected citrus hosts in the world, and this characterization could help to develop strategies to rehabilitate citrus plant through nutrient application in diseased plants. Especially, considering that scanty of literature is available on the biochemical-based characterization of HLB in Pakistan, the research was conducted to characterize these HLB-infected varieties biochemically in citrus orchards of the Punjab, Pakistan.

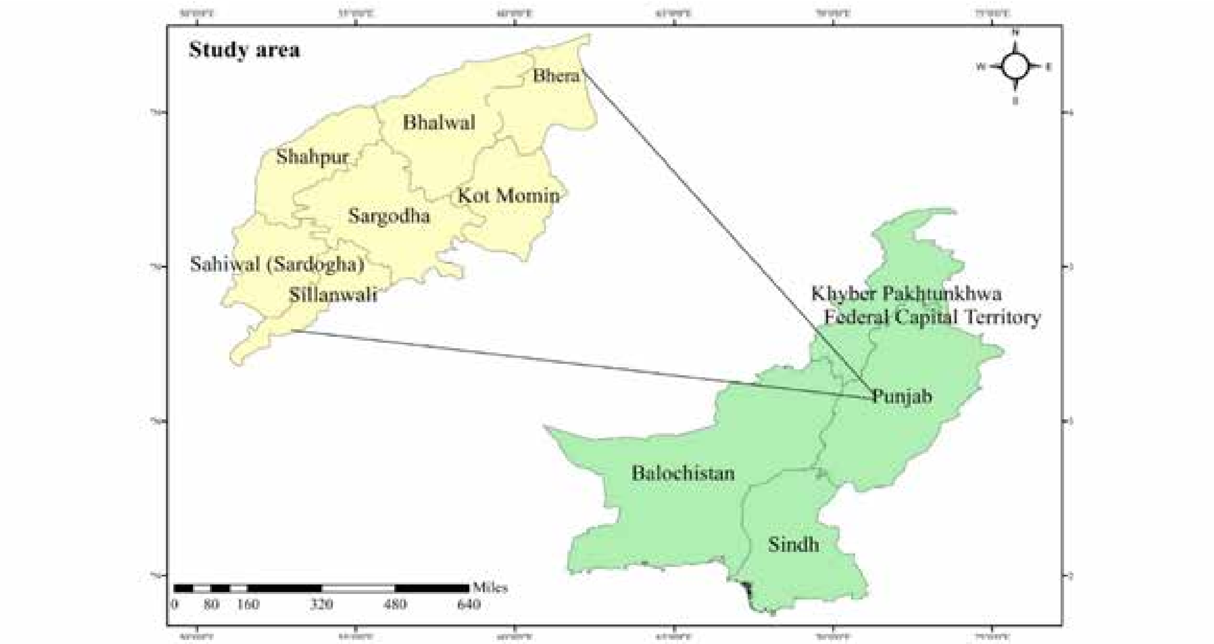
MATERIALS AND METHODS

Samples of 'Kinnow' mandarin (*Citrus reticulata* Blanco) and 'Mosambi' orange (*C. sinensis* (L.) Osbeck) grafted on rough lemon (*C. jambhiri* Lush.) were collected from citrus trees 13-20 yr old in five orchards in the major citrus growing areas of district Sargodha, Punjab Province, Pakistan (Figure 1). Morphological characters of the diseased samples (leaves and fruits) of 'Kinnow' and 'Mosambi' were observed and characterized according to the disease severity rating scale developed by Akhtar and Ahmad (1999). All the morphological and biochemical characterization was done on 200 samples with different disease symptoms and severity levels from trees with available fruits and marked symptoms. Healthy fruits and leaves samples were also compared with diseased samples taken from 200 samples from 10 trees which were PCR assessed by using protocols and primers described by Sajid et al. (2021). The primer pairs used were A2 (F) (TATAAAGGTTGACCTTTCGAGTTT) and J5 (R) (ACAAAAGCAGAAATAGCACGAACAA) described by Hocquellet et al. (1999), which generate an amplicon DNA band of 703 bp. PCR conditions used to confirm the citrus greening disease (CGD) (syn. Huanglongbing, HLB) in infected samples of citrus varieties considered that samples were initial denatured at 94 °C for 2 min for one cycle followed by denaturation at 94 °C for 30 s. Annealing was done at 58 °C for 1 min and extension at 72 °C for 1 min (35 cycles). Final extension was carried out at 72 °C for 10 min (1 cycle). The reaction was held at 4 °C for infinity (Sajid et al., 2021).

Shape of leaves, leaf area, and size of leaves

The visual observations were made on shape and size of 20 infected leaves. Leaf area was measured through leaf area meter (HM-YMJB; Shandong Hengmei Electronic Technology, Shandong, China). Infected leaves with blotchy pattern were collected from PCR-confirmed diseased trees. Leaves with clear symptoms were collected from the branches having infected fruits with prominent symptoms. Twenty infected leaf samples with three replicates were observed.

Figure 1. Map of the study areas where citrus orchards were sampled show location of the sampled citrus orchards.



Fruit weight, fruit diameter, and rind thickness

At the time of harvesting from symptomatic and healthy trees, fruits were collected to assess diameter, weight, rind thickness and biochemical parameters (Hussain and Singh, 2020). Twenty infected fruits from the already PCR-confirmed pooled samples obtained from the citrus orchards in the growing area of Sargodha (Sajid et al., 2021), with three replicates, were collected and observed. Fruit weight, diameter and rind thickness were measured by vernier caliper. The collected infected fruits, along with healthy fruits, were also used to observe fruit shape and color. Number of seeds was also registered.

Biochemical parameters

Different biochemical parameters such as chlorophyll content, starch contents, juice percentage, total soluble solids (°Brix), total sugars, titratable acidity and ascorbic acid were measured from the collected fruits and leaves (for starch contents only). The established protocols were followed as described below. Total chlorophyll content was measured through chlorophyll meter (model CL-01; Hansatech Instruments Ltd., King's Lynn, Norfolk, UK) by using manufacture protocols. Starch content was measured according to protocol described by Thimmaiah (2004). Briefly, 1 g leaves was suspended in 5 mL 80% ethanol and centrifuged at 10000 rpm for 10 min at 4 °C. The collected pellet was again washed with ethanol and centrifuged. The pellet was suspended in 5 mL water followed by addition of 6.5 mL perchloric acid, after drying. The mixture was extracted through shaking and centrifuged at 10000 rpm and 4 °C for 10 min. Again, 6.5 mL perchloric acid were added and centrifuged. The two mixtures were pooled and made volume 1 mL by adding distilled water. Anthrone reagent (4 mL) was added and heated at 80 °C for 8 min. Absorbance was measured at 630 nm using spectrophotometer (U2020; Irmeco, Lütjensee, Germany). To evaluate total soluble solids, 20 fruits were cut into two halves and juice was collected by squeezing with juice squeezer (WF 550, WestPoint, Karachi, Pakistan) in a glass jar and placed the jar at 4 °C until use. One drop of juice was put on the digital refractometer (Palette PR-101α, ATAGO, Tokyo, Japan).

Titratable acidity and juice percentage were calculated according to the materials and method used by Hussain (2014) and Iftikhar et al. (2021) using the following formulae:

$$\text{Titratable acidity (\%)} = (\text{Milliequivalent factor} \times \text{Volume of titrant} \times \text{Volume of NaOH})/(\text{mL juice} \times \text{Volume aliquot}) \times 100$$

where milliequivalent factor for citric acid is 0.0064.

$$\text{Juice (\%)} = (\text{Juice weight per fruit}/\text{Average fruit weight}) \times 100$$

To determine ascorbic acid (vitamin C) in fruit juice, citrus samples of each symptomatic and asymptomatic (healthy) trees were collected to determine ascorbic acid. The ascorbic acid content of juice was determined following the technique depicted by Ruck (1961). Ascorbic acid content (%) was calculated by following formula:

$$\text{Ascorbic acid (\%)} = (D1 \times V1 \times V2/D2 \times W) \times 100$$

where, D1 is volume of 2,6-dichlorophenol indophenol dye used to titrate aliquot (sample reading); V1 is volume of aliquot taken for titration (mL); V2 is volume of the aliquot made by 0.4% oxalic acid (mL); D2 is volume of 2,6-dichlorophenol indophenol dye used to titrate against 2.5 mL (1 mL standard ascorbic acid + 1.5 mL 0.4% oxalic acid) of reference solution (standard reading); and W is volume of the juice used (mL).

Total sugars in juice were determined through the method described by Hortwitz (1960). Briefly, in 250 mL flask, 10 mL each fruit sample were taken and 100 mL distilled water, lead acetate and potassium oxalate were added. Distilled water was used to make volume and then filtered. Different forms of sugars were estimated through this filtrate. Total sugars were calculated by following formula:

$$\text{Total sugars (\%)} = 25 \text{ X } \frac{x}{z}$$

where, x is volume standard sugar against 10 mL Fehling solution; z is volume aliquot (sample) against 10 mL Fehling solution; Fehling solution was prepared based on method of Lane and Eynon in 1923 described by Hortwitz (1960).

Statistical analysis

The data was collected and subjected to factorial ANOVA to compare the response of two varieties using the software Statistix 8.1 (Analytical Software, Tallahassee, Florida, USA). The means were compared through LSD.

RESULTS

All the samples in our study were confirmed though PCR prior to undergo the morphological, physical and biochemical parameters, then obtained results were in concordance with those literatures cited above.

Morphological and physical parameters

Leaves and fruits samples from HLB-infected trees along with healthy trees as control showed the characteristic symptoms of HLB and morpho-physical parameters (Figures 2 and 3). There was significant difference in the morphological parameters of fruit and leaves, especially fruit color and shape of leaves in HLB infected samples of mandarin and orange (Figure 4). Orange had more lopsided fruits (80%) than mandarin (70%) in HLB-infected samples as compared to 20% and 30% in healthy samples, respectively. Similarly, fruit colors were categorized in three categories such as full green and half green for HLB infected-samples and light green to orange for healthy samples. In the HLB-infected samples, 10% of mandarin was full green as compared to orange with the mean value of 30%, while 60% mandarin was half green as compared to orange that was 50% half green, whereas, 30% mandarin and 20% orange were light orange as healthy. The HLB infected leaves showed that orange leaves were smaller and narrower than mandarin, orange showed mean values of 90% and 10%, respectively, as compared to mandarin with 85% small and 15% and 10% narrow leaves (Figure 4). Physical parameters of HLB-infected and healthy samples of both the local citrus varieties were compared (Table 1). Among the physical parameters, fruit diameter was significantly reduced to 35.56 cm in orange as compared to mandarin (46.77 cm) in infected samples as compared to healthy samples of orange and mandarin with the mean values of 67.12 and 72.54 cm, respectively ($p < 0.05$). Although fruit weight of infected mandarin (86.75 g) was higher than of infected orange (78.23 g) but nonsignificant differences were observed between each other. The similar case was in healthy samples of mandarin and orange, 140.38 and 134.91 g, respectively. Rind thickness was significantly less in HLB infected samples of mandarin (5.7 mm) as compared to orange (8.0 mm), whereas rind thickness of healthy mandarin was also significantly less (2.19 mm) as compared to healthy orange (2.95 mm). Similarly, juice weight was significantly low in HLB infected orange (11.26 g) as compared to mandarin (19.04 g). Similar trend was also observed in healthy samples of both varieties. There was nonsignificant difference in the numbers of aborted seeds in HLB infected mandarin and orange fruits (14-85 and 13.28, respectively) and nonsignificant differences were observed for healthy mandarin (3.60) and orange (2.25) samples. Leaf area of infected mandarin with the mean value of 8.52 cm^2 was noticeably lesser than orange (10.9 cm^2) as compared to healthy mandarin and orange with the mean values of 14.04 and 17.48 cm^2 respectively (Table 1).

Figure 2. Morphological parameters (mottling and smalling of leaves, lopsided and unripening of fruits) of huanglongbing infected 'Kinnow' mandarin (A, B, C, D and F), and 'Mosambi' orange (E).

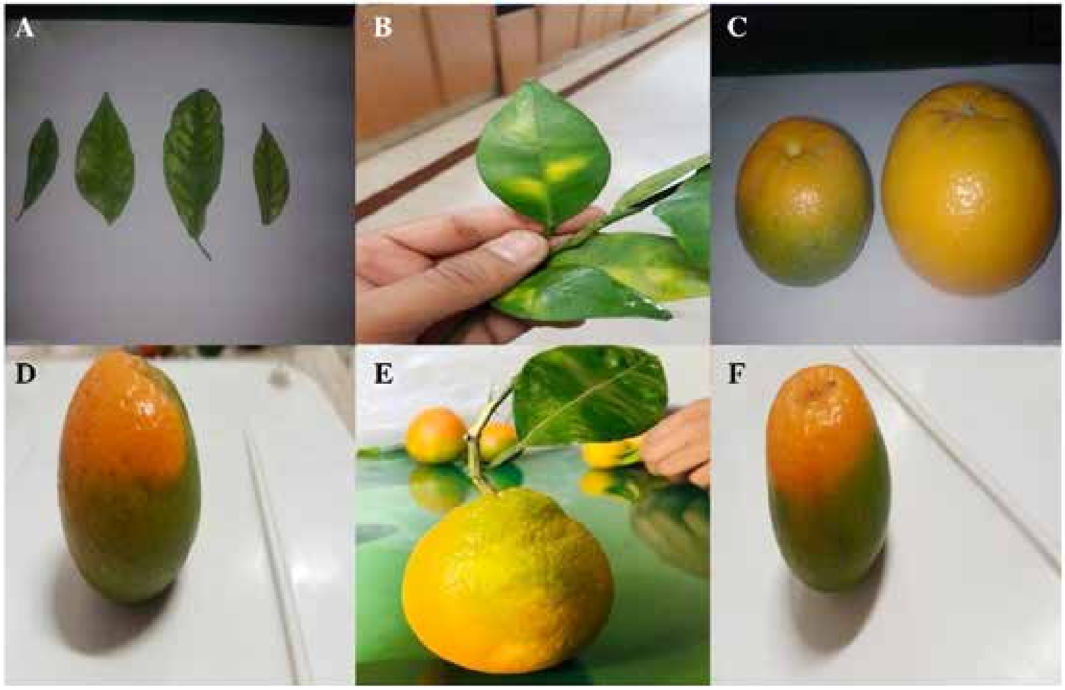


Figure 3. Seed abortion (white arrows) and rind thickness in huanglongbing infected samples.

Figure 4. Fruit shape, fruit color and shape of leaves in citrus 'Kinnow' mandarin and 'Mosambi' orange samples affected by huanglongbing.

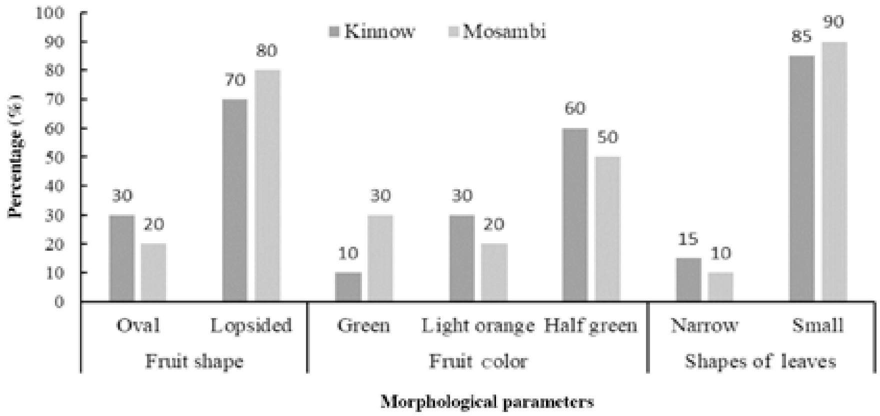


Table 1. Physical parameters of huanglongbing infected samples of two citrus varieties.

	Physical parameters					
Cultivars	FD	FW	RT	JW	NAS	LA
	cm	g	mm	g		cm^2
Kinnow (infected)	46.77c	86.75b	5.68b	19.04c	14.85a	8.52d
Kinnow (healthy)	72.54a	140.38a	2.18d	53.40a	3.60b	14.04b
Mosambi (infected)	35.56d	78.23b	8.00a	11.26d	13.28a	10.97c
Mosambi (healthy)	67.12b	134.91a	2.95c	42.15b	2.25b	17.48a
Variation coefficient	7.14	12.57	15.64	12.47	42.99	7.56
p-value	0.0015	0.6213	< 0.0001	0.0499	0.8954	0.0263

Means sharing same letter in the columns are non-significantly different.
FD: Fruit diameter; FW: fruit weight; RT: rind thickness; JW: juice weight; NAS: number of aborted seeds; LA: leaf area.

Biochemical parameters

Biochemical parameters of HLB infected fruits of two local cultivars also showed the significant difference in infected citrus samples among the varieties (Table 2). Starch accumulation, which was the characteristic feature of HLB in infected samples of both local citrus varieties, was higher than the healthy samples. Starch accumulation in HLB infected leaves samples of orange was significantly higher with the mean value of 6.12 µg mL^{-1} as compared to mandarin (5.54 µg mL^{-1}). Juice percentage of HLB infected mandarin was higher (22.73%) than orange (14.70%) but significantly less than healthy samples (38.22% and 31.30%, respectively). Among the two species acidity percentage of infected mandarin (0.99%) was significantly higher than infected orange (0.90%). Whereas, acidity was significantly lower in healthy samples of mandarin and orange with the mean values of 0.57% and 0.53%, respectively. HLB significantly reduced total sugars (%) in infected mandarin (5.16%) compared to infected orange (4.79%). Similarly, total soluble solids (%) among infected mandarin (8.54%) and orange (8.28%) were not significantly different but significantly higher in healthy mandarin and orange. The difference in total chlorophyll (mg mL^{-1}) in infected mandarin (13.50 mg mL^{-1}) and orange (14.92 mg mL^{-1}) were nonsignificant but total chlorophyll was significantly higher in healthy samples of both cultivars. Ascorbic acid (%) in infected samples of mandarin (34.28%) and orange (34.12%) had nonsignificant difference but this parameter was significantly lower in healthy samples (Table 2).

Table 2. Biochemical parameters of huanglongbing infected samples of two citrus varieties.

	Biochemical parameters						
Cultivars	Starch	Juice	Acidity	TS	TSS	ChI	AA
	µg mL^{-1}		%			mg mL^{-1}	%
Kinnow (infected)	5.54b	22.73c	0.99a	5.16c	8.54b	13.50c	32.50b
Kinnow (healthy)	2.56d	38.22a	0.57c	8.78b	13.06a	27.15b	38.23a
Mosambi (infected)	6.12a	14.70d	0.90b	4.79c	8.28b	14.92c	32.91b
Mosambi (healthy)	3.18c	31.30b	0.53c	9.94a	12.44a	33.12a	38.13a
Variation coefficient	10.08	17.28	13.68	8.47	9.21	15.10	3.59
p-value	0.8312	0.5900	0.2449	< 0.0001	0.4010	0.0031	0.38

Means sharing same letter in the columns are non-significantly different.
TS: Total sugars; TSS: total soluble solids; Chl: total chlorophyll; AA: ascorbic acid.

DISCUSSION

Typical symptoms of HLB were caused by alteration in physiological and biochemical changes on infected citrus plants (Dala-Paula et al., 2019) and these alterations were typical to infected plants. Abnormality in fruits and blotchy mottling of leaves are the typical symptoms of HLB. These symptoms lead to the decline of citrus tree and ultimate death of the plant in the severe cases (Albrecht et al., 2016). It has been observed that symptomatic trees exhibited small leaves, upright growth, chlorosis and blotchy mottling (McCollum and Baldwin, 2017). Aborted seeds were also produced in greening affected fruits (Saifullah et al., 2015). They also observed more aborted seeds in infected samples as compared to healthy samples. Dark and aborted seeds have also been observed in greening affected fruits which have abnormal peel color yellow to orange at peduncular end and green at styler end (Dala-Paula et al., 2019).

Lopsided fruits and mottling of leaves have been discussed by many researchers who attributed these typical symptoms with HLB (Bove, 2006; Bassanezi et al., 2009; Gottwald, 2010; Gottwald et al., 2012). The results of morphological and physical parameters of local cultivars *viz*., mandarin and orange infected with HLB in our study had the same trends and in accordance with those symptoms reported in the literature. Abundant starch in aerial part of citrus plant is one of the major and prominent characteristics of HLB affected tree (Whitaker et al., 2014). This high level of starch in leaves was associated with HLB symptoms (Takushi et al., 2007; Etxeberria et al., 2007). Our study concluded the high starch contents were observed in leaves infected with HLB. Excessive starch in leaves depicted the abnormality in regulation of glucose-phosphate transport (Martinelli and Dandekar, 2017). This unbalance also affected the sugar transport (Etxeberria et al., 2007; Zheng et al., 2018). The phloem transport of HLB infected plants is inhibited by phloem plugging and alter the starch level in leaves and resulting is extraordinary starch accumulation (Etxeberria et al., 2007; Tian et al., 2014). We observed the alteration in physical and biochemical parameters like; chlorosis, more aborted seeds, small leaves and unripening of fruits in HLB infected samples of mandarin and orange. Moreover, there was starch accumulation in HLB infected leaves. Other biochemical parameters like, more acidity, less soluble solids and fewer total sugars were observed in infected samples as compared to healthy ones in both cultivars, mandarin and orange. Similarly, fruit weight and juice weight and percentage were badly affected by HLB that makes the fruit and fruit juice unmarketable (Mattos-Jr et al., 2020). The fruit had bitter taste because of high acidity (Baldwin et al., 2010; 2018; Dala-Paula et al., 2019). In this research level of acidity increased to 0.90%-0.99% between fruits collected from HLB infected and healthy plants, which showed the big problem with commercialization of mandarins and sweet oranges in Pakistan if disease is more recurrent. The juice had high acidity and less juice weight in infected fruit samples as compared to healthy in our study. Therefore, results of juice quality in our study were in accordance with McCollum and Baldwin (2017) where similar reduction in juice and fruit weight were observed.

Our results regarding the physical and biochemical parameters were in concordance with the studies of Bassanezi et al. (2009), Baldwin et al. (2010) and Shokrollah et al. (2011). Physical and biochemical parameters of HLB affected citrus plants were also observed by Shokrollah et al. (2011) during the management of greening disease in Malaysia. They found low titratable acidity, high peel thickness, low fruit weight, and low juice percentage in HLB-infected fruit which were similar to our results. Physiology and fruit juice quality of HLB affected samples were also determined by Bassanezi et al. (2009) and Baldwin et al. (2010). They also observed the significant change in sugar, acidity, juice percentage, fruit diameter, fruit weight and °Brix in HLB infected fruits. They found that HLB affected fruit is less in diameter and weight, had high acidity percentage and low sugars similar to our results. They further observed that low juice percentage in greening samples. The greening disease not only alter the physiology but also the symptomatic response of citrus plants towards HLB, but this disease also favors citrus fruits become unmarketable due to this disease. It is concluded that HLB have a vital role in altering the physiology of plants and this could be overcome by application of nutrients (Dala-Paula et al., 2019). The understanding of biochemistry of HLB affected citrus plants suggest to explore of phenomenon of masking of symptoms in different environmental conditions.

CONCLUSIONS

Among the citrus species, the two local varieties 'Kinnow' mandarin and 'Mosambi' orange have been ranked highly nutritional and tasteful not only in Pakistan but also in Asian region. These two citrus varieties are widely cultivated in the region and these have been found very responsive against the different infectious diseases of citrus. Therefore, these two local cultivars were targeted to characterize morpho-physical and biochemically against the huanglongbing (HLB) which is a threatening element in the citrus industry in Pakistan. This disease deteriorates the quality and quantity of citrus fruit and by-products. Among the two local varieties, infected mandarin showed the more marked symptoms of HLB among the physical parameters, fruit diameter, fruit weight, juice weight, and leaf area were significantly lowered in HLB infected citrus samples of both varieties. Whereas, number of aborted seeds and rind thickness were significantly higher in HLB infected citrus samples as compared to healthy. Similarly, more starch was found in the HLB infected samples of both local citrus varieties. Juice percentage, acidity, total sugars and total soluble solids, chlorophyll contents, and ascorbic acid were significantly lower in HLB infected samples. Compared to infected orange, infected mandarin had higher values of physical parameters except rind thickness and leaf area, and also significantly higher values of all the studied biochemical parameters except starch accumulation.

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