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Influence of plant growth promoting rhizobacteria on fruit yield, pomological characteristics and chemical contents in cucumber mosaic virus-infected tomato plants

NARJES H. DASHTI, MAGDY S. MONTASSER, NEDAA Y. A. ALI AND VINEETHA M. CHERIAN

Department of Biological Sciences, College of Science
Kuwait University, P.O. Box 5969, Safat 13060, Kuwait
Corresponding Author: E-mail: magdy.montasser@ku.edu

ABSTRACT

The influence of two plant growth promoting rhizobacteria (PGPR) strains, *Pseudomonas aeruginosa* and *Stenotrophomonas rhizophilia*, on fruit yields, pomological traits and chemical contents on cucumber mosaic virus (CMV) infected-tomato (*Solanum lycopersicum*) fruits were studied. Two CMV strains were used to infect tomato plants. CMV-KU1 is a locally isolated CMV associated with a benign viral satellite RNA, while CMV-16 is a satellite-free virus that causes severe stunting, manifested by vegetative and fruit yield loss in tomato. The study was conducted in parallel on two different cultivar varieties of tomato, namely Supermarmande and UC82B and results obtained for each were compared. The results indicated that the presence of the PGPR almost doubled the average yield per plant, even of those plants infected by the CMV viruses, compared to the healthy control treatments. There was also a significant improvement in the pomological qualities of the PGPR-treated tomato fruits compared to the corresponding healthy and virus infected treatments, especially, in the average of weight, hight and volume of fruits. The chemical analysis of tomato fruits revealed that the presence of the PGPRs increased the total protein, lycopene, alkalinity and phenol content of the tomato fruits compared to the healthy controls. However, PGPRs had no influence on reduced sugars, total soluble solids or the titerable acid content, but reduced the amount of ascorbic acid in tomato fruits of infected plants compared to healthy controls.

Keywords: PGPR; tomato; fruit quality; CMV.

INTRODUCTION

Tomato (*Solanum lycopersicum L.*), is rated by the Food and Agriculture Organization (FAO) as the second most cultivated vegetable in the world with an annual production of nearly 108 tons of fresh tomato in 3.7 $\times 10^6$ hectares worldwide (Kacjan Marsic *et al.*, 2011; Ordookhani *et al.*, 2010; Olaniyi *et al.*,

2010). Many reports have indicated that viruses have become an increased threat to the growth and cultivation of tomatoes resulting in severe and recurring yield losses all over the world especially in Kuwait (Dashti *et al*., 2012; 2007; Montasser *et al*., 2006 a).

Cucumber mosaic virus (CMV), belonging to the genus *Cucumovirus* of the *Bromoviridae* family, considered to be one of the most economically damaging virus among the field grown vegetables worldwide (Montasser *et al*., 2006a) causes severe yield losses, yellowing, ring spots, deformed fruit and poor fruit set of tomatoes. In Kuwait, a CMV outbreak resulted in high losses in tomato crops over the past years (Montasser *et al*., 2006 a). CMV-16 is a satellite RNA-free CMV strain, which causes severe stunting, chlorosis and malformation of fruits in tomato plants (Sayama *et al*., 1993). CMV-KU1 is a mild strain associated with a benign satellite RNA (345bp long) that induces mosaic symptoms on squash leaves (Montasser *et al*., 2006a).

Plant growth promoting rhizobacteria are beneficial microorganisms that inhabit the rhizosphere and promote plant growth (Sahran & Nehra, 2011; Dashti *et al*., 2012). Many PGPRs protect plants by at least one of the following mechanisms: suppression of plant disease by induction of systemic resistance or antibiotic production (Bioprotectants), improved nutrition acquisition (Biofertilizers) and production of phytohormones (Bio-stimulants) (Siddiqui & Akhtar, 2007; Sahran & Nehra, 2011).

This present research aims to: a) compare the influence of a mixture of two PGPR strains namely *Pseudomonas aeruginosa* and *Stenotrophomonas rhizophilia* in enhancing yield and pomological characteristics of tomato fruits in two different cultivars of tomato namely Supermarmande and UC82B; b) determine the influence of the PGPR and the viral strains on the chemical fruit quality of both test tomato cultivars.

MATERIALS AND METHODS

PGPR source and inoculums preparations

Two strains of PGPRs were used in this study, one from each of the bacterial species of Pseudomonas (*P. aeruginosa)* and Stenotrophomonas (*S. rhizophilia*). The inoculum mixture of the two strains was prepared by culturing each of the two PGPR strains in nutrient broth and incubating at 20-25C with constant shaking at 125 rpm. When the cultures reached log phase, each of the strains was adjusted with distilled water at A_{420} giving a cell density of 10^8 CFU /ml. Equal volumes (1:1) of the two strains were mixed and allowed to stand approximately for half an hour at room temperature without shaking (Dashti *et al*., 2012).

Virus source, maintenance and inoculums preparations

The CMV strain associated with a benign viral satellite RNA (CMV-KU1) was isolated in Kuwait (Montasser 2012 United States patent # US 8,138,390 B2). CMV-16, subgroup II, is a Japanese isolate from tomato (kindly provided by H. Sayama, Kikko Foods Corporation) (Sayama *et al*., 1993). The viral isolates were invigorated by mechanical passage into fresh squash (*Cucurbita pepo* L.) and tomato (*Solanum lycopersicon* L.) plants. The infected leaves were ground in neutral 0.01M potassium phosphate buffer with a mortar and pestle and the crude sap was used to inoculate the tomato test plants.

Greenhouse experiments

Seeds of tomato cultivars 'Supermamande' and 'UC82B' were surface sterilized in sodium hypochlorite (2% solution containing 4ml L^{-1} Tween 20) and then rinsed several times with distilled water before planting them by hand into pots containing sterilized soil-less growth media (Peat moss with Perlite in a ratio of 3:1). Three independent experiments were conducted. Plants were divided into the following treatments: (A) PGPR treated plants subjected to (1) KU1, (2) 16, (3) KU1 + 16, and (4) non-treated; (B) Non-PGPR treated plants subjected to (1) KU1, (2) 16, (3) KU1 + 16, and (4) non-treated. Treatments were arranged in a randomized complete block design with 20 plants for each treatment. PGPRs were applied to the plants at the dicotyledonary stage. The PGPRs were applied under sterile conditions to the base of the plants close to the roots to ensure better colonization. The virus was applied onto the plants by mechanical sap transmission (Montasser *et al*., 2006a). Plants were maintained under greenhouse conditions at a temperature of 25°C with watering carried out every alternate days using a sterile Hoagland solution.

Evaluation of fruit yield and pomological characteristics of tomato fruits

Fruits collected from each plant of different treatments were counted to determine the average fruit number per plant. The fruit yield was determined by weighing all the fruits obtained per plant for each treatment on a weigh balance with 0.01 g sensitivity (Dashti *et al*., 2007). Mean fruit weight, height and diameter were determined for 10 fruits randomly selected from each treatment. Fruit volume was determined by calculating the over flowing water (Karakurt *et al*., 2011). Specific gravity of each fruit was determined as $d = m/V$ where'd' is the specific gravity, 'm' is the average weight of the fruit in each treatment and 'V' is the average volume of the fruit in each treatment (Karakurt *et al*., 2011).

Biochemical analysis of the tomato fruits

Chemical analyses were done on the same fruits used for pomological analysis. The pH of the fruits was determined using a pH meter. The total soluble solids in tomato was determined as per the procedure described by (Karakurt *et al.*, 2011). The total protein content of the fruits was determined by the Bradford method (Montasser *et al.*, 2012) using Bovine serum albumin as the protein standard. Titrable acidity was determined by acid-base titration using 0.1N NaOH as a measurement of the amount of citric acid present in the juice with drops of phenolphthalein added as end point indicator (Karakurt *et al.*, 2011) and ascorbic acid by redox titration with 0.005 mol L^{-1} iodine solution with starch solution as end point indicator. Phenol estimation was done following the method described by Kacjan Marsic *et al.* (2011) using Folin-Ciocalteu and 5.3 % sodium carbonate solution with caffeic acid as the standard. The absorbance was determined at 725 nm. Reducing sugars were estimated by the phenol-sulfuric acid method (Montasser *et al.*, 2012). The sugar standards were prepared by mixing equal portions of glucose, galactose and arabinose in saturated benzoic acid solution. The lycopene content in the tomato fruits was estimated by the procedure described by Ordookhani *et al.* (2010). Lycopene was extracted from fresh tissues with extraction solution consisting of hexane, butylated hydroxy-toluene in acetone and ethanol in the ratio 2:1:1. The absorbance of hexane layer on the top was measured at 503 nm and the total amount of lycopene was determined using the following formula:

Lycopene ($mgkg^{-1}$) = (x/y) x A503 x 3.12

(x: amount of hexane used for extraction (ml), y: the weight of the fruit tissue (g) and A503: the absorbance at 503 nm).

Electron microscopy

Both scanning and transmission electron microscopy were used to detect the presence of PGPR and virus particles.

Thin sectioning

Tissue fragments excised from infected leaves and roots were fixed in a drop of 3% gluteraldehyde in 0.01 M potassium phosphate buffer pH 7.0 and were kept in the fixative for three days at room temperature under slight vacuum, followed by four washes with 0.01 M potassium phosphate buffer, pH 7.0, for 15 min each. The samples were kept in pure Spurs resin overnight then embedded in labeled capsules. After decapsulation, blocks were ready for sectioning. Thin sections were double stained with uranylacetate and lead citrate before examining in the electron microscope (Montasser *et al.*, 2006 a).

Scanning electron microscopy (SEM)

Root cells and pure cultures of the PGPR were fixed with 2.5 % glutaraldehyde in sodium cacodylate buffer for 24 h. The materials were washed thrice with sodium cacodylate buffer, post fixed with 1% osmium tetraoxide for 1 h and finally washed thrice with sodium cacodylate buffer. The materials were dried in ascending concentration series of acetone, starting with 30 % and ending with 100 % acetone. The dry materials were coated and examined (Dashti *et al.*, 2007).

Detection of CMV viruses from treated tissues

Enzyme-linked immunosorbent assay (ELISA) for CMV detection

ELISA was performed as described by Montasser *et al.* (2006 a). Each plant was sampled by collection of three terminal leaflets from three young non inoculated leaves. 0.5g of plant tissue was homogenized with 10x (w/v) coating buffer (15mM sodium carbonate, 35mM sodium bicarbonate, pH 9.6, containing 2% polyvinyl pyrolidone: CB-PVP) in a mortar and pistol for sap extraction. Extracted crude sap was filtered through cheesecloth and centrifuged at 6,000 g for 2 min. The clarified extract was pipetted into microtiter wells and incubated at 4 ^{o}C over night, or at 37 ^{o}C for 3 h. The wells were washed thrice for 3 min each with phosphate-buffered saline (PBS) containing 0.5% Tween-20 (PBS-T), and blocked by incubation in 1% bovine serum albumin (BSA) in PBS, for 30-60 min. CMV-specific polyclonal antibody was incubated at 37 ^{o}C for 60 min, washed thrice with PBS-T, followed by addition of goat anti-mouse alkaline phosphatase conjugate diluted in PBS buffer (1:1000) and incubated at 37 ^{o}C for 3-4 h. After 3 washes with PBS-T, p-nitrophenyl phosphate was added in the substrate buffer (pH 9.8). The absorbance was measured at 405nm, 15-60 min after the addition of the substrate. Values that exceeded twice that of the untreated/healthy samples and/or the buffer controls were considered positive.

Nucleic acid analysis of CMV

Total nucleic acids (TNA) were extracted by the method described by Montasser *et al.*, 2006a. 0.25 g of infected tissue was powdered in liquid nitrogen using a sterile pre-cooled mortar and pestle and extracted using 1x extraction buffer (0.1 M glycine, 0.01 M EDTA, 0.1 M NaCl, pH 9.0 with 10% SDS and 10% n-Lauryl Sarcosine), equilibrated water saturated phenol (containing 0.1% 8-hydroxyquinoline) and chloroform. The nucleic acids present in the upper aqueous layer were precipitated by addition of 3 volumes of 95% ethanol and kept at ^{o}C for 30 min followed by centrifugation for 10 min. Pellets were washed twice with 70% ethanol, dried under vacuum, re-suspended in nuclease free water

and then examined by ethidium bromide stained 6% gel electrophoresis (39:1 acrylamide:bisacrylamide) in TRIS-borate (TBE) buffer, at 300 V for 1.5 hr.

Statistical analysis

Analysis of variance (ANOVA) at $P = 0.05$ was performed on all the data using the SPSS (Statistical Package for Social sciences) -PASW statistics 18 software and the means were separated with Duncan's Multiple Range Test (DMRT) using PASW statics 18 and the Michigan University Statistical Package (MSTAT-C, version 2.1, Michigan) software.

RESULTS

Evaluation of fruit yield and pomological characteristics

There were significant differences in fruit yield ($P < 0.05$) among the treatments and between the two cultivars. The average fruit yield of the cultivar UC82B was comparatively higher than that of Supermarmande variety. The presence of PGPRs significantly increased the fruit yield of the tomato plants compared to the healthy controls and the plants infected with the CMV strains (Table 1) in both cultivars. The lowest yields were obtained from plants infected with CMV-16 alone in both tomato varieties (Table 1). CMV-KU1 also reduced the average yield of the infected plants but not as severely as CMV-16. The fruit number per plant was not directly dependent on the presence of any of the PGPR or the CMV strains (Table 1). However, it inversely varied to a certain extent to the individual fruit weight. The larger the weight of the fruits, lesser the fruit number per plant (Table 1). This trend was observed in both the cultivars. The largest fruit weight was obtained for plants treated with PGPRs alone, followed closely by the healthy controls. The other pomological parameters such as fruit diameter, fruit height, fruit volume and specific gravity were found to be proportional with individual fruit weight of the tomatoes in both cultivars. Plants treated only with the PGPR mixture showed the highest values for each of these parameters while non-PGPR plants infected only with CMV-16 showed the lowest values (Table 1).

Table 1. PGPR effects on fruit yield, number and pomological traits of tomato cultivars

Treatments [v]	Fruit yield/plant (g)	Fruit number/ plant	Fruit weight (g)	Fruit diameter (cm)	Fruit height (cm)	Fruit volume (ml)	Specific gravity of fruit (gml^{-1})
Tomato cultivar UC82B							
H	161.3 (±4.8)[w] e [x]	5 (± 0.5) f	35.2 (± 1.1) b	5.2 (± 0.1) b	4.8 (± 0.4) b	54.5 (± 4.1) b	0.64 b
PGPR	285.2 (± 5.5) a	8 (± 0.7) e	40.7 (± 1.4) a	6.1 (± 0.4) a	5.6 (± 0.3) a	56.7(± 2.3) a	0.71 a
KU1	129.3 (±3.8) f	10 (± 0.4) c	14.9 (± 2.5) e	2.3(± 0.8) g	1.5 (± 0.2) f	27.4 (± 1.3) g	0.54 c
PGPR + KU1	259.6 (± 4.1) b	11(± 0.2) b	28.1(± 3.9) c	4.5 (± 0.1) c	3.9 (± 0.7) c	44.6 (± 1.1) c	0.63 b
16	96.1 (± 5.6) h	10 (± 0.5) c	11.6 (± 3.2) f	1.5 (± 0.3) h	1.2 (± 0.06) g	23.2 (± 3.8) h	0.5 d
PGPR + 16	253.7 (± 4.4) c	12 (± 0.5) a	21.9 (± 1.8) d	4.2 (± 0.2) d	3.7 (± 0.1) c	42.1 (± 2.4) d	0.52 c
KU1/16	117.0 (± 4.2) g	9 (± 0.2) d	17.3 (± 1.5) e	2.6 (± 0.3) f	1.8 (± 0.03) e	36.4 (± 1.9) f	0.47 e
PGPR + KU1/16	227.4 (± 3.9) d	11(± 1)3 b	21.3 (± 2.4) d	3.8 (± 0.1) e	3.1 (± 0.1) d	40 (± 3.2) e	0.53 c
Tomato cultivar Supermarmande							
H	140.8 (+ 4.1) e	3 (+ 0.2) e	50.2 (+ 1.7) b	5.4 (+ 0.5) b	5.0 (± 0.2) b	57.3 (± 3.7) b	0.57 b
PGPR	268.3 (± 2.3) a	5 (± 0.3) d	57.7 (± 1.3) a	6.4(± 0.4) a	6.0 (± 0.7) a	64.1(± 2.3) a	0.90 a
KU1	106.5 (± 3.7)f	7 (±0..2) b	18.2(± 2.1) e	2.8(± 0.9) g	2.1 (± 0.1) f	35.5 (± 1.3) f	0.52 c
PGPR + KU1	185.2(±4.3) b	7(± 0.7) b	27.2 (± 1.9) c	4.7 (± 0.5) c	4.2 (± 0.3) c	50.1 (± 1.1) c	0.54 d
16	63.8 (± 2.3) h	5 (± 0.7) d	13.1(± 2.4) f	2 (± 0.4) h	1.8 (± 0.2) g	27.24(± 2.4) h	0.48 e
PGPR + 16	174.1(± 4.6) c	8 (± 0.1) a	23.2 (± 2.4) d	5(± 0.2) d	4.5 (± 0.1) c	44.3 (± 1.3) e	0.52 c
KU1/16	84.4 (± 3.8) g	6 (±0.1) c	1 5.2(± 2.3) f	3.4 (± 0.8) f	2.7 (± 0.6) e	29.9 (± 1.9) g	0.5 c
PGPR + KU1/16	162.2 (± 2.0) d	7(± 0.4) b	23.6 (± 2.4) d	4.8 (± 0.4) e	3.9 (± 0.1) d	47.6 (± 3.2) d	0.49 e

[v] **:** Means are the average of three experiments (*n* = 10) **H:** healthy control plants; **PGPRs used**: a mixture of plant growth promoting *Pseudomonas aeruginosa* and S*tenotrophomonas rhizophilia*; **KU1:** CMV-KU1 associated with satellite viral RNA; **16:** Challenge virus CMV-16

[w]**:** Average values from the different treatments rounded off to one decimal place or two decimal places + numbers in parenthesis = standard deviation

[x]**:** Different letters next to the values indicate differences (*P < 0.05)* between means. Means with same letter are not significantly different at probability level $P < 0.05$ according to Duncan's multiple range test (DMRT)

Influence of the PGPRs on the chemical content of the tomato fruits

Both bacterial and viral treatments had a significant effect (P < 0.05) on the chemical content of the tomato fruits (Table 2). The pH values were generally higher for Supermarmande compared to UC82B. Treatments containing PGPRs alone were more alkaline compared to the healthy controls. The treatments containing the viruses alone were more acidic compared to the healthy controls (Table 2). The total soluble solids of the tomato ranged between 2-6 % for both Supermarmande and UC82B. The PGPRs had no effect on the total soluble content in the tomato fruits of both the cultivars. The viruses on the other hand slightly reduced the total soluble content of the fruits. The presence of PGPRs increased the total protein content in tomatoes. The total protein content was higher for plants of the Supermarmande variety compared to that of UC82B. The presence of the viruses CMV-KU1 and CMV-16, both individually and together, decreased the protein content in tomatoes of both the cultivars (Table 2). The presence of the PGPRs did not have any

significant effect on the titerable acidity of the tomato fruits. However, it had a negative impact on the ascorbic acid content in the fruits. Both CMV-16 and CMV-KU1 increased titerable acidity of the tomato fruits. CMV-KU1 did not have any significant effect on the ascorbic acid content. CMV-16, however, caused a significant reduction of ascorbic acid levels (Table 2). PGPRs did not significantly affect the reduced sugar content of tomatoes. On the other hand, both CMV-KU1 and CMV-16 significantly decreased the reduced sugar content of the infected plants (Table 2). The total phenol content and the lycopene content of the tomato fruits increased in the presence of PGPRs. CMV-KU1 significantly reduced the total phenol content of tomatoes; however, it did not have any significant effect on the lycopene content of the tomato fruits. CMV-16 significantly reduced both phenol as well as lycopene content (Table 2). The reduced sugar, phenol and lycopene content were higher in general for Supermarmande compared to UC82B.

Table 2. PGPR effects on fruit content of tomato cultivars

Treatments[v]	pH	Total soluble solids (%)	Total proteins (mg/100g)	Titrable acidity (%)	Ascorbic acid (mg/100ml)	Reduced sugar (%)	Total phenols (mg/100g)	Lycopene (mg/kg)
Tomato cultivar UC82B								
H	5.2 (± 0.04)[w] b[x]	5.4 (± 0.3) a	921.6(± 6) c	0.61 (± 0.02) d	4.4 (± 0.04) a	2.9 (± 0.01) a	53.3(± 0.88) b	84.6(± 0.6) c
PGPR	5.4 (± 0.02) a	5.3 (± 0.3) a	928.3(± 3.8) a	0.6 (± 0.05) d	3.1 (± 0.34) b	2.9 (± 0.09) a	63(± 1.52) a	96.4(± 0.7) a
KU1	5.1(± 0.01) d	4(± 0.1) b	908 (± 4.2) d	0.75 (±0.02) b	4.4(± 0.1) a	2.2(± 0.1) b	45.7(± 1.56) c	84.2(± 0.5) c
PGPR + KU1	5.1 (± 0.01) c	3.9(± 0.1) b	924.6(± 4.3) b	0.76 (± 0.01) b	3.3(± 0.08) b	2.3(± 0.2) b	52.3 (± 2.84)b	96.2 (+ 1.5)a
16	5.1 (± 0.02) c	3.1(± 0.2) c	780.6 (± 2.8) f	0.72 (± 0.01) c	2.9(+ 0.05) c	2 (+ 0.1) c	44.7 (+ 1.4) c	74.1 (+ 2.1) c
PGPR + 16	5.2 (+ 0.02) b	3(+ 0.2) c	894 (+ 6.1) e	0. 71 (+ 0.01) c	2.4 (+ 0.07) d	2.1 (+ 0.1) c	49 (+ 0.98) d	90.7 (+ 0.8) b
KU1/16	5.0(+ 0.02) e	2.2 (+ 0.2) d	726 (+ 5.2) h	0.83 (+ 0.02) a	3 (+ 0.03) b	1.3(+ 0.2) d	36.6 (+ 1.2) e	74.9 (+ 0.9) d
PGPR + KU1/16	5.1(+ 0.01) d	2.3 (+ 0.2) d	836.3(+ 3.4) g	0.84 (+ 0.01) a	2.5(+ 0.1) d	1.33 (+ 0.2) d	43.3 (+ 1.7) d	90.1 (+ 2.5) b
Tomato cultivar Supermarmande								
H	5.5 (± 0.02) b	5.8 (± 0.2) a	952 (± 2.3) c	0.74 (± 0.02) c	4.8 (± 0.05) a	3.3(± 0.2) a	56.7 (±1.45) b	88.2(±1.1) c
PGPR	6.1 (± 0.02) a	5.8(± 0.2) a	977(± 2.1) a	0.75 (± 0.02) c	3(± 1.1) b	3.3 (± 0.2) a	66.3 (± 1.66) a	97.6 (± 1.2) a
KU1	5.2 (± 0.01)e	5(± 0.3) b	922.7 (± 2.4) d	0.84(± 0.02) b	4.8(± 0.08) a	2.6 (± 0.1)b	49.6 (± 1.4) c	88(± 0.7) c
PGPR + KU1	5.5(± 0.02) c	4.8(± 0.3) b	955.6(± 3) b	0.83 (± 0.01) b	2.9 (± 0.11) b	2.63 (± 0.2) b	54.7 (± 1.2) b	97.1 (± 0.8) a
16	5.2 (± 0.01) f	3.9(± 0.1) c	904.3 (± 3) f	0.83(± 0.03) b	2.6 (± 0.03) c	2.2(± 0.1) c	43 (± 2.4) e	82.5(± 1.1) d
PGPR + 16	5.4 (± 0.01) d	3.7(± 0.1) c	916(± 2.1) e	0.81 (± 0.01) b	2.1(± 0.1) d	2.2 (± 0.3) c	55.7 (± 1.7) b	91.5(± 2.2) b
KU1/16	5 (±0.1) h	2.6(± 0.2) d	877.3 (± 2.2) h	0.88(± 0.01) a	2.7(± 0.2) b	1.5(± 0.2) d	39.3 (±1.45) f	82.9 (±1) d
PGPR + KU1/16	5.1 (± 0.1) g	2.8 (± 0.2) d	898.7(± 2)g	0.9 (± 0.01) a	2.2 (± 0.2) d	1.5(±0.3) d	46.7(± 0.88) d	91.1(± 3.5) b

[v] **:** Means are the average of three experiments ($n = 10$) **H:** healthy control plants; **PGPRs used**: a mixture of plant growth promoting *Pseudomonas aeruginosa* and S*tenotrophomonas rhizophilia*; **KU1:** CMV-KU1 associated with satellite viral RNA; **16:** Challenge virus CMV-16

[w]**:** Average values from the different treatments rounded off to one decimal place or two decimal places + numbers in parenthesis = standard deviation

[x]: Different letters next to the values indicate differences (*P* < *0.05)* between means. Means with same letter are not significantly different at probability level $P < 0.05$ according to Duncan's multiple range test (DMRT)

Electron microscopy

Micrographs obtained from scanning electron microscopy of root tissues indicated that a considerable population of the inoculated PGPRs (Fig. 1A and B) were present in the rhizosphere. Transmission microscopy studies have indicated the presence of PGPRs inside the root cells as well (Fig. 1C &D). The cell size of the *P. aeruginosa* strain used was found to be around 0.6-0.8 micrometer while that of *S. rhizophilia* was 1.2-1.4 micrometer. The population of the inoculated PGPRs were greater outside the roots than on the inside. Electron micrographs of the CMV strains were also obtained (Fig. 2A &B). Ultra-thin sections of infected leaves showed virus clusters present in plant tissue cells (Fig. 3A and B). Particle diameters ranged from 28.9 to 30 nanometer for both CMV-KU1 and CMV-16. The CMV strains were not distinguishable using electron micrographs alone.

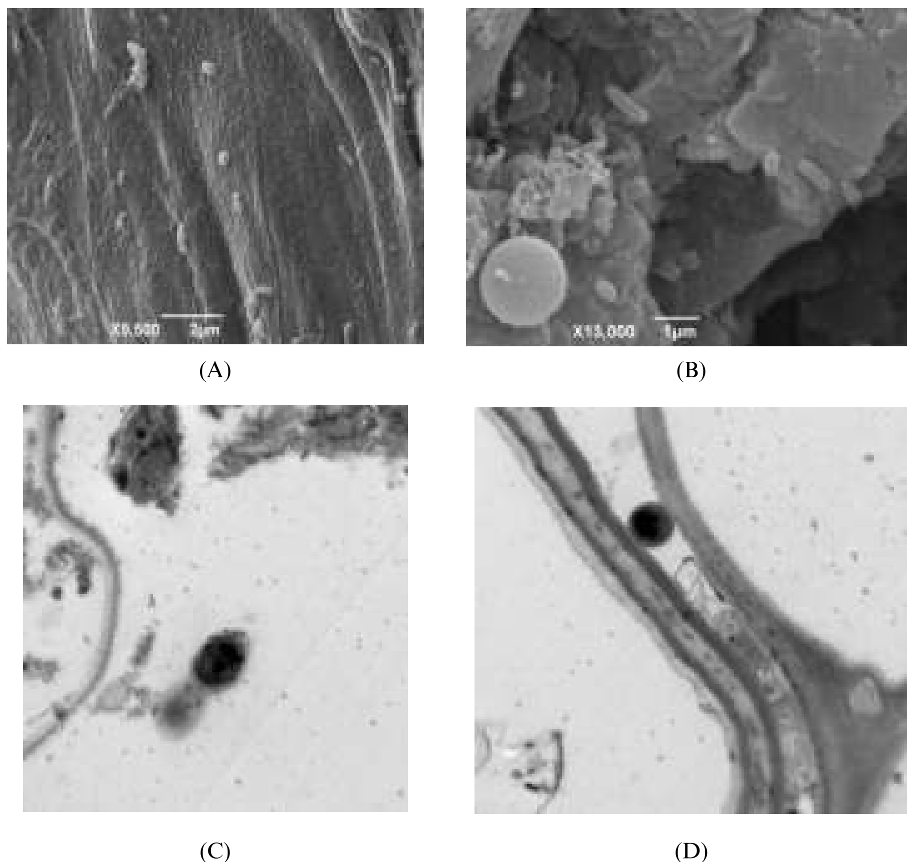

Fig. 1. Detection of Plant growth promoting rhizobacteria (PGPRs) using Scanning and Transmission Electron Microscopy. A & B) PGPRs associated with the root surfaces of tomato (*Solanum lycopersicum*) plants by SEM. C) TEM micrograph showing a dividing *Pseudomonas aeruginosa* cell. C) micrograph showing a *S.rhizophilia* cell wedged between two tomato cells.

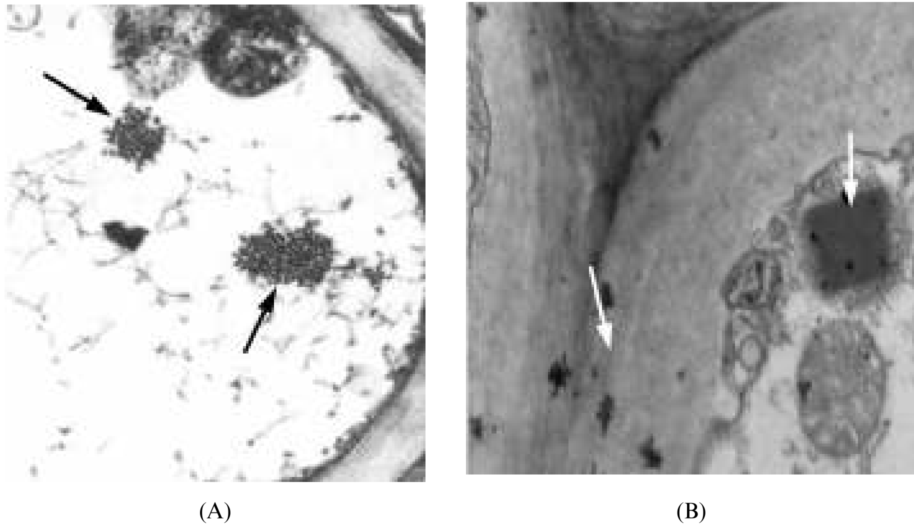

Fig. 2. Ultra-thin sections of infected stem (A) and root tissue (B) of tomato plants containing a cluster of *Cucumber mosaic virus* (CMV) particles both as aggregates and virus crystal, indicated by arrows, double stained with 2% uranyl acetate.

Detection of CMV viruses in treated plant tissues

Enzyme-linked immunosorbent assay (ELISA)

ELISA readings (Table 3) indicated that plants treated with PGPRS had lower infections compared to CMV-KU1 and CMV-16 treatments. The absorbance values are shown in Table 3.

Table 3. Detection of CMV-KU1 and CMV-16 based on Enzyme Linked Immunosorbent Assay (ELISA)

Treatments[v]	Absorbance Values[w]	Diseased/ Healthy Ratio	Virus Titer
Tomato cultivar UC82B			
H	0.24 (+ 0.03) f [x]	1	- [y]
KU1	0.75 (+ 0.03) d	3.1	+
PGPR + KU1	0.63(+ 0.12) e	2.6	+
16	1.54 (+ 0.21) a	6.4	+ + +
PGPRs + 16	1.08 (+0.20) b	4.5	+ +
PGPRs + KU1/16	0.88 (+ 0.05) c	3.7	+
KU1/ 16	1.10 (+ 0.18) b	4.6	+ +

Table 3. Detection of CMV-KU1 and CMV-16 based on Enzyme Linked Immunosorbent Assay (ELISA)

Treatments[v]	Absorbance Values[w]	Diseased/ Healthy Ratio	Virus Titer
Tomato cultivar Supermarmande			
H	0.32 (+ 0.01) k	1	-
KU1	0.93 (+ 0.03) i	2.9	+
PGPR + KU1	0.74(+ 0.05) j	2.3	+
16	1.97 (+ 0.12) g	6.2	+ + +
PGPRs + 16	1.20 (+0.20) h	3.8	+ +
PGPRs + KU1/16	0.97 (+ 0.08) i	3.0	+
KU1/ 16	1.21 (+ 0.12) h	3.8	+ +

[v] **:** Means are the average of three experiments ($n = 10$) **H:** healthy control plants; **PGPRs used**: a mixture of plant growth promoting *Pseudomonas aeruginosa* and S*tenotrophomonas rhizophilia*; **KU1:** CMV-KU1 associated with satellite viral RNA; **16:** Challenge virus CMV-16

[w]**:** Average of absorbance values measured at 405nm, from the different treatments + numbers in parenthesis = standard error

[x]**:** Different letters next to the values indicate differences ($P < 0.05$) between means. Means with same letter are not significantly different at probability level $P < 0.05$ according to Duncan's multiple range test (DMRT)

y: Positive reaction is defined as an absorbance greater than twice that of the healthy tissues; - = no virus, + = virus titer more than healthy control (The more +s the higher the titer of the virus)

Polyacrylamide gel electrophoresis

Total nucleic acids (TNA) analyses on 6% polyacrylamide gels resulted in detection and visualization of both CMV-KU1 and CMV-16 (Fig. 4).

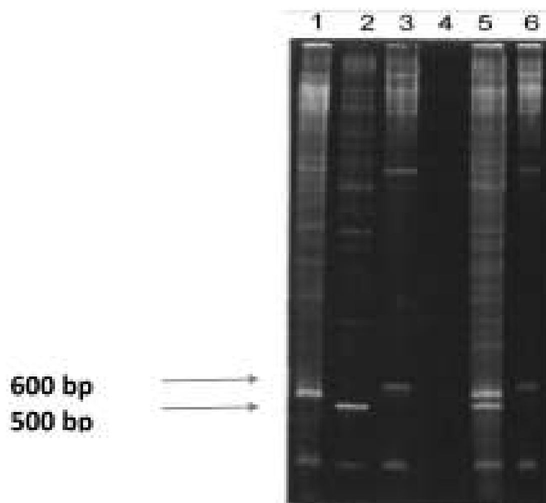

Fig. 3. Analysis of total nucleic acids (TNA) of infected plants on 6% ethidium bromide stained polyacrylamide gel to differentiate between CMV-KU1 and CMV-16. Lane 1: TNA of tomato plants infected with CMV-KU1, viral band seen between 500-600 bp (arrows); Lane 2: TNA of tomato plants infected with CMV-16, viral band seen between 500-600 bp (arrows); Lanes 3and 6: TNA from healthy tomatoes; Lane 4: blank and Lane 5: TNA of tomato plants infected with both CMV-KU1 and CMV-16.

DISCUSSION

PGPRs were able to successfully promote plant growth and fruit yield in both Supermarmande and UC82B even in the presence of CMV viruses. The beneficial ability of the PGPRs may vary with the PGPR strain(s) used and its mechanism in promoting plant growth (Dashti *et al.*, 2007). Mixtures of biocontrol agents with different plant colonization patterns may be useful for the biocontrol of several plant pathogens via multiple mechanisms of disease suppression (Murphy *et al.*, 2003; Siddiqui & Akhtar, 2009). Moreover, consortia of bio-control agents with taxonomically different organisms that require different optimum temperature, pH and moisture conditions may colonize roots more aggressively, thus improving plant growth and the efficacy of the bio-control agents (Siddiqui & Akhtar, 2009). The fruit number per plant of the different treatments was shown to decrease with increased fruit size. This negative correlation between fruit weight and fruit number have been reported previously in many cultivars of tomato (Blay *et al.*, 1999). The specific gravity of tomato fruits were higher for plants treated with PGPRs compared to the non-PGPR treated healthy and virus treated plants. The co-relation between specific gravity and the firmness of the fruits has been reported earlier (Karakurt *et al.*, 2011). Higher the specific gravity, greater the fruit firmness**.** The sugar content

and the acidity of the tomato fruits determine the tomato flavor. The better flavors are obtained with high sugar content. The total sugar content of ripe tomato is usually between 1.7 and 4.7% (Turhan & Seniz, 2009). Although, the PGPRs used in this study had no effect on the sugar content of the tomato fruits, percentage of sugar content in the non-virus treated fruits were as high as 2.9 for UC82B and 3.3 for Supermarmande. This agrees with the findings of Turhan & Seniz (2009). The pH of the tomato fruits for this study ranged between 5-5.4 for the UC82B and between 5- 6 for Supermarmande. Although this falls within the acceptable pH range for tomatoes, the values obtained were slightly higher than the ideal pH value of 4.5 (Turhan & Seniz, 2009). Karakurt *et al*. (2011) observed that titerable acidity of the different fruits varied with the PGPR strains used. The PGPRs in this study had no effect on the titratable acidity of the tomato fruits. On the other hand, the PGPRs were shown to generally reduce ascorbic acid. This is in accordance with the findings of the Karakurt *et al*.(2011). PGPR treatments increased lycopene contents of tomato fruits, which agrees with the findings of Ordookhani *et al*. (2010). The PGPRs also increased phenolic content of tomato fruits. Lycopene is known to be responsible for the reddening of the tomato, due to the differentiation of the chloroplasts and chromoplasts; so this carotenoid is very important in terms of the nutritional and marketable quality of tomatoes. Also, lycopene is beneficial for human health (Ordookhani *et al*., 2010). Many of the flavinoids and antioxidants present in tomato belong to the group of polyphenols (Kacjan Marsic *et al*., 2011). Any increase in the levels of phenolic content will improve the overall quality of the tomato fruits. The ELISA results indicated the presence of virus in the infected plants. The PGPR treated plants showed a lower absorbance reading compared to the non-PGPR treated virus controls. This may be an indication that the PGPR activity in the roots may have some effect in inhibiting the virus propagation and multiplication. PGPR-mediated bio-control can be extended to foliar and systemic diseases, even when the PGPRs are applied to seeds and roots, if the mechanism for control involves induction of host defenses (Murphy *et al*., 2003). The successful establishment of PGPRs in the roots, promote plant growth and yield and fruit quality, even in the presence of damaging plant viruses such as CMV which enables it to be used as an effective bio-control agent. A cost effective and easily available solution to this problem, such as this technique, can save large economic losses both to the Kuwaiti farmers and the Government.

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تأثير بكتيريا الجذور المنشطة لنمو النبات على محصول الثمار والخصائص الكيماوية فى نباتات الطماطم المصابة بفيروس تبرقش الخيار

نرجس دشتى، مجدى منتصر، نداء على و فينيسا شيريان

قسم العلوم البيولوجية - كلية العلوم - جامعة الكويت

ادارة الأبحاث - بحث رقم SL02/07

خلاصة

تم دراسة تأثير سلالتين من بكتيريا محفزة للنمو (Pseudomonas aeruginosa & Stenotrophomonas) على محصول ثمار الطماطم Solanum Lycopersicum وكذلك على المحتوى الكيميائي للطماطم المصابة بفيروس فسيفساء الخيار (CMV) ولقد تم اختيار سلالتين من هذا الفيروس للتجربة على نباتات الطماطم منهم سلالة جديدة تم عزلها من الكويت (CMV-KU1) ووجد أن التركيب الجيني الخاص بها يرافقه حمض نووي حميد لا يسبب أي أعراض على أوراق نباتات الطماطم إلا أنه قد يسبب خسارة في المحصول بنسبة 10٪ إلى 15٪. أما الفيروس الثاني وهو عبارة عن سلالة عرفت بإسم CMV-16 وهي خالية من الحمض النووي المرافق ولكنها تسبب تقزم شديد في نباتات الطماطم وكذلك خسارة كبيرة في محصول الثمار وضعف المجموع الخضري للطماطم وقد تم إجراء هذه التجارب بصورة منفصلة على صنفين مختلفين من نباتات الطماطم. (Supermarmande & UC82B). وبمقارنة النتائج تبين أن وجود البكتيريا المحفزة للنمو قد ضاعفت المحصول حتى في النباتات المصابة بالفيروس مقارنة بالنباتات السليمة. وقد لوحظ تحسناً ملحوظاً في جودة الثمار في النباتات المعاملة بالبكتيريا المحفزة للنمو مقارنة بالنباتات الأخرى السليمة الغير معاملة بالبكتيريا المحفزة وخصوصاً بمتوسط وزن وحجم الثمار، وطول النباتات. وقد تم إجراء دراسات مقارنة للتحاليل الكيميائية لثمار الطماطم التي أسفرت في وجود البكتيريا المحفزة إلى زيادة كم البروتين الكلي واللايكوبين ومحتوى الفينول وقلوية ثمار الطماطم مقارنة بالنباتات الغير معالجة بالبكتيريا المحفزة. ولقد تبين غياب تأثير واضح للبكتيريا المحفزة على السكريات المختزلة أومحتوى الجوامد أوالحموضة مع إنخفاض واضح في كمية حمض الأسكوربيك (فيتامين C) في ثمار الطماطم مقارنة بالنباتات السليمة.

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