

Effect of Phytoplasma Infection on Primary and Secondary Metabolites and Antioxidative Enzyme Activities of Sweet Orange (*Citrus sinensis* L.)

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ABSTRACT

Sweet orange (*Citrus sinensis* L.) is one of the most economically important citrus crops in the world and is the most commonly grown citrus fruit in the world. Nonetheless, its production is constantly threatened by pathogens that cause considerable economic losses and severe social impacts including phytoplasma. Phytoplasma is an emerging threat to sweet orange production which leads to severe yield losses worldwide. Phytoplasmas are phloem-limited pleomorphic bacteria, mainly transmitted through leafhoppers but also by plant propagation materials and seeds. This study aimed to evaluate the biochemical alterations in sweet orange plant as a response to phytoplasma infection. Phytoplasma-infected plants in this study, showed symptoms, such as yellowing of leaves, stunted and rolled foliage, unripened shoots and fruits, stunted roots or plant and “witches' broom”. Nested PCR confirmed the presence of phytoplasma in all the infected plants. Primary metabolites including chlorophyll a, b and total chlorophyll contents were significantly reduced. Meanwhile, carotenoid, proline and soluble protein were increased while soluble sugar, hydrogen peroxide and malondialdehyde were decreased in phytoplasma-infected plants. The secondary metabolites including phenolics, glycine betaine and anthocyanin were increased while ascorbic acid was decreased in the phytoplasma-infected plants. The activities of the antioxidative enzymes: ascorbate peroxidase, superoxide dismutase, catalase, and peroxidase activities were increased in phytoplasma-infected plants. On the basis of comparing our findings with previous reports, it is clear that the responses of host plants to phytoplasma infection are complex and may vary among plants.

Keywords: *Citrus sinensis* L.; Phytoplasma infection; Primary metabolites; Secondary metabolites; Antioxidative enzymes

INTRODUCTION

Sweet orange (*Citrus sinensis* L.) belongs to the family Rutaceae. It is a small, shallow-rooted evergreen shrub or tree growing about 6 - 13 meters tall with an enclosed conical top and mostly spiny branches [1]. Oranges are very wellknown fruit, widely available in countries around the world. The tree is commonly cultivated for its fruit in warm temperate, subtropical and tropical zones. It prefers a prominent change in the seasons and so is not so suited to the tropics, where it is grown more as a garden tree, but is widely grown commercially in the subtropics. Orange starts flowering and bearing fruit after 3-5 years. Trees aged 3-4 years produce 2.5-5 t/ha of fruit and 8-12 year old trees produce 20-40 t/ha of fruit. Single trees may live up to 100 years, but the economic is 30 years [1]. Orange fruits contain high concentrations of flavonoids beneficial to human health [2].

Citrus is the most economically important tree fruit crop in the world and orange is the most commonly grown citrus fruit in the world [3]. In 2018, 11.6 tonnes per hectare of oranges were grown in Pakistan [4]. The majority of citrus arrives at market in the form of processed products, such as single-strength orange juice and frozen juice concentrate [3]. Nonetheless, citrus production is constantly threatened by pathogens that cause considerable economic losses and severe social impacts. Among the major citrus diseases are phytoplasma-associated diseases [5]. The primary visible symptoms of these diseases are yellowing leaves, stunted and rolled foliage and unripened shoots and fruits. Other symptoms of phytoplasma infection might be stunted plants, stunted roots, aerial tubers, a “witches' broom” appearance on terminal new bud growth, and even die back of entire portions of the plant [6]. Witches' broom was first observed in Oman, and later was found to be present in United Arab Emirates [7], India [8] and Iran [9]. It occurs as an

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abnormal brushlike cluster of dwarfed weak shoots arising at or near the same point; twigs and branches of woody plants may die back. Its evocative name, dates to the middle ages, when brooms were often made of twigs and odd things in nature were assumed to be caused by bad magic. Witches' broom is caused by phytoplasma [6].

Phytoplasma-associated diseases are a major limiting factor to quality and productivity of many ornamentals, horticultural and other economically important agriculture crops worldwide [10]. Phytoplasma comprises approximately 30 distinct clades based on 16S rRNA gene sequence analyses of ~200 phytoplasmas. They can be found in monocot and dicot plant species in most parts of the world. Phytoplasma constitute a large monophyletic group within the class Mollicutes which have variable sizes and shapes and survive and multiply in the isotonic environments provided by plant phloem and insect hemolymph [11]. They are non-helical and wall-less prokaryotes transmitted by insects [12]. Phytoplasmas are mostly dependent on insect transmission for their spread and survival. The phytoplasma life cycle involves replication in insects and plants. They infect the insect but are phloem-limited in plants. The phloem-sucking leafhopper, planthoppers and some psyllids are responsible for the transmission of phytoplasma [13]. Phytoplasmas can be pathogenic to some insect hosts, but generally do not negatively affect the fitness of their major insect vector (s). In fact, phytoplasmas can increase fecundity and survival of insect vectors, and may influence flight behaviour and plant host preference of their insect hosts [14].

In plants, phytoplasmas induce symptoms that suggest interference with plant development [11]. Hameed et al. [15] studied the changes in total phenolic compounds, total soluble proteins, peroxidase (POD), catalase (CAT), polyphenol oxidase (PPO), Chlorophyll *a*, Chlorophyll *b*, and total chlorophyll in leaves of phytoplasma-infected and healthy plants of six mungbean genotypes. Total phenols were decreased significantly in four but increased in one genotype. Protein contents were increased significantly in two genotypes, POD in four, PPO in one and PAL in two genotypes. However, activity of CAT and amount of *Chl a*, *Chl b* and total *Chl* were decreased significantly in all genotypes. Hence, phytoplasma can interfere with host metabolism and photosynthesis to induce disease. Huseynova et al. [16] analyzed the changes in the activity of metabolic and antioxidant enzymes, as well as in the content of total soluble sugars, tocopherols, and total phenolic compounds in field-grown pepper plants under the influence of phytoplasma infection. The activities of benzidine peroxidase (BPO) and guaiacol peroxidase (GPO) were observed to increase in infected pepper leaves in comparison with the healthy control. The levels of studied antioxidant enzyme isoforms were enhanced under pathogenesis. Activities of aspartate aminotransferase (AsAT) and alanine aminotransferase (ALAT) were also increased in the infected plants compared with healthy plants. The amount of tocopherols, soluble sugars, and total phenols was significantly higher in leaves due to the phytoplasma infection. Ahmad et al. [17] mentioned that phytoplasmas are one of the most destructive plant pathogens associated with different morphological, anatomical and physiological changes leading to huge losses in oil seed crop production. Various primary and secondary metabolites produced in response to phytoplasma infection were quantified and studied to understand how phytoplasma affects the physiology and metabolic processes of infected plants. In the infected sesame plants, increased catalase, superoxide dismutase and peroxidase

activity along with phenolic contents and soluble proteins was detected. The levels of proline and glycinebetaine also increased. Higher levels of malondialdehyde and hydrogen peroxide were measured and infected plants showed lower contents. Thus, diverse antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT) contribute in ROS metabolism, when phytoplasma attack attack the plants.

Phytoplasma (16SrX) is related to pigeon pea witches broom in Brazil [18] and citrus huanglongbing is related to aster yellows disease in China (16SrI) [19]. According to recent classification, bushy stunt maize phytoplasma is an agent of group 16srI and subgroup B [20]. The analysis was based upon indications of phytoplasma infection confirmed by PCR technique [21]. Studies on physiological relationships between some host plants and phytoplasma have been reported [22]. However, studies related to the physiological and biochemical changes caused by phytoplasma in oranges are rare and has never been done before. This study aimed to molecularly detect phytoplasma infection in *C. sinensis* and determine the physiological and biochemical changes caused by the infection by evaluating the pigments, protein, soluble sugar, proline, phenolic contents, carbohydrates, glycine, betaine, amino acid and antioxidative enzymes of infected plant.

MATERIALS AND METHODS

Effect of phytoplasma infection on primary and secondary metabolites

To determine the concentration of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids of orange plant, the methods from Arnon [23] followed. A 0.5 g leaf sample was homogenized with mortar and pestle in 80% acetone and filtered. The absorbance of the filtrate was read at 645 and 663 nm for chlorophyll *a* and *b*, respectively, using a picodrop spectrophotometer (Hitachi-U-2001, Japan). Chlorophyll *a*, and *b* were calculated as described by Yoshida et al. [24], as given below:

$$\text{Chl. } a \text{ (mg/g)} = [12.7 (\text{OD}_{663}) - 2.69 (\text{OD}_{645})] \times V/1000 \times W$$

$$\text{Chl. } b \text{ (mg/g)} = [22.9 (\text{OD}_{645}) - 4.68 (\text{OD}_{663})] \times V/1000 \times W$$

To determine the soluble sugar contents, the plant samples were added with anthrone reagent. The hydrogen peroxide (H₂O₂) was estimated following the method of Velikova et al. [25]. A 0.5 g leaf sample was centrifuged at 12,000 rpm for 15 minutes and the supernatant was separated using a micropipette. About 0.5 ml (500 µl) supernatant was taken in microfuge tube and added 0.5 ml (500 µl) potassium phosphate buffer (pH 7.0) and 1 ml (1000 µl) 1 Molar potassium iodide (KI) solution. Tubes were mixed with vortex. The absorbance values for hydrogen peroxide were measured by using the picodrop spectrophotometer at 390 nm wavelength. The concentration of malondialdehyde (MDA) was determined following the protocol of Heath and Packer [26].

Free proline was determined using the protocol of Bates et al. [27]. A 0.5 g leaf sample was homogenized in 5 ml of 3% of aqueous sulphosalicylic acid and the homogenate was filtered using a Whatman No.2 filter paper. One ml of filtrate was taken and mixed with 1 ml of acid ninhydrin (1.25 g ninhydrine in 30 ml glacial acetic acid) and 1 ml of glacial acetic acid in a test tube. The mixture was briefly mixed in a vortex and heated at 100°C in a water bath for 1 h and then transferred in the ice bath. Four ml of

toluene was added to the solution and mixed by a vortex for 15-20 seconds until it cools down. The chromophore containing proline was aspirated from aqueous phase in a test tube and incubated at room temperature. The absorbance was measured at 520 nm using a spectrophotometer. The same procedure was followed to make a blank using 2 ml of toluene. The amount of free proline was calculated following the formula:

$$\text{Proline } (\mu \text{ moles})/\text{g fresh weight} = [(\mu\text{g proline}/\text{ml} \times \text{ml toluene})]/[115.5 \mu\text{g}/\mu\text{mole}/(\text{g sample}/5)]$$

The phenolic contents were determined following Julkenon-Titto. A 0.5 g leaf sample were ground in 5 ml 80% acetone and centrifuged at 12,000 rpm for 15 minutes. The 0.5 ml (500 μ l) of Folin-ciocalteu' phenol reagent was used to measure the absorbance at 750 nm using a picodrop spectrophotometer. An 80% acetone was used to calibrate the instrument. Standard curve was prepared by using the standard solutions of tannic acid (100 μ g/ml stock). Total soluble proteins of the samples were determined based from the methods of Bradford [28].

To determine the ascorbic acid content, plant samples were added with 2% dinitrophenyl hydrazine and 1 drop of 10% thiourea at 535 nm. Glycine betaine was determined based from the study of Grieve and Grattan [29]. A 0.5 g of plant material was homogenized and was mixed with in 20 ml of de-ionized water and shaken for 24 h at 25°C. The extracts were diluted using 1:1 ratio with 2N H₂SO₄. A 0.5 ml of the diluted extract was transferred into centrifuged tubes. The tubes were cooled down in ice bath for 1 h and a cold IK-I₂ reagent was then added. The contents of the tubes were gently mixed with vortex. The tubes were stored at 4°C for 16 h and were centrifuged at 0°C for 15 min at 1,000 rpm. The supernatant was removed using the fine tipped glass tube. The per iodide crystals were dissolved in 9 ml of 1,2-dichloroethane followed by vigorous mixing in a vortex until complete solubilization of crystals was achieved. The solution was kept at room temperature for 2 to 2.5 h and the absorbance was measured at 365 nm. The absorbance readings were compared with standard curve.

Anthocyanin was measured using the Wagner [30] method. About 0.5 g plant tissue was ground in acidified solution. For the preparation of acidified solution, 0.5 ml of HCL was dissolved in 49.5 ml of distilled water and 49.5 ml of methanol. The plant extracts were heated for 1 hour at 50°C in a water bath and were centrifuged for 10 minutes at 12,000 rpm. Then, the supernatant was removed using a micropipette. Anthocyanin concentration was determined by reading its absorbance at 535 nm.

Effect of phytoplasma infection on antioxidative enzyme activities

Plant samples were centrifuged at 15,000 rpm for 15 min at 4°C. Antioxidants like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) were then extracted. The activity of SOD was determined by measuring its ability to inhibit the photoreduction of nitrobluetetrazolium (NBT) following the method of Giannopolitis and Ries [31]. CAT and POD were measured using the method of Chance and Machly [32] with some modifications while the protein weight of the extracts was measured based from the method of Bradford [28].

Molecular detection of phytoplasma

Genomic DNA was isolated from 0.5 g fresh leaf sample from healthy and phytoplasma-infected plants using the cetyl-trimethyl ammonium bromide method as described by Ahmad et al. [33]. CTAB protocol was used to isolate DNA by dissolving the samples in TE buffer (100 μ l) and stored at -20°C. A 2 μ l of (1 μ g/ μ l) RNase was added and tubes were centrifuged at 3,000 rpm for 30 seconds. About 1 by 10th vol (10 μ l) of 2.5 M sodium acetate was added to precipitate the RNase A and 2 volumes (200 μ l) of isopropanol was later added. The concentration of DNA was determined by picodrop and gel electrophoresis using 0.8% agarose with known concentration of DNA. DNA concentration was calculated using OD values at 260 nm using the following formula:

$$\text{Concentration of DNA } (\mu\text{l}/\text{ml}) = \text{OD at } 260 \text{ nm} \times 50$$

To confirm the quality of DNA, samples were run in 0.8% agarose gel in 1x TAE buffer and stained with ethidium bromide. DNA was evaluated by comparing it with a standard DNA sample. RNase was added to the genomic DNA to remove contaminants. The DNA was subjected to PCR and the PCR products were stored at 4°C. The PCR products were later run in gel electrophoresis to obtain molecular bands. Data were statistically analyzed using analysis of variance (ANOVA) by a COSTAT software.

RESULTS

Effect of phytoplasma infection on primary and secondary metabolites

As shown in Table 1, primary metabolites including chlorophyll a, b and total chlorophyll contents were significantly reduced in phytoplasma-infected plants. Chl a/b ratio was also decreased. Meanwhile, carotenoid was increased while soluble sugar decreased in concentration. On the other hand, amino acid was the same between healthy and phytoplasma-infected plants. Hydrogen peroxide and malondialdehyde were lower in the phytoplasma-infected plants while proline and soluble protein were higher in the phytoplasma-infected plants. Meanwhile, secondary metabolites including phenolics, glycine betaine and anthocyanin were increased while ascorbic acid was decreased in the phytoplasma-infected plants.

Effect of phytoplasma infection on antioxidative enzyme activities

As shown in Table 2, all the anti-oxidants: ascorbate peroxidase, superoxide dismutase, catalase, and peroxidase activities were increased in the phytoplasma-infected plants.

Molecular detection of phytoplasma

Phytoplasma-infected plants in this study, showed symptoms, such as yellowing of leaves, stunted and rolled foliage, unripened shoots and fruits, stunted roots or plant and "witches' broom". Phytoplasma was confirmed to be present in all of the infected plants using nested PCR [34,35]. Healthy plants did not contain genomic DNA of phytoplasma.

DISCUSSION

Primary metabolites are essential for plant growth, development,

Table 1: Changes in the concentration of primary and secondary metabolites in phytoplasma-infected citrus (*Citrus sinensis* L.) plants.

Primary Metabolites	Healthy Plants (mg kg ⁻¹)	Infected Plants (mg kg ⁻¹)
Chl a	0.0897 ± 0.008	0.046 ± 0.006
Chl b	0.125 ± 0.017	0.063 ± 0.013
Total chl	0.215 ± 0.025	0.110 ± 0.013
Carotenoids	0.132 ± 0.029	0.275 ± 0.018
Soluble sugar	1.699 ± 0.183	0.609 ± 0.238
Amino acid	0.587 ± 0.043	0.587 ± 0.085
Hydrogen peroxide	25.506 ± 32.780	0.959 ± 0.638
Malondialdehyde	111.192 ± 21.692	345.042 ± 61.292
Proline	0.665 ± 0.061	1.237 ± 0.099
Soluble protein	0.665 ± 0.061	1.237 ± 0.099
Secondary Metabolites	Healthy Plants (mg kg ⁻¹)	Infected Plants (mg kg ⁻¹)
Phenolics	167.202 ± 2.661	300.533 ± 5.366
Anthocyanin	1.549 ± 0.047	1.583 ± 0.080
Ascorbic acid	25.527 ± 1.438	13.703 ± 1.931
Glycine betaine	12.965 ± 2.922	38.357 ± 1.204

Table 2: Changes in the concentration of anti-oxidants in phytoplasma-infected citrus (*Citrus sinensis* L.) plants.

Anti-oxidants	Healthy Plants (mg kg ⁻¹)	Infected Plants (mg kg ⁻¹)
Ascorbate peroxidase	0.0422 ± 0.035	0.161 ± 0.077
Superoxide dismutase	25.633 ± 2.572	370.093 ± 0.336
Catalase	0.479 ± 0.161	2.143 ± 0.632
Peroxidase	0.189 ± 0.052	1.033 ± 0.280

stress adaptation, and defense. Chlorophyll a, b and total chlorophyll contents were significantly reduced in phytoplasma-infected sweet orange plants. The subsequent Chl a/b ratio was also decreased. Liu et al. [36] mentioned that many pathogen infections in plants are closely related to reducing rate of photosynthesis, and affect the chloroplast number, ultrastructure and chlorophyll metabolism. Deterioration of chloroplast structure, pigment composition and electron transport can be attributed to the damage in PSII mostly caused by infection stress [36]. The decreased chlorophyll contents were the reason why the leaves appeared yellow among the phytoplasma-infected plants.

Meanwhile, carotenoid was increased in the phytoplasma-infected plants. Carotenoids are a group of natural tetraterpenoid pigments distributed widely in plants. Many flowers, fruits, and roots owe their vivid orange, yellow, and red hues to carotenoids. Carotenoids play essential roles in photosynthesis and photoprotection and provide precursors for the biosynthesis of phytohormones abscisic acids (ABA) and strigolactones [37]. Sun et al. [37] further mentioned that carotenoid derivatives also act as signaling molecules to mediate plant development and responses to environmental cues. Carotenoids in chloroplasts function mainly as photosynthetic pigments and photoprotectors. Without carotenoids, chloroplasts could not function properly and thus plants would not survive. The increased concentration of carotenoids in phytoplasma-infected plants would mean that carotenogenic genes were activated in response to phytoplasma infection [23].

On the other hand, soluble sugar was decreased in phytoplasma-infected plants. Couée, et al. [38] emphasized that soluble sugars, especially sucrose, glucose, and fructose, play an obviously central role in plant structure and metabolism at the cellular and whole-organism levels. They are involved in the responses to a number

of stresses, and they act as nutrient and metabolite signalling molecules that activate specific or hormone-cross-talk transduction pathways, thus resulting in important modifications of gene expression and proteomic patterns. Various metabolic reactions and regulations directly link soluble sugars with the production rates of reactive oxygen species, such as mitochondrial respiration or photosynthesis regulation, and, conversely, with anti-oxidative processes, such as the oxidative pentose-phosphate pathway and carotenoid biosynthesis. Moreover, stress situations where soluble sugars are involved, such as phytoplasma infection, are related to important changes in reactive oxygen species balance. These converging or antagonistic relationships between soluble sugars, reactive oxygen species production, and anti-oxidant processes are generally confirmed [38]. Xue et al. [39] reported that phytoplasma infection inhibited photosynthesis. Results showed that soluble sugars were reduced during phytoplasma infection. The excessive damage to the structure and basal metabolism of the phytoplasma-infected plants in this study significantly reduced the subsequent photosynthetic products including soluble sugars. After being infected by phytoplasma, the sweet orange plant exhibited increased respiration, cell wall enhancement, and synthesis of some defense complexes in which more carbohydrates are essential for these metabolic activities to provide energy to the phytoplasma-infected plant [39]. Photosynthetic products are first used for the synthesis of soluble sugars such as sucrose.

On the other hand, amino acid was the same between healthy and phytoplasma-infected plants which implies that the amino acid metabolism was not affected during phytoplasma infection. Zeier [40] mentioned that distinct amino acid metabolic pathways constitute integral parts of the plant immune system. Amino acids can have profound impact on plant resistance to pathogens. Lys produces the immune signal pipecolic acid (Pip) that amplifies

plant defence responses and acts as a critical regulator of plant systemic acquired resistance, defence priming and local resistance to bacterial pathogens including phytoplasmas, which are obligate bacterial parasites. Asp-derived pyridine nucleotides influence both pre- and post-invasion immunity, and the catabolism of branched chain amino acids appears to affect plant resistance to distinct pathogen. Moreover, the acylation of amino acids can control plant resistance to pathogens and pests by the formation of protective plant metabolites or by the modulation of plant hormone activity [40].

Hydrogen peroxide was decreased in phytoplasma-infected plants. Ismail et al. [41] stated that hydrogen peroxide is produced predominantly in plant cells during photosynthesis and photorespiration and also in respiration processes. It is most stable of so-called Reactive Oxygen Species (ROS) and therefore, plays crucial role as signaling molecule in various physiological process. In this study, H_2O_2 over-production does not occur in the phytoplasma-infected plants. H_2O_2 production is low because it can be 'quenched' and counteracted by the induction of glutathione peroxidase activity and, at the same time, by the probable oxidation of glutathione to glutathione disulfide [42].

Malondialdehyde (MDA) was also decreased in the phytoplasma-infected plants. Abdollahi et al. [43] mentioned that the changes in MDA serve as an indicator of the extent of oxidative damage under stress, with a lower MDA level, a characteristic of intolerant plants which did not come up with elevated levels of stress due to phytoplasma infection. MDA content was higher in healthy plants than in infected plants. MDA production is mostly triggered by hydroxyl radicals, although the peroxidation can be caused by other reactive oxygen species as well.

Proline was increased in phytoplasma-infected plants which were also found by Ahmad et al. [17]. Vives-Peris et al. [44] also observed that citrus plants exude proline when stressed. Proline plays an important role in plants by protecting them from various stresses and also helps plants to recover from stress more rapidly [45]. Proline metabolism is involved in the oxidative burst and the hypersensitive response associated with avirulent pathogen recognition [40].

Secondary metabolites serve: (i) as competitive weapons used against other bacteria, fungi, amoebae, plants, insects, and large animals; (ii) as metal transporting agents; (iii) as agents of symbiosis between microbes and plants, nematodes, insects, and higher animals; (iv) as sexual hormones; and (v) as differentiation effectors [46]. Phenolics were higher in the phytoplasma-infected plants than in healthy plants. Junqueira et al. [47] emphasized that increased phenolics points out changes in host metabolism due to the phytoplasma. The study of Torres et al. [48] revealed that, in general, plants tend to a progressive increase in total phenols related to the plants exposure to phytoplasma infection. Hameed et al. [15] further mentioned that phenolics are well-known antimicrobial compounds occurring in plants and function as precursors to structural polymers such as lignin, or serve as signal molecules to activate plant defense genes. In phytoplasma-infected plants, total phenols were significantly higher in leaves. Phenolic compounds are thought to be involved in the defense in plants and their increased accumulation after infection could be related to the resistance mechanism of the plant. Hence, the phenolic content of the plants increases as a response of the defence mechanism, which reflects its condition and resistance.

Some plant varieties (genotypes) including sweet orange are capable of biosynthesizing a unique secondary metabolite, glycine betaine (GB). Glycine betaine was increased in phytoplasma-infected sweet orange plants. Kurepin et al. [49] mentioned that GB accumulation by plant tissues has been shown to occur in a range of abiotic stresses and it has often been directly correlated with a plant's increasing tolerance to the abiotic stress. Meena et al. [50] emphasized that pathogenic diseases which affect the plants throughout their life cycle leads to changes in cellular level, secondary stresses comprising protein denaturation, membrane injury, destruction of reactive species and osmotic stress and the accumulation of glycine betaine.

Anthocyanin was increased in phytoplasma-infected plants. Himeno et al. [51] mentioned that anthocyanins are plant secondary metabolites synthesized by the flavonoid pathway and play important roles in flower pigmentation, allelopathy, or UV protection. They are also important as antioxidant molecules to protect plant cells against damage by reactive oxygen species. The production of anthocyanins in phytoplasma-infected leaves reduces the risk of photo-oxidative damage and delays leaf senescence.

Ascorbic acid was decreased in phytoplasma-infected plants. Ascorbic acid is an abundant component of plants. It reaches a concentration of over 20 mM in chloroplasts and occurs in all cell compartments, including the cell wall [52]. Smirnoff and Wheeler [52] stated that ascorbic acid has proposed functions in photosynthesis as an enzyme cofactor (including synthesis of ethylene, gibberellins and anthocyanins) and in control of cell growth. Khan et al. [53] mentioned that ascorbic acid produced in plants as indirect response against pathogenic attack at different sites in plants and its intertwined network cause changes in nuclear gene expression via retrograde signaling pathways, or even into systemic responses, all of which are associated with pathogenic resistance. Indeed, ascorbic acid plays an important role in resistance to pathogenesis. Smirnoff [54] stated that ascorbic acid accumulation is induced by low and high temperature and nitrogen deficiency; stresses which limit photosynthesis rate and, like high light intensity, give rise to excess excitation energy. To maintain the appropriate ascorbic acid concentration, its rate of synthesis is repressed as it increases and the rate of breakdown increases.

All the antioxidative enzymes, ascorbate peroxidase, superoxide dismutase, catalase and peroxidase were increased in phytoplasma-infected plants. Ascorbate peroxidase (APX) exists as isoenzymes and plays an important role in the metabolism of H_2O_2 in higher plants [55]. Pathak et al. [56] mentioned that APX catalyzes reduction of H_2O_2 to H_2O utilizing the reducing power of ascorbate. Caverzan et al. [57] stated that antioxidant defenses, which can detoxify ROS, are present in plants including APX enzymes that play a key role catalyzing the conversion of H_2O_2 into H_2O , using ascorbate as a specific electron donor. Different APX isoforms are present in distinct subcellular compartments, such as chloroplasts, mitochondria, peroxisome, and cytosol. The expression of APX genes is regulated in response to biotic and abiotic stresses as well as during plant development. The APX responses are directly involved in the protection of plant cells against phytoplasma infection.

Superoxide dismutase (SOD) was increased in phytoplasma-infected plants. Bowler et al. [58] reported that SODs are metal-containing enzymes that catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide. In plants, three forms of the enzyme exist, as classified by their active site metal ion: copper/

zinc, manganese, and iron forms. It is only in plants that all three different types of SOD coexist. To protect against the cytotoxic ROS, it is important to inactivate ROS by employing SOD [59]. Zafari et al. [60] studied the activities of SOD and observed to be greater in infected leaves than the healthy control. In infected leaves, SOD isoforms were stronger than that of the healthy control. These results suggest that SODs can be activated in response to infection by phytoplasma.

Catalase is an oxygen-scavenging enzyme that removes toxic substrates (H_2O_2) during development, which are otherwise lethal [15]. In this study, catalase was increased in phytoplasma-infected plants. Zafari et al. [60] mentioned that the development of an antioxidant defense system in plants protect them against oxidative stress damage, by either the partial suppression of ROS production, or the scavenging of ROS which has already been produced. Thus, catalase participates in ROS metabolism during phytoplasma infection.

Peroxidase was increased in phytoplasma-infected plants. Our results are in agreement with the findings of Junqueira et al. [61] and Zafari et al. [60]. Peroxidases are involved in the scavenging of Reactive Oxygen Species (ROS), which are partially reduced forms of atmospheric oxygen, highly reactive, and capable of causing oxidative damage to the cell. Peroxidases can be a source of hydrogen peroxide (H_2O_2) but also are capable of scavenging it. Pandey et al. [62] stated that plants protect themselves, after pathogen attack, through the passive and active defense mechanisms. The passive defense mechanisms involve structural barriers or existing anti-microbial compounds which prevent colonization in the tissue, while, the active or induced defense responses include the hypersensitive response (HR) and systemic acquired resistance (SAR) i.e., production of phytoalexins and pathogenesis-related (PR) proteins, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (oxidative bursts), ion fluxes across the plasma membrane, lignification and the reinforcement of the cell wall through both the cross-linking of cell wall structural proteins. The active defense responses are regulated through a complex and interconnected network of signaling pathways mediated by salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Among the proteins induced during the plant defense, the class III plant peroxidases are well known and they play roles through (1) reinforcement of cell wall physical barriers comprising lignin, suberin, feruloylated polysaccharides and hydroxyproline-rich glycoproteins; (2) enhanced production of reactive oxygen species as signal mediators and antimicrobial agents; and (3) enhanced production of phytoalexin. Peroxidases have been reported to be induced by phytoplasma infection.

Phytoplasma-infected plants in this study, showed symptoms, such as yellowing of leaves, stunted and rolled foliage, unripened shoots and fruits, stunted roots or plant and "witches' broom". When phytoplasma infect sweet orange plants, the physiology of the plant is changed. They produce reactive oxygen species and activate antioxidative enzymes that play important roles in defense system against phytoplasma.

CONCLUSION AND RECOMMENDATIONS

On the basis of comparing our findings with previous reports, it is clear that the responses of host plants to phytoplasma infection are complex and may vary among plants. In sweet orange, primary

metabolites including chlorophyll a, b and total chlorophyll contents were significantly reduced as well as the Chl a/b ratio. Meanwhile, carotenoid was increased while soluble sugar decreased in concentration. On the other hand, amino acid was the same between healthy and phytoplasma-infected plants. Hydrogen peroxide and malondialdehyde were lower in the phytoplasma-infected plants while proline and soluble protein were higher in the phytoplasma-infected plants. The secondary metabolites including phenolics, glycine betaine and anthocyanin were increased while ascorbic acid was decreased in the phytoplasma-infected plants. All the antioxidative enzymes: ascorbate peroxidase, superoxide dismutase, catalase, and peroxidase activities were increased in the phytoplasma-infected plants.

Phytoplasma-infected plants in this study, showed symptoms, such as yellowing of leaves, stunted and rolled foliage, unripened shoots and fruits, stunted roots or plant and "witches' broom". Nested PCR confirmed the presence of phytoplasma in all the infected plants. The production of phytoplasma-resistant sweet orange is very relevant to improve its quality and protect it from the future threat of phytoplasma diseases. This can be achieved by understanding its adaptive mechanism and response to phytoplasma infection.

Since phytoplasma cannot be cultured in our laboratory, it is recommended for future research to determine the biochemistry, physiology and anatomy of phytoplasma. It is moreover essential to understand the molecular basis of phytoplasma-vector interaction, epidemiology and other factors involved in disease development in order to reduce the disease outbreaks. In addition to phytoplasma diagnostics, future research priorities should be focused on vector-phytoplasma interactions; vector biology; role of weather parameters in disease epidemics; development of resistant varieties; and crop and region specific integrated disease management modules. Priorities for future research should be based on mechanisms of spread of the vector (s), verification of seed transmission and development of resistant varieties to control phytoplasma-associated diseases.

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