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Disease-Induced Changes in Biochemical Composition of Kinnow Plants (*Citrus nobilis x Citrus deliciosa*): A Comparative Study of Healthy and Citrus Canker-Infected Plants

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1. Abstract

Kinnow mandarin (*Citrus nobilis x Citrus deliciosa*) is a commercially important citrus cultivar, widely grown in the Indian subcontinent, particularly in northern India. However, like many citrus varieties, Kinnow plants are susceptible to various diseases that can severely impact crop yield and quality. One of the most devastating diseases affecting citrus crops worldwide is citrus canker, caused by the bacterium *Xanthomonas citri* subsp. *citri* (Xcc). In the current study, it was attempted to explore the selected biochemical parameters in comparative manner between healthy and infected plants. The study found that Kinnow plants infected with citrus canker exhibit significant biochemical changes, including reduced chlorophyll content, and increased levels of phenolic compounds, alkaloids, terpenoids, proline, MDA, and ROS. These changes suggest that the disease induces a complex response in the plants, affecting both primary and secondary metabolism. The observed biochemical alterations highlighted the plant's efforts to combat the infection and mitigate the stress caused by the pathogen.

Keywords: Kinnow mandarin, Citrus Canker, Plant metabolites.

1. Introduction

Kinnow mandarin (*Citrus nobilis x Citrus deliciosa*) is a commercially important citrus cultivar, widely grown in the Indian subcontinent, particularly in Pakistan and northern India (Sharma *et al.,* 2020). Known for its sweet flavour, easy-to-peel nature, and high juice content, Kinnow mandarins have gained significant economic importance in the region's horticultural sector (Khan *et al.,* 2015). However, like many citrus varieties, Kinnow plants are susceptible to various diseases that can severely impact crop yield and quality.

One of the most devastating diseases affecting citrus crops worldwide is citrus canker, caused by the bacterium *Xanthomonas citri* subsp. *citri* (Xcc) (Graham *et al.,* 2004). This disease is characterised by the formation of necrotic lesions on leaves, fruits, and stems, leading to defoliation, fruit drop, and overall decline in plant health (Gottwald *et al.,* 2002). Citrus canker is particularly problematic due to its rapid spread through wind-driven rain and its ability to survive in infected plant material, making eradication challenging once established in an area (Behlau *et al.,* 2010).

Studying the biochemical changes induced by citrus canker in Kinnow plants is crucial for several reasons. First, understanding the plant's response to pathogen infection at the molecular level can provide insights into the mechanisms of disease resistance and susceptibility (Talon and Gmitter, 2008). Second, identifying specific biochemical markers associated with infection can aid in early disease detection and monitoring (Martinelli *et al.,* 2016). Third, elucidating the changes in plant metabolism during infection can guide the development of targeted disease management strategies and inform breeding programs for disease-resistant cultivars (Killiny and Hijaz, 2016).

The biochemical composition of plants is known to undergo significant alterations in response to pathogen attack. These changes can include modifications in primary metabolites such as sugars and amino acids, which are essential for plant growth and development (Rojas *et al.,* 2014). Additionally, secondary metabolites, including phenolic compounds and flavonoids, often play a role in plant defence mechanisms (Zacarés *et al.,* 2007). Changes in enzymatic activities, hormone levels, and mineral content can also significantly impact the plant's ability to resist or succumb to disease (Albrecht and Bowman, 2008).

The current research seeks to conduct a comprehensive comparative biochemical analysis of healthy Kinnow plants and infected with citrus canker. Specifically, it takes to quantify and compare the levels of primary and secondary metabolites in healthy and infected Kinnow plants. The research work hypothesise that citrus canker infection will lead to significant alterations in the primary metabolite and secondary metabolites profile of Kinnow plants.

By elucidating these disease-induced biochemical changes, this study aims to contribute to our understanding of the Kinnow plant's response to citrus canker and provide valuable insights for developing improved disease management strategies and breeding programmes.

2. Materials and Methods

2.1 Study Area and Plant Material

The study was conducted in Sri Ganganagar, Rajasthan, India and sampling was performed in the 5 marked Kinnow Orchards (sampling sites) between the year 2021 to 2022. Healthy and citrus canker-infected Kinnow mandarin (*Citrus nobilis x Citrus deliciosa*) plants were selected from these orchards.

Before the sampling, the disease confirmation was performed through visual inspection of characteristic canker lesions on plant leaves (Mavrodieva *et al.,* 2004).

2.2 Sample Collection and Preparation

In the sample collection, leaf samples were collected from both healthy (10 leaves) and infected plants (10 leaves) at each sampling site, ensuring similar age and leave position on the plant. Further, in the field, samples were marked and immediately transferred to ice box. In the lab the leaves were stored in the refrigeration facility at -12°C to protect from any water loss and biochemical changes until further analysis. Prior to each biochemical assay, samples were homogenised by using glass homogeniser.

2.3 Biochemical Analyses

2.3.1 Total Chlorophyll Content

Total Chlorophyll content was determined using the method described by Lichtenthaler and Buschmann (2001). In which, 0.1 g of fresh leaf tissue was homogenized in 10 ml of 80% acetone. The homogenate was centrifuged at 3,000 rpm for 5 minutes, and the absorbance of the supernatant was measured at 663 nm and 645 nm using a UV-visible spectrophotometer.

2.3.2 Total Phenolic Content

Total phenolic content was measured using the Folin-Ciocalteu method (Singleton *et al.,* 1999). Fresh leaf samples (0.5 g) were extracted with 80% methanol, and the extract was reacted with Folin-Ciocalteu reagent. Absorbance was measured at 765 nm, and results were expressed as µg gallic acid equivalent per gram of fresh weight.

2.3.3 Protein Content

Protein content was determined using the Bradford method (Bradford, 1976). Fresh leaf tissue (0.5 g) was homogenised in phosphate buffer (pH 7.0), and the extract was reacted with Bradford reagent. Absorbance was measured at 595 nm, and bovine serum albumin was used as a standard.

2.3.4 Total Alkaloid Content

Total alkaloid content was measured using the method described by Sreevidya and Mehrotra (2003). Fresh leaf samples were extracted with ethanol, and the extract was reacted with Bromocresol Green solution. Absorbance was measured at 470 nm.

2.3.5 Terpenoid Content

Terpenoid content was determined using the method of Ghorai *et al.* (2012). Fresh leaf samples were extracted with methanol, and the extract was mixed with vanillin-sulfuric acid reagent. Absorbance was measured at 608 nm.

2.3.6 Proline Content

Proline content was measured using the Ninhydrin method (Bates *et al.,* 1973). Fresh leaf samples (0.5 g) were homogenised in Sulfosalicylic acid and reacted with acid Ninhydrin. Absorbance was measured at 520 nm.

2.3.7 Malondialdehyde (MDA) Content

Lipid peroxidation was assessed by measuring MDA content using the Thiobarbituric acid (TBA) method (Heath and Packer, 1968). Fresh leaf tissue (0.5 g) was homogenised in trichloroacetic acid and centrifuged. The supernatant was mixed with TBA and heated. Absorbance was measured at 532 nm and 600 nm.

2.3.8 Reactive Oxygen Species (ROS)

The Reactive Oxygen Species (ROS) were viewed as the amount of H_2O_2 content. Thus, H_2O_2 content, as a measure of ROS, was determined using the method of Velikova *et al.* (2000). Fresh leaf samples (0.5 g) were homogenised in trichloroacetic acid and centrifuged. The supernatant was mixed with potassium phosphate buffer and potassium iodide. Absorbance was measured at 390 nm.

2.4 Statistical Analysis

Data were analysed using Student's t-test to compare healthy and infected samples. Statistical significance was set at $p < 0.05$. Analysis was performed using *PaSt* software (version 4.17) (Hammer *et al.,* 2001; Hammer & Harper, 2024).

3. Observations

The investigation of the biochemical parameters in healthy Kinnow plants and citrus canker infected plants revealed the subsequent observation (Table-1). The study captured the eight biochemical parameters at five different sampling sites with the combination of healthy and infected plants. To develop the deductions, average value of particular biochemical parameter for healthy and infected plats were also calculated.

Table 1: Biochemical parameters of healthy and infected plants at different sampling sites

S.N.	Biochemical Parameter		S-1	S-2	S-3	S-4	S-5	Average
1.	Total Chlorophyll Content (μg/g)	Healthy Plant	680	730	690	670	610	676
		Infected Plant	360	350	450	410	420	398
2.	Total Phenolic Content (μg GAE/g)	Healthy Plant	33.0	39.50	33.50	36.0	41.50	36.7
		Infected Plant	51.50	48.50	48.0	54.50	51.0	50.7
3.	Protein Content (μg/g)	Healthy Plant	1.25	1.15	1.10	1.10	1.10	1.14
		Infected Plant	1.30	1.20	1.10	1.05	1.10	1.15
4.	Total Alkaloid Content (μg/g)	Healthy Plant	2.20	2.10	2.10	2.0	2.0	2.08
		Infected Plant	3.40	3.10	2.90	2.90	2.80	3.02
5.	Terpenoid Content (μg/g)	Healthy Plant	840	730	790	650	660	734
		Infected Plant	890	820	890	730	950	856
6.	Proline Content (μg/g)	Healthy Plant	3.30	2.90	2.50	2.60	3.10	2.88

		Infected Plant	7.10	5.20	4.80	4.80	6.50	5.68
7.	Malondialdehyde (MDA) Content (µg/g)	Healthy Plant	0.02	0.01	0.01	0.01	0.01	0.012
		Infected Plant	0.02	0.02	0.02	0.01	0.01	0.016
8.	Reactive Oxygen Species (ROS) (µmol/g)	Healthy Plant	2.20	1.65	1.50	2.0	1.40	1.75
		Infected Plant	5.10	3.70	3.30	4.10	4.0	4.04

4. Result and Discussion

This study examined the biochemical modifications in Kinnow plants caused by citrus canker disease, comparing key biochemical parameters between healthy and infected plants (Table-1). The data reveal significant alterations in several biochemical markers, indicating a profound impact of the disease on the metabolic processes of plants (Figure-1, 2, 3, and 4). Further, these elevated parameters were examined for statistical significance through t-test. The t-values and significance of the biochemical tests were highlighted in the table (Table-2).

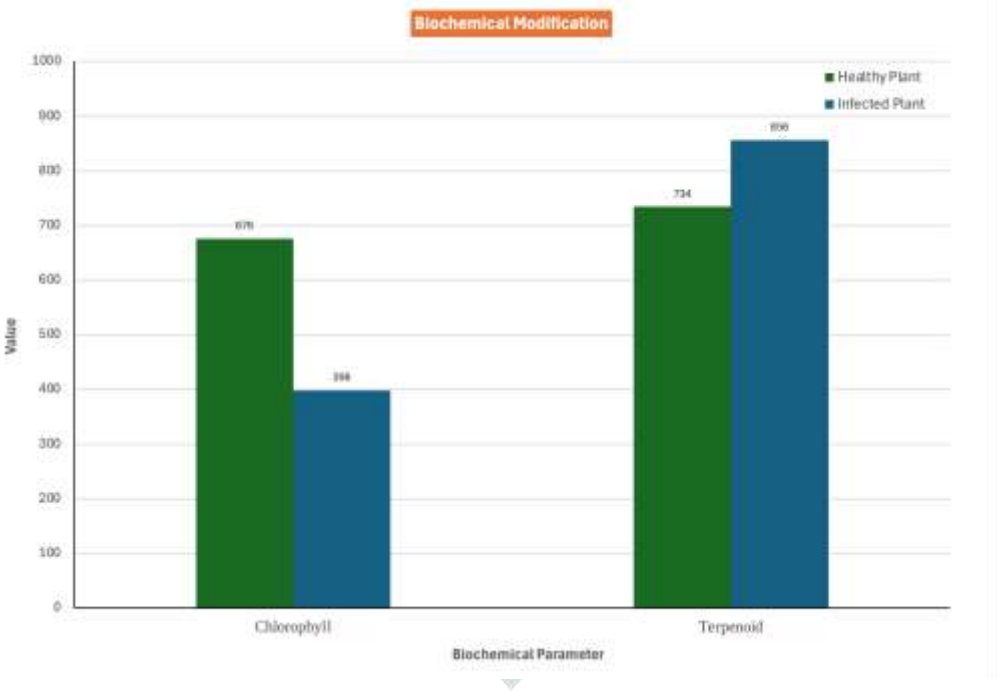

Figure 1: Modification in biochemical parameters of healthy and infected plants (Chlorophyll, Terpenoid)

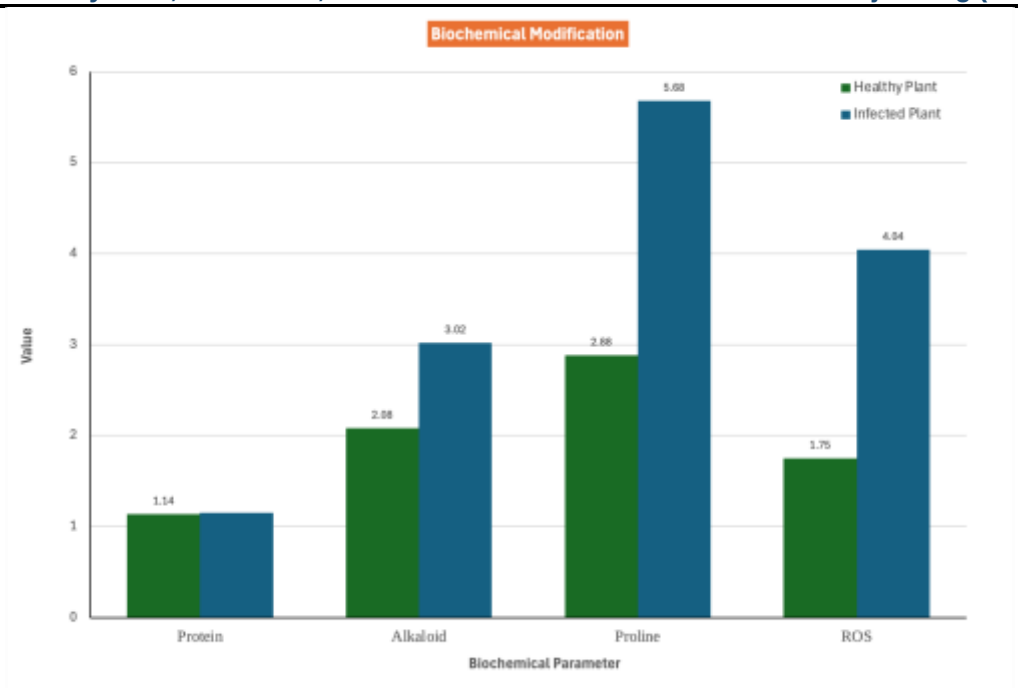

Figure 2:Modification in biochemical parameters of healthy and infected plants (Protein, Alkaloid, Proline, ROS)

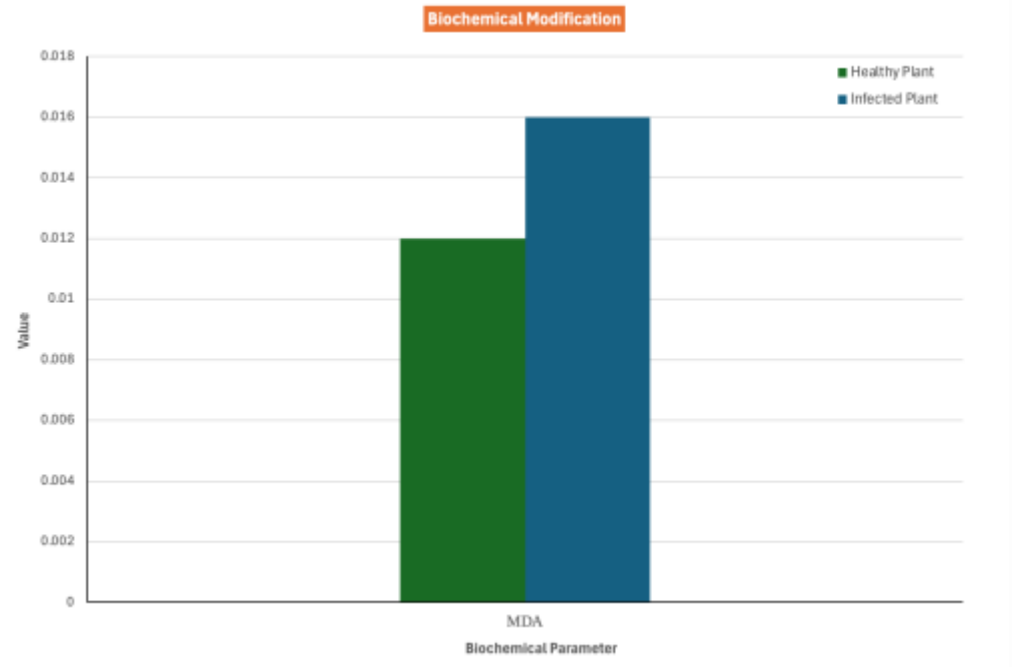

Figure 3: Modification in biochemical parameters of healthy and infected plants (MDA)

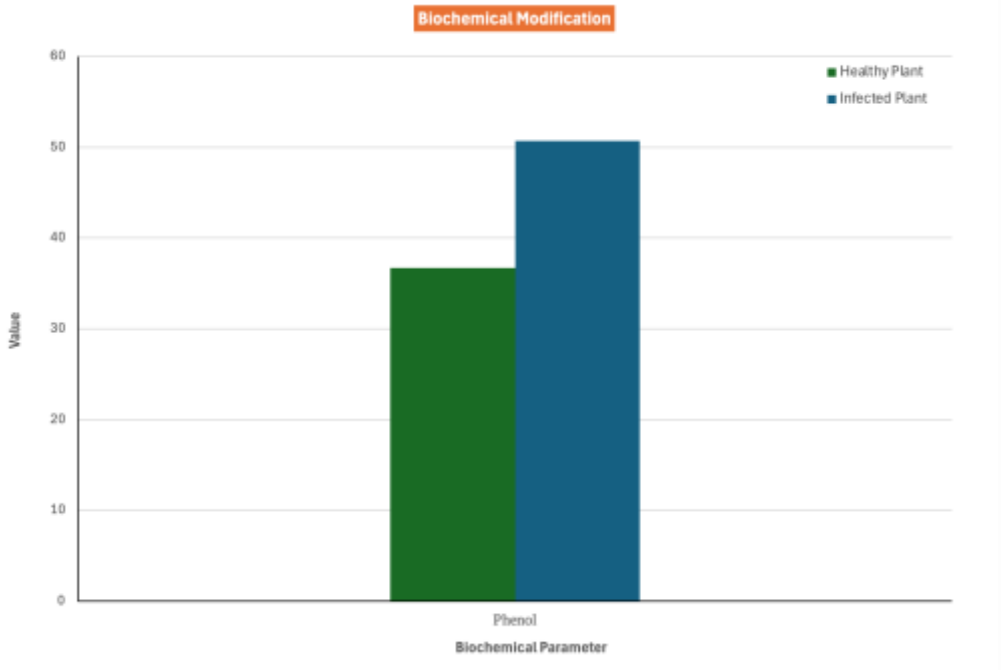

Figure 4: Modification in biochemical parameters of healthy and infected plants (Phenol)

Table 2: Biochemical parameters and their statistical significance test

Biochemical Parameter	Average Value (Healthy Plant)	Average Value (Infected Plant)	t (Cal)	t (Crit)	Conclusion
Total Chlorophyll Content (µg/g)	676	398	10.29	2.306	Significant Difference
Total Phenolic Content (µg GAE/g)	36.7	50.7	6.89	2.306	Significant Difference
Protein Content (µg/g)	1.14	1.15	0.19	2.306	Non- Significant Difference
Total Alkaloid Content (µg/g)	2.08	3.02	8.31	2.306	Significant Difference
Terpenoid Content (µg/g)	734	856	2.32	2.306	Significant Difference
Proline Content (µg/g)	2.88	5.68	5.65	2.306	Significant Difference
Malondialdehyde (MDA) Content (µg/g)	0.012	0.016	1.26	2.306	Non- Significant Difference
Reactive Oxygen Species (ROS) (µmol/g)	1.75	4.04	6.82	2.306	Significant Difference

4.1 Total Chlorophyll Content (µg/g)

The study found a significant decrease in total chlorophyll content in infected plants (398 µg/g) compared to healthy plants (676 µg/g). The calculated t-value, 10.29 (greater than the t critical value, 2.306), suggests a disruption in the photosynthetic apparatus. Similar reductions in chlorophyll content have been reported in other studies on pathogen-infected plants, where oxidative stress and chloroplast degradation are common outcomes of infection (Kumar & Sharma, 2020; Hasanuzzaman *et al.,* 2013). The reduction in chlorophyll is likely due to increased production of reactive oxygen species (ROS), which can damage cellular components, including chlorophyll, leading to impaired photosynthesis and reduced plant vitality. This substantial reduction in chlorophyll content suggests a marked decrease in photosynthetic efficiency, which is a common symptom of stress in plants under

pathogen attack (Arnon, 1949). The decline in chlorophyll could be attributed to the oxidative stress and cellular damage induced by the citrus canker infection.

4.2 Total Phenolic Content (µg GAE/g)

Phenolic compounds are crucial in plant defence mechanisms. The total phenolic content in infected plants (50.7 µg GAE/g) compared to healthy plants (36.7 µg GAE/g) was found amplified. This elevation of total phenolic content is statistically significant as calculated t-value received, 6.89 (greater than the critical value of 2.306). It aligns with findings from other studies where increased phenolic content is a typical plant response to pathogen attack (Treutter, 2010; Mithöfer & Boland, 2012). Phenolics contribute to the reinforcement of cell walls and act as antimicrobial agents, thus inhibiting pathogen proliferation. The increased phenolic content in infected plants suggests an active defence response, potentially enhancing resistance to the citrus canker pathogen.

4.3 Protein Content (µg/g)

The protein content exhibited negligible changes between healthy (1.14 µg/g) and infected plants (1.15 µg/g). Further, the significance test revealed t-value as 0.19, which is smaller than the critical value of 2.306, indicating no statistical significant difference. This stability in protein content contrasts with some reports where pathogen infection leads to either an increase or decrease in protein levels, depending on the specific plant-pathogen interaction (Van Loon, 1985; Görlach *et al.,* 1996). The minimal change in this study suggests that the overall protein synthesis or degradation was not markedly affected by the citrus canker disease, indicating that basic metabolic functions remained relatively stable despite the infection.

4.4 Total Alkaloid Content (µg/g)

Alkaloids are secondary metabolites that are often associated with plant defence, and their increase suggests an active biochemical response to pathogen invasion (Harborne, 1973). The study found, a significant increase in total alkaloid content in infected plants (3.02 µg/g) compared to healthy plants (2.08 µg/g). The t-value of test denotes 8.31, which is greater than the critical value (2.306). It supports the hypothesis that alkaloids tend to increase and play a defensive role in plant responses to biotic stress (Zhang et al., 2018; Wink, 1999). Alkaloids, as secondary metabolites, can prevent herbivores and pathogens through toxicity or deterrence. The increased alkaloid content in infected Kinnow plants suggests an upregulated defence mechanism, which may contribute to the plant's ability to withstand or limit the spread of the infection.

4.5 Terpenoid Content (µg/g)

The terpenoid content also significantly increased in infected plants, as study revealed an elevation in terpenoid content in infected plants (856 µg/g) compared to healthy plants (734 µg/g). The statistical significance test also revealed the same inference as t-value (2.32) found greater than the critical value (2.306). Terpenoids are another class of secondary metabolites involved in plant

defence, often functioning as antimicrobial agents or as precursors to signalling molecules that modulate plant immune responses (Gershenzon & Dudareva, 2007; Tholl, 2015). The increased terpenoid levels indicate that the infected plants are mobilising additional resources towards strengthening their defence mechanisms in response to the citrus canker infection.

4.6 Proline Content (µg/g)

Similarly, proline content was found significantly higher in infected plants (5.68 µg/g) compared to healthy plants (2.88 µg/g) and t-value (5.65) found greater than the critical value (2.306). It is suggesting that proline content tend to increase by the pathogen attack and environmental stress. Earlier studies also confirmed that proline accumulation is a common response to various stress conditions, including pathogen attack, where it functions as an osmoprotectant, stabilising proteins and membranes, and scavenging free radicals (Szabados & Savouré, 2010; Verbruggen & Hermans, 2008). The elevated levels of proline in infected plants indicated a stress response, potentially contributing to the plant's ability to cope with the oxidative stress associated with infection.

4.7 Malondialdehyde (MDA) Content (µg/g)

Malondialdehyde (MDA) is a marker of lipid peroxidation, indicating oxidative stress in plants. The MDA content was slightly higher in infected plants (0.016 µg/g) compared to healthy plants (0.012 µg/g) and statistical significance test revealed no significant difference as t-value (1.26) received lower than the critical value (2.306). Although some studies have reported significant increases in MDA content under pathogen-induced oxidative stress (Mittler, 2002), the non-significant difference in this study suggests that lipid peroxidation may not be a dominant response in Kinnow plants to citrus canker infection, or that the plants might possess effective mechanisms to mitigate lipid peroxidation, such as enhanced antioxidant activity.

4.8 Reactive Oxygen Species (ROS) (µmol/g)

The study found reactive oxygen species (ROS) levels significantly elevated in infected plants (4.04 µmol/g) compared to healthy plants (1.75 µmol/g). The inference was supported by the statistical significance test as t-value (6.82) found greater than the critical value (2.306). The significant increase in ROS indicates a heightened oxidative stress response due to the infection. ROS are commonly produced in response to pathogen attack and can lead to oxidative damage; however, they also play a crucial role in signalling pathways that activate defence mechanisms in plants (Apel & Hirt, 2004; Foyer & Noctor, 2005). The elevated ROS levels in infected plants underscore the dual role of ROS as both damaging agents and signalling molecules in the plant's response to citrus canker.

5. Conclusion

The comparative analysis of the biochemical parameters between healthy and citrus canker-infected Kinnow plants demonstrates the extensive impact of the disease on the metabolism of plants. The significant reduction in chlorophyll content indicates impaired photosynthesis, while the increase in

secondary metabolites such as phenolics, alkaloids, and terpenoids highlights an enhanced defence response. The elevation in proline and ROS levels further underscores the stress experienced by the infected plants. These findings are consistent with earlier studies on plant-pathogen interactions and contribute to our understanding of the biochemical changes induced by citrus canker in Kinnow plants, offering insights that could inform future strategies for disease management.

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