



MOLECULAR DIAGNOSIS FOR THE (*CUCUMOVIRUS*) CUCUMBER MOSAIC VIRUS AND EVALUATING THE EFFICACY OF INOCULATION WITH BACTERIA (*BRADYRHIZOBIUM SPP.*) AND SOME DIETARY SUPPLEMENTS AND THEIR COMPATIBILITY IN ITS BIOLOGICAL CONTROL ON CHARD PLANT (*BETA VULGARIS*)

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Abstract

The study aimed to diagnose the local strain for CMV from infected plants with it for the first time in the Salah al-Din province, depending on the RT-PCR technique where it gave a clear band with a size of bp207. The current experiment included 42 treatments and each of them are repeated 3 times to evaluate the efficacy of the bacteria (*Rhizobium spp.*) and Organic dietary supplements, Which included MycoVeggie, Zhi Mint, and ROS Solutions and their interactions in resistance Cucumber mosaic virus CMV and the growth of Chard plant, The results showed plant tolerance to the trait of the intensity with CMV infection in plants treated with the three dietary supplements with bacteria, where their intensity of infection reduced to 25% compared to the control treatment, where the intensity of infection with the virus increased to 74%, while the significant difference between the treatments and their effects was observed in the trait of the leaf area and the fourth treatment was excelled in this compared to the rest of the treatments. There were also significant differences between the treatments in the trait of the amount of chlorophyll. In this trait, the treating with supplements (MycoVeggie, Zhi Mint, and ROS Solutions, and bacteria) was excelled, which amounted to (14.5 SPAD) and followed by the treating with MycoVeggie and bacteria which amounted to (11 SPAD) compared to the control treatment which amounted to (5.5 SPAD).

Key words : Cucumber Mosaic virus (*Cucumovirus*), *Bradyrhizobium spp.*, MycoVeggie, mint, dietary supplements

Introduction

Chard plant (*Beta vulgaris*) belongs to the Chenopodiaceae family. It is a foliar plant that is found in many countries of the world. Its leaves are eaten where it contains many important nutrients, especially calcium and vitamin K (Hassan 2001; Baloch *et al.*, 2008). Chard plant is cultivated in most of Iraq's provinces and the production amounted to 3641 tons in 2017 (Central Statistical Organization of Iraq, 2017). Cucumber Mosaic virus (*Cucumovirus*) is considered the most important viral disease around the world, and in addition to cucumber, spinach, bananas, and tomatoes, it causes the reduction of production, especially in greenhouses (Hamed, 2012; Stoimenovs and Mahjabeen *et al.*, 2011). Farmers (Madhusudhan *et al.* 2008), where the virus affects more

than 800 plant species (Ashfaq *et al.*, 1992). Virus resistance by Biological methods has become one of the strategic plans for research institutions and companies interested in resistance to plant diseases. This was characterized by the use of *Rhizobium spp.* Where it is considered from a soil Organisms capable of coexistence with the host under the Symbiosis system. This property has been invested in the resistance of viral plant diseases, as mentioned by (Al-Ani *et al.*, 2009) that these bacteria reduce the intensity of infection with the Cowpea mosaic virus. It also reduced the concentration of BYMV particles when treating the seeds of the broad bean plants with it. Khadr and Al-Fahad (2018) concluded that reducing the percentage and intensity of infection with an average (37.67%, 15.31%), respectively, compared to the control treatment which amounted to (50%, 65.92%), respectively. The researchers also focused on

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the effect of fungi and algae in the resistance of plant diseases, although the studies limited in that to limit the health aspect of humans and animals more than their positive effects on plants, Sivanandhan (2017) concluded that the Mycelium and fruit bodies for *Ganoderma lucidum* fungus contain 400 compounds of antifungal, antiviruses, and antibacterial. Gao *et al.*, (2003) noted that many Polysaccharide and Triterpenes have a role in stopping the replication of viruses. Ayed and Al-Fahad (2018) obtained significant results in reducing the intensity of the infection with TMV using dietary supplement produced from *Spirulina platensis*. Samari *et al.*, (2019) confirmed that the interaction between *S. platensis* and the *Cordyceps Sinensis* fungus stimulates systemic resistance against Maize Dwarf Mosaic Virus (MDMV), increases the leaf area, and the amount of chlorophyll for the infected plants with the virus. Some studies have indicated that the Roselle (*Hibiscus sabdorriffa*) extract can inhibit certain microbial resistant to Antimicrobial (Salman 2009; Khafaji and Safawi, 2008). Other studies have indicated the ability of the mint extract to inhibit certain plant pathogens, including Fungi and pathogenic bacteria (Luma *et al.*, 2013). Due to the importance of Chard crop from the nutritional and medical side, and the seriousness of the CMV virus through its spread by mechanical and biological means, the absence of studies in the field of resistance to viruses using organic supplements, we decided to conduct this study.

Materials and Methods

Molecular Diagnosis: After the collection of infected plant samples from the fields, the molecular diagnosis was performed in the central laboratory for the University of Tikrit and according to the Primer sent from the United States of America (Iqbal *et al.*, 2017), By adopting the following method:

1. Isolation of DNA from infected plants: It was isolated according to the mentioned method by (Iqbal *et al.*, 2017) and measuring the concentration and purity of RNA extracted using DNA concentration and purity device (NANO DROP 2000).
2. Reverse transcription polymerase chain reaction (RT-PCR) for the DNA for the virus. The following materials are added to the kit RT-PCR type and according to the method of work mentioned by (Iqbal *et al.*, 2017) and with the presence the used primer in diagnosis the virus by the following sequence: Left forward (TGATTCTACCGTGTGGGTGA and (AGCACGGCGTACTTTTCAT) Right primer. The kit sample was placed into the PCR device to conduct the polymerase chain reaction according to

the following program: Denaturation is 94° for 30 sec, Recombinant is 52.5° for 30 sec and the elongation is 72° for 10 min and for 35 cycles. The cDNA is detected through conducting electrophoresis on agarose with the concentration of 0.8% and dying with 10 µl of Ethidium bromide for 20 min and then transfer them to the electrophoretic display device for the purpose of imaging and reading the distances between the formed bands compared to the distances of the Guide bands.

Soil sterilization and the preparation of the plants used for the experiment

Soil sterilization with Formaldehyde where 62 ml of Formaldehyde was added to 10 L of water. The dilution was 1% (Formaldehyde is used to control some pathogenic fungi for plants in greenhouses. It is also used in the sterilization and cleaning of surgical instruments and equipment in hospitals. It is composed of formaldehyde gas dissolved in distilled water. It is used for the preservation of long-term tissue samples. It protects the tissue from rotting and decomposition caused by some bacteria). The soil was then covered with polyethylene for a week and the soil was then removed and stirred daily for 5 days. The sterile soil was then mixed with 1:1 peat moss and placed in the pots. The pots were cultivated with three seeds per section and then sprayed with water every two days.

Acquiring pure viral isolation and conducting the process of mechanical inoculation

This method was conducted as described by (Alani *et al.*, 2009), The leaves were taken from eggplant and tomato, which showed the infection clearly and then cut into small pieces by a blade and then placed in a sterile ceramic mortar, then crushed the leaves well by a pestle to prepare the virus vaccine in the plant extract and with the presence of buffer solution Phosphate Regulator KH_2PO_4 with ratio of 10 g leaves: 20 ml From the solution, the extract was filtered through two layers of malleable cloth and the leachate was used for inoculation. The buffer solution was prepared using 3.20 g of KH_2PO_4 and dissolving it in a quarter of distilled water. Infection was conducted in the fourth real leaf stage by thumb method which conducted by wearing a plastic glove in each vaccination, where the thumb is immersed in the pre-mentioned vaccinate and passing it on the upper leaf surfaces and then spraying it with water by sprayer, and monitoring the appearance of symptoms regularly daily on the plant.

Obtaining bacterial isolation: *Bradyrhizobium* bacteria

The isolates were obtained from the laboratory of bacteria, University of Samarra, College of Science, Department of Life Sciences which licensed by Dr. Wael Mohammed Mehdi, Molecules isolated from cowpea plants classified *Bradyrhizobium* spp. the isolate was multiplied on the media of Yeast Extract Manitol Congo Reed Agar. This media consists of: 10 g.L⁻¹ Manitol, 0.1 g.L⁻¹ NaCl, 0.2 g.L⁻¹ MgSO₄.7H₂O, 0.5 g.L⁻¹ K₂HPO₄, 1 g.L⁻¹ Yeast Extract, 0.25 g.L⁻¹ Congo Reed, 15-20 g.L⁻¹ Agar Agar. This media is sterilized by the autoclave at 121 °C for 15 min after being cooled, it was then poured into dishes until it is solidified and then used. It was taken a swab and marked by a planning method and incubated the dishes at 28 °C inverted (Howieson and Dilworth, 2016). Ten cowpea plants were cultivated, Rhizobium was tested on it, All plants showed a bacterial node. The bacteria multiplied for the vaccine on the Nutrient broth media for the vaccine used for media.

Preparation of dietary supplements used in the experiment

Organic supplements included the following preparations:

The dietary supplement was added during three stages of the plant life, as follows: MycoVeggie (5 g.L⁻¹ water), mint after milling (5 g.L⁻¹ water), ROS Solutions

(5 ml.L⁻¹ water).

Field experiment

The experiment was conducted at the research station of the College of Agriculture, University of Tikrit according to The Randomized Complete Block Design (RCBD) system as described in the following field plan.

The used Criteria for the detection of the efficiency of the used treatments in the resistance of the virus

Some indicators of the CMV effect were measured and stimulated resistance to the Saline plant, which included:

Measuring the intensity of infection with CMV

To Satisfactory evidence for the intensity of the infection with CMV infection established based on the appearance of symptoms intensity and the intensity of infection for plants was calculated by calculating the number of infected plants and the degree of each plant and then applied the following equation (Mckinney, 1923).

The intensity of infection % =

$$\frac{\begin{aligned} &(\text{The No. of infected plants} \times 1) \\ &+ (\text{The No. of infected plants} \times 2) \\ &+ \dots + (\text{The number of infected plants} \times 6) \end{aligned}}{\text{The No. of taken samples} \times 5 (\text{higher class})} \times 100$$

| No. | Name of commercial product (supplement) | Effective Material | Physical Body | Source |
|-----|---|--|---------------|------------------------------------|
| 1 | MycoVeggie (Macrophage) | A mixture of medicinal plants fiber, medicinal fungus, and Spirulina | Coarse powder | Factories of DXN company, Malaysia |
| 2 | Zhi mint | Ganoderma mushroom and natural mint oil | Disks | |
| 3 | ROS Solution | Roselle Flower Extract | juice | |

| Treatments | Symbols (T) | Number of replicates (R) |
|---|-------------|--------------------------|
| MycoVeggie | MF | 3 |
| Zhi mint | ZM | 3 |
| ROS Solution | ROS | 3 |
| <i>Bradyrhizobium</i> spp | B | 3 |
| MycoVeggie + Zhi mint | MF+ZM | 3 |
| MycoVeggie + ROS Solution | MF+ROS | 3 |
| Zhi mint + ROS Solution | ZM+ROS | 3 |
| MycoVeggie + bacteria | MF+B | 3 |
| Zhement + bacteria | ZM+B | 3 |
| ROS Solution + bacteria | ROS+B | 3 |
| MycoVeggie + Zhi mint + ROS Solution + bacteria | MF+ZM+ROS+B | 3 |
| Control 1 healthy | CON1 | 3 |
| Control 2 is only infected | CON2 | 3 |
| Control 3 (healthy + Materials) | CON3 | 3 |

Measuring the amount of chlorophyll in the direct method

The relative content of total chlorophyll in the leaves was measured by Chlorophyll meter (SPAD type) with units (SPAD units) by taking three leaves from the top and the middle and bottom of the plant from each treatment randomly and their amount of chlorophyll was estimated and the rate of the three readings were then taken.

Measuring the leaf area (cm².plant fresh weight)

It was measured according to the known-area disks method by taking three leaves randomly from each treatment during the stage of vegetative growth at the age of the eighth real leaf, it was then weighed and recorded the weight of the disks from each leaf, and the following area was then calculated

which described by Dovrnic (1965).

$$\text{Leaf area (cm}^2\text{.plant}^{-1}\text{)} = \frac{\text{Total disk weight} \times \text{The area of the disk known area}}{\text{The weight of the disk known area}}$$

Statistical analysis: It was conducted according to the statistical program for the year 2010.

Results and Discussion

Molecular Diagnosis: CMV was diagnosed on the Chard plants using the PCR-RT technique. After all the steps and diagnostic experiments were performed, the used primers showed their results according to the number (1) bands size 207 bp, These results agree with (Iqbal, 2017). This study mentioned above confirmed that the diagnosis was accurate and that the local virus strain is approaching the same strain that was diagnosed in Pakistan by (Iqbal, 2017).

Fig. 2 shows that Bearing infection with the virus in plants treated with the three dietary supplements with bacteria, where the intensity of infection reduced to 25% compared to the control treatment, where the intensity of infection with the virus increased in it to 74%. The

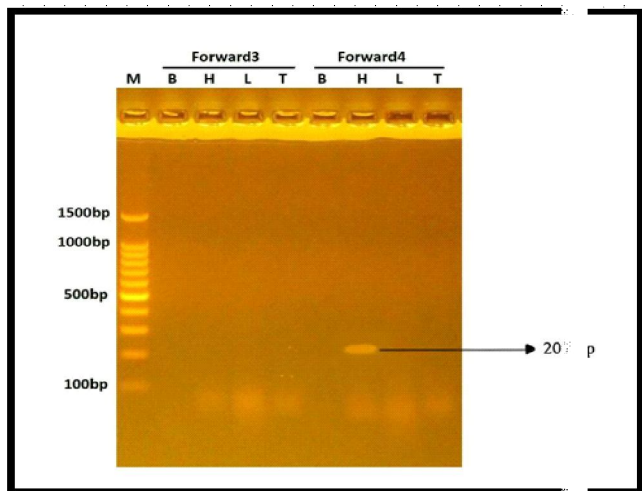


Fig. 1: Detection of the CMV virus on a Chard plant using a primer with size 207 bp.

dietary supplement (MycoVeggie) contains algae (cyanobacter), which is characterized by its production of many effective and bio-active compounds in the biological system for various plant pathogens such as viruses, fungi, and bacteria. The most important characteristic is that it is rich in protein, vitamins, and minerals, as well as other compounds such as fatty acids, polyphenols, polysaccharides and pigments such as Carotenoids and chlorophyll that stimulate plants with different mechanisms to resist pathogens. This result agrees with (Usharani *et al.*, 2015; Abbassy *et al.*, 2014).

It is noted that there is a significant effect for treatments containing *G. Lucidium* fungus. This is due to the fact that it is a fungus that has a microbiological antibody and to control various diseases. where It consists of a fruit body, mycelium and spores containing about 400 active compounds that have different effects, including antimicrobial activity, mainly including polysaccharides, fatty acids, flavonoids, protein, sterols and organic germanium Vitamins, minerals and other essential elements such as Triterpenoids (Mehta and Savita, 2012; Kim, 1999, Mizuno, 1995). Most of the results were enhanced by a significant increase for the studied traits in the bi-treatments and then quadruple treatments. This indicates compatibility between the products, especially the MycoVeggie extract and bacteria. Treatments have also led to an increase in the growth and yield parameters, thus It is believed to have a nutritional role as well as its Anti-viral role. Shail, (2017) explained the reason for the difference between the healthy plant and the infected (treating with bacteria extract) in stimulating the resistance by the plant content of Leghaemoglobin in the infected plant, where the infected plant has a smaller amount and this negatively affects the nitrogen fixation for the infected plants and this is reflected in the production of chlorophyll, thus reduce the intensity of infection with the virus. These results agree with (Khadr, 2018) who concluded that treating with the extracts gives longer time for the plants to stimulate their resistance against the virus. This explains the effect of natural dietary supplements and *Bradyrhizobium spp* in the reduction of CMV damage, especially the Integrated quadratic treatment. Salman and Al-Khafaji (2009) found that the extract of the Roselle plant had an inhibitory effect on the various bacterial species compared with the antibiotics. Ohkawa (1991) attributed the effectiveness of the Roselle flower extract to its containment of effective chemicals, especially the Protocatechuic Acid (PCA). Al-Safawi (2008) said that the Roselle extract has the ability to dissolve the active substances in the plant and influence the growth of bacteria through the effect on the cell wall and its ability to penetrate and its impact on DNA and ribosomes and various activities for Bacteria.

Effect of the used treatments on the amount of chlorophyll (SPAD) for healthy Chard plant and the infected plants with CMV

Fig. 3 shows that there were significant differences between the treatments in the amount of chlorophyll. In this treatment, the treating with (MycoVeggie, Zhi Mint, and ROS Solutions, and bacteria) was excelled by giving it (14.5 SPAD) and followed by the effect of the treating with MycoVeggie and bacteria which amounted to (11

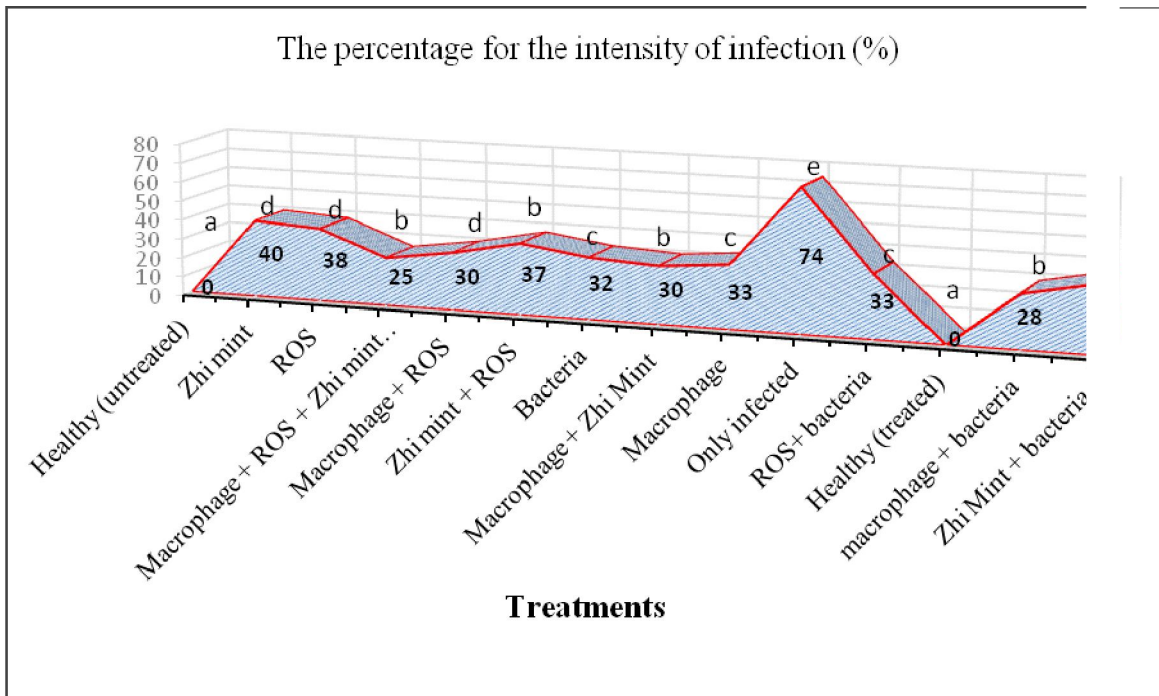


Fig. 2: Effect of the used treatments in the percentage for the intensity of infection (%) for healthy Chard plant and the infected plants with CMV.

* Similar letters between the treatments indicate that there are no significant differences according to Duncan's New Multiple tests at 5% level.

SPAD) compared to the control treatment which amounted to (5.5 SPAD). The reason for the superiority of the quadriplegic treatment may be due to the presence of more than one mechanism for resistance to the virus and according to the used materials. The reason for the effect of the MycoVeggie can be attributed to its containment of *Spirulina*, which in turn is known to stimulate plant resistance against viral diseases. This result agrees with (Youssef, 2018). The effect of the ROS extract may be due to its high content of vitamin C, which is known to play the inhibitory role of viruses. The Zhi Mint powder contains *Ganoderma lucidium* extract, which has high results in inhibiting viruses (Eid and Al-Fahd, 2018). The *G. lucidium* fungus has the ability to inhibit the infection with the TMV virus, with the percentage of 70-65% because they possess compounds effective in inhibition of viral infection, where there are barriers preventing the virus from penetrating the plant cell. It has proven its ability to resist viral infection even at high temperatures. Among the compounds that have proved effective in this fungus are polysaccharides such as GXM (Mehta and Jandaik, 2012). This may explain the increase in the amount of chlorophyll for the role of bacteria in the stabilization of nitrogen, which has a significant role in the process of building and increasing the amount of chlorophyll despite the presence of viral infection and this agrees with (Lima *et al.*, 2005).

Effect of the used treatments on the leaf area (cm²) for healthy Chard plant and the infected plants with CMV

Fig. 4 shows the widening of the significant difference between treatments and their effects on the leaf area, In this treatment, the treating with (MycoVeggie, Zhi Mint, and ROS Solutions, and bacteria) was excelled by giving it compared to other treatments. As shown from the figure, there is a significant increase for healthy plants and treating with supplements and bacteria, which amounted to (43.3 cm²) compared to the control treatment, which amounted to (12.27 cm²). The reason for the increase in growth may be due to the role of most treatments in stimulating growth in general and supplying the plant with elements necessary to build cells, These results agree with (EAthab, 2009) that the treating the infected plants with the Cowpea severe mosaic *comovirus* gave the dry and fresh weight for the total vegetative amounted to (0.74 g, 4.36 g), respectively, compared to the infected plants and treating with bacteria amounted to (2.06 g, 9.28 g), respectively, This indicates that the virus reduces the weight of total vegetative while the bacteria compensate for the shortfall in the dry and fresh weight of the total vegetative. A number of researchers have indicated that the work of algae (found in MycoVeggie) in the plant is similar to that of growth

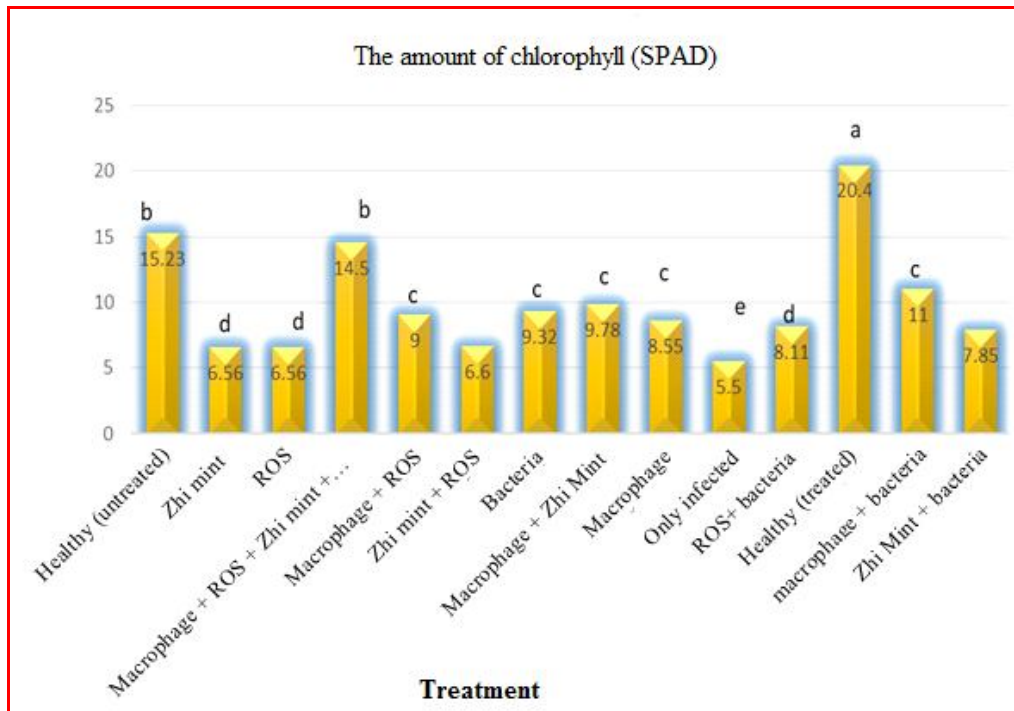


Fig. 3: Effect of the used treatments in the amount of chlorophyll (SPAD) for healthy Chard plant and the infected plants with CMV.

* Similar letters between the treatments indicate that there are no significant differences according to Duncan’s New Multiple tests at 5% level.

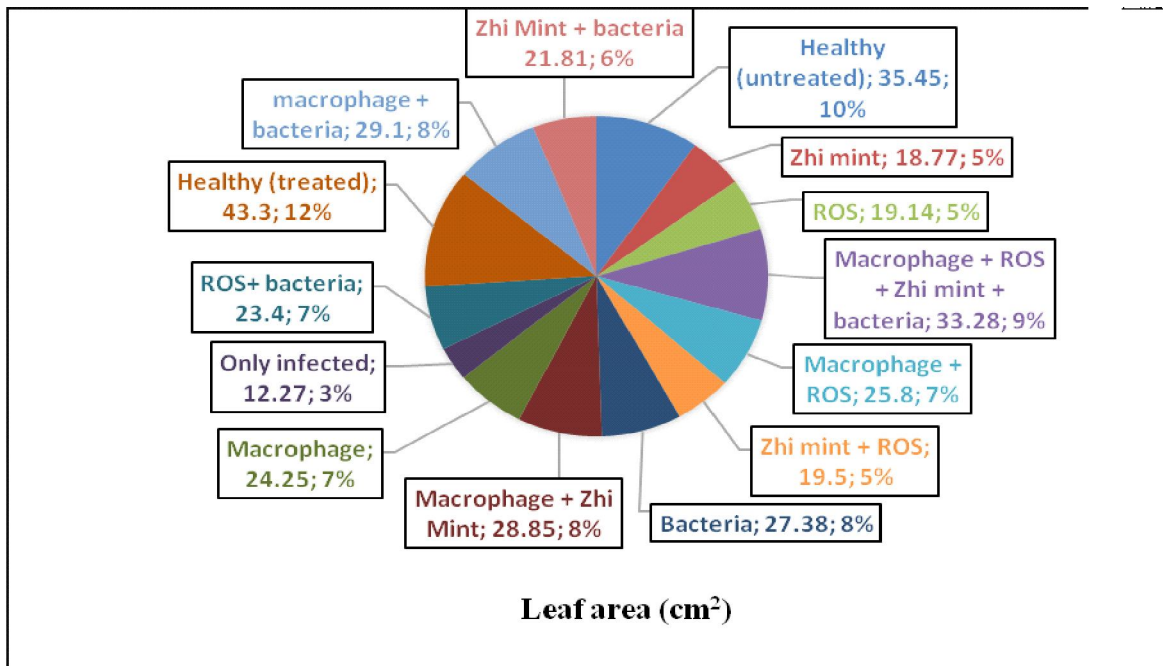


Fig. 4: Effect of the used treatments in the leaf area (cm²) for healthy Chard plant and the infected plants with CMV.

* Similar percentages between the treatments indicate that there are no significant differences according to Duncan’s New Multiple tests at 5% level.

regulators because they contain Auxins and Gibberellins, which increase nutrients absorption, cell division, and elongation, as well as activation of enzymes that stimulate

plant growth and its resistance to disease (Verkleij, 2002; Jensen, 2004). This effect was reinforced by the effect of lingzhi mushroom (found in Zhi Mint) of effective

compounds with the same effect, The increase in the leaf area of the fungus treatment can be explained by the fact that it contains adenosine and phosphorus and the important nutrients for the synthesis of adenosine triphosphate in the mitochondria inside the cell. Al-Nuaimi (2011) noted that the above substances play an important role in chemical reactions, which in turn reduces the infection.

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