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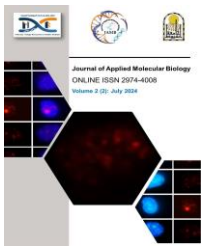

Management of *Watermelon Mosaic Virus* Infecting Squash Plants through Application of Certain Fungal Bioagents

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ABSTRACT

Watermelon mosaic virus (WMV) poses a significant threat to cucurbit crops. Leaves of squash plants showing viral like symptoms similar to the typical symptoms of WMV were collected from Assiut governorate, Egypt and mechanically inoculated into healthy squash plants. Total RNA was extracted from symptomatic infected squash leaves and reverse-transcription polymerase chain reaction (RT-PCR) was conducted using specific primers to amplify Coat Protein (CP) of WMV. The results of RT-PCR confirmed the identification of WMV. To assess the impact of application of six fungal isolates to control WMV, two experiments were conducted in two different seasons, employing three distinct application methods, namely seed coating, soil drenching, and foliar spray. The results indicated a noteworthy reduction in disease severity of WMV based on symptom development, with an average reduction of 52.0% compared to the infected control group. Different fungi varied in their ability to reduce the disease severity. The efficacy of fungal bioagents in controlling WMV was also influenced by the application method. On average, Seed coating with fungal bioagents exhibited the highest reduction in disease severity (54.2%), followed by foliar spray (51.5%) and soil drenching (50.0%). In addition, these treatments had an impact on the chlorophyll content, where a significant increase in chlorophyll content was observed in the treated plants compared to the infected control group, with an averaged increase of 62.7%. These results suggested that using fungal bioagents could be useful to reduce the disease severity of WMV in squash plants.

1-INTRODUCTION

Squash (*Cucurbita pepo*) is a popular and important vegetable crop in Egypt, with two distinct types, winter and summer squash; it is being cultivated either in greenhouses or in the field. Squash could be used as an ingredient in a wide range of dishes, and it is an essential component of Egyptian cuisine. The cultivation of squash is a crucial part of the country's agricultural industry and plays a significant role in its economy.

Plant diseases, fungal and viral diseases, are responsible for causing significant crop losses in crop production worldwide [1, 2, and 3]. These losses have far-reaching consequences, creating critical challenges for global food security. Squash and other cucurbit crops are susceptible to various viral diseases. More than 35 viruses have been isolated and characterized from cucurbits [4]. Among these viruses, *Watermelon mosaic virus* (WMV) is considered as an important pathogen and responsible for the reduction of the quality and quantity of fruit production in squash, leading to substantial yield reduction. WMV is a member of the genus *Potyvirus* in the family *Potyviridae*, with a worldwide distribution, mostly in temperate and mediterranean regions. Potyviruses are single-stranded, positive-stranded RNA viruses that represent about 25% of all known plant viruses and are responsible for important losses in most major crops worldwide. Although WMV has long been known in many parts of the world, it has not been studied extensively at the molecular level. However, WMV presents a broader host range than most potyviruses. Cucurbit plants that are susceptible to WMV may exhibit a range of symptoms, including leaf mottling and mosaic, chlorosis, tip stunting or bunching, and lead to severe reduction in fruit yield and quality [5, 6].

It is known for its distinct long flexuous filamentous particles that are approximately 750 nanometers in length, and it is being transmitted through aphids. WMV can infect over 170 plant species belonging to 27 families, including several weed species that can become carriers of the virus and infect crops [7] and can cause significant losses to various crops, especially cucurbits, and legumes, leading to a severe reduction in both yield and fruit quality [8]. The WMV genome contains about 10035 nucleotides and encoded for one polyprotein comprises of 3217 amino acids, this polyprotein could be subsequently divided into different functional and structural proteins [9].

Many attempts have been conducted to reduce the disease severity of WMV. Including development of resistant cultivars or management of insect vectors. Recently many researchers reported that application of certain bioagent can sufficiently reduce the disease severity of plant viruses. Various microorganisms have proved to be potential control factors against plant viruses [10, 11].

The use of biological agents in plant disease control is a highly effective and promising tool. By harnessing the power of beneficial microorganisms, we can manage plant pathogenic diseases while promoting environmental sustainability.

One category of these methods is using bioagents fungi to control plant pathogens. Using fungal bioagents has gained significant attention in agricultural research due to their potential to enhance plant growth, improve nutrient uptake, and provide protection against pathogens. Moreover, using bioagents strains can induce systemic resistance in plants, which can reduce the damage caused by various pathogens, including viruses. They do so by triggering the plant's defense mechanisms, such as the production of pathogenesis-related proteins and activation of signaling pathways. This induced resistance can reduce disease severity and improve plant health. However, when it comes

to controlling viral diseases like WMV, the role of fungal bioagents is primarily focused on enhancing plant resistance rather than directly targeting the virus itself. Fungal bioagents, including *Trichoderma*, can induce systemic resistance in plants, activating defense mechanisms that can limit the spread and severity of viral infections. Many fungi and microorganisms have been applied to control plant viruses [12, 13, and 14].

Therefore, the objective of the present study was to assess the effectiveness of certain fungal isolates (bioagents) to reduce the disease severity of *Watermelon mosaic virus* infecting squash plants.

2. MATERIALS AND METHODS

2.1. Source of the virus:

Symptomatic zucchini plants (*Cucurbita pepo*) showing viral-like symptoms including mosaic, yellowing, and distorted leaves were collected from the Experimental Farm of the Faculty of Agriculture, Assuit University, Egypt.

2.2. Mechanical Inoculation:

Seeds of zucchini (*C. Pepo*) HYTECH HYBRID SEED SQUASH cultivar were sown in pots (30 cm in diameter) containing sterilized soil (clay to sand in a 1:1 ratio) and were kept in insect-proof greenhouse conditions. One week after planting cotyledon leaves of zucchini seedlings were gently washed with distilled sterilized water, the leaves were dusted with carborundum powder (600 mesh), and then each cotyledon leaf was gently rubbed with 100 ml of plant sap from symptomatic zucchini leaves.

Plant sap of symptomatic leaves was prepared by crushing 100 mg of symptomatic zucchini leaf in 100 ml of sterilized phosphate buffer pH 7 in sterilized and cold mortar and pestle. The phosphate buffer pH 7.2 was prepared by mixing 87.09g of 1M K2HPO4 with 68.04 of 1M KH2PO4 then sterilized and kept in the refrigerator for further use.

After rubbing cotyledon leaves with a phosphate buffer with plant-infected sap, the leaves were gently washed with distilled sterilized water then kept under insect-proof greenhouse conditions. And monitor the development of symptoms. The symptoms were recorded on a weekly basis.

2.3. Virus Identification:

In the present study, a two-step Reverse-Transcription Polymerase Chain Reaction (RT-PCR) method was used for molecular identification of WMV using specific primers to amplify Coat Protein (CP) of WMV. The first step includes the synthesis of complementary DNA (cDNA), followed by amplification of the resulting cDNA (RT) by PCR in a separate tube.

2.4. Total RNA Extraction:

To synthesize cDNA for RT-PCR, total RNA was extracted from fresh symptomatic zucchini leaves **(***C. pepo*) as well as healthy leaves using the ABT Total RNA Mini Extraction Kit according to the manufacturer's instructions (Applied Biotechnology, EGYPT). Extracted RNA was then stored at −20 ◦C until use.

2.5. Synthesis of complementary DNA (cDNA):

In brief, synthesis of cDNA for RT-PCR was performed using ABT H-minus cDNA synthesis kit according to the instructions of manufacturer. RNA template was incubated

with reverse transcriptase enzyme, dNTPs, WMV reverse primer and ribonuclease inhibitor at 50 C for 60 min.

2.6. Reverse-Transcription Polymerase Chain Reaction (RT-PCR):

The RT-PCR assay was conducted using specific primers to amplify the Coat Protein (CP) gene of WMV, namely WMV-F2: 5′-CACATTACTTGGAGCTAAAG-3′ and WMV-R2: 5′-ATATGCTTCCGCTGCATCTG-3′, as described by [17].

The PCR reaction was performed in a total volume of 25µl containing: 1µl DNA template, 12.5 µl PCR master mix, and 0.5 µl each of forward and reverse WMV primer (10µM), 0.5 µl DMSO and 10 µl nuclease free water.

PCR amplifications were performed in a Biometra thermal cycler (Biometra, Göttingen, Germany) using the following PCR profile: initial denaturation at 95°C for 5 min, followed by 25 cycles each consisting of 1 min at 94°C, 1 min at 55°C, followed by 2 min at 72°C, with a final extension at 72°C for 10 min.

PCR products were separated using horizontal gel electrophoresis unit on 1% (w/v) agarose gels in 1X TAE buffer. A 1 kb DNA Ladder was used to estimate the size of amplified DNA fragments. The gel was run for 30 minutes using constant voltage of around 100 V. The gel was then soaked in Cyber Green stain for 30 minutes, and then visualized and photographed under UV light.

2.7. Isolation and purification of fungi:

Soil samples were collected from various medicinal and ornamental plants grown in greenhouses at Assuit University's farm. Soil samples were collected at depth of around 10 cm nearby the roots of plants. Soil was left for air dry at room temperature for four days. One gram of each sample was placed in flasks containing 100 ml sterile water and the flasks were shook again Serial dilutions (10^{-1}, 10^{-3}) and 1 mL of each dilution was spread on a petri dish containing Potato Dextrose Agar media (PDA) [14] supplemented with antibiotic Chloramphenicol 200mg. Three plates were made from each concentration. The dishes were rotated by hand to ensure homogenous distribution of soil suspension dilution and then incubated at 28 C till colonies appeared. Then, the colonies were purified to a new plate by the hyphal tip technique [15].

2.8. Morphological identification of fungal isolates:

In the present study, six fungal isolates were identified according to their morphological distinctive characteristics at Assuit University's AUMMC (Assuit University Moubasher Mycological Center). Identification of fungal isolates was carried out on 4-10 days old culture using morphological and microscopic characteristics of mycelium and spores according to [16].

2.9. Assessment of the efficiency of certain microorganisms to control WMV under greenhouse conditions:

In the present study, the efficiency of six fungal microorganisms (isolates) to reduce the disease severity of WMV on zucchini plants was evaluated in two different season (Spring and Summer of 2023) in a greenhouse of the Faculty of Agriculture, Assuit University, Egypt.

The application of these isolates was implemented in three different methods, namely:

a- Soil drench:

The suspension was prepared by the cultivation of the isolates on a PDA plate and incubated at 28 C for 7 days. After that, the spores and mycelium were harvested and conveyed to flasks containing distilled sterilized water, and the concentration was adjusted at 2-3x10-5 CFU. Each fungal microorganism was applied to three different pots.

After that zucchini seeds (H. TECH) were surface sterilized, and three seeds were sown in each pot. 200 ml of each microorganisms' suspension was added to each one of three pots at the time of planting .

One week after germination, the cotyledon leaves were mechanically inoculated with WMV as described before, and zucchini plants were inoculated with H2O as a mock inoculation to serve as a healthy control. Two weeks after inoculation, the application of these microorganisms (6 fungal isolates) was repeated for a second time by preparing suspension as described before, and 200 ml of each suspension was added to each pot. Zucchini plants inoculated with WMV without adding any microorganism's suspension were used as infected control, while plants with mock inoculation served as healthy control.

b- Seed treatment:

The suspension of each one of 6 fungal isolates was prepared as described before, then the seeds of the zucchini plant (*C. Pepo*) HYTECH HYBRID SEED SQUASH cultivar were soaked separately in 50ml of each suspension at concentration ($2x10^5$) for four hours at room temperature. After the three seeds were sown in each pot, and three pots were made for each microorganism, healthy and infected control was made as aforementioned.

c- Foliar spray:

The suspension of each one of the 6 of mentioned microorganisms was prepared and adjusted to the desired concentration as described before. Then 100 ml of each suspension was sprayed on three zucchini cotyledon leaves one day after inoculation, using a sprinkler. The foliar spray was repeated again two weeks after inoculation.

2.10. Assessment of Disease severity

The developments of viral symptoms were observed in all treated and non-treated plants and the symptoms of WMV were recorded three and six weeks after inoculation using the following rating scale for WMV as described by [17].

0 = no symptoms at all.
1 = yellowing in lower leaves.
2 = Yellowing and mosaic.
3 = severe mosaic and severe mottling.
4 = malformed leaves, stunting plant growth, severe mosaic, or death of the plant.

The percentage of disease severity infection was calculated using the following formula:

$$\text{Disease severity (\%)} = \frac{\sum(Disease\ grade \times Number\ of\ plants\ in\ each\ grade)}{(Total\ number\ of\ plants)\times(The\ highest\ diease\ grade)} \times 100$$

2.11. Assessment of total chlorophyll content in plant leaves:

In order to evaluate the direct influence of WMV infection on chlorophyll content in leaves of squash plants infected with WMV and treated by certain fungal isolates (Bioagents) using different treatments (application method) in the first (Spring 2023) and the second (Summer 2023) season, the total chlorophyll content of a leaf was measured using a Soil Plant Analysis and Development (SPAD) meter, which is widely used to rapidly measure SPAD values as a proxy for chlorophyll content [18].

2.12. Statistical analysis:

Statistical analysis of phenotypic data was carried out using two-way ANOVA using CoStat statistical software, and Fisher's Least Significant Difference (LSD) test was carried out using CoHort software at 0.05 level of probability.

3-RESULTS

3.1. Identification of *Watermelon mosaic virus*:

3.1.1. Mechanical inoculation:

Inoculated squash seedling at age of cotyledon leaves with infected plant sap led to the development of the typical symptoms of *Watermelon mosaic virus* infection, that include plant virus, as A= Mosaic, B = Mottling and C= leaf deformation **(Figure 1).**

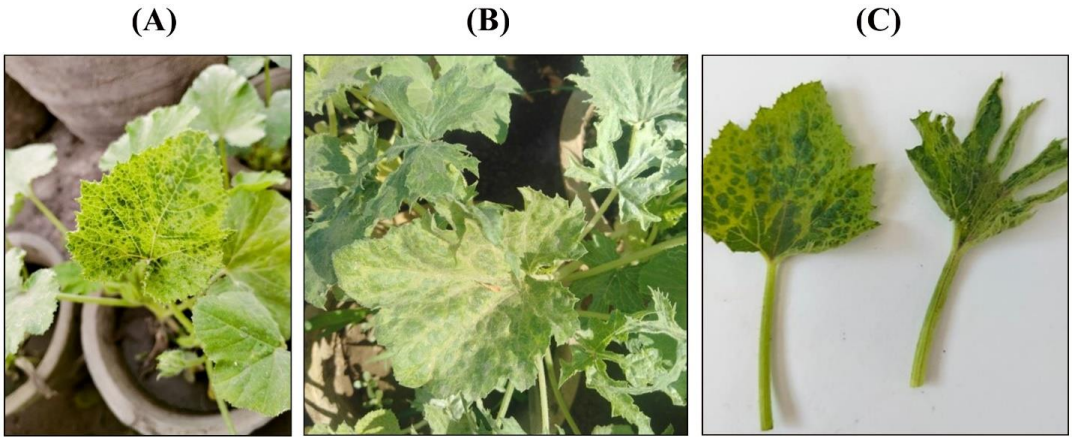

Figure 1. Symptoms induced on zucchini plants that are mechanically inoculated by sap from infected zucchini showing mosaic symptoms; discoloration and slight deformation are observed. A= Mosaic, B = Mottling and C= leaf deformation.

3.1.2. Reverse –Transcription Polymerase Chain Reaction (RT-PCR):

The result of RT-PCR assay used for molecular identification and confirmation of the presence of WMV in the studied plants showed that using specific forward and reverse primers targeting the CP gene of WMV resulted in a specific PCR band with the expected amplicon size of WMV, confirming molecular identification and the presence of infection with WMV.

3.1.3. Morphological identification of fungal isolates:

In this study, six fungal isolates were identified according to their morphological distinctive characteristics at Assuit University's AUMMC (Assiut University Moubasher Mycological Center). Four isolates namely, F1, F2, F3 and F4 were identified as

Trichoderma longibrachiatum, while F5 isolate was identified as *Aspergillus quadrilineatus,* and F6 isolate was identified as *Penicillium purpurogenum* (**Table 1).**

Table 1. Identification of six fungal isolates used in the study according to their morphological distinctive characteristics.

Fungal isolate	**Identification**
F1	*Trichoderma longibrachiatum*
F2	*Trichoderma longibrachiatum*
F3	*Trichoderma longibrachiatum*
F4	*Trichoderma longibrachiatum*
F5	*Aspergillus quadrilineatus*
F6	*Penicillium purpurogenum*

3.1.4. Effect of application with fungal isolates on development of WMV symptoms:

In the present study, the effectiveness of six fungal isolates to reduce the disease severity of WMV was assessed under greenhouse conditions in two successive seasons (Spring and Summer 2023). These fungal isolates were applied with three different application methods (i.e. seed treatment, soil drench and foliar spray), and the disease severity (%) were then recorded three and six weeks after inoculation.

The disease severity (%) observed on leaves of squash plants infected with WMV and treated by certain fungal isolates (bioagents) using different treatments (application method) after three and six weeks of inoculation in the first (Spring 2023) and second (Summer 2023) season is presented in **Table 2** to **Table 5.**

The disease severity (%) after three weeks of inoculation during the first season (**Table 2)** ranged from 5.0 (foliar spray with F2 isolate) to 25.0% (soil drench with F3 isolate), with an average of 15.2% compared to 50.0% disease severity for control (untreated) infected plants. Whereas higher disease severity (%) was observed after six weeks of inoculation during the first season (**Table 3)** which ranged from 27.5 (seed treatment of F1 isolate) to 79.1% (foliar spray with F6 isolate), with an average of 51.7% compared to 91.6% disease severity for control (untreated) infected plants.

In the second season, the disease severity (%) after three weeks of inoculation (**Table 4)** ranged from 13.8 (seed treatment with F3 isolate) to 38.8% (soil drench with F5 isolate), with an average of 26.5% compared to 58.0% disease severity for control (untreated) infected plants. Whereas higher disease severity (%) was observed after six weeks of inoculation (**Table 5)** which ranged from 33.3 (foliar spray with F4 isolate) to 50.0% (soil drench with F6 isolate), with an average of 43.4% compared to 85.0% disease severity for control (untreated) infected plants.

The results showed significant reductions in the disease severity of WMV on the studied plants due to different treatments of fungal bioagent during the first and second seasons (**Figure 2** and **Figure 3**, respectively). Higher reductions in the disease severity were observed after three weeks of inoculation compared to six weeks of inoculation,

with an average of 69.6 and 43.5%, respectively, in the first season (**Figure 2**) and 54.3 and 49.0%, respectively, in the second season (**Figure 3**).

Table 2. The disease severity (%) of WMV on squash plants treated by fungal isolates (bioagents) using different treatments (application method) after three weeks of inoculation in the first season (Spring 2023).

Bioagents (BIO)	**Treatments (TREAT)**			
	Seed treatment	**Soil drench**	**Foliar spray**	**Mean BIO**
F1	19.2	8.3	13.3	**13.5**
F2	7.5	23.3	5.0	**11.9**
F3	19.2	25.0	15.0	**19.7**
F4	10.0	10.0	19.1	**13.1**
F5	7.5	13.3	21.9	**14.2**
F6	13.3	23.3	20.0	**18.8**
Mean TREAT	**12.8**	**17.2**	**15.7**	**15.2**
CON. H	0.83			
CON. INF	50.0			
LSD (0.05) (BIO)	8.7			
LSD (0.05) (TREAT)	5.4			
LSD (0.05) (BIO*TREAT)	15.2			

Table 3. The disease severity (%) of WMV on squash plants treated by fungal isolates (bioagents) using different treatments (application method) after six weeks of inoculation in the first season (Spring 2023).

Bioagents (BIO)	**Treatments (TREAT)**			
	Seed treatment	**Soil drench**	**Foliar spray**	**Mean BIO**
F1	27.5	50.0	72.2	**49.9**
F2	43.9	41.6	66.6	**50.7**
F3	35.8	63.9	41.6	**47.1**
F4	35.8	44.1	56.6	**45.5**
F5	65.8	45.8	36.0	**49.2**
F6	58.3	66.4	79.1	**67.9**
Mean TRAT	**44.5**	**52.0**	**58.7**	**51.7**
CON. H	1.6			
CON. INF	91.6			
LSD (0.05) (BIO)	14.4			
LSD (0.05) (TREAT)	8.8			
LSD (0.05) (BIO*TREAT)	24.9			

CON. H indicates control (untreated) of healthy plants and CON. INF indicates control (untreated) of infected plants.

Table 4. The disease severity (%) of WMV on squash plants treated by fungal isolates (bioagents) using different treatments (application method) after three weeks of inoculation in the second season (Summer 2023).

Bioagents (BIO)	**Treatments (TREAT)**			
	Seed treatment	**Soil drench**	**Foliar spray**	**Mean BIO**
F1	27.7	16.5	27.8	**24.0**
F2	19.5	27.8	19.4	**22.2**
F3	13.8	33.3	22.2	**23.1**
F4	36.1	30.5	16.6	**27.7**
F5	36.1	38.8	25.0	**33.3**
F6	33.3	25.0	27.7	**28.6**
Mean TREAT	**27.8**	**28.7**	**23.1**	**26.5**
CON. H	0.0			
CON. INF	58.0			
LSD (0.05) (BIO)	10.3			
LSD (0.05) (TREAT)	6.4			
LSD (0.05) (BIO*TREAT)	17.9			

Table 5. The disease severity (%) of WMV on squash plants treated by fungal isolates (bioagents) using different treatments (application method) after six weeks of inoculation in the second season (Summer 2023).

Bioagents (BIO)	**Treatments (TREAT)**			
	Seed treatment	**Soil drench**	**Foliar spray**	**Mean BIO**
F1	41.6	41.7	41.1	**41.5**
F2	46.9	47.3	36.1	**43.4**
F3	47.2	41.6	36.1	**41.7**
F4	41.6	41.6	33.3	**38.3**
F5	47.2	44.5	46.9	**46.2**
F6	47.2	50.0	49.7	**49.0**
Mean TREAT	**45.3**	**44.5**	**40.5**	**43.4**
CON. H	2.7			
CON. INF	85.0			
LSD (0.05) (BIO)	9.3			
LSD (0.05) (TREAT)	5.7			
LSD (0.05) (BIO*TREAT)	16.1			

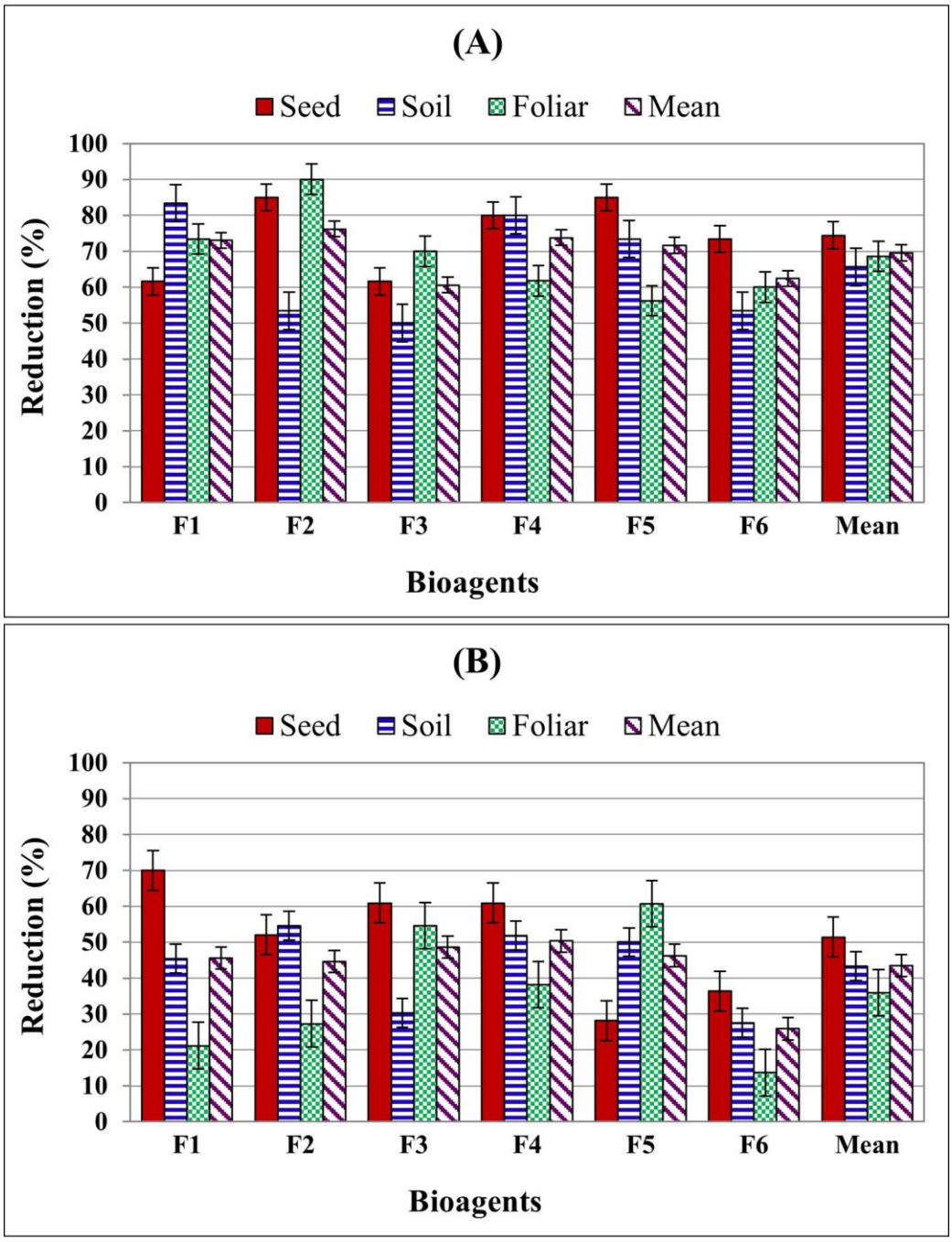

Figure 2. The percentage of reduction in disease severity of WMV on squash plants due to application with certain fungal isolates using different treatments during the first season (Spring 2023) after three weeks (A) and six weeks (B) of inoculation.

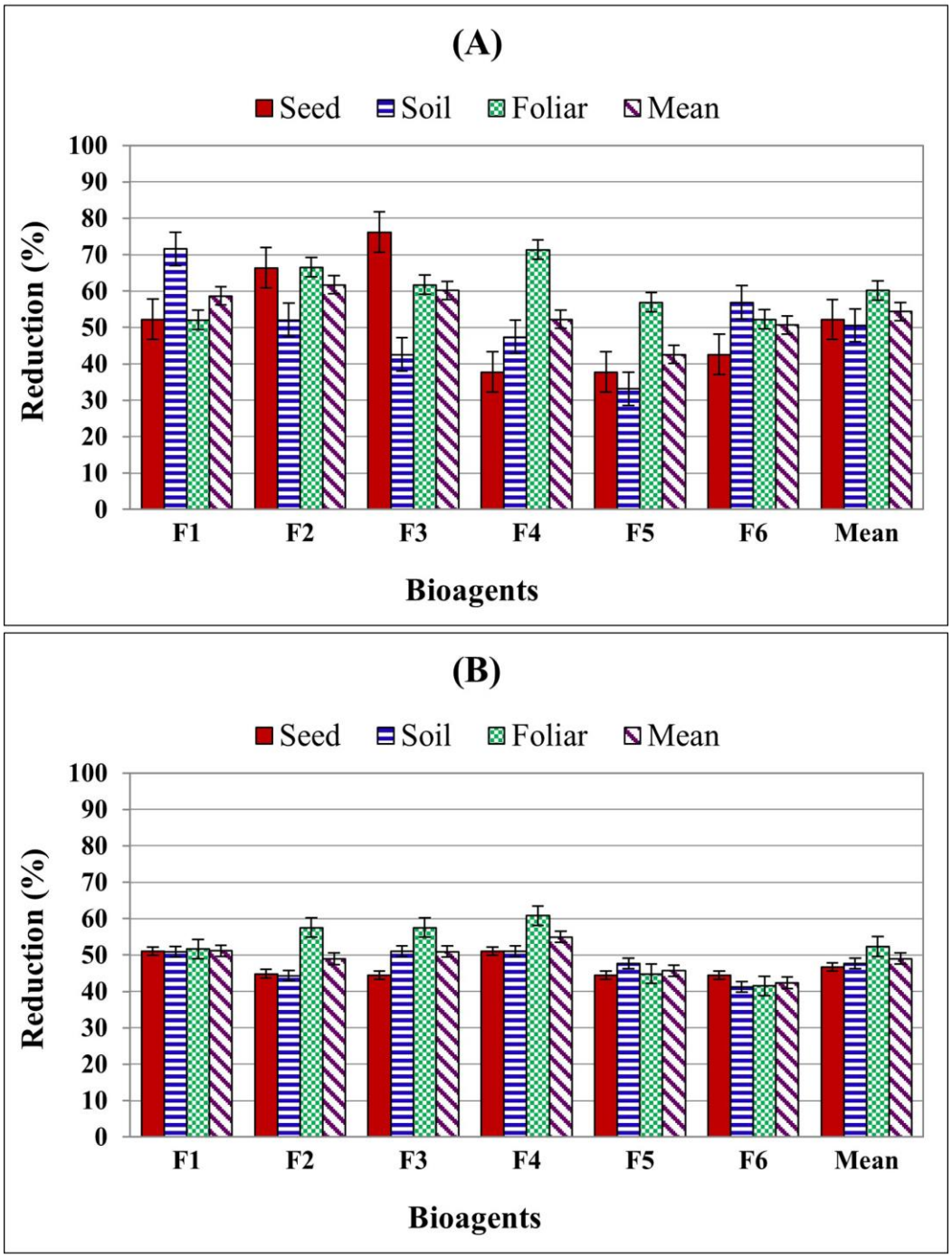

Figure 3. The percentage of reduction in disease severity of WMV on squash plants due to application with certain fungal isolates using different treatments during the second season (Spring 2023) after three weeks (A) and six weeks (B) of inoculation.

The highest reduction in disease severity three weeks after inoculation during first season was observed in the case of application of F2 isolate, as F2 isolate showed the lowest disease severity (11.9%) with a higher reduction of 76.2% averaged all application methods, followed by the application of F4 and F1, as their averaged disease severity was

13.1, and 13.5, respectively, with a reduction in disease severity of 73.8% and 73.0%, respectively (**Table 2** and **Figure 2**).

Meantime, the highest reduction in the disease severity six weeks after inoculation during first season was observed in the case of application of F4 isolate, as the mean of disease severity was 45.5% compared to 91.6% disease severity in the control infected plants, with a reduction of 50.3%. In additions, higher reductions in the disease severity six weeks after inoculation during the first season were also observed with F3 followed by F5 isolates, as the disease severity of these treatments were 47.1, and 49.2, with 48.6%, and 46.3% reductions in the disease severity, respectively (**Table 3** and **Figure 2**).

During the second season, the highest reduction in disease severity three weeks after inoculation was observed in the case of application of F2 isolate, as the mean disease severity was 22.2% compared to 58.0% in infected control plants, with 61.7% reduction in the disease severity, followed by F3 and F1 isolates, as the disease severity of these treatments were 23.1, and 24.0, with 60.2 and 58.6% reductions in the disease severity, respectively (**Table 4** and **Figure 3**).

Meantime, the highest reduction in the disease severity six weeks after inoculation during the second season was observed in the case of application of F4 isolate, as the mean disease severity was 38.3% compared to 85.0% in infected control plants, with 54.9% reduction in the disease severity, followed by F1 and F3 isolates, as the disease severity of these treatments were 41.5, and 41.7, with 51.2 and 50.9% reductions in the disease severity, respectively (**Table 5** and **Figure 3**).

Regarding the interaction between bioagents and application method, the results revealed that the most effective treatment observed after three weeks of inoculation in the first season was recorded in the case of foliar application by F2 bioagent, followed by seed treatment of F2 and F5, with 90.0, 85.0 and 85.0 reductions in the disease severity, respectively. Meanwhile, seed treatment displayed the most effective application method after six weeks of inoculation when applied with F1 bioagent (70.0% reduction in the disease severity) followed by F3 and F4, with 60.9% reduction in the disease severity, respectively (**Figure 2)**. In the second season, the highest reduction in the disease severity observed after three weeks of inoculation (76.2%) was observed when seeds were treated by F3 bioagent, indicating that seed treatment of F3 isolate was considered as the most effective treatment, while soil drench with F5 isolate showed the lowest reduction in the disease severity (33.1%). Meantime, foliar spray with F4 isolate displayed the highest reduction in the disease severity (60.8%), while soil drench with F6 isolate displayed lowest reduction (41.2%) after six weeks of inoculation (**Figure 3).**

Consistently, averaging all fungal isolates, seed treatment displayed the highest reductions in the disease severity after three and six weeks of inoculation, (74.4 and 51.4%, respectively) during the first season (**Figure 2**). Whereas foliar spray of fungal

isolates displayed the highest reductions in the disease severity after three and six weeks of inoculation (60.1 and 52.3%, respectively) during the second season (**Figure 3**).

On average and across seasons and time of inoculation, the treatments with F4 bioagent displayed the highest reduction in the disease severity (56.3%) followed by F2 (55.0%), F1 (54.7%) and F3 (53.8%), while F5 and F6 bioagents displayed the lowest reductions (49.8 and 42.3%, respectively). Interestingly, seed treatment displayed the highest reduction in the disease severity (54.2%) followed by foliar spray (51.5%) and soil drench (50.0%), with an average of 52.0% (**Figure 4**).

3.1.5. Effect of application with fungal isolates on total chlorophyll content:

Total chlorophyll content in leaves of squash plants infected with WMV and treated by fungal isolates (bioagents) using different treatments in the first (Spring 2023) and second (Summer 2023) season are presented in **Table 6** and **Table 7**, respectively.

Total chlorophyll content (SPAD unit) of plant leaves recorded in the first season ranged from 23.2 (foliar spray with F6 isolate) to 39.4 (soil drench with F1 isolate), with an average of 31.1 compared to 18.8 for control (untreated) infected plants (**Table 6**). Whereas in the second season, total chlorophyll content (SPAD unit) of plant leaves ranged from 23.1 (soil drench with F1 isolate) to 36.9 (soil drench with F5 isolate), with an average of 31.1 compared to 19.3 for control (untreated) infected plants (**Table 7**).

Table 6. Total chlorophyll content (SPAD unit) in leaves of squash plants infected with WMV and treated by certain fungal isolates (Bioagents) using different treatments (application method) in the first season (Spring 2023).

Bioagents (BIO)	**Treatments (TREAT)**			
	Seed treatment	**Soil drench**	**Foliar spray**	**Mean BIO**
F1	35.8	39.4	26.5	**33.9**
F2	32.1	30.1	26.8	**29.6**
F3	26.7	29.3	34.9	**30.3**
F4	26.4	34.5	34.8	**31.8**
F5	31.4	33.2	25.6	**30.1**
F6	33.8	35.6	23.2	**30.9**
Mean TREAT	**31.0**	**33.7**	**28.6**	**31.1**
CON. H	38.6			
CON. INF	18.8			
LSD (0.05) (BIO)	4.5			
LSD (0.05) (TREAT)	7.8			
LSD (0.05) (BIO*TREAT)	2.7			

Table 7. Total chlorophyll content (SPAD unit) in leaves of squash plants infected with WMV and treated by certain fungal isolates (Bioagents) using different treatments (application method) in the second season (Summer 2023).

Bioagents (BIO)	Treatments (TREAT)			
	Seed treatment	Soil drench	Foliar spray	Mean
F1	31.7	23.1	27.6	**27.5**
F2	32.3	35.1	27.8	**31.7**
F3	33.8	27.3	34.9	**32.0**
F4	34.2	27.5	32.2	**31.3**
F5	34.6	36.9	27.9	**33.2**
F6	31.8	32.1	28.2	**30.7**
Mean TREAT	**33.1**	**30.3**	**29.8**	**31.1**
CON. H	39.9			
CON. INF	19.3			
LSD (0.05) (BIO)	4.39			
LSD (0.05) (TREAT)	2.69			
LSD (0.05) (BIO*TREAT)	7.61			

CON. H indicates control (untreated) of healthy plants and CON. INF indicates control (untreated) of infected plants. Total chlorophyll content of a plant leaf was measured using a Soil Plant Analysis and Development (SPAD) method.

The results indicated that the treatments with fungal bioagents with different treatments had a positive impact on the total chlorophyll content of plant leaves with a significant average increase of 62.7% in the treated plants compared to the infected control group (**Figure 4).** Seed treatment and soil drench with all fungal bioagents displayed higher increase in total chlorophyll content (averaged 67.8 and 67.6, respectively), while foliar spray showed the lowest increase in total chlorophyll content (52.9). Averaged all application methods, the fungal isolates F5, F4 and F3 showed higher increase in the total chlorophyll content (65.6, 65.2 and 63.1, respectively).

4 - DISCUSSION

Cucurbits are important crops worldwide, and squash is considered as an important vegetable crop in Egypt and worldwide. It has been reported that cucurbits are being infected with many plant viruses. Therefore, cucurbits viral disease has been described as a problem worldwide. In this regard, some cucurbits growers consider aphid transmitted viruses as one of the most important limiting factors that can cause losses as high as 100% [16]. *Watermelon mosaic virus* is considered as the most common among these viruses infecting cucurbits [19].

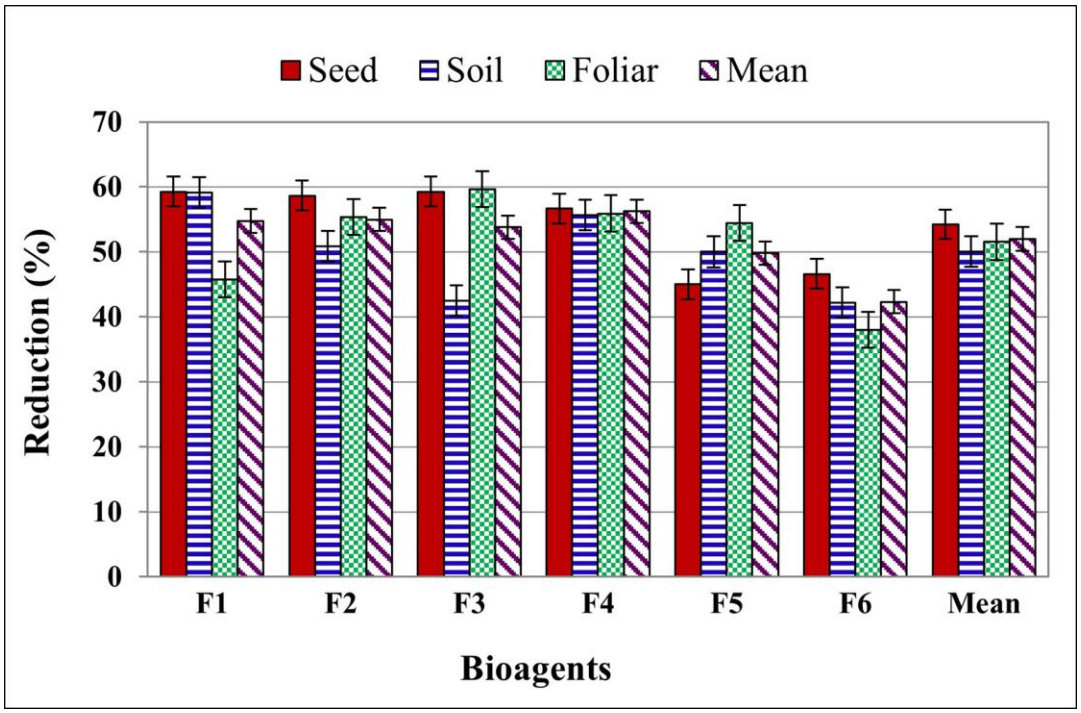

Figure 4. Overall reduction in disease severity of WMV on squash plants due to application with certain fungal isolates (bioagents) using different treatments (application method).

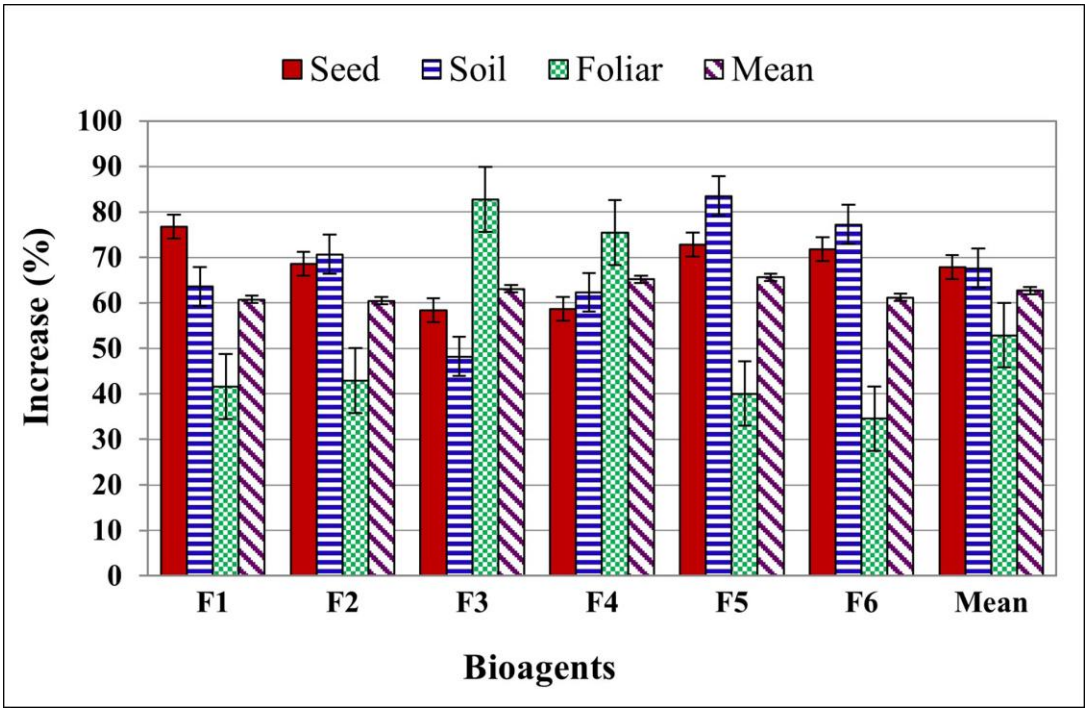

Figure 5. Overall increase in total chlorophyll content in leaves of squash plants due to application with certain fungal isolates (bioagents) using different treatments.

The main strategy to control *Watermelon mosaic virus* is through application of insecticides to control insect vectors, but this strategy poses many hazards to humans and environment. In addition, this virus is being transmitted in a non-persistent manner and thus using insecticide is not too useful for controlling this disease. Therefore, there is an urgent need to reduce reliance on agrochemicals and attempt to establish a new method to control *Watermelon mosaic virus*.

Biological control is known as a safe method to control plant disease and reduce economic losses, avoiding the bad effects of synthetic chemicals on environment and human health [20]. Microorganisms can be used to control viral diseases and enhance the growth of the plant [21] and can reduce the disease severity of plant viruses [22].

Beneficial microorganisms have received a lot of attention regarding management of plant viral diseases, as they provide a harmless and secure means of suppressing viruses [23]. These bioagent include both fungi and bacteria [23, 25 - 27].

Results of the present study showed significant reductions in the disease severity of WMV on the studied plants due to different treatments of fungal bioagents during the first and second seasons, comparing to control (untreated) infected plant. These findings demonstrated that these beneficial microorganisms are efficient agents to reduce the disease severity resulting by plant viruses [28 - 31].

Notably, after three weeks of inoculation in the first season, the application of F2 yielded the highest reduction in disease severity, with a remarkable 76.2% decrease compared to infected control plants. Following closely were treatments with F4 and F1, which exhibited higher reductions of 73.8% and 73.0% in disease severity, respectively. Meantime, higher reductions in the disease severity were observed after six weeks of inoculation by F4, F3 and F5 isolates (50.3, 48.6 and 46.3%, respectively). In addition, higher reductions in disease severity three weeks after inoculation were observed in the second season by F2, F3 and F1 isolates (61.7, 60.2 and 58.6% respectively). Meantime, higher reductions were observed after six weeks of inoculation by F4, F1 and F3 isolates (54.9, 51.2 and 50.9%, respectively). These findings were consistent with those obtained by [28] and [29], as they reported that application of certain fungal isolates like *Trichoderma sp.* is an efficient biocontrol agent to control plant viruses. In addition, [30] reported that using *Penicillium simplicissimum* is an efficient method to control plant viruses, and [29] demonstrated the ability of the rhizospheric fungus *Trichoderma harzianum* to control *Cucumber mosaic virus.*

Trichoderma spp. are important for their benefits on agriculture like enhancement the plant growth and increase the plant defense against pathogens [32 - 34] Moreover, its secondary metabolites could be used in management of plant viral diseases [35] , and this could achieved through the ability of *Trichoderma* spp. to induce systemic acquired resistance in plants [34]. *Trichoderma spp* can also induce the production of phytohormons, these products could be considered as biofertilization [32].

The mechanism by which microorganism and fungi can reduce the disease severity of WMV is not clear, but previous studies have suggested that it may include the activation of certain defense genes [36, 37], while some studies focused on metabolites of certain fungi and bacteria which have been found to prevent or reduce the lesion produced by TMV [38].

Consistently, seed treatment displayed highest reductions in the disease severity after three and six weeks of inoculation in the first season (74.4 and 51.4%, respectively), whereas foliar spray displayed highest reductions in the disease severity after three and six weeks of inoculation (60.1 and 52.3%, respectively) in the second season. However, on average and across seasons and time of inoculation, seed treatment displayed the highest reduction in the disease severity (54.2%) followed by foliar spray (51.5%) and soil drench (50.0%), These finding revealed that application method can significantly affect the efficiency of fungi to reduce the disease severity of WMV. In general, seed treatment led to better management compared to soil and foliar treatment. In this regard, our findings partially agree with previous observations by [39] and [40] which reported seed treatment with microorganism is a common method to control plant viruses.

Furthermore, the results indicated that the treatments with fungal bioagents had a positive impact on total chlorophyll content of plant leaves with a significant average increase of 62.7%. Seed treatment and soil drench displayed higher increase in total chlorophyll content (averaged 67.8 and 67.6, respectively), while foliar spray showed the lowest increase in total chlorophyll content (52.9). These findings revealed a positive association between severity of the disease and the plant contents of chlorophyll, and thus reductions of the disease severity led to enhancement of chlorophyll content of plant leaves. This finding was consistent with the observation reported by [41]' who found that virus infections can lead to chlorosis due to impaired photosynthesis and resource distribution, and thus reduction of the disease severity resulting from plant viruses can significantly improve chlorophyll content.

Interestingly, our study has shown that certain microorganisms can reduce the severity of WMV and positively impact chlorophyll levels. Seed and soil treatments were found to be the most effective application methods, followed foliar application. These findings are consistent with those of [42] and [43], as they also reported enhanced chlorophyll content in various crops with PGPR treatment.

This study found that *Watermelon mosaic virus* infection can affect the plant contents of carbohydrates in zucchini plants, and this result is in the same line with previous observations that plants infected with *Watermelon mosaic virus* showed increasing protein contents comparing with uninfected plants [44, 45].

CONCLUSION

The present study reported that application of certain fungal microorganisms as bioagents significantly reduced the disease severity resulting by WMV on squash plants. Moreover, different fungi varied in their ability to reduce the disease severity. Application methods also played a critical role in the efficiency of these bioagents to control viral disease. Seed treatment led to better management to reduce the disease severity. Enhanced chlorophyll content was observed by the treatments with fungal bioagents. Thus, fungi can be successfully used as bioagents to control viral diseases and enhance plant growth by reducing the disease severity. Therefore, the study suggested that beneficial fungi could have great attention in agricultural research due to their potential to enhance plant growth, improve nutrient uptake, and provide protection against pathogens. However, further studies are still required to investigate the mechanisms by which microorganisms can protect plants from infection.

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