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MONITORING THE LETHAL IMPACT OF HEAT ON CANDIDATUS LIBERIBACTER ASIATICUS (CLAS) PROTEOBACTERIUM, THE CONTRIBUTORY MEANS OF HUANGLONGBING IN PUNJAB, PAKISTAN

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Abstract

Citrus ranked first out of all the fruits that are produced in Pakistan with a major share (~ 80%) of kinnow. To check the potential therapeutic impact of higher temperatures on *Candidatus Liberibacter asiaticus* (CLas) development on five citrus hosts (*Citrus reticulata*, *Citrus paradisi*, *Citrus limon*, *Citrus limetta* and *Citrus sinensis*), the proteobacterium CLas titers were monitored in psyllid specimens and naturally infected leaves using qPCR techniques and primers for two and half years by collecting samples from symptomatic citrus trees from three district study districts of Punjab i.e., Faisalabad, Sargodha and Toba Tek Singh. Apart from molecular detection of CLas in psyllid and leaf samples, the citrus trees were also observed visually and ranked on a disease scale from 0 to 5 based on symptoms percentage on citrus plants. The monthly mean temperatures at the three study districts were different but the variation among these temperatures was not big enough. The mean cyclic threshold (Ct value) of the greening bacterium was highest (37.02) in 2021 than the previous years in psyllid specimens that were collected from *C. reticulata* in Toba Tek Singh which represented lowest disease incidence while the lowest Ct value (12.07) in psyllid specimens that were collected from *C. sinensis* of Sargodha which represented highest disease incidence. The highest Ct values in leaf samples were recorded for *C. reticulata* (33.36) while the lowest Ct values for the *C. sinensis* (13.34) were recorded in comparison with *C. paradisi*, *C. limon* and *C. limetta*. Maximum disease index (DI) symptoms based on visual observations were observed on *C. sinensis* (3.85) in Sargodha during 2020 while minimum (1.40) on *C. reticulata* in Toba Tek Singh during 2019. The Ct values significantly increased in summer month e.g., May-June which show that minimum bacterium titer was found during the summer months due to heat impact, but heat is not enough to cure this citrus greening bacterium throughout the year. To reduce greening bacterium titer to minimum there is need to further improve management practices and manage psyllid populations using different IPM tactics.

Keywords: citrus greening, disease index, therapeutic impact, heat, proteobacterium, citrus, bacterium titer, qPCR technique, psyllid

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1. INTRODUCTION

Citrus ranked first out of all the fruits that are produced in Pakistan with a major share (~ 80%) of kinnow (*Citrus raticulata* cv. Kinnow). Owing to favorable climatic conditions, major citrus cultivars including kinnow mandarin (*Citrus reticulata*), sweet orange (*Citrus sinensis*), grapefruit (*Citrus paradisi*), lemon (*Citrus limon*) and lime (*Citrus limetta*) are commercially grown in Pakistan [1].

Citrus cultivation and production has been seriously influenced by different biotic factors including pests (Asian citrus psyllid, whiteflies, citrus leaf miners, fruit flies) and diseases (citrus greening, citrus canker, citrus decline, citrus sudden death, etc.) as well as abiotic factors (temperature, relative humidity, rainfall, photoperiod) [2]. Among all these factors, the citrus greening disease or Huanglongbing (HLB) is very important and spread by the phloem-limited proteobacterium *Candidatus Liberibacter asiaticus* (CLas). This disease inflicts massive systemic loss of citrus plantations which results in severe economic yield loss, deteriorated quality fruit, and the complete decline of trees. [3]. This disease is vectored by the phloem-sucking insect pest Asian citrus psyllid (*Diaphorina citri*) during the feeding process. The psyllid adults feed on mature leaves as well as tender flush growth. Female egg-laying and subsequent development of psyllid nymphs are limited to the young flush on the citrus trees [4]. Both adults and nymphs are capable of proteobacterium pathogen transmission in the phloem of infected trees [5]. Once the pathogen reaches the phloem section, it blocks the sieve tube and starch starts to accumulate inside the infected leaves which results in disease symptoms and other disorders [6], [7]. Being incurable, this disease has different disease symptoms including chlorotic interveinal leaf appearance or/and blotching of symptomatic leaves, bright, yellowish-colored branches or growth, and dark greenish leaf islands [8] while the fruit symptoms include misshapen and lopsided fruit, skeletonized tree canopy, improper color development at maturity, sour taste and premature fruit drop [9].

Typical symptoms of Huanglongbing were first reported in the Indian sub-continent (now the Punjab region of Pakistan) by the investigations of Husain and Nath, in 1927 [10] but the disease was not given any name at that time. Later, up to ≈ 90% yield losses have been reported due to psyllid-disease association in the Multan district of Punjab [11]. Presently, this disease is a major cause of reducing tree life by making citrus trees skeletonized and unproductive over a period of 7-10 years, that's why the average yield of citrus (12 tons/hectare) in Pakistan is about 40% less than the production in the United States [12].

The capability of the psyllid-CLas pathosystem to infest citrus trees is greatly affected by the prevailing climatic conditions. The life cycle and development of psyllid pests are dependent upon the temperature with a lower threshold (0°C) and upper threshold (40°C) with higher rates of mortality were observed from 35°C onward to 41°C [13]. In certain areas, the developmental cycle of the psyllid completes in only eleven days and up to eight generations in a year, but the same psyllid species could not develop huge populations under cooler environmental conditions [14]. Different phenological and

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empirical investigations documented that the temperature affects psyllid population ecology by influencing flush appearances on citrus hosts [15], [16]. Furthermore, the higher temperature also influences the CLas development and its acquisition rates by the psyllid pest [17], [18], [19].

Various empirical investigations in different parts of the world including Florida (United States), Brazil and India documented heat impact on CLas titers in citrus leaves and reported that lower CLas titers were found in naturally infected mature leaves in hotter months [12], [17],[18],[19]. Likewise, the potted plants from the greenhouse were artificially inoculated with CLas then they were exposed to varying temperatures from 24°C to 35°C and lower titer values were reported at the higher temperatures as compared to lower temperatures [20], [21]. The lower CLas titers were reported in mature and fully expanded leaves of citrus [20], [21] as well as in young immature young flush leaves [17]. The decreased CLas titers in young flush leaves were constantly linked with reduced pathogen acquisition by adult *D. citri*. Likewise, the bacterium-positive *D. citri* adults were consistently exposed to 14, 20, 26, 32 and 38°C temperatures and the lowest CLas titers were reported at 38 and 14°C in comparison with other temperatures [17]. The Punjab region of Pakistan is versatile regarding climatic conditions because summer months are very hot and the temperature reaches up to 45°C while the winter months are cool and the minimum temperature drops up to 1 to 2°C while spring and autumn (fall) seasons have favorable temperatures for psyllid development and peak psyllid populations have been recorded during spring and fall seasons [12], [15]. It was hypothesized that higher temperatures during summer months may be a reason for the successful survival of citrus cultivars especially kinnow mandarin especially in Punjab, Pakistan.

To check the potential therapeutic impact of higher temperatures on CLas development in different citrus hosts, the proteobacterium CLas titers were monitored in psyllid specimens and naturally infected leaves using qPCR techniques and primers for two and half years by collecting samples from symptomatic citrus trees from three district study districts of Punjab i.e., Faisalabad, Sargodha and Toba Tek Singh. Apart from molecular detection of CLas in psyllid and leaf samples, the citrus trees were also observed visually and ranked on a disease scale from 0 to 5 based on symptoms percentage on citrus plants.

2. MATERIALS AND METHODS

2.1. Psyllid collection

Five psyllid samples from five greening infected citrus trees were collected each month for each citrus cultivar (*Citrus reticulata*, *Citrus paradisi*, *Citrus limon*, *Citrus limetta* and *Citrus sinensis*) from three major citrus growing and study districts i.e., Faisalabad, Sargodha and Toba Tek Singh with the help of an aspirator. Psyllid specimens from greening infected trees were preserved in small vials containing 95% ethanol and later stored at -20°C in a low-temperature freezer in the IPM lab. In the entomology

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department, the University of Agriculture, Faisalabad (UAF). Each psyllid sample consisted of 5 to 150 specimens; on average, most of the samples had approximately 40-50 specimens.

2.2. Plant sample collection

Samples of leaves having typical symptoms of citrus greening or huanglongbing (HLB) disease i.e., chlorotic interveinal leaf appearance or/and blotching of symptomatic leaves, bright, yellowish-colored branches, growth, dark greenish islands were collected each month from five citrus cultivars (*C. reticulata*, *C. paradisi*, *C. limon*, *C. limetta* and *C. sinensis*) from three citrus growing and study districts of the citrus belt. A total of 10 leaf samples were randomly collected from each citrus cultivar from five (5) surveyed trees. A total of 50 symptomatic leaves were collected for each leaf sample. These leaves were wrapped in aluminum foil, properly labeled, and placed in a cooling box containing dry ice. When back in the laboratory these leaves were put in storage at a temperature of -80^{0}C and DNA was extracted in the same week. Asymptomatic leaves were collected as a negative control. Before extraction of DNA, experimental leaves were cleaned with the help of distilled water and then dried using tissue paper. Infected midrib portions of leaves were cut, put in an Eppendorf tube, and instantly placed on ice and properly labeled. DNA was extracted during the same week of sample collection.

2.3. DNA extraction and real-time PCR for Las quantification

The DNA of psyllid specimens was extracted using the DNeasy Blood and Tissue kit (Qiagen, China) in accordance with the maker's animal tissue procedure with some alterations to isolate the proteobacterium DNA of psyllid specimens [22]. The *D. citri* specimens were taken from the preserved samples in small vials containing 95% ethanol, the specimens were dried in the air at ambient temperature. The Psyllid DNA was extracted from the field-collected specimens with the help of the Qiagen DNeasy blood and tissue toolkit using an animal tissue procedure with minor variations. Firstly, the ATL buffer with an amount of 180 microliters was mixed with proteinase K in the amount of 20 microliters. Then 20 microliters were taken from this mixed solution and added into the microcentrifuge tubes having 1.5 milliliters capacity along with ground psyllid specimens. This mixture was then homogenized using a micro pestle. Finally, with the help of AE buffer (pH 9.0, 0.5 mM EDTA, 10 mM Tris) in the amount of 35 microliters, the elution of DNA was executed. After that quantification of extracted DNA was performed by spectrophotometer. All the working surface areas were applied with decontaminants to keep the chance of cross-contamination minimum. The Sterile, RNase and DNase-free micropipette and microcentrifuge tube were utilized to perform all the DNA extraction steps. Additionally, the DNA extractions were executed using a laminar flow hood chamber.

Similarly, the plant samples were tested for greening bacterium presence and quantification. The midrib part of the five sampled leaves was incised by using a sterilized sharp razor blade for each citrus cultivar. The chopped midribs were pooled, and a subsample of 100 mg was taken for DNA extraction purposes. The liquid nitrogen was

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used to freeze the obtained samples and subsequently these samples were grounded with stainless steel beads and bead mills (TissueLyzer II, Qiagen). The DNA extraction from plant symptomatic leaf samples was done by DNeasy Plant Kit (Qiagen, China) with some modifications as described by [22], [23]. Quantification of the *CLas* concentration was performed with the help of qPCR-based and definite primer sequences; the sequence of forward primer (5′-TGAATTCTTCGAGGTTGGTGAGC-3′) while the sequence of reverse primer (5′-AGAATTCGACTTAATCCCCACCT-3′) made by using specific sequence from proteobacterium (M94319) from GenBank which were utilized for executing PCR in 25 µL volume of reaction [24].

Quantitative real-time qPCR was executed by using the 16S ribosomal DNA (rDNA) *CLas* specific primers Taq polymerase, Taq mix, and qPCR master mix (Qiagen, China) and DNA from psyllid specimens (1 µL) were manipulated by fast real-time PCR Biosystems in a 20 µL qPCR reaction volume. In addition to aimed DNA, internal control primers were also manipulated in multiplex qPCR amplification of plant and psyllid specimens as explained by [22]. The quantitative PCR protocol settings were set to I) 2 minutes at 50^0C, II) 10 minutes at 95^0C, and 40 cycles with (15 seconds at 95^0C, and 60 seconds at 60^0C). Duplicate reactions were performed for each sample in a real-time PCR system (CFX Bio-Rad Connect™ module from Singapore), and positive control and negative control sequences of the *CLas* bacterium were included for each set of amplifications.

The DNA extraction from plants and psyllid samples was executed by using DNeasy Plant Kit (Qiagen, China) and adopted the protocol as per instructions of the manufacturer with some modifications for extraction of psyllid DNA and qPCR were executed by using the CLas specific primers Taq polymerase, qPCR master mix and Taq mix (Qiagen, China) and *D. citri* DNA template (1 µL) were manipulated using fast real-time PCR Biosystems in a 20 µL qPCR reaction volume. The real-time qRT-PCR values were interpreted as the cyclic threshold value (Ct value). The *CLas* quantification (Ct value) measurements in different citrus cultivars and psyllid samples were repeated every month for a period of two and half years to monitor the impact of heat on greening bacterium titer the year around.

2.4. Greening disease index rating with visual observations

The citrus tree canopy was divided into two hemispheres and each hemisphere was individually scored from 0 to 5 on a ranking scale representing the proportion of tree branches showing greening symptoms within each hemisphere section (0 = No branch with HLB signs, 1 = 10% branches with HLB indications, 2 = 25% branches with HLB appearance, 3 = 50% branches are with HLB indications, 4 = 75% branches are with HLB appearance, and 5 = All the tree branches having symptoms of HLB disease). The mean value of both scores was manipulated for each tree per sampling date to rate the disease index (DI) for a specific cultivar. The disease index (DI) was calculated individually for all five citrus cultivars to make comparisons over time.

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2.5. Weather data

The weather data of each study location (Faisalabad, Sargodha and Toba Tek Singh) was taken from the meteorological department of Pakistan website on a fortnightly basis. The minimum and maximum temperatures were also recorded but only average temperatures were represented in making graph.

2.6. Statistical analysis

The Ct values from the psyllid specimens and leaf samples as well as disease index (DI) values from visual observations of the citrus trees were inserted into the excel. All the statistical analyses (analysis of variances) were executed using Statistix 8.1 software under a three-way factor arrangement with four replications and treatments means were compared. Since this experiment was performed in three study districts in different months for two and a half years, the treatments were separated using Tukey's honest significance difference test (HSD). The level of significance was set at 5%. The fortnightly mean temperatures were pooled together to calculate the monthly mean temperatures. The graph of monthly mean temperature was made using GraphPad Prism software version 9.

3. RESULTS AND DISCUSSION

3.1. Temperature variation in experimental locations

The monthly mean temperatures at the three study districts were different but the variation among these temperatures was not big enough. The lower temperature peaks were recorded at the district Sargodha (maximum monthly mean temperature was recorded in the range of 31.84-34.62°C throughout the study period) while the higher temperature peaks were recorded at Toba Tek Singh district (maximum monthly mean temperature was recorded in the range of 33.98-36.69°C throughout the study period). The temperature peaks of district Faisalabad were in between the Sargodha and Toba Tek districts (Figure 1). January was the coolest month (monthly mean minimum temperature was dropped up to 2.70°C) while June (mean monthly maximum temperature rose to 42.02°C) was the hottest month throughout the study duration.

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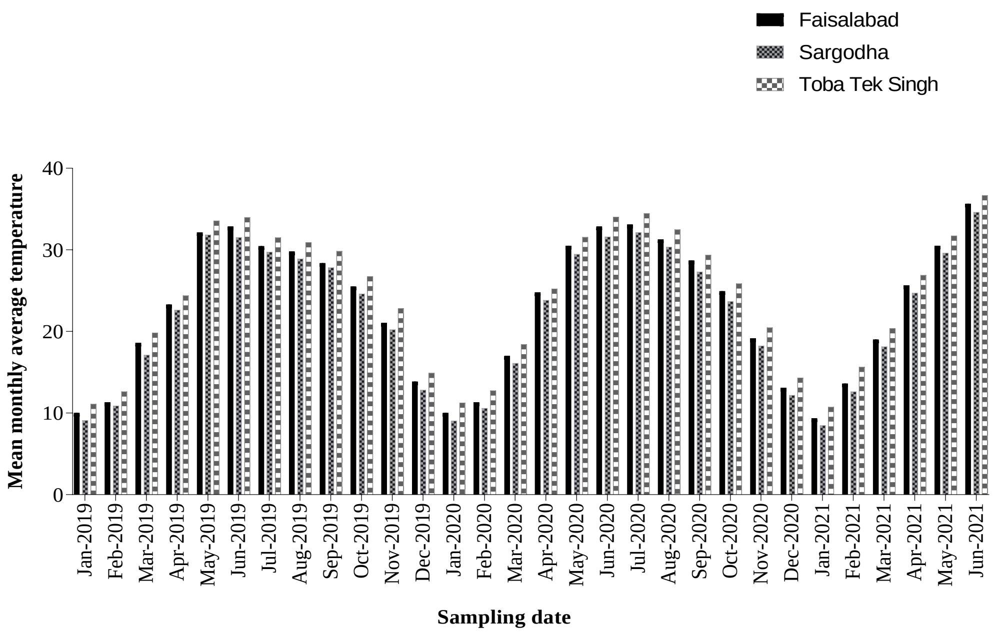

Figure 1: The monthly mean temperature (°C) of three study districts (Faisalabad, Sargodha and Toba Tek Singh) for two and a half research period (January 2019 to June 2021).

3.2. Bacterium quantification in psyllid samples

During 2019 the disease incidence results from Tukey's HSD means test showed the effect of seasons on causal bacterium presence in psyllid samples and on average the proteobacterium presence (Ct values) was maximum during hot summer months of May (23.263) and June (25.697) months when the temperature was highest which indicates that the greening titer was reduced significantly while minimum Ct values were documented during winter months i.e. December (17.400) and January (16.254) which indicates that CLas titer was significantly higher than the other months in psyllid samples (F=6606.37, df=11, P=0.0000). The impact of prevailing temperature throughout the year was also investigated across different citrus cultivars in Punjab and found that Ct values were significantly different on all the experimental cultivars throughout the study period (F=36197.5, df=4, P=0.0000). The cyclic threshold values were also significantly different across three studies districts (F= 12739.2, df=2, P=0.0000). Maximum Ct values was documented psyllid specimens collected from *C. reticulata* (26.039) which indicates the minimum disease incidence while minimum Ct value (17.160) in *C. sinensis* which represent the maximum proteobacterium presence in psyllid population. The greening bacterium titer on *C. limetta* (22.844), *C. limon* (18.249) and *C. paradisi* (17.670) was in decreasing order and disease incidence was vice versa (Table 1). The bacterium presence in psyllid specimens were minimum in district Toba Tek Singh with highest Ct value (22.253) while highest bacterium titer or lowest Ct value (18.695) was documented

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in district Sargodha because the average temperature in Toba Tek Singh was higher than other districts (Table 1).

In 2020 the greening bacterium titer in psyllid specimens was tested on monthly basis. The analysis of variance and subsequent Tukey HSD test results demonstrated that the Ct values in psyllid specimens were significantly varied across all the tested citrus cultivars (F = 35885.2, df = 4, P = 0.0000) with maximum Ct value on *C. reticulata* (26.818) which shows that minimum bacterium presence is found in psyllid specimens collected from this cultivar. The minimum (18.000) Ct value was documented on the *C. sinensis* cultivar which represents the maximum disease incidence was found in the psyllid samples on this citrus cultivar. The impact of average monthly temperature was also observed on CLas titer in five citrus cultivars and found that Ct values were significantly different throughout the year (F = 6362.33, df = 11, P = 0.0000) with maximum Ct values were found in psyllid specimens during the summer months May (23.962) and June (26.501) which represent the minimum disease incidence in psyllids while the minimum Ct values were noted in December (18.230) and January (17.143) which indicates the maximum disease incidence in psyllids. The bacterium presence was also tested in psyllids across different study districts, and it was found that the disease incidence was significant changed in study districts (F = 12995.8, df = 2, P = 0.0000) with maximum Ct value (lowest disease incidence) in psyllids collected from district Toba Tek Singh (23.163) and minimum Ct value (highest disease incidence) in specimens from Sargodha (19.552) owing to relatively higher average temperatures (Table 2).

In 2021, similar trends were documented but the presence of the proteobacterium was also higher as compared to previous years. Overall, the disease incidence in psyllid specimens increased with every passing year. The Ct values were significantly different across all the citrus cultivars (F = 20125.5, df = 4, P = 0.0000) with maximum Ct values in *C. reticulata* (30.024) while the minimum greening titer in *C. sinensis* (19.661). The CLas titer was also significantly varied across study months (F = 5264.95, df = 5, P = 0.0000) with maximum Ct values May (25.011) and June (27.566) which represented the minimum disease incidence due to impact of high temperature while the minimum Ct value (20.545) was documented in psyllid specimens during the January (winter). The disease incidence also significantly varied across all the study districts (F = 4634.73, df = 2, P = 0.0000) with maximum Ct value (lowest disease incidence) in Toba Tek Singh (25.270) while minimum Ct value (highest disease presence) in Sargodha (22.014) (Table 3).

3.3. Bacterium quantification in leaf samples

During 2019 the bacterium titer was also checked using incised midrib portions of the symptomatic greening leaves collected from five citrus cultivars and three study districts and disease severity was significantly different across all the investigated citrus cultivars (F = 14658.1, df = 4, P = 0.0000). The maximum disease prevalence (minimum Ct values) in tested plant samples were recorded for *C. paradisi* (18.374) while the lowest disease incidence (maximum Ct values) was documented for the *C. reticulata* (26.169) in

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comparison with *C. limetta* (23.495), *C. limon* (22.586) and *C. sinensis* (19.445). The Ct values increased from February (19.567) to June (25.438) when the temperature was moderate during the spring and early summer season and then increased during the hot summer months of May (23.822) and June (25.438) when the maximum average temperatures were recorded (Table 1). When the average temperature started to decrease after the month of June then psyllid infestation started to increase, and corresponding Ct values started to decrease again till next spring summer in a consistent fashion (Table 1). The disease severity was also significantly different across different study districts of Punjab (F=8902.54, df =2, P=0.0000) with maximum Ct values in district Toba Tek Singh (23.786) and minimum in Sargodha (20.007). The district Sargodha is in the central Punjab with somewhat mild climate while the Toba Tek Singh is also located in the central Punjab but is closer to the southern part of Punjab which is somewhat hotter. Overall, the higher temperatures were recorded in the Toba Tek Singh as compared to the other two districts (Table 1).

In 2020 the disease prevalence was found significantly different across all the study districts of Punjab (F=14911.0, df=2, P=0.0000) (Table 2) with minimum disease indications in district Toba Tek Singh with maximum Ct value (24.709) while maximum symptoms in district Sargodha with minimum Ct value (20.943). All the investigated citrus cultivars significantly represented disease severity (F=25264.5, df=4, P=0.0000) with maximum bacterium presence was tested in *C. paradisi* with lowest Ct values (19.247) leaves while minimum bacterium concentrations in *C. reticulata* (27.098) in comparison with *C. limetta* (24.399), *C. limon* (23.370) and *C. sinensis* (20.306) in a consistent and decreasing fashion (Table 2). When Ct values were compared in different months and they were significantly different regarding disease incidence (F = 3963.05, df = 11, P = 0.0000) then it was clearly revealed that Ct values were maximum in May (24.707) and June (26.206) with least disease incidence while minimum in December (20.345) and January (19.176) when the minimum monthly average temperature was prevailing (Table 2).

The disease severity was also investigated on a year-to-year basis and increased throughout the research duration and similar trends of bacterium titers were reported in the third year (Table 3). All the investigated citrus cultivars were significantly different in disease indications (F = 11773.5, df = 4, P = 0.0000) with maximum incidence in *C. paradisi* with minimum Ct value (20.746) and minimum in *C. reticulata* with maximum Ct value (29.208) compared to *C. sinensis* (22.590), *C. limon* (25.257) and *C. limetta* (27.489). The greening bacterium titer was also significantly varied across different months (F=2023.77, df =5, P=0.0000) in 2021 (Table 3) with minimum incidence was found in June with maximum Ct value (27.328) while maximum in January with lowest Ct value (22.510). Likewise, different districts expressed significantly different Ct values (F = 6680.66, df = 2, P = 0.0000) (Table 5.3) with maximum greening bacterium in district Sargodha with minimum Ct values (22.941) and minimum in district Toba Tek Singh with highest Ct value (26.961). In most of the tested plant samples, the titer of the greening bacterium was higher than in the tested psyllid samples (Table 1-3).

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Table 1: Mean Cyclic threshold (Ct) values of *Candidatus Liberibacter asiaticus* in tested psyllid specimens and plant samples on five citrus cultivars throughout the year during 2019

Districts	Months	Citrus Cultivars									
		C. paradisi		*C. sinensis*		*C. reticulata*		*C. limon*		*C. limetta*	
		Ct value ACP	Ct value Plant	Ct value ACP	Ct value Plant	Ct value ACP	Ct value Plant	Ct value ACP	Ct value Plant	Ct value ACP	Ct value Plant
Faisalabad	January	14.14	15.15	13.63	17.36	20.32	21.86	14.64	19.45	18.08	19.05
	February	15.17	17.31	14.66	16.66	24.65	23.63	16.91	19.56	22.06	21.55
	March	17.27	21.23	17.58	22.04	25.39	27.88	18.07	24.66	22.66	26.84
	April	18.67	18.58	18.16	20.37	27.45	28.44	19.00	22.35	23.72	25.05
	May	19.33	20.61	18.82	21.69	29.67	28.69	22.69	23.47	25.04	26.12
	June	21.02	22.33	20.51	22.55	33.54	29.64	25.72	25.63	26.84	27.92
	July	18.94	19.63	18.43	19.07	27.64	27.83	18.83	23.07	23.83	24.50
	August	18.09	19.52	17.56	20.41	26.65	26.77	17.58	23.95	23.61	22.20
	September	18.07	20.63	16.76	21.28	26.42	25.33	16.98	23.41	23.37	25.55
	October	17.14	17.58	16.63	19.18	25.75	25.08	16.55	22.82	22.32	23.74
	November	15.78	16.55	15.27	18.85	23.63	24.36	16.47	24.80	20.28	24.08
	December	14.85	15.63	14.34	18.47	21.61	23.55	15.33	20.05	19.63	19.43
Toba Tek Singh	January	16.20	16.30	15.69	18.06	22.80	23.61	16.27	21.85	20.64	19.37
	February	17.12	15.41	16.61	18.64	25.92	25.30	18.09	23.51	23.56	23.49
	March	18.95	22.52	18.44	24.03	28.75	28.10	19.35	21.44	25.58	27.38
	April	19.97	19.41	19.46	22.14	29.40	29.21	20.36	26.52	25.64	26.86
	May	20.93	21.06	20.42	23.58	31.60	30.61	24.52	25.25	27.55	28.55
	June	23.25	23.36	22.74	24.72	35.63	31.72	27.84	27.52	28.53	29.52
	July	21.17	21.88	20.66	22.40	29.55	30.02	21.41	23.07	26.61	24.32
	August	20.58	21.20	20.07	21.44	28.08	27.90	20.43	24.55	25.95	25.80
	September	20.09	21.08	19.58	23.16	27.66	29.53	19.61	26.12	24.63	23.70
	October	19.01	20.17	18.50	20.58	27.66	27.34	18.76	25.32	24.29	28.28
	November	18.21	19.44	17.70	20.40	25.64	26.44	18.60	26.55	22.53	21.08
	December	17.20	18.52	16.69	19.51	23.54	25.52	17.50	22.31	21.57	20.49
Sargodha	January	12.58	13.87	12.07	13.34	18.39	21.07	11.66	17.45	16.71	17.58
	February	13.87	11.44	13.36	14.42	21.44	23.47	14.61	19.44	20.17	19.69
	March	17.12	13.55	15.61	19.56	23.06	21.38	15.68	17.39	21.17	24.47
	April	17.57	18.44	16.61	18.58	25.64	25.42	16.45	22.35	22.08	23.44
	May	18.15	15.52	17.64	19.71	27.52	26.68	20.83	21.45	24.23	24.36
	June	20.21	19.63	19.70	20.63	31.07	27.59	23.74	23.37	25.12	25.44
	July	17.92	19.40	17.41	17.39	25.72	26.22	17.47	19.49	22.63	23.32
	August	17.03	17.55	17.06	16.08	24.72	25.83	16.56	20.29	22.08	22.43
	September	16.12	18.39	16.52	18.77	24.04	24.30	15.72	22.08	21.55	21.34
	October	15.62	17.52	15.11	15.72	23.58	23.64	15.05	21.07	20.61	20.36
	November	14.90	16.42	14.39	14.96	23.66	25.08	14.45	23.12	19.06	19.69
	December	13.93	14.66	13.42	14.32	19.67	23.09	13.28	18.40	18.47	18.87

3.4. Measurement of disease index (DI)

The symptomatic citrus trees were also visually observed for greening related symptoms such as chlorotic interveinal leaf appearance or/and blotching of symptomatic leaves, bright, yellowish-colored branches, growth, dark greenish islands and then ranked these

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cultivars on a 1-5 scale based on percent branch infestations. All the citrus cultivars expressed disease symptoms significantly different from each other (Table 4). Maximum disease symptoms were observed on *C. sinensis* while the minimum disease indications on the *C. reticulata* in comparison with *C. limetta*, *C. limon* and *C. paradisi*. When the district means of Ct values were compared then it is clearly indicated that more disease symptoms were noted in district Sargodha while minimum disease symptoms were observed in Toba Tek Singh. The frequency of disease symptoms also significantly varied across different months (Table 4). Maximum disease symptoms were observed during winter months of December and January while minimum symptoms were noted during hot summer months of May and June. The disease symptoms decreased consistently and gradually from February to June and then started to increase from July to December. The year-to-year wise comparison of disease index values represents that disease manifestations increased consistently throughout the study period from 2019 to 2021 in all the citrus cultivars and in all the study districts (Table. 4).

Table 2: Mean Cyclic threshold (Ct) values of *Candidatus Liberibacter asiaticus* in tested psyllid specimens and plant samples on five citrus cultivars throughout the year during 2020

Districts	Months	Citrus Cultivars									
		C. paradisi		***C. sinensis***		***C. reticulata***		***C. limon***		***C. limetta***	
		Ct value ACP	**Ct value Plant**	**Ct value ACP**	**Ct value Plant**	**Ct value ACP**	**Ct value Plant**	**Ct value ACP**	**Ct value Plant**	**Ct value ACP**	**Ct value Plant**
Faisalabad	January	14.92	15.60	14.27	18.14	21.23	22.33	15.23	20.21	19.31	19.58
	February	16.08	17.94	15.27	17.44	24.34	25.04	17.34	26.19	22.58	22.08
	March	18.31	22.51	17.23	22.08	25.23	24.57	17.64	20.26	24.27	26.12
	April	18.99	20.51	18.33	23.23	28.23	28.49	20.41	22.35	24.58	25.72
	May	20.19	21.45	19.32	22.08	30.47	29.72	23.08	24.08	25.96	26.80
	June	21.64	23.37	21.28	23.09	34.33	30.40	26.09	26.25	27.53	28.48
	July	19.09	21.14	19.32	20.14	28.25	28.61	19.39	23.74	24.61	23.12
	August	19.85	20.28	19.00	21.09	26.28	28.44	19.08	25.09	24.07	25.45
	September	18.91	19.45	18.28	21.09	27.69	27.25	18.24	25.07	23.69	24.43
	October	17.74	18.45	17.36	20.40	27.28	26.39	17.71	24.37	23.56	27.39
	November	16.32	17.09	16.39	19.42	26.31	25.83	17.50	23.07	21.63	25.73
	December	15.53	16.30	15.34	18.97	22.40	24.63	16.34	21.32	20.59	20.41
Toba Tek Singh	January	17.47	17.55	16.64	19.50	23.45	26.56	17.16	22.19	21.83	20.04
	February	18.24	16.53	17.28	19.52	27.45	30.42	19.53	27.04	24.67	24.37
	March	20.97	20.40	19.51	24.86	30.17	30.42	20.22	22.07	25.83	29.58
	April	21.13	22.07	20.25	23.08	30.17	31.80	21.27	24.22	26.61	28.39
	May	21.32	22.07	21.37	24.58	32.19	32.45	25.28	26.17	28.78	29.67
	June	24.42	24.22	23.58	25.94	36.36	30.66	28.21	28.28	29.55	30.33
	July	22.20	22.58	21.56	23.33	30.34	29.44	21.08	25.12	26.83	26.65
	August	21.21	22.22	20.98	22.20	29.69	28.61	21.16	27.34	27.75	25.42
	September	19.58	23.35	20.38	24.08	29.05	30.13	20.39	26.72	26.15	27.80
	October	19.44	21.30	19.53	21.08	28.34	27.08	19.53	26.08	25.75	24.77
	November	18.86	20.20	18.60	20.85	26.09	26.39	19.66	24.15	23.62	22.23
	December	13.12	19.47	17.49	20.16	24.12	24.63	18.47	23.16	22.73	21.09
Sargodha	January	14.55	14.30	13.07	14.07	19.11	22.54	12.46	18.39	17.89	18.56
	February	17.38	12.40	13.88	15.52	22.09	24.79	15.21	24.51	21.23	20.94
	March	18.69	19.46	16.59	20.33	24.07	22.29	16.08	18.16	22.06	25.28

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	April	19.43	14.45	17.48	19.47	26.07	26.15	17.88	20.20	23.53	24.50
	May	21.39	16.42	18.54	20.66	28.15	27.61	19.83	22.29	25.53	25.22
	June	19.06	20.32	20.30	21.04	32.07	28.48	24.72	24.16	26.07	26.30
	July	18.38	20.44	18.54	18.58	26.08	27.08	19.64	23.29	23.75	24.60
	August	18.13	18.58	17.94	17.37	24.24	26.83	17.20	21.18	23.06	23.52
	September	16.39	19.42	17.37	19.64	25.25	25.94	16.07	22.08	22.69	22.66
	October	15.48	18.25	16.52	16.69	24.66	25.04	16.42	23.19	21.42	21.50
	November	14.41	17.46	15.09	16.13	24.08	24.37	15.21	20.29	19.97	20.20
	December	13.12	15.37	14.12	15.22	20.18	24.38	14.26	19.07	19.13	19.44

Table 3: Mean Cyclic threshold (Ct) values of *Candidatus Liberibacter asiaticus* in tested psyllid specimens and plant samples on five citrus cultivars throughout the year during 2021

Districts	**Months**	**Citrus Cultivars**									
		C. paradisi		***C. sinensis***		***C. reticulata***		***C. limon***		***C. limetta***	
		Ct value ACP	**Ct value Plant**	**Ct value ACP**	**Ct value Plant**	**Ct value ACP**	**Ct value Plant**	**Ct value ACP**	**Ct value Plant**	**Ct value ACP**	**Ct value Plant**
Faisalabad	January	17.46	18.51	16.31	18.66	26.41	25.04	18.31	27.58	23.78	23.91
	February	19.45	23.24	18.47	23.25	27.51	28.78	18.84	21.39	24.48	28.67
	March	20.17	22.07	19.31	22.23	29.36	29.61	20.34	26.40	25.22	26.80
	April	20.24	21.42	19.42	24.58	29.44	29.18	21.21	23.39	25.61	27.67
	May	21.28	22.40	20.25	23.67	31.56	30.21	24.39	25.35	26.81	27.41
	June	22.32	23.95	22.41	24.46	35.65	31.22	27.52	27.58	28.36	29.58
Toba Tek Singh	January	19.43	17.45	18.48	20.27	28.69	27.36	20.17	28.55	25.12	25.81
	February	20.16	24.08	20.20	25.13	29.41	30.76	21.41	23.36	26.33	30.38
	March	21.82	23.06	20.56	24.44	31.31	31.29	22.19	28.50	27.50	28.61
	April	21.28	21.41	21.13	24.88	31.43	31.42	22.91	25.50	27.58	29.74
	May	22.08	23.51	22.33	26.06	33.65	32.69	26.40	27.53	29.69	30.54
	June	25.44	25.08	24.46	26.98	37.02	33.36	29.52	29.55	30.42	31.56
Sargodha	January	15.28	13.32	14.66	16.22	25.39	27.90	16.32	25.43	22.39	21.66
	February	18.41	20.36	17.63	21.50	25.52	23.61	17.55	19.58	23.16	26.56
	March	19.29	15.36	18.54	20.25	27.41	27.33	18.48	21.34	24.50	25.63
	April	19.41	19.55	18.66	20.25	27.61	27.96	18.47	24.61	24.66	25.87
	May	20.34	17.36	19.58	21.45	29.55	28.37	20.67	23.52	26.61	26.66
	June	22.38	21.32	21.50	22.36	33.53	29.69	25.51	25.50	27.46	27.75

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Table 4: Mean disease index (DI) values of surveyed symptomatic citrus plants on five citrus cultivars for two and a half years period

Districts	Months	Citrus Cultivars														
		C. paradisi			*C. sinensis*			*C. reticulata*			*C. limon*			*C. limetta*		
		2019	2020	2021	2019	2020	2021	2019	2020	2021	2019	2020	2021	2019	2020	2021
Faisalabad	January	3.01	3.13	3.24	3.15	3.27	3.38	1.34	1.49	1.66	3.08	3.26	3.43	3.17	3.31	3.39
	February	2.67	2.77	2.87	2.98	3.05	3.17	1.16	1.24	1.36	2.87	2.96	3.13	2.90	2.97	3.07
	March	2.15	2.26	2.45	2.57	2.75	2.88	1.08	1.16	1.26	2.54	2.66	2.85	2.46	2.67	2.88
	April	1.93	2.04	2.16	2.20	2.47	2.67	0.86	0.96	1.08	2.15	2.37	2.48	2.13	2.47	2.67
	May	1.51	1.68	1.84	1.96	2.09	2.16	0.69	0.75	0.92	1.97	2.06	2.15	1.96	2.13	2.26
	June	1.67	1.77	1.96	1.76	1.83	1.94	0.79	0.83	0.84	1.76	1.82	1.92	1.46	1.55	1.90
	July	1.90	1.98	-	2.03	2.12	-	0.89	0.93	-	1.97	2.06	-	1.69	1.77	-
	August	2.13	2.16	-	2.27	2.35	-	0.98	1.07	-	2.21	2.27	-	1.95	2.06	-
	September	2.27	2.35	-	2.65	2.76	-	1.14	1.19	-	2.51	2.63	-	2.25	2.35	-
	October	2.74	2.84	-	2.88	2.94	-	1.29	1.32	-	2.76	2.83	-	2.55	2.64	-
	November	2.97	3.05	-	3.10	3.16	-	1.45	1.51	-	3.02	3.10	-	2.75	2.85	-
	December	3.18	3.26	-	3.36	3.42	-	1.59	1.65	-	3.23	3.34	-	3.32	3.40	-
Sargodha	January	3.13	3.27	3.42	3.24	3.37	3.45	1.65	0.40	0.47	3.15	3.29	3.44	3.15	3.24	3.35
	February	3.03	3.08	3.16	3.02	3.13	3.24	1.46	0.52	0.60	3.06	3.15	3.24	2.92	2.97	3.08
	March	2.67	2.76	2.87	2.64	2.87	2.97	1.27	0.62	0.76	2.77	2.88	3.06	2.48	2.76	2.89
	April	2.37	2.48	2.67	2.30	2.53	2.87	1.14	0.76	0.85	2.45	2.47	2.67	2.25	2.54	2.77
	May	2.16	1.42	1.72	1.85	1.95	2.17	0.98	0.84	0.94	2.15	2.22	2.37	1.96	2.14	2.33
	June	1.76	1.83	1.98	2.15	2.18	2.60	0.87	0.94	1.07	1.76	1.84	1.96	1.67	1.74	1.88
	July	1.96	2.04	-	2.46	2.52	-	0.98	1.03	-	1.97	2.06	-	1.92	1.53	-
	August	2.18	2.23	-	2.77	2.83	-	1.14	1.19	-	2.30	2.38	-	2.17	2.22	-
	September	2.36	2.40	-	3.06	3.13	-	1.27	1.31	-	2.53	2.62	-	2.53	2.63	-
	October	2.53	2.59	-	3.27	3.33	-	1.45	1.50	-	2.76	2.83	-	2.79	2.86	-
	November	2.83	2.87	-	3.49	3.54	-	1.57	1.64	-	3.03	3.08	-	3.15	3.24	-
	December	3.20	3.25	-	3.77	3.85	-	1.74	1.81	-	3.35	3.42	-	3.57	3.64	-
Toba Tek Singh	January	2.87	2.95	3.07	3.05	3.13	3.48	1.25	1.35	1.46	2.39	2.47	2.58	2.48	2.56	2.78
	February	2.56	2.77	2.85	2.78	2.94	3.16	1.13	1.18	1.27	2.08	2.25	2.42	2.11	2.23	2.36
	March	2.15	2.45	2.58	2.45	2.63	2.87	1.03	1.07	1.16	1.87	1.97	2.06	1.97	2.10	2.21
	April	1.95	2.15	2.25	2.14	2.34	2.48	0.93	0.98	1.08	1.47	1.74	1.88	1.67	1.77	1.96
	May	1.66	1.87	1.95	1.84	1.95	2.14	0.81	0.88	0.96	1.17	1.37	1.57	1.37	1.53	1.74
	June	1.33	1.37	1.64	1.63	1.83	1.96	0.69	0.75	0.83	1.32	1.43	1.48	1.25	1.41	1.55
	July	1.54	1.62	-	2.03	2.08	-	0.79	0.84	-	1.57	1.63	-	1.53	1.62	-
	August	1.77	1.84	-	2.27	2.33	-	0.89	0.97	-	1.78	1.86	-	1.76	1.84	-
	September	1.96	2.04	-	2.49	2.50	-	1.06	1.07	-	2.07	2.14	-	1.97	2.08	-
	October	2.25	2.30	-	2.70	2.74	-	1.16	0.94	-	2.34	2.44	-	2.18	2.26	-
	November	2.66	2.72	-	3.05	3.15	-	1.28	1.34	-	2.65	2.74	-	2.53	2.65	-
	December	2.97	3.05	-	3.27	3.35	-	1.40	1.45	-	2.96	3.07	-	2.84	2.91	-

DISCUSSION

In this study, we monitored the CLas bacterium titer in psyllid specimens and symptomatic leaf samples and assessed the impact of heat on CLas titer over time. The citrus producers in Pakistan are curiously looking for better plant health and subsequent better citrus yields because per hectare yield and plant life is about 40% less than top citrus growers in the world [12]. There are several reasons behind the reduced citrus plant life including intercultural with other crops, climatic extremes, pest and disease incidence, low-quality rootstocks and nursery plants, and poor-quality soil. In comparison with non-infested citrus trees, insect pests increase the chances of a citrus orchard's decline by up to 52% [25]. Different cultural practices and deep ploughing cause root injury and subsequent pathogen transmission. Irregular fertilizer applications and other orchard management practices affect the timings of flush growth presence and subsequently the buildup of psyllid populations. The peak psyllid populations during the spring and autum

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seasons are the main factors behind bacterium transmission in citrus plantations [15]. The greening bacterium (CLas) titer was assessed using qPCR technique from psyllid specimens collected from different citrus cultivars as well as from incised midrib portion of the symptomatic leaves collected from the infected citrus plants and it was found that maximum Ct values were found during summer months (May-June) which represented the minimum disease due to high prevailing temperatures because these months have highest temperature > 40°C especially during the day which is enough to reduce bacterium presence in the citrus cultivars. When the temperature decreased from July onward then the proteobacterium started to multiply and disease incidence increased during the autumn (September-October) and reached maximum during winter months (December-January). The impact of heat also represented in different study districts because somewhat hotter district Toba Tek Singh showed less disease symptoms while the district Sargodha documented maximum disease incidence which is represented in low Ct values (low Ct value represented the high concentration of the CLas bacterium). The experiment was also confirmed by the disease index experiment in which visual symptoms noted on the citrus leaves which were maximum during the autumn and winter months while minimum during the hot summer months. The results of our studies are consistent with the findings of the [18] who reported the increased disease severity of the during study period in Brazil. Similarly, Ct values significantly varied across the months, locations, and years. Similarly, the study of greening titer in different citrus cultivar in India reported the maximum Ct values in *C. reticulata* and these results supported our research findings [26]. Another study documented the effect of thermotherapy on bacterium acquisition, transmission and its titer in psyllid specimens and infected leaves. This study reported that temperature > 40°C reduced the bacterium to minimum but bacterium was detectable after exposure to higher temperatures under lab conditions. These findings confirmed our results because the lowest bacterium presence was recorded during May-June [27]. Another investigation reported that heat therapy is applicable in reducing greening bacterium titer during summer months. These results confirmed our research findings that heat reduced greening bacterium titers during hot summer months [28]. An important study in Punjab, Pakistan reported reduced disease incidence which was considered was a main reason behind the survival of citrus industry during summer months and these results were in consistent with our results [12]. The overall results expressed that natural climatic conditions during summer months were effective for reducing greening bacterium titer in Pakistan, but the bacterium is still detectable. Likewise, the highest Ct values were reported in *C. reticulata* which indicated that this variety was less susceptible to this bacterium as well as psyllid infestations. This reason may possibly be one of the main reasons behind the success of Kinnow mandarin (*C. reticulata*) and survival of citrus industry in Pakistan. By reducing psyllid infestations can further reduce psyllid infestations on the citrus and subsequent bacterium transmission during psyllid feeding.

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4. CONCLUSION

The citrus is a serious citrus disease in Pakistan and around the globe. There are number of reasons behind this bacterium transmission including psyllid vector transmission. The Ct value using qPCR is an important parameter to indicate the CLas titer. Maximum Ct values were observed during the summer months with minimum disease incidence while lowest Ct values were reported during winter months which indicates the highest disease incidence. Naturally high temperatures were effective in greening titer reduction only during the summer months. These findings may be helpful in reducing bacterium titer throughout year which may be of assistance in IPM-based psyllid vector and greening disease management.

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