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COMPARATIVE ANALYSIS OF FLAVONOIDS IN *XANTHOMONAS AXONOPODUS* L. AFFECTED CITRUS VARIETIES OF PUNJAB, PAKISTAN

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A B S T R A C T

The current study examined the correlation between the prevalence of *Xanthomonas axonopodis* L. and flavonoid content in the leaves and pulp of various citrus cultivars (both healthy and *Xac*-infected) across different regions of Pakistan. The study considered environmental factors such as temperature (°C), relative humidity (%), total precipitation (mm), and wind speed (km/h). Flavonoid levels were quantified using liquid chromatography-mass spectrometry. Statistical analysis revealed the presence of 12 different flavonoids in the citrus cultivars. The concentrations of these flavonoids were found to be lower in *Xac*-infected plants compared to healthy ones. Additionally, the amounts of each flavonoid varied under different climatic conditions. The findings of this study could be utilized to promote the commercialization of citrus varieties that are either resistant to *Xac* infection or possess high flavonoid content.

Keywords: *Xanthomonas axonopodis* pv. *citri,* Citrus canker, *Rutaceae,* Citrus, Flavonoids.

INTRODUCTION

Citrus, a pivotal fruit crop within the *Rutaceae* family, holds significant importance globally in terms of production and trade. The quality of citrus fruits varies across countries due to diverse climatic conditions and cultivars. Despite its global prominence, citrus faces the devastating citrus canker disease, affecting almost all commercial varieties (Gmitter *et al.,*1990; Liu *et al.,* 2020). This gram-negative, aerobic, rod-shaped bacterium (*Xanthomonas axonopodis* pv. *citri*) originates from South Asian countries and annually destroys 90% of citrus in Pakistan due to inadequate management and postharvest treatments (Naveed *et al.,* 2023).

Citrus canker remains a significant threat in Pakistan, caused by *X. axonopodis* pv. *citri* (*Xac*), which causes substantial economic losses, reducing export value and fruit production. The disease manifests through lesions on leaves and fruits, leading to premature fruit drop and other economic losses (Siddique *et al.* 2017). Citrus canker's impact is e*Xac*erbated by the bacterium's ability to survive for several months. The disease spread is influenced by environmental factors and can be increased by the citrus leaf miner insect. *X. axonopodis* pv. *citri* is classified into three main groups based on various pathovars, with distinct types affecting different citrus varieties. Understanding and managing citrus canker is crucial for sustaining the industry's growth and minimizing economic losses (Faostat, 2016).

Plant metabolite profiling is a valuable tool for understanding the structure, function, and biosynthetic pathways of metabolites, providing insights into how plants respond to both biotic and abiotic stressors. Recent studies have emphasized the impact of *X. axonopodis* pv. *citri* (*Xac*) infection on the metabolome, specifically on flavonols, flavones, and flavonoids (Canteros *et al.,* 2017; Singh and Prevalence, 2014). Despite numerous investigations into citrus canker disease, the mechanisms underlying citrus infection by *Xac* remain unclear. Deciphering these mechanisms is essential for developing economically important *Xac* -infected varieties.

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Metabolomics, as demonstrated in this study, holds diagnostic potential for distinguishing between healthy and infected cases and offers insights into the pathogenic mechanisms for future studies (Derso *et al.,* 2007). Focusing on flavones and flavanones, major flavonoid groups in the citrus family, this study explores their role in determining the bitter taste in citrus fruits. Naringenin, a dominant flavonoid backbone, undergoes various modifications to form derivatives like hesperetin. Bitter compounds such as naringin and limonoid (nomilin and limonin) contribute to the bitter taste in citrus fruits[17-21]. Citrus fruits, rich in essential nutrients like vitamins B and C, sugars, niacin, thiamin, copper, riboflavin, potassium, carbohydrates, and calcium, play a vital role in human health. The study highlights the nutritional richness of edible citrus parts, particularly in vitamin C2. Orange juice, for example, contains titrable acidity, sugars, vitamin C, and citric acid (Roeschlin *et al.* ,2017; Li *et al.,* 2021).

The study not only explores the physical parameters influencing citrus canker prevalence in different citrus-growing areas of Punjab but also hypothesizes that higher flavonoid levels may be present in young fruit/leaves or expanding stages, decreasing in concentration as fruit/leaves mature. To test this hypothesis, flavonoid profiling was conducted in fifteen citrus *Xac*-infected cultivars compared to healthy cultivars using Liquid Chromatography-Mass Spectrometer (LC-MS) samples collected from various regions of Punjab. The findings have the potential to contribute to the production of high-quality citrus, aiding in overcoming citrus canker disease in Pakistan.

MATERIAL AND METHODS

The current study described the diagnostic information of citrus infected with *X. axonopodius* Quantitative PCR technique was used for the identification of *Xac* from HLB-symptomatic and asymptomatic citrus samples compared with the healthy (control). Global Spectroscopy- Mass Spectroscopy (LC-MS) analysis were directed to identify the secondary metabolites from healthy and *Xac*-affected citrus varieties. Healthy and *Xac*-infected varieties of citrus plants were collected from different regions of Punjab as mentioned in Table 1.

The DNA extraction procedure followed was based on the method outlined by Llop *et al.* (1990). Total DNA was extracted from both pure bacterial suspensions and lesions present on citrus peel pulp and leaves. RNA removal and subsequent DNA extraction were conducted according to the Qiagen protocol supplied with the kit (Qiagen Inc., Valencia, CA). Bacterial suspensions at a concentration of 10^8 CFU ml−1 were obtained from the lesions, and 1000 µl of these suspensions were centrifuged at 8000 g for 5 minutes. The resulting pellet was re-suspended in 500 µl of extraction buffer (composed of 0.2 mol l−1 Tris–HCl pH 7.5, 0.25 mol l−1 NaCl, 0.025 mol l−1 EDTA, 0.5% SDS, 0.5% PVP, and sterilized by filtration) and shaken for 60 minutes at room temperature. Subsequently, the suspension was centrifuged at 1000 g for 5 minutes, and 450 µl of the supernatant was mixed with 450 µl of isopropanol, allowing it to stand for 30 minutes at room temperature. Following centrifugation at 8000 g for 10 minutes, the supernatant was discarded, and the pellet was air-dried before being resuspended in 100 µl of sterile water (Golmohammad *et al.* 2007).

Real-time PCR was conducted using SYBR Green and a TaqMan probe, as described by Cubero and Graham, (2007). The assays were carried out in a final volume of 25 µl, comprising PCR universal master mix (Quantimix SYBR Green or Quantimix Easy Probes; Biotools), 10 µmol l−1 of primers J-RTpth3 and J-RTpth4, and 5 µmol l−1 of TaqMan probe (J-Taqpth2b). Two microliters of extracted DNA from leaf samples, along with water control and positive control, were included. The amplification, detection, and data analysis processes were executed, and the thermal profile for both SYBR Green and TaqMan probe involved an initial activation step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15s and 60°C for 1 minute.

For metabolomic analysis, samples were extracted using a 1:1 (v: v) mixture of methanol and 10 mM ammonium acetate. After vortexing and centrifugation, the supernatant was transferred to Liquid Chromatography vials for analysis. All samples were initially run in positive mode followed by negative mode. The sequence commenced with three blanks, followed by a neat quality control (QC). Between each sample's technical replicates, another blank and neat QC were run. Global metabolomics profiling was conducted using a Thermo Q-E*Xac*tive Oribtrap mass spectrometer with Dionex UHPLC and autosampler. Analysis was performed in positive and negative heated electrospray ionization modes, with a mass resolution of 35,000 at m/z 200 using polarity switching. Separation was achieved on an Angiotensin I-Converting Enzyme, 2 18-pfp with 100x2.1mm, 2µm column, with mobile phase A as 0.1% formic acid in water

and mobile phase B as acetonitrile. The flow rate was 350 µL/min, and a 4 µl sample was injected.

Statistical analyses were carried out using Microsoft Excel and SAS (https://www.sas.com/ en_us/ software/ stat.html).

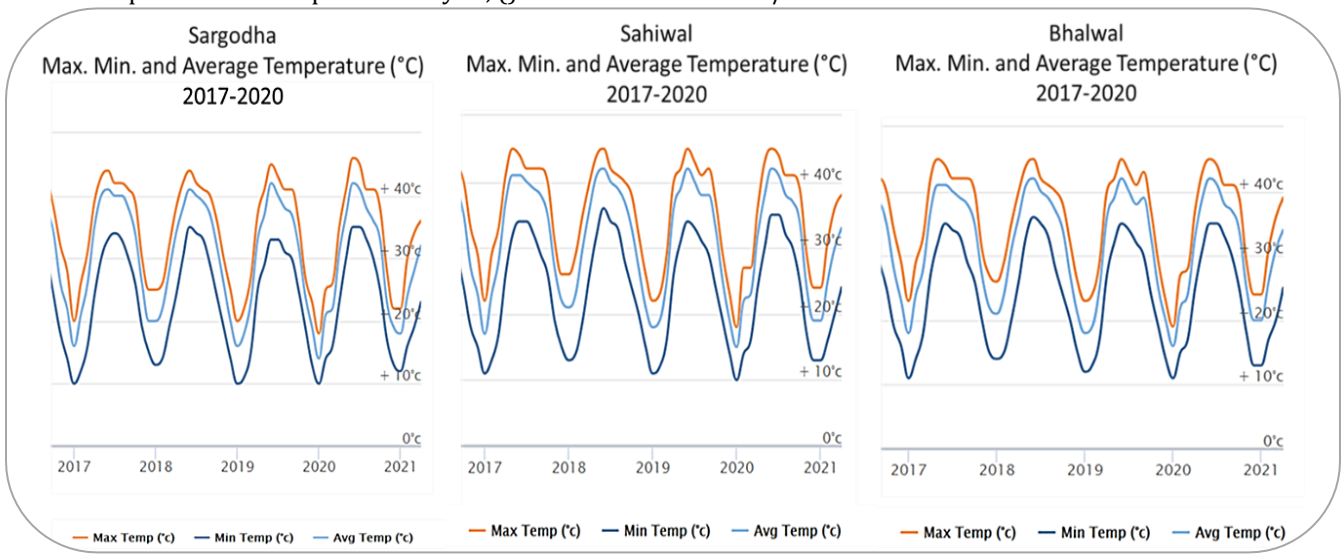
RESULTS

In different citrus cultivars in different areas of Sargodha, Sahiwal and Bhalwal, Punjab, Pakistan showed that at temperature of 33- 42 °C,43- 58 % relative humidity, with total precipitation of 174.41- 179 mm and maximum wind speed of 15.5- 18.6 Km/h), *Xac* incidence was 94.01- 95.98 % while *Xac* index was 27.38- 29.17 % (Table 1; Figure 1). The study of Khan *et al.* (2020) also supported our findings.

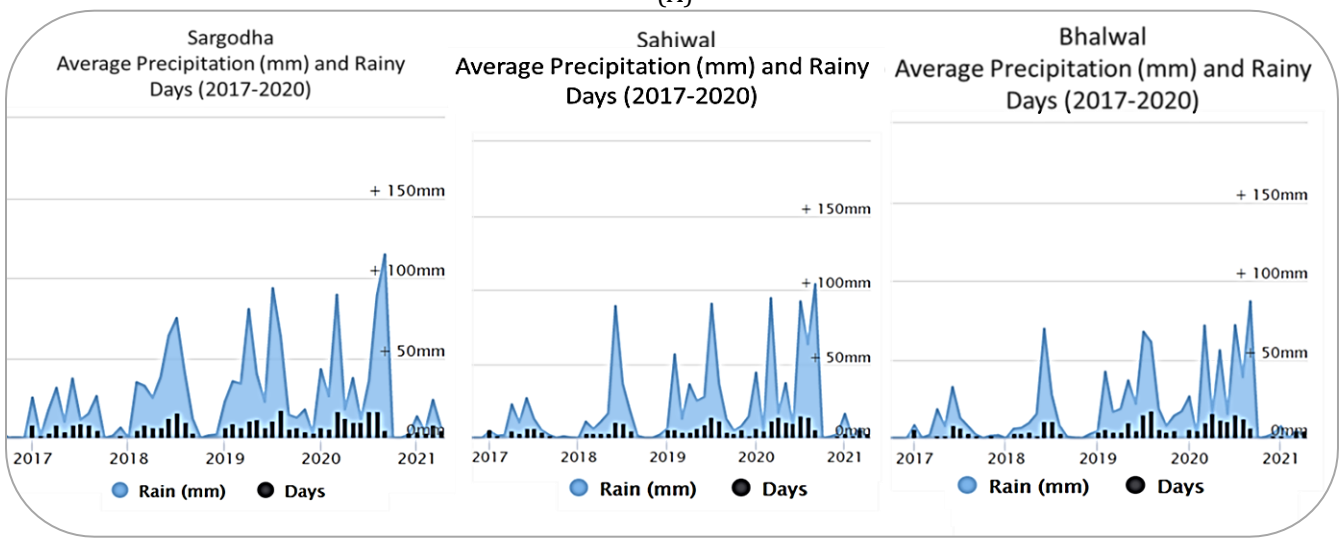
Table 1. Correlation of environmental factor on *Xac* Index and Incidence on citrus cultivars from 2017- 2020

Cities	Annual Temperature (°C)			Total Precipitation	Max. wind	Relative Humidity	*Xac* index	*Xac* Incidence
	Average	Maximum	Minimum	(mm)	(Km/h)	(%)	(%)	(%)
Sargodha	41±0.71 (54.67)	44±1.10 (55)	34±1.00 (45.33)	175.99±1.79 (234.65)	17.025±1.10 (22.7)	50.5±5.32 (67.33)	28.26±0.64 (37.68)	94.78±0.81 (126.37)
Sahiwal	41.25±0.83 (55.00)	44±1.22 (58.67)	34.25±0.43 (45.67)	178.71±1.69 (238.28)	18.32±1.99 (24.43)	46.75±9.12 (62.33)	19.73±0.54 (26.31)	73.20±1.91 (97.61)
Bhalwal	44.75±0.43 (59.67)	35.25±0.43 (47.00)	174.71±0.75 (232.95)	20.025±2.91 (26.70)	43.5±7.70 (58.00)	23.59±0.49 (31.45)	71.9±0.59 (95.87)	41.75±0.43 (55.67)

±= Data expressed after triplicate analysis, ()= Data shows variance/ S^2



(A)



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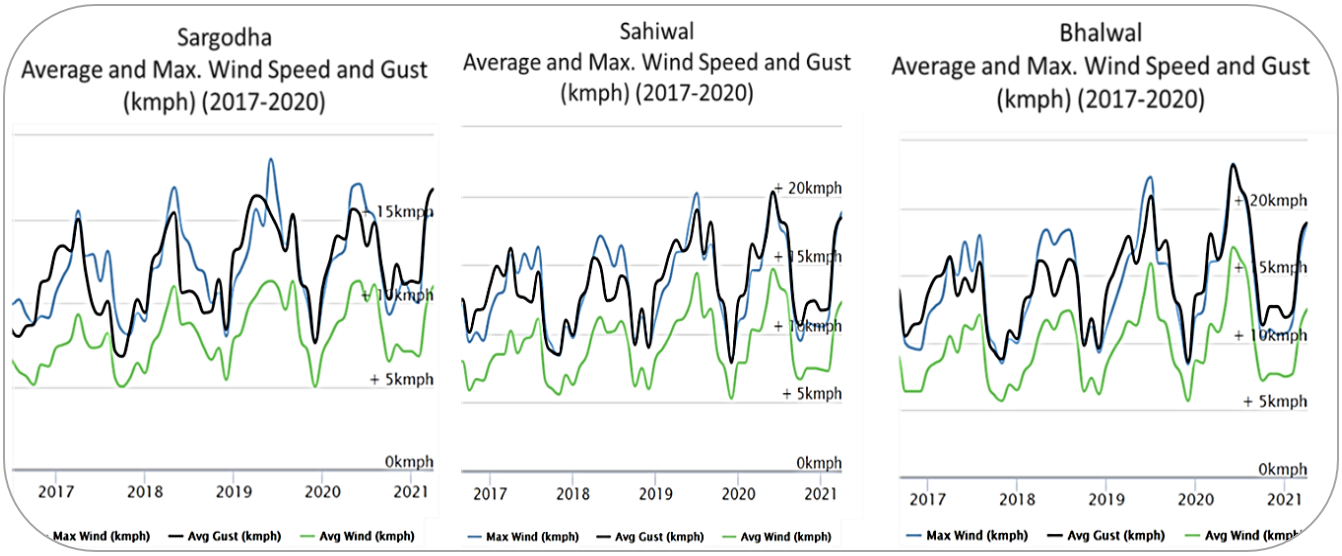

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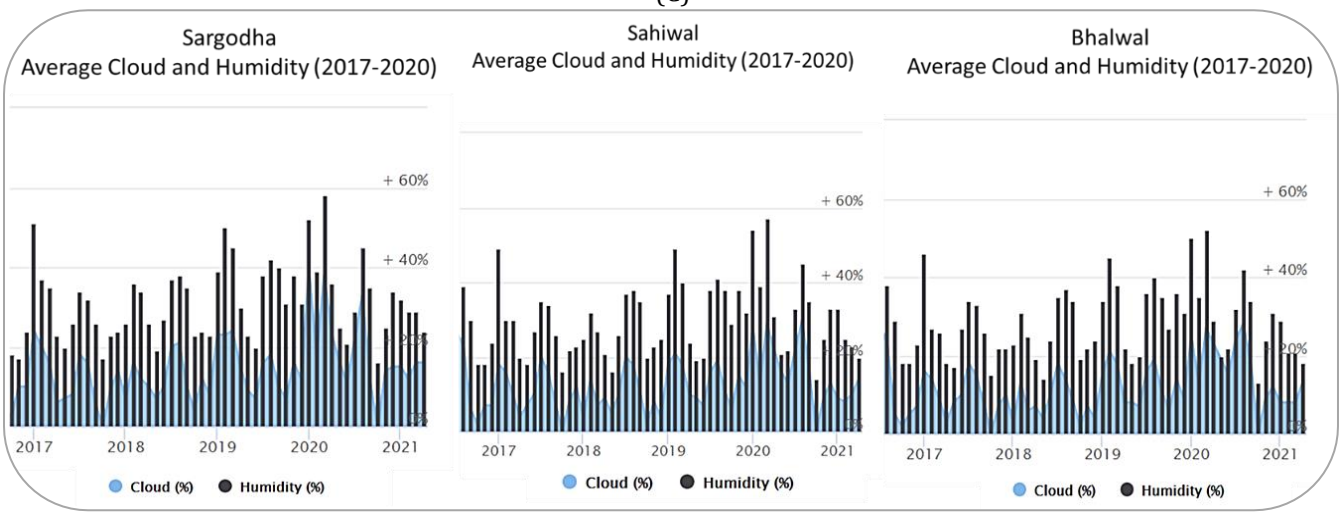

(D)

Figure 1. (A) Temperature (°C), (B) Precepitation (mm) and Rainy days, (C) Wind Speed and Gust (Km/h) and (D) Average Cloud and Humidity (%) in Sargodha, Sahiwal and Bhalwal from 2017-2020. Max= Maximum, Min= Minimum, Ave= Average, Temp= Temperature

Among the citrus-producing regions in Pakistan, Sargodha exhibits a higher incidence of *Xanthomonas axonopodis* infection. The occurrence of two types of bitterness (immediate and delayed) in citrus fruits is attributed to the presence of specific flavonoids, particularly neohesperidin and naringin, for immediate bitterness, and limonin of limonoids for delayed bitterness. The delayed bitterness is particularly pronounced when the fruit is mechanically damaged or undergoes certain processes, affecting citrus juices.

Flavones and flavanones, as major constituents of the citrus flavonoids, play a pivotal role in determining the bitterness in citrus fruits. The bitterness is primarily influenced by flavanone neohesperidosides. Naringenin, a dominant flavonoid backbone in certain citrus species, undergoes various modifications such as hydroxylation and methylation to produce derivatives like hesperetin, contributing to the bitter taste. In grapefruit, naringin is a major contributor to bitterness, while sour orange is characterized by neohesperidin as a key component. Limonoid, including nomilin and limonin, represents another major category of bitter compounds in citrus fruits.

Studies have indicated that limonoids generally accumulate at higher levels in young or expanding fruit stages, decreasing to very low concentrations or becoming undetectable in mature fruit, although exceptions to this trend have been observed. In the

current study, elevated concentrations of polyphenolic compounds, such as flavonoids (Eujambolin, 4-coumarate, Naringin, neohesperidin Flavone), were observed in infected leaves and pulp, with the exception of 4-Hydroxy-5,6,7,8-Tetramethoxy, which exhibited higher concentrations in healthy plants. This aligns with findings from a previous study that reported higher levels of phenols and polyphenols in infected citrus plants. Similarly, increased levels of hesperidin and neohesperidin were detected in *Xanthomonas axonopodis*-infected citrus leaves and pulp, consistent with findings from other researchers34,35.

Table 2. Content of flavonoids (mg/g) in healthy and *Xac*-Infected citrus samples of Pakistan.

Parts of plant	Flavonoids	Healthy	Infected	Tukey Pr>|t|
Leaves	4 –Coumarate	0.128±0.006	0.105±0.069	<0.327
	Eujambolin*	0.060±0.012	0.318±0.123	<0.0001
	Vanilloloside	0.187±0.031	0.278±0.198	<0.193
	4' -Hydroxy -5,6,7,8 -tetramethoxy flavone*	7.176±0.481	0.481±2.339	<0.0001
	Neohesperdin*	1.569±0.361	2.345±0.534	0.001
	Naringin*	1.847±0.877	2.545±0.425	0.015
Pulp	4 -Coumarate	0.020±0.009	0.018±0.006	<0.724
	Eujambolin	0.001±0.001	0.001±0.001	<0.825
	Vanilloloside*	0.250±0.105	0.523±0.155	<0.120
	4' -Hydroxy -5,6,7,8 - tetramethoxyflavone*	0.051±0.051	0.054±0.050	<0.907
	Neohesperdin*	1.923±0.243	2.541±0.091	2.87E-07
	Naringin*	0.363±0.197	0.555±0.245	0.016

Note: ±= Results obtained after triplicate analysis, and SD= Standard Deviation, Flavonoids marked with an asterisk (*) exhibit predominance in citrus samples between the healthy and *Xanthomonas axonopodis* (*Xac*)-infected conditions.

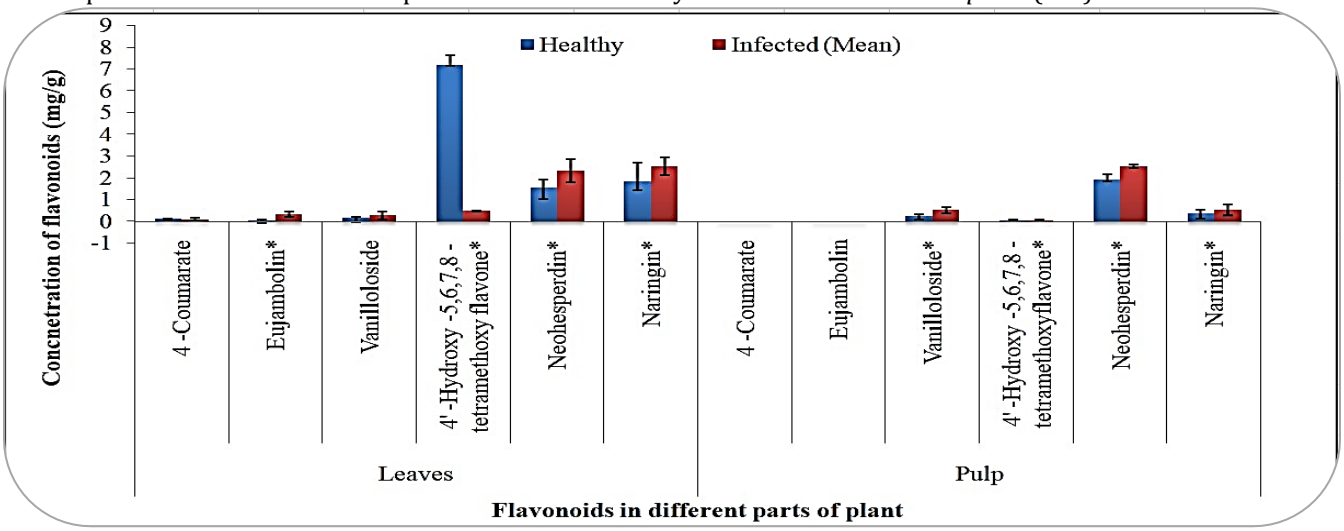

Figure 2. Comparison of flavonoids content in healthy and *Xac*-infected citrus pulp

DISCUSSION

Citrus is an important fruit crop constituting a wide range of species and famous for its nutritional value. Its cultivation is spread over a large area and it ranked first in fruit production of Pakistan. The major losses of citrus are done by various microbial diseases such as, citrus canker. It is caused by *Xanthomonas axonopodis* pv. *citri* which is the major threat to citrus production. Citrus leaf miner feed on the leaves which facilitate the spread of pathogen from roots to fruits. Citrus canker is severe disease which affects the local and international market badly. Citrus canker is caused by *Xanthomonas axonopodius* which is a gram negative, aerobic, rod-shaped bacterium. The favorable condition for citrus canker is high humidity and warm temperature. At early stage the growth of lesion starts from one side of leaves and then moves to other side of the leaf. Usually it takes 10 days to for the lesions to become prominent. Symptoms of citrus canker can be seen on leaves, stem and fruit of the plant. Flavonoids are the important class of natural products; particularly, they belong to a class of plant secondary metabolites having a polyphenolic

structure, widely found in fruits, vegetables and certain beverages. They are also known to be potent inhibitors for several enzymes, such as xanthine oxidase (XO), cyclo-oxygenase (COX), lipoxygenase and phosphoinositide 3-kinase. In nature, flavonoid compounds are products extracted from plants and they are found in several parts of the plant. Flavonoids are used by vegetables for their growth and defense against plaques. They have a place with a class of low-atomic weight phenolic intensifies that are generally appropriated in the plant realm.

This study comprises the data of citrus infected with *Xanthomonas axonopodis* in three main cities of Pakistan, named; Sargodha, Sahiwal and Bhalwal. The located regions of these three main cities were examined in this study are listed in Table 1. This study gives the information of *Xac* prevalence in different citrus cultivars in different areas of Sargodha, Sahiwal and Bhalwal from year 2017-2020) based on an average, maximum and minimum temperature (°C), relative humidity (%), total precipitation (mm), and maximum wind speed (kmph). The study of (Khan *et al.,* 2020) also support out findings.

There were many citrus producing areas present in Pakistan. However, Sargodha have found greater incidence percentage of *X. axonopodis.* With the prevalence of *Xac*-affected areas, we detect the flavonoid content in citrus cultivars. There are distinctive two types of bitterness seen in citrus fruit; the immediate bitterness and the delayed bitterness, dependent on the types of flavonoids (Panche *et al.,* 2016). The immediate bitterness is largely deliberated by neohesperidin and naringin, and the delayed bitterness is mostly manufactured by limonins of limonoids (Breska *et al.,* 2006). Delayed bitterness is steadily established upon fruit and mechanically damaged the fruit and citrus juices (Panche *et al.,* 2016). Flavones and flavanones are the major group of flavonoids in citrus family and the primary cause of bitterness in citrus fruit is determined by flavanone, neohesperidosides. The flavanone naringenin is the dominant flavonoid backbone in some citrus species, while it might undergo a variety of modifications to form other flavanones, for instance, hydroxylation and methylation to generate its derivative hesperetin (Jaganath and Crozier, 2011) for the bitter tasting. Naringin is the major contributor to the bitter taste in grapefruit and neohesperidin in sour orange. Limonoid is the other major type of bitter compound in addition to naringin in citrus fruit, including nomilin and limonin (Breksa *et al.,* 2006). Flavonoids play a crucial role in the defense mechanisms of citrus plants against citrus canker disease, primarily through their involvement in specific biochemical pathways that enhance plant resistance. These compounds are known for their antioxidant properties, which help mitigate oxidative stress induced by the pathogen *Xanthomonas axonopodis* pv. *citri*. Flavonoids such as naringin and neohesperidin accumulate in higher concentrations in infected tissues, suggesting their role in strengthening the plant's cell walls and inhibiting pathogen proliferation. Additionally, flavonoids can act as signaling molecules, triggering the activation of defense-related genes and the production of phytoalexins, which are antimicrobial substances that further deter pathogen invasion. By modulating these pathways, flavonoids enhance the overall resilience of citrus plants, reducing the severity and spread of citrus canker disease. A study has shown that generally accumulate relatively high flavonoid concentration at young fruit or fruit expanding stage and then fall to very low in concentration or under detectable levels in mature fruit but sometimes this theory was reversible (Wang and Liu, 2020; Kausik *et al.,* 2024).

The practical implications of the role of flavonoids in combating citrus canker disease are significant for citrus cultivation and agricultural practices. Understanding the defense mechanisms mediated by flavonoids can lead to the development of more resistant citrus cultivars through selective breeding or genetic engineering, enhancing crop resilience against *Xanthomonas axonopodis* pv. *citri*. By identifying and promoting cultivars with naturally higher flavonoid content, farmers can reduce the reliance on chemical pesticides, leading to more sustainable and eco-friendly farming practices. Moreover, the application of flavonoid-rich extracts or formulations as natural plant protectants could be an effective strategy for managing citrus canker. These natural treatments could serve as an alternative or supplement to traditional chemical treatments, potentially reducing environmental impact and improving fruit safety for consumers. Additionally, monitoring flavonoid levels in citrus plants could serve as an early diagnostic tool for detecting disease onset and severity, allowing for timely and targeted interventions. By integrating flavonoid analysis into routine agricultural practices, farmers can better

manage crop health, ultimately leading to improved yield, quality, and economic value of citrus fruits (Mabogoane *et al.,* 2024).

CONCLUSION

This study explores the bacterial disease citrus canker, its causal agent, symptomatology, and its prevalence in Pakistan. It also provides detailed information on the flavonoid content in different citrus cultivars from the regions of Sargodha, Sahiwal, and Bhalwal (Table 1). Additionally, the study examines the environmental parameters of these areas, including average, maximum, and minimum temperatures (°C), relative humidity (%), total precipitation (mm), and maximum wind speed (km/h) (Table 2). These parameters were also discussed by Khan *et al.*[29] in relation to the prevalence of *Xanthomonas axonopodis* pv. *citri*. The study concluded that the incidence and index of *Xanthomonas axonopodis* pv. citri were relatively higher in Sargodha compared to Sahiwal and Bhalwal, as shown in Table 2 for the years 2017-2020. It was found that the levels of naringin and neohesperidin were higher in *Xac*-infected leaves and pulp compared to healthy (control) samples, as illustrated in Table 3 and Figures 2-3. These findings are supported by several previous studies. [24,34,36]

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Khadija Gilani	:	Performed complete experimental analysis and wrote the first draft and give final approval for the publication
Rehana Badar	:	Revised the draft and give final shape to the manuscript
Nazia Kanwal	:	Revised the draft and provide technical support
Asma Ahmed	:	Provide technical support
Samina Khurshid	:	Provide technical support
Muqadas Asghar	:	Provide technical support