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# EFFECT OF PHYTOPLASMA INFECTION ON PLANT HORMONES, ENZYMES AND THEIR ROLE IN INFECTED SESAME

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## ABSTRACT

Sesame (*Sesamum indicum* L.) from the family of *Pedaliaceae* is one of the most ancient crops and oilseeds. It was a major oilseed crop in the ancient world due to its easiness of extraction, great stability, and resistance to drought. One of the most dangerous pathogens that affect sesame and other plants worldwide is phytoplasma. Phytoplasma can cause different character of symptoms such as: phyllody, the common symptom caused by phytoplasma infection, stunting, changing the color of leaves from green to yellow and alteration of the floral parts into leafy structures bearing no capsule and seeds, which consider a serious threat for the production of sesame in many countries. On the other hand, effect of the phytoplasma infection on plant enzymes such as Peroxidase and Polyphenol oxidase and hormones like Auxins, Cytokinin, Gibberellins and Abscisic acid were studied. Electron microscopy assay was used to verify the cellular changes which conducted as a result of phytoplasma infection. Infected sesame samples which examined by PCR were used as an exporter of phytoplasma and directly applied for electron microscopy test and the analysis of enzymes and hormones. Transmission electron microscopy (TEM) was used to detect ultra-structural changes in infected sesame plants.

## KEYWORDS:

Sesame, Phytoplasma, plant hormones, enzymes, PCR, Transmission electron microscopy.

## INTRODUCTION

Sesame (*Sesamum indicum* L.), family of *Pedaliaceae*, which consists of sixteen genera and sixty species, genus *Sesamum* belongs to the system of Tupiflora. It was a major oilseed crop in the ancient world due to its easiness of extraction, great stability, and resistance to drought [1]. It is also known as benni seed, gingelly, simsim, ajonjoli and sesamo. Sesame is considered to have both nutritional and

medicinal values. The seeds are used either decorticated or whole in sweets such as sesame bars and halva, in baked products, or milled to get high-grade edible oil or tahini, an oily paste [2]. Sesame seed contains two lignins, sesamin and sesamol which converted to sesamol. Sesamol has been found to have anti-oxidative effects and to induce growth arrest and apoptosis in cancer cells [3, 4]. Sesame plants suffer from several fungal, viral, bacterial and phytoplasma disease. Phytoplasmas are cell-wall less bacteria that have very small genome sizes and are amongst the smallest self replicating living organisms [5]. The phytoplasmas are normally considered pathogens of plants and it found in the phloem cells of host plants. The fact that phytoplasmas cannot be cultured on artificial media and can only be maintained in their plant host has made the study of phytoplasmas very laborious and difficult.

During the last ten years, PCR has been applied for the detection of a large number of microorganisms, also including phytoplasmas. Several methods have been developed for the universal detection of phytoplasmas, these differ in principle only in the choice of primers which are normally designed so that they will amplify a region of the well characterized and highly conserved ribosomal (rDNA) genes. Nested-PCR assay, designed to increase both sensitivity and specificity, is the leading method for the amplification of phytoplasmas from samples in which unusually low titer, or inhibitors are present that may interfere the PCR efficacy [6]. Plant diseases associated with the presence of phytoplasmas typically exhibit a number of symptoms that are suggestive of disturbances in the normal balance of plant enzymes and hormones [7].

Plants formed many defense mechanisms. Enzymes play one of the most important roles in the defense infection process and pathogenesis. Peroxidase was one of the key enzyme, controlling plant growth and development. It involves in construction and eventual lignifications of cell walls, protection of tissue from damage and infection by pathogenic microorganisms [8, 9]. The most frequently suggested role for polyphenol oxidase in plants has been in defense against herbivores and pathogens [10].

**TABLE 1**  
**Primers used in PCR reactions to identify phytoplasma on sesame plants**

Primer	Sequence	Size (bp)	Reference
P1	5'-AAGAGTTGATCCTGGATT-3'	1800	Sinclair et al., [16]
P7	5'-CGTCCTTCATCGGCTCTT-3'		
R16F2n	5'-GAAACGACTGCTAAGACTTGG-3'	1200	Wang and Hiruki [17]
R16R2	5'-TGACGGGCGGTGTGTACAAACCCC-3'		

Plant hormones (additionally called phytohormones) are chemicals that direct plant development, it is a flag atoms delivered in the plant and happen at low concentrations. In target cells, hormones organize cellular processes locally, and move to other sites in other functional parts of the plant [11]. Hormones also determine the formation of flowers, stems, leaves, leaves, as well as the development and maturation of fruits. They affect the tissues that grow and grow down, the formation of leaf and stem growth, the development and maturity of fruits, the longevity of plants and even the death of plants. Hormones are vital for plant growth and, if not, the plants will mainly consist of a mass of undifferentiated cells. They are also known as growth factors [12].

Phytoplasmas can be detected by using ultrathin-section EM [13]. The technique is sensitive, but phytoplasmas are not always easily distinguishable from plant material or from other plant-pathogenic bacteria inhabiting the plant phloem. The method is very hard and applicable only in specialized laboratories with a very expensive electron microscope. It is therefore not suitable for high throughput analysis. PCR has been used during the last ten years for the detection of a large number of microorganisms, also including phytoplasmas. Several methods have been advanced for the universal detection of phytoplasmas, these vary in principle only in the choice of primers which are normally designed so that they will amplify a region of the well distinguished and highly protected ribosomal (rDNA) genes. Nested-PCR assay, designed to increase both sensitivity and specificity, is the leading method for the amplification of phytoplasmas from samples in which unusually low titer, or inhibitors are present that may interfere the PCR efficacy [14]. The aim of this research was conducted to be able to identify phytoplasma of infected sesame plants by using polymerase chain reaction (PCR) with degenerate primer P1 / P7 followed by nested primer R16R2/ R16F2n to overcome problems concerned to sensitivity of phytoplasma detection.

## MATERIALS AND METHODS

**Source of plant material.** Leaves from symptomatic sesame plants were collected from Agriculture Research Center, Giza-Egypt. The samples maintained under greenhouse condition and used for further tests.

### DNA Extraction and PCR Amplification.

DNA was extracted from fresh samples of sesame plants exhibiting phyllody, witches broom and symptoms belong to phytoplasma infection using the modified Dellaporta extraction method [15]. Viral DNA, from infected and healthy plants was amplified using degenerate and specific PCR primers. The concentration of the primers was adjusted to 10 mM with high quality Milli-Q water. Degenerate PCR primers P1/P7 (Table 1) was applied for the first PCR reaction followed by nested (specific) PCR primer R16F2n/R16R2 (Table 1). PCR reaction mixture of 50µl contained 250 ng (10µl) extracted DNA, 10 mM of dNTPs and 0.1 µM of each primer and 1.25 mM MgCl<sub>2</sub>. Taq DNA polymerase (Thermo Scientific). Viral DNA was amplified in a Bio-Rad DNA Thermal Cycler (PTC100) by 35 cycles of melting, annealing and DNA extension conditions of 1 cycle of 3 min at 94°, 30 cycles of 1 min at 94°C, 2 min at 53°C and 55°C for nested primer, 2 min at 72°C and final extension 10 min at 72°. The temperature then was decreased to 4°C until the reaction mixtures were removed. The amplified DNA was electrophoresed in 1% agarose gel in 0.5TBE buffer at 120V for 1 hour, stained with ethidium bromide (0.5µl/ml) and photographed using gel-documentation system (Bio-Rad, GelDoc XR). VC 100bp Plus DNA ladder (Vivantis) and VC 1Kb Plus DNA ladder (Vivantis) were used as PCR Markers.

**Determination of total soluble protein.** Soluble proteins were measured by the Bio-Rad micro assay modification of the Bradford [18] procedure using crystalline bovine serum albumin as a reference.

**Determination of enzymes activity. Preparation of enzymes extract.** Plant fresh tissues were ground using cold mortar and pestle and homogenized with cold sodium phosphate buffer (100mM, pH 7) containing 1% (w/v) polyvinylpyrrolidone (PVP) and 0.1 mM EDTA. The extraction ratio was 4 ml extraction buffer for each one gram of plant tissues. The homogenate was centrifuged at 4500 rpm at 4° C for 15 min. The supernatant was used for measurement of peroxidase (POD) and polyphenol oxidase (PPO), activities.

**Peroxidase activity (POD).** The assay mixture (100 ml) contained 10 ml of 1% (v/v) guaiacol, 10 ml of 0.3 % H<sub>2</sub>O<sub>2</sub> and 80 ml of 50mM phosphate buffer (pH 6.6). Volume of 100 µl of crude enzyme was added to 2.9 ml of the assay mixture to start the

reaction. The absorbance was recorded every 30 sec for 3 min at 470 nm using spectrophotometer. The enzyme activity was expressed as unit.  $\text{mg}^{-1}$  protein [19].

**Polyphenol oxidase activity (PPO).** The reaction mixture consisted of 100  $\mu\text{l}$  crude enzyme, 600  $\mu\text{l}$  catechol and 2.3 ml phosphate buffer (0.1 M, pH 6.5). The absorbance at 420 nm was recorded at zero time and after 1 min using spectrophotometer. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001 per min at 420 nm. The enzyme activity was expressed as unit.  $\text{mg}^{-1}$  protein [20].

**Hormonal analysis.** The HPLC analysis was used to determine the phytohormones as follows: ten grams of fresh tissue per sample was homogenized with 70% (v/v) methanol and stirred overnight at 4°C. The extract was filtered through a Whatman filter and the methanol evaporated under vacuum. The aqueous phase was adjusted to pH 8.5 with 0.1 M phosphate buffer and then partitioned with ethyl acetate 3 times. After removal of the ethyl acetate phase, the pH of the aqueous phase was adjusted to 2.5 with 1 N HCl. The solution was partitioned with diethyl ether 3 times, and then passed through anhydrous sodium sulfate. After that the diethyl ether phase was evaporated under vacuum and the dry residue containing hormones was dissolved in 2.0 ml of methanol and stored in vials at 4°C. The chromatographic analysis was performed on an Agilent Model 1260 [21]. The hormones content was expressed as  $\mu\text{g/g}$  fresh weight.

**Chromatographic procedure.** Acid was adjusted to different pH values with sodium hydroxide. The Luna C18 column was equilibrated for each mobile phase condition with a time limit of 30 min. The column temperature was maintained at constant 25°C. The separation was carried out by isocratic elution with a flow rate of 1.0 mL/min. An injection volume of 10  $\mu\text{L}$  was used for each analysis. The standard solution of the individual acid was prepared in the mobile phase and chromatographed separately to determine the retention time for each acid at Wavelength at: 210 nm [22].

**Morphology of phytoplasma particles.** Phytoplasma dip preparation technique from sesame infected plants was applied. Carbon coated copper grids (400mesh) were dipped in sap expressed and then negatively stained by 2% phosphotungstic acid (PTA) for 2min then air dried. The grids were examined using transmission electron microscope JEOL (JEM-1400TEM, Japan) at the candidate magnification. Images were captured using CCD Camera Model AMT at 50000X. This work was done in the TEM lab, Faculty of Agriculture, Cairo University, Research Park (FARP).

### Transmission electron microscopy (TEM).

Transmission electron microscopy (TEM) was carried out to check the ultrastructural changes inside the infected young leaves of sesame plants in addition to healthy ones. Pieces about 2x2 mm, were taken from leaf samples, cut and transferred to a separated vial to be fixed in 2.5% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.4) for an hour. After removing the fixative solution, the tissues were washed in sodium phosphate buffer three times for 30 min each. After washing, the buffer was pulled out, and 1% of osmium-tetroxide ( $\text{OsO}_4$ ) was added to the tube and allowed for 1/5 hours at 4°C. After removing the fixative solution, the samples were dehydrated in an ethanol series of 15%, 30%, 50%, 70%, 80% and 95% before exposing to 100% for 15% min for every step expect the step of 100% ethanol, which was repeated twice. Infiltration with Spurr's epoxy resin, one large drops into the sample tube every 15 min, until at 75% resin overnight with rotating. Samples were put into 100% resin, for at least a day, then samples were placed into flat BEEM capsule molds, the resin was incubated overnight in an oven at 60°C.

Samples were sectioned (90- 100 nm thick) with ultra- microtome (Leica model EM-UC6) at thickness 60 $\mu\text{m}$ , mounted on copper grids (400 mesh). Ultra-thin sections were double stained with 2% uranyl acetate and 10% lead citrate, and then allowed to dry well for 10 min. followed by lead citrate for 5 min. Stained sections were examined by transmission electron microscope JEOL (JEM- 1400 TEM, Japan) at the candidate magnification and images were captured using CCD camera model AMT. Optronics camera with 1632x 1632 pixel formats as side mount configuration. This work was carried out at the TEM lab, Cairo University, Research Park (CURP), Faculty of Agriculture.

## RESULTS

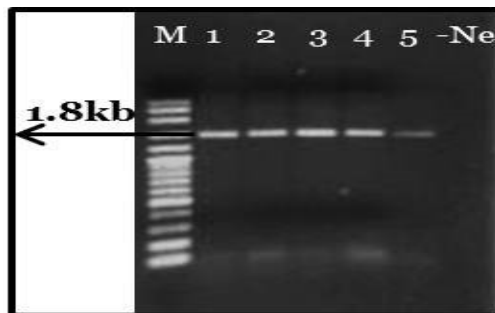
**Source of samples.** Sesame infected and healthy samples were collected from Giza Governorate, Egypt. All collected samples were symptomatic with different phytoplasmas like such as phyllody, green leaf like floral, proliferation and virescence except the healthy plant as shown in Fig.(1). Symptoms were recorded and all sampled applied directly for DNA extraction and molecular detection.

**Molecular Detection.** PCR amplification was used for phytoplasma detection using universal phytoplasma PCR primer P1/P7 (Fig.2). Nested primer R16F2n/R16R2 was used to confirm the infection with a product size range about 1250 bp for all infected samples, while no results were shown in health plants (Fig.3).



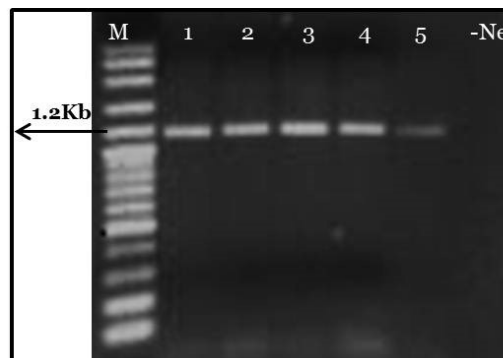
**FIGURE 1**

Symptoms collected from different sesame plant with the main phytoplasma like: (A) Healthy sesame plant, (B) Virescence with floral proliferation, (C) Phyllody and (D) Cracked capsules with germinated seeds.



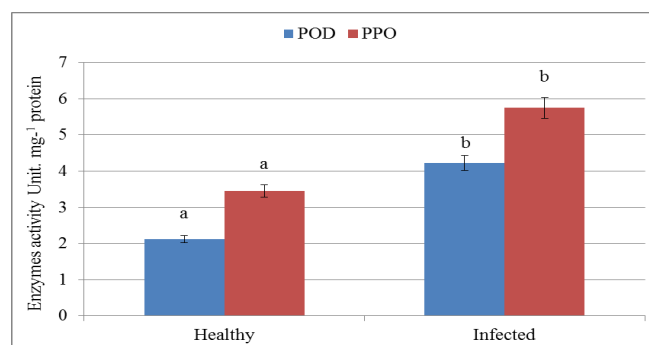
**FIGURE 2**

Ethidium bromide-stained gel of PCR amplification products obtained by using universal primer pair P1/P7 for phytoplasma detection. DNA was extracted from sesame plant samples showed symptoms phytoplasma like. M=100 bp DNA marker, Lanes (1-5) = different sesame samples with phytoplasma like symptoms; -Ne= Healthy sesame plant.



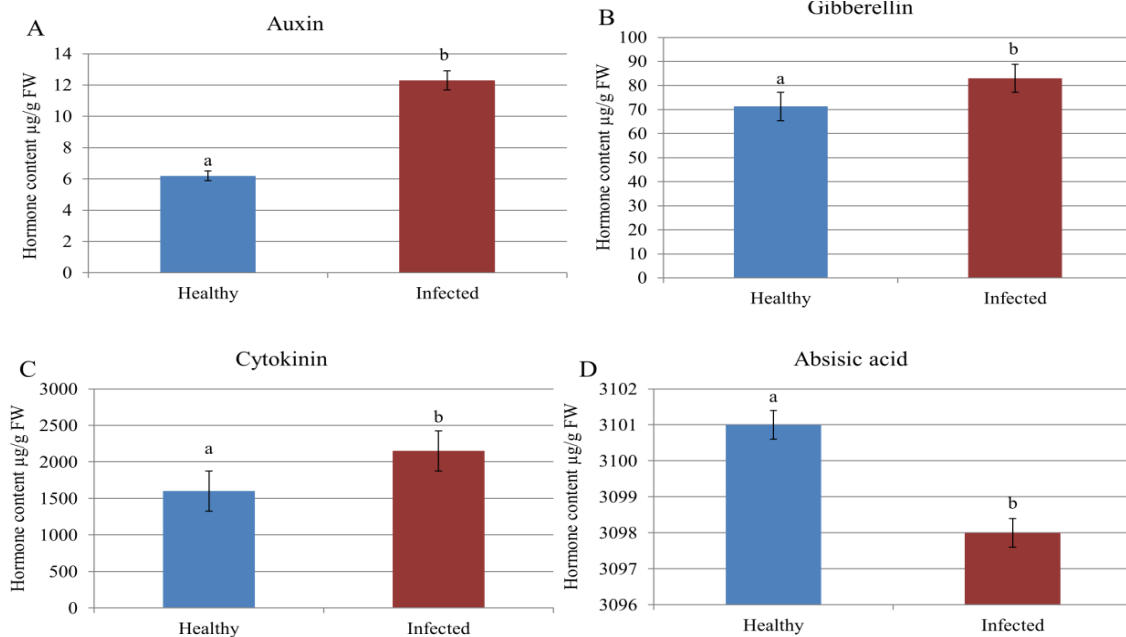
**FIGURE 3**

Ethidium bromide-stained gel of PCR amplification products obtained by using nested primer pair R16F2n/R16R2 for phytoplasma detection. DNA was extracted from sesame plant samples showed symptoms phytoplasma like. M=100 bp DNA marker, Lanes (1-5) = different sesame samples with phytoplasma like symptoms; -Ne= Healthy sesame plant.



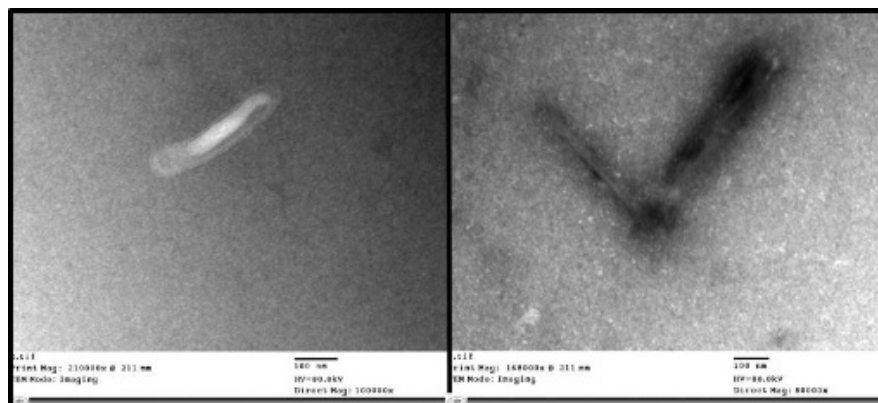
**FIGURE 4**

Effect of phytoplasmas infection on sesame enzymes activity



**FIGURE 5**

Effect of phytoplasma infections on some hormones ( $\mu\text{g/g FW}$ ) in sesame plant : (A) IAA, (B) GIB and (C) Cyto showed significant increase in infected plant than the healthy one. While, in case of (D) ABA was significantly lower in infected leaves than in healthy ones.



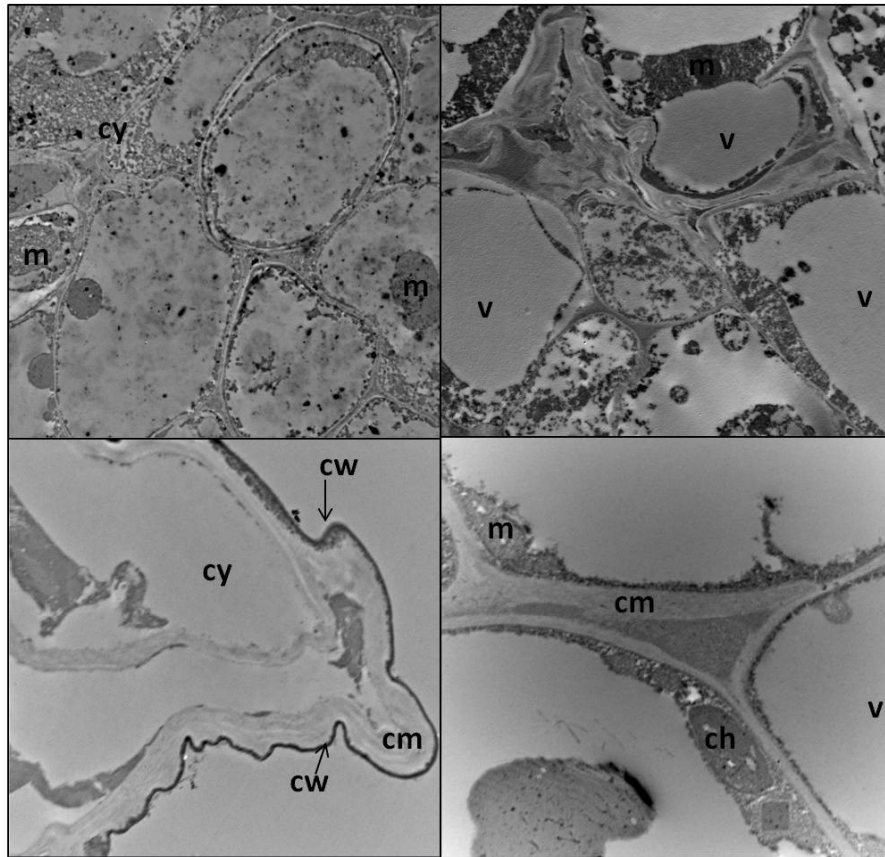
**FIGURE 6**

Electron microscopy showed pleomorphic phytoplasma particles in infected sesame plant samples.

**Enzymes activity.** A study of the activity of peroxidase and polyphenols oxidase found significant differences between infected plants and healthy plants. The activity of the antioxidant enzymes in the affected plants was higher than the control level as shown in (Fig. 4). The activity of peroxidase activity on the infected sesame was higher in the plant sesame health; this indicates an attempt to resist the plant to disease so this indicates of enzymatic changes of the plant. The activity of polyphenols oxidase on the infected sesame was higher than in the healthy sesame plant and this indicates the attempt to resist the plant disease this indicates the changes in the enzyme in the plant.

**Hormonal analysis.** The result of hormonal analysis due to the phytoplasma infection observed

that the Auxin hormone (IAA) in (Fig. 5A) indicates a high proportion of the hormone in the infected plant compared to the healthy plant. As shown in (Fig.4B), when the Gibberellin (GIB) hormone was analyzed, a significant increase was observed in the infected plant compared to the control, their role in plant defense role of these hormones in modulating plant defense responses against the disease. The data in (Fig.4C) show an increase in cytokine (Cyto) in the infected plant compared with control. Data on (Fig.4D) showed that the contents of Abscisic acid (ABA) were reduced in the infected plant when compared with the healthy samples. These discoveries showed the relationship of a phytoplasmas with sesame displaying distinctive sort of indication, for example, phyllody, shoot multiplication, internodes shortening, and little leaves and blooms.



**FIGURE 7**

An electron micrograph of ultrathin section in sesame leaf infected with phytoplasma showing that cell arrangement of the mesophyll is disordered cw=cell wall, m=mitochondria, n=nucleus, v=vacuole and ch=chloroplast representing the uneven thick and disorganization of the cell wall on the cell membrane. Necrotization and vacuolation of the cytoplasm is obvious, also representing the nucleus of palisade cell as severe dilation and disturbance the nuclear membrane is observed with full disappearance of the nucleus. The chromatin material is condensed preferring. The mitochondria are also swollen. The cytoplasm is necrotized and vacuolated.

**Morphology of phytoplasma particles.** Electron microscopy of the phytoplasma particles in dip preparation from sesame infected plants inspection after negative staining with 2% phosphor-tungsten acid (PTA). The examination revealed the presence of phytoplasma particles measure from 80-800 nm. They may be round or elongated (Fig.6), hence are termed pleomorphic in shape.

**Ultrathin Section Transmission Electron microscopy.** Ultra-thin sections of sesame leaves infected with phytoplasma were investigated by electron microscope. Regarding the ultrastructural cytological changes observed in in mesophyll layer as it was disorganized and disordered. Chloroplasts were missing sharpen, continued abnormal and the cell wall was irregular. Uneven thickness of the cell wall, disorganization of the cell membrane was clear. The cell membrane was obliterated and invaginated in several positions. Narcotization and vacuolization of the cytoplasm is also obvious as a result of infection. The palisade cells were irregular in shape contained

multiple chloroplast which was arranged along the cell wall. The nucleus disappeared leaving goast shape, the cytoplasm was lapsed leaving the cell cavity empty but containing few numbers of small vacuoles. Cell wall invagination was also observed. On the other hand, examination of phloem parenchyma cells revealed great cytopathological changes. The cell wall showed high degree of degeneration. The endoplasmic reticulum showed dilation. Regarding the chloroplast of the phloem parenchyma cell, the most obvious was the degradation of grana and lamina in a dissociation manner. The cytoplasm also contained electron dense materials which might be aggregated. Degradation of chloroplast membrane is also clear and the chloroplast lost its normal structure. The psychological changes expended to the mitochondria, which were swollen and misshapen (elongated) as shown in Fig (7).

## DISCUSSION

It was observed that some symptoms in sesame are like to phytoplasma symptoms such as, grouping of branches of developing tissues, virescence which is pigmentation of non-green flower parts to green, phyllody, formation of bunched fibrous secondary roots, weakness of plants, reddening of leaves and stems generalized yellowing, and phloem necrosis. These symptoms are also observed by Jomantiene et al., [23] who noticed the same symptoms in strawberry. The observation of phytoplasma was confirmed by using PCR primers P1/P7 and the result was 1.8 kb bands in gel electrophoresis [24]. The PCR method easily distinguishes plant and phytoplasma so that a screening of varieties for the presence of phytoplasma could take place [25, 26]. The confirmation step is then done by using nested PCR with specific primers R16R2/ R16F2n, this assay has been widely used for the detection of phytoplasma and is probably the most thoroughly investigated. It detects all strains of phytoplasma whereas healthy plants do not react [27]. Nested-PCR with a combination of different universal primers can improve the diagnosis of unknown phytoplasma present with low titer in the symptomatic host [28]. PCR has been widely used in the detection of many organisms including viruses and virus like. The results have greatly contributed to determine the distribution and diversity of the phytoplasma in sesame plants. Among all economically important diseases affecting sesame Phytoplasma is a standout amongst the most vital infections of sesame plants and causes critical losses around the world. The phytoplasma show a variety of side effects that proposes significant aggravations in the ordinary adjust of hormones and the activity of antioxidant enzymes characterized by stunting of the plants and change of the floral parts into leafy structures bearing no container and seeds are currently a genuine danger for the production of sesames in many countries. Therefore, the hormonal changes and the activity of the antioxidant enzymes were analyzed on the proper sesame plant and the plant infected with the phytoplasmas. The activity of enzymes was analyzed and significant differences were observed between infected plants and healthy plants. The activity of antioxidant enzymes in the infected plants was higher than the control level as shown in (Table 2) which agreement with the results of previous studies who found a relationship between increased enzyme antioxidant activity and pathogen resistance in plants [29-31]. The activity peroxidase activity and polyphenol oxidase activity on infected sesame was higher than in the healthy sesame plant this indicates the attempt to resist the plant to disease so this indicate of enzymatic changes of the plant in agreement with others [32-34] who found the activity of peroxidase and polyphenol oxidase increased with infected plant compared by

Healthy plant. To study the effect of phytoplasma infection on the sesame plant hormones, several hormones, Auxin, Gibberellins, Cytokinin, Abscisic acid were analyzed. In this study the result of hormonal analysis of each hormone. First, the analysis of the Auxin hormone in (Fig. 4A) indicates a high proportion of the hormone in the infected plant compared to the healthy plant. In contrast with the result of Zafari et al. [30] who found Auxin (IAA) content decreased significantly in sesame infected by phytoplasma comparing to that of control. As shown in (Fig. 4B), when the Gibberellins hormone was analyzed, a significant increase was observed in the infected plant compared to the control. In contrast with the result of Ding et al. [35] who found a decrease in the proportion of the Gibberellins hormone in the infected plant compared to the control. The data in (Fig. 4C) shows an increase in cytokinin in the infected plant compared with control. Data on (Fig. 4D) showed that the contents of ABA were reduced in the infected plant when compared with the healthy samples. These discoveries showed the relationship of a phytoplasmas with sesame displaying distinctive sort of indications, for example, phyllody, shoot multiplication, internodes shortening, and little leaves and blooms. By using negative stain method, the phytoplasma was captured. The electron microscopy was used then to detect the cellular transformation of the infected sesame plant. The cytoplasm narcotized and vacuolized these results were same to Paltrinieri et al. [36] in kiwi plant. The palisade cells were observed, which appeared containing a lot of chloroplast and changed in shape. The disappearance of nucleus was totally clear as shown in Lebsky and Poghosyan [37] who showed the same cellular transformation in tomato sample. The cytoplasm was lyses and the cell cavity remained empty [38]. The degradation of chloroplast membrane also appeared clear. The mitochondria appeared different were swelled and elongated [39].

## CONCLUSION

From this study it could be concluded that, Plants formed many defense mechanisms and enzymes such as Peroxidase and Polyphenol oxidase play one of the most important roles in the infection process and pathogenesis. Plant hormones like Auxins, Cytokinin, Gibberellins and Abscisic acid assume a significant part in controlling the manner by which plants develop and create. In addition polymerase chain reaction (PCR) with degenerate primer P1 / P7 followed by nested primer R16R2/ R16F2n able to identify phytoplasma of infected sesame plants to overcome problems concerned to sensitivity of phytoplasma detection. Further studies are needed to confirm this finding.

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