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(Original Article)

Application of Certain Bacterial Bioagents to Control Watermelon Mosaic Virus

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Abstract

Squash is an important vegetable crop in Egypt. Squash can be infected with many plant viruses; among these viruses Watermelon mosaic virus (WMV) is considered as the most prevalent one under Egypt conditions. Traditional methods to control WMV are not very useful. In the current study, six bacterial isolates (two isolates of *Bacillus amyloliquefaciens* and one isolate from each *B. velezensis, B. cereus, Brevundimonas diminuta*, and *Streptomyces enissocaesilis*) were applied to squash plants as bioagents to control WMV in three different application methods (soil drench, seed treatment and foliar spray) during two consecutive seasons under greenhouse conditions. The results revealed that all 6 bacterial bioagents significantly reduced the disease severity of WMV either 3 or 6 weeks after inoculation. During the first season, the most efficient bioagents were *B.cereus* (B6) and *Brevundimonas diminuta* (B5). While the bioagents *B. amyloliquefaciens* (B1) and *Streptomyces enissocaesilis* (B2) were the most efficient isolates to control WMV in the second season. Evidently, the application of bacterial isolate led to a significant increase in chlorophyll content in treated plants compared to infected control ones. These results proved that the application of bacterial bioagents can be a useful method to control WMV in squash plants.

Keywords: *Bacterial bioagent, Disease severity, Squash and Watermelon mosaic virus*

Introduction

Squash belongs to the cucurbit family (Lecoq, 2012) is a popular vegetable crop in Egypt. Summer and winter squash are grown either in greenhouse or open fields including (Fathy *et al*., 2024). Plant viruses are responsible for causing considerable losses in both yield and quality of a wide variety of economically significant crops (Zitter *et al*.,1996). Squash is being infected by many viruses (Abdalla *et al*., 2017a). It is known that there are at least 59 viruses infecting squash (Nicaise, 2014). Among these viruses, Watermelon mosaic virus (WMV) is considered as one of the most important viruses infecting squash under Egypt conditions (Fathy *et al*., 2024). WMV is widely prevalent in temperate climates and Mediterranean countries (Webb and Scott, 1965) and there are many strains

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belonging to different regions of the world (Alonso-Prados *et al*., 2003; Gibbs *et al*., 2008). WMV can infect over 170 plant species in 27 families, including *Cucurbitaceae*, *Fabaceae*, *Malvaceae*, and *Chenopodiaceae* (Desbiez and Lecoq, 2004). The losses of crops caused by WMV can go up to 100%, depending on the growing season (Demski and Chalkley, 1972).

WMV belongs to the genus *Potyvirus* and *Potyviridae* family. The particle of WMV is flexuous filamentous particles measure around 750 nm in length (Purcifull *et al*., 1984). It is transmitted by various aphid species in a non-persistent manner, especially melon or cotton aphid (*Aphis gossypii*) and the green peach aphid (*Myzus persicae*). However, there are at least 38 aphid species able to transmit potyviruses (Lecoq *et al*., 2017; Provvidenti, 2017).

Management of WMV depends mainly on using insecticide to control the aphid vector. This virus is being transmitted in a non-persistent manner, so using insecticides is not efficient enough to control this disease. In addition, the excessive application of insecticide has many negative effects on humans and the environment. Therefore, there is an urgent need to establish another alternative to manage WMV infection. One alternative could be the application of certain bacterial bioagents to control this disease. Several biological control agents (BCAs), including *Bacillus*, *Pantoea*, *Streptomyces*, *Trichoderma*, *Clonostachys*, *Pseudomonas*, *Burkholderia*, and certain yeasts, have been reported before for their efficiency to control plant pathogen (Lahlali *et al*., 2022; Fathy *et al*., 2024). Bioagents can effectively suppress viral activity in a safe and efficient manner (Clay 1988; Siegel and Latch 1991; Li *et al*., 2016; Ramzan *et al*., 2016; Lecoq *et al*. 2017). Several substances that are produced by bioagent have been identified as powerful inhibitors of plant viruses (Abd El-Shafi 2005; Oka *et al*., 2008).

Therefore, the objective of this study is to evaluate the effectiveness of certain bacterial bioagents including *Bacillus cereus*, *Brevundimonas* diminuta, *Bacillus amyloliquefaciens*, *Streptomyces enissocaesilis*, and *Bacillus velezensis* as potential bioagents to reduce the disease severity of Watermelon mosaic virus in squash plant.

Materials and Methods

1. Source of the virus and its identification

Watermelon mosaic virus isolate obtained in this study was previously identified by Fathy *et al*. (2024) using specific primers to amplify the coat protein (CP) gene of WMV using Reverse Transcription- Polymerase Chain Reaction (RT-PCR), and the isolate was kept in living squash plant under protected greenhouse condition at the Faculty of Agriculture, Assiut University, Egypt.

Mechanical Inoculation

Mechanical inoculation was conducted as previously described by Fathy *et al*. (2024). Zucchini (*C. Pepo*) Hytech Hybrid cultivar seeds were planted in pots (30 cm in diameter) filled with sterilized soil in a greenhouse free of insects. One-week post planting, the cotyledon leaves of the zucchini seedlings were gently washed with distilled and sterilized water. Then, carborundum powder (600 mesh) was dusted on the leaves, and each cotyledon leaf was gently rubbed with 100 µl of plant sap (prepared by crushing 1 g of symptomatic zucchini leaves in 1 ml of cold phosphate buffer pH 7.4).

2. Source of bacterial bioagent isolates

Four bacterial isolates (B1 to B4) which previously proved their capacity to control plant pathogens were kindly provided by the Department of Plant Pathology of Faculty of Agriculture, Assiut University. Two bacterial isolates (B5 & B6) were isolated as follows:

Isolation of bacterial bioagents

The soil samples were gathered from medicinal plants grown in greenhouse at Assiut University's Farm and left to dry naturally for four days. Once dried, they were ground in a sterilized mortar and sifted through a 4mm mesh screen. One gram of the prepared soil was then stirred in 100 ml of sterilized water for about 5 minutes in a 250 ml Erlenmeyer flask. The mixture was then left to stand for 30 minutes (Li *et al*., 2016). A series of dilutions was prepared with a concentration range of 10-1 to 10-7. One milliliter of each dilution was taken and spread on a petri dish containing Nutrient Agar media (NA). Three plates were made for each concentration. The plates were then incubated at 37°C for 48 hours until colonies emerged. As soon as the emerging colonies started to appear, they were transferred to a new plate in order to get pure cultures.

3. Identification of bacterial bioagents

Pure bacterial isolates were cultivated in 10 ml test tubes containing nutrient broth medium (Clay, 1988). The culture was incubated at 28ºC for 48 hours and the culture was then boiled in Eppendorf tubes. The tubes were then subjected to centrifugation for 2 min at 4000 rpm. The supernatant was discarded, and the remaining dead bacterial cells were shipped to SolGent Company, Daejeon South Korea for performing the polymerase chain reaction (PCR) and gene sequencing. The PCR amplifications and sequencing were conducted using two universal primers 27F (5'-AGAGTTTGATCC TGGCTCAG-3') and 1492R (5'-Gto GTTACCTTGTTA CGACTT-3').

The obtained sequences were subjected to the gene bank (NCBI) website to molecularly identify these bacterial bioagents using Basic Local Alignment Search Tool (BLAST).

4. The efficiency of certain microorganisms to control WMV under greenhouse conditions

The efficiency of 6 Bacterial isolates to control WMV on squash plants was evaluated in two different trials during two successive seasons (2022 and 2023) in a greenhouse of the Faculty of Agriculture, Assiut University. The application of these microorganisms was implemented in three different methods (soil drench, seed coating and foliar spray), as previously described by Fathy *et al*. (2024).

The development of viral symptoms was observed in all treated and non-treated plants, and the symptoms of WMV were recorded three- and six-weeks post inoculation using the following rating scale for WMV as described by Abdalla *et al.* (2017a)

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 was calculated using the following formula:

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

5. Effect of Bacterial bioagents application on the total chlorophyll content in zucchini plants infected with WMV

Chlorophyll content in infected squash plants with WMV (treated and non-treated with bacterial bioagents) was measured using a Soil Plant Analysis and Development chlorophyll SPAD meter (SPAD-505 plus) (Khadka *et al*., 2020).

Statistical analysis

Statistical analysis of the phenotypic data was carried out using 2-way ANOVA using CoStat statistical software, and Least Significant Difference (L.S.D) at 0.05 was calculated for comparing means using CoHort software.

Results

1. The source of the virus and its identification

Watermelon mosaic virus isolate obtained in this study was obtained from symptomatic zucchini plants grown at Experimental Farm at Faculty of Agriculture, Assiut university, and identified using specific primers to amplify the CP gene of WMV in Reverse Transcription- Polymerase Chain Reaction (RT-PCR) as previously described by Fathy, *et al*. (2024).

2. Identification of bacterial bioagents

Six bacterial isolates were molecularly identified through using Basic Local Alignment Search Tool (BLAST) available in Genebank website, as presented in Table (1).

Table 1. Molecular identification of bacterial bioagents isolates

Bacterial isolate	Identification
B1	*Bacillus amyloliquefaciens*
B2	*Streptomyces enissocaesilis*
B3	*Bacillus amyloliquefaciens*
B4	*Bacillus velezensis*
B5	*Bacillus cereus*
B6	*Brevundimonas diminuta*

3. Effect of certain bacterial isolates on WMV disease severity in season 2022

Results in Table 2 showed that all bacterial bioagents significantly reduced the disease severity of WMV either three or six weeks after inoculation. The most efficient bioagent to reduce WMV disease severity three weeks after inoculation was B6 followed by B5, as the disease severity were 6.7 and 9.7%, respectively (averaged the three application methods). Meanwhile, the most efficient bioagent to reduce WMV disease severity six weeks after inoculation was B6 followed by B5, as the disease severity were 42.8 and 47.2%, respectively. Data also showed that the application method can affect the effectiveness of these isolates to control WMV either three or six weeks after inoculation. The best application methods were seed and soil treatment in both three or six weeks after inoculation, but there was no significant difference between seed and soil application. In general, the least disease severity three weeks after inoculation occurred in the case of seed treatment with the bioagent B6 followed by soil treatment with B5, as the disease severity was 2.5 and 5%, respectively. While, in case of six weeks after inoculation it occurred in case of seed treatment with B4 followed by seed treatment with B2, as the disease severity were 25 and 31.3 %, respectively.

Table 2. Effect of certain bacterial isolates on WMV disease severity in season 2022

Bioagents (BIO)	Disease severity (%)							
	Three Weeks after inoculation				Six Weeks after inoculation			
	Seed coating	Soil drench	Foliar spray	Mean	Seed coating	Soil drench	Foliar spray	Mean
B1	2.5	20.8	31.6	18.3	50	54.2	91.6	65.3
B2	20.8	18.3	20.8	20	31.3	57.5	58.3	49.0
B3	5.0	15.0	19.2	13.1	40.8	58.3	68.8	55.9
B4	7.5	4.2	21.6	11.1	46.7	25.0	87.5	53.0
B5	15.0	5.0	9.2	9.7	50	54.2	37.5	47.2
B6	2.5	10	7.5	6.7	37.5	35.8	55	42.8
Mean	8.8	12.2	18.3	13.2	42.7	47.5	66.5	52.2
Control (healthy)	0.8				1.6			
Control (Infected)	56.6				91.7			
L. S. D at 0.05								
Bioagent (A)	9.3				18.4			
Application Method (B)	5.7				11.3			
Interaction (AB)	16.1				31.8			

4. Effect of certain bacterial isolates on WMV disease severity in season 2023

Results in Table 3 showed that application of certain bacterial bioagents can significantly reduce the disease severity of WMV either three or six weeks after inoculation during 2023. The most efficient bioagent reduced the disease severity in both three and six weeks after inoculation were B1 followed by B2 as the disease severity were 13.8 and 28.5 in case of three weeks were 29.6 and 34.1 % in case of six weeks, respectively. Data also showed that the application method can affect the effectiveness of these bacterial isolates to control WMV either three or six weeks after inoculation. The best application method was foliar spray followed by seed treatment in case of three weeks after inoculation as the mean disease severity were 12.5 and 18.5 %, respectively while in the best application method six weeks after inoculation were seed and foliar spray with disease severity about 34.7 and 36.1, respectively.

Table 3. Effect of certain Bacterial bioagents to reduce disease severity of WMV: second season 2023

Bioagents (BIO)	Disease severity (%)							
	Three Weeks after inoculation				Six Weeks after inoculation			
	Seed treatment	Soil drench	Foliar spray	Mean	Seed treatment	Soil drench	Foliar spray	Mean
B1	11.1	22.2	8.3	13.8	30.6	36.1	22.3	29.6
B2	22.3	22.2	11.1	18.5	33.1	38.6	30.6	34.1
B3	22.3	38.9	11.1	24.1	33.3	58.3	27.8	39.8
B4	13.9	22.2	13.8	16.6	33.3	35.6	50	39.6
B5	19.4	25.1	13.9	19.4	30.6	63.3	55	49.6
B6	22.3	33.3	16.6	24.1	47.2	49.4	30.6	42.4
Mean	18.5	27.3	12.5	19.4	34.7	46.8	36.1	39.2
Control (Healthy)	0.0				2.8			
Control (Infected)	58.4				85			

L. S. D at 0.05			
Bioagent	(A)	10.8	14.8
Application Method	(B)	6.6	9
Interaction	(AB)	18.78	25.7

5. Effect of Bacterial bioagents on the total chlorophyll content in zucchini plants infected with WMV

Results in Table (4) showed that application with bacterial bioagent can significantly increase the chlorophyll content in zucchini plants infected with WMV in two consecutive seasons (2022 and 2023). Data indicated that there was no significant difference in chlorophyll content between healthy plants and treated infected plants with bioagent. The best bioagent led to highest chlorophyll content occurred in case of bioagent B5 followed by B6 in the first season as the chlorophyll content were 35.4 and 34.9, respectively, while they were bioagent B1 followed by B4 in the second season with chlorophyll content of 35.9 and 32.4, respectively. Data in Table (4) revealed also that application method of these bioagent affected the chlorophyll content. During the first season the highest chlorophyll content was observed in case of seed followed by soil treatment but there was no significant difference between them as the chlorophyll content were 36.6 and 36.4, respectively, while in the second season the application method did not affect significantly in the chlorophyll content. In general, the highest chlorophyll content was observed in case of seed treatment with B4 in the first season and seed treatment with bioagent B1 in the second season as chlorophyll content were 43.6 and 39.7, respectively.

Table 4. Effect of bacterial bioagents on the total chlorophyll content in zucchini plants infected with WMV

Bioagents (BIO)	**Total chlorophyll content** (SPAD unit)							
	Season 2022				Season 2023			
	Seed treatment	Soil drench	Foliar spray	Mean	Seed treatment	Soil drench	Foliar spray	Mean
B1	28.0	43.2	28.1	33.1	39.7	36.1	31.9	35.9
B2	37.2	33.3	25.7	32.1	32.3	29.9	27.2	31.6
B3	36.4	29.1	26.2	30.5	27.8	27.3	37.1	30.8
B4	43.6	40.0	22.5	35.4	35.8	27.2	34.2	32.4
B5	37.5	35.7	26.7	33.3	28.9	29.9	29.8	29.5
B6	35.6	38.4	30.7	34.9	27.1	32.0	23.7	27.6
Mean TREAT	36.4	36.6	22.8	33.2	31.9	30.4	30.7	31.3
Control (Healthy)	39.9				38.6			
Control (Infected)	19.3				18.8			
L. S. D at 0.05								
Bioagent (A)	5.3				5.1			
Application Method (B)	3.2				3.1			
Interaction (AB)	9.1				8.8			

Discussion

Viruses can cause severe losses in vegetable production worldwide (Abdalla *et al*., 2017a). Control of plant viruses is not an easy task, because the available options to control plant viruses are limited ones. Although using resistant cultivars may present a potential method to control plant viruses, the absence of resistant cultivars against many viruses made the use of this method limited to a few cases. Using insecticides to control insect vectors of these viruses presents another alternative method to control plant viruses, but this method could be useful only to control plant viruses that are being transmitted in persistent manner, while their efficiency to control plant viruses transmitted in non-persistent manner is limited. So, there is a need to apply another efficient method to control plant viruses. Application of beneficial bacterial isolates may present a safe method to control plant viruses (Abdalla *et al*., 2017a) and this method has been investigated against many viruses (Srinivasan and Mathivana 2009). Biological control offers a safe approach to control plant pathogens including many viral diseases (Vinodkumar *et al*., 2018; Worsley *et al*., 2020) and avoiding the undesired effects of synthetic chemicals at the same time (Tucci *et al*., 2011).

This study attempted to investigate the potential of using certain bacterial bioagents to control Watermelon mosaic virus infecting squash plants during two consecutive seasons under greenhouse conditions. Four *Bacillus sp*. isolates (two isolates of B. *amyloliquefaciens* and one isolate from each *B. velezensis* & *B. cereus* &) in addition to one isolate of *Brevundimonas diminuta* and *Streptomyces enissocaesilis* were applied in three different methods (soil drench, seed treatment and foliar spray). The results of this experiment revealed that all 6 bacterial bioagent can significantly reduce the disease severity of WMV. The most efficient bioagent to reduce WMV disease severity was B6 *Bacillus cereus* followed by *Brevundimonas diminuta*. These data are in agreement with previous studies that using bacterial isolates are an efficient method to control plant viruses (Srinivasan and Mathivana 2009; Abdalla *et al*., 2017a and b).

The utilization of various bacterial agents has proven to be successful in controlling plant pathogens. *Bacillus* species, in particular, gained attention due to their efficiency, widespread presence in different environments, and their ability to enhance plant growth (Montesinos and Bonaterra *et al*., 1996; Nakkeeran *et al*. 2006). According to previous studies *Bacillus* species has been used to control viruses, including Tomato mosaic virus (Saharan and Nehra 2011), Cucumber mosaic virus and Banana bunchy top virus, (Harish *et al*., 2009), and, Tomato mottle virus (Murphy *et al*., 2000), and Sunflower necrosis virus (Srinivasan and Mathivanan 2009). The findings of this study are the line with Zehnder *et al*., (2000) who stated that *B. subtilis*, *B. pumilus*, and *B. amyloliquefaciens* are potential agents to control Cucumber mosaic virus infecting tomato plants.

The current study demonstrated that the method of application can significantly affect the efficiency of bacteria in decreasing the severity of Watermelon mosaic virus. This partially agrees with previous studies that confirmed application method can affect the effectiveness of bioagents to control plant viruses (Kloepper *et al*., 2004 ; Abdalla *et al*., 2017a).

Previously, most experiments performed to control viruses using microorganisms were conducted by soil application method (Maurhofer *et al*. 1994; Raupach *et al*., 1996; Raupach *et al*., 1998). While Murphy *et al*. (2003) mentioned that soil or seed application of *B. subtilis* and *B. pumilus*, resulted in significant reduction in disease severity of viral symptoms. Additionally, Zehnder *et al*., (2000) reported that seed treatment and soil drenching with *Bacillus* species induced resistance against Cucumber mosaic virus in tomato plants. While, application of *B. amyloliquefaciens* through foliar spray can be useful to reduce the disease severity of Cucumber mosaic virus in pepper and Tomato mottle virus in tomatoes, and Sunflower necrosis virus (Murphy *et al*. 2000; Harish *et al*., 2009; Srinivasan and Mathivanan 2009; Lee and Ryu 2016).

This study found that application of certain bacterial bioagent led to significant increase in chlorophyll contents compared with infected control with a significant average increase. These results agree with previous studies that found treatment with bacterial bioagents can induce physiological changes in plants (Maurhofer *et al*., 1994).

Previous studies had suggested that there are many mechanisms by which bacterial bioagents can provide protection against viral plant pathogens. These mechanisms include: induction of host plant resistance (Halfeld-Vieira *et al*., 2006), enhance the production of pathogenesis-related protein (Ryu *et al*., 2004), activation of plant enzymes (Murphy *et al*., 2000), and induction of physiological changes in the plants (Maurhofer *et al*., 1994). Additionally, studies have shown that *Bacillus spp*. have been effective in enhancing growth, which help to manage many viral diseases in various economically significant crops (Wang *et al*., 2015; Saharan and Nehra 2011; Tahir *et al*., 2017).

In conclusion, in this study six bacterial isolates including Bacillus *amyloliquefaciens, Streptomyces enissocaesilis, Bacillus velezensis, Bacillus cereus*, and *Brevundimonas diminuta*, in three different application methods, reduced the disease severity of WMV. Application of bacterial bioagents to control WMV and can be integrated with other methods to control this virus. Further studies are required to study the effect of application combination of different isolates and study the factors which may improve the ability of these bacterial isolates to control WMV.

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تطبيق بعض العوامل الحيوية البكتيرية لمكافحة فيروس تبرقش البطيخ

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الملخص

محصول الكوسة هو أحد محاصيل الخضر الهامة في مصر. تصاب الكوسة بالعديد من الفيروسات. يعتبر فيروس تبرقش البطيخ من أكثر الفيروسات شيوعاً تحت ظروف مصر. طرق المكافحة التقليدية لفيروس تبرقش البطيخ ليست عالية الفعالية. في تلك الدراسة تم استخدام ستة عزلات بكتيرية (عزلتين من *Bacillus amyloliquefaciens* ، وعزلة واحدة من كلا من *Bacillus cereus*، *Bacillus. velezensis*، *Brevundimonas diminuta*، *Streptomyces enissocaesilis*) لمكافحة فيروس تبرقش البطيخ. وتمت المعاملة بتلك العزلات البكتيرية عن طريق ثلاث طرق مختلفة تضمنت معاملة البذور، غمر التربة، رش المجموع الورقي خلال موسمين زراعيين متتاليين تحت ظروف الصوبة. أوضحت النتائج التي تحصل عليها من خلال تلك الدراسة أن جميع العزلات البكتيرية نجحت في تقليل شدة الإصابة المرضية بفيروس موازييك البطيخ بدرجة معنوية. خلال الموسم الأول كانت بكتريا *Bacillus cereus* وبكتريا *Brevundimonas diminuta* هي أكثر العزلات البكتيرية كفاءة في تقليل شدة الإصابة المرضية بفيروس تبرقش البطيخ، بينما في الموسم الثاني فإن بكتريا *B. amyloliquefaciens* *وبكتريا* *Streptomyces enissocaesilis* كانت هي الأفضل. أوضحت نتائج تلك الدراسة أن معاملة نباتات الكوسة المصابة بفيروس تبرقش البطيخ بتلك العزلات البكتيرية أدت إلى زيادة معنوية في محتوى الأوراق من الكلوروفيل مقارنة بالنباتات غير المعاملة. هذه النتائج تشير إلى أن استخدام بعض الأنواع البكتيرية كعوامل حيوية يمكن أن يفيد في تخفيض شدة الإصابة المرضية بفيروس تبرقش البطيخ.

الكلمات المفتاحية: *العامل الحيوي البكتيري، الشدة المرضية، الكوسة، فيروس تبرقش البطيخ*