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Antiviral activities of three *Streptomyces* spp. against *Zucchini yellow mosaic virus* (ZYMV) infecting squash (*Cucurbita pepo* L.) plants

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Abstract

Background *Zucchini yellow mosaic virus* (ZYMV) is the major devastating disease worldwide, which leads to substantial economic losses (up to 100%) to yield and fruits quality produced of squash plants. Application of agro-pesticides is efficient and incompatible with organic agriculture and reportedly has harmful effects on human health and ecosystem. Nowadays, *Streptomyces* spp., a rich source of potential bioactive secondary metabolites, is extensively used to manage various biotic stresses for sustainable agriculture and considered to be eco-friendly.

Results An isolate of ZYMV was isolated from squash plants and identified based on biological and molecular characterization using RT-PCR for several genes, i.e., coat protein gene (*CP*), *DAG*, *P1* and *P3* coding regions in the virus RNA, and then, nucleotide sequences were compared to other isolates submitted in GenBank having accession numbers, i.e., OM925548.1, OM925549.1, OM925550.1 and OM925551.1, respectively. Phylogenetic trees of *CP*, *DAG*, *P1* and *P3* sequences compared to other ZYMV nucleotide sequences presented in the GenBank. In order to determine new efficient substances elicitors derived from *Streptomyces* spp. to control ZYMV, greenhouse trials were designed with seven treatments including culture broth of three *Streptomyces* spp. (*S. sampsonii*, *S. rochei* and *S. griseus*) individually or in combinations. Early application of *Streptomyces* spp. revealed potent antiviral activity against ZYMV infection, inhibited virus replication and promoted plant growth as well as induced systemic resistance. Moreover, physiological stress markers as indicators for systemic acquired resistance were distinguished via significantly enhanced proline, phenols and defense-related enzymes, i.e., catalase, superoxide dismutase and glutathione peroxidase by culture broth treatments, despite the presence of infection. Real-time qPCR assay was a more reliable and accurate detection for quantification ZYMV than conventional PCR. The results revealed that the three *Streptomyces* spp. novel biocontrol agents produced Behenic alcohol (Docosanol) which provided clues to be potential antiviral mechanisms capable to down-regulate *P1* gene expression responsible for virus replication and movement from cell to cell to induce systemic infection as well as safe eco-friendly candidates for the controlling approaches against plant viral pathogens.

Conclusion Results suggest that the three *Streptomyces* spp. provided clues as a novel biocontrol agent having potential antiviral with protective activity and eco-friendly alternative pesticides for managing plant viruses.

Keywords Antiviral, Behenic alcohol, *P1* gene expression, Streptomyces, Biocontrol, RT-qPCR

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Background

Zucchini yellow mosaic virus (ZYMV; genus *Potyvirus* and family *Potyviridae*) is one of the most prevalent and destructive viral diseases that induces considerable economic losses in the major cucurbit crops production worldwide. Early ZYMV infection causes severe fruit yield losses and up to 100% yield reduction of marketable fruit in the tropical and subtropical regions (Clarke et al. 2020). The ZYMV genome consists of positive-sense single-stranded ssRNA of approximately 9600 nucleotides and encodes a polyprotein that is proteolytically processed into several mature proteins. These proteins included protease (*P1*), helper component/protease (*HC*), *P3*, cylindrical inclusions (*CI*), nuclear inclusion a, (NIa), viral protein linked genome (*VPg*), nuclear inclusion b (*NIb*), *CP* and *DAG* which have a highly-conserved region consisting of three amino acids Asp-Ala-Gly, sited in the N-terminus of the *Potyvirus CP* related to aphid transmission (Moradi et al. 2019). Various molecular techniques have been used to detect viral genomes, i.e., reverse-transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) (Singhal et al. 2021). Both PCR and RT-qPCR provide significantly greater specificity, sensitivity and rapid method for detecting virus than other methods. Additionally, RT-qPCR has the additional advantage that the virus can be quantified at low titer in the infected samples (Rodríguez-Verástegui et al. 2022).

Nowadays, overusing synthetic agro-pesticides to manage the pathogen and its vectors has caused many deleterious effects including environmental pollution, harm to human health and the emergence of resistance in the pathogen. At the same time, sustainable agriculture is considering for novel biocontrol agents and economic techniques to apply the agents or their natural bioactive metabolites in disease management strategies. Recently, *Streptomyces* has become an attractive potential agent and eco-friendly for sustainable agriculture, being a promising applicant for the biocontrol of several phytopathogens, i.e., fungi (Ayed et al. 2021), bacteria (Kaari et al. 2022), nematodes (Sholkamy et al. 2020) and viruses (Silva et al. 2022). *Streptomyces* spp. are well known as potential biological agents for controlling plant pathogens, since they are capable of producing various secondary metabolites including extracellular proteases enzymes, herbicides and huge number of antibiotics, antifungal, antibacterial, antiviral, antibiotics (Taha et al. 2021) and plant growth promoters, i.e., auxin, cytokinin and gibberellin (Boukhatem et al. 2022). Many researchers revealed that the applications of *Streptomyces* spp. for bio-controlling plant viruses are limited, and their potential mechanisms as antiviral agents are still unknown (Chen et al. 2022). Therefore, the present research aimed to: (a) characterize molecularly ZYMV, (b) evaluate efficiency of three *Streptomyces* spp. as a biocontrol agent against ZYMV infection and (c) investigate their efficacy in promote plant growth and inducing systemic resistance under greenhouse conditions.

Methods

The present work was conducted at the greenhouses of Dept. of Plant Pathology, Fac. of Agric. Two major experiments were conducted as part of the study methodology as follows: (a) survey, samples collection and characterization of *Zucchini yellow mosaic virus* (ZYMV) isolate in Giza Governorate and (b) evaluation the impact of culture broth of three *Streptomyces* species on ZYMV-inhibition and activates' plant defense responses in squash plants.

Source of virus isolate and detection

Samples suspected of being ZYMV naturally infected squash (*Cucurbita pepo* L. cv. Yara F_1) plants showing symptoms indicative of virus and heavily infected with insect aphids (*Myzus persicae*) were collected during the spring and fall seasons of 2019–20 from different locations, i.e., Mansouriya, Abu Ghalib, Al-Waraq and Dahshur belong to Giza Governorate, Egypt. *Chenopodium amaranticolor* as a diagnostic host plant was used for biological purification of the virus through a single local lesion technique repeated three times. Then, infectious sap was inoculated into squash plants and maintained in the greenhouse at 25 °C. Also, the virus was detected molecularly using reverse transcription polymerase chain reactions (RT–PCR), then inoculated into squash plants and kept as a source continuously maintained under greenhouse conditions.

Molecular characterization of ZYMV isolate using RT-PCR

Extraction of RNA and RT-PCR

Total RNA was extracted from ZYMV infected squash leaves cv. Yara F_1, according to the manufacturer's protocol of RNA Kit (Geneaid, Taiwan). To amplify various genomic regions, *Verso* one-step RT-PCR Reddy Mix Kit protocol (Thermo Scientific, USA) was used according to the manufacturer's instructions. Four primer pairs were used for the detection of four genes as shown in Table 1. Three different RT-PCR approaches were used to detect ZYMV, through amplification of the *CP*, *P1*, *P3* and *DAG* coding targeting regions in RNA. To detect *P1* and *P3* coding regions, the primers used for PCR amplification were ZY229F/ZY838R and ZY2715F/ZY3385R, respectively, as designated by Glasa et al. (2007). RT-PCR reactions were performed using the following cycling conditions:

Table 1 The used primers in RT-PCR amplification

Primer	Sequence (5′ → 3′)	Position in genome	Reference
ZY229F	AGTGGCACCTGGCCACATGGC	*P1* (nt 229–838)	Glasa et al. (2007)
ZY838R	CATCTCAGTGTGCCGCATTCG		
ZY2715F	TGATAAGCGAAGCTTCACCAC	*P3* (nt 2,715–3,385)	
ZY3385R	GTATATGGGCGCATCATCCTG		
DAG-F	ATT TGC GCT GCG ATG	*DAG* (nt 8735–749)	Hosseini et al. (2007)
DAG-R	GCG TGG CAA TGA CAT		
ZYU-F	CTCCATACATAGCTGAGACAGC	*CP* (nt 8372–9593)	Choi et al. (2002)
ZYD-R	AGGCTTGCAAACGGAGTCTAATC		

cDNA synthetize was performed at 50 °C/15 min, followed by initial denaturation at 94 °C/5 min, 35 cycles at 94 °C/min, 54 °C/45 s, 72 °C/1 min, and final extension at 72 °C/10 min, while to detect ZYMV sequences for *DAG* coding region, the primers were used for RT-PCR amplification according to Hosseini et al. (2007). RT-PCR reactions were performed at 50 °C/15 min to synthetize cDNA, 94 °C/3 min traced by 35 cycles at 94 °C/30 s, 43 °C/30 s and 72 °C/30 s, then a final elongation step at 72 °C/7 min. Meanwhile, to detect ZYMV sequences for coat protein-coding region (*CP*), the ZYUF/ZYDR primer pairs were used for RT-PCR targeting amplicons as designated by Choi et al. (2002). Amplicons of four genes separately were electrophoresed in agarose gel (1.5%) using 50 bp DNA Ladder for *CP*, *P1* and *P3* genes (GeneDireX, USA) or 100 DNA Ladder for *DAG* gene (Biomatik, USA) as molecular weight markers, then stained with EZview stain (Biomatik, USA), analyzed by electrophoresis and visualized as well as photographed under UV illumination.

Nucleotide sequence and phylogenetic analysis

PCR amplicons of the ZYMV isolate were purified using the Geneaid Gel and PCR Clean-Up System (Geneaid, Taiwan) for sequencing. Sequences of the nucleotides for *P1*, *P3*, *DAG* and *CP* genes, i.e., ~600, ~670, ~458 and ~1221 bp, respectively, were performed using 3500 Genetic Analyzer (Applied Biosystems) at Colors Medical Labs for Research, Cairo, Egypt. Sequences analyses of the resulting nucleotides were assembled and analyzed using DNAMan Ver.7 program. Deduced amino acid sequences were obtained using an online translation tool (https://web.expasy.org/translate). Nucleotide and protein sequence data were subjected to sequence similarity searches against the GenBank database using the BLAST program. Phylogenetic trees were constructed after multiple sequence alignments using Clustal W embedded in the DNAMan Ver.7 program.

Impact of *Streptomyces*-derived substances in activation plant defense responses against ZYMV infection

In this context, three *Streptomyces* spp., i.e., *S. sampsonii* (MN700191 "DG1"), *S. rochei* (MN700192 "DG4") and *S. griseus* (MT210913 "DG5"), were previously characterized molecularly (Gebily et al. 2021) and by Gas chromatography–mass (GC–Mass) analysis, as well as applied in the field as described by Ghanem et al. (2022). These *Streptomyces* spp. were applied to induce systemic resistance, activate plant defense responses against ZYMV infection and promote squash growth.

Inocula preparations of Streptomyces species

The inoculum of each Streptomyces isolate was prepared as follows: *S. griseus*, *S. rochei* and *S. sampsonii* were grown for 7-days on liquid starch casein medium to prepare culture broth. Culture broth was contained all the components of media, i.e., cell-free extract, mycelia and spores. All *Streptomyces* spp. were applied at the rate of 50 ml/1-l water (1 ml contained 15×10^6 cfu). To prepare 1 Liter of spray solution, add 50 ml culture broth, potassium soap (0.5%) and 5% acacia gum (Arabic gum) and then complete using sterilized distilled water (Gebily et al. 2021).

Greenhouse experimental design and Streptomyces bioactivity assay

This experiment was carried out in a greenhouse during two successive spring seasons (2021 and 2022). The purpose of these trials was to assess the efficiency of Streptomyces as a bioagent for foliar application and/or as bio-priming of seeds when treated either individually or in mixtures on squash cv. Yara F_1. Squash seeds were treated with culture broth for 16 h before sowing. Mechanically inoculated squash plants exhibiting ZYMV symptoms were used as virus source. Plants were applied by Streptomyces culture broth and then kept for 48 h

before inoculation with ZYMV-infectious sap. Application of culture broth was carried out 2 days before ZYMV inoculation on the first true leaf and then reapplied 3 times (each 10 days). Randomized Complete Block Design (RCBD) was conducted using ten replicates/treatment. Each experimental unit comprised three pots (two plants/pot). The experiment was designed eight treatments as follows:

- T1: Seed priming in a 5% suspension for *S. sampsonii*, then spray the plants with the same concentration.
- T2: Seed priming in a 5% suspension for *S. rochei*, then spray the plants with the same concentration.
- T3: Seed priming in a 5% suspension for *S. griseus*, then spray the plants with the same concentration.
- T4: Seed priming in a 5% suspension of the three species combination, then spray the plants with the same concentration.
- T5: Seed priming in water, then spray the growing plants with a 5% mixture suspension of the three species.
- T6: Seed priming in a 5% suspension of the three species combination, then spray the plants with water.
- T7: Soak the seeds in water, and then, spray the growing ZYMV-infected plants with water as a positive control (P.C.).

Evaluation of disease severity and plant characteristics

All the following measurements, i.e., disease severity, morphological characteristics, physiological and metabolic changes, were conducted on the ZYMV-infected squash plants.

Disease severity (DS) and infection rate (IR)

The severity of ZYMV symptoms was evaluated 15 days after inoculation and was repeated weekly on a 0–5 scale as described by Sofy et al. (2014), while infection rate for each treatment was estimated weekly according to the following equations (Yang et al. 1996):

$$\mathrm{DS}(\%) = \frac{\sum(\text{Number of plants of each grade} \times \text{disease grade})}{(\text{Total number of plants} \times \text{the highest grade})} \times 100$$

$$\mathrm{IR}(\%) = \frac{\text{Number of infected plants}}{\text{Number of total evaluated plants}} \times 100$$

Morphological characteristics of the treated plants

Plant parameters such root length, leaves number, stem diameter and length were measured 25 days post-inoculation. Furthermore, a pot from the first replicate was taken for each treatment, both plants were totally removed and rinsed and the roots were completely displaced from the soil to measure their lengths and the height and number of leaves/plant.

Physiological and metabolic changes of the treated plants

In this context, leaf content of photosynthetic activity and biosynthesis of assimilatory pigment chlorophyll content (total chlorophyll, Chlorophyll a (Chl a) and chlorophyll b (Chl b) as well as metabolic changes containing proline contents and total phenolic content were measured along with the biosynthesis of antioxidant enzymes, i.e., catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the ZYMV-infected squash plants (Table 2). Enzymes measurement and Real-time PCR were conducted in Hormone & Immunology and Biotechnology Labs., respectively, at Research Park (CURP), Fac. of Agri., Cairo Univ., Egypt.

Real-time qPCR assay and data analysis

One-step real-time qRT-PCR assay

This trial was conducted to measure virus titer inside the Streptomyces-treated and ZYMV-infected squash plants. The synthesis of cDNA was performed using Thermo Scientific RevertAid Reverse Transcriptase kit (Thermo Scientific, USA) according to the manufacturer's instructions. Total RNA was extracted using Plant Virus RNA Kit (Geneaid-Taiwan) according to the manufacturer's protocol. The extracted RNA concentration was measured using NanoDrop™ One/One^{C} Microvolume UV–Vis Spectrophotometer (Thermo Scientific, USA). Three microliters (µl) from both *P1* forward primer (ZY229F) and *P1* reverse primer (ZY838R), two µl distilled water and 4.5 µl extracted RNA were added together in Eppendorf for each sample. In the second step, 5X Reaction Buffer 4 µl, Thermo Scientific™ RiboLock RNase Inhibitor (#EO0381) 0.5 µl (20U), dNTP Mix, 10 mM each (#R0191) 2 µl (1 mM final concentration), RevertAid Reverse Transcriptase 1 µl (200U) were added and mixed gently. The samples were incubated at 42 °C/60 min, then terminated the reaction by heating at 70 °C/10 min and kept in ice. Direct PCR assays were performed with *P1* primer pair (ZY229F and ZY838R) for cDNA. One µl of cDNA synthetase was used in 25 µl total PCR reaction mixture contained 25 pmol of each primer; 12.5 µl ama*R* OnePCR master mix (GeneDirex ready-to-use) Cat. No. SM213-0250. The DNA amplification was started with a denaturation step at 94 °C/3 min

Table 2 Formula equations and reference of the metabolic changes used in the present manuscript

Metabolic change and Formula equation	References
Photosynthetic pigments Total Chlorophyll (mg/gFW) = Chlorophyll a + Chlorophyll b Chlorophyll a (mg/gFW) = (12 * A663) – (3.11 * A646) Chlorophyll b (mg/gFW) = (20.78 * A646) – (4.88 * A663) Carotenoid (mg/gFW) = (1000 * A480 – 1.12 * Chl a – 34.07 * Chl b)/245	Wellburn (1994)
Proline content μmoles Proline/g of fresh weight = $\frac{\mu g Proline/ml\ X\ ml\ Toluene}{115.5 \mu g/\mu mole}$ Xg sample/5	Bates et al. (1973)
Catalase Activity (CAT) CAT Activity (U/L) = $\frac{^{A}standard - ^{A}sample}{^{A}standard}$ X 1000	Aebi (1984)
Superoxide dismutase (SOD) Percent inhibition = $\frac{^{A}_{\Delta}control - ^{A}_{\Delta}sample}{^{A}_{\Delta}control}$ X 100 Where: $\Delta\ ^{A}$control = The change in absorbance at 560nm over 5min. following the addition of PMS to the reaction mixture in the absence of sample: $\Delta\ ^{A}$ sample = The change in absorbance at 560nm over 5min. following the addition of PMS to the reaction mixture in the presence of sample SOD Activity (U/ml) assessed according to that equation: U/ml = % Inhibition *3.75	Nishikimi et al. (1972)
Glutathione peroxidase (GPx) GPx Activity (mU/ml) = $\frac{A\ 340\ /\ min}{0.00622}$ X 121 X Dilution factor	Paglia and Valentine (1967)

followed by 35 cycles consisting of denaturation at 94 °C/1 min, then annealing at 52 °C/1 min and extension at 72 °C/2 min followed by 72 °C for 5 min. All PCR products were electrophoresed in 1.5% agarose gel with 50 bp DNA Ladder (Biomatik-USA) as a marker, then stained with EZview stain and analyzed by electrophoresis. The gel was visualized and photographed on UV-illuminator.

Real-time PCR process

A reaction master mix was prepared by adding the components (except template DNA) for each 20 µl reaction in a tube at room temperature as follows: ten µl Maxima SYBR Green/ROX qPCR Master Mix (2X), 1 µl from both forward primer and 1 µl reverse primer, 2 µl template cDNA and 6 µl distilled water, nuclease-free for total volume 20 µl. The master mix was mixed thoroughly and dispensed appropriate volumes into PCR tubes or plates and then gently mixed the reactions. Centrifuge briefly if needed. The samples were placed in the thermal cycler following programmed the device (thermal cycler BIO-RAD T100 Thermal Cycler, Singapore) according to the recommendations. Two-step cycling protocol was done as follows: one cycle for 10 min to initial denaturation at 95 °C, followed by 40 cycles at 43 °C/15 s. Annealing/extension time and temperature were 60 s/60 °C. Fold change for each sample was calculated using ΔΔCt equation as the following**:**

$$\text{Fold change for each sample} = 2^{-\Delta\Delta Ct}\ \text{value}$$

Data analysis

Collected data of the two seasons (2021 and 2022) were checked for normality using the Shapiro and Wilk test (1965). According to Wickens and Keppel (2004), ANOVA of the four-replicate randomized complete block (RCBD) was performed for each season. Significant differences among combined means of treatments were evaluated at a 5% probability ($p \leq 0.05$) level using Duncan's multiple range tests (Wickens and Keppel 2004). Statistically analysis of data was calculated by MSTAT-C V.2.1 (Michigan State Univ., Michigan, USA).

Results

The naturally infected squash plants grown at different locations within Giza governorate, Egypt, showed various symptoms of Zucchini yellow mosaic disease including mottling, vein banding, mild and severe mosaic, blisters, malformation and stunting (Fig. 1a). The virus was identified as ZYMV based on symptomology reaction of diagnostic host and virus particles using transmission electron microscopy (TEM) as well as DNA-based technique such reverse transcription polymerase chain reaction (RT-PCR).

Biological and physical detection of ZYMV

The isolated virus proved to be infectious to many cucurbit crops. Further, it produced systemic symptoms, i.e., blisters on leaves and fruits as well as stunting on *Cucurbit pepo* L., cvs. Yara F1 (Fig. 1b) and Eskandrani,

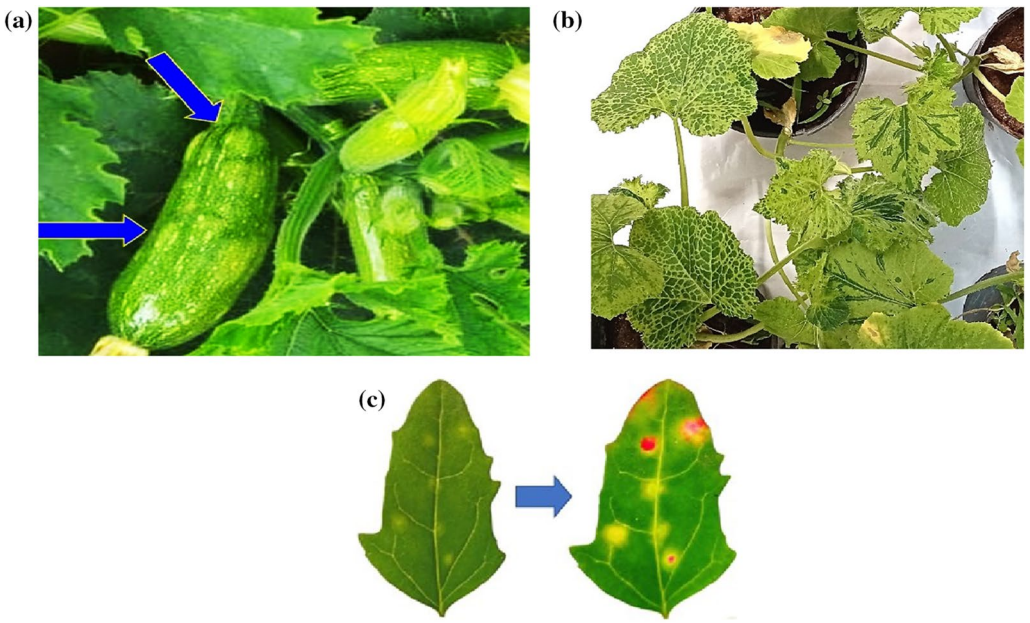


Fig. 1 a *Zucchini yellow mosaic virus* naturally infected plants exhibited several symptoms including blisters on the fruits. **b** *Zucchini yellow mosaic virus* produced vein banding, mosaic and systemic raised green blisters on leaves of mechanically inoculated squash *Cucurbita pepo* cv. Yara F1. **c** *Zucchini yellow mosaic virus* developed chlorotic local lesions symptoms on test plants Ch. Amaranticolor

mosaic on *Cucumis sativus* L. cv. Beta Alpha, *Cucurbita moschata* Duchesne "Pumpkin" and *Luffa aegyptiaca* Mill. ZYMV developed chlorotic local lesions symptoms on diagnostic host plants *Ch. amaranticolor* (Fig. 1c). Regarding physical detection, electron micrographs of partially purified preparation of ZYMV revealed the presence of flexuous particles (Fig. 2). The modal size of particles stained with 2% phosphotungestic acid was found to be 763X12.8 nm in diameter.

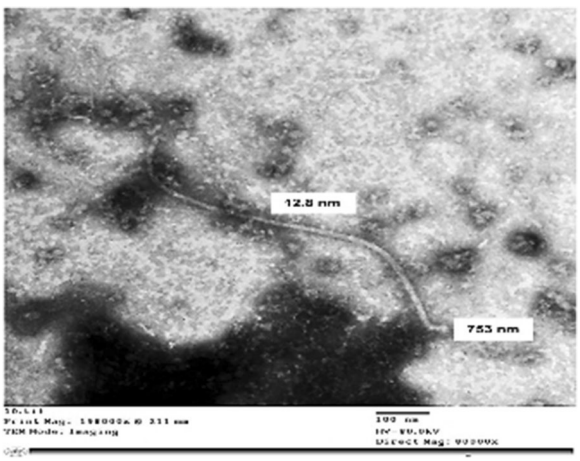


Fig. 2 Transmission electron microscopy reveals the presence of filamentous flexuous virus particles measuring approximately 750 × 13 nm, which are typical of the genus Potyvirus

Detection of ZYMV using RT-PCR

RT-PCR approach was conducted in total RNA extracted from ZYMV-infected squash leaf using specific primers pairs for each of the *CP, DAG, P1* and *P3* genes. The molecular length of the amplicons were approximately 1221, 458, 600 and 670 bp for partially *CP*, *DAG, P1* and *P3* genes, respectively (Fig. 3a, b, c and d).

Sequence alignment, primer specificity and phylogenetic tree

The sequences of the isolate's four genes (*CP, DAG, P1* and *P3*) were deposited to NCBI GenBank (National Central for Biotechnology Information) under accession numbers, i.e., OM925548.1, OM925549.1, OM925550.1 and OM925551.1, respectively. Phylogenetic tree of genes' sequences such *CP, DAG, P1* and *P3* was designed to compare the ZYMV isolate with other isolates either in Egypt or in other countries. Phylogenetic tree and alignment program using Clustal W inserted in the DNA-Man Ver.7 program based on the above-mentioned gene sequences *CP* (1221nt), *DAG* (458nt), *P1* (600nt) and *P3* (670nt) showed the close and distance relationships between the nucleotide sequences in the present study with the nucleotide sequences of available strains published in the NCBI GenBank as shown in Fig. 4a–d. Phylogenetic tree of the isolate identified in the present study shared range of nucleotide sequence identity between 87.1–95.6% for *P1* and 93.3–97.7% for *P3* with the other

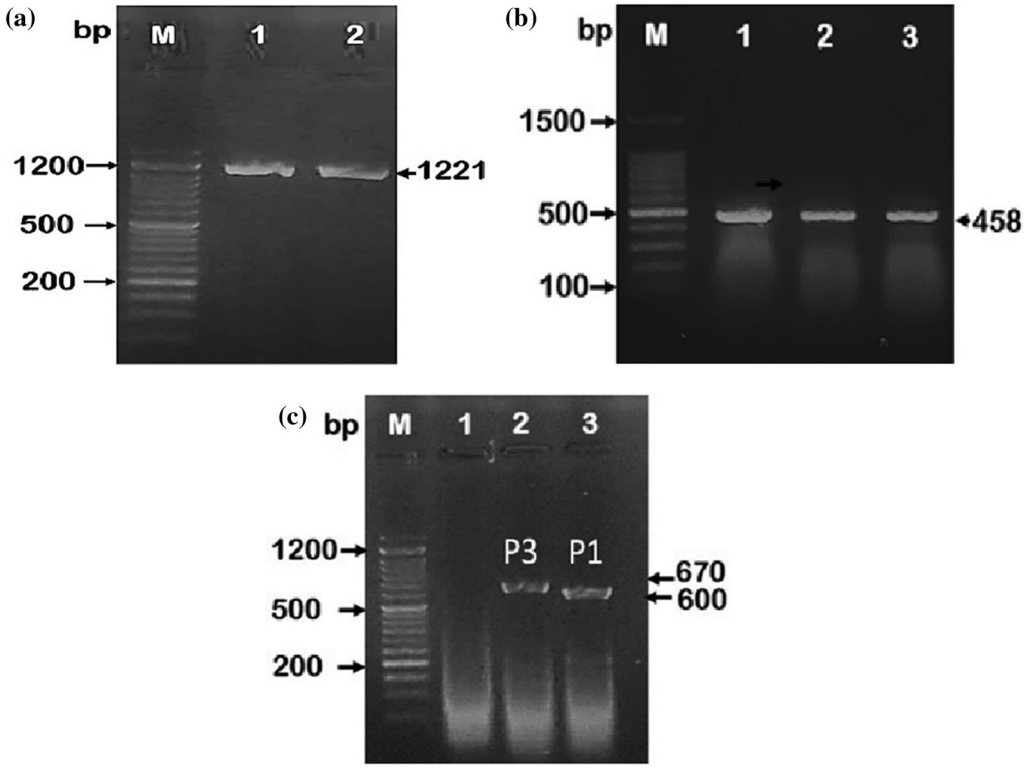


Fig. 3 **a** Electrophoretic gel analysis of PCR products of RT-PCR product amplified with ZYU-F/ZYD-R primer pairs for artificially infected plants with *Zucchini yellow mosaic virus*. Lanes 1 and 2 are PCR amplicons of partially coat protein gene (1221 bp) and M indicates 50 bp DNA ladder. **b** Electrophoretic gel analysis of PCR products of RT-PCR amplicons with AGF/DAG-R primer pairs for artificially infected plants with *Zucchini yellow mosaic virus*. Lanes 1, 2 and 3 are PCR amplicons of DAG gene (458 bp), and M indicates 100 bp DNA Ladder. **c** Electrophoretic gel analysis of PCR products of RT-PCR of *Zucchini yellow mosaic virus* P1 and P3 genes from artificially infected plants with ZYMV. Lane 1 healthy plant. Lane 2 and 3 are PCR amplicons of genes *P1*(600 bp) and *P3* (670 bp). M indicates 50 bp DNA Ladder

representative isolates submitted in the GenBank, while it shared 75.9–100 and 38.2–97.7% sequence identity for *CP* and *DAG* genes with the other isolates published in the GenBank.

Impact of Streptomyces substances application on inducing systemic resistance in squash plants against ZYMV infection

Three *Streptomyces* spp. substances (culture broth, elicitor) were applied either individually or in mixtures to induce systemic resistance (SR), activate plant defense responses against ZYMV infection and promote squash growth under greenhouse conditions. Then, disease severity (DS), infection rate, (IR) morphological characteristics, physiological and metabolic changes were measured in the Streptomyces-treated squash plants and infected with ZYMV. Finally, qRT-PCR was utilized to measure virus titer within squash plants.

Disease severity, infection rate and growth parameters

Virus symptoms development started 5 days post-ZYMV inoculation (dpi) and continuously observed up to 30 dpi, compared to very weak symptoms development 10 days dpi in the *Streptomyces*-treated/ZYMV-infected plants, indicating the protective role of *Streptomyces* substances. The result of three *Streptomyces* spp. application either individually or in mixtures decreased the percentages of disease severity (DS) caused by ZYMV. Result of T4 application (seed treated with mixture of three *Streptomyces* culture broths plus foliar application) decreased the percentages DS to 23.18 and 17.06% than 92.19 and 94.31% for T7 (control treatment only ZYMV-infected plant) in both seasons 2021 and 2022, respectively. Application of T3 (*S. griseus*) was the best second treatment, followed by T2 (*S. rochei*), which decreased percentages of disease severity to 42.81, 32.50% and 45.91, and 32.08% than T7 (control) in 2021 and 2022, respectively. Regarding infection rate (IR), the results indicated that the infection rates were reduced to 12.60, 24.38 and 40.31% in season 2021 and 32.90, 12.50 and 25.16% in season 2022, when ZYMV-infected squash plants were treated by T4, T3 and T2, compared to T7 (control) 99.56 and 95.63%, respectively. The individual treatments (T5 and T6) either spraying with *Streptomyces* spp. or seed priming

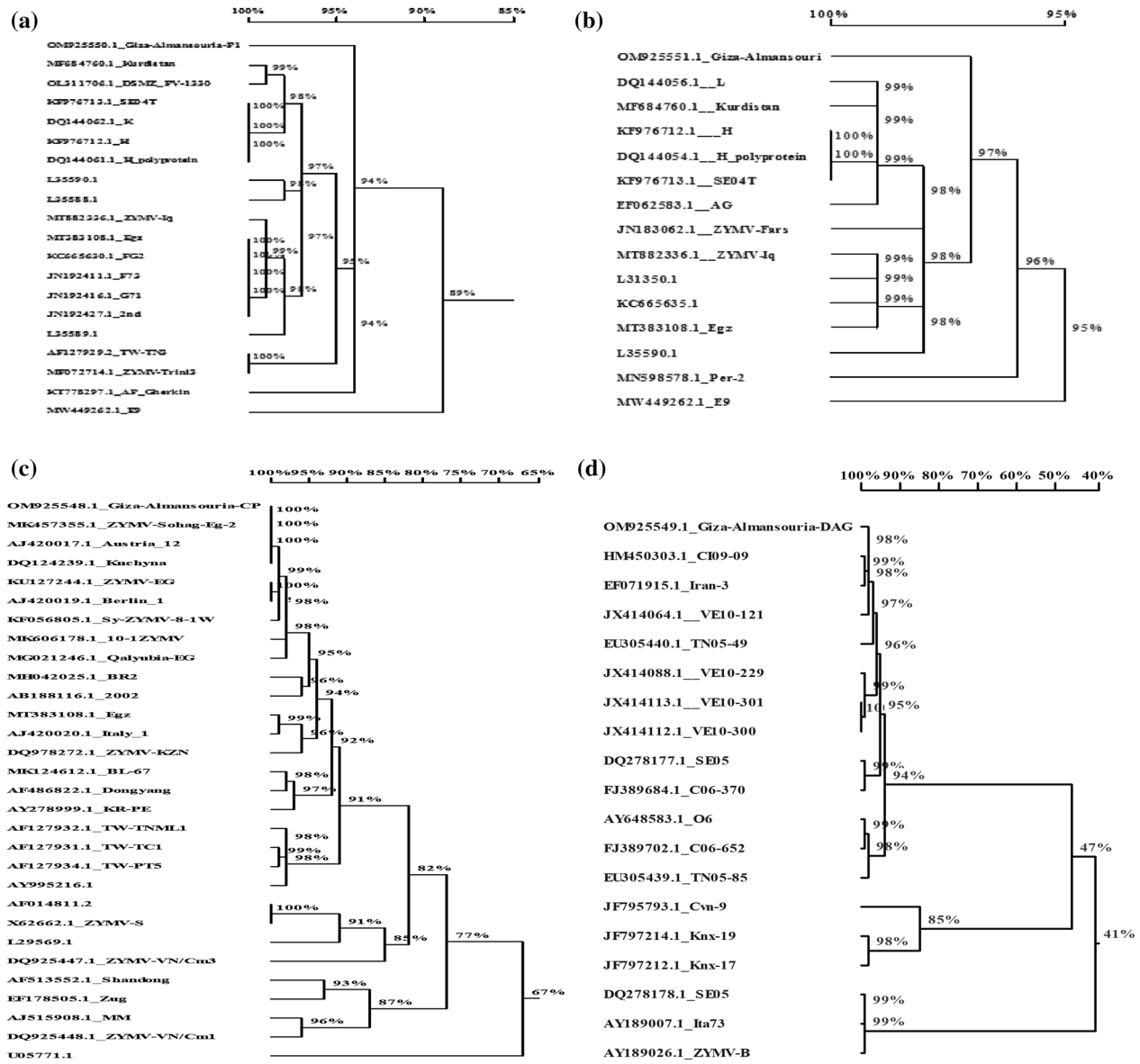


Fig. 4 **a** Phylogenetic tree of *Zucchini yellow mosaic virus* P1 gene nucleotide sequences (600nt) of ZYMV. The tree was generated using DNAMan V.7 program using method of neighbor joining (1,000 bootstrap replicates) (accession number = OM925550.1). **b** Phylogenetic tree of *Zucchini yellow mosaic virus P3* gene nucleotide sequences (670nt) was conducted using DNAMan V.7 program using method of neighbor joining (1,000 bootstrap replicates) (accession number = OM925551.1). **c** Phylogenetic tree derived from the CP nucleotide sequences (1221nt) of ZYMV. The tree was generated using DNAMan V.7 program by the neighbor-joining method with 1,000 bootstrap replicates. (CP gene: OM925548.1). **d** Phylogenetic tree derived from the DAG nucleotide sequences (458nt) of ZYMV. The tree was generated using DNAMan V.7 program by the neighbor-joining method with 1,000 bootstrap replicates. (DAG gene: OM925549.1)

only were not effective in decreasing ZYMV infection in both of IF and DS during two seasons compared to T7 (control) (Figs. 5 and 6).

Concerning measurements of morphological characteristics of the treated plants, the result revealed that T4 followed by T3 were the best treatments caused a significant increase in plant growth through the two seasons (2021 and 2022) in root length, plant length and leaves number of ZYMV-infected squash plants (Figs. 7, 8, 9 and 10).

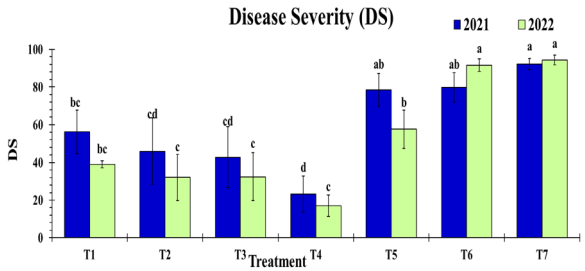


Fig. 5 Influence of applying *Streptomyces* spp. on disease severity of ZYMV-inoculated plants

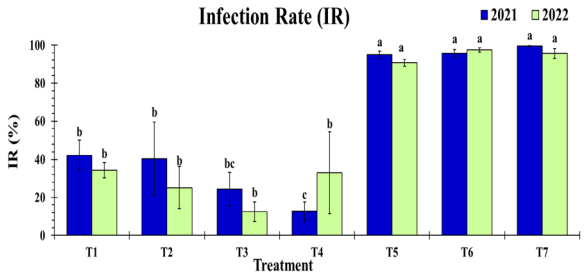


Fig. 6 Influence of applying *Streptomyces* spp. on infection rate in inoculated plants with ZYMV

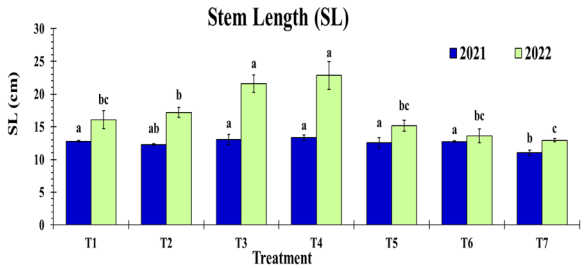


Fig. 7 Impact of three Streptomyces species treatments on morphological characteristics (stem length) of squash plants (Yara f1) with ZYMV infection

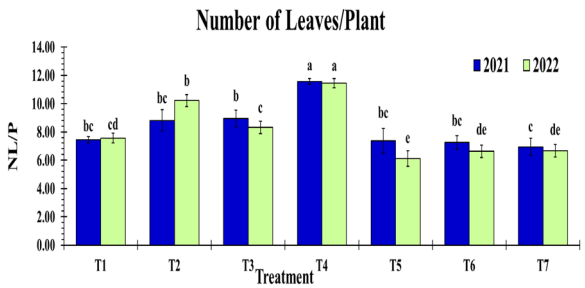


Fig. 8 Impact of three Streptomyces species treatments on number of leaves/plant of squash plants (Yara f1) with ZYMV infection

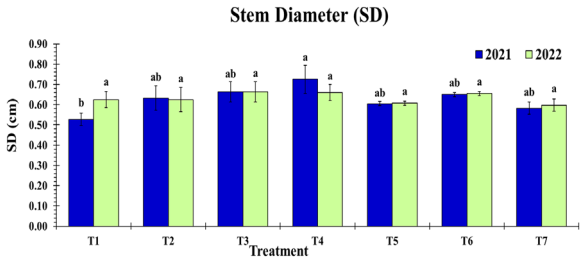


Fig. 9 Influence of three Streptomyces species treatments on stem diameter of squash plants (Yara f1) with ZYMV infection

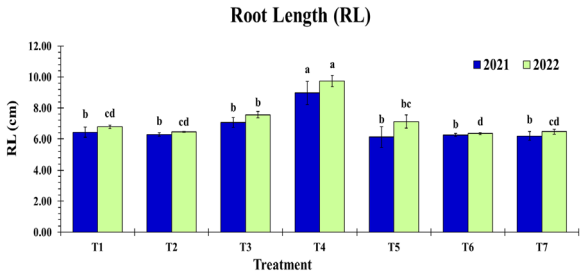


Fig. 10 Effect of three Streptomyces species treatments on root length of squash plants (Yara f1) with ZYMV infection

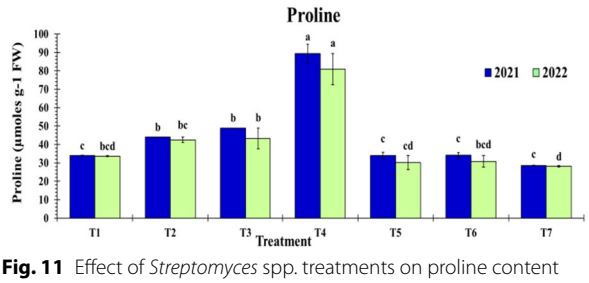


Fig. 11 Effect of *Streptomyces* spp. treatments on proline content in squash plants (Yara F1) at one month post-mechanical inoculation (Pmi) with ZYMV

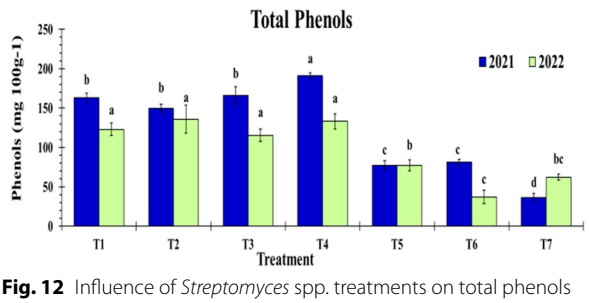


Fig. 12 Influence of *Streptomyces* spp. treatments on total phenols content in squash plants (Yara F1) one month after mechanical inoculation (Pmi) with *Zucchini yellow mosaic virus*

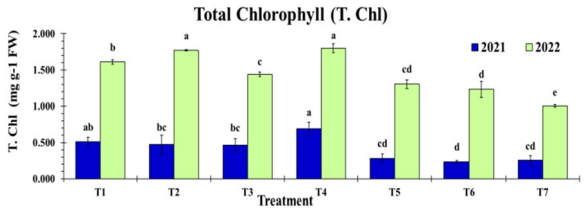


Fig. 13 Effect of *Streptomyces* spp. treatments on the quantity of leaves total chlorophyll in squash plants (Yara F1) one month Pmi with *Zucchini yellow mosaic virus*

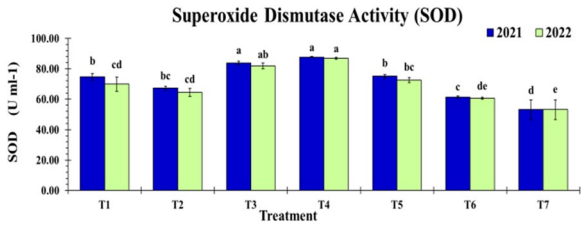


Fig. 14 Effect of *Streptomyces* spp. treatments on the enzyme activity of SOD in squash plants (Yara F1) one month Pmi with *Zucchini yellow mosaic virus*

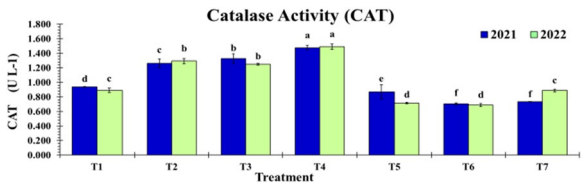


Fig. 15 Impact of *Streptomyces* spp. treatments on the enzyme activity of catalase in squash plants (Yara F1) at one month Pmi with *Zucchini yellow mosaic virus*

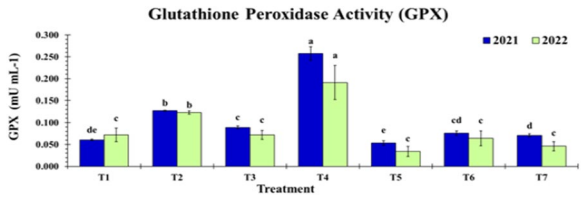


Fig. 16 Influence of *Streptomyces* spp. treatments on enzyme activity of GPX in squash plants (Yara F1) one month Pmi with *Zucchini yellow mosaic virus*

Physiological and metabolic changes

Physiological and metabolic changes of squash plants were measured after the application either individually or in mixture of three *Streptomyces* spp. The changes of some physiological and metabolic parameters included: proline, total phenolic, chlorophyllous pigments and antioxidant enzymes activities (CAT, SOD and GPx), were measured in the ZYMV-infected plants and are illustrated in Figs. 11, 12, 13, 14, 15 and 16.

Impact of Streptomyces treatments on proline level Regarding amino acid proline content (µmoles g^{-1} FW), the *Streptomyces*-treated infected had the highest values when they were applied with T4 (89.27 ± 5.1 and 80.91 ± 8.4), followed by T3 (48.8 ± 0.13 and 43.12 ± 5.6), T2 (43.96 ± 0.03 and 42.43 ± 1.5) and T1 (33.97 ± 0.16 and 33.64 ± 0.31), respectively, in both seasons (2021 and 2022) than T7 (control, 28.45 ± 0.20) (Fig. 11).

Determination of total phenols and chlorophyll The results of the plant analyses included total phenolic compounds (mg/100 g^{-1}) are illustrated in Fig. 12. Results revealed that the best treatments induced a significant increase were T4 (191.2 ± 3.26 and 133.1 ± 9.73), followed by T3 (165.8 ± 11.0 and 115.4 ± 7.86), T1 (162.8 ± 6.33 and 122.9 ± 7.9) and T2 (149.7 ± 5.0 and 135.9 ± 17.66), respectively, through the two seasons (2021 and 2022) than T7 (control, 62.26 ± 3.99). Concerning, the measurements of total chlorophyll (mg/g^{-1} FW) in the *Streptomyces*-treated/ZYMV-infected plants compared to the control treatment (T7) are displayed in Fig. 13. Applying the mixture treatment (T4) revealed the highest quantity of (1.800 ± 0.06 mg/g^{-1} FW), followed by T2 (1.770 ± 5.0), T1 (1.610 ± 0.03) and T2 (1.438 ± 0.01) in season 2022 than the control T7 (1.003 ± 0.02).

Effect of Streptomyces treatments on antioxidant enzymes activity The activity of enzymes (CAT, GPx and SOD) increased significantly after application of *Streptomyces* culture broth. Applying T4 (mixture of the three *Streptomyces* culture broth) achieved the highest activity of SOD, reaching 87, 86 U/ml than 53 U/ml (in both seasons) for the control (T7) during seasons 2021 and 2022, respectively. Also, applying each of *Streptomyces* culture broth separately, i.e., T3, T1 and T2 resulted in 83, 81; 74, 69 and 67, 64 U/ml in the seasons 2021 and 2022, respectively (Fig. 14). Regarding CAT activity (U/l), spraying each of T4, T3, T1 and T2 increased CAT activity to 1.475, 1.327, 0.940 and 1.263 U/l than 0.733 (T7) in season 2021, respectively. Meanwhile, same treatments in season 2022 resulted in 1.490, 1.250, 0.890 and 1.293 U/l compared to 0.888 (T7), respectively (Fig. 15). Conferring, GPx activity was recorded as 0.258 ± 0.0154, 0.128 ± 0.001, 0.089 ± 0.003 and 0.061 ± 0.001 mU/ml in Streptomyces-treated infected plants, i.e., T4, T2, T3 and T1, respectively, in season 2021. Approximately, similar results were recorded in season 2022 (Fig. 16).

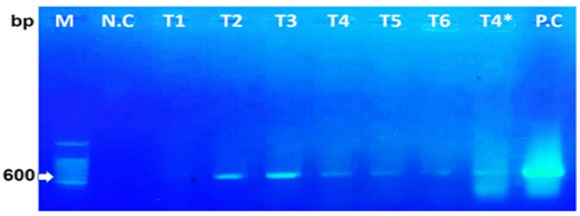


Fig. 17 RT-PCR detection of *Zucchini yellow mosaic virus—P1* gene expression in *Streptomyces*-treated resulted in virus titer among different treatments. M indicates 50 bp DNA Ladder, Lanes = T1-T6 amplicons of *P1* genes (600 bp) of the Streptomyces-treated plant, T4* = the second sample to ensure the effect of Streptomyces mixture. N.C and P.C as negative and positive control

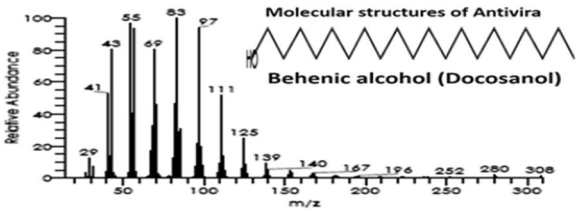


Fig. 18 A chromatogram of GC–MS analysis of *Streptomyces griseus* Molecular structures of the GC–MS analyzed compounds. Mass spectrum of the compounds obtained of three *Streptomyces* spp. was compared to the mass spectral databases of WILEY 09 and NIST14

Influence of Streptomyces spp. on development of viral disease Remarkably, the result as mentioned earlier of disease severity and development showed that the symptoms of ZYMV-infected squash completely disappeared after treatment with the three *Streptomyces* spp. substances compared to the infected plant which had severe disease symptoms. This trial was designated to evaluate the role of antiviral activity of substances derived from *Streptomyces* spp. on expression of *P1* gene, which play a vital role in virus replication and symptom expression of *Potyvirus* species (Fig. 17). As mentioned in our previous article, the GC–MS fractionation exposed 44, 47 and 54 substances derived from *S. sampsonii* DG1; *S. griseus* DG5; and *S. rochei* DG4, respectively. These strains were able to produce an antiviral termed Behenic alcohol (fatty alcohol, Docosanol) having the same characteristics (RT = 23.87, Molecular Weight = 326, 661-19-8, Molecular Formula $C_{22}H_{46}O$, MF = 926, Area % = 9.89, Cas # = 661-19-8) as presented in (Fig. 18). For this reason, *Streptomyces* spp. substances having antiviral activity of Behenic alcohol against ZYMV-replication were applied. In this context, PCR amplification with the primers pair (ZY229F/ZY838R) resulted in an approximately 600 bp product within *P1* gene expression in the Streptomyces-untreated/ZYMV-infected plant as a positive control (P.C). Meanwhile, the application of culture broth substances including antiviral Behenic alcohol was capable to down-regulate *P1* gene expression in all *Streptomyces*-treated/ZYMV-infected plants. The culture broth treatments (T1, T4, T5, T6 and T4*) prior/post to ZYMV infection resulted in an amplified faint band of *P1* gene product (600 bp) compared to the prominent one in the P.C. Also, T2 and T3 resulted in moderately faint band, while there is no amplified product in the healthy plant (N.C.).

Evaluation of antiviral activity of *Streptomyces* spp. and quantification of ZYMV titer by Real-time qPCR

In addition to PCR, real-time-qPCR was assayed to ensure the potential activity of Behenic alcohol derived from *Streptomyces* spp. as an antiviral against ZYMV infection in squash plants. The assessment of Behenic alcohol potential activity is based on the virus presence in the leaves, disease severity and the expression of *P1* gene, its efficacy and accurateness in quantification of ZYMV. In real-time quantification of PCR assay, the cycle threshold (Ct) value is a constraint reflecting the quantity of templates existing in the reaction. Applying *Streptomyces* spp. significantly reduced the development of virus symptoms and disease severity in the *Streptomyces*-treated/ZYMV-infected plant compared to the control. Total RNA was extracted from each treatment and control and detected by qPCR using *P1* primers pair (ZY229F/ZY838R). Fold change for each sample was calculated using ΔΔCt equation, and the results revealed that T4, T2, T1 and T3 gradually achieved down-regulation in ZYMV titer and decreased expression of ZYMV-*P1* gene in comparison with control (T7, "P.C").

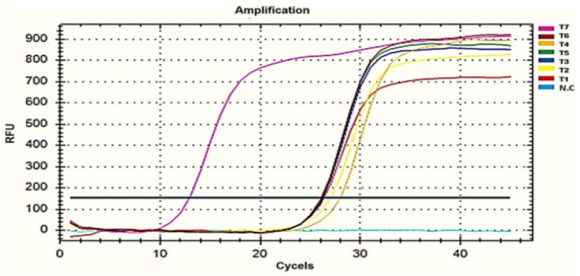


Fig. 19 *Zucchini yellow mosaic virus*—Quantification using real-time qPCR. Shape of curve T7 (P.C) similar fluorescence one, and P.C began fluorescing at cycle 10 compared to cycles 20–26 for other treated samples

Table 3 Values for the cycle threshold (Ct) housekeeping (reference gene from cucumber plants) and Ct *P1* gene responsible for virus replication and symptom expression

No	Ct h.k*	Ct P1 gene**
T1	23.73	26.60
T2	26.08	27.27
T3	26.90	26.45
T4	25.18	28.21
T5	25.73	26.38
T6	27.38	26.31
T7	12.92	13.15

*Ct h.k = Ct housekeeping (reference gene from cucumber plants), and Ct *P1* gene = Ct values reflect the cycles number that are requested till the fluorescent signal rises above background threshold to be detected

Likewise, T5 and T6 caused down-regulation for *P1* gene expression. Ct data for both *P1* and housekeeping genes (cucumber plant) are shown in Table 3, and values lower than 1 refer to down-regulation of *P1* gene expression, whereas values higher than 1 refer to up-regulation. Fold change of ZYMV titer and amplification of each sample are shown in Fig. 19, the shape of the control treatment curve (T7, "P.C") was similar to those obtained with fluorescence, and it started fluorescing at cycle 10 compared to cycles 20–26 for other treated samples. Also, the curve did not reach the threshold. Low Ct value refers to the highest target DNA presents in the sample, and the fewer cycles are required until the fluorescence signal crosses the background threshold since it amplifies faster, while a high Ct value refers to the highest number cycles that occur before fluorescent signal can pass through the background threshold, reflected to the lowest target DNA in the sample. Data indicated that reliable results could be acquired when total RNA was as low titer when using real-time RT-PCR assay.

Discussion

Zucchini yellow mosaic virus (ZYMV) is considered a destructive disease causing severe losses of fruit yield and up to 100% yield reduction of marketable cucurbit crops worldwide (Clarke et al. 2020). Extensive application of agrochemicals to control the transmitting vector and reduce the virus dissemination causes several problems, i.e., the existence of toxic pesticide, environmental pollutions, and aphid resistance to pesticide. Therefore, the aforementioned problems in controlling viral diseases have stimulated renewed interest in biological control as alternative methods to manage disease. Nowadays, *Streptomyces* spp. is an efficient, eco-friendly agent and benign during disease management. They are one of the most fascinating candidates, economically sustainable sources having various antifungal, antibacterial antiviral, antitumor and anticancer substances capable to inhibit several phytopathogens (Ghanem et al. 2022).

In the present work, firstly ZYMV-suspected infected leaf samples from different cucurbits crops including squash symptomatic with mild and severe mosaic, malformation of leaves and fruit, blisters were collected from several major cucurbits growing locations. Biological (depending on the diagnostic host *Ch. amaranticolor*) and molecular detection using PCR proved to be infected with samples were infected with ZYMV. Consistent results are described by many investigators that ZYMV often produces wide diversity symptoms including blisters, deformation of the fruit and yield losses (Morteza et al. 2021).

Concerning ZYMV molecular detection, the *CP, DAG, P1 and P3* genes of ZYMV isolate were successfully amplified using RT-PCR. A total RNA preparation from the propagative host squash as a template and the generation of single RT-PCR amplified product of 1221, 458, 600 and 670 bp lengths when tested specific primers pair of the present ZYMV isolate. These findings agree with several investigators (Alinizi et al. 2021).

Phylogenetic tree of the isolate identified in the present study shared range of nucleotide sequence identity (87.1–95.6) for *P1* and (93.3–97.7%) for *P3* with the other representative isolates submitted in the GenBank, while it shared 75.9–100 and 38.2–97.7% sequence identity for *CP* and *DAG* genes with the other isolates published from different countries in the GenBank and other sequences are presented "*CP* and *DAG* genes." These results revealed that phylogenetic tree analyses based on the *CP*, *P1* and *P3* genes grouped ZYMV isolate of the present study together with isolates from the Middle East, the European isolates and some isolates from China and Japan in the subgroup (AI) as well as other geographically distributed mosaic isolates worldwide. The above result suggests that the similarity between the viral isolates sampled from neighboring locations in the region is always closely related due to the biogeographically structure of environmental conditions, vectors and viral isolates spread in this region and international trading of infected seeds between different countries. Such described symptoms are similar to those declared by other investigators working with ZYMV (Alinizi et al. 2021).

Secondly, the purpose of this part depends on the potential activity of substances derived from the three *Streptomyces* spp. (*S. griseus*, *S. sampsonii* and *S. rochei*) as bio-inducers, also having an antiviral activity, which were applied due to their ability to enhance the SAR in squash plants cv. Yara F1 against ZYMV infection under greenhouse conditions. The evaluation of their activity

is based on the disease severity (DS), infection rate (IR), morphological characteristics, photosynthetic pigment, promoting squash growth and activities of antioxidant enzymes as well as virus titer within the treated infected plant's one month post-treatment.

Data presented revealed that seed priming process with substances derived from *Streptomyces* spp. either individually or in mixtures and subsequent virus inoculation, then applying *Streptomyces* on foliar part after 48 h, significantly reduced percent of both disease severity and infection rate. Regarding, the application of *Streptomyces* strains resulted in the disappearance of symptoms or the development of a very weak symptoms. However, untreated (control) plants *Streptomyces* reacted with severe mosaic, vein banding and blisters causing up to 95% yield losses in squash fruits.

Concerning plant growth parameters, the *Streptomyces* spp. treatment was observed to sustain normal morphological development among ZYMV-infected by enhancing root length, plant length and leaves number compared to the control. The obtained result affirms that the application of the three strains significantly promoted plant growth and development as well as reduced the RNA replication in the infected plants, resulting in mild disease symptoms than the untreated plants. These results agree with those confirmed by Boukhatem et al. (2022) who discussed that *Streptomyces* strains produce various metabolites capable to play promote plant growth. Applied *S. rochei* and *S. griseus* were previously characterized by GC-Mass as producers of auxins and gibberellin in their secondary metabolites substances as plant growth regulators (Ghanem et al. 2022). Thus, they proved to be efficient bioagents to control phytopathogens and promote plant growth. In agreement to our result, Vurukonda et al. (2018) recorded that the *S. rochei* produced secondary metabolites including cytokinins, auxins and gibberellin were able to promote plant growth. Similarly, Myo et al. (2019) demonstrated that *S. fradiae* NKZ-259 had a potential activity for promoting tomato plant growth and production of Indole-3-acetic acid (IAA). Furthermore, Al-Tammar and Khalifa (2023) revealed that that *Streptomyces* substances including phytohormones (auxin, gibberellin and cytokinin) were efficient in improving plant growth and yield and recognized as environmentally benign, as bio-remediators, bio-stimulators, biological agents, bio-fertilizers for promoting plant growth.

In the present study, *Streptomyces* application also induced physiological and metabolic changes within the *Streptomyces*-treated/ZYMV-infected squash plants causing alteration of some molecules, i.e., proline, total phenols and accumulation or reduction in various metabolites photosynthetic pigments (chlorophyll) antioxidant enzymes activity such CAT, SOD and GPx. In this context, the present result confirmed that the increase in proline content is considered to be an efficient compatible molecule and initial mechanism that produces in squash leaves during signaling network of plant-pathogen interaction and play a central role to activate all the defense responses against invading ZYMV. Likewise, Singhal et al. (2021) indicated that the response of proline metabolizing pathway plays an essential role in imparting resistance to the black gram (*Vigna mungo*) plants against *Yellow mosaic virus* (YMV) infection. They suggested that proline accumulation was initiated as a part of induced defense response against the YMV infection and as an essential component of defense mechanisms in plants playing vital role as osmolyte and a powerful non-enzymatic antioxidant. The results of the present study are in harmony with many investigators who reported that amino acid proline plays various important roles, i.e., acts as stress-related signal revealing cross-tolerance to different abiotic and biotic stresses and as osmo-regulator molecule could stabilize the effect of stresses at cellular level conditions as well as plays important role in limitation of the plant development and growth (Christgen and Backer (2019).

Additionally, the results conferring the total phenolic and chlorophyll contents among virus-infected plants recorded significantly increasing in all treatments (T1-T6) than T7 (control treatment), describing that quantitative changes of total phenols and chlorophyll contents play a vital role in several physiological processes to improve squash plant defense against ZYMV infection. This finding clearly revealed that *Streptomyces* secondary metabolites play a dynamic role as precursors to many biological functions and are frequently associated with elevated chlorophyll levels and phenolic compounds. Such phenolic compounds or phytoalexins which play essential roles in the plant's defense against pathogen. The result clearly agrees with Lamb and Dixon (1997) who revealed that the phenolic compounds or their derivatives quinone produced through peroxidase oxidation could inhibit viral RNA replication. A similar accumulation of total phenols and chlorophyll contents was observed within different plants species in response to various *Streptomyces* treatments such as *Streptomyces* spp.-treated cucumber (Latake and Borkar 2017), *Streptomyces* spp.-treated potato (Nasr-Eldin et al. 2019), *S. cellulosae*-treated tomato (Abo-Zaid et al. 2020) and *S. chromofuscus*-treated tomato (Chen et al. 2022).

In consistency with the present results, *Streptomyces* spp. as bio-elicitors for seed priming and foliar applications increased the enzymes activity such CAT, SOD and GPx in the *Streptomyces*-treated plants comparable with the control. It is evident that the use of *Streptomyces*

spp. substances as a bio-elicitor has potential effects on the fastest of seed germination, development and quality of the plant during the pathogenesis process, due to its roles in the plant's defense against disease. Further, the noticeable efficiency of *Streptomyces* spp. in protecting of squash plants against ZYMV infection could be related to improvement of activity of these defense-related enzymes. These results are in harmony with those by Yang et al. (2022) who declared that the priming seed process can stimulate the immune system in the plant and capable to induce epigenetic deviances such as physiological and metabolic changes which is efficient to the maintenance and establishment of plant immune memory. In accordance with our current findings, many researches indicated the vital role of plant antioxidative enzymes in defense against pathogen infections. Siddique et al. (2014) recorded a marked elevation in total phenols concentration and both SOD and polyphenol oxidase (PPO) activities in cotton plants following infection with *Cotton leaf curl burewala virus* (CLCuBuV). Moreover, Taha et al. (2021) affirmed that the treated plant with *S. ovatisporus* LC597360 culture filtrate (CF) resulted in a significant increase in activity of peroxidase (POD), polyphenol oxidase (PPO), catalase (CAT) relative to control untreated plants. These findings clearly indicate that *S. ovatisporus*-treated tomato plant significantly enhanced both antioxidant enzymes activity and protein content that could have a role in defense against *Tomato mosaic virus* (ToMV) infection through elicitation of systemic resistance and prompting plant growth. They declared that the elicitation of SAR by *S. ovatisporus* is mainly attributed to defense-related enzymes due to their potency to manage viral infections in plants without inducing any environmental risks. Furthermore, Chen et al. (2022) applied two strains of *S. chromofuscus* (RSF-23 and CTF-20) against *Tomato yellow leaf curl virus* (TYLCV) infection. They revealed that the RSF-23 strain successfully enhanced the activities of defense-related enzymes, including SOD, CAT, peroxidase (POD). They indicated that the measured enzymatic activities were significantly higher among RSF-23-treated plants, as compared to those observed in the plants treated with CTF-20 strain. Comparable observations to our results have been declared by others investigators, i.e., Taha et al. (2021) working with *Streptomyces* species, and displayed that the plant defense is related to enzymes activity, due to the elevation of reactive oxygen species (ROS) scavenging toxic molecules capacity during both virus infection and replication. In the site of infection, the plant initiates a response to pathogen attack pathogen including extreme peers of ROS including superoxide hydrogen peroxide, and peroxides causing oxidative burst associated with local and systemic signal translocation to prevent virus movement and replication within plant. Furthermore, Meena et al. (2022) professed that the elicitor substances produced by microorganisms act as a fungal pathogenicity molecule, while elicitor substances derived from *Streptomyces* spp. are capable to initiate plant defense responses and inducing systemic resistance up to 85%.

Regarding, reduction of the infection percentage among Streptomyces-treated plants is to minimize the intensity, development of ZYMV symptoms and replication as well as accumulation. Our observation about disease development indicated that the *Streptomyces* spp. had a strong impact on the appearance and development of viral symptoms than the control. The above result suggests that induced resistance is due to enhancement of protective capability developed by a plant when appropriately stimulated by substances derived from *Streptomyces* spp. It is evident that the antiviral compound Behenic alcohol (Docosanol, fatty alcohol) produced from the three *Streptomyces* spp. has potent antiviral property against ZYMV infection. Illustrated result of the present work indicates that the *Streptomyces*-derived substances included Behenic alcohol (Docosanol) that provided clues as a vital role in reducing or/and preventing viral replication and accumulation, due to its inhibition of the *P1* viral protein, which involves ZYMV evolution, virus–host adaptation and symptom expression. Therefore, our early application of squash plants with secondary metabolites could significantly inhibit *P1* responsible for virus replication and movement from cell to cell to induce systemic infection and reflected in the disappearing ZYMV symptoms. In accordance with the present results, many investigators discussed that *P1* has multifunction in the pathogenicity of potyviral including ZYMV, i.e., *P1* gene may be involved in other non-proteolytic functions such as viral amplification or cell-to-cell transportation (Qiuyue 2016) and *P1* is involved in virus replication and pathogenicity and it plays a vital role as a regulator in pathogen–host adaptation and expression of the symptoms (Alinizi et al. 2021).

In accordance to the present results, Behenic alcohol provided evidence for its capability in inhibiting a wide range of human RNA or DNA viruses as medicinal chemotherapeutic agents. Morsy et al. (2023) applied Behenic alcohol (Docosanol) to control recurrent oral-facial herpes simplex virus (fever blisters and cold sores, "HSV-1") and other viral infections such as acquired immune deficiency syndrome, hepatitis viruses (HBV "B" and HCV "C") and COVID-19 pandemic virus or severe acute respiratory syndrome. They observed that Docosanol has potent antiviral activity since it inhibited the virus attachment and entry into host cells, inactivated

viral replication cycle and avoidance of inducing drugs-resistance strains. Nevertheless, Latake and Borkar (2017) assessed secondary metabolites of *S. olivaceus* to control *Cucumber mosaic virus* (CMV) and proved to be efficient against CMV infection under glasshouse and field conditions when applying both of seed priming and foliar spraying treatments. Similarly, the above-mentioned results are consistent with those affirmed by many researchers who declared that the substances derived from various strains of *Streptomyces* spp. play an efficient role as antiviral against RNA and DNA plant viruses, respectively, such as Potato virus-Y (PVY^{NTN}), Tobacco mosaic virus (TMV) (Abo-Zaid et al. 2020) and Tomato yellow leaf curl virus (TYLCV) and Tomato mosaic virus (ToMV) (Chen et al. 2022).

SYBR green-based real-time RT-PCR assay combined with melt curve examination was developed for the detection of ZYMV and proved to be cost effective and time saving as well as does not harmful. In real-time quantification of PCR assay, the Ct value is a parameter reflecting the quantity of templates existed in the reaction. The result consistently showed that the dCt values amplified gradually relative with reducing virus titer. These results clearly display that as the virus titer decreased, the dCt values consistently increased. This is a compelling suggestion of a reduction in *P1* gene expression which establishes the antivirus's highly effective capability to prevent virus replication and disease development. Moreover, real-time RT-PCR was a more sensitive, faster and reliable assay as it could detect the very lowest ZYMV virus titer in the sample. The results are consistent with many investigators working in plant pathology who confirmed that RT-qPCR is not only a rapid and accurate, safer, reliable assay method ensuring results of conventional PCR, but also a more sensitive method for the detection and quantification very low titer of plant pathogens (Rodríguez-Verástegui et al. 2022). The above-mentioned findings suggest that the three used strains provided clues as a novel biocontrol agent and have potential protective activity as an antiviral substances and candidates for the management of plant viral infections. Further, these results affirmed their superiority in physiological and metabolic changes such as promoting growth plant, induction of proline, total phenols and antioxidant enzymes activity in squash. The present research is the first one that reveals the potentiality of the three species (*S. sampsonii*, *S. rochei* and *S. griseus*) in biocontrol of ZYMV and demonstrated its antiviral mechanisms.

Conclusion

Our conclusions reveal that the strains of *Streptomyces* spp. (*S. sampsonii*, *S. rochei*, and *S. griseus*) produce various potential active substances including antiviral (Behenic alcohol) efficiently restricted ZYMV infection rate among squash plants and limited disease development by decreasing disease symptoms, virus replication and accumulation. This bioagent has increased proline values, total phenols and chlorophyll content as well as promoted plant growth. Applying *Streptomyces* spp. resulted in activation of significantly high defense responses, representing elevated SR through high expression of antioxidant enzymes (SOD, CAT and GPx), among virus-infected plants. The present study suggests that application of *Streptomyces* spp. culture broth is a promising approach as an alternative for the eco-friendly management of ZYMV in squash plantations and resulting in a significant economic return for growers.

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Author contributions
GG is supervisor, led the study design, led the study design, and followed-up laboratory works and writing—original draft, and review and editing the manuscript, AM contributed to data curing and application of Streptomyces; AK was involved in molecular biology techniques; DG contributed to preparation of *Streptomyces* spp. culture broth; and AO carried out laboratory works of methodology.

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All data are available in the manuscript. Some figures and tables are only attached in the supplementary materials file.

Declarations

Ethics approval and consent to participate
All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Consent for publication
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Competing interests
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