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# CITRUS PLANT NUTRITIONAL PROFILE IN RELATION TO *HUANGLONGBING* PREVALENCE IN PAKISTAN

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Citrus is an important fruit crop in Pakistan that requires proper crop nutrition and disease management strategies as it is a tree crop and withstands harsh seasonal conditions for decades. Huanglongbing (HLB) is a century old, devastating disease of citrus caused by phloem limiting bacteria of the α-proteobacteria subdivision. As disease has no known cure, so, effective prevention methods are useful in crop management. Improper crop nutrition impairs plant genetic resistance to invasive pathogens, decreases yield and reduces productive life of the plant. In this study we selected 116 citrus trees from 43 orchards of Punjab for a nutritional assessment. All the trees were showing HLB symptoms and were subjected to NPK and Zn analysis as well as molecular detection of *Candidatus* L. asiaticus, the pathogen associated with HLB. Nitrogen and Zn were significantly higher ($P \leq 0.05$) in HLB infected trees. Out of 48 diseased trees, 19, 43 and 27 were deficient in nitrogen, phosphorous and potash, respectively. Our study concludes that there is no relationship between nutritional deficiency status and HLB incidence in citrus; however, nutritional treatments may help in stress relief to infected plants.
**Keywords:** Citrus, huanglongbing, crop nutrition, disease management.

## INTRODUCTION

Citrus is the most important fruit crop in Pakistan both in the area under cultivation (199.9 thousand hectares) and fruit production (2.132 MMT) (Anonymous, 2010). Crop nutrition and disease management are of vital importance for plant health and orchard viability in terms of yield and quality. Plant nutrient and pathogen interactions have been complex to understand and it is likely that nutrient deficiencies in crop plants increase susceptibility to a variety of diseases. The disease severity can be reduced by enhancing chemical, biological and genetic control of many plant pathogens through proper nutrition (Huber and Haneklaus, 2007). Number of factors including soil type, soil nutritional status, type of rootstock and disease status affect nutrient uptake in fruit crops. Phosphorus (P) availability is known to be intrinsically limited in tropical soils of Brazil impairing citrus production (Quaggio *et al.,* 1998). Sufficient P supply is necessary for both bearing and non-bearing citrus trees for better growth and fruit production irrespective of the rootstock (Mattos *et al.,* 2006). *Xylella fastidiosa* infected citrus plants have been reported to show nutritional deficiencies due to probable extracellular polymer production by the bacteria, obstructing normal flow of nutrients in the xylem tissues. Leaf nutritional analysis confirmed P and K deficiency in *X. fastidiosa* infected citrus trees, whereas, Fe, Mn and Zn contents were found to be higher in chlorotic areas (Stenico *et al.,* 2009). Different studies have shown the potential of some mineral nutrients to induce activation of specific plant defense mechanisms under disease stress conditions resulting in beneficial systemic acquired resistance (SAR) against pathogen attacks (Reuveni and Reuveni, 1998). Ferric chloride and di-potassium hydrogen phosphate are reported to induce resistance in rice against blast disease (Manandhar *et al.,* 1998). Phosphoric acid salts effectively controlled *Phytophtora spp.* infections in different plant species (Guest and Grant, 1991). In response to gummosis disease, metal-phosphite and K-Phosphite were found to be inefficient (Tuset *et al.,* 2003); however, *Eurofit* mineral complex conferred significant disease control (Ferrari, 2001; Garcia-Mina, 2006).

Leaf N contents were reported to be 2.18, 2.31, 2.43 and 2.44 percent in citrus samples from Sargodha, Sillanwali, Bhalwal and Kotmomen tehsils respectively. Phosphorus contents were found to be 0.018% in Sillanwali and 0.16% in the other 3 three areas, whereas, K contents were recorded as 0.693, 0.607, 0.865 and 0.819 % respectively in the three sampling areas (Khan *et al.,* 2011). In Sahiwal district of Pakistan, citrus leaf analysis showed N contents ranging from 1.3 to 2.8 percent indicating that leaf nitrogen content was deficient in 40% of the samples and low in 24% of the samples; 32% of the plants possessed adequate nitrogen and only 4% plants showed high N levels. Similarly, leaf P contents varied from 0.19 to 0.35% indicating sufficient quantity of P in 68% plants and high P content in 32 % plant samples. K contents ranged from 0.78 to 1.71% of dry matter, indicating that 60% trees were with low K and 40% samples contained sufficient leaf K contents. Zn contents were found to be deficient in 28%, low in 68% and satisfactory in only 4% of the citrus leaf samples (Ranjha, 2002). The potash concentration was found to be 1.15 and

1.07%; 1.20 and 1.15% in Rhode Red Valencia and Valencia Late respectively during two years study, however, the Zn contents were found to be lower than recommended levels (Toplu *et al.,* 2008). Nutritional status of Kinnow mandarin on nine exotic and local rootstocks revealed a significant difference regarding leaf NPK contents on different rootstocks. Leaf N contents were found to be maximum (2.60 and 2.67%) on Rough lemon and minimum (2.20 and 2.21%) with Troyer citrange rootstock during two different years of study; whereas, P contents ranged from 0.09%to 0.16% and K concentration was recorded from 1.15 to 1.65% (Ahmad *et al.,* 2007).

In avocado, N, K, Na, and Cu increased in leaves of *Phytophthora* infected plants whereas, P, Mg, Mn, and Fe concentrations decreased as compared to the healthy controls. However, Ca, Cl, and Zn concentration in leaves was not influenced by the *P. cinnamomi* infestation in roots suggesting fungal presence in the soil system to increase N, P, K, Ca, Mg, Na, Cl, Cu and Fe concentrations in plant stems than in comparison to the root system of healthy controls. Further, the total individual nutrient uptake in the plants was lower in infected trees as compared to controls (Labanauskas *et al.,* 1977).

Many of the citrus growers disagree to remove HLB infected trees, leading to investigations about tree nutrition for sustainable production (Timothy *et al*, 2010; Timothy *et al.*, 2011). Researchers are trying nutrition as an alternative to tree removal as a part of HLB management program with sustainable productivity of infected trees (Hall and Gottwald, 2011). The roots of HLB infected plants could not transport minerals efficiently (Pustika *et al*., 2008). In addition to phloem degeneration, re-translocation of elements is thought to be affected by the toxin or siderophore produced by '*Ca*. L. asiaticus' that chelates Fe and Zn (Beattie *et al.,* 2010). Potash conc. increased and calcium and magnesium decreased (Koen and Langenegger, 1970), whereas, Ca, Mn and Zn concentrations were lower in HLB infected plants (Aubert, 1979). PCR based HLB infected trees showed 12%, 21% and 42% lower foliar concentrations of N, Mg and Fe respectively than in PCR-negative trees, whereas, K, P and Zn concentrations did not show any difference between both of the groups. Foliar fertilizer treatments affected symptom severity as infected plants showed severity score of 36%, about 40% less than the trees fertilized through soil. But similar results could not be confirmed on different soil types (Beattie *et al*., 2010).

Citrus greening or Huanglingbing is the most devastating citrus disease with no known control. Early disease symptoms have been similar to Zn, Mn or Fe deficiency making the disease identification more complicated. Further, Pakistani soils have been deficient in a number of plant nutrients limiting plant health, yield and produce quality. This study provides nutritional profile of selected nutrient elements of citrus plantations in relation to Huanglongbing disease in central parts of Pakistani Punjab.

## MATERIALS AND METHODS

Plants with persisting HLB symptoms were sampled from different citrus growing areas. From 43 orchards in 9 tehsils (district administrative subdivision) of 4 districts; 116 leaf samples were collected for molecular detection of HLB and plant nutritional studies. Leaf samples were analyzed for N, P, K and Zn contents.

***Leaf Sampling*:** Five to six months old leaves were preferred for sampling. From each tree 80 to 100 leaves with no apparent insect or any other physical damage were collected, packed in polythene zip lock plastic bags, labeled and carried to the laboratory. The leaves were washed carefully with detergent and distilled water to remove dust and contaminants, first air-dried in the shade for a couple of days followed by oven drying, ground to fine powder form and stored in air-tight plastic bottles at room temperature before digestion for nutrient analysis.

***Total Nitrogen*:** Method described previously (Chapman and Parker, 1961) was used for total nitrogen estimation which involves digestion of plant material with concentrated sulfuric acid and the digestion mixture comprised of potassium sulfate, copper sulfate and ferrous sulfate in a ratio of 10:0.5:10. Briefly, one gram of oven dried leaf material was transferred to Kjeldhal's digestion flask along with 30 ml of concentrated sulfuric acid and 5 gm digestion mixture. The mix was first heated slowly and then vigorously till a transparent green liquid material resulted. On cooling, the contents were transferred to a 100 ml volumetric flask to make volume up to the mark by adding distilled water. From the aliquot, 10 ml was taken and distilled in micro kjeldhal's apparatus using 40% sodium hydroxide, 4% boric acid and mixed indicator of bromocresol green and methyl red.

The distillate was titrated against N/10 sulfuric acid till the original color of methyl red restored. From the quantity of acid used in the titration; the percent element nitrogen was calculated by using the following formula.

$$N (\%) = \frac{A - B \times 100 \times 100 \times 0.014}{\text{Volume of digested samples used (ml)}}$$

Where;

A = Quantity of N/10 sulfuric acid used

B = Blank reading

Blank reading was taken for estimating the actual percentage of nitrogen in the sample. All the procedures of digestion, distillation and titration were the same for the blank sample.

***Wet Digestion for Elements Other Than Nitrogen*:** The wet digestion method for estimating elements other than nitrogen was described previously (Yoshida *et al.,* 1976) and used for determining P, K and Zn contents of the plant samples. Briefly, one gram of oven dried leaf material was transferred to a 100 ml beaker, 10 ml of triacid mixture ($HNO_3$, $HClO_4$ and $H_2SO_4$ in ratio of 5:2:1) was added. Then the beaker was covered with watch glass and allowed to stand still for about four hours till all the initial reaction subsided. It was then heated gently until the solid material disappeared, followed by a vigorous heating till a clear colorless solution resulted. When the volume was reduced to 1.5 ml, it was removed from hot plate and cooled. Then distilled water was added to make volume to 100 ml in a volumetric flask. The filtrate was stored in clean plastic bottles and was used for P, K and Zn estimation.

***Phosphorus Estimation*:** Chapman and Parker (1961) described phosphorus estimation method using spectrophotometer. Color of the samples was developed by adding following reagents in a 100 ml volumetric.

i. 5 mL of digested leaf material
ii. 5 mL of $H_2SO_4$ and water (1:6)
iii. 5 mL of Ammonium molbydate (5%)
iv. 5 mL of ammonium vandate (0.25%)
v. Distilled water to make volume up to the mark

Standard curve was obtained by using different known concentrations of potassium di-hydrogen phosphate. Then the samples were analyzed using a spectrophotometer at a wavelength of 470 nm and the color intensity was noted and compared to that of standard curve to find out the P contents (ppm) which was then converted into percentage by using the following formula.

$$P\ (\%) = \frac{\text{ppm on graph x dilution}}{10^6} \text{ x } 100$$

***Potassium Estimation*:** Potassium was also determined by Chapman and Parker (1961) method using flame photometer. Standard curve was prepared by using different known concentrations of Potassium chloride (KCl). Standard solutions and the quantity of element were found in ppm by comparing the emission of flame photometer with that of standard curve, which was then converted to percentage by using the formula:

$$K\ (\%) = \frac{\text{ppm on graph x dilution}}{10^6} \text{ x } 100$$

***Zinc Estimation*:** Concentration of Zinc (Zn) was determined in citrus leaf samples after wet digestion. Analytical studies were performed with Atomic Absorption Spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200 Japan) following the conditions described in AOAC (1990). The instrument was operated at 213.9 nm wavelength with a slit width of 1.3nm at 10mA lamp current with standard type burner head. Flame was produced using Air + $C_2H_2$ at 7.5 mm burner height with 160 kpa and 6 kpa oxidant gas and fuel gas pressure, respectively.

***Standards Preparation*:** Calibrated standards were prepared by using the commercially available stock solution (Applichem®, Germany) in the form of an aqueous solution at 500 ppm. Working standards were prepared by dilution with highly purified de-ionized water. All the glassware used during analytical work, was immersed in 8N $HNO_3$ overnight followed by several washings with de-ionized water prior to use. The required calibrated standards were prepared by the following formula:

C1V1 = C2 V2

Where:

C1= Concentration of stock solution
V1= Volume of stock solution
C2= Concentration of standard solution
V2= Volume of standard solution

## RESULTS AND DISCUSSION

Leaf DNA extracts was tested for ‘*Candidatus* Liberibacter asiaticus’ detection through quantitative real-time PCR (qPCR). Out of 116 samples analyzed, 48 (41.4%) showed presence of *Ca.* L. asiaticus (Data not shown). Nutritional status of plant samples used for HLB associated pathogen detection was also studied by estimating N P K and Zn concentrations in symptomatic leaves. The data was clustered manually into nutritionally deficient and not deficient (satisfactory) groups for individual nutritional elements using Florida leaf nutritional standards for citrus (http://edis.ifas.ufl.edu/ss442). It was estimated that out of 116 plants studied, 83 (71.55%), 99 (85.34%) and 70 (60.34%) plants were deficient in N, P and K, respectively (Table 1) whereas, none of the samples studied was found to be Zn deficient. Regarding analysis on the basis of qPCR results, 19 out of 48 HLB infected trees were deficient in leaf nitrogen, whereas, 29 showed satisfactory nitrogen levels. Similarly, 43 of 48 plants infected with HLB were also deficient in leaf P contents, whereas 5 HLB infected trees did not show any P deficiency. Further, among 48 infected trees, 27 were deficient in leaf K contents and the rest of 21 showed optimum level of leaf K contents. None of 116 samples analyzed including 48 HLB positive trees showed any Zn deficiency (Table 2).

**Table 1. Overall NPK and Zn status in plants tested for HLB**

| Element | Status | No. of Plants | % |
|---|---|---|---|
| **N** | Deficient | 83 | 71.55 |
| | Satisfactory | 33 | 28.24 |
| **P** | Deficient | 99 | 85.34 |
| | Satisfactory | 17 | 14.65 |
| **K** | Deficient | 70 | 60.34 |
| | Satisfactory | 46 | 39.65 |
| **Zn** | Deficient | Nil | Nil |
| | Satisfactory | 116 | 100 |

**Table 2. Nutritional status of plants tested for HLB**

| Nutrient | Deficient | | Not deficient | | Total |
|---|---|---|---|---|---|
| | HLB Positive | HLB Negative | HLB Positive | HLB Negative | |
| **N** | 29 | 54 | 19 | 14 | 116 |
| **P** | 43 | 56 | 5 | 12 | 116 |
| **K** | 27 | 43 | 21 | 25 | 116 |
| **Zn** | NIL | NIL | 48 | 68 | 116 |

***General Leaf Nutritional Status in different areas*****:** Data was grouped in to their respective areas for mean N P K and Zn estimation with respect to sampling areas and was statistically analyzed using Welch Test as it is best suited for data comprising of unequal group sizes. Significant difference was noted in individual nutrient elements with respect to different areas. Leaf N contents varied significantly from one to other area (Fig. 1A) with the highest (2.333%) and the lowest (1.283%) values in Sillanwali and Toba Tek Singh regions respectively. Similarly mean leaf P contents varied significantly among different areas at P= 0.05, where the highest (0.0946%) and the lowest (0.0607%) P contents were recorded in Kotmomen and Lalian areas, respectively (Fig. 1C). With respect to leaf K concentration, none of the areas studied showed any significant difference. The maximum (0.7788%) and minimum (0.586%) K contents were noted in Kot Momen and Bhalwal regions respectively (Fig. 1B). The highest (49.15 ppm) Zn concentration in Sargodha area showed significant difference from all of the other areas including Shamasabad where the lowest (30.66 ppm) Zn concentration was recorded (Fig. 1D). However, in none of the areas studied, leaf zinc was found deficient as per Florida citrus nutrition standards.

***HLB and Plant Nutrients Interaction*****:** Nutrient data from qPCR tested HLB positive and negative groups and respective N, P, K and Zn values were subjected to statistical analysis using Welch Test as mentioned earlier. Regarding P and K, there was no significant difference between both of the groups, however, leaf N and Zn contents showed significant statistical difference between both of the groups (Fig. 2). It is interesting to note that 78.18% of infected plants were deficient in P contents but there was no statistical difference between the groups with respect to leaf P concentration as out of total 68 HLB free plants 56 were deficient in phosphorous only, indicating no relation with P deficiency and disease incidence.

More than 50% samples showed multi-nutritional deficiency, whereas, Zn level in leaves were at satisfactory levels in all the tested plants. By large, nitrogen and phosphorus leaf contents were deficient in all of the areas covered in this study, which may be due to lower fertilizer inputs. There are number of factors affecting nutrients uptake from the soil, such as soil pH, plant disease status and

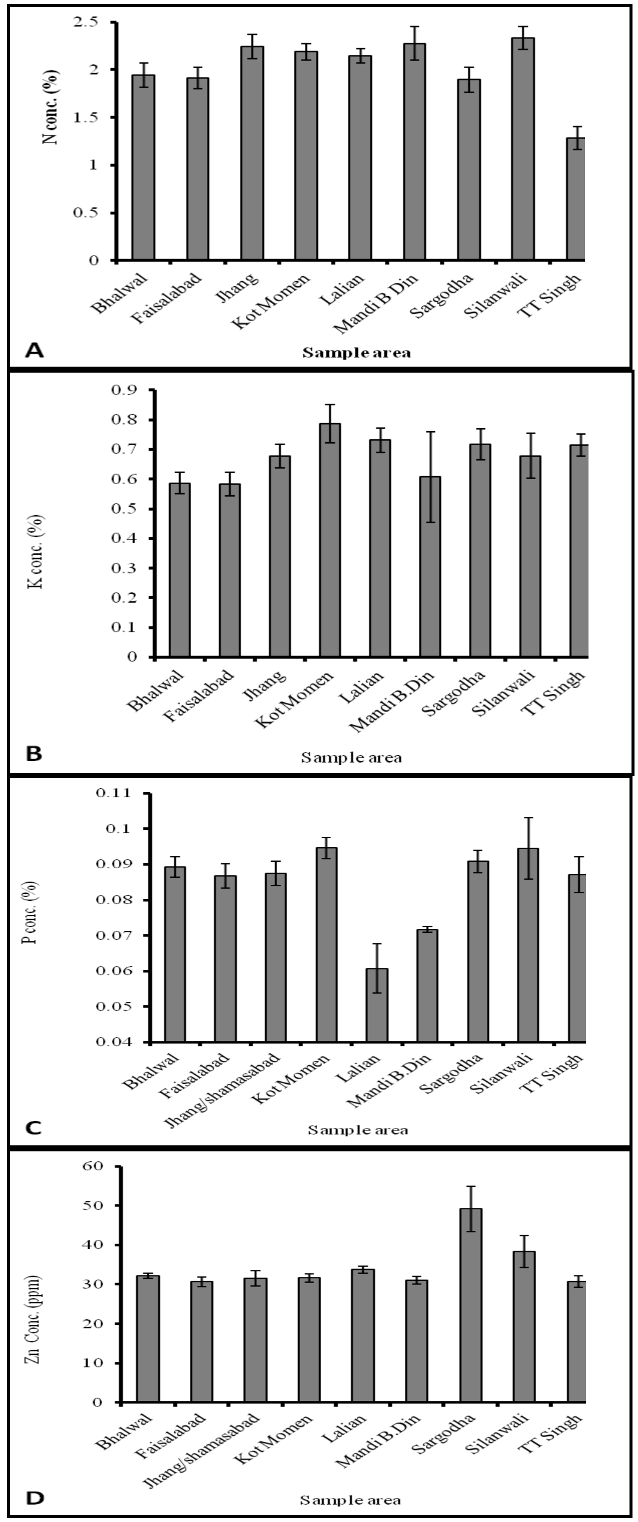


**Figure 1. Mean Nitrogen (%±SE) (A), Potash (%±SE) (B), Phosphorus (%±SE) (C) and Zinc (ppm±SE) (D) concentration in different areas studied for HLB detection**

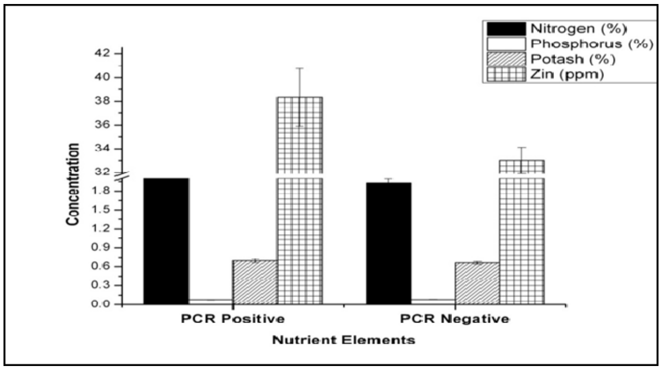


**Figure 2. Mean leaf N (%±SE), P (%±SE), K (%±SE) and Zn (ppm±SE) concentration of qPCR tested HLB positive and negative citrus plant groups**

presence of nematodes. In most of the citrus soils in Pakistan, pH ranges from 8.00 to 8.50, which may reduce Phosphorus uptake. Soil pH is reported to limit P uptake in Brazil, reducing citrus production (Quaggio *et al.,* 1998), However, high prices of Phosphorus fertilizers may lead to low inputs at farm level. Certain diseases such as *Xylella fastidiosa* are reported to decrease phosphorus and potassium uptake, inducing deficiency of these nutrients in leaves (Stenico *et al.,* 2009), whereas, effects of HLB have not been studied so far. However, in Avocado fruits; N, K, Na, and Cu are reported to increase in leaves of *Phytophthora* infected plants whereas, P, Mg, Mn, and Fe concentrations are decreased (Labanauskas *et al.,* 1977). *Phytophthora* has been a disease of crucial importance in Pakistan and is thought to be present in almost every orchard. Its coexistence with HLB or coexistence of HLB with other diseases is not well studied. As, surprisingly higher nitrogen and Zinc contents were detected in HLB infected trees, it may be due to HLB induced physiological changes in plants or coexistence of *Phytophthora* and HLB, although care was taken to avoid sampling from any tree showing *Phytophthora* symptoms. Another reason for higher N and Zn concentrations may be that, most of the farmers try to recover their declined trees by adding extra doses of fertilizers, which may build up these nutrients in leaves of HLB infected trees.

Many Florida citrus growers use various foliar nutritional products such as micronutrients, to maintain health and productivity of HLB infected trees (Timothy *et al*, 2010). Substantial evidence is available about the positive effects of improved and balanced mineral nutrition in annual crops and foliar fungal and bacterial diseases (Timothy *et al*., 2010). However, it will be less conclusive to assume the same for systemic vascular diseases, like HLB. The beneficial effects of nutrition do not extend to situations of unnecessary and copious fertilization, which can in fact increase disease severity. Prolonging the life of infected trees through improved nutrition seems to increase the probability of transmission of the pathogen by *D. citri* and might not be advisable to small farmers in the absence of efficient area-wide management approach for the disease (Beattie et al., 2010) and vector. This study suggests to improve orchard nutritional status and to consider steps to improve overall tree health and minimize tree stress. As more than half of the trees have been deficient in two or more macronutrients, both soil and foliar nutrients application should be emphasized including micronutrients, even in the absence of deficiency symptoms.

Further research in this respect, may answer the questions of significant importance. Role of nutrient deficiency in increased disease susceptibility in plants have been discussed by several researchers (Evans *et al*., 2006; Datnoff *et al*., 2006; Graham and Webb, 1991; Huber and Graham, 1999). Our findings suggest that nutritional deficiency is not likely to affect disease incidence and susceptibility; however, it misleads while sampling for *Ca*. L. asiaticus detection. Further, balanced and improved plant nutrition might have some role in survival of HLB infected plants with sustained production for some period.

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