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QUICK INDEXING OF HUANGLONGBING ON THE BASIS OF SYMPTOMOLOGY AND IODO-STARCH TEST IN RELATION TO ENVIRONMENTAL FACTORS

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Huanglongbing (HLB) is one of the devastating diseases in major citrus growing areas of the world. The disease has posed a threat for citrus industry with a considerable loss in production. A survey was carried out for monitoring of HLB in citrus growing areas of Sargodha district. Symptomology and iodo-starch test were two parameters for quick indexing of disease in the field. The disease incidence was in the range of 8.2 to 11.4%. Characteristic symptoms such as mottling and Zn-deficiency like symptoms were observed in the leaves suspected to be infected of citrus samples. Iodo-starch test was found to be reliable for starch accumulation in HLB infected samples in the field. The correlation of maximum, minimum temperature and relative humidity with symptomatic expression was found significant while the rainfall had negative impact on progression of disease.
Keywords: HLB, citrus diseases, citrus psylla, biochemical test, symptomatic expression.

INTRODUCTION

Huanglongbing (HLB), also known as citrus greening disease (CGD) is a century old disease in citrus plantations which greatly not only reduces quality of citrus fruits but also fruit bearing age of citrus trees. It is also known with other names in different regions of the world such as "yellow dragon disease" in China, "dieback" in India, "mottle leaf" in Philippines, "vein phloem degeneration" in Indonesia and "greening" in Africa. The term "Huanglongbing" was first time used by Lin and was adopted as official name of this disease in 1995 during the 13th conference of International organization of citrus virologist (IOCV).

HLB has been reported in major citrus growing area of the world and destroyed about 60 million of citrus trees in Asia and Africa (Timmer *et al*., 2003; Bove, 2006; Batool *et al*., 2007). The causal agent of this disease is non-culturable, phloem limited bacterium which was characterized for the first time in 1994 on the basis of rDNA sequence of its genome and was suggested that it belonged to a genus of α-proteobacteria subdivision. The citrus greening bacterium is 2µm long and 0.2µm in diameter. Three main strains of the pathogen i.e., *Candidatus* liberibacter asiaticus (Las), *Ca*. liberibacter africanus (Laf) and *Ca*. liberibacter americanus (Lam) have been identified from the HLB infected citrus trees from all over the world on the basis of 16s r DNA sequence. Among them *Las* is the most destructive and prevalent species in the infected trees (Jagoueix *et al*., 1994; Bove, 2006; Teixeira *et al*., 2005). This bacterium was defined and named by Murray and Schlifer (1994) as *'Candidatus'* (a common generic name for all non-culturable bacteria) 'Liberibacter'. The pathogen is transmitted by insect vector "psyllid" and grafting in nature. African citrus Psylla transmits the only *Laf* while Asian citrus psyllid (*Diaphorina citri*) can transmit both *Las* and *Lam*.

The incidence of this disease along with prevalence of insect vector (*D. citri*) has already been confirmed in Pakistan (Catara *et al*., 1991). The incidence in Punjab was reported from 16 to 62% (Akhtar and Ahmed, 1999). The molecular evidence of this disease has also been confirmed through PCR in Pakistan (Chohan *et al*., 2007). The detection plays an important role in formulating disease management strategies. Preliminary diagnosis of citrus greening disease is based on visual symptoms expressed under natural conditions. Characteristic symptoms of HLB have been described by many scientists (Graca 1991; Bove, 2006; Batool 2007). The detection of Pathogen is difficult because of its very low concentration in host plant parts (McClean, 1970; Huang 1979). Hung *et al*. (1999) reported the trouble shooting in detection of this disease through Polymerase chain reaction (PCR), Enzyme linked immunosorbant assay (ELISA) and electron microscopy (EM). The disease sometimes also confuses with the symptoms of nutritional deficiency which is another one of the major problems in citrus orchards (Khan *et al*., 2012). Although nutritional deficiency has no correlation with HLB disease incidence but availability of nutrition in proper way helps citrus plant to face the infection stress (Razi *et al*., 2011). Conventionally HLB is characterized mainly on this basis of symptoms expression prior to molecular techniques. Therefore, this research was

carried out with an objective for the quick indexing of HLB disease. For this purpose an iodine starch test was used that plays an important role in indexing procedure on the principal of accumulation of starch. This method will not only be helpful in quick identification of HLB infected plants in field but also in formulation of management strategies. The correlation of environmental factors with disease severity was also analyzed which would be helpful in developing a prediction model for this disease.

MATERIALS AND METHODS

***Monitoring of HLB*:** An extensive survey was carried out to monitor the HLB in all six tehsils of in Sargodha district; Sargodha, Bhalwal, Shahpur, Sahiwal, Sillanwali and Kot Momin. Five hundred plants were observed in each tehsil. Samples from the trees suspected to be infected were collected on the basis of symptoms. Each sample consisted of affected fruits, leaves and twigs was carefully packed in sterilized polythene bags and quickly transferred to ice bucket right after detaching from the tree. Prior demarcations for collection of samples the selected trees were sprayed by $ZnSO_4$ to avoid the chances of Zn deficiency. The disease incidence was calculated as follows:

***Characterization of symptoms expression*:** Symptoms on fruits, twigs and leaves (Batool *et al*., (2007) were recorded for sample collection. Symptoms expression on different plant parts were used as scale to record disease severity. The disease rating scale developed by Akhtar and Ahmad (1999) with slight modifications was used to record the disease severity as follows.

0 = No symptom
1 = Blotchy mottle on leaves (Up to 25% leaves with symptoms)
2 = Lopsided fruit and color inversion (Up to 26-50% canopy with symptoms)
3 = Partially declined tree (51-75% damage)
4 = Severely declined tree (>75%)

***Iodo-starch test*:** Followings are the key-points to perform Iodo-starch test (Etxeberria *et al*., 2007).

1. Symptomatic leaves were selected.
2. Samples were selected from healthy branches of suspected trees to avoid the production of misleading results due to damaged branches.
3. Leaves were collected from outer canopy.

***Iodine solution*:** Iodine solution (2%), without sodium or potassium iodide, was prepared and diluted to 10 times to avoid the false result due to strong reaction of pure iodine solution. The solution was kept in dark tinted bottle.

***Procedure*:** Cleaned razor small thin sections of the leaf blades on either sides of midrib were made. Each section contained blotchy mottled and normal green portions. These cut sections of the leaves were immersed in the diluted tincture of iodine solution for two minutes followed by rinsing with distilled water for removal of excess dye. The stained sections were observed with the help of magnifying glass.

***Statistical analysis*:** The data of disease severity was correlated to environmental factor using Pearson's correlation through R software for the statistical analysis.

RESULTS

***Symptomology*:** Symptoms expression was used as a basic criterion for the initial diagnosis of HLB. All the plant parts were observed for the symptoms. Infected trees showed the leaf dropping and stunting along with characteristic symptoms such as Mottling, Zn deficiency like symptoms. Fruits were lopsided and colour inversion was also observed. Inside layer of infected fruits were thicker than normal. Aborted seeds were observed when the fruit cut into two halves. Fruits were poorly developed and fail to attain the normal orange colour (Fig.1).

***Iodo Starch Test: Distribution of starch was observed with the help of Iodo-starch test*:** Symptomatic leaves from healthy branches were detached and treated with iodine solution as described earlier in materials and methods. Iodo-starch test showed that HLB positive leaves stained very intense dark grey to black throughout the entire cut surface. Healthy leaves exhibited only some portion of the edges stained which were much less stained as compared to HLB positive leaves samples (Fig. 2). This test provided the quick indexing for the citrus greening disease in the field for further characterization.

Disease incidence of HLB in district Sargodha: The disease incidence in all six tehsils of Sargodha was in the range from 8.2% to 11.4% with the mean percent incidence of 9.8% (Fig. 3). The pie chart showed that the highest disease incidence of 11.40% was in Sargodha tehsil followed by Silanwali (10.6%) and Bhalwal (10%). The least incidence was recorded in Sahiwal (8.2%).

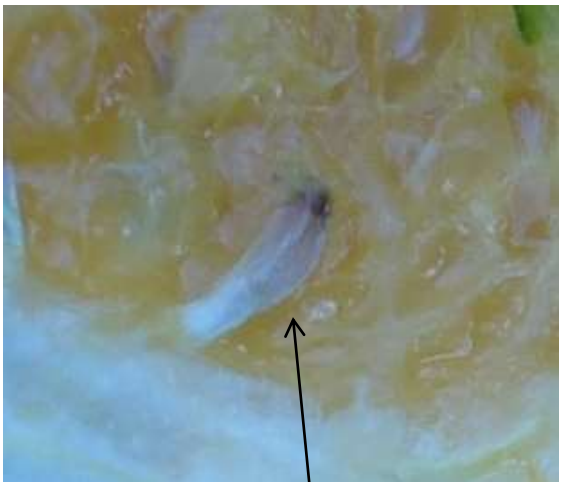
***Disease severity in relation to Environmental factor*:** Correlation of disease severity to different environmental factors (Rain fall, maximum temperature, minimum temperature and relative humidity) has been presented in Table 1. Overall maximum and minimum temperature was significantly correlated with disease severity. There was non-significant correlation between rainfall, relative humidity and disease severity.

***Progression of disease in relation to environmental factors*:** Progression of disease was regarded as disease severity and observed on the basis of symptomatic expression on monthly basis. A disease incidence change over a short period of time in case of citrus greening is very much slow and least notable

Mottling/Zn deficiency like

Colour inversion

Lopsided fruit with aborted



Aborted seed

Figure 1. Characteristic symptoms expression of citrus samples infected with HLB.

because of perennial nature of citrus host trees and very irregular and uneven distribution of greening bacterium inside infected host. Therefore disease severity was measured according to disease rating scale formulated by Akhtar and Ahamd (1999) with slight modifications and correlated to environmental factors. Figure 4 showed the disease severity in all tehsils of Sargodha in relation to maximum, minimum temperature and rainfall. The disease severity was high from October to February when the maximum and minimum temperatures were at low level. The rainfall had negative impact that the disease incidence was least at high rainfall and high when low or no rainfall. Statistically the impact of rainfall was non-significant.

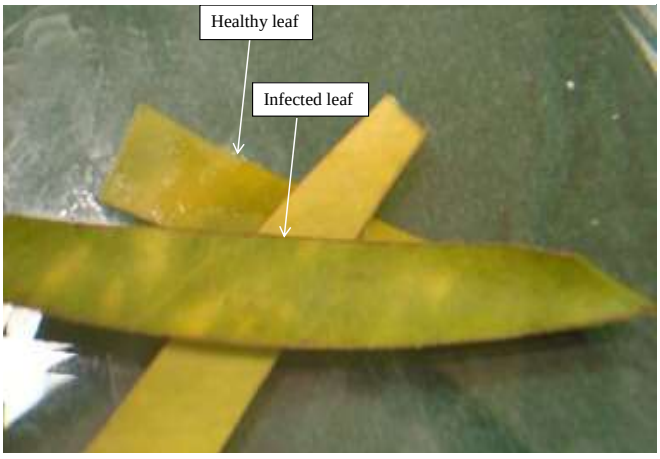


Figure 2. HLB-positive leaves with intense dark to grey stain.

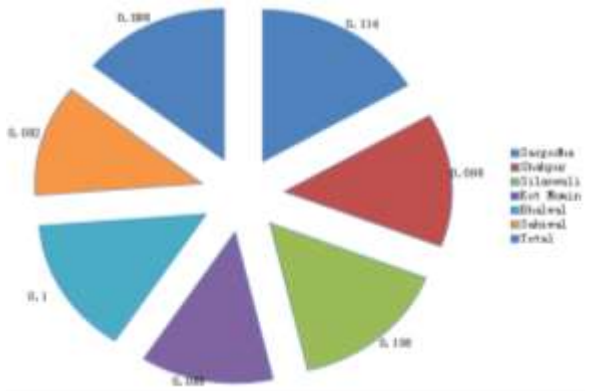


Figure 3. Disease incidence of HLB in district Sargodha.

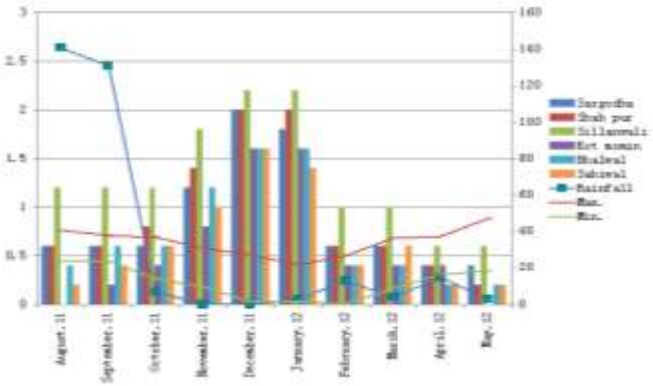


Figure 4. Disease severity recorded in six tehsils of Sargodha district based on rating scale.

Table 1. Correlation between disease severity and different environmental factors for different tehsils of Sargodha.

Tehsil	Rainfall (mm)	Temperature (°C)		Relative humidity (%)
		Max.	Min.	
Sargodha	-0.306	-0.762**	-0.654*	0.275
	0.389	0.010	0.040	0.442
Shahpur	-0.311	-0.802**	-0.655*	0.372
	0.383	0.005	0.040	0.289
Sillanwali	-0.148	-0.758*	-0.558	0.493
	0.684	0.011	0.094	0.148
Kotmomin	-0.472	-0.835**	-0.759*	0.231
	0.168	0.003	0.011	0.520
Bhalwal	-0.273	-0.761*	-0.600	0.368
	0.445	0.011	0.067	0.296
Sahiwal	-0.431	-0.768**	-0.712*	0.281
	0.213	0.010	0.021	0.431
Overall	-0.327	-0.795**	-0.667*	0.344
	0.356	0.006	0.035	0.331

Upper values indicated Pearson's correlation coefficient; Lower values indicated level of significance at 5% probability.* = Significant (P<0.05); ** = Highly significant (P<0.01)

DISCUSSION

Huanglongbing, caused by *Candidatus liberibacter* is one of the notorious diseases in citrus orchards of more than 40 countries (Iftikhar *et al*., 2014). Characteristics symptoms such as mottling, Zn deficiency like symptoms, lopsided fruits and aborted seeds were observed. Our results on the observations of symptoms were in accordance with Graca (1991) and Batool *et al*., (2007). The iodo-starch test was performed for quick indexing in the field. The accumulation of starch was confirmed with blackening of edges of infected leaves samples after treating with iodine solution. The similar results were reported by Etxeberria *et al*., (2007). After visual observations aiding with confirmatory test the incidence of HLB disease in different tehsils of Sargodha was found in range from 8.2% to 11.4%. The highest incidence was recorded in Sargodha tehsil (11.4%), followed by Silanwali 10.6%, Bhalwal 10.0%, Shahpur 9.8%, Kot Momoin 8.8% and Sahiwal 8.2%. The mean disease incidence was 9.8%. Similar results were presented by Akhtar and Ahmed in 1999. They carried out a survey in Punjab and Khyber Pakhton Khwoa (KPK). They observed the citrus greening disease (CGD) incidence in Punjab from 16-66% and 90% in KPK. Disease severity (symptoms expression) was recorded according to scale (Akhtar and Ahmad, 1999) with slight modifications. In the survey areas, none of the orchard visited showed severity level of 3 or above except one tree in an orchard from Sillanwali. Environmental factors like temperature and rainfall plays an important role in symptomatic expression. Therefore, there was significant change in symptoms expression in summer and winter seasons (Ahmed *et al*., 2011; Gottwald *et al*., 2012). The correlation analysis of disease severity to the environmental factors showed mostly non-significant results. However maximum temperature had highly significant but negative correlation with disease severity. It means that disease severity observed at maximum, the maximum temperature was at minimum level relative to other months of year. Disease severity was highest in December when temperature is at minimum level. Similar results reported by (Gottwald *et al.*, 2007) which stated that symptoms expression is severe in autumn and winter seasons while they are less prominent in spring and summer. Similarly rainfall showed significant negative correlation for disease severity. The reason behind, there is better availability of nutrients and development of new flush which gives the tree more vigor and health to fight against HLB infection and reduce disease severity.

***Conclusion*:** Huanglongbing is one of the serious diseases in the citrus groves not only of the world but also Pakistan. It is responsible for low citrus production. Quick indexing gave the positive results in detection of HLB in the field. This assay is also the need of time for the formulation of control strategies against this disease. Quick indexing will also serve

as general assessment of disease during screening process on large scale prior to molecular confirmation. Environmental factors also play an important role for the symptomatic expression of HLB. Therefore, keep these factors in mind during management of this disease. Nutritional supplements at appropriate time during the season are also helpful to combat with this disease. In future, there is a need for the development of assay which should be quick and more reliable for the detection of HLB in field.

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