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Antiviral Effects of the Liquid Culture, Cell Free Supernatants and Extracellular Products from *Serratia marcescens* subsp. *marcescens* against Watermelon Mosaic Virus (WMV)

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THIS STUDY was to specify the potential antiviral action of *Serratia marcescens* subsp. *marcescens* and its extracellular products against watermelon mosaic virus (WMV). The highest level of protection observed occurred in experiments, where bacteria were mixed with virus *in vitro* before inoculation, resulting in gave 100% viral inhibition. *S. marcescens* significantly improved ($p< 0.05$) several growth parameters in all experiments compared to viral control. The highest level of virus inhibition obtained when a mixture of all compounds extracted and purified from *S. marcescens* subsp. *marcescens* was applied to cucumber plants reducing infection by 95% . The results of RT-PCR showed that *in vitro* liquid bacterial culture treatment and applied 24 hr after virus inoculation treatment highly reduced the accumulation of WMV in treated plant leaves . The usage of *S. marcescens in vitro* and *in vivo* led to increase in total protein, polyphenoloxidase and phenolic compounds compared to viral-infected and healthy controls, while reducing glutathione oxidase. *S. marcescens*, and its metabolic products found in culture filtrates show promise as an effective biocontrol agent for WMV infection in plants and appear to promote plant growth even in the absence of virus.

Keywords: *Serratia*, Watermelon mosaic virus, Systemic and local resistance, RT-PCR, Antioxidant enzymes

Viruses affecting plant health are chronic threats to food production and ecosystem stability worldwide. Viruses affect many economically important crops: tomato mottle virus infects tomato (Murphy & Zehnder, 2000) while cucumber mosaic virus (CMV) infects over 800 plants species (Palukaitis *et al*., 1992). Zucchini yellow mosaic virus (ZYMV) infects cucumber (Abd El- Shafi, 2005) and watermelon mosaic virus (WMV) naturally infects members of *Cucurbitaceae*, *Chenopodiaceae*, *Malvaceae* & *Legumonsae* families (Shukla *et al.*, 1994).

Watermelon mosaic virus (WMV) is a member of genus *Potyvirus* (family: Potyviridae) and consists of flexuous, filamentous particles, approximately 750 nm long. WMV can experimentally infect more than 170 plant species belonging to 27 families, including many weeds that can host the virus between crops (Desbiez

et al., 2007). Watermelon mosaic virus-1 (WMV-1) is synonymous with papaya ring spot virus strain W (Purcifull *et al.*, 1984). It was first reported in India by Bharagava and Joshi in 1960 (Sharma *et al.*, 2010). Watermelon mosaic virus (WMV) [formerly watermelon mosaic virus-2] was first described by Webb & Scott (1965). WMV causes mosaic patches on foliage and fruits of infected plants. The plants are especially damaged when infected as young plants because the yields are reduced and the fruit quality is often affected by bumps and discoloration (Hausbeck, 2011).

Host defenses in plants can be stimulated by various means, including plant extracts, synthetic chemicals and rhizobacteria. Nonpathogenic rhizobacteria can induce protection against a wide variety of pathogens, a process known as induced systemic resistance. Among *Serratia* species, *S. marcescens* subsp. *marcescens* is an important bacterium which induces systemic resistance to various pathogens. The strain *S. marcescens* 90-166 protects plants against cucumber mosaic virus (Raupach *et al*., 1995 and Ryu *et al*., 2004). *Serratia marcescens* (a Gram-negative bacterium, *Enterobacteriaceae,* soil inhabitant) produces a variety of chitinolytic enzymes, DNAase, lipase and gelatinase (Ulhoq & Peberdy, 1991 and Giri *et al*., 2004). *S. marcescens* subsp. *marcescens* was considered originally to be an innocuous, non-pathogen and was used as a biological marker. The ability to form red pigment (prodigiosin) is characteristics of it (Hejazi& Falkiner, 1997).

The objective of the present study was to specify the potential antiviral action of *Serratia marcescens* subsp. *marcescens* and its extracellular products against WMV.

Materials and Methods

Bacteria and Virus

The plant growth promoting bacteria *Serratia marcescens* subsp. *marcescens* was isolated from Sharkia Governorate, Egypt and identified according to Bergy's Manual systematic of bacteriology. A total of 50 presumptive *Serratia* isolates which produced red pigments were isolated from the different soil samples using nutrient agar (Difco Manual, 1994). Subsequent confirmation using biochemical tests showed that 95 % of the isolates had the typical morphology and biochemical reactions expected of *S. marcescens*. The cultures were stored in glycerol (20 %) at - 20 ˚C in the research collection of the Laboratory of Microbiology, Faculty of Agriculture, Zagazig University, Zagazig, Egypt. The identification was confirmed in the Egyptian culture collection at Cairo Mercin, Ain Shams University, Cairo. The cultures of *S. marcescens* were maintained on nutrient agar slopes at 4 ˚C and subcultured every 4 weeks. Aliquots (100 µl) of fresh culture of *S. marcescens* was inoculated into Erlenmeyer flasks, 250 ml capacity, each containing 100 ml of nutrient broth pH 7 and incubated at 37 ˚C in a shaking incubator at 150 rpm for 3 days for producing sufficient inoculum. A final inoculum was prepared by serially dilutions in Ringer's solution to reach a final level of 7 log CFU ml^{-1} as determined by optical density at 600 nm and confirmed by plate counting on nutrient agar. The cell free supernatant (CFS) was then prepared by filtration through a 0.45µm filter. The filtrate obtained and the

liquid culture were both used for antiviral assays. Watermelon mosaic virus (WMV-Egyptian isolate) used in all the experiments was isolated from naturally infected squash fruit. This virus was identified based on the external symptoms on naturally infected squash fruit; on symptoms of mechanically infected cucumber plants and on real time- polymerase chain reaction (RT-PCR) analysis. The seeds of *Cucumis sativus* L. cultivar Beit Alpha F-1 were obtained from Agriculture Research Institute, Giza, Egypt and used as viral host plants. A fertile cultivated soil was obtained from Sharkia Governorate for cultivation of cucumber seeds in plastic pots (1000 cm^3) one cm depth below the soil surface and kept under the natural day light in the greenhouse of Faculty of Science, Zagazig University. Irrigation of cultivated seeds was carried out as required until the end of each experiment. The virus was propagated and maintained in cucumber plants according to Faccioli & Capponi (1983): 5g of naturally infected zucchini fruits were ground in a sterile mortar with a pestle in 5 ml of 0.01 M phosphate buffer solution of pH 7.2 then filtered through cotton. The volume was made up to 20 ml with phosphate buffer then 100 µl of viral sap were mechanically inoculated into coteledonary leaves and first leaf of squash dusted by carborundum (600 mesh-Prolab). The inoculated leaves were then washed with distilled water (Yarwood, 1955). After 28 days the symptoms were recorded and the infected leaves were frozen and used as a source of inocula in further experiments.

Antiviral bioassay

Two experiments were carried out :

Treatment with bacterial culture or CFS against WMV (In vitro)

In this experiment, equal volumes of the liquid bacterial culture or separately CFS and WMV were mixed together (2 ml of sap containing virus + 2 ml of liquid culture in a test tube for 10 min and then 100 µl of the mixture, inoculated directly into the cotyledonary first leaves of *Cucumis sativus* L. previously dusted with carborundum (600 mesh, Prolab). The inoculated leaves were then washed with distilled water according to Yarwood (1955). The number of plants with symptoms were counted after 28 days and the mean of 20 plants per each treatment was calculated. General control plants (healthy plants) were inoculated with buffer only. Viral control plants were inoculated with 2ml buffer + 2ml viral sap. The percentage of inhibition was calculated according to the equation: % of viral inhibition = number of symptomatic plants in viral control – number of symptomatic plants in treatment / number of symptomatic plants in viral control x 100. Plants were harvested and then number of leaves, shoot length and fresh weight were determined.

Post inoculation experiment (treatment after virus infection) (In vivo)

The cotyledonary leaves and first leaf of *Cucumis sativus* L. plants were inoculated with virus inoculum (100 µl / leaf) after dusting the leaves with carborundum, then the inoculated leaves were washed with distilled water. After 6 and 24 hr, the leaves were treated with the liquid bacterial culture or CFS 100 ul/leaf.

The developing symptoms were recorded after 28 days (20 plants for each treatment) and the percentages of inhibition were calculated as mentioned before. The viral control (cucumber plants inoculated with WMV only) and the general control (healthy plants treated with phosphate buffer) were done. Plants were harvested and then number of leaves, shoot length and fresh weight were determined.

Seed treatment experiment

The cucumber seeds were soaked in liquid bacterial culture and CFS for 12, 24 and 36 hr, then the seeds were germinated in pots. After germination, the cotyledonary leaves and first leaf were dusted by carborundum prior to inoculation with WMV inoculum (100 µl/leaf), after which the inoculated leaves were rinsed with distilled water. The symptoms were observed and recorded up to 28 days from inoculation. The percentages of inhibition were calculated. Healthy control seeds were done by soaking seeds in distilled water for each period. The viral control is cucumber seeds soaked in distilled water for 6, 12 and 24 hr, then germinated and mechanically inoculated with virus inoculum only. Plants were harvested and then number of leaves, shoot length and fresh weight were determined.

Antiviral activity of Serratia marcescens subsp. marcescens substances

Production of sufficient amount of antiviral compounds was carried out according to method described by Abd El-Shafi (2005). *S. marcescens* was cultured in 20 Erlenmeyer flasks , 250 ml capacity, each containing 100 ml of nutrient broth pH 7, then inoculated and incubated at 37 °C in a shaking incubator 150 rpm for 3 days. At the expiry of the incubation period, 9 organic solvents, namely: benzene, ethyl alcohol, ethyl acetate, methanol, pentanol, petrolium ether, chloroform, acetone and diethyl ether (v/v) were tested for their ability to extract antiviral compounds from broth. The extract was obtained by shaking the extracts in a separating funnel 3 times. The organic phase was collected and evaporated under reduced pressure by using a roatary evaporator. The evaporation was continued until less than 5ml remained. The extract was spotted on thin layer chromatography (TLC) silica gel plates using butanol: acetic acid: water (2:1:1, v: v: v) as the solvent developing system, then examined under UV light revealing and 6 spots (Fig. 1). The R_f values for the six spots and their antiviral activity were determined. Each spot was separately collected, combined and eluted in chloroform and filtered. The eluate was concentrated till dryness by vacuum rotary evaporation. The dry film of each spot dissolved in 1 ml methanol, then the eluate diluted by adding 5 ml of distilled water. The antiviral activity was carried out after mixing the eluted compounds with sap containing WMV (v: v) and the percent of viral inhibition was calculated after 28 days

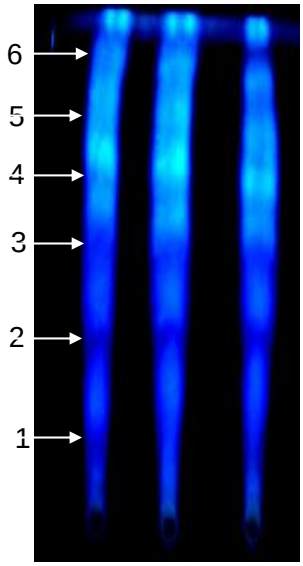

Fig. 1. TLC Profile of the extracted antiviral compounds from *Serratia marscecens* .

Antioxidant activity

Total antioxidants concentrations of the plant in response to viral and bacterial treatments were determined by the method of Gupta *et al*. (2004) with slight modifications by El-Sayed *et al*. (2012). Briefly, 1 gm of plant leaves was homogenized in a mortar containing 20 ml protein extraction buffer (50mM Tris-HCl pH 7.5, 20 mM EDTA) with 0.5 g sterile sea sand. After 10 min of homogenization in an ice bath, the mixtures were filtered, then the filtrate was centrifuged at 5000 rpm for 10 min. The supernatant was used as the source of antioxidants and intracellular compounds. For antioxidant concentration, 0.5ml of the plant extract was mixed with 100 µl of 20mM ferrous chloride and 100 µl of 30 % ammonium thiocyanate. The developed red complex was measured at 500nm.

Total soluble protein

The total soluble protein was determined by the method of Lowry *et al*. (1951), using bovine serum albumin as standard.

Polyphenol oxidase activity

Polyphenol oxidase activity was estimated as described by Sarvesh & Reddy (1988) with slight modifications. Briefly, 200 µl of enzyme extract was incubated with 800 µl of 0.2M O-catechol, dissolved in 2M carbonate- bicarbonate buffer (pH 8.0), for 30 min at 30 °C. After stopping of the reaction by addition of 500µl of 5% H_2SO_4, the developed color was measured at 420 nm. One unit of ploy phenol oxidase was expressed by the amount of enzyme releasing 1 µM of quinine /min/ ml, under standard assay conditions.

Glutathione oxidase activity

Glutathione oxidase activity was determined according to Bergmeyer *et al.* (1974) with minor modifications. The reaction contains 200 µl of enzyme preparation with 0.1 M glutathione in potassium phosphate buffer (pH 8.0), 0.2 mM guaiacol and 2U horseradish peroxidase. After 30 min incubation at 30°C, the reaction was stopped by freezing for 15 min, then the developed color was measured at 436nm. Glutathione oxidase activity was calculated from the following formula; One unit = A 436/min x 4/25.5 (Extinction co-efficient of tetra-guaiacol).

Phenolic compounds analysis

Extraction of free and bound cell wall phenols

Free phenols were extracted from cucumber plants according to the method of Campbell & Ellis (1992). The fresh samples were weighed, then frozen. These samples were powdered in liquid nitrogen using mortar and pestle. The powder was transformed into 5ml polystrene tubes with caps. Extraction was carried out with 2 vol. of 50% methanol for 1.5 hr. at 80±1°C in a water bath. The mixture was centrifuged for 5 min at 3000 rpm and the supernatant used for the Folin-Ciocalteau assays. From the remaining pellets, phenolic acids esterified to the cell wall by ester linkage were saponified for 24 hr at room temperature, according to the method of Funk & Brodelius (1990), 0.5 M NaOH in the ratio of 1 g original sample: 4ml. The mixtures were neutralized with one- quarter vol. of 2M HCL, centrifuged and the supernatants also used for the Folin-Ciocalteau assays according to method described by Julkunen-Tiitto (1985).

RT-PCR deteciton of WMV from Cucumis sativus plants

Total nucleic acids were extracted from virus infected leaves as described by Yoon & Ryu (2002), and used as templates for RT-PCR detection. RT was performed in a reaction mixture (20 µl) containing 2.5 mM $MgCl_2$, 0.5 mM of each dNTP, 1 µl containing 50 pM of reverse primer, 1× buffer, 1 U RNasin (Roche, USA), and 2.5 U SSTII reverse transcriptase (Invitrogen, USA) at 42°C for 60 min. PCR was performed using 5 µl of the synthesized cDNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.04 U DNA polymerase Mix Taq (Roche, USA), and 1 µl 50 pM of virus primers (Reverse 5´> AGATGTTAATCCATGTATACC<3´ and Forward 5´ > GCCAAAACAGCAACGCCA< 3´) (Lee & Ryu, 2009). PCR was performed in a thermal cycler (BioRad, USA). Denaturation was executed at 94°C for 3 min before starting PCR cycling. Each PCR cycle consisted of 30 s at 94°C, 30 s at 52°C, and 40 s at 68°C. A total of 35 cycles was performed, and cycling ended with final extension at 68°C for 10 min.

Statistical analysis

Data of all trials were statistically analyzed using the General Linear Model Program of SAS (1996). Differences among means were tested by Duncan's multiple range test (Duncan, 1955).

Results

Screening of antiviral activity of S. marcescens subsp. marcescens on WMV

S. marcescens subsp. *marcescens* showed antiviral activities against WMV on cucumber plants compared to mechanically infected viral control plants. After four weeks under WMV stress, the cucumber plants showed severe symptoms including malformation, stunt, mosaic, dark green blisters and leaf crinkling . Data in Table 1 reveal that the liquid bacterial cultures were the most potent *in vitro* treatment (100% viral inhibition), while cell free supernatant (CFS) gave 75% inhibition of viral infection. (Treatment at 24 hr with liquid. culture was the most effective post inoculation treatment at 90% viral inhibition). However, cell free supernatant gave 50% inhibition post viral inoculation by 6 hr. Viral infected control plants (mechanically inoculated with WMV) were characterized by mosaic patches, green blistering, leaf curl, malformation and reduction in growth. Moreover, bacterial treated plants not only reduced the symptoms but also improved the plant growth (Photos 1 & 2) and (Tables 2 &3). The liquid culture of *S. marcescens* and its CFS effectively inhibited the production of local lesions on the leaves of *Chenopodium amaranticolor* compared to untreated leaves, indicating that the bacterium or its secreted metabolites are capable of reducing WMV infection substantially (Photo 3). Also, results in Table 1 showed that soaking of cucumber seeds in liquid culture of *S. marcescens* for 24 hr also was effective in limiting subsequent viral infection (85% reduction) while soaking in CFS for 24 hr gave 70% viral inhibition.

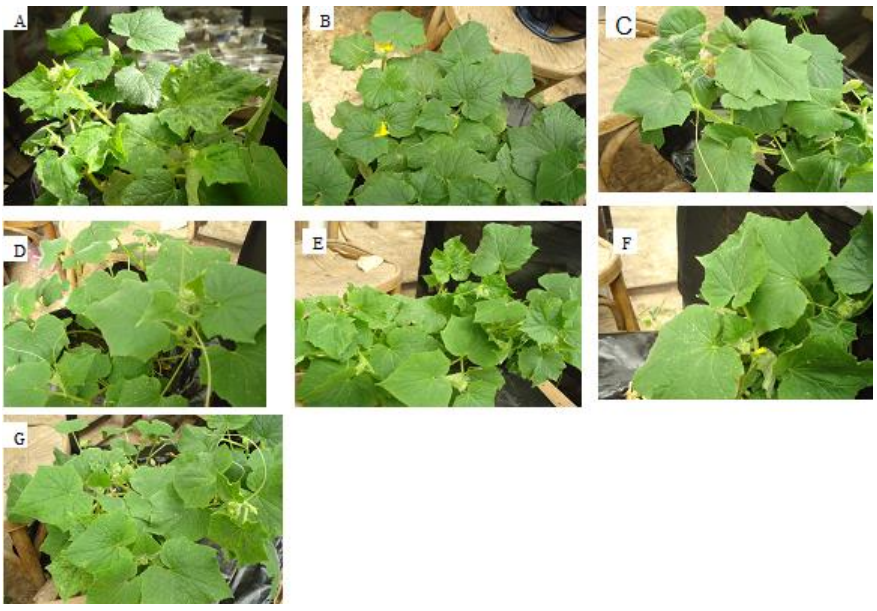

Photo 1. (A) . ***Viral*** **control; (B): Healthy control; (C): Bacterial control; (D):** ***in vitro*** **bacterial culture; (E):** ***in vitro*** **bacterial supernatant; (F): post 24 hr bacterial culture; (G): post 24 hr bacterial supernatant.**

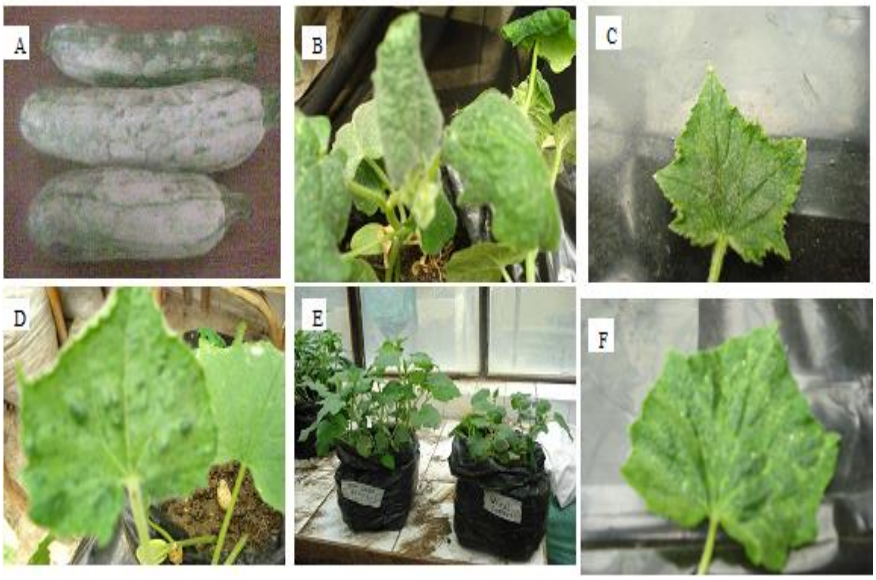

Photo 2. WMV symptoms (A) : Naturally infected squash fruits; (B): Leaf curl; (C): Malformation; (D) Dark green blisters; (E): Reduction in growth; (F): Mosaic.

Photo 3. Local lesions (LLs) on the leaves of *C. amaranticolor* by WMV against *S. marcescens* strain ss *marcescens.* A) The leaves were coated with a mixture of WMV and crude liquid culture of *S. marcescens*. There are no LL. B) the leaves were coated with a mixture of WMV and CFS of *S. marcescens*. One chlorotic LL turned necrotic. C) The leaves were coated with WMV only. There are 14 chlorotic and necrotic LL.

TABLE 1. Effect of liquid bacterial culture and cell free supernatant on the infectivity of watermelon mosaic virus (by %) on cucumber plants .

Treatments	*% of viral inhibition by liquid bacterial culture of *S. marcescens*	*% of viral inhibition by cell free supernatants of *S. marcescens*
Treatment with bacterial culture or CFS against WMV (*In vitro*) :	100	75
In vivo, post 6 hr	57	50
In vivo, post 24 hr	90	31
Seed soaking for12 hr	81	65
Seed soaking for 24 hr	85	70
Seed soaking for 36 hr	60	60

General control= twenty plants normal (without any treatments) all are healthy.
Viral control= cucumber seedling mechanically inoculated with WMV on cotyledonary and first stage leaves.
*=% of viral inhibition = number of symptomatic plants in viral control – number of symptomatic plants in treatment/ number of symptomatic plants in viral control x 100

TABLE 2. Effect of liquid bacterial culture and cell free supernatant of *S. marcescens* on some growth parameters of mechanically infected cucumber plants by WMV.(Each value is the mean for 20 plants ± SD).

Treatments	Healthy control	Viral control	Liquid bacterial culture	Cell free supernatant
In vitro				
No. of leaves	5.6 ± 0.97a	3.7 ± 0.82b	6.1 ± 0.15a	6.2 ± 0.79a
Shoot length (cm)	25.1 ± 3.51c a	15.1 ± 3.14d	30.3 ± 3.56b	33.5± 3.34a
Fresh weight (g)	8.94 ± 0.96a	2.96 ± 1.56b	9.28 ± 1.92a	10.22 ± 2.12a
Post experiment (6hr)				
No. of leaves	5.6 ± 0.97b	3.7 ± 0.82c	5.1 ± 0.74b c	6.5 ± 1.08a
Shoot length (cm)	25.1 ± 3.51b a	15.1 ± 3.14d	21.5 ± 2.76c	29.8± 3.49a
Fresh weight (g)	8.94 ± 0.96a	2.96 ± 1.56c	5.97 ± 0.69b	9.22 ± 1.67a
Post experiment (24hr)				
No. of leaves	5.6 ± 0.97ab	3.7 ± 0.82c	6.3 ± 1.34a	5.2 ± 1.03b
Shoot length (cm)	25.1 ± 3.51b	15.1 ± 3.14c	30.9 ± 3.34a	25.7 ± 3.59b
Fresh weight (g)	8.94 ± 0.96ab	2.96 ± 1.56c	9.73 ± 1.16a	8.06 ± 1.33b

Healthy control= twenty plants normal (without any treatments) all are healthy.
Viral control= cucumber seedling mechanically inoculated with WMV on cotyledonary leaves and first stage leaves.
a,b,c,d means in the same row with different superscript differ significantly ($p<0.05$).

TABLE 3. Effect of soaking cucumber seeds for different times in liquid culture of *S. marcescens* or cell free supernatant of those cultures on some growth parameters of treated plants inoculated with WMV at first stage leaf (Each value is the mean twenty reading ± SE) .

Treatments	Healthy control	Viral control	Liquid bacterial culture	Cell free supernatant
Soaking for 12 hr				
No. of leaves	6.2 ± 0.79a	5.2 ± 0.77b	6.8 ± 1.03a	7.2 ± 1.48a
Shoot length (cm)	39.0 ± 3.16a	28.7 ± 1.64b	43.5 ± 4.1a	43.0± 8.95a
Fresh weight (g)	9.77 ± 1.456b	7.31 ± 0.93c	9.41 ± 1.52b	12.04 ± 2.73a
Soaking for 24 hr				
No. of leaves	6.2 ± 0.79a	5.2 ± 0.77b	6.7 ± 0.67a	6.6 ± 1.17a
Shoot length (cm)	39.0 ± 3.16b	28.7 ± 1.64c	54.1 ± 4.36a	37.6± 4.53b
Fresh weight (g)	9.77 ± 1.456a	7.31 ± 0.93b	10.44 ± 1.48a	10.15 ± 1.06a
Soaking for 36 hr				
No. of leaves	6.2 ± 0.79b	5.2 ± 0.77c	6.0 ± 0.67bc	7.1 ± 1.45a
Shoot length (cm)	39.0 ± 3.16b	28.7 ± 1.64c	46.70 ± 3.09a	40.1 ± 7.23b
Fresh weight (g)	9.77 ± 1.456ab	7.31 ± 0.93c	8.58 ± 1.51bc	11.31 ± 3.73a

Healthy control= twenty plants were soaked in water (all are healthy).
Viral control= cucumber seedling mechanically inoculated with WMV on cotyledonary and the first true leaves.
a,b,c,d means in the same row with different superscript differ significantly ($p<0.05$).

The growth parameters of post inoculation and in vitro experiments

Growth parameters of plants subjected to post inoculation treatment (*in vivo*) and *in vitro* treatments (the bacterial culture or CFS was mixed with WMV) are represented in Table 2. Generally, the data reveal that foliar application of bacterial cultures and CFS significantly ($p< 0.05$) increases the growth of plants over virus infected controls. The virus had significantly ($p< 0.05$) detrimental effects on all growth parameters measured. An unexpected effect is the observation that *in vitro* treatment with bacterial cultures or filtrates also significantly ($p< 0.05$) led to increases of shoot length over the healthy control.

Effect of soaking cucumber seeds in liquid culture of S. marcescens and CFS

In this experiment, *S. marcescens* increased seed germination and seedling vigour where the treated plants germinated after 4 days, while untreated germinated after 6 days. The results on the growth parameters of plants in these experiments are shown in Table 3. In each case, the number of leaves, shoot length and fresh weight increased significantly ($p< 0.05$) more than the viral control. Moreover, soaking the seeds for 24 hr in liquid culture increased the measured growth parameters above those of the healthy control. These results demonstrate that *S. marcescens* in the rhizosphere promote plant growth.

Phenolic compounds and enzyme activity

The results in Table 4 show that virus infection decreased the total protein and polyphenol oxidase compared to healthy control, while increasing phenolics and glutsthione oxidase. The application of *S. marcescens in vitro* and *in vivo* increased the total protein, polyphenoloxidase and phenolic compound compared to viral and healthy control while reducing the measured activity of glutathione oxidase.

TABLE 4. The protein profile, enzyme activity and phenolic compounds of cucumber plants treated in *vitro* and *in vivo* with *S. marcesens.*

Treatments / Measurements	Viral control	Healthy control	**In vitro* exp.	*In vivo* post 24 hr.
Total protein (mg/ml)	0. 417	0.441	0.651	0.676
Glutathione oxidase (U/ml)	6.10	5.23	3.79	5.20
Polyphenol oxidase (POx)U/ml	6.17	6.75	7.23	7.74
Phenolic compounds cpd (ug/ml)	94.7	67.7	96.7	96

**In vitro:* Treatment with bacterial culture or CFS against WMV (*in vitro*)

Antiviral substances produced by S. marcescens

The results of extractability of the antiviral substances showed that benzene, ethyl acetate, petroleum ether, chloroform and pentanol, were capable of extracting antiviral compounds from the culture of *S. marcescens*, while ethyl alcohol, methanol, acetone and diethyl ether were not suitable in the extraction process. The R_f of the compound 1, 2, 3, 4, 5, and 6 were 0.27, 0.44, 0.59, 0.69, 0.81, and 0.93, respectively. All compounds inhibited WMV. Compound 1 was the most potent compound, as it gave 90% inhibition from equivalently diluted samples. Mixture of all compounds showed 95% inhibition (Table 5 and Fig. 1).

TABLE 5. Effect of purified bacterial compounds on the infectivity of watermelon mosaic virus (by %) on cucumber plants .

Treatments	The Rf	**% inhibition of viral infection by compounds of S. marcescens*
Compound 1	0.27	90
Compound 2	0.44	85
Compound 3	0.59	85
Compound 4	0.69	70
Compound 5	0.81	65
Compound 6	0.93	60
Mixture of all	-	95

General control= twenty plants normal (without any treatments) all are healthy.
Viral control= cucumber seedling mechanically inoculated with WMV on cotyledonary leaves and first stage leaves.
*=% of viral inhibition = number of symptomatic plants in viral control – number of symptomatic plants in treatment/ number of symptomatic plants in viral control x 100

RT-PCR quantification of WMV

From the data of RT-PCR the titre of WMV was reduced by about 62.9 and 39.7 % using *S. marcescens* co-inoculated *in vitro* or 24hr post virus inoculation, respectively compared to positive viral controls. This suggests the bacterial treatment has a deleterious effect on the viral structures as protein coat or on virus replication. Or does it mean the bacterium induces host defences that also lower virus replication capacity (Fig. 2).

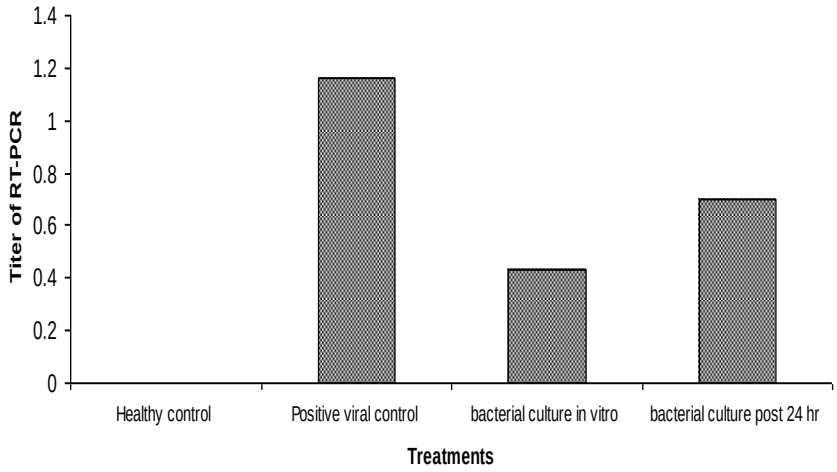

Fig. 2. RT-PCR titer for positive, nega tive and treated plants.

Discussion

Watermelon mosaic virus (WMV) causes serious economic losses in many cucurbits. Cucurbitaceous crops are grown commercially throughout the world (Yoon *et al*., 2008). Increasing use of chemical inputs can cause negative effects, such as development of strains of pathogen resistant to the chemical or changes that overcome host resistance genes (Gerhardson, 2002). Furthermore, the growing cost of pesticides and consumer demand for pesticide-free food has led to a search for substitutes for these products. There are also a number of fastidious diseases, *e.g.*, virus & viroid diseases, for which chemical solutions are few, ineffective or nonexistent (Gerhardson, 2002). Biological control is being considered as an alternative or a supplemental way to reduce the use of chemicals in agriculture (Postma *et al*., 2003). The present study shows that the crude culture of *S. marcescens*, as well as 6 extracellular products recovered from culture filtrates *(*CFS) are potent inhibitors of WMV infection. The *S. marcescens* was used to enhance cucumber growth and protection against WMV infection by foliar spraying and seed soaking; treatment with either liquid culture of *S. marcescens* and its CFS were in harmony with results described by Raupach *et al*. (1996), Helmy & Maklad (2003) and Fletcher *et al*. (2006).

WMV infection was inhibited up to 100% when plants were treated simultaneously with a mixture of crude liquid culture + viral sap *in vitro* for 10 min (foliar treatment). The liquid culture of *S. marcescens* and its CFS inhibited the production of local lesions on the leaves of *Chenopodium amaranticolor* effectively when compared to untreated leaves, indicating that *S. marcescens* is capable of preventing WMV infection. These results agree with those obtained by Thapa *et al.* (2009), where they found that culture filtrate of *S. marcescens* strain Gsm01 is a potent inhibitor to CMV-Y on a *Chenopodium amaranticolor* and on *N. tobacum* cv. Xanthi-nc plants. Treatment of *S. marcescens* crude culture or its CFS 24hr after WMV inoculation in cucumber plants educed symptoms formation up to 90% and 31%, respectively as compared to the viral control plants. This could be due to some form of induced systemic resistance.

Results here are in accordance with previous studies, where Maurhofer *et al*. (1994) successfully studied the resistance effect of *P. fluorescens* on tobacco against TNV. Also, the PGPR strains of *Serratia* spp. have been reported to mediate induced systemic resistance (ISR) in protection against CMV infection (Raupach *et al*., 1996; Ryu *et al*., 2004 and Thapa *et al*., 2009).

Seeds soaked with crude culture or CFS for 24 hr showed WMV infection reductions of 85 and 70%, respectively and also led to increases in several growth parameters of cucumber plants (number of leaves, shoot length and fresh weight). Increases were significant ($p< 0.05$) in comparison to virus infected control plants. These results agree with those of Shehata & El-Borollosy (2008) who showed that *S. marcescens* crude culture treatment inhibited ZYMV if present for 72 hr at germination or prevented infection for 72 hr and enhanced the growth of squash plants. Investigators studied the effect of PGPR on plant growth when used in the form of biofertilizer which is mainly attributed to: i) Fixation of atmospheric nitrogen, ii) Solubilization of minerals such as phosphorus, and iii) Synthesis of phytohormones (Murphy *et al*., 2003; Lucas Garcia *et al*., 2004 and Han *et al*., 2005).

The RT-PCR technique is highly sensitive and is considered a reliable method for quantifying virus presence in comparison with biological indexing. RT-PCR became widely used as a diagnostic method for detecting infecting plant viruses for several different groups, such as the geminiviruses, luteoviruses and potyviruses (Al-Saleh *et al*., 2010). Data shown in this study represent the inhibition of WMV infection based on number of asymptomatic plants and RT-PCR diagnosis. In the present study RT-PCR confirmed that not all of plants giving negative external symptoms were virus-free, as it gives reading in virus amplification. These results revealed that virus replication was inhibited with bacterial treatment, but still some virus particles were present. These results agree with previous study, *e.g.*Shehata & El-Borollosy (2008).

Using gel electrophoresis, a new protein band (22.4 KDa) was detected in treated cucumber plants and it may be a signal for induced systemic resistance in

plants (data not shown). This results agree with that obtained by Abdel-Shafi (2005) and Shehata & El-Borollosy (2008).

Extracts of virus infected plants had decreased total protein and polyphenoloxidase compared to healthy controls, but increased levels of phenolic compounds and glutathione oxidase. These results are similar to Zinati (2009) who showed total protein reduction and increases in phenolic compounds in Matvdaht infected with wheat streak mosaic virus. The loss of leaf soluble protein in viral infected leaves may be due to either degenerated chloroplasts or inhibition porotein synthesis (Bertamini *et al*., 2005). The usage of *S. marcescens in vitro* and *in vivo* increased the total protein, polyphenoloxidase and phenolic compound compared to viral and untreated healthy control plants, while reducing the glutathione oxidase. These results may indicate that plants respond to infection by synthesis of phenolic compounds to prevent proliferation and spreading of virus as part of an active defense response (Nicholson & Hammerschmidt, 1992). Also, bacterial treatments increase the phenolic compounds more than viral control emphasize the role of phenolic compounds in preventing movement and reducing symptoms on plants.

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التأثيرات الضد فيروسية للمزرعة السائلة و العالق بدون الخلايا) (CFS) و المنتجات الخارجية لبكتيريا *سيراشيا مارسينس* سلالة SS *مارسينس* ضد فيروس موزيك البطيخ الاحمر WMV.

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تم دراسة التأثيرات الضد فيروسية للمزرعة السائلة و CFS و المنتجات الخارجية لبكتيريا *سيراشيا مارسينس* سلالة SS *مارسينس* ضد فيروس موزيك البطيخ الأحمر WMV. اظهرت البيانات فى هذه الدراسة وجود فيروس موزيك البطيخ الأحمر بناء على الأعراض الخارجية لثمرة الكوسة المصابة طبيعيا واعراض نباتات الخيار المصابة ميكانيكيا و تحليلات RT-PCR. بعد اربع اسابيع من الأصابة بفيروس WMV اظهرت نباتات الخيار اعراض مثل التشوه، التقزم ,التبرقش , بقع خضراء غامقة و التفاف الأوراق. و علاوة على ذلك حدث انخفاض معنوى فى مقاييس النمو مثل انخفاض طول المجموع الخضرى, عدد الأوراق و الوزن الطازج. بينما نباتات الخيار المعاملة ببكتيريا تحفيز النمو(*سيراشيا مارسينس* سلالة SS *مارسينس*) اظهرت مقاومة ضد الفيروس واعلى نتيجة لتثبيط الفيروس كانت فى تجربة *in vitro* (حيث تم خلط البكتيريا مع الفيروس بكميات متساوية) حيث اعطت ١٠٠% تثبيط. وفى تجربة المعاملة بعد الحقن ثبطت البكتريا الفيروس ب ٩٠% عند فترة ٢٤ ساعة.

عند نقع بذور الخيار فى المزرعة السائلة و CFS للبكتريا زاد الأنبات و ثبط الفيروس بنسبة ٨٥ , ٧٠% على التوالى.

معاملة نباتات الخيار بالبكتريا أدى لزيادة مقاييس النمو زيادة معنوية فى كل التجارب مقارنة بالنباتات المصابة بالفيروس (الكنترول) .

تم استخلاص و تنقية ٦ مواد ضد فيروسية من البكتريا و اختبار الانشطة الضد فيروسية لهم وكانت اعلى نتيجة تثبيط عندما تم خلط هذه المواد مع بعضها حيث اعطت ٩٥% تثبيط.

واوضحت نتائج RT-PCR ان معاملة البكتريا لنباتات الخيار فى تجربتى in vitro, post 24 h. قللت تراكم الفيروس فى النباتات المعاملة.

ان استخدام البكتريا in vitro, in vivo عمل على زيادة البروتين الكلى فى النباتات وزيادة انزيم بولى فينول اوكسيديز وايضا المركبات الفينولية مقارنة بالنباتات المصابة بالفيروس فقط و النباتات السليمة بينما انخفض انزيم glutathione.