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Research Article/Araştırma Makalesi

Biochemical alterations in lettuce (*Lactuca sativa* L.) infected with '*Candidatus* Phytoplasma asteris' related strain (16SrI-B subgroup)

*'*Candidatus *Phytoplasma asteris' (16SrI-B alt grup) ile infekteli marul (*Lactuca sativa *L.)'da biyokimyasal değişimler"*

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ABSTRACT

Phytoplasma infections are able to limit the lettuce growth around the world. The alterations of biochemical contents in the host physiology following phytoplasma infection in lettuce remain to be elucidated. In this study, changes in total protein and chlorophyll content, proline, malondialdehyde (MDA) accumulation, peroxidase (POD) and catalase (CAT) enzyme levels were investigated in leaves of lettuce plant after *Candidatus* Phytoplasma asteris infection. Symptoms observed in plants infected with phytoplasma were yellowing, little leaf, stunting, and a general decline. Phytoplasma agent detected in all infected lettuce by PCR-RFLP studies. Total protein and chlorophyll contents of phytoplasma-infected plants were lower than those of healthy control. Proline, MDA accumulation, POX and CAT enzyme activities were increased in infected plants as compared to those of control. The results show that phytoplasma infection can modify the host physiology of lettuce. In conclusion, this study indicated that the previously identified *Ca.* P. asteris was still pathogen with no changes in its DNA sequence and it was able to reduce the quality parameters of the lettuce plant and possess potential danger to the lettuce growing areas.

Key Words: Biochemical alterations, Phytoplasma, Lettuce, PCR,

ÖZ

Dünya genelinde marulda verimi sınırlayan fitoplazma infeksiyonları görülmektedir. Marul bitkisinde fitoplazma etmeninin konukçu fizyolojisinde meydana getirdiği biyokimyasal bileşenlerindeki değişimler anlaşılmaya devam etmektedir. Bu çalışmada *Candidatus* Phytoplasma asteris tarafından etkilenen marul bitkisinin yapraklarındaki total protein ve klorofil içeriği, prolin, malondialdehid (MDA) birikimi, peroksidaz (POD) ve katalaz (CAT) enzimlerindeki değişimler incelenmiştir. Fitoplazma ile infekteli bitkilerde gözlenen simptomlar sararma, küçük yaprak, bodurlaşma ve genel olarak bitkinin ölümü biçiminde olmuştur. PCR-RFLP çalışmalarıyla tüm infekteli marul bitkilerinde fitoplazmanın varlığı doğrulanmıştır. Fitoplazma ile infekteli bitkilerde toplam protein ve klorofil içeriği sağlıklı kontrol bitkilerine kıyasla azalmıştır. Prolin, MDA birikimi, POX ve CAT enzim aktivitesi infekteli bitkilerde artış gösterirken; sağlıklı kontrol bitkilerinde önemli düzeyde azalmıştır. Sonuçlar, fitoplazma infeksiyonunun konukçu fizyolojisini modifiye edebileceğini göstermiştir. Sonuç olarak bu çalışma, *Ca.* P. asteris'in marulu infekteleyen DNA dizisi değişmeyen bir patojen olduğu, etmenin marul bitkisinde kalite parametrelerini düşürdüğü ve marul yetiştirilen alanlarda potansiyel tehlike olabileceğini ortaya koymuştur.

Anahtar Kelimeler: Biyokimyasal değişimler, Fitoplazma, Marul, PCR,

Introduction

Lettuce (*Lactuca sativa* L.) is a broadleaf herbaceous cold climate vegetable belonging to the Lactuca genus in the Asteraceae family that plays a significant role in human nutrition. It is very rich in vitamins A, C, E, and K and contains high amounts of fiber, sugar, Ca, Fe, Mg, and K minerals (Kim et al., 2016). The most important place among the morphological features of lettuce is its leaves which are the consuming part. Flat or curly leaves and leaf colour are the important distinguishing factors. In addition to leaf shape and color, features such as core and head formation, leaf fleshiness, friability and earliness can be counted among the distinguishing factors (Křístková et al., 2008). Considering these features, salads and lettuces; curly-leaf salads, oily salads and lettuce are grouped under three main groups (Vural et al., 2000; Lebeda et al., 2007). Lettuce and salad group, which can be found in markets throughout the year, is one of the most consumed vegetables (Aybak, 2002; Shatilov et al., 2019). Due to these features, lettuce is grown in open, greenhouse and low plastic tunnels in almost every region of Turkey depending on the climate. The production of lettuce, which has a very short production period (2-3 months), is usually the second or third product culture in our country before or after the main vegetable production (Akinci et al., 2003).

However, lettuce production is adversely affected by some abiotic and biotic factors. According to Soylu et al. (2017), *Sclerotinia sclerotiorum, Botrytis cinerea, Golovinomyces cichoracearum* and *Bremia lactucae* are the most important fungal agents observed in lettuce cultivated areas. Among the bacterial agents, *Pectobacterium carotovorum* subsp. *carotovorum,* and *Enterobacter cloacae*, *Xanthomonas campestris* pv. *vitians* could be counted as significant pathogens. In addition, *Beet western yellows, Lettuce mosaic, Lettuce dieback, Lettuce big vein, Tomato spotted wilt, Turnip mosaic* virus agents were also detected in lettuce (Koike et al., 2007; Sertkaya, 2015).

In addition to these factors, phytoplasmas, could also be counted as bacterial agents, leading to symptoms such as yellowing, wilting, chlorotic local lesion, deformity, witches' broom formation and stunting in lettuce plants (Akkurak et al., 2021). Phytoplasmas are located in the Mollicute class of the Prokaryotae kingdom. They are limited to the phloem, they do not possess cell walls, therefore, they are very sensitive to tetracycline group of antibiotics. Phytoplasmas infect over 1000 plant species worldwide, including vegetables, fruits, and weeds, and are spread from infected plant to healthy plant by insects feeding on phloem tissues. (Bertaccini et al., 2014; Namba, 2019). Small leaves, phyllody, virescence, large buds and witches' broom are among the most common symptoms observed in vegetables infected by phytoplasma agents (Kumari et al., 2019).

Phytoplasmas affect plant species which mostly belong to Apiaceae, Asteraceae, Cucurbitaceae, Fabaceae and Solanaceae families from those 16 different ribosomal groups have been reported worldwide in 16SrI-A, 16SrI-B, 16SrIII-J, 16SrIX and 16SrXII-A (Kumari et al., 2019). For example, Lin et al. (2014) reported that *Ca.* P. asteris (16SrI) was associated with the most common symptoms such as chlorosis and wilting on infected lettuce plants were. It has been reported that the transfer from diseased lettuce plants to healthy plants could be via phloem-feeding leafhopper (*Macrosteles striifrons*) (Lin et al., 2020). *Ca.* P. trifolii in cabbage and pepper, *Ca.* P. solani and *Ca.* P. trifolii in tomatoes and potatoes, *Ca.* P. asteris in lettuce are the important phytoplasmas that have been transferred via insects (Çağlar et al., 2010; Yılmaz et al., 2019; Ulubaş Serçe and Yılmaz, 2019; Güller and Usta, 2020; Akkurak et al., 2021).

Phytoplasmas live in the phloem tissue of infected plants and accordingly initiate physiological deterioration in the host plant leading to the production of defense proteins (Musetti et al., 2005; Huseynova et al., 2017). Rasool et al. (2020) found that the activity of peroxidase (POD), superoxide dismutase and catalase (CAT) enzymes responsible for defense

responses increased while Chlorophyll *a*, Chlorophyll *b* and total Chlorophyll levels reduced in phytoplasma-infected plants in sweet orange (*Citrus sinenses* L.). Decreased chlorophyll content in plants infected with phytoplasma is thought to be a possible reason for the leaf color turning from green to yellow. In another study, malondialdehyde (MDA) and hydrogen peroxide levels were found higher in *Sesamum indicum* plants infected with phytoplasma than those of healthy plants (Ahmad et al., 2019). Zafari et al. (2012) also stated that a decrease in total protein content was evident in the leaves of the lime plant infected with *Ca.* Phytoplasma aurantifoliae.

We had previously performed DNA isolation and PCR-RFLP on lettuce plants that showed signs such as yellowing, tiny leaves, and stunting. We then stated that the component producing these symptoms was discovered to be associated to the *Candidatus* Phytoplasma asteris (16SrI-B) subgroup as a consequence of the analysis. (Akkurak et al., 2021). In this study, we aimed to examine the biochemical changes in antioxidant enzymes (POD and CAT) along with chlorophyll, protein, malondialdehyde (MDA) and proline contents in lettuce plant infected by *Ca.* P. asteris. These possible changes in terms of physiological and biochemical patterns are not explored in lettuce plants to the best of our knowledge.

Material and Methods

Plant material

Previously reported causal agent including symptoms such as yellowing, small leaf, necrosis, stunting, and a general decline in lettuce plants had been related to *Ca.* Phytoplasma asteris through PCR tests (Akkurak et al., 2021). In this study, healthy control (n=6) and symptomatic (n=8) leaves of *L. sativa* were collected for DNA extraction to confirm our previous findings if the symptomatic lettuce plants still possess the confirmed phytoplasma agent. The samples were tested again via two-step polymerase chain reaction (PCR) assays.

DNA isolation and PCR analysis

Total DNA isolation was made from symptomatic and healthy lettuce plants as described by Ahrens and Seemüller (1992). Tissue leaf midrib (0.5 g) was homogenized in 2 ml of CTAB buffer and 2 ml aliquots of the extract were incubated at 65°C for 35 min. Afterward, DNA purification was made through chloroform-isoamyl alcohol (24:1) extraction method and precipitated. Total DNA was melted in 50 µl of TE buffer and maintained at -20 ºC until use. Total DNAs obtained here was used as template in our PCR studies for phytoplasma diagnosis.

Template DNA was tested with two-step PCR using R16F1/RO primers in the first round PCR followed by R16F2n/R2 primers in the second round to amplify the 1.250 bp DNA fragment from the 16S rRNA gene (Gundersen and Lee, 1996; Duduk et al., 2013).

The following conditions were used for PCR: denaturation at 94°C for 3 minutes, annealing at 54°C (F1/R0) or 55°C (F2n/R2) for 2 minutes, and primer extension at 72°C for 3 minutes. Both PCRs were subjected to a 10-minute extension cycle at 72 °C. Ten µl of F2n/R2 amplified products were electrophoresed in a 1% agarose gel and visualized on a UV transilluminator.

Protein contents

Bradford's method was used to determine total protein content (1976). Approximately, 0.5 g healthy and symptomatic lettuce leaf samples were taken and homogenized with 5 ml of sodium phosphate buffer, pH 7. Afterwards, 5 ml of Coomassie Brilliant Blue G-250 was mixed with 100 µl of plant extract and read at 595 nm in a spectrophometer (Shimadzu, UV-1280). Bovine Serum Albumin Fraction V (Sigma), at different concentrations (10-100 µg ml^{-1}) was used for the protein standard curve.

Chlorophyll measurement

Chlorophyll *a* and chlorophyll *b* analyses in healthy and infected lettuce leaves were made according to the method of Arnon (1949). Approximately 0.5 g leaf was homogenized in 5 ml

acetone/water (80% v/v) mix, then filtered and placed in Eppendorf tubes. Readings were made against 80% acetone control at 663.5 nm for chl *a* and 645 nm for chl *b*. Chlorophyll calculation was made on the basis of mg L^{-1} on a fresh weight (FW) basis.

Total chlorophyll (mg L^{-1})= 20.2 A_{645}+ 8.02 $A_{663.5}$
Chlorophyll *a* (mg L^{-1})= 12.7 $A_{663.5}$- 2.69 A_{645}
Chlorophyll *b* (mg L^{-1})= 22.9 A_{645}- 4.68 $A_{663.5}$

Proline analysis

Proline activity was employed with minor modifications according to Bates et al. (1973) (Karakas et al. 2013). The compound consisting of an acid-ninhydrin mixture was used as a color agent in the analysis. Nearly 0.5 g leaf sample was taken from healthy and infected the fresh leaf, grinding in liquid nitrogen, and solubilized by adding 10 ml of 3% sulfosalicylic acid. The extract was passed through filter paper Whatman No:1 and 2 ml of the mixture was added to 2 ml of acid ninhydrin solution, and the mix was boiled for 1 hour at 100 ˚C. The reaction was then stopped in ice cold water. Afterwards, toluene (5 ml) was added to the reaction mix and vortexed for 30 seconds. Then, the upper phase was taken, and the absorbance reading was evaluated at 520 nm in a spectrophotometer (Shimadzu, UV-1280).

Malondialdehyde (MDA) analysis

MDA was measured according to Heath and Packer (1968) with some modifications (Karakas et al. 2013). A fresh leaf sample of about 0.5 g was taken from healthy and infected plants, homogenized with 10 ml of 0.1 percent trichloroacetic acid (TCA), and centrifuged for 5 minutes at 10,000 *g*. After centrifugation, 4 ml of 20% TCA [containing 5% thiobarbituric acid (TBA)] was added to 1 ml of extract. The resulting mixture was incubated for 30 minutes at 95 ˚C for 30 minutes. It was then rapidly cooled with the aid of an ice bath and then centrifugation at 10.000 *g* for 10 minutes, 300 µl of supernatant was taken, the absorbance readings at 532 and 600 nm in a spectrophotometer were used for the MDA calculation using the following formula.

MDA (nmol g^{-1} fresh g.) = [Extraction volume (ml) x [(A_{532}-A_{600}) /(155 mmol L^{-1} cm^{-1})] / Sample quantity (g)]x 10^3

Catalase (CAT) activity (E.C. 1.11.1.6)

The protocol prepared by Milosevic and Slusarenko (1996) was used to measure catalase (CAT) activity. A 0.5 g of fresh leaf sample was collected from the plants and homogenized with Na-phosphate buffer, pH 7.0. Then, 50 µl of the obtained supernatant was added to 2.95 ml of the reaction mix, then it was read in the spectrophotometer at 240 nm for 30 seconds. One unit of catalase enzyme activity expressed as the amount of enzyme that breaks down 1 µmol H_2O_2 in 1 minute was used to express the enzyme in terms of unit mg^{-1} protein (Wang and Han, 2009).

Peroxidase (POD) activity (E.C. 1.11.1.7)

Peroxidase measurement was performed following protocol prepared by Cvikorova et al. (1994) with some modifications (Karakas et al., 2013). Approximately 0.5 leaf samples were taken from the healthy and infected plants, homogenized in phosphate buffer, pH 7.0. Then, 100 µl of the obtained leaf extract was taken and 3 ml of reaction mix (13 mmol L^{-1} guaiacol, 5 mmol L^{-1} H_2O_2 and 50 mmol L^{-1} Na-phosphate, pH 6.5) was added. The reaction was started with the addition of H_2O_2 and read 3 times at 25˚C at 470 nm within 1 minute intervals. One unit of peroxidase enzyme was defined as 0.1 absorbance min^{-1} at AA470 nm. Results are expressed in units mg^{-1} protein.

Statistical analysis

The data obtained as a result of the analyses were analyzed using Tukey's Test Method using one-way analysis of variance (ANOVA) in Minitab Statistical Software-20. Variations between groups were significant at $p< 0.05$. The error bars in the graphs show the mean ± error.

Results and Discussion

Morphological observations in infected lettuce plants

We observed remarkable symptoms in lettuce plants infected with *Ca.* P. asteris such as yellowing, small leaf, stunting and decline (Fig. 1).

We confirmed that the symptoms in lettuce plants were associated with *Ca.* Phytoplasma asteris through PCR-RFLP studies. No DNA bands associated with *Ca.* Phytoplasma asteris in the healthy lettuce plants were detected (Fig. 2).

Fig. 1. Symptoms of lettuce plants infected with phytoplasma yellowing and small leaf (a), stunting (b) and decline (c).

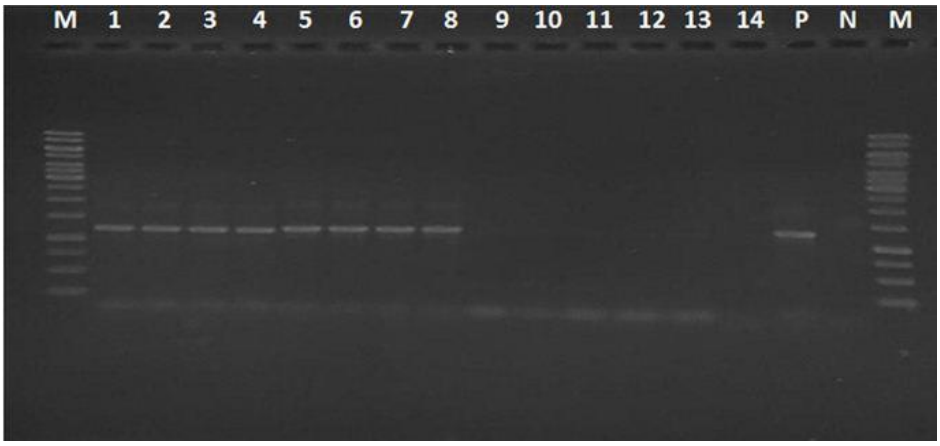

Fig. 2. Gel electrophoresis of DNA amplified by PCR. *Lane 1-8* phytoplasma-infected lettuce leaves, lane 9-14 non-infected lettuce leaves, *N;* negative control or no DNA, *P;* positive control, *M;* 1 kb DNA size markers.

Effect of phytoplasma infection on total protein and chlorophyll contents

Soluble protein content was significantly lower in infected leaves when compared to those of healthy plants (Fig. 3). Similar conclusions were observed by Nasir et al. (2017), who indicate that chickpea plants are infected with *Ca.* P. australasia reduced the soluble contents of leaves.

Decreased in protein content in most diseased plants are commonly observed. In this study, we detected soluble protein decline in infected lettuce plants following phytoplasma infection in which the lettuce plants exhibited defensive responses throughout the growing season.

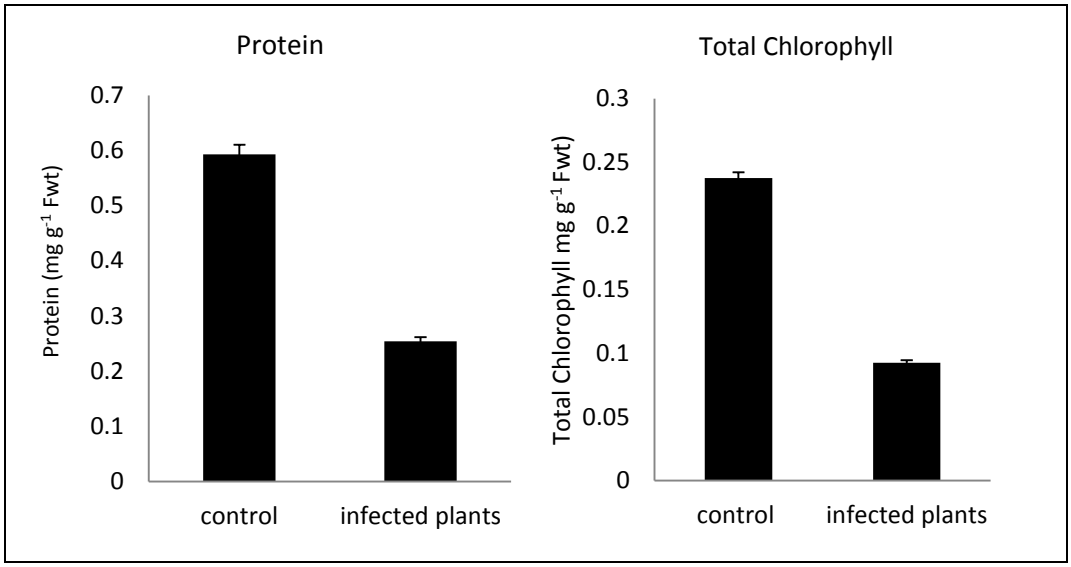

Fig. 3. Total protein and chlorophyll levels in control and phytoplasma-infected lettuce leaves.

The significant decline was also observed with that of total chlorophyll content (Fig. 3, p≤0.05). Previous reports were in line with our findings in which the phytoplasma infection decreased the chlorophyll content of Chinese jujube (Liu et al., 2016), corn beans (Hameed et al., 2017) and lime (Zafari et al., 2012) crops. In the present study, the low chlorophyll content is thought to be related to the yellowing and chlorosis symptoms. Although the detailed mechanism for the chlorophyll degradation following phytoplasmas is not revealed yet, we assume that phytoplasmic agents interfere with Mg^{2+}/Fe^{2+} molecules through the production of reactive oxygen species (ROS) and other stress metabolites.

Effect of phytoplasma infection on proline and MDA content

When we examined proline and MDA levels, both metabolites exhibited remarkable differences from those of control plants, p≤0.05. Proline has significant roles widely of biological mechanism, stress, signaling, energy production. Its role in the pathogenicity of microorganisms has been subject to be elucidated (Christgen and Becker, 2019). The amount of proline in lettuce leaves infected with phytoplasma increased as compared to the healthy ones (Fig. 4). Similar findings were also obtained in *Citrus aurantifolia* by Abdolahi et al. (2012) who reported that phytoplasma infection resulted in high accumulation of proline in the leaf. For instance, Dikilitas et al. (2020) indicated that the determination of the proline content in plants infected with phytoplasma agent is a good marker for measuring the effects of the disease as well as resistance levels of the host plants.

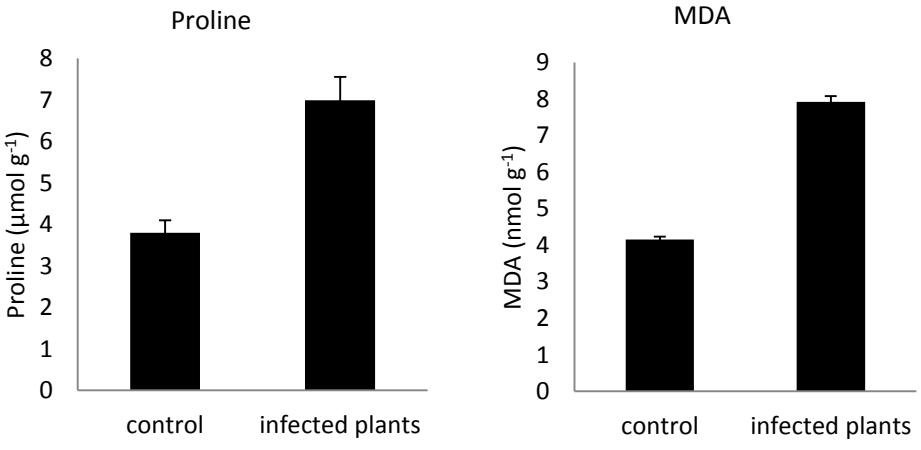

Fig. 4. Proline and MDA levels in control and phytoplasma-infected lettuce leaves.

One of the most important effects of free radicals formed under abiotic and biotic stress conditions is the peroxidation of lipids. With the reaction of polyunsaturated fatty acids and oxidants, lipids in the cell membrane are oxidized and malondialdehyde (MDA) is composed as the end product of lipid peroxidation (Morales and Munné-Bosch, 2019). Weber et al. (2004) reported a remarkable increase in MDA level in *Arabidopsis* spp. under oxidative stress conditions. In this study, the MDA content of the plants infected with *Ca.* P. asteris was found higher than the healthy plants (Fig. 4). Similarly, Ahmad et al. (2019) stated that increased MDA levels in *Sesamum indicum* following phytoplasmas infection. However, Rasool et al. (2020) also reported that MDA level in *Citrus sinensis* L. increased following phytoplasma infection. It could be possible that MDA level may not increase upon infection. The host plant could tolerate the stress or suppress MDA activity depending on its capacity in terms of genetic background and the infection period of the phytoplasmic agent. However, in general, MDA content appear to elicit different responses in the plant depending on the plant or the phytoplasma species. Therefore, increases in MDA are a widely used marker for the determination of biotic and abiotic stress levels and it serves as a sign of the extent of damage under stress.

Effect of phytoplasma infection on enzyme activities (POD and CAT)

When the level of antioxidant enzymes was the elucidated, level of both enzymes increased following *Ca.* Phytoplasma asteris infection.

Peroxidases are one of the antioxidant enzymes that play an important role in cell wall formation, lignification, removal of ROS, and inducing plant defense system (Passardi et al., 2005; Prasannath, 2017). In this study, we determined that POD and CAT activities increased significantly in lettuce leaves infected with phytoplasma (Fig. 5, $p \leq 0.05$). Similarly, Junqueira et al. (2011) reported that phytoplasma infection in the maize plants resulted in a significant increase in POD and CAT enzymes.

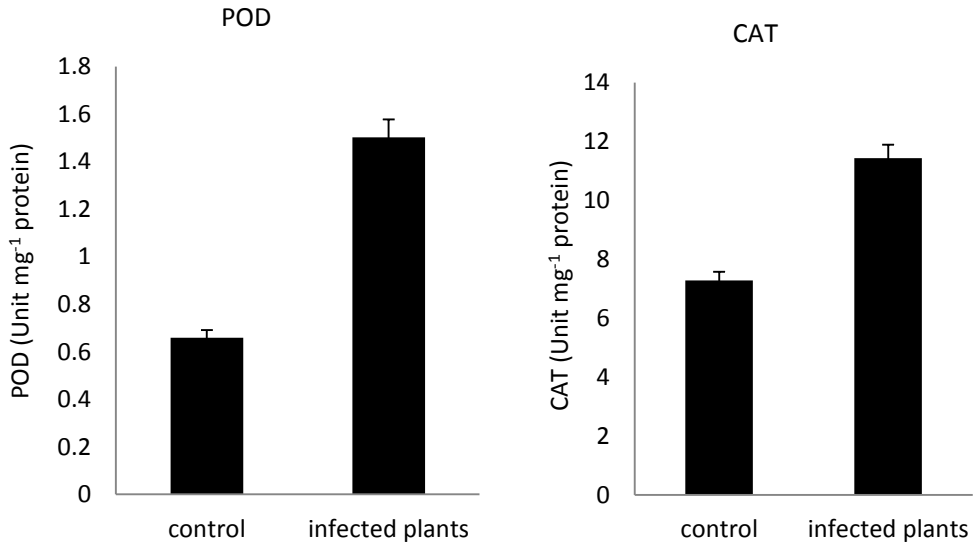

Fig. 5. POD and CAT enzyme levels in control and phytoplasma-infected lettuce leaves.

Catalase is also one of the essential enzymes capable of protecting biological systems against free radical attack (Ighodaro and Akinloye, 2018) and is the most effective enzyme, which reduces H_2O_2 into H_2O and O_2 (Mhamdi et al., 2010; Alam et al., 2014; Veronica et al., 2017). Catalase activity is mostly elevated against plant pathogen attack (Magbanua et al., 2007). Hameed et al. (2017) in mungbean (*Vigna radiata*), Zafari et al. (2012) in lime (*Citrus aurantifolia*) also reported that increased CAT activity in plants following phytoplasma infection.

Conclusions

This study indicated that phytoplasma could damage the infected lettuce plant via deteriorating the physiological and biochemical processes. Compared to uninfected plants, chlorophyll and protein contents decreased in diseased plants. The morphological changes (yellowing, small leaf, stunting and decline) observed in the infected-plant indicated that the phytoplasma has the potential to reduce photosynthesis rate and protein synthesis.

The increase in proline, MDA, CAT and POD enzyme activities with phytoplasmic infection showed that the lettuce plant became defensive against the agent. Knowing the biochemical changes that occur in the host plant provides an understanding of the responses of the phytoplasma infection and can provide information about the recovery that activates the resistance mechanism. These results provide important clues for developing control strategies against phytoplasma diseases.

Conflict of interest: All the authors declare that there is no conflict of interest in this study.

Author contributions: HA was responsible for designing the Nested-PCR and characterization and biochemical analyses. All authors contributed sections to the manuscript and approved the final version of the text.

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