

## Induction of Resistance Against Zucchini Yellow Mosaic *Potyvirus* and Growth Enhancement of Squash Plants Using Some Plant Growth-promoting Rhizobacteria

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**Abstract:** During the investigation three isolates from the predominant rhizobacteria were isolated from squash plants rhizosphere, and identified morphologically and physiologically to be related to *Bacillus subtilis*, *Serratia marcescens* and *Pseudomonas fluorescens* species. The bacterial liquid crude cultures (48 h of age) and their Millipore® filtrates were tested for the ability to induce systemic resistance within squash plants (*Cucurbita pepo* var. Eskandarany) against zucchini yellow mosaic *potyvirus* (ZYMV) infection. Two types of treatment were carried out: (1) germination of squash seeds on cotton witted with crude culture or its filtrate, left to grow to the age of 4-5 leaves in pots, then inoculated with constant inoculums of ZYMV, (2) spraying of healthy squash plants (carrying 4-5 leaves) and challenged by mechanical ZYMV inoculation at time intervals. Data proved that best results were obtained by treatment of seeds germination with the *Serratia* crude culture for 72 h, as number of symptomless plants were 9 out of 30 plants inoculated, followed by *Pseudomonas* treated plants which gave 7 asymptomatic plants. The induced resistance was tested using indirect enzyme linked immunosorbant assay (I-ELISA) and immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) for the detection of ZYMV helper component proteinase gene (*Hc-pro*), which proved that the mentioned symptomless plant were virus-free or with a low concentration of virus infection. Plants giving virus-free results revealed the higher peroxidase enzyme activity (6 U/g). Using gel electrophoresis and in comparison with control plants, a new protein band was detected in the protected squash plants extracts (molecular weight of about 30 KDa), assuming to be a plant pathogen related protein. Increase in growth measures was observed for *Serratia* and *Pseudomonas* treated squash plants, as the higher plant fresh weights were 106.1 and 100.2 gm, respectively. Statistical lowest significant differences test (LSD) showed significant differences between *Serratia* results and those of *Bacillus* for biological data of virus infections and plant growth measures, while lower differences were found between *Serratia* and *Pseudomonas*.

**Key words:** *Cucurbita pepo*, Plant growth-promoting rhizobacteria, *Bacillus*, *Serratia*, *Pseudomonas*, Zucchini yellow mosaic *potyvirus*, I-ELISA, IC-RT-PCR, Pathogen related protein.

### INTRODUCTION

Virus infections cause great damage to economical crops, this loss is so clear especially in developing countries. Investigators were aiming to control such incurable pathogen using an alternative biological controlling strategy depending on a clean agriculture system (Murphy *et al* 2003 and Fletcher *et al* 2006).

The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria to increase plant growth and controlling different pathogens has shown considerable promise and found to be safe on human health compared with chemical fertilizers (Katan, 1993 and Han *et al* 2005 and Jing *et al* 2007).

Systemic resistance for virus infections can be induced in plants treated with certain bacteria or with bacterial products, and also by treatment with some chemicals which may be more risky when compared with bacteria (Bakker *et al* 2003 and Shoman *et al* 2003).

Many investigators study the effect plant growth-promoting rhizobacteria (PGPR) on controlling plant viruses infection, i.e., Bergstrom *et al* (1982) showed that resistance in cucumber against cucumber mosaic virus (CMV) could be induced by previous treating of plants with *Colletotrichum orbiculare* or *Pseudomonas*

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*syringae*. Maurhofer *et al* (1994) studied successfully the effect of *Pseudomonas fluorescens* on the resistance of tobacco against tobacco necrosis virus (TNV). De Meyer *et al* (1999) enhanced the resistance of tobacco plants against tobacco mosaic virus (TMV) using *Pseudomonas aeruginosa* and Ryu *et al* (2004) protected *Arabidopsis thaliana* plants against CMV infection using *Serratia marcescens*.

Studies indicated that some PGPR can act as an inducer of systemic resistance within treated plants, i.e., *Pseudomonas fluorescens*, *Serratia marcescens*, *Bacillus* sp, *Streptomyces gibsonii*, *Curtobacterium flaccumfaciens* and *Burkholderia gladioli* (Compant *et al* 2005).

The role of such induced systemic resistance described by the enhancement of the production of plant antioxidant protective enzyme, peroxidase (Shoman *et al* 2003), besides the activation of some plant defense genes producing pathogenic related proteins (PR-Ps), which are not well studied yet for its mode of action (Shehata and El-Borollosy, 2007).

Plant growth-promoting rhizobacteria enhance plant growth by direct and indirect means, but the specific mechanisms involved have not all been well-characterized (Glick, 1995). PGPR have been reported to directly enhance plant growth by a variety of mechanisms: fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus, and synthesis of phytohormones such as auxins (Glick, 1995; Lucas Garcia *et al* 2004 and Han *et al* 2005)

Therefore, the objective of this investigation is to study the effect of treating squash plants with some isolates of PGPR, on controlling ZYMV infection and enhancement of plants growth. Study the enhancement of peroxidase production as an antioxidant plant protecting enzyme, and the induction of PR-Ps plant genes for protein production.

## MATERIALS AND METHODS

### **Isolation of Virus and Bacteria:**

Zucchini yellow mosaic *potyvirus* was isolated from squash plants showing virus like symptoms (collected from the open fields of Mansoura, El-Dakahlia, Egypt). Isolation was performed depending on I-ELISA (according to Koenig, 1981) using specific polyclonal antibodies (Agdia Inc., USA), and *Chenopodium amaranticolor* & *C. quinoa* as local lesion hosts. To prove the presence of viral filamentous particles, electron microscopic examination of negatively stained leaf-dip preparation was carried out according to Pospieszny and Cajza (2004). On the other hand, three of the predominant rhizobacteria were isolated from the mentioned squash plants rhizosphere. Media used for isolation were nutrient agar (Thiery and Francon, 1997) and King's medium B (KMB) (King *et al*, 1954) depending on the agar plate dilution method.

### **Bacterial Cultures:**

Three isolates of the most predominant squash rhizosphere bacteria were selected and isolated as pure cultures. Bacterial colonies properties were determined, and cells were observed under microscope after proper staining (Gram and spore staining). Essential biochemical tests were carried out according to Collins and Lyne (1984). Finally bacteria were identified according to Bergey's Manual (Farmer, 1984; Sneath, 1984 and Holt *et al*, 1994).

Bacteria were grown separately in nutrient broth for 48 hr/30°C, and then liquid cultures were used directly for plants treatment. Bacteria were used either in the form of crude culture or as a filtrate (obtained using 0.45 µm Millipore® filtrates).

### **Squash Plants Treatment:**

Treatment of squash plants (*Cucurbita pepo* var. Eskandarany) (30 plants for each treatment) with either crude bacterial cultures (*Bacillus*, *Serratia* and *Pseudomonas*) (48 h age) or their filtrates was carried out as follows:

### **Germination:**

Squash plant seeds were disinfected using 4% sodium hypochlorite for 5 min, then were germinated on cotton witted with sterilized water inside Petri dishes for 5 days at 28°C±2. After that cotton was witted with either crude bacterial cultures or its filtrate for 48 and 72 h (controls were always germinated with water). Post treatments seedlings were washed with sterilized water and planted in sterilized 1:1 soil:peat moss, and challenged with constant inoculums of ZYMV by mechanical inoculation at the age of 5 leaves.

**Spraying:**

Healthy squash plants carrying 5 leaves were sprayed with bacterial cultures or their filtrates, then inoculated with ZYMV at different time intervals (5 and 10 days) post spraying.

Note that plants were always irrigated with 200 ml of either crude bacterial culture or its filtrate (diluted 1:1 with sterilized water) every 5 days (irrigation source always resembled treatment), while control plants were irrigated with water.

**Evaluation of Plants Induced Systemic Resistance and Growth:**

**Biologically:**

After 15 days from virus inoculation plants were categorized according to symptoms development to: plants showing severe, mild or asymptomatic plants (in comparison with control plants which was treated with water instead of bacterial treatment).

**I-ELISA:**

Plants showing different symptoms degrees (representing the best treatment) and controls were evaluated for virus level using I-ELISA test, and with the aid of ZYMV specific polyclonal antibodies (Agdia Inc., USA).

**IC-RT-PCR:**

Plants which gave negative or low I-ELISA values were tested for virus presence using IC-RT-PCR by detecting of ZYMV *Hc-pro*. Immunocapturing and cDNA synthesis was carried out as described by Minafera and Hadidi (1994). The following primers (from Invitrogen Corp., USA) were designed using Primer Premier software (PREMIER Biosoft International, USA) depending on the nucleotide sequence of ZYMV strain KR-PA (AY278998) (Kwon *et al.*, 2005): 5'ATGTCGTCGCAACCGGAAGTTCAGTTCTTC3' (Forward) and TTACCAACTCTG TAATGCTTCATCTCGC3' (Reverse). PCR procedure was carried out as performed by Ghosh *et al.* (2002) for *Hc-pro* gene isolation and amplification.

**Electrophoresis:**

Squash plants giving negative I-ELISA and PCR results in addition to control plants were analyzed for soluble proteins by polyacrylamide gel electrophoresis (PAGE). Leaf tissues were grind with cold phosphate-citrate buffer (30 mM Na<sub>2</sub>HPO<sub>4</sub>, 80 mM Citrate, 5mM Ascorbate, 14 mM Mercaptoethanol, pH 2.8), (1:1 w/v). The homogenate was filtered through cheesecloth and centrifuged for 30 min at 15000 rpm. The supernatant was dialyzed against Tris HCl buffer (50mM Tris, 1mM EDTA, and 3mM Mercaptoethanol, pH 8.0). The product was subjected to 15% polyacrylamide gel electrophoresis in the presence of 0.2% SDS according to Laemmli (1970). The bands molecular weights were determined using Gel-pro Analyzer software (Media Cybernetics, USA).

**Peroxidase Activity:**

Squash plants with different symptoms degrees and untreated plants were evaluated for peroxidase activity according to Kim & Yoo (1996). Leaves were extracted in 0.1 M phosphate buffer (pH 6.0) with the rate of 0.1 gm leaves per 1 ml buffer, and then centrifuged at 15000 rpm for 5 min at 4°C. Formation of tetraguaiacol was performed in 3 ml reaction mixture containing: 1ml of 0.1 M phosphate buffer (pH 6.0), 1 ml of 15 mM 2-methoxyphenol (guaiacol), 1 ml of 3 mM H<sub>2</sub>O<sub>2</sub>, and 15 µl of enzyme extract. Peroxidase activity was determined at 470 nm with a spectrophotometer (Shimadzu UV 1201). One unit of peroxidase activity (U) represents the amount of enzyme catalyzing the oxidation of 1 mol of guaiacol in 1 min.

Note that plants resistance studies were performed after symptoms appearance (about 15 days post viral inoculation).

**Plant Growth:**

Plants from different treatments were left to grow to 50 days of age for determination of growth measures (fresh or dry weights). Plants growth measures differences were statistically analyzed using LSD test with the aid of SPSS® software (SPSS Inc., USA).

## RESULTS AND DISCUSSIONS

**Isolation of Virus and Bacteria:**

Zucchini yellow mosaic *potyvirus* was isolated from naturally infected squash plants (showing mainly mosaic and malformation) (Figure 1, A). Samples which gave positive I-ELISA results with ZYMV specific

antiserum produced chlorotic local lesions on *Ch. Amaranticolor* (Figure 1, B) and *C. quinoa*. Three cycles of local lesion isolation were performed and the last produced lesions were inoculated on *Nicotiana tabacum* cv. White Burley plants. Electron microscope micrographs of infected tobacco leaf-dip preparations revealed flexuous filamentous virus particles (Figure 1, C).



**Fig. 1:** Squash leaf used for ZYMV isolation showing mild mosaic, and blisters (A), chlorotic local lesions produced on *Ch. Amaranticolor* leaf 15 days post inoculation with ZYMV infected squash sap (B). Electron micrograph for infected squash leaf-dip preparation showing filamentous virus particle (C) (60000 X).

Concerning bacterial isolation, three of the predominant bacteria were isolated from squash rhizosphere and identified to the degree of genus according to Bergey's Manual depending on colonies, morphological and physiological characteristics (Table 1).

**Table 1:** Differential characteristics of the isolated rhizobacteria

Characteristics & Biochemical tests	Isolate number		
	1	2	3
Morphology	Long rods in chains	Short rods	Short rods
Spores	-	-	-
Gram staining	+	-	-
Motility	Motile	Motile by polar flagellum	Motile
Pigments	-	Fluorescent bluish diffusible pigment	Red undifusible pigment
Oxygen requirements	Aerobic, facultative	Aerobic	Aerobic & facultatively anaerobic
Growth temp. (°C):			
4	-	+	-
30	+	+	+
37	+	+	+
41	+	+	-
50	-	ND	-
Growth pH:			
4	+	-	±
5.7	+	+	-
6.8	+	+	±
9.0	+	-	-
Growth in NaCl (%):			
5	+	+	-
7	+	-	-
10	-	-	-
Gelatin hydrolysis	+	-	+
Starch hydrolysis	+	-	-
Glucose	+	+	+
Maltose	+	-	+
Manitol	+	-	+
Sucrose	+	±	+
Citrate	+	+	+
Voges Proskauers (VP)	+	ND	+
Indol	-	-	-
Nitrate reduction	+	+	-

+: Good growth, ±: Moderate growth, -: No growth and ND: Not detected.

Results clearly showed that isolate no. 1 tends to be similar in characters to genus *Bacillus* and nearly towards *B. subtilis* (Sneath, 1984). While isolate no. 2 considered to be *Pseudomonas fluorescens* (Holt *et al*, 1994; Banjo *et al*, 2006) and isolate no. 3 was assumed to be *Serratia marcescens* (Farmer, 1984; Banjo *et al*, 2006).

**Evaluation of Plants Induced Systemic Resistance and Growth:****Biologically:**

Fifteen days post virus inoculation plants were studied for symptoms development and degrees. Results are demonstrated in Table (2) proving that *Serratia* crude culture treatment was the best as 9 plants out of 30 were asymptomatic (germination for 72 hr treatment), followed by *Pseudomonas* which gave 7 asymptomatic plants for the same treatment. LSD test values showed significant differences between *Serratia* data compared with other bacteria for all treatments, and with germination compared with spraying treatments.

**Table 2:** Effect of rhizobacteria treatments on squash plants ZYMV symptoms

Symptoms* & treatment	Germination						Spraying						
	48			72			5			10			
Bacteria	S	M	N	S	M	N	S	M	N	S	M	N	
<i>B. subtilis</i>	C	22	5	3	20	5	5	23	7	0	19	10	1
	F	23	5	2	21	6	3	25	5	0	24	4	2
<i>P. fluorescens</i>	C	19	6	5	17	6	7	19	8	3	18	9	3
	F	20	6	4	19	6	5	22	7	1	20	7	3
<i>S. marcescens</i>	C	13	9	8	11	10	9	19	7	4	17	8	5
	F	17	7	6	15	8	7	20	8	2	19	8	3
Control**	S: 26 M: 3 N: 1						27 2 1			25 4 1			

LSD at the level of all treatments results

Between	<i>Serratia</i> and <i>Bacillus</i>	<i>Serratia</i> and <i>Pseudomonas</i>
5%	15.7	9.3
1%	22.08	13.0

Note: Treatments were germination with crude culture (C) or its filtrate (F) for 48 & 72 hr and

Spraying with (C) or (F) followed by inoculation with ZYMV at time intervals: 5 & 10 days. Thirty plants were used for each treatment.

\*: (S) Severe, (M) Mild and (N) NO symptoms.

\*\* : Controls were plants treated with water.

**I-ELISA:**

Plants showing mild or no symptoms from *Serratia* and *Pseudomonas* crude culture germination treatment (for 72 hr) were selected for I-ELISA test (Table 3). Controls were squash plants germinated with water. Five plants out of 9 and 3 out of 7 gave negative I-ELISA values, for *Serratia* and *Pseudomonas*, respectively.

**Table 3:** I-ELISA values for mild and symptomless squash plants treated by germination with crude bacterial cultures for 72 hr

Symptoms* & Bacteria	I-ELISA values at 405 nm						
<i>P. fluorescens</i>	mild	0.919 +	0.801 +	0.794 +	0.708 +	0.858 +	0.811 +
	no	0.651 +	0.598 +	0.495 +	0.456 +	0.300 -	0.241 -
<i>S. marcescens</i>	mild	0.500 +	0.710 +	0.665 +	0.681 +	0.598 +	0.777 +
	no	0.541 +	0.491 +	0.581 +	0.448 +	0.320 -	0.318 -
<i>P. fluorescens</i>	no	0.251 -					
<i>S. marcescens</i>	mild	0.841 +	0.691 +	0.719 +	0.781 +		
	no	0.351 -	0.400 -	0.291 -			
Controls**	severe	0.941 +	1.056 +	0.900 +			
	mild	0.815 +	0.791 +	0.921 +			
	no	0.568 +					
	healthy	0.195 -	0.200 -	0.184 -			

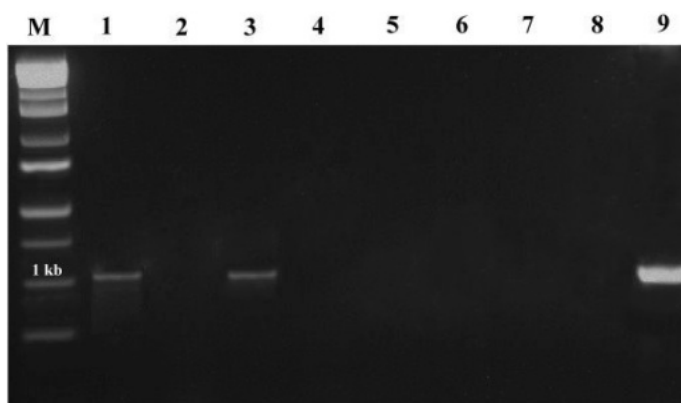
Each I-ELISA value was the average of three numbers. +: positive and -: negative.

**IC-RT-PCR:**

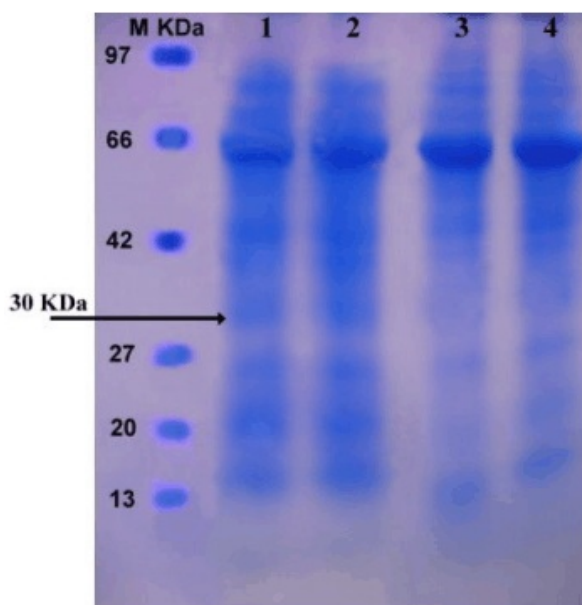
Plants which gave negative I-ELISA values were subjected to IC-RT-PCR in addition to control plants which gave severe symptoms. Three plants out of 5 and from *Serratia* treated squash plants were found to be virus-free, while two plants gave the bands of *Hc-pro* for ZYMV (1.3 kbp) but with a lower density compared with control plants. All *Pseudomonas* treated plants giving negative I-ELISA values were confirmed to be virus-free as no bands were observed within gels.

**Electrophoresis:**

This was carried out on squash plants giving negative I-ELISA and PCR results, compared with untreated plants which gave severe symptoms and healthy plants. Results proved the presence of a pathogen related protein (bands of about 30 KDa) which was not observed through control lanes (Figure 3).



**Fig. 2:** IC-RT-PCR for detection of ZYMV *Hc-pro* in treated and control squash plants. Lane 1-5 & 6-8 plants treated with germination with *Serratia* and *Pseudomonas* crude culture for 72 hr, respectively. Lane 9: control plants with severe symptoms. M: Lambda DNA/*EcoRI*+*HindIII* marker (Promega, USA).



**Fig. 3:** PAGE for *Serratia* and *Pseudomonas* treated squash plants giving negative results with I-ELISA and PCR, Lanes 1 & 2, respectively, control healthy plant (Lane 3) and infected plant (Lane 4). M: marker protein.

**Peroxidase Activity:**

The peroxidase activity test was performed on squash plants treated by germination (for 72 hr) and irrigation with *Serratia* and *Pseudomonas* crude cultures, in addition to untreated severely infected and healthy plants. Results proved that plants giving negative I-ELISA and PCR results (*Serratia* treated plants) have the higher enzyme activity: 6 U/ gm, while *Pseudomonas* treated plants gave 4 U/ gm. Infected untreated plants gave value of 2 U/ gm, while no clear enzyme activity was observed with healthy plants.

**Plant Growth:**

Five plants giving different symptoms degrees from *Serratia* and *Pseudomonas* germination (for 72 hr with crude culture) and irrigation treatment were left to grow to 50 days of age. Squash plants were then tested for fresh and dry weights compared with infected and healthy controls. Data in Table (4) revealed that *Serratia* treatment gave the highest growth measures values.

**Table 4:** Effect of bacterial treatments on squash plants fresh and dry weights

Plant no*		<i>Serratia</i>			<i>Pseudomonas</i>			Controls	
		Severe	Mild	No	Severe	Mild	No	Infected	Healthy
1	F	75.1	80.8	102.5	58.1	73.4	98.0	40.9	70.1
	D	10.0	10.5	15.0	7.6	9.5	12.1	5.2	9.0
2	F	59.5	87.3	100.9	55.5	80.1	91.4	60.7	80.3
	D	8.0	11.5	14.5	7.1	10.2	12.1	8.0	10.1
3	F	73.4	79.2	96.8	63.1	75.1	100.2	45.3	73.2
	D	9.6	10.0	13.0	8.3	10.0	14.1	5.7	9.5
4	F	70.2	82.0	106.1	60.8	81.0	85.0	50.8	69.6
	D	9.3	11.2	15.2	8.0	10.6	11.2	6.5	9.0
5	F	65.0	83.7	90.0	61.2	77.6	90.1	59.1	81.0
	D	8.7	10.9	12.5	8.2	9.7	11.8	7.8	10.2

LSD between *Serratia* and *Pseudomonas*: 5%=10.5, 1%=14.9.

\*: Squash plants of 50 days age. F= fresh and D= dry weights in gm.

### Discussion:

Pathogenic microorganisms affecting plant are a major and chronic threat to food production and ecosystem stability worldwide. Producers became more dependent on agrochemicals as a relatively reliable method of crop protection and fertilization (Compant *et al*, 2005). However, increasing use of chemical inputs causes several negative effects, i.e., development of pathogen resistance strains (Gerhardson 2002). Furthermore, the growing cost of pesticides, particularly in less-affluent regions of the world, 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, i.e., virus and viroid diseases, for which chemical solutions are few, ineffective, or nonexistent (Gerhardson 2002). Biological control and fertilization is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Postma *et al*, 2003).

Three of PGPR were isolated from squash rhizosphere and assumed to be *B. subtilis*, *P. fluorescens* and *S. marcescens* depending on some physiological tests and their colony and morphological characteristics. The obtained results were compared with what found by Farmer (1984), Sneath (1984), Holt *et al* (1994) & Banjo *et al* (2006) and found to be in harmony.

The bacteria were used to enhance squash growth and protection against ZYMV infection by two kinds of treatments, i.e., seeds germination and spraying, these ways of treatment with either crude bacterial culture or its filtrate were in harmony with what was described by Raupach *et al* (1996), Shoman *et al* (2003) and Fletcher *et al* (2006).

Protection against ZYMV was evaluated by studying number of asymptomatic plants and the confirmation of virus absence by I-ELISA and RT-PCR, *Serratia* and *Pseudomonas* gave promising results in decreasing symptoms degrees and production of acceptable number of virus free-plants. Raupach *et al* (1996) and Murphy *et al* (2003) found that tomato plants treated with PGPR (*P. fluorescens* and *S. marcescens*) did not develop initial symptoms 14 days after CMV inoculation and remained asymptomatic throughout the experimental period. They proved that no virus antigen could be detected by ELISA in any asymptomatic treated plant.

In the present study PCR confirmed that not all of plants giving I-ELISA negative values were virus-free, as some of them produced weak *Hc-pro* band. This result revealed that virus replication was inhibited with bacterial, but still some virus particles present.

The induction of peroxidase activity in plants occurs in response to numerous biotic and abiotic stimuli, including exposure to pathogens or elicitor preparations, chemical oxidizing agents, red light, and mechanical stimuli (Casal *et al*, 1994; Donald and Cipollini, 1998). Peroxidase is an important enzyme in the reinforcement of plant cell walls, and helps in protein extension to generate a firmer matrix material to be a part of the activated defence response (Jabs *et al*, 1996; Shoman *et al*, 2003). Peroxidase enzyme activity was studied in plants giving virus-free results, higher values was obtained with *Serratia* followed by *Pseudomonas* treatments, 7-5 U/gm, respectively, the results were in harmony with what found by Shoman *et al* (2003).

Investigators studied the effect of PGPR on plant growth when used in the form of biofertilizer which is mainly performed by indirect means, i.e., fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, and synthesis of phytohormones (Murphy *et al*, 2003; Lucas Garcia *et al* 2004 and Han *et al* 2005). Results of squash plants fresh and dry weights were much higher in treated plants compared with controls, *Serratia* and *Pseudomonas* were so much closer in their results, with fresh weights higher values of 106.1 and 100.2 gm, respectively.

Statistical analysis of biological experiments showed lower level of significant differences between *Serratia* and *Pseudomonas*, while higher differences were found between *Serratia* and *Bacillus*.

The obtained results predominated the usage of either *Serratia* or *Pseudomonas* for plant growth promotion and as systemic resistance inducers against pathogens. *Serratia* may not be preferred as some investigators reported its pathogenicity to human causing inflammation of the urinary tract, but especially at hospitals and with patients with lower degree of immunity (Civen *et al*, 2006).

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