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ORIGINAL PAPER

Witches' Broom Disease: Biochemical Changes in Hibiscus Leaf and Insect Vector Control

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Abstract

In 2013, a witches' broom phytoplasma was detected in Hibiscus (*rosa-sinensis*) shrubs using nested polymerase chain reaction (nested-PCR) in five Egyptian governorates, Alexandria, Fayoum, Giza, Mansoura, and Qalyubia. In the present study, phytoplasma of the same kind was detected in other samples from the same shrubs and locations. The predicted PCR product size was ~1200 bp. Quantitative measurements of plant metabolism can provide a broad view of the biochemical status of the plant. Carotenoid and chlorophyll content was decreased while starch content and soluble sugar increased, indicating the decline in photosynthetic efficiency and metabolic disorders in the leaves. Levels of antioxidant enzymes and non-enzymatic antioxidants were increased more than 100% and 200% in the diseased leaves respectively, compared to healthy plants. These results seem to confirm that more enzymatic and non-enzymatic antioxidants occurring together might positively affect defense activities and cell wall enhancement to deal with the over-production of reactive oxygen species and pathogen stress. Increasing these antioxidative defense systems, in turn, may affect the stability of hibiscus shrubs, as shrubs may pass the infection to other crops over the years through phloem-sucking insects such as leafhoppers. Five different concentrations of hydrophilic nano-silica (NS), from 100 ppm to 500 ppm, were tested under laboratory conditions on mortality and survival rates of phytoplasma vector, green leafhopper, *Empoasca decipiens* Paoli. Two different applications were used to determine the effectiveness of NS-concentrations. Among these concentrations, 500 ppm had the highest effect on mortality (0% survival) for both nymphs and adults of *E. decipiens*.

Keywords: Hibiscus, Phytoplasma Witches'-broom, PCR, ROS, *Empoasca decipiens*.

Abbreviations: Witches' broom; WB, Reactive oxygen species; ROS, Chlorophyll; Chl, Carotenoid; Car, Total phenolic content; TP, Total flavonoid content; TF, Superoxide dismutase; SOD, Ascorbate peroxidase; APX, Polyphenol oxidase; PPO, Peroxidase; POD, Glutathione; GSH, Malondialdehyde; MDA, Phenylalanine ammonia-lyase; PAL, Nano-silica; NS, Imidacloprid; IM, Parts per million; ppm.

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Introduction

Phytoplasmas, members of the class Mollicutes, are polymorphic plant pathogenic bacteria lacking the cell wall; mainly inhabit the phloem of plants and hemolymph of insect hosts. They have small genomes and are therefore highly dependent on the intake of the nutrients from their hosts. They are transmitted by phloem-sucking insects such as leafhoppers (*Empoasca* spp.), belonging to the family Cicadellidae (Bertaccini and Duduk, 2009). This species increasingly found both in greenhouses and in the field. The genus is widely distributed in the northern hemisphere, in the Mediterranean region, and North Africa as well as, mainly distributed in Southern Europe, and Central Asia (Naseri *et al*., 2007), where both nymphs and adults damage plants directly by feeding on fruit and foliage (Raupach *et al*., 2002).

Since the early 1990s, controlling various species of insects has been mainly by the use of neonicotinoids such as Imidacloprid (IM). They act by strongly binding to nicotinic acetylcholine receptors in the central nervous system of insects and stimulate the nervous system at low concentrations, whereas higher concentrations cause receptor blockage, paralysis, and death (Kundoo *et al*., 2018). However, the most common insecticides can cause many serious problems threatening human health, such as respiratory diseases and lung cancer (Budzinski and Couderchet, 2018). Meanwhile, their residual build-up infects non-target organisms such as beneficial insects, birds, domestic animals and kill them. Furthermore, many species from the family Cicadellidae show resistance to chemical insecticides (Grass *et al*., 2008).

In another approach, biological control agents of green leafhoppers (*E. decipiens*) through the release of predators such as *Orius* species in the family Anthocoridae is often difficult and not successful, since the winged adults and nymphs move too quickly to capture (Helyer and Talbaghi, 1994). Currently, the use of nano-based products such as hydrophilic nano-silica (NS), led to the control of a wide range of insect pests and increasing the plant resistance (El-Bendary and El-Helaly, 2013; Abd El-Wahab *et al*., 2016). On the other side, many chemical compounds were tested to control the causal agent of witches' broom (WB) disease, such as tetracycline and chloramphenicol. It is found that these compounds may reduce the phytoplasma disease symptoms, while the associated symptoms occur again with stopped these treatments (Laimer and Bertaccini, 2019).

Reactive oxygen species (ROS), it includes many reactive species such as free radicals, for example, superoxide ($O_2^{\bullet}$), singlet oxygen (O), nitric oxide (NO), peroxyl ($ROO^{\bullet}$), and hydroxyl radicals ($OH^{\bullet}$), or non-radical like hydrogen peroxide (H_2O_2), are natural byproducts of cellular oxidative metabolism. ROS plays several vital roles in cellular processes and physiological functions, including plant cell proliferation, adenosine triphosphate (ATP) generation during oxidative phosphorylation, cell signaling, and host-defense activities. ROS-generation takes place at low levels in different subcellular compartments including chloroplasts, mitochondria, and peroxisomes. During pathogen stress, the accumulation of ROS can lead to the disruption of the redox signaling mechanisms, as well as cause toxic effects through the overproduction of free radicals that damage all components of the plant cell (Luti *et al*., 2016; Collin, 2019).

Detoxification of ROS or the excess of free radicals is scavenged endogenously by enzymatic antioxidants such as superoxide dismutase (SOD), Catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX), by converting them to fewer reactive-products. Enzyme SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide (H_2O_2), which is subsequently decomposed into oxygen and water (H_2O) by catalase or peroxidase (Fukai and Ushio-Fukai 2011; Tenhaken, 2014). Ascorbate is an important molecule also functioning in several vital processes in plant cells, including the ascorbate-glutathione cycle. It is involved in detoxification reactions of ROS, where two ascorbate molecules are used by the enzyme ascorbate peroxidase (APX) to reduce H_2O_2 to H_2O (Presnell *et al*., 2013). Besides the functions of enzymatic antioxidants, several non-enzymatic antioxidants (like phenols and glutathione) are valuable biomarkers for the defense response in plants. Also, reactive carbonyl species, like malondialdehyde (MDA) is considered a general indicator of cellular damage at the cell membrane by a pathogenic infection (Dallagnol *et al*., 2011). As an alternative, carbohydrates are central to many essential metabolic pathways and can use as fast indications of pathogen infection, including phytoplasma infection (Maust *et al*., 2003; Zafari *et al*., 2012).

Hibiscus (China rose) shrubs of the Malvaceae family are grown as ornamental shrubs throughout Egypt, also as planted as a fence or hedge plants to protect crops from the force winds. The WB disease of *Candidatus* Phytoplasma brasiliense (Acc. No. KF716175) was reported in Egypt (Mokbel *et al*., 2013), as well as its vector (*E. decipiens*) (Ahmed *et al*., 2014). Previous studies have shown that WB-phytoplasma infection causes numerous biochemical changes such as the inhibition of the host-photosynthetic or stimulation of carbohydrate and energy metabolisms of diseased trees. As a result, the trees exhaust the nutrient supply and die after 3 years or some years of the onset of symptoms (Xue *et al*., 2018). However, over seven years, the disease still exists in host hibiscus plants and persists as a source of infection. Hence, the present study performed firstly to determine the biochemical changes and investigating the antioxidant defense systems that occur because of interactions between phytoplasma and host components. Secondly, to determine the efficient concentration among five concentrations from 100 ppm to 500 ppm of hydrophilic NS to control *E. decipiens* under the laboratory conditions.

Materials and Methods

Plant materials and disease symptoms

On January 10, 2019, healthy and infected samples were collected from hibiscus shrubs that are growing in Alexandria, Fayoum, Giza, Mansoura, and Qalyubia governorates.

The diseased plants had symptoms that were stunted with shortened internodes, the proliferation of axillary shoots, and the yellowing of leaves. The samples included in this study were originated naturally from hibiscus shrubs that previously infected with the witches' broom disease of phytoplasma (Mokbel *et al*., 2013).

Nested PCR for detection of phytoplasma

Total DNA was extracted from naturally infected hibiscus leaves as well as the healthy ones using plant DNA extraction kit (Sigma, USA). The DNA extracted from symptomatic and/or asymptomatic hibiscus plants was used as a template for direct PCR. Nested PCR assay was performed using universal phytoplasma-specific primers, P1/P7, and R16F2n/R16R2 (Lee *et al*., 2004; Bhat *et al*., 2006). Primer pair P1/P7 was used in the first step PCR for the amplification of 1800 pb product of the 16S rRNA gene. One µl DNA extracted from hibiscus plants was used in 25µl total PCR mixture contained 25 pmol of each primer; 200 µM of each dNTP; 1x polymerase reaction buffer; 2.5 mM $MgCl_2$; 1.25 U of dream-Taq polymerase (Fermentas) and sterile water to a final volume of 25 µl. The amplification consisted of a denaturation phase at 94°C for 120 seconds, followed by 35 cycles of 94°C for 30 seconds, annealing at 55°C for 60 seconds, and primer extension at 72°C for 90 seconds. A final extension step was added for 10 min at 72°C. The primer pair R16F2n/R16R2 used to amplify a 1.2 kb fragment of the 16S rRNA gene in the second step nested-PCR as described by (Wang and Hiruki, 2001). One µl of DNA amplified by direct PCR with primer pair P1/P7 from hibiscus samples was used at 1:10 dilution as a template for nested-PCR. The nested PCR was performed with the same thermo-program, except that the annealing step was at 50°C for 2 min. The PCR products were analyzed using Agarose Gel Electrophoresis by staining 1% agarose gel with EZ-View stain (Biomatik, USA) and visualized by UV illumination.

Chemical analysis of leaves

Sugars content and starch

Total soluble sugars and reducing sugars were spectrophotometrically determined at 540 nm using the picric acid method as described by Thomas and Dutcher (1924).

Non-reducing sugar was calculated as the difference between total sugars and reducing sugars. Starch was determined according to Salo and Salmi (1968).

Photosynthetic pigments

Chlorophyll a, b, and carotenoids were determined after extraction with acetone (80%), according to Holden (1965).

Total flavonoids and phenolic compounds

The TF content was determined according to the aluminum chloride colorimetric method of Chang *et al*. (2002). The Folin-Ciocalteu method as described (Meda *et al*., 2005) was used to determine the TP content.

Samples preparation of the cytosolic protein fractions

Leaf samples, represented by 1 g fresh tissue each, were homogenized in 4 ml of ice-cold buffer (1 M Tris–HCl, pH 7.2) containing 0.25 M sucrose using a pre-chilled mortar and pestle. The homogenates were centrifuged at 12000 g for 15 min at 4°C. The resulting supernatants were stored as cytosolic extracts and used for later analyses.

Oxidation stress assay

The level of lipid peroxidation in the leaf tissue was calculated in terms of MDA content (as a byproduct product of lipid peroxidation and as a marker of oxidative stress), through by the formation of thiobarbituric acid reactive substances (TBARS), and expressed in µM according to the method described by Haraguchi *et al*. (1997). The extinction coefficient of TBARS was taken as 1.56×105 mM at wavelength 532nm.

Enzyme assays

The activity of cytosolic SOD was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) following the method of Giannopolitis and Ries (1977). The POD, PAL, APX, and Polyphenol oxidase (PPO) activities were assayed spectrophotometrically using methods and the reaction conditions of Fielding and Hall (1978), Dickerson *et al*. (1984), Amako *et al*. (1994), and Ngadze *et al*. (2012), respectively. Protein was measured by the method of Bradford (1976), and Bovine serum albumin (BSA) was used as a protein standard.

Activity assay for glutathione (GSH)

The GSH level in homogenates was measured by acid-

soluble sulfhydryl level determination as described by La Vecchia *et al*. (1992).

Statistical analysis

Each assay was performed in triplicate. Data were subjected to analysis of variance using the WASP 2.0 software package (Agri. Stat., ICAR). Least Significant Difference (LSD) method was used to test the differences between means at a 5% level of probability.

Phytoplasma-vector control

Host plant cultivation

Broad bean (*Vicia faba* L.) plants were grown from seed under the greenhouse conditions at 24°C with 60% relative humidity and a 16 h photoperiod. Ten days after germination, plants transplanted into 10-cm plastic pots. Each pot was then covered with a cylindrical glass cage (25 cm high and 8 cm diameter), and muslin covering on top tightened with rubber bands (Fig. 1A).

Insects rearing

The male and female adults of the green leafhoppers of about 3 mm to 4.5 mm in size were collected by sweep net from diseased hibiscus shrubs (leaves and around stems). The identity of the leafhoppers was confirmed as an *E. decipiens* (Paoli) (Fig. 1B) using the morphological keys, as described by Paoli (1930) and Dworakowska (1973). Leafhoppers in pairs of 20 individuals were reared on potted broad bean seedlings, at the 5-7 leaf stage, to feed and mate under the following conditions 24 °C ± 1 with 16-h light/8-h dark cycles and 60-70% relative humidity (Raupach *et al*., 2002). A group of newly emerged leafhopper nymphs was then collected from the cage and transferred to a new insectary room to perform experiments, leaving behind another group of young nymphs per the same plant to develop until adulthood.

Bioassay tests

Different concentrations of hydrophilic nano-silica (NS) (100, 200, 300, 400, and 500 ppm) were tested, and supplied by NanoTech-Egypt. Two methods of in vitro bioassay, the direct and the indirect methods, were used to determine the most effective concentration of an NS against the newly emerged nymphs and adults. Ten pests were used per replication with three replicates per concentration. Distilled water (DW) was applied as negative control while, IM, the recommended concentration 500 ppm provided with pesticide (WHO, 2005), was applied as a positive control on the target insect.

For both assays and each of the treatments, a 5% agar solution was poured into Petri dishes (7 cm diameter, 1.3 cm deep), and left to solidify at room temperature (Fig. 1C).

For direct application, the fresh leaves of broad bean were provided in each petri dish then the insects were gently transferred on it than two hours later was sprayed directly with 5 ml of the different concentrations previously mentioned above. The treatments were daily observed for mortality rate and survived individuals (Fig. 1D).

For an indirect application, broad bean plants were grown in new single 10 cm diameter pots in the greenhouse under the same conditions. Plants at the 5-6 leaf stage were sprayed with the different concentrations of NS as mentioned above, NS applied again after one week and then used for treatment. For each treatment, two leaves of each foliage treatment were offered to 10 nymphs or 10 adults in each replicate (Fig. 1E). The mortality rate and survived individuals were daily observed among different treatments which have equal numbers of individuals (10), and calculated using the formula $M\ \% = [d / 10] \times 100$ where M is the mortality rate; d is the number of death determined by each concentration to live individuals.

Results and Discussion

Symptoms and PCR detection of pathogens

Symptomatic leaf samples were collected from different five governorates in Egypt and exhibiting mass deformation of twigs and branches, crinkled leaves, short internodes, the proliferation of axillary shoots, and leaf yellowing (Fig. 2). Nested-PCR assay confirmed the presence of phytoplasma, and the PCR products of 1200 bp were obtained from phytoplasma-infected samples, but not from healthy controls (Fig. 3). In molecular diagnosis, the nested-PCR method is one of the highly sensitive tools used to quantify and amplify phytoplasma-specific DNA with low levels in diseased samples (Gundersen and Lee, 1996). Generally, plant phenotype influenced by phytoplasma-mediated alterations in hormone pathways. Several studies that focus on the interaction of phytoplasma with plants suggest that phytoplasma effectors, such as TENGU and SWP1, inhibiting auxin production pathways in the host plant, resulting in undesirable traits such as dwarfism and witches' broom symptoms (Sugawara *et al*., 2013; Wang *et al*., 2018). While SAP11 responsible for witches' broom symptoms and curled leaves (Sugio *et al*., 2011).

Biochemical analysis

To determine the biochemical changes that cause by phytoplasma in hibiscus plants, as well as the plant defense pathways; hibiscus-infected leaves (PCR positive) and healthy leaves (PCR negative), were used to prepare samples for chemical analysis, as shown in Tables 1 to 5.

Fig. 1 Host plant cultivation and bioassay tests. Broad bean seeds (*Vicia faba* L.) were planted into plastic pots; the produced plants in each pot were kept with a cylindrical glass cage and a muslin fabric before inserting the insects into the cage (A). The green leafhoppers, *E. decipiens* (Paoli); have been found to range in size from just over 3 mm to about 4.5mm (B). All the NS-concentrations was tested *in vitro* petri dish assays by spraying leafhoppers as a direct application, and the broad bean leaves as an indirect application to identify the potential toxicity of NS (C, D, and E).

Fig. 2 Naturally infected hibiscus plants from different locations in Egypt showing disease symptoms of witches' broom, shoot proliferation, short internodes, stunting, and yellowing of leaves (A). Healthy leaves (B).

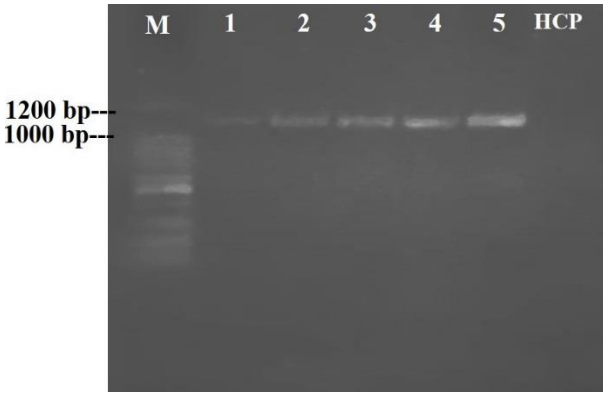

Fig. 3 Agarose gel showing bands of 1.2 kb by nested PCR obtained of phytoplasma-infected hibiscus samples from different locations: 1) Alexandria, 2) Fayoum, 3) Giza, 4) Mansoura, and 5) Qalyubia. M: 100 bp plus DNA Ladder. HCP: Healthy control plant.

Sugars, starch, and pigment analysis

As shown in Table 1, the starch and soluble sugar content increased significantly in phytoplasma-infected plants as compared to the healthy samples by 63.39% and 58.19 %, respectively. The results of this study were similar to those reported by Maust *et al*. (2003) and Zafari *et al*. (2012). In this study, an increase (29.7%) in the content of non-reducing sugar also was observed in the phytoplasma-infected leaves, meaning that more sucrose was produced, presumably, by increasing the activity of the sucrose enzymes involved in the metabolism as sucrose phosphate synthase (SPS), which can catalyze sucrose synthesis (Xue *et al*., 2018). Consequently, the total chlorophyll and carotenoid content significantly decreased in phytoplasma-infected leaves. The total chlorophyll and carotenoid content were reduced around 58.25 % and 44.74 % of their original values respectively, as compared to healthy samples (Table 2). Luti *et al*. (2016) have reported that the accumulation of carbohydrates could inhibit photosynthesis through the loss of enzymatic activity of ribulose-1, 5-bisphosphate carboxylase-oxygenase (RuBisCO), and other Calvin-cycle enzymes. The breakdown of chlorophyll and carotenoid appeared in the presence of several phytoplasmas in different plant species (Bertamini and Nedunchezhian, 2001; Zafari *et al*., 2012). These authors hypothesize that the inhibition in photosynthetic pigment accumulation because of enhanced chlorophyllase activity in the phytoplasma infected leaves.

Therefore, the changes in factors controlling photosynthesis and carbohydrate metabolism in leaves reflect phytoplasma growth requirements. Wherefrom the companion cells, the sugar diffuses into the phloem sieve-tube elements through the plasmodesmata that link the companion cell to the sieve tube elements. Phytoplasma inhabits phloem sieve elements in infected plants, which documented by several researchers by electron microscopy (El-Banna *et al*., 2007; El-Banna *et al*., 2015; Ahmed *et al*., 2014). Besides, mitochondria and chloroplasts are responsible for the synthesis of adenosine triphosphate (ATP) within the cell; however, mitochondria may be a source of ATP and energy for phytoplasmas, which lack both ATP synthase gene and sucrose synthase gene (Hogenhout *et al*., 2008; Hren *et al*., 2009). This pattern appears in the form of a system towards providing a favorable condition for multiplication of the phytoplasma in phloem sieve elements in infected plants or their supply of intense energy and the growth requirements. However, sugars as primary metabolites providing energy and structural material such as lignin for firs defense response in plants as well, they act as signal molecules interacting with the hormonal signaling network activating the plant's innate immune system. This fact, in addition to a strong correlation between higher soluble sugar concentration

and stress tolerance (Morkunas and Ratajczak, 2014). It is accepted to conclude that this one of the associated phytoplasma-mechanisms for their plant-colonization, meanwhile the soluble sugar concentration may play a role in disease tolerance.

Table 1 Concentrations of sugar and starch (mg/g fresh weight) in healthy and phytoplasma-infected hibiscus leaves.

Parameter	Healthy	Infected	Percentage of change (%)	LSD at 0.05
Soluble sugar	**9.640**	**15.250**	**+ 58.19**	**1.458**
Reduced sugar	**6.310**	**10.930**	**+ 73.20**	**5.921**
Non-reduced sugar	**3.330**	**4.320**	**+ 29.70**	**2.965**
Starch	**8.55**	**13.97**	**+ 63.39**	**1.186**

Data are means of three independent measurements (n=3).

Table 2 Concentrations of chlorophyll a, chlorophyll b, and carotenoids (mg/g fresh weight) in healthy and phytoplasma-infected hibiscus leaves.

Parameter	Healthy	Infected	Percentage of change (%)	LSD at 0.05
Chlorophyll a	**84.096**	**36.390**	**- 56.70**	**6.075**
Chlorophyll b	**74.663**	**30.000**	**- 59.80**	**5.816**
Chlorophyll (a+b)	**158.760**	**66.270**	**- 58.25**	**7.049**
Carotenoids	**16.380**	**9.050**	**- 44.74**	**1.646**

Data are means of three independent measurements (n=3).

Total flavonoid (TF), total phenolic (TP) contents, and PAL activity

Illustrated results in Table 3 indicate a lower content of TF by 35.6% and higher content of TP by 200.8% in phytoplasma-infected plants as compared with healthy plants. The increase of TP levels due to phytoplasma thus seems to relate to increase the defense response of the hibiscus plants against oxidative stress effect and inhibition of the pathogen inside the phloem. This assumption was also confirmed by the investigation of the accumulation of phenolic substances that have been found in infected plum and apple trees where the presence of phytoplasma triggered an increase (three-fold) in phenolic compounds which related to defense involving the inhibition of the pathogen inside the phloem vessels (Musetti *et al*., 2000; 2004). Ngadze *et al*. (2012) have reported that the phenolic compounds as secondary metabolic pathways may lead to enhancing the mechanical strength of host cell walls by the synthesis of lignin and suberin that are involved in the formation of physical barriers and blocked the spread of pathogens.

Phenylalanine ammonia-lyase activity also plays a significant role in the synthesis of several defense-related secondary compounds such as phenols, phytoalexins, and lignin (Halpin, 2004). Data in Table 3 reveal that the activity of PAL increased by about 251.8 % over the healthy control plants. The significant level of PAL with total soluble phenols during pathogen invasion reflects the importance of the enzyme in plant defense mechanisms. Romanazzi *et al*. (2007) reported that grapevines affected by Bois noir (BN) phytoplasma and those that had recovered from the disease showed an up-regulation of PAL that responsible for the biosynthesis of phenol derivatives, as compared to healthy plants.

Another study suggested that the activation of PAL and the subsequent increase in phenolic content in plants might be involved in the defense mechanisms against *Candidatus* phytoplasma *solani* in the infected plants (Huseynova *et al*., 2017). Therefore, it is highly likely that the high TP concentration or the high activity of PAL may play a critical role in serving the host in disease resistance.

Malondialdehyde (MDA) level, ROS-scavenging mechanisms, and glutathione (GSH) content

In the present investigation, MDA was analyzed as an indication of cellular stress–induced lipid peroxidation before assessing the level of antioxidant enzymes generated within the cells. Where, ROS like hydroxyl radical ($OH^{\bullet}$), and hydroperoxyl ($HOO^{\bullet}$, the simplest form of peroxyl radical, $ROO^{\bullet}$), can react with the unsaturated fatty acids in the cell membranes and induce lipid peroxidation process, which can start with the oxidation of only a few lipids but may spread, thus resulting in significant cell damage (Dallagnol *et al*., 2011; Lee *et al*., 2016). As shown in Table 4, the level of MDA found to be significantly increased with infection (204 %), indicating that there is oxidative stress that can mediate damage to cell structures during host-pathogen interaction. In the majority of cell

types, physiological levels of oxidative stress and the damage elicited by lipid peroxidation can be countered through the activation of antioxidant defenses and regulation of the production of free radicals to maintain cellular homeostasis (Mihalas *et al*., 2017). Where, the termination of the lipid peroxidation process starts when two radicals react and produce a non-radical species (Lee *et al*., 2016); this happens when the concentration of radical species is high enough for them to collide, and the high concentration of antioxidants like superoxide dismutase and glutathione can speed up this process.

Table 3 Total flavonoid content, phenolic content (mg/g fresh weight), and the phenylalanine ammonia-lyase activity (PAL) (nKat/g protein) in healthy and phytoplasma-infected hibiscus leaves.

Parameter	Healthy	Infected	Percentage of change (%)	LSD at 0.05
Total flavonoids	**105.790**	**68.10300**	**- 35.620**	**2.193**
Total soluble phenols	**383.353**	**1151.903**	**+ 200.48**	**5.175**
PAL activity	**10.5760**	**37.21300**	**+ 251.86**	**3.614**

Data are means of three independent measurements (n=3).

Table 4 Malondialdehyde level and Glutathione content in healthy and phytoplasma-infected hibiscus leaves.

Parameter (µM)	Healthy	Infected	Percentage of change (%)	LSD at 0.05
Malondialdehyde	**18.986**	**57.803**	**+ 204**	**4.370**
Glutathione	**2.180**	**6.566**	**+ 201**	**1.192**

Data are means of three independent measurements (n=3).

Results in Table 5 show that the enzymatic activity of APX, PPO, POD, and SOD in hibiscus leaves infected with phytoplasma was more than two-fold higher than that of healthy ones. The obtained results were similar to those reported by Zafari *et al*. (2012) through their studying the effect of *Candidatus* Phytoplasma aurantifolia on the activity of antioxidant enzymes in lime plants. These results generally suggest that the enzymatic antioxidant system, as ROS scavengers, can activate by several types of phytoplasma. Having established that the levels of antioxidant enzymes were significantly elevated upon exposure of cells to oxidative stress induced by a phytoplasma, also, the high level of the GSH (201%) found almost parallel to that of MDA (204%) in infected leaves of hibiscus shrubs with phytoplasma (Table 4). Therefore, possibly the high concentration of GSH may be resulting in the maintaining of the redox state of the host plant and subsequently enhance growth in hibiscus shrubs or their stability and serving in the tolerance of phytoplasma at the same time. A previous study indicated that plants use the non-enzymatic antioxidant system such as thiols to balance oxidative stress, letting ROS concentration free to act as a signaling defense inducer without excessive induction of cell damage (Luti *et al*., 2016).

Table 5 Activity of antioxidant enzymes (U min^{-1} mg^{-1} protein) in healthy and phytoplasma-infected hibiscus leaves.

Parameter	Healthy	Infected	Percentage of change (%)	LSD at 0.05
Superoxide dismutase (SOD)	**18.543**	**37.280**	**+ 101**	**1.470**
Ascorbate peroxidase (APX)	**4.8460**	**10.336**	**+ 113**	**2.111**
Polyphenol oxidase (PPO)	**20.100**	**42.860**	**+ 113**	**3.988**
Peroxidase (POD)	**19.243**	**52.650**	**+ 173**	**3.789**

Data are means of three independent measurements (n=3).

A growing body of evidence also suggests that plants can activate many enzymes such as PPO in the first stage of plant defense where PPO catalyzes the oxidation of phenolics to free radicals that can react with biological molecules, thus creating an unfavorable environment for plant pathogen development (Mohamed *et al*., 2012). According to Ray *et al*. (1998), POD and PPO enzymes may participate in the plant defense reactions, leading to increase plant resistance against pathogenic agents. It was also interesting to note that hibiscus plants that showed significant PPO and POD activities during a phytoplasma infection also recorded a high concentration of phenolic compounds in the leaves. Thus, the elevated levels of both GSH and phenolic compounds (non-enzymatic antioxidant system) along with the enzymatic antioxidant system (PPO and POD) can contribute to the maintaining the redox balance, neutralizing the toxicity of ROS, quenching singlet oxygen ($^1O_2$), and dealing with the undesirable level of lipid peroxidation caused by ROS to avoid the cell damage (Bell and Wheeller, 1986).

However, some plant pathogens can benefit from the increase in the ROS levels produced in host plants during the resistance of infection to facilitate their colonization

and nutrient uptake from the host (Govrin and Levine, 2000). It is important to stress that the presence of superoxide scavenging enzyme, or SOD. The SOD plays a defensive role to restrict pathogen colonization (Govrin and Levine, 2000). At the same time, enhancing antioxidant enzymes, in particular SOD, can lower the oxidative degradation of lipids and thus, indirectly help in DNA-damage repair that occurs because of lipid peroxide process or fatty acid radicals (Ighodaroab and Akinloyeb, 2018). Further, the SOD activity plays the main role in ROS-detoxification pathways that catalyze the dismutation of superoxide radicals to H_2O_2 with reducing its toxicity, however, antioxidant-scavenging enzymes, such as POD needs H_2O_2 as a co-substrate to function (Tenhaken, 2014).

Although the change in the H_2O_2 level is not evaluated in the present work, it can suppose that POD was involved in strengthening cell walls of hibiscus plants instead of the scavenging of H_2O_2, which allowed the accumulation of a higher level of H_2O_2, as noted from the function of the SOD enzyme or POD enzyme's requirement. Thus, excess of SOD or POD activity along with an accumulation of H_2O_2 in the same tissue could increasing plant resistance to oxidative stress and maintain the stability of the cell by cross-linking of cell wall components (Wakabayashi *et al*., 2012 and Tenhaken, 2014). According to another study (Musetti *et al*., 2004), the accumulation of H_2O_2 contributed to the recovery of the apple trees from apple proliferation phytoplasma. These authors hypothesize that the overproduction of H_2O_2 localized in the phloem tissues could limit the multiplication of phytoplasma. This hypothesis is intriguing because the effect of the recovery process phenomenon may relate to the stability and existence of hibiscus shrubs as a source of infection over the years.

Therefore, it is highly likely that the high level of SOD of particular value to hibiscus shrubs in DNA repair and entire defense strategies, especially in scavenging or stabilizing, or deactivating superoxide anion radical ($O_2^{-\bullet}$) and fatty acid-radicals before they attack the cellular components such as DNA, proteins, and lipids.

Nano-silica (NS) and phytoplasma-vector control

The results presented in Fig. (4) and summarized in Tables (6, 7, and 8) indicate that the direct and indirect application of high NS concentrations was more efficient than low concentrations against nymphs and adults of *E. decipiens*. Further, the effect of NS on the green leafhoppers' nymphs and adults was found to be dependent on dose and time. Where, the highest tested NS concentration 500 ppm showed high toxicity against green leafhoppers' nymphs and adults with maximum mortality (100%) at the first and 6th days in both direct and indirect treatment, respectively, compared to IM treatment.

The mechanism of insect pest control using NS is based on the fact that this type of nanoparticles is absorbed into cuticular lipids (which insects use to prevent the drying out of their cells) by physisorption and leads to the death of the insect when it is applied on the surfaces of leaves and stem (Barik *et al*., 2008).

Although there are no studies available on NS against leafhoppers (*E. decipiens*), several studies evaluated the toxic potential of hydrophilic NS on a wide range of insects. Debnath (2012) tested three types of NS (hydrophilic, hydrophobic, and lipophilic) on *Sitophilus oryzae* and *Spodoptera litura*, and found the highly toxic effect of hydrophobic and lipophilic NS reached 100 % after 7 and 14 days, respectively against *S. oryzae*, whereas mean mortality reached 100 % within 24 hours after hydrophilic NS treatment against *S. litura*. El-Bendary and El-Helaly (2013) also evaluated the effects of NS (100, 150, 200, 250, 300, and 350 ppm) on the resistance of tomato plants to *S. littoralis*. Their results indicated high toxic action at all concentrations. Moreover, they found high resistance in tomato plants against this insect pest, especially at 300 and 350 ppm. Also, Barik *et al*. (2012) found that the application of NS at 112.5 ppm was effective against the tested mosquito species (*Anopheles*, *Aedes*, and *Culex*). Furthermore, El-Samahy and Galal (2012) found that the reduction percentages in the number of *Aphis craccivora* or *Liriomyza trifolii* increased by increasing the NS concentration from 100 ppm to 500 ppm. Besides, El-Samahy *et al*. (2014) compared the efficacy of NS, neem oil extract, and IM against *Tuta absoluta* (Meyrick). Results revealed the high efficacy of NS, which did not differ significantly than IM. Moreover, Abd El-Wahab *et al*. (2016) recommended the NS hydrophilic at a 500-ppm concentration as a promising control method for

Myzus persica, *Acyrthosiphon pisum*, and *A. craccivora*. Therefore, this treatment (hydrophilic NS at 500-ppm) can be used in the integrated pest management program (IPM) for the control of the insect vectors of phytoplasma and to reduce the use of toxic pesticides to human health.

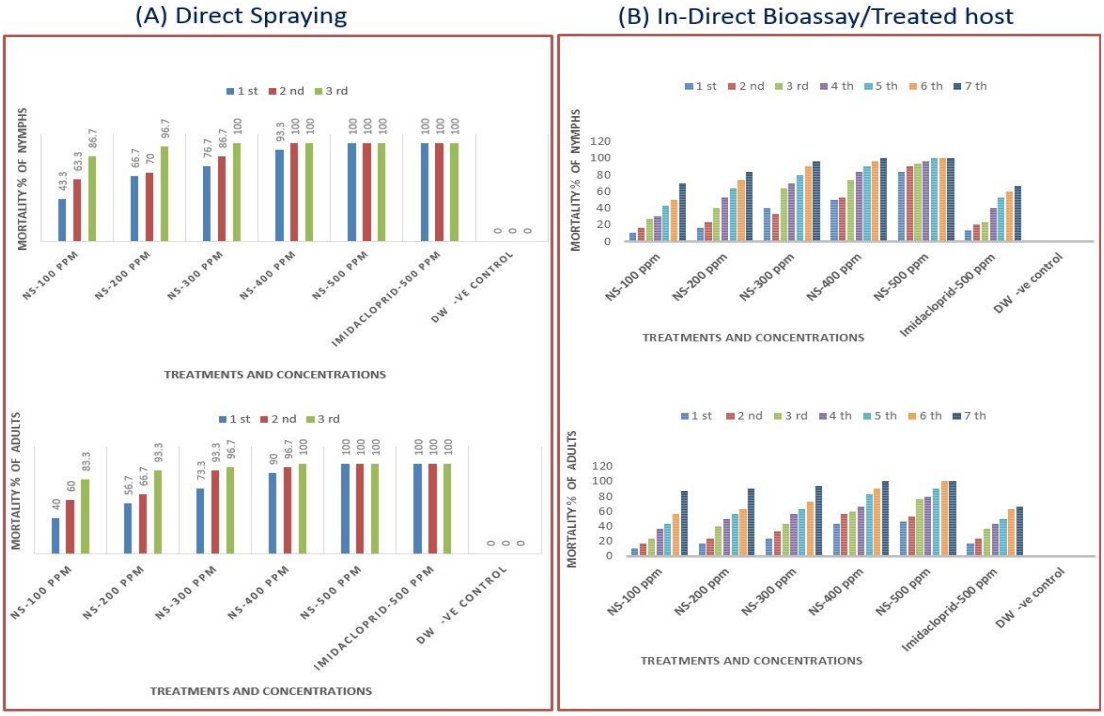

Fig. 4 Influence of Nano-silica (NS) type (hydrophilic) and Imidacloprid treatment at 500 ppm on the mortality rate of the phytoplasma-vector (green leafhopper: *Empoasca decipiens* Paoli) under laboratory conditions. (A): Direct application assay on leafhoppers. (B): Indirect application assay on leafhoppers by continuous feeding on the leaves of the treated host plant (*Vicia faba* L.).

Table 6 Percentage of mortality rates of phytoplasma-vector (green leafhopper: *Empoasca decipiens* Paoli) treated or sprayed directly with Nano-silica (NS) type (Hydrophilic) or Imidacloprid (IM), and daily follow up to 3rd day.

Treatment (ppm)	1st day				2nd day				3rd day			
	Nymphs		Adults		Nymphs		Adults		Nymphs		Adults	
	N	%	N	%	N	%	N	%	N	%	N	%
NS 100	13	43.3	12	40	19	63.3	18	60	26	86.7	25	83.3
NS 200	20	66.7	17	56.7	21	70	20	66.7	29	96.7	28	93.3
NS 300	23	76.7	22	73.3	26	86.7	28	93.3	30	100	29	96.7
NS 400	28	93.3	27	90	30	100	29	96.7	30	100	30	100
NS 500	30	100	30	100	30	100	30	100	30	100	30	100
IM 500	30	100	30	100	30	100	30	100	30	100	30	100
DW	00	000	00	000	01	3.30	00	000	01	3.30	00	0.00

Table 7 Percentage of mortality rates of phytoplasma vector (Nymphs of *Empoasca decipiens* Paoli) recorded using host leaves previously treated with Nano-silica (NS) type (Hydrophilic) or Imidacloprid (IM), and daily follow up to 7th day.

Treatment (ppm)	1st day		2nd day		3rd day		4th day		5th day		6th day		7th day	
	Nymphs		Nymphs		Nymphs		Nymphs		Nymphs		Nymphs		Nymphs	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%

NS 100	3	10.0	5	16.7	8	26.7	9	30.0	13	43.3	15	50.0	21	70.0
NS 200	5	16.7	7	23.3	12	40.0	16	53.3	19	63.7	22	73.3	25	83.3
NS 300	12	40.0	16	53.3	19	63.7	21	70.0	24	80.0	27	90.0	29	96.7
NS 400	15	50.0	17	56.7	22	73.3	25	83.3	27	90.0	29	96.7	30	100
NS 500	25	83.3	27	90.0	28	93.3	29	96.7	30	100	30	100	30	100
IM 500	4	13.3	6	20.0	7	23.7	12	40.0	16	53.3	18	60.0	20	66.7
DW	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 8 Percentage of mortality rates of phytoplasma vector (Adults of *Empoasca decipiens* Paoli) recorded using host leaves previously treated with Nano-silica (NS) type (Hydrophilic) or Imidacloprid (IM), and daily follow up to 7th day.

Treatment (ppm)	1st day		2nd day		3rd day		4th day		5th day		6th day		7th day	
	Adults		Adults		Adults		Adults		Adults		Adults		Adults	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
NS 100	3	10.0	5	16.7	7	23.3	11	36.7	13	43.3	17	56.7	26	86.7
NS 200	5	16.7	7	23.3	12	40.0	15	50.0	17	56.7	19	63.3	27	90.0
NS 300	7	23.3	10	33.3	13	43.3	17	56.7	19	63.3	22	96.7	28	93.3
NS 400	13	43.3	17	56.7	18	60.0	20	66.7	25	83.3	27	70.0	30	100
NS 500	14	46.7	16	53.3	23	76.7	24	80.0	27	90.0	30	100	30	100
IM 500	5	16.7	7	23.7	11	36.7	13	43.3	15	50.0	19	63.3	20	66.7
DW	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Conclusion

According to the results of this research, hibiscus plants can tolerate the existence of a phytoplasma and protect themselves against oxidative stress caused by the WB-disease by starting different anti-oxidative defense systems. Hibiscus plants applied two defensive strategies to deal with ROS under phytoplasma WB-disease stress. The first system comprises antioxidant enzymes (high molecular weight) such as superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO), and ascorbate peroxidase (APX), along with phenylalanine ammonia-lyase (PAL). The second mechanism comprises non-enzymatic antioxidants (low molecular weight) such as phenols and glutathione. It is highly likely that the leaf soluble sugar concentration or the high level of glutathione may play a critical role in serving the host in disease tolerance. The phytoplasma can manipulate the hosts to exploit them and possibly to help them simultaneously. It is tempting to speculate that mitochondria may play a role in phytoplasma-disease development, given their importance in ATP production. The hydrophilic NS applications also could help with the integrated environment-friendly management strategies for the control of the insect vectors of phytoplasma.

Conflict of interest

The authors declare that they have no competing interests.

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