GRAFT TRANSMISSION AND BIOLOGICAL INDEXING OF HUANGLONGBING ON LOCAL CITRUS GERMPLASM

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ABSTRACT: Huanglongbing is the most devastating disease of citrus with no known cure and uncertain disease latency period. The disease was explained in association with its psylla (Diaphorina citri) vector in Indo-Pak subcontinent by 1927, but was ignored in Pakistan for misconception of natural thermotherapy. Biological indexing has proven value for detection of several plant diseases including HLB and helps understanding epidemiological aspects of the disease. Current study relates disease transmission and symptoms expression rate using different indicator plants inoculated with a variety of the pathogen sources from field, transmitted through different budwood types in a specified timeline. Kinnow mandarin proved to be a better indicator plant with moderate to severe disease symptoms on 76.92% of the inoculated plants in comparison to 42.31% transmission success in rough lemon. Similarly, stick grafting proved to better than leaf piece grafting both showing wide presence of HLB in citrus groves of the Punjab.

Key words: Citrus greening, graft transmission, biological indexing, Huanglongbing

INTRODUCTION

Citrus is the biggest fruit crop in Pakistan with 199.9 thousand hectares of area, 2.132 MMT annual fruit production and 13th position among citrus growing countries. Average yield in Pakistan is about 12 tons per hectare; about 40% of per hectare production in USA [1]. Punjab shares 95.6% of the total citrus production of the country with predomination of Kinnow mandarin (approx. 80%) mainly concentrated in Sargodha and its peripheral areas along with districts of Toba Tek Singh, Faisalabad and Sahiwal [2]. Huanglongbing or citrus greening is about a century old and the most lethal disease of citrus caused by a phloem limited bacteria of α-proteobacteria subdivision. The disease is reported to be present in Indo-Pak subcontinent as early as 1927 [3], however, it was largely ignored leading to a regular uprooting of citrus groves with low and poor quality fruit production. Despite of vast presence of disease vector; the Asian citrus psylla (*Diaphorina citri*) the misconception of natural thermotherapy led wide spread of the disease in Punjab.

Biological indexing has been a classic method of HLB confirmation using different types of indicator plants. The first report of graft transmission of HLB came from Lin [4] using Ponkan mandarin, resulting disease expression in 83 of 94 inoculated trees and 4 of 22 uninoculated guard row plants confirming graft transmission of HLB pathogen. Lin's contribution to HLB was acknowledged in the 13th conference of International Organization of Citrus Virologists (IOCV) held in Fuzhou (Fujian, China) by affirming the official name of the disease to be *huanglongbing* (HLB) for all types of citrus greening disease [5]. Roistacher, in 1991[6] listed factors affecting the success of graft transmission including kind of the tissue used, age of the plant tissue, type of indicator plants, and the season of the year for inoculum collection. In the presence of CTV, good symptom expression in indicator plants may not be easy due to interference. He also preferred Ponkan or other mandarins as indicator seedlings as a differential host to make a distinction in tristeza and greening. He also recommended sweet orange and Orlando tangelo for African greening, and sweet orange or Ponkan mandarin seedlings for Asian greening as indicator and Grapefruit seedlings in the absence of severe tristeza isolates [6].

African greening symptoms appear primarily under cool conditions (below 25°C), does not show symptoms above 27°C under glasshouse conditions and are more prominent in winter than in summer. Further, the African citrus greening symptoms are severe in elevations above 700 m and absent in low lying hot areas. However, Asian greening appears and persists well in both hot and cool climates [7]. In Indo-Pak subcontinent, greening does well in hot conditions, above 25°C [8] at low altitudes. The symptoms of Asian citrus greening do not appear very well or disappear above 1500 m, probably because of vector's absence [9]. While working under controlled laboratory conditions, symptoms of African citrus greening were moderate to severe at 22° to 24°C and disappeared at 27° to 32°C, whereas symptoms of Asian citrus greening from India and Philippines strongly expressed at both temperature regimes [10].

In graft inoculated plant material HLB symptoms develop in about 20% plants within 3 to 12 months of inoculation under greenhouse conditions [11,12]. Both African and Asian greening pathogens are graft transmisible [12,13]; however, graft transmission of *Candidatus* Liberibacter spp. varies with the plant part used for grafting, the amount of tissue used, and the pathogen isolate. Single buds transmission of African greening varied from 0 to 50%, with type of isolate used [12] whereas side grafts with twigs were found to be even more effective for pathogen transmission with very little or no effectiveness of fruit stems and bark strips [12]. Early experiments reported 58% graft survival with disease symptoms expression in 20% plants, whereas, 10-16% and 40% disease expression from asymptomatic and symptomatic branches respectively from infected trees was recorded after 3 to 9 months of inoculation [14]. Graft transmission induced severe vein yellowing and leaf mottle on the emerging shoots of cv. Orlando tangelo within 12 weeks after grafting on two different experimental temperatures for *Ca*. L. asiaticus [15].

ISSN 1013-5316; CODEN: SINTE 8Sci.Int.(Lahore),24(2),155-159,2012

Disease symptoms appeared on citrus plants in 4 to 5 months after inoculation or pruning and were characterized by yellowing of the apical leaves similar to manganese and iron deficiencies. Whereas, blotchy mottle on leaves appeared approximately 6 months after grafting or pruning and continued to appear up to 1 year. Significant variation was noted in pathogen transmission efficiencies with different tissue types used for disease transmission through grafting. Transmission success was achieved only when xylem containing tissues were used. Disease transmission success was 2.5, 10.0, and 25.0%; and 2.6, 17.1, and 19.0% for individual buds and 2 or 4 cm twigs, respectively. First disease symptom was observed as yellow leaves on the new shoots 4 months after pruning. Pathogen transmission varied significantly among citrus cultivars and '*Candidatus* species. The disease transmission percentage in different citrus species varied from 2% to 65.2% [16]. The infected trees maintained under "warm" (27 to 32°C) conditions, failed to show the characteristic HLB leaf mottle, irrespective of the fact that these plants were showing such symptoms under cool conditions [17]. '*Ca.* L. asiaticus' was detected in 71% newly growing flushes after 3 months of inoculation, however, disease symptoms were not clearly visible. Graft inoculation of the disease was 70 to 90% efficient for different citrus cultivars with field tree inoculum sources maintained under greenhouse conditions. Inoculation responses of 30 different citrus genotypes to CTV-free '*Ca.* L. asiaticus' demonstrated that, '*Ca.* L. asiaticus' multiplied in most of the plants, with a wide range of reactions among the different indicator plants. Sweet orange and grapefruit were highly sensitive to respond the infection with severe yellowing of young leaves, stunted growth, and ultimate death, however, Mexican lime, Sun Chu Sha mandarin, and Eureka lemon better tolerated the infection. Incubation under continuous light showed much greater chlorosis with significantly lower incubation period for symptom expression with few exceptions, whereas, three different temperature (20°C, 27°C, or 32°C) regimes did not affect disease expression and qPCR detection [18]. Characteristic HLB chlorosis started to appear on foliage after 5 months of graft inoculation and was fully clear after 9 months followed by an extensive decline within 2 years [19]. Coletta-Filho *et al.,* 2010 [20] reported 100% disease transmission through grafting in 120 days post-inoculation. There was a direct relationship between the concentration of pathogen and symptom expression suggesting yellowed leaves or shoots may be the first symptom of HLB appearing in trees with even a low amount of bacteria, whereas, blotchy mottle to appear when pathogen was about 1000 times higher in the plant tissue after 180 day of infection [20].

Biological indexing of several citrus graft transmissible diseases has been of crucial importance in better understanding the nature of these diseases under controlled conditions. It further adds to identification of resistance and tolerance in different varieties and species against a number of plant diseases. It also helps understanding disease symptoms for better disease identification under field conditions, where symptoms become more diversified under changing field conditions. Current experiment was designed to study graft transmission of HLB and symptoms expression on specific citrus germplasm of high commercial importance in the local citrus industry, under greenhouse conditions.

MATERIALS AND METHODS

Sources of plant material for inoculation and disease transmission are given in Table 1. All the field sources were confirmed to be Citrus Tristeza Virus (CTV) free through DAS-ELISA prior to graft transmission of HLB to the indicator plants. Method described by [5,21] was used for disease inoculation and transmission of HLB on indicator plants under controlled conditions. The experiment was laid out in three factors under completely randomized design (CRD) including type of indicator plants (TIP), budwood type (BT) and months after inoculation (MAI); each at two levels with different source plants (SP) as treatments with 4 replications. Rough lemon (*Citrus jambhiri* Lush) and Kinnow mandarin (*Citrus reticulata* Blanco) were used as TIP, BT included semi-hard wood stick and leaf piece grafts whereas 4 month and 7 month as MAI. One year old potted disease free plants of both of the indicator species were maintained at 25 ± 2 ^{o}C before and after graft inoculation of the disease during the whole study period. Apart from 13 infected field plants, a couple of healthy Kinnow mandarin tree and a couple of replication sets with self grafting were used as negative controls.

Bud-wood was collected during the cool periods of the year (November-December). More than 20 bud sticks and/or six young shoots with sufficient bud sticks or 20 to 25 leaves were collected from inoculum sources, put in plastic bags and transferred immediately to a cool box to avoid budwood desiccation. Both side grafts and leaf-piece grafts with T-budding or veneer grafting (only when indicator plants did not show sufficient sap flow) were used for disease transmission.

Four side grafts were mounted to every indicator plant; each piece of graft tissue consisted of a 4-5 mm thick and 10-12 cm long stick or about 2 cm long rectangular leaf sections containing sliced midrib tissue for semi-hard wood and leaf piece grafting respectively.

Healthy controls were also grafted in the similar manner including two healthy control plants from the field and two sets of replications from greenhouse grown disease free self graft controls to elaborate the comparative transmission and indexing success. The data was collected after 4 and 7 months of disease inoculation on basis of criteria given in table 2.

RESULTS AND DISCUSSION

The disease symptoms expression showed a successful pathogen transmission which was quantified on a scale of 1 to 5. The data was analyzed in three factors factorial experiment under CRD. The factors included budwood type, type of indicator plants (TIP) and months after inoculation (MAI). All the factors showed significant effect on disease symptoms expression indicating Kinnow mandarin (*C. reticulata*) to be better indicator plant as compared to rough lemon with 40.38% plants with severe HLB symptoms after 7 months of inoculation. Only 6 (11.54%) rough lemon (*C. jambhiri*) plants showed severe disease symptoms after 7 months of inoculation. Similarly 13 (25%) and 5 (9.61%)

plants of rough lemon and Kinnow mandarin expressed very little or no disease symptoms after 7 months of inoculation

Table 1: Source plants species/ varieties with areas of origin for inoculum transmission to the indicator plants**.**

Species/ Variety	Number of source plants	Area	Number of Source plants
Kinnow Mandarin	8	Faisalabad	6
F. Early	2	Jhang	2
Grapefruit	1	T. T. Singh	1
Pineapple Orange	1	Sargodha	2
Valentia Late	1	Bhalwal	2
Total	13		13

Table 2. Biological indexing Criteria for graft transmission of HLB under controlled conditions**.**

Symptom index	Symptom level	Criteria
5	**Severe**	Irregular and asymmetric leaf mottle on 7 or more leaves Irregular and asymmetric leaf mottle on 4 or more leaves + Zn deficiency on 6 leaves Irregular and asymmetric leaf mottle on 4 or more leaves + Zn deficiency on 4 leaves + up to 40% leaf area reduction on visual basis
4	**Moderate**	Irregular and asymmetric leaf mottle on 5 or more leaves no other symptoms Zn deficiency on 7 leaves + Mn/ Fe. Deficiency on other 7 leaves Zn, Mn, Fe deficiencies on 15 leaves + corky and yellow veins in 20% leaves of post inoculation flush Leaf area reduction by 30% with Zn, Fe, and Mn deficiency on 10 leaves
3	**Light moderate**	Irregular and asymmetric leaf mottle on 2 leaves + 3 leaves with Zn deficiency 5 leaves with Zn deficiency Combined deficiency symptoms of Zn, Mn and Fe on up to 10 leaves Stunted growth of new leaves with any of the single deficiency of Zn, Fe and Mn on 7 leaves
2	**Light**	No asymmetric leaf mottle but Fe, Zn or Mn deficiencies symptoms on at least 5 leaves with stunted plant growth 30-35% reduction in leaf area on visual basis on post inoculation flush Leaf drop + corky and yellow veins in 20% leaves of post inoculation flush No new growth + corky and yellow veins in 20% leaves of post inoculation flush
1	**No symptoms**	No visual difference when compared with self controls

(Table 3) indicating long disease latency period. The symptoms expression rate was also affected by different inoculums source varieties (Fig. 1A) indicating grapefruit (*C. grandis*) and Valencia late (*C. sinensis*) to be better inoculums sources. Further, Kinnow mandarin (*C. reticulata*) always proved to be a better indicator plant as compared to rough lemon (*C. jambiri*) irrespective of inoculums sources and type of inoculation tissue used (Fig. 1B). The linear Trend line with respect to severe disease symptoms expression through leaf midrib grafting on rough lemon (Fig. 1C) shows increasing symptoms severity with reference to time. It identifies a long time period for disease symptoms expression. Similarly, stick grafting showed better disease transmission as compared to leaf graft on both types of indicator plants. These results prove that disease latency period may not be uniform irrespective of any genetic resistance in any specific citrus species or variety and symptoms may or may not appear for a long period of time even under controlled conditions.

Data was also analyzed applying t-test (Table 4) and results showed significant effects of source plants, months after inoculation (MAI) and type of indicator plants (TIP) disease symptoms expression; however, statistical analysis proved that there was no effect of type of budwood used for disease transmission on symptoms expression. Regarding interaction of different factors only interaction between source plants and indicator plant types showed significant effect on disease expression, whereas, no interaction was found for all the other interactions studied.

Distribution of pathogen has not been uniform [8,11,17] and impart to symptoms expression. Other findings [20] indicate 100% graft transmission, whereas our finding did not reflect such a high percentage. This is most probably because of difference in varieties used, time of budwood collection and initial bacterial population in the budwood, which has been variable under open field conditions. The variability in symptoms expression emphasizes molecular detection of *Ca.* L. asiaticus graft inoculated indicator plants to estimate exact transmission success %age under greenhouse condition, irrespective of level of symptoms expression.

Table 3: Level of disease symptoms (LDS) expression on number of indicator plants with percent of inoculated with different budwood types after different post inoculation periods.

MAI	LDS*	Rough Lemon		Kinnow mandarin	
		Stick Graft	Leaf Graft	Stick Graft	Leaf Graft
4 Months	5	2	2	8	9
	4	5	4	15	9
	3	18	13	16	21
	2	11	17	6	9
	1	16	16	7	4
	Total	52	52	52	52
7 Months	5	6	5	28	21
	4	16	11	12	19

ISSN 1013-5316; CODEN: SINTE 8Sci.Int.(Lahore),24(2),155-159,2012

	3	16	21	5	7
	2	9	6	3	3
	1	5	9	4	2
	Total	52	52	52	52

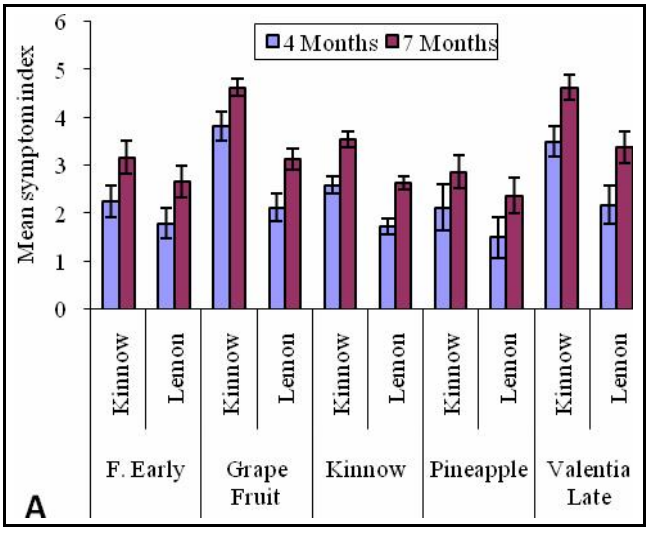
*Level of disease symptoms

Table 4: Analysis of variance table for effect of source plant, months after inoculation (MAI), Type of indicator plants (TIP) types of budwood used and their interactions on graft transmission and disease expression

Source of variation	df	Mean Square	F	Sig.
SP	15	30.543	24.598	**
MAI	1	110.168	88.724	**
TIP	1	94.102	75.785	**
BT	1	0.668	0.538	NS
SP x MAI	15	0.356	0.287	NS
SP x TIP	15	5.173	4.166	**
MAI x TIP	1	0	0	NS
SP x MAI x TIP	15	0.146	0.118	NS
SP x BT	15	1.735	1.397	NS
MAI x BT	1	0.004	0.004	NS
SP x MAI x BT	15	0.121	0.097	NS
TIP x BT	1	1.817	1.463	NS
SP x TIP x BT	15	0.85	0.685	NS
MAI x TIP x BT	1	0.012	0.01	NS
SP x MAI x TIP x BT	15	0.146	0.117	NS

NS = non significant **highly significant

Due to lack of resources, molecular detection for *Ca.* L. asiaticus in the indicator plants could not be conducted. However, all the source plants from the field were subjected to real time PCR and found to be infected with *Ca.* L. asiaticus. These results further suggest that disease transmission success may not be completely reflected through biologically indexed symptoms expression [18] and infected plants may remain symptomless for long periods, suggesting alternate [8].



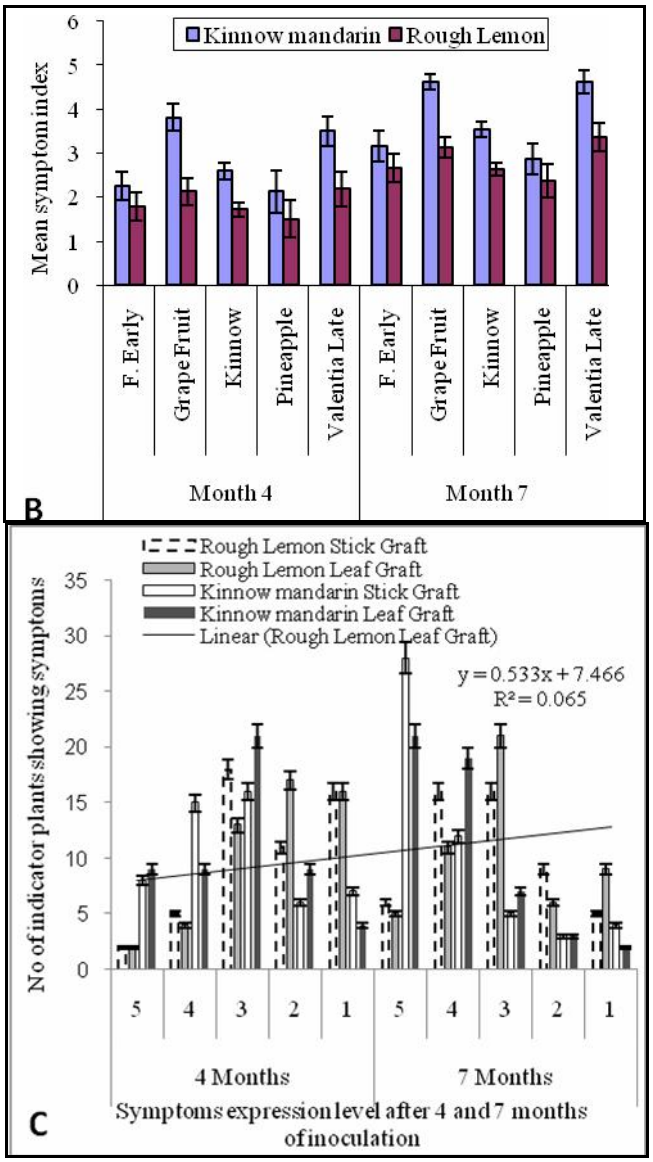


Figure 1: Effect of different HLB source plants on disease symptoms expression for different indicator plants (A), effect of source plants for disease expression level with respect to different time periods (B) and number of indicator plants with different disease expression levels for biological indexing of HLB (C).

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