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Biological Control of a severe Viral Strain of Cucumber Mosaic Virus(CMV) using a Mild Strain of CMV associated with Viral satRNA combined with a mixture of plant growth promoting Rhizobacteria (PGPRs)

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Abstract

The effect of using plant growth promoting rhizobacteria (PGPR) as compensatory agents to nullify the detrimental growth effects that occur when using satRNA mediated protective technology were studied in greenhouse grown chilli pepper. A benign cucumber mosaic virus strain (CMV) associated with satRNA, CMV-KU1, was successfully used as a biological control agent to combat infections caused by another severe strain of CMV, CMV-16. However, despite its protective capability, CMV-KU1 caused certain vegetative and yield loss to the plants to which it was applied. A mixture of two known PGPR strains, *Pseudomonas aeruginosa* and *Stenotrophomonas rhizophilia* were used in this investigative study to see if this loss can be reversed or compensated. Additionally, three different routes of PGPR application to the plants were also tested to determine which would provide the best result in promoting plant growth and reducing infections. The three PGPR application pathways tested were i) application directly to the rhizosphere of the plants by injection to the roots, ii) coating of the seeds with PGPRs before sowing, and iii) mixing of the PGPRs with the soil before sowing. Observations were made based on the manifested symptoms of the infected plants, fruit yield and ELISA readings. The resulting data were statistically analysed. The results indicated that the presence of PGPRs significantly improved the plant growth, yield, fruit number and fruit set rate in chilli pepper compared to using satRNA alone. Moreover, the best results were obtained when PGPRs were directly inoculated into the rhizosphere compared to the two other alternative application methods tested.

Keywords: PGPR, CMV, Virus Biological Control, Chilli Pepper

Introduction

Pepper (*Capsicum annuum* L.), member of the family Solanaceae is currently cultivated worldwide under various environmental and climatic conditions. In fact, it is the second most important crop among Solanaceous fruits. The chilli production has an economical impact in local as well as export markets in Asia and other parts of the world. More than one billion people consume chilli in one or another form on a daily basis. Viral diseases, especially the cucumber mosaic, are the major limiting factors for successful pepper cultivation (Tan et al., 2012).

Cucumber mosaic virus (CMV), belonging to the genus *Cucumovirus* of the Bromoviridae family, is considered to be one

of the five most widespread and economically damaging virus (Gallitelli, 2000; Lin et al., 2003; Montasser et al., 2006a; Tan et al., 2012) affecting numerous vegetable and horticulture crops all over the world. CMV symptoms generally vary with the viral strain and hosts. Some common symptoms which are characteristic to a CMV epidemic include a) sudden progressive necrosis and death of the whole plant; b) severe deformation of leaves (fern leaf and shoestring) accompanied by stunting of plants and bushy growth; c) internal browning of fruits without deformation and discoloration of the foliage; and d) leaf curly and sun blotch-like spots on fruits (Gallitelli, 2000).

CMV causes widespread diseases in pepper plants. CMV is

transmitted in pepper by numerous species of aphid in a non-persistent manner (Zitikaite & Samuitiene, 2009). In Bulgaria, CMV is the widest-spread pathogens, causing the biggest economical losses in pepper crops. It was found to be the causative agent of about 80-90% of pepper crop destruction in the country (Stoimenova et al., 2005). CMV can sometimes cause severe infections in synergy with other viruses in pepper plants. Extremely severe but nonlethal symptoms were observed in pepper plants co-infected with CMV and Pepper Mottle Virus relative to infection with either virus alone in experiments carried out under greenhouse conditions (Murphy & Bowen, 2005).

Viral satellite RNAs (satRNAs) are small nucleic acids whose nucleotide sequences are unrelated to, but are dependent upon the viral genome for replication, encapsidation and dispersion; they have a mutualistic relationship (Xu et al., 2000). Satellite RNAs are capable of altering the virus to such an extent that they can modulate (attenuate or exacerbate) the symptoms caused by their cognate helper viruses (Hu et al., 2009). Most of their replication is limited to co-infected cells (Simon et al., 2004). Satellite RNAs have garnered global interest over the past decades mainly because a) They can alter symptoms of their helper viruses b) they do not encode any RNA polymerase for self-replication but depend on the host machinery c) they are molecular parasites as they can modulate the accumulation of their cognate helper viruses in plant cells co-infected with the virus d) they can be employed as high-level expression vectors for foreign genes and e) they serve surrogate models for the molecular biological study of their associated helper viruses (Hu et al., 2009).

CMV KU1, a CMV strain associated with a benign, naturally occurring viral satellite RNA was isolated in Kuwait (United States Patent no. US 8,138,390 B2; Montasser et al., 2006b). This virus does not cause any mosaic symptoms in both tomato and pepper except for mild stunting and 30-40% yield loss (Dashti et al., 2012). As a result, it has been successfully used as a biological control agent against more damaging viruses that cause total destruction of crop plants. CMV-KU1 has been successfully tested as a protective virus against many lethal CMV viruses such as CMV-D, CMV-F (Montasser et al., 2006b) and CMV-16 (DQ018288.1). CMV-16, subgroup II, is a Japanese isolate from tomato (Sayama et al., 1993) that contains no viral satellite. It causes severe stunting and fruit malformation in pepper plants. Despite its success to contain outright damage due to severe viral infections by other necrogenic CMV virus, the yield and the growth loss caused by CMV-KU1 limits its commercial use as a biological control agent (Montasser et al., 2006b).

Plant growth promoting rhizobacteria (PGPR) are a mixture of beneficial microorganisms which increase crop yield, plant growth and also protect against pathogens (Sahran & Nehra, 2011). PGPRs generally protect the plants against CMV infection by improving the plant's natural resistance to fight infection (Murphy et al., 2000). PGPRs induce systemic resistance either through salicylic acid-dependent systemic acquired resistance pathway (SAR) or by jasmonic acid and ethylene perception induced systemic resistance (ISR) pathway (Nelson, 2004; Sahran & Nehra, 2011;

Beneduzi et al., 2012). Dashti et al., (2012) have previously shown that using both PGPRs and CMV-KU1 together can reduce CMV-16 infections in tomatoes and promote plant growth to a value equivalent to uninfected plants.

With such ground breaking finding, the major objectives of this study was to i) further investigate the PGPR ability to compensate for the yield loss caused by satRNA associated CMV-KU1 in chilli pepper, ii) to test how the PGPR ability to stimulate growth influences the overall protective capability of the benign satRNA associated virus (CMV-KU1) and see how this compares with using either CMV-KU1 and the PGPRs alone without the other, and iii) to compare the efficacy of three different methods PGPR application onto plants based on plant growth and fruit yield.

Materials and methods

Virus source, maintenance and inoculum preparations

Both the protective viral isolate, CMV-KU1 and the challenge strain, CMV-16, were revitalized from preserved frozen tissues by mechanical inoculation into fresh squash (*Cucurbita pepo* L.) and tomato (*Solanum lycopersicon* L.) plants.

Virus purification

Virus purification were performed as per the protocol described by Montasser et al. (2006a). The virus particles were precipitated using 10% Polyethylene glycol (PEG) solution. The precipitate was further clarified by ultracentrifugation at 27,000 rpm for 4 hours. The purified extract was stored at 4°C.

PGPR source and inoculum preparation

Two strains of PGPRs were used in this study: *Pseudomonas aeruginosa* and *Stenotrophomonas rhizophilia*. The PGPRs strains that used were isolated in a previous work from the *Vicia faba* rhizosphere (Radwan et al. 2005). Diluted soil suspensions from the *Vicia faba* rhizosphere were plated on solid *Pseudomonas* medium and Yeast-mannitol agar for *P. aeruginosa* and *S. rhizophilia* respectively. These were incubated at 30 °C for 7 days and pure colonies were sub cultured. The organisms were identified by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) Braunschweig (Germany) and by the various biochemical tests performed at the Kuwait University (Radwan et al., 2005). The inoculum mixture of the two strains were prepared by culturing the two PGPR strains in nutrient broth and incubating at 20- 25 °C with constant shaking at 125 rpm. When the cultures reached the log phase, each of the strains were adjusted with distilled water at A420 giving a cell density of 10⁸ 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).

Preparation of seeds and soil

Seeds of pepper were surface sterilized as previously described (Radwan et al., 2007). Half the total number of seeds from were soaked with the PGPR mixture for 48 hours before being planted. The seeds were planted by hand into pots, washed with sodium hypochlorite solution containing sterilized garden soil (PlantafLOUR). Following germinations, the seedlings were thinned to one plant per pot to ensure better growth.

Greenhouse set-up

Three independent simultaneous experiments were conducted for the different PGPR application methods. For each experiment, test pepper plants were divided into 6 groups based on the different treatments as follows: a) Plants treated with satellite RNA associated virus CMV-KU1 alone (referred to as KU1), b) Plants treated only with PGPR mixture (referred to as PGPR); c) Plants treated with a combination of CMV-KU1 and the PGPR mixture (referred to as PGPR+KU1), d) Plants treated with satellite virus CMV-KU1 alone and challenged with CMV-16 (referred to as KU1/16), e) Plants treated only with PGPR mixture and challenged with CMV-16 (referred to as PGPR/16) and f) Plants treated with a combination of CMV-KU1 and the PGPR mixture and challenged with CMV-16 (referred to as PGPR+KU1/16). Two control treatments, one positive (challenged only with CMV-16), and the other negative (healthy plants without any bacterial or viral inoculations) were also included. Treatments were arranged in a randomized complete block design with 10 plants of each treatment (10 plants per treatment \times 8 treatments = 80 plants per one method of PGPR application). The entire study was confirmed with three consecutive sets of experiments. The first trial was conducted between November (2012) - February (2013). The second trial was conducted between April (2013) - June (2013) and the third trial was conducted between September (2013) - November (2013). Challenge viruses CMV-16 was inoculated at 14 days post-inoculation (dpi) with treatments. CMV-KU1 viruses was applied to the plants at the dicotyledonary stage.

PGPR inoculation and maintenance

The PGPRs were applied in three different ways. To the first group, the PGPRs were applied directly under sterile conditions to base of the plants close to the roots at the dicotyledonary stage. For the second group, the PGPR mixtures were directly mixed with the sterile soil in the ratio 1:3 before sowing. For the third group, the seeds were coated with PGPRs by incubating them with the culture flasks containing PGPR mixtures for 48 hours before sowing. A few test seeds were placed in agar plates to check for PGPR growth. The virus applied onto the plants by mechanical sap transmission (Montasser et al., 2006b). The leaves of the plants were dusted with carborundum powder as an abrasive material, and then rubbed with crude sap extracted from infected leaves grounded in 0.01M phosphate buffer by using a sterile cotton swab. Plants were maintained under greenhouse conditions with at a temperature of 25° C with alternating 16 hours light and 8 hours dark periods. The watering was carried out every alternate days using sterile Hoagland solution. Perforated pots were used to ensure proper drainage of excess amount of solutions. Twenty one days after inoculation the plants were re-potted. Plants were scored for symptoms at 18, 21, 28, 35 and 42 days post inoculation with the biological treatments.

Evaluation of plant growth characteristics

Forty two days post inoculation with the different treatments, the plants were harvested as per the procedure demonstrated by (Radwan et al., 2007; Dashti et al., 2014)). Plants were carefully dislodged from the soil, taking special care not to sever the fine

root hairs. After washing the roots of plants, their heights, fresh weights and fruit yield were measured. The plants were then placed in paper bags and kept in the sun for 2-3 days to remove all moisture content for dry weight determination. Fruit set rates were obtained by counting were calculated by flower number and obtained percent values (Karakurt & Aslantas, 2010).

Approximately 10 g of the soil attached to the root of each treatment were transferred into 90 ml of sterile distilled water. This was shaken for 10-20 minutes after which 1 ml aliquot from this mixture was taken, serially diluted (10 fold) and finally plated by spread plating on nutrient agar. Incubation was for 48 hours at 20-25° C. Eight dilutions were prepared per treatment with two replicates for each dilution. Control plates were also incubated. The purity of the strains were confirmed by gram staining and biochemical analysis using API strips.

Disease assessments

Enzyme-linked Immunosorbent assay (ELISA)

The presence of CMV-16 in the foliar tissue of untreated and treated test plants was investigated using the indirect ELISA method (Dashti et al., 2014). Each plant were sampled at the end of 18, 21, 28 days by collection of three terminal leaflets from three young non-inoculated leaves. ELISA plates were prepared as described previously (Dashti et al., 2012). The absorbance was measured at 405 nm, 15-60 min after the addition of the substrate, using a Biotek Model EL307 (Burlington, VT) or a Dynatech MR700 ELISA Reader (Bio-Rad Laboratories, Inc. U.K.). Values that exceeded twice that of the untreated/healthy samples and/or the buffer controls were considered positive. Based on the ELISA values, the percentage infection of plants in each treatment were also calculated.

Bioassay

Based on the visibility of symptoms appearing on the plants, the plants were scored from a scale of 0 to 10 where, where 0 = no symptoms and 10 = severe symptoms) (Zehnder et al., 2000). A logistic model was fitted to assess disease intensity, area under disease progression curve and disease prevention. This model is given by the following equations: Disease Intensity = $100(\sum sn/SN)$, where s is the disease score, S is the highest s grade, n is number of plants with the same s value, and N is total number of test plants indexed (Zehnder et al., 2000). Area under disease progression curve (AUDPC) calculated using the formula: $\sum (0.5)(Y_i + Y_{i+1})(T_i + T_{i+1})$ where Y = disease severity at time T , and i = the time of the assessment in days (Zehnder et al., 2000). Disease prevention was calculated using the formula $100[(C - T)/C]$, where C = disease intensity of control plants inoculated only with CMV-16 and T = disease intensity of three treatments plants challenged with CMV-16 (Montasser et al., 2006b). The results obtained for the plant growth parameters were statistically analysed using Microsoft excel and the graphs were constructed using the GraphPad prism (v. 6.0). Analysis of variance (ANOVA) at $P = 0.05$ was performed on the data obtained from ELISA 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 (MSTATC) software.

Detection of satRNA in replicated tissues

The fate of the satRNA and its intactness in the replicated tissues of the different inoculums were determined using PCR. The samples were amplified using satRNA specific primers (Forward:5'-CCTCCGCGGATGCTAACTT-3'and Reverse: 5'CGGAATCAGACTGGGAGCA-3'-R) with the cycle set-up as follows:10 cycles at 94 ° C for 15 sec (denaturation), 63 ° C for 30 sec (annealing) and 72 ° C for 40 sec (extension) followed by 40 cycles at94 ° C for 30 sec (denaturation), 55 ° C for 30 sec (annealing) and 72 ° C for 40 sec (extension)

Results

Effect of CMV-KU1/PGPR combinations on improving growth, fruit yield and fruit set rate in pepper

The effect of PGPR/CMV-KU1 treatments on plant development was determined by measuring various growth parameters such as plant height, plant weight (both fresh and dry), fruit number, fruit yield and fruit set rate (Fig. 1 & 2). Both CMV-KU1 and CMV-16 adversely affect the growth and yield of pepper. However the symptoms caused by CMV-KU1 were milder in pepper compared to CMV-16. Plants treated with both CMV-KU1 and CMV-16 together showed slight stunting and light mosaic symptoms. This was however better compared to effect of CMV-16 alone on the plants. The presence of the PGPRs were found to compensate for the yield losses caused by both CMV-KU1 and CMV-16. Plants inoculated with the PGPRs showed reduced level of symptoms when infected by CMV-16 and were able to grow almost as tall as healthy control plants (Fig. 2).

Plants protected by CMV-KU1 alone reduced the overall growth of the plants by around 50% (Fig. 2). The average yield of CMV-KU1 treated plants was reduced by three times the value of the healthy control plants. The plants themselves appeared slightly stunted with weak stems, fewer and narrower leaves. CMV-KU1

strain however, showed no visible mosaic symptoms on pepper leaves (Fig. 1A).The positive control, that is, plants inoculated only with the challenge virus CMV-16 showed severe signs of CMV infection. Severely infected CM-16 plants were characterized by stunting, with significant yield losses compared to both the healthy control plants and the KU1 treated plants. Additionally, the leaves showed slight mosaic symptoms like yellowing, chlorosis and curling (Fig. 1 A). For the treatments where both CMV-KU1 and CMV-16 were present (KU1/16), the growth parameter values lay in between that of the KU1 treatments and 16 treatments. The presence of CMV-KU1 reduced the severity of the CMV-16 infection. The yield of these treatments were tripled compared to the 16 treatments (Fig. 1B and Fig. 2).

The growth parameters of the plants treated with PGPRs without the addition of any virus showed significantly higher values compared to the healthy. These plants appeared taller with broader leaves and thick strong stems (Fig. 1A). The fruit yield was also better than those of the healthy (Fig. 1B). The presence of either CMV-KU1 or CMV-16 caused a slight decrease in the overall growth and yield of the plant. This reduction was even more prominent when CMV-16 and CMV-KU1were present together in the plants. This reduction however was within or slightly less than the growth range of the healthy control plants. The flower abscissions for the CMV-16 treated plants were quite high and there was significant delay in the fruit setting and ripening compared to the healthy controls and KU1 treated plants. The fruit set rate of the CMV-16 treated plants were only 10%. That is only 10% of the total flowers present actually ripened into fruits (Fig. 2). The fruit set rate was determined as an average percent of the total number of flowers that develop into fruits per week. In comparison, the average fruit



Fig. 1. A) Greenhouse experiment to determine the protective effect of CMV-KU1 (V) and PGPR mixture (A+B) on the growth of pepper plants, then challenged with severe viral strain of CMV-16 (16). B) A fraction of yields from the greenhouse experiments obtained from pepper plants from the various treatments. H: Healthy control, V: CMV associated with Viral satellite RNA (CMV-KU1), 16: Challenge severe strain of CMV-16; V+16: plants vaccinated with CMV-KU1 and challenged with CMV-16. A+B: Plants inoculated with PGPR mixture of *Pseudomonas aeruginosa* and *Stenotrophomonas rhizophilia*; A+B+V: Plants treated with both PGPR and CMV-KU1; A+B+V+16: Plants treated with PGPR and CMV-KU1 before being challenged with CMV-16.

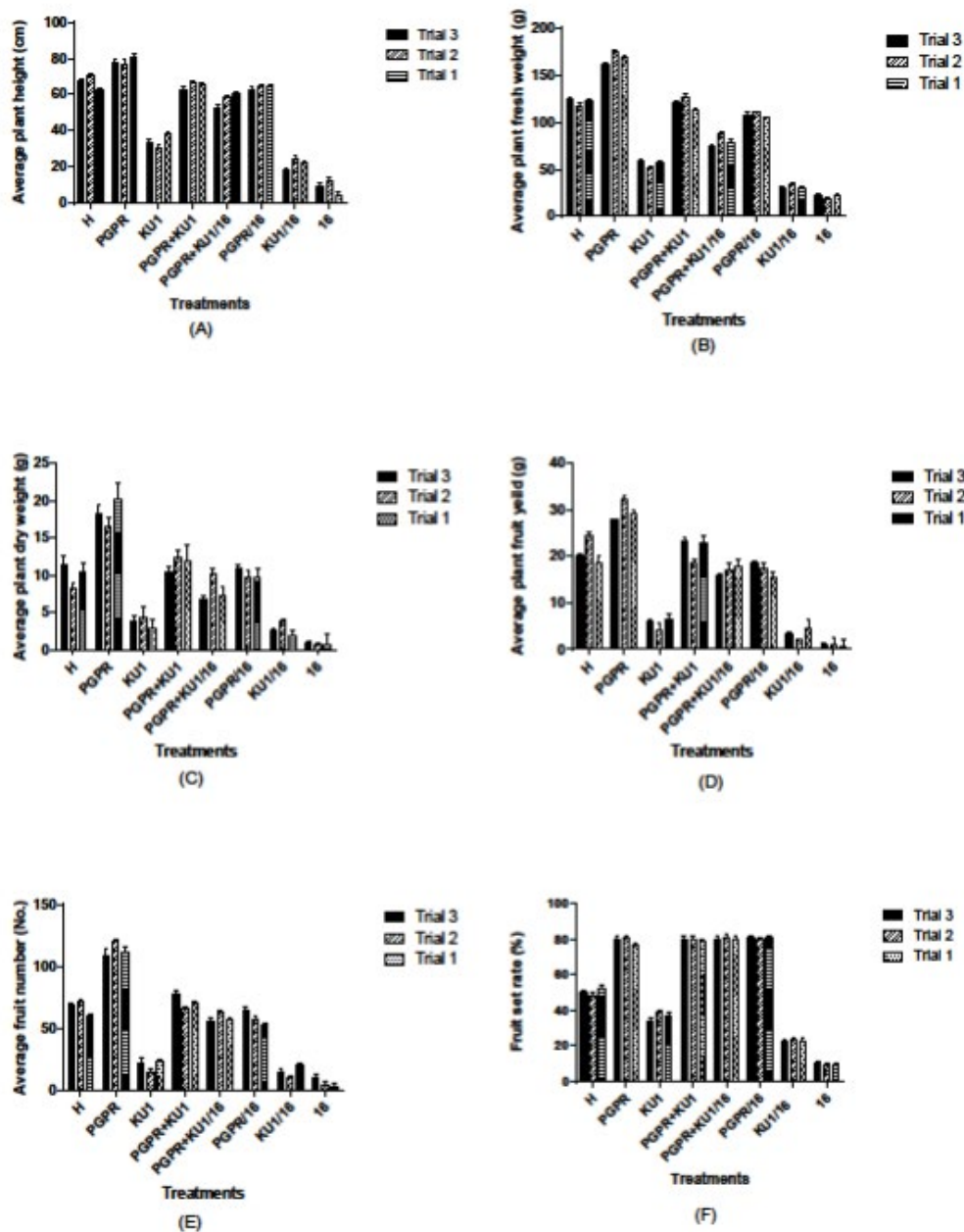


Fig. 2

Fig. 2. Comparisons of the efficacy of CMV-KU1/PGPR mediated protection on growth parameters such as A) plant height, B) Plant fresh weight, C) Plant dry weight, D) Fruit yield, E) Fruit number and F) Fruit set rate of pepper plants against infection by a severe strain of CMV-16. Data collected 42 days post-inoculation with CMV-KU1 and confirmed with three consecutive experiments (n = 10) H: healthy control plants; KU1: CMV-KU1 strain associated with satellite viral RNA; 16: Challenge virus CMV-16; KU1/16: plants pre-treated with CMV-KU1 and challenged with CMV-16; PGPR: Plants treated with the PGPR mixture consisting of *Pseudomonas aeruginosa* and *Stenotrophomonas rhizophilia*; PGPR+KU1: Plants treated with the PGPR mixture together with CMV-KU1; PGPR+KU1/16: Plants treated with the PGPR mixture and CMV-KU1 before being challenged with CMV-16; PGPR/16: Plants treated with PGPRs and challenged with CMV-16 virus. Standard error is represented by error bars.

set rate of the healthy was about 50 % and that of KU1 and KU1/16 were 34.2 and 22.7 respectively (Fig. 2). The average fruit size of the CMV-16 treatments were also smaller compared to the other treatments. The addition of PGPRs improved the fruit set rate of the plants. About 80% of the flowers formed developed into fruits in all of the PGPR treated plants. The colour of the fruits were not dependent on the treatments. However there was a higher occurrence of yellowish-green fruits among the virus treated plants compared to the healthy and the PGPR treated plants, where most of the fruits were dark green (Fig.1B).

Disease prevention of the CMV infection in pepper plants by CMV-KU1/PGPR

The disease prevention capability of the CMV-KU1/ PGPR combinations was estimated based on the visible symptoms and ELISA. Symptoms observed were scored on a scale from 0-10 where 0 indicates no visible symptoms and 10 indicates the highest form of the disease. Based on the scores of the various treatments, the disease severity, percentage prevention and area under the disease progression curve (AUDPC) were calculated (Fig. 3).

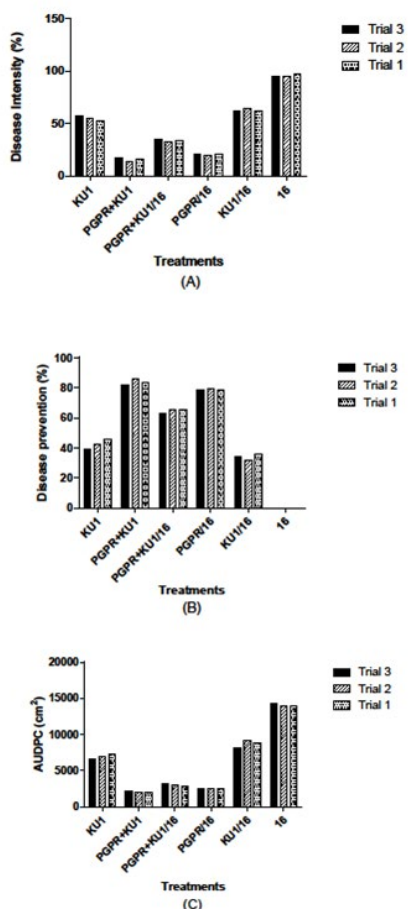


Fig. 3

Fig. 3. Comparison of A) disease severity, B) Percent disease prevention and C) area under the disease progression curve (AUDPC) for the various treatments. Data collected 42 days post-inoculation with CMV-KU1 and confirmed with three consecutive experiments (n = 10) KU1: CMV-KU1 strain associated with satellite viral RNA; 16: Challenge virus CMV-16; KU1/16: plants pre-treated with CMV-KU1 and challenged with CMV-16; PGPR+KU1: Plants treated with the PGPR mixture together with CMV-KU1; PGPR+KU1/16: Plants treated with the PGPR mixture and CMV-KU1 before being challenged with CMV-16; PGPR/16: Plants treated with PGPRs and challenged with CMV-16 virus.

The CMV-KU1 treated plants expressed a lower disease severity rating compared to the control treatments challenged with CMV-16. The control plants challenged only with the CMV 16, developed severe symptoms within seven days following inoculations. Disease severity values of the positive control treatments (16) at the end of 42 weeks was around 95% indicating a high rate of disease incidence and progression. The disease prevention capacity of CMV-KU1 was 34.2%. In addition to this, the appearance of symptoms on plants inoculated with CMV-KU1 was delayed compared to the 16 treatments. The presence of PGPRs further reduced the severity of the disease. Plants treated with the PGPRs reduced the disease by about 70-85%. The plants treated with CMV-KU1 were mildstunted. When challenged together with CMV 16, this stunting became even more pronounced. However, the presence of the PGPRs prevented stunting and the treated plants were able to grow as tall as the healthy control plants. Many of the plants inoculated with PGPR treatments were completely able protect themselves against the CMV-16 virus. The AUDPC values, calculated over three weeks, of the different treatments are also shown in Fig.3. The values were highest for the positive control treatments and lowest for the PGPR treated plants.

CMV accumulation in the plant tissues was determined by ELISA. ELISA readings showed that the absorbance values at 405 nm were lower for plants pre-treated with CMV-KU1/PGPR combinations before challenging them with CMV-16. In fact, the values observed were only slightly higher than that of the healthy control values.

The ELISA reading for the different treatments during the three different trial periods are shown in Table 1. The average absorbance value for the healthy controls was between 0.2-0.35. Absorbance values that exceed twice that of the healthy control values are considered positive. The average ELISA readings for treatments having only the CMV-KU1 virus was much lower compared to the positive controls, i.e. plants infected with CMV-16 virus alone (Table 1). Treatments with the PGPR were mostly negative for the CMV antigen. All positive reactions were indicated by the '+' sign, the number of which was increased with the intensity of the infection (Table 1) Based on the disease to healthy ratio values recorded, the incidence of disease in positive controls (16) was about 8 times that of the healthy values, while those infected with CMV-KU1 alone (KU1) was two times more than the healthy. Plants infected with both the CMV-KU1 and CMV-16 viruses (KU1/16), were 5 times more diseased than the healthy. With the addition of PGPR, the values of KU1/16 reduced significantly (Table1).

Effects of different methods of PGPR application

Three different techniques by which PGPRs were applied to the plants, inoculating PGPRs directly to the rhizosphere (PGPR (roots)) was found to be the most effective method compared to coating the PGPRs onto the seeds or applying them to the soil (Fig. 4 & 5). Both PGPR (roots) and PGPR(seeds) both showed a better growth statistics compared to the healthy, while PGPR(soil) negatively impacted the growth of the plants. The yield from this treatment was also poor. The average fruit yield of PGPR (soil) were between 11-12.9g compared to the 27.9-32.3 g of PGPR(roots) and 22-25.9g of PGPR(seeds). The fruit yield of the healthy (H) were between 18-24 g (Fig. 5). All the PGPR treatments were able to produce better yield compared to CMV-KU1. The growth of the treated plants was also better. The PGPR count in the soil for plants inoculated at the rhizosphere was around 107cells/ml. For treatments wherein the PGPRs were coated onto the seeds before sowing, the counts reduced to around 105cells/ml. For treatments, where PGPRs were mixed with the soil, final count obtained after pour plating was 103cells/ml. All these values were lower than the initial count of the PGPRs applied i.e. 108 cells/ml.



Fig 4.

Fig. 4. A comparative study showing the effect of PGPRs on the growth of pepper plants when they are applied to the plants in three different ways A) Plants after one week of PGPR application; B) plants after 3 weeks of PGPR application. H: healthy control plants; 16: positive control plants inoculated with the severe CMV-16 virus; AB (roots): plants inoculated with the PGPRs by injecting into their rhizosphere; AB(seeds): plants whose seeds were coated with the PGPRs before sowing; AB(soil): PGPR treated pepper plants wherein the PGPRs were first mixed with the soil in the 1:3 ratio before planting.

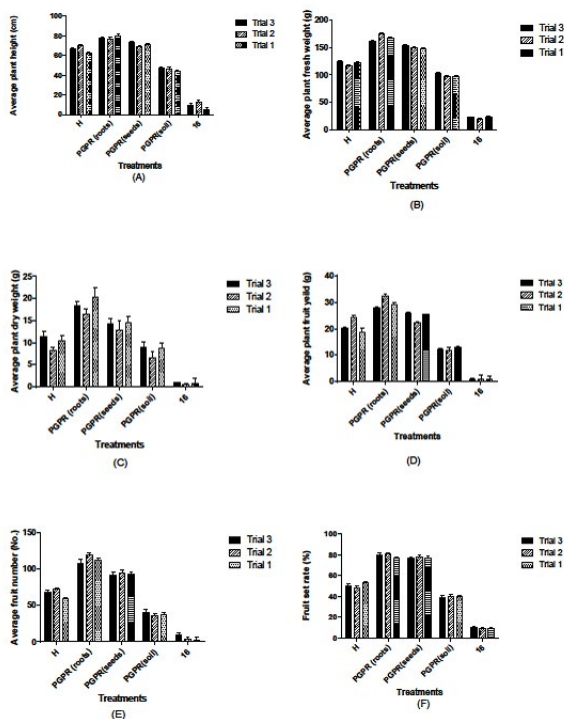


Fig. 5

Fig. 5. Comparison of the various growth parameters for the different methods of application of PGPRs in pepper plants A) plant height, B) Plant fresh weight, C) Plant dry weight, D) Fruit yield, E) Fruit number and F) Fruit set rate. Data collected 42 days post-inoculation with CMV-KU1 and confirmed with three consecutive experiments (n = 10) H: Healthy control plants; 16: Challenge virus CMV-16; PGPR (roots): Plants inoculated with the PGPR mixture directly into the rhizosphere close to the roots at the di-cotyledon stage of the plants; PGPR (seeds): PGPR coated on to the seeds before sowing; PGPR (soil): PGPRs mixed with the soil (1:3) before sowing. Standard error is represented by error bars.

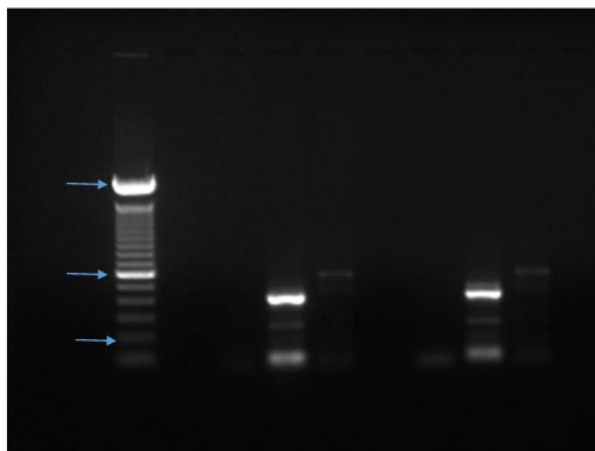


Fig. 6. PCR product of healthy and infected plants. PCR were performed (40 cycles) using specific pair of primers CMV-F/CMV-R. PCR product were separated by electrophoresis through 1% agarose gel. Lane 1, DNA marker 1 kb; lane 2, healthy pepper plants; lane 3, Pepper infected with CMV-KU1; lane 4, Pepper infected with CMV-16; lane 5, Pepper plants infected with PGPRs alone; lane 6, Pepper plants infected with PGPRs and CMV-KU1; lane 7, Pepper plants infected with PGPRs and CMV-16.

Discussion

This study revealed that the use of satRNA associated CMV-KU1 combined with PGPRs provided considerable protection to pepper plants against severe CMV infection, damage and yield loss when compared to the satRNA alone. The results have also indicated that there is no significant difference in the growth parameters of PGPR + KU1/16 treated plants and PGPR/16 treated plants according to the statistical analysis. These findings are in agreement with Murphy et al., 2000 who reported that PGPRs act mainly by reducing the external symptoms by promoting plant growth and enhancing systemic defence. even in remote sites far away from their own site of action (Nelson, 2004). Although each of them has individually proven to be effective in plant protection, using them together make them more potent as biological control agents. Together they not only protect the plant from infection, but also increase the growth and yield of the treated plants to values comparable to or better than the healthy control plants (Dashti et al., 2012). The disease prevention capacity of CMV-KU1 against CMV-16 virus only about 34% compared to PGPR/CMV-KU1 combination, which was 63%. Even the yield values for the combination were higher compared to CMV-KU1 alone. These findings are concordant with previous work done by Dashti et al (2012; 2014) on tomatoes. The findings indicate that the detrimental effects of the satRNA on the growth and the yield of the pepper plants can be minimized by adding PGPR inoculums. Together they serve as effective biological control agents against CMV infection.

satRNA and mediated protection using CMV-KU1 has been successfully tested before on tomatoes, melon, squash and tobacco (Montasser et al., 2006b; Dashti et al., 2007, 2012). The application of mild strains of other CMV containing satellite RNA to greenhouse and field crops has been evaluated (Roossinck et al., 1992). In several cases, CMV containing satellite RNA was able to protect plants to various extents against infection by more virulent strains either applied to the plants or introduced by natural infestation via the aphid vectors of CMV-sat RNA. Our ELISA confirm this as plants treated with satRNA showed reduced virus titers. The satellite RNA is believed to parasitize the viral genome at the molecular level. The viral satellite out competes the viral genome replication of the challenged virus (CMV-16) that depends on the host plant's enzymatic machinery and replicative enzymes. As a result, the ELISA titers in the are significantly reduced. However, the external symptoms of the disease is not completely removed (Hu et al, 2009; Montasser et al., 2006 b). Thus, one can obtain variable levels of protection against CMV and pathogenic

satellite RNAs in the field by using a CMV-plus-satellite "vaccine". Both *Pseudomonas* and *Stenotrophomonas* have also been used previously to promote growth and protection of crop plants. (Adesemoye & Ugoji, 2009). *Pseudomonas florescens* have been successfully used as a biological control agent against foot rot and also to enhance root proliferation in black pepper (Paul & Sarma, 2006). *S. rhizophilia* is a xylose utilizing, non-lypolytic, non β -glucosidase producing *Stenotrophomonas* species that is capable of growth even at low temperatures (4°C). These properties offer a great advantage for symbiotic association with plants. *S. rhizophilia* is also known to have remarkable antifungal activity against plant-pathogenic fungi (Wolf et al., 2002). This ability to produce resistance to disease and hence promote plant growth is largely due to the ability of *Stenotrophomonas* species to produce siderophores for ion chelation, antibiosis and production of lytic enzymes (Berg et al., 1996). *S. rhizophila* is able to colonize various plant sections in tomato, sweet pepper, cotton and oilseed rape. *S. rhizophila* colonies have been observed in the endophyte of tomato root hairs. The plant growth promoting effect of *S. rhizophila* is mostly via the suppression of pathogens and deleterious microbes, which could lead to a better growth environment for the plant. (Schmidt et al., 2010)

The application of PGPRs directly to the plant roots was found to be more effective compared to coating the seeds or applying them directly into the soil. This might be due to that application of PGPRs directly into the roots might allow them to colonize the plant roots more effectively compared to the other two techniques. It might be more difficult to colonize the hard coats of the seeds compared to the softer tissues of the roots (Nelson, 2004). PGPRs applied to the soil may not be effectively able to attach to the roots and colonize them. Moreover, many of the PGPRs may secrete secondary metabolites to survive in the soil and this may antagonistically affect the growth and the development of plants (Joseph et al., 2007). The ability of enhancing plant growth is largely dependent on: a) their genetic traits such as motility, b) chemotaxis to seed and root exudates, c) production of pili and fimbriae, d) production of specific cell surface components, e) ability to use certain cell surface components of root exudates, protein secretion, and f) quorum sensing (Nelson, 2004).

It has been observed that the CMV-KU1 associated with a viral satellite RNA requires a minimum of three weeks to establish themselves and provide protection. This is in agreement with the results obtained by Montasser et al., 2006b. This is the time taken by the CMV-KU1 virus to successfully multiply and spread in the roots and leaves. Plants treated with CMV-KU1 and challenged with CMV-16 virus at 1 week and 2 weeks after inoculation with the protective virus was as infected as the positive control treatments (Dashti et al., 2012).

Although satRNA and PGPRs together provide effective protection and yield improvement, certain limitations are encountered when using them repeatedly over a large scale. One of the major disadvantages of using satRNA as a biological control agent is the inherent risk of mutation of the satRNA to a pathogenic form (Tepfer, 2003). This mutation might increase the disease severity

of the infected plants and may lead to severe economic loss. The pathogenicity of the satRNA was found to vary between the cognate helper viruses as well. For instance, A variant of the B5 satRNA was observed to cause severe infection when the helper virus is subgroup II strain and alleviated the disease when the helper virus is of the sub group I strain (Simon et al., 2004). Additionally, the sequence variations occurring in satRNA is extremely high. This adds to the risk of using them as biological control agents. Another disadvantage is that even though satRNA attenuates the symptoms of the host plants they do not completely prevent the onset of the disease. Moreover, they cause a certain amount of yield and vegetative loss in the inoculated plants (Montasser et al., 2006 a; b; Dashti et al., 2012). The occurrence of some virus strains that do not support the replication of satRNAs in certain host plants may present an obstacle to satRNA-mediated disease management (Hu et al., 2009). The beneficial ability of the PGPRs is largely dependent on the choice of the strain used. The mode of action of the PGPRs is varying with strain used. Using PGPRs in mixtures have been found to be more effective. However, the individual strains might antagonistically compete with each other reducing their overall effect (Murphy et al., 2003; Siddiqui & Akhtar, 2009).

Conclusion

Research conducted on the viral satellite-mediated protection technology as a biological control agent is still in its nascent phase. Much research work has still to be conducted before the losses incurred by CMV viruses can be completely prevented and viruses can completely be brought under control.

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