Effect of *Cucumber mosaic virus* (CMV) infection on antineoplastic alkaloids from periwinkle (*Catharanthus roseus* L.) cultured in the Mecca region and resistance induction by plant-growth-promoting rhizobacteria (PGPR)

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**ABSTRACT**

More than 100 alkaloids have been found in periwinkle, of which vincristine and vinblastine are the most notable for the treatment of diseases such as leukaemia. In this study, characterization of naturally occurring *Cucumber mosaic virus* (CMV) infection showed mosaic, leaf deformation and stunting of plants. Viral identification was confirmed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with a specific CMV polyclonal antibody and reverse transcription PCR with a specific primer for the CMV-RdRp-gene, which yielded a 513 base pair DNA fragment. The effect of CMV infection on the antineoplastic alkaloids in periwinkle leaves was determined. We also studied the effect of using plant-growth-promoting rhizobacteria (PGPR) isolates against virus infection to stimulate resistance induction in host plants. *Bacillus subtilis* 281 and *B. pumilus* 293 were examined individually (B1 and B2) and in a mixture (B1&B2) for their effectiveness against infection with CMV. The results of greenhouse experiments were confirmed by artificial mechanical inoculation. The PGPR strain treatments and results were reinforced by analysing the protein patterns as well as determining the total phenol, total flavonoid and total alkaloid contents. PGPR treatment evidently lowered the virus concentrations, the percentage of infected plants and the disease severity compared with healthy and infected controls. Seedlings treated with the B1&B2 strain mixture yielded significantly lower levels of virus infection than B1 or B2 individually in all experiments compared to controls. Our findings demonstrate the possibility of using selected *Bacillus* spp. strains to induce systemic resistance for CMV infection control.

**Introduction**

The *Apocynaceae* family, which includes periwinkle (*Catharanthus roseus*, also known as *Vinca rosea*), produces active compounds such as alkaloids and tannins that have anticancer activities [1,2]. The active alkaloid compounds (vincristine and vinblastine) that are produced in the stems and leaves are necessary for cancer drugs, while the roots produce anti-hypertensive compounds [3–5]. Some viruses, viroids and phytoplasmas that infect *V. rosea* are widely distributed around the world and cause significant damage and economic losses [6].

Due to a very little highlighting of these plant diseases, very limited records of virus infections are available. *Cucumber mosaic virus* (CMV) infection causes specific symptoms in *C. roseus*, including chlorosis, mild mosaic and plant distortion [7]. CMV belongs to the family *Bromoviridae*, genus *Cucumovirus*. CMV is a +ssRNA virus and has been illustrated in more than a few hosts [8–11].

Vinblastine and vincristine have traditionally been obtained from *C. roseus*, and these antimicrotubule drugs are used to treat more than one type of cancer [5]. Viral infection leads to a decrease in total photosynthesis pigment, total soluble intracellular protein, total phenol content and the alkaloids catharanthine and vindoline in *C. roseus* leaves compared to healthy controls. To protect periwinkle plants from CMV infection and reduce alkaloid and vindoline losses, several plant protection approaches have been investigated such as biological control [12], systemically acquired resistance inducers and plant-growth-promoting rhizobacteria (PGPR) to manage plant pathogens [13–15].

PGPR induce plant growth promotion either directly or indirectly, and they may also exhibit biological activity against plant pathogens. The activity of PGPR against periwinkle viruses has not yet been investigated. Exploring new methods such as using PGPR to manage viral disease started in the last few years, and several reports
have already been published [16–20]. This work was carried out to identify viral infection via symptoms. Reverse transcription polymerase chain reaction (RT-PCR) was used to identify the CMV isolated from RNA extracts of infected periwinkle plants compared with non-infected plants. The effects of periwinkle CMV infection on total photosynthesis pigments, total soluble intracellular protein and total phenol content and the alkaloids catharanthine and vindoline in periwinkle leaves were compared to healthy control seedlings. In addition, we summarize the roles of PGPR on their effect on naturally and artificially infected periwinkle plants and their ability to protect healthy commercial periwinkle orchards in the Mecca region, as listed below.

The management of plants with PGPR can lead to induced systemic resistance (ISR) to stop viral multiplication and inhibit disease symptoms compared with healthy plants. This investigation was undertaken to evaluate the effects of two different PGPR strains, B1 and B2, and a B1&B2 mixture on virus multiplication and disease development in CMV-infected periwinkle plants in the Mecca region; to explore the possibilities for increasing the systemic resistance induced in periwinkle plants against CMV infection by PGPR in the Mecca region; increasing the productivity of periwinkle plants for alkaloids and vindoline; and to determine how to apply and use different concentrations of different PGPR isolates to curb the spread of this virus on periwinkle plants in the periwinkle orchards of various Mecca region areas.

Materials and methods

Diagnosis of the virus isolate by serological assay

No special permission to access periwinkle locations was needed. It is noteworthy to mention that the fields in the Mecca region that were the subject of this study did not include endangered or protected species.

CMV is a naturally occurring virus that infects periwinkle in nurseries, gardens, orchards and landscape plantings in the Mecca region. Samples of periwinkle plants showing mosaic, leaf deformation and plant stunting were collected during September of 2016 from several nurseries, orchards and landscape plantings growing in the Mecca region. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed according to the manufacturer’s instructions using CMV-specific antiserum (Bioreba AG Christoph Merian-Ring 7, CH-4153 Reinach BL1, Switzerland), as described by [21]. All samples were examined three times for confirmation. Plant samples that yielded a positive reaction were used as a source of CMV infection.

The extracted diluted sap of infected samples was used to inoculate the indicator host Chenopodium amaranticolor Coste & Reyn in preparation for CMV separation. To obtain the virus isolate in a pure form, the single local lesion technique was performed according to [22]. Periwinkle seedlings were inoculated with the infected juice of CMV as systemic hosts. Inoculated periwinkle seedlings were kept in separate cages and observed for symptom expression after 3–4 weeks from inoculation as a source of virus infection. Samples that yielded negative reactions on the ELISA assay were discarded.

Molecular detection of CMV by RT-PCR

Total nucleic acids (TNAs) extraction

Total nucleic acids (TNAs) were extracted from 100 mg of leaf veins ground in 1 mL of grinding buffer and silica purified according to [23].

cDNA synthesis

Samples of 8–10 μL of TNA were mixed with 1 μL of random hexamer primers. Reverse transcription was carried out for 1 h at 39 °C by adding 4 μL of 5 × M-MLV buffer, 2 μL of 10 mmol/L dithiothreitol (DTT), 0.5 μL of 10 mmol/L deoxyribonucleoside triphosphates (dNTPs), and 200 units of M-MLV reverse transcriptase (Bethesda Research Laboratories, USA) in a final volume of 20 μL.

PCR

The detection of CMV by RT-PCR was carried out using a set of specific primers, CMV-s 5′-TAACCTCCCGTCTTACCCGT-3′ and CMV-a 5′-CCATACCCATTAGCTCAGTGT-3′ for the CMV-RdRp gene, whose nucleotide sequences and use-conditions have previously been described [24]. Briefly, 2.5 μL of reverse-transcribed TNA mixtures were subjected to amplification with the addition of 2.5 μL of 10 × Taq polymerase buffer (Promega Corporation, USA), with a final concentration of 1.5 mmol/L MgCl₂ for a total volume of 25 μL. PCR products were analysed with electrophoresis, stained with ethidium bromide and examined on an ultraviolet (UV) transilluminator [25].

PGPR strains and inoculum preparation

A selected PGPR strain, B1, was isolated from a healthy tomato plant rhizosphere soil, and strain B2 was isolated from healthy cucumber rhizosphere soil [26]. The PGPR isolates were identified at the Biotechnology Lab, Agricultural Research Institute, Giza, Egypt. All bacterial isolates were grown in nutrient agar medium. The bacterial cells were transferred to nutrient-broth (NB) medium and incubated for 24 h at 37 °C, sub-cultured and re-suspended in 10 mL of sterile distilled water. The
concentration of bacteria was titrated to $10^8$ colony-forming units (CFU)/mL based on absorbance at 660 nm. The PGPR strains were stored in tryptic soy broth (TSB) adjusted with 15% glycerol at $-80^\circ C$ until use.

**Efficiency evaluation of specific PGPR isolates on periwinkle virus**

Periwinkle seeds of the Pacifica Polka Dot cultivar were germinated and developed in plastic pots (30 cm × 30 cm). The soil was mixed with the PGPR suspension ($1 \times 10^8$ CFU/cm³) at a ratio of 1:50 (v/v) immediately after seed planting (using a handheld sprayer until run-off) and then drenched once with 20 mL of the PGPR strain suspensions again when the plants reached the true-leaf stage (approximately 14 days from sowing the seeds).

Periwinkle seedlings were inoculated with CMV after seven days from the initial PGPR soil drenching. The CMV inocula were prepared from CMV-infected top periwinkle leaves that yielded a positive reaction on ELISA. The periwinkle seedlings were conserved in a greenhouse at 24–27°C. ELISA was used in addition to symptom observation to check for the presence of the virus.

Seedling pots were placed in a completely randomized block design with three seedlings per pot, three pots per replicate and three replicates per treatment, giving a total of 27 seedlings per treatment. The treatments were named as follows: (T1) healthy controls; (T2) healthy seedlings treated with B1; (T3) healthy seedlings treated with B2; (T4) healthy seedlings treated with B1 + B2 (at a ratio of 1:1); (T5) control seedlings infected with CMV; (T6) seedlings infected with CMV and treated with B1; (T7) seedlings infected with CMV and treated with B2; and (T8) seedlings infected with CMV and treated with B1+B2. At five weeks after CMV inoculation, similarly sized plants were selected and examined for growth and physiological parameters.

**Evaluation of changes in plants following viral infection after treatment with the virus and PGPR isolates**

Infection percentage and disease severity (DS) were recorded at one month after inoculation, according to the following scale: 0 = symptomless; 1 = light mottling and crinkling; 2 = mild mosaic and crinkling; 3 = severe mosaic, crinkling and size reduction; and 4 = severe mosaic and malformation. DS values were calculated using the following formula according to [18]:

$$DS(\%) = \frac{\text{disease grade} \times \text{number of plants in each grade}}{\text{total number of plants} \times \text{highest disease grade}} \times 100$$

The youngest leaves of both control and treated periwinkle seedlings were collected at one month after inoculation for analysis of changes.

**Protein pattern analyses**

**Total soluble protein extraction**

The leaves of periwinkle were collected from infected, healthy and treated plants and soil. Total soluble proteins were extracted in sodium dodecyl sulphate (SDS) reducing buffer. The sample was diluted at least 1:4 with sample buffer and centrifuged at 10,000 rpm for 20 min. Then, 10 μL of total soluble protein was taken for electrophoresis.

**Protein patterns**

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 10% acrylamide slab gels following the system of [27]. Electrophoresis was carried out with a current of 25 mA and 130 V per gel until the bromophenol blue marker reached the bottom of the gel after 3 h. After electrophoresis, the Coomassie Brilliant R250 staining method was used for protein bands and polypeptides.

**Total phenol content**

Total phenolic concentration was estimated by the Folin–Ciocalteu colourimetric method with methanolic plant extracts and absorbance at 765 nm [28]. The standard curve was prepared using different dilutions of gallic acid in methanol:water (50:50, v/v). The results were expressed in terms of gallic acid equivalent (GAE) mg/g.

**Total flavonoid content**

Flavonoid concentration was determined by an aluminium chloride colorimetric method with methanolic plant extracts and absorbance at 415 nm [29]. Total flavonoid content was determined by preparing a standard curve of quercetin at different concentrations in methanol (g/mL).

**Alkaloids**

Plant extracts (1 mg) were dissolved in dimethyl sulfoxide (DMSO). A set of reference standard solutions of atropine (20, 40, 60, 80 and 100 μg/mL) were prepared in the same manner, as described earlier. The absorbances of
the test and standard solutions were determined against the reagent blank at 470 nm with a UV/Visible spectrophotometer. The total alkaloid content was expressed as milligrams of GAE per gram of extract [30,31].

**Statistical analyses**

All statistical data were focused on the analysis of variance (ANOVA). All statistical tests were carried out using SPSS software.

**Results and discussion**

**Detection of CMV source and propagation**

Periwinkle leaves showing evidence of infection with typical symptoms of CMV infection, i.e. mosaic, leaf distortion and stunting of growth, were collected from several nurseries, orchards and landscape plantings of periwinkle plants growing in the Mecca region (Figure 1). Samples that yielded a positive reaction for CMV were used as a source of virus infection, and samples that yielded a negative reaction were discarded. Following consecutive single local lesions transfer on Chenopodium amaranticolor Coste & Reyn, the resulting virus isolate was propagated as a pure CMV source in a proliferation host such as periwinkle (paciifica Polka Dot cultivar). The symptoms that developed were observed on periwinkle leaves. This result was in agreement with [6,8,11].

**RT-PCR**

We detected the CMV-RdRp-gene in infected periwinkle seedlings by RT-PCR. The PCR fragment of the expected size of 513 bp was amplified. Electrophoresis of the PCR fragments amplified is shown in Figure 2. No bands were found in samples from healthy plants [9–11].

**PGPR application and CMV-infection symptoms**

The plants under study were observed for symptom propagation and severity at one month after inoculation. All the results of the PGPR treatments showed effective repression of CMV infection symptoms compared to the controls over two repeated experiments. Moreover, periwinkle leaves treated with the mixture of B1+B2 showed fewer symptoms than leaves treated with the B1 strain or the B2 strain individually. ISR has been documented as a promising means for managing plant diseases due to the efficiency of induced resistance against various pathogens occurring under authentic crop field conditions [32]. PGPR are among the different groups of plant-associated bacteria that can stimulate natural plant defences against bacterial and fungal plant pathogens [33–35]. However, only a small number of researchers have explored their activity against viral plant pathogens [36,37], and CMV has not been one of them.

**Virus concentration and DS**

Periwinkle infection by CMV caused increased virus concentration, percentage of infection and disease severity, while soil drenched with PGPR showed a significant reduction in CMV-related parameters compared to non-treated healthy and infected controls in all replicated greenhouse assays (Table 1). In addition, the infected
controls had an average significant disease severity that was higher than any of the other treatments, whereas treatment with the mixture of B1+B2 resulted in disease suppression of 85.18%, which was significantly higher than plants treated with B2 (74.07%) or B1 (66.67%) individually. Table 1 also shows the ELISA measured CMV concentration in young leaves of periwinkle seedlings at 21 days after inoculation. The indicated ELISA values for the leaf samples collected from each PGPR treatment were significantly lower than for the infected plants. The mixture of B1 + B2 and B2 treatments yielded a significantly lower concentration of CMV compared with B1 treatment.

Decreasing of virus symptoms by PGPR application has been previously reported [15]. According to Murphy et al. [38], PGPR can potentially induce ISR against CMV to reduce symptoms or reduce viral accumulation in infected periwinkle plants. Based on the obtained data, the two PGPR isolates were able to reduce virus infection by reducing CMV disease symptoms and severity, which indicates ISR. Inoculation with a mixture of the B1+B2 strains or individually provided satisfactory disease suppression.

**Protein pattern analyses**

The effects of CMV infection and PGPR treatments on many of the protein patterns in different periwinkle (pacific Polka Dot) cultivars were observed (Figure 3(A, B) and Table 2). All the PGPR treatments of healthy seedlings demonstrated novel protein subunit band numbers (21, 53 and 64) compared with healthy controls. The running factors and molecular weights (kDa) of these proteins were, respectively, 0.125, 185; 0.416, 65; and 0.647, 28. The existence of these protein bands in healthy plants treated with PGPR and their absence in healthy controls could be attributed to the effects of treatment with PGPR. In contrast, bands number 57 (0.537, 42), 65 (0.663, 27) and 72 (0.925, 10), respectively, were found in all healthy and all PGPR-treated healthy seedlings and were absent in all control infected and PGPR-treated infected seedlings. Infected plants and virus infection after PGPR treatment yielded novel bands of 42 (0.309, 96), 55 (0.503, 42) and 61 (0.622, 31), respectively, that were present in infected controls and all infected seedlings treated with PGPR, but were absent from all healthy seedlings treated with PGPR. The percentage of protein bands in infected and healthy seedlings treated with PGPR was higher than in untreated seedlings. The polypeptides accumulated to a greater extent depending on the PGPR treatments.

A high increase in the density of several newly synthesized polypeptide bands was detected in CMV-infected leaves that were believed to be pathogenesis-related proteins (PRPs) and were altered in response to virus infection and PGPR treatment in periwinkle leaves. Applications of PGPR treatment induced both resistance to CMV and the accumulation of PRPs in periwinkle leaves [20,39].

**Total phenol, flavonoid and alkaloid contents**

The total phenol and flavonoid contents reached 495.83, 477.31 and 441.63 mg/g fresh weight and 46.07, 44.37 and 40.15 mg/g fresh weight, respectively, in healthy periwinkle seedlings treated with B1+B2, B2 and B1, respectively, compared with healthy controls (294.29 and 36.90 mg/g fresh weight). In contrast, infected seedlings treated with different PGPR (B1+B2, B2 and B1) reached 467.51, 419.43 and 299.63 mg/g fresh weight and 43.17, 37.43 and 34.25 mg/g fresh weight, respectively, in infected leaves treated with PGPR higher than infected control seedlings (Figure 4(A,B)). Leaf cultivars treated with the B1 + B2 mixture showed a highly significant increase in phenolics and flavonoids in healthy and infected periwinkle seedlings that accumulated phenolics and flavonoids less than seedlings infected by virus and more than control seedlings (Figure 4(A)).

The data shown in Figure 4(C) show that the application of PGPR led to an increase in the alkaloid content of periwinkle leaf cultivars (pacific Polka Dot) compared to the viral control leaves (71.12 and 62.43 mg of AE/g of extract, respectively). The application of PGPR (B1+B2, B2 and B1) increased the alkaloid content in healthy and infected seedlings treated with PGPR compared to healthy and viral control seedlings (116.52, 111.29 and 103.48 in healthy treated seedlings compared to 110.03, 97.37 and 67.16 mg of AE/g of extract, respectively).

Phenolic, flavonoid and alkaloid compounds are important secondary metabolites present in plants, and possess high antioxidant activities that can help in the

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**Table 1. Effects of PGPR strains B1, B2 and B1+B2 on virus concentration, disease severity percentage and percentage of infection caused by CMV in periwinkle seedlings under greenhouse conditions.**

| Treatment          | Virus concentration | R1  | R2  | R3  | Percentage of infection (%) | DS % |
|--------------------|---------------------|-----|-----|-----|----------------------------|------|
| Control healthy    | 0.000               | 0/9 | 0/9 | 0/9 | 0.00                        | 0.00 |
| Control infected   | 1.475               | 9/9 | 9/9 | 9/9 | 100                         | 100  |
| CMV + B1           | 0.311               | 2/9 | 4/9 | 3/9 | 33.33                       | 17.59|
| CMV + B2           | 0.258               | 3/9 | 2/9 | 2/9 | 25.93                       | 10.18|
| CMV + (B1+B2)      | 0.181               | 1/9 | 1/9 | 2/9 | 14.82                       | 3.71 |

**Note:** Data are presented as the means of two trials each with 27 seedlings per treatment. In ELISA test for virus concentration, the positive and negative controls are 1.492 and 0.113, respectively. Positive control means of infected leaves showed symptoms typically, and negative control means of infected leaves showed no symptoms.
Figure 3. SDS–PAGE profile (A and B) showing the changes in protein patterns of periwinkle seedlings, healthy and infected with different species of PGPR. M, protein ladder marker; L1, control healthy periwinkle; L2, healthy seedling treated with \((1 \times 10^8 \text{ CFU/cm}^3)\) B1; L3, healthy seedling treated with \((1 \times 10^8 \text{ CFU/cm}^3)\) B2; L4, healthy seedling treated with \((1 \times 10^8 \text{ CFU/cm}^3)\) B1+B2; L5, infected periwinkle; L6, infected seedling treated with \((1 \times 10^8 \text{ CFU/cm}^3)\) B1; L7, infected seedling treated with \((1 \times 10^8 \text{ CFU/cm}^3)\) B2; and L8, infected seedling treated with \((1 \times 10^8 \text{ CFU/cm}^3)\) B1+B2.

Table 2. Hypothesized protein markers for determined effect of different PGPR treatments on protein bands in healthy and infected *Vinca rosea* seedlings.

| Bands No. | RF     | MW | Control healthy | Healthy treated with PGPR | Control infected | Infected treated with PGPR |
|----------|--------|----|----------------|---------------------------|-----------------|---------------------------|
| 21       | 0.125  | 185| -              | +                         | +               | -                         | -                         |
| 42       | 0.309  | 96 | -              | -                         | -               | -                         | +                         |
| 50       | 0.416  | 65 | -              | -                         | -               | -                         | +                         |
| 53       | 0.474  | 53 | -              | +                         | +               | -                         | -                         |
| 55       | 0.503  | 48 | -              | -                         | -               | -                         | +                         |
| 57       | 0.537  | 42 | +              | +                         | +               | +                         | +                         |
| 61       | 0.622  | 31 | -              | -                         | -               | -                         | -                         |
| 64       | 0.647  | 28 | -              | +                         | +               | -                         | -                         |
| 65       | 0.663  | 27 | +              | +                         | +               | -                         | -                         |
| 66       | 0.673  | 26 | -              | -                         | -               | +                         | +                         |
| 72       | 0.925  | 10 | +              | +                         | +               | -                         | -                         |
termination of free radicals. In addition, they play an important role in defence mechanisms against microbial pathogens based on their toxicity and repellence to microbes [40–43].

PGPRs are well recognized biotic elicitors owing to their ability to induce secondary metabolite synthesis in plants. Recent studies largely deal with the major role of PGPRs to improve the production of secondary metabolites, including terpenoids, phenolics, alkaloids and essential oils, including monoterpenes, sesquiterpenes and phenylpropanoids in plants [44,45].

Figure 4. Effects of PGPR strains treatments on alkaloids (A), total phenol (B) and flavonoids content (C) of periwinkle seedlings infected by CMV under greenhouse conditions.
Conclusions

In conclusion, two PGPR strains, B1 and B2, were examined for their capacity to induce resistance to virus infection in periwinkle seedlings. Individual treatment with B2 or in combination with B1 showed inhibitory activity against virus symptoms according to virus concentration and disease severity. Evaluation of total soluble proteins, novel protein bands; and total phenol, flavonoid and alkaloid contents revealed that the application of PGPR to leaves challenged with CMV did not decrease the total soluble proteins, although they were able to protect the plants. The results obtained suggest the potential for the two PGPR isolates to protect periwinkle seedlings against CMV through stimulating plants to produce PR proteins and activating ISR against virus infection by accumulating secondary metabolites including terpenoids, phenolics, alkaloids and essential oils.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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