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Extraction, Purification and Characterization of Two Antiphytoviral Substance (s) Produced by Zucchini Yellow Mosaic Virus

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ABSTRACT

The ability of *Pseudomonas viridilivida* strain Zagazig University, Faculty of Science (ZUFS) to inhibit Zucchini Yellow Mosaic Virus (ZYMV) in cucumber plants was studied *in vitro* by mixing it with sap containing virus and *in vivo* by seed and soil treatments. The production, extraction, purification and characterization of the antiviral substance (s) from strain ZUFS was done. *P. viridilivida* stimulated the growth of cucumber (*Cucumis sativus* L.) and inhibit the symptoms of zucchini yellow mosaic virus (ZYMV) by 90%, when mixed with virus suspension in equal volumes (v/v). Treatment of cucumber seeds with bacterial pellets increase the plant growth and showed 100% viral inhibition. The virus concentration was detected by DAS-ELISA technique in leaves of cucumber plants, whose seeds were treated with *P. viridilivida*. The ELISA value was 0.805 near to the negative control of ZYMV (0.697), while the positive virus control was 1.884. The *P. viridilivida* detected by electron microscopy inside the root tissue of seed treated cucumber plants, (root-colonizing). The treatment of natural and sterile soil by liquid bacterial culture gave 70 and 89.3% inhibition, the ELISA value of cucumber leaves grown on treated sterile soil was 1.231 compared to (1.884) for positive ZYMV control. ZYMV-infection induced severe modification in number and ultra structure of chloroplast, where lower number and irregular structure of chloroplasts were observed in ZYMV infected leaf in comparison with control and treated plants. Scrolls, pin-wheels, bundles and laminated aggregates were induced in the cytoplasm of infected cells. Physical properties of ZYMV showed that the virus was sap transmissible, its thermal inactivation point is 65°C, dilution end point is 10^{-3} and longevity *in vitro* is 24 h at room temperature. The antiviral substance (s) produced by culture of *P. viridilivida* were extracted by using different solvents followed by thin layer chromatography (TLC) purification. Two spots blue and green detected under UV lamp had R_f of 0.80 and 0.93, respectively. The IR, UV, NMR, mass spectrum and elemental analysis of blue and green compounds showed that the molecular formula of these compounds suggested to be $C_{19}H_{35}O_4N$ and $C_{17}H_{34}O_4N$ and molecular weight 341 and 316, respectively. Purified substance (s), were tested against ZYMV in cucumber plants giving inhibition 100%. The ELISA values were 0.975, 0.928 for virus concentration in cucumber leaves extract treated with blue and green compounds, respectively in comparison with 1.884 for mechanically inoculated ZYMV.

Key Words: Bio-control; Zucchini yellow mosaic virus; *Pseudomonas viridilivida*; ELISA; Electron microscopy; Microchemical analysis

INTRODUCTION

Zucchini yellow mosaic virus (ZYMV), genus *Potyvirus*, is one of the most widespread and destructive viral agent through-out the world and causes severe economic loss to cucurbit crops every year (Lisa & Lecoq, 1984). ZYMV was first rereported by (Lisa *et al*., 1981).

In Egypt squash crop is subjected to severe losses in both the yield and the quality of the fruits due to ZYMV (Provvidenti *et al*., 1984a b). This virus causes severe prominent foliage mosaic, severe malformation, plant stunting and fruits with knobbey areas Cucumber, like other cucurbits, suffers.

A disease incidence of 80% or more, together with severe symptoms and abnormalities on foliage and fruits, caused by ZYMV in cucumber (*cucumis sativus* L.) and zucchini squash, has been reported worldwide (Al-Shahwan, 1990; Stobbs & Van Schagen, 1990). Some isolates of ZYMV induced pin-wheels, scrolls, bundles and laminated aggregates (Petersen *et al*., 1991).

Plant growth promoting rhizobacteria (PGPR) are plant root colonizers and belong to different genera and species, most reported strains are from *Pseudomonas* and *Bacillus spp*. (Kloepper, 1991). The beneficial effects of most reported PGPR strains indicated that these bacteria increased growth indirectly by changing the microbial balance in the rhizospher, iron-chelating siderophores, antibiotics and HCN production (Kloepper & Schroth, 1981; Ahl *et al*., 1986; Schippers, 1988; Weller, 1988). The *Pseudomonas spp*. are important for biological control.

Zhou and Paulitz (1994) reported that strains of *Pseudomonas spp*. induced systemic resistance in cucumber against *Pythium aphanidermatum*. Also, the *P. fluorescenes* protected the cucumber from cucumber mosaic virus (CMV) infection (Wang *et al*., 1992; Desbeiz & Lecoq, 1997; Helmy & Maklad, 2003). Recent reports have shown that several strains of *Pseudomonas putida* produce N-acylhomoserine lactones (Steidle *et al*., 2002). Nevertheless little is know about *P. viridilivida,* which is very related species to *P. flourcent* biotype C. the *P. viridilivida* designated ZUFS chosen in this study for biological control of ZYMV.

The objective of research was to obtain *P. virirdilivida* isolate as bio-control agent of ZYMV, which can promote growth of cucumber plants and to extract natural antiphytoviral substance (s) from this strain of bacteria as well.

MATERIALS AND METHODS

Isolation, identification and culture condition. The bacterial isolate was isolated from rich garden soil, purified, grown on soil extract broth pH 7 (James, 1958) for 3 days at 37°C and screened for its antiviral activity against ZYMV in cucumber plants in greenhouse. The isolate identified by the physiological reaction according to Bergey's manual of determinative bacteriology (Holt, *et al.*, 1986), and by Biolog-microplate system (Bochner, 1989) in Identification of Microorganisms, Biological Control of Plant Diseases and Evaluation of Bio-fungicides Unit, Plant Pathology Research Institute, ARC-Giza Egypt. Cucumber plants (*Cucumis sativus* L.) cv. Beit-Alpha as systemic host of ZYMV were used. Seeds were obtained from the Agricultural Research Institute, Dokki, Giza, Egypt. The seeds were cultivated in plastic pots (1000 cm^3) in greenhouse of Faculty of Science, Zagazig University. Plants were irrigated as required till the end of the experiment.

Virus. Zucchini yellow mosaic virus (ZYMV-El-Sharkia isolate) isolated from naturally infected zucchini fruit with severe symptoms (Fig. 1a) The virus was identified by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) as described by Clark and Adams (1977) using the ZYMV-ELISA kits in Agricultural Research Center, Agricural Genetic Engineering Research Institute, Giza, Egypt. The virus was propagated and maintained in cucumber plants according to Faccioli and Capponi (1983), 5 g of naturally infected (*Cucurbita pepo* L.) fruits were ground in sterile mortar with a pestle in 5 mL of 0.01 m phosphate buffer solution of pH 7.2 Then filtered by Whatman filter paper No. 1 or by cotton piece. The volume was made up to 20 mL with phosphate buffer pH 7.2 then 100 µL of sap were mechanically inoculated in to cotyledonary leaves and first leaf of cucumber dusted by carborundum (600 mesh-Prolab). Then wash the inoculated leaves by distilled water according to Yarwood (1955). After 21 days the infected leaves were frozen and used as inocula in further experiments.

The stability of ZYMV-Sharkia isolate (ZYMV-Sh_1)

1- Thermal inactivation point [TIP]. The tubes containing the virus were kept in water bath for 10 min at different temperatures (30, 40, 50, 60, 70, 80 & 90^0C), then mechanically inoculated in to carborundum-dusted leaves of cucumber and the TIP was determined.

2- Dilution end point [DEP]. Crude extracted of sap containing virus from diseased cucumber leaves was serially diluted from 10^{-1}: 10^{-5}, then each dilution mechanically inoculated to cucumber seedling and the DEP was determined.

3- Longevity *in vitro* [LIV]. The effect of aging on the infectivity of the virus under test was determined at room temperature (25 – 30°C) after time 3, 6, 12, 24 and 48 h.

Antiviral activity of *Pseudomonas viridilivida* strain ZUFS

i. *In vitro* study. Liquid bacterial culture mixed with sap containing virus in equal volumes (V/V) then 100 µL/leaf were inoculated.

ii. Seed treatment experiment (*in vivo* study). Cucumber seeds were mixed for 2 h with bacterial pellets then air dried for 24 h. The seeds cultivated in plastic pots containing a fertile soil. Control pots contained un-treated seeds. After complete germination, carborundum-dusted leaves inoculated with virus suspension. All pots were watered as required with tap water. After 3 weeks the number of symptomatic plants, were recorded and the % of viral inhibition was determined. The number of leaves, shoot length and fresh weight were also determined. The colonization of bacteria in cucumber roots was detected by transmission electron microscopy. The virus concentration was detected by ELISA technique in the leaves of cucumber plants of the above treatment.

iii. Soil treatment experiment (*in vivo* study). Plastic pots were filled with 750 g of sterilized and non-sterilized soil then the bacterial culture was added at 10^7 CFU/g of soil to each pots (10 mL bacteria/1 kg soil). The bacterial cultural did not add to the pots of viral control and the healthy control. Two days after soil treatment with bacteria, cucumber seeds were cultivated. After germination, the sap mechanically inoculated in to the leaves. The % of viral inhibition, were calculated. The morphological character like number of leaves, shoot length and fresh weight were determined. A highly sensitive test called (DAS-ELISA) was used to detect the virus in cucumber leaves of the above experiment.

Ultra-structure investigation. The infected cucumber leaves with ZYMV as well as the treated and healthy were processed for transmission electron microscopy (TEM) by method adopted by Perera and Gay (1976). Under binocular dissecting microscope, the desired area on the block was then trimmed in to flat stopped pyramid using a razor blade, the sides of pyramid were kept parallel. Semithin section (0.5 - 1 µm thick) were stained with toludine blue and

examined with the light microscope and photographed using Motic apparatus (Hong Kong model, 2003). Ultrathin sections were cut by the same Richter ultra-microtome. Silver or gold sections were picked up on a dull surface of formvar (polyvinyl/formaldehyde) coated copper grids, which were prepared by a method outlined by Weakley (1971**)** using 0.6% (W/V) formvar in chloroform. Then double-stained on a wax plate placed in a Petri dish. A drop of 2% aqueous uranyl acetate (Juniper *et al*., 1970) was pipette on the wax plate and the mounted grades were gently floated, with the sections facing down on the drop of the stain. The dish was immediately covered with a lid. The sections were left for 30 min in uranyl acetate and then washed in a gently stream of a glass distilled water and dried on filter paper. The sections were examined and photographed using JEM-100 CX JEOL, electron microscope (Japan) in Faculty of Science, Zagazig University.

Cytopathology. Using the above electron microscope, various inclusion bodies can be observed in ultra-thin sections (Edwardson & Christie, 1978). Systematically ZYMV-infected leaves of *Cucumis sativus* L. 21 days post virus inoculation were subjected to electron microscopic examination. The ultra-thin sections were prepared as given by Abdel-Ghaffar (1994), cutting the infected leaf in to 1 x 1 mm pieces followed by pre-fixing in 2.5% glutraaldhyde and 2% paraformaldehyde for 4 h, then post-fixing in 1% osmium tetraoxide for 1 h and half then dehydrating in ascending grade series of ethanol. The specimens were treated with propylene oxide and then embedded in fresh resin in embedding capsules. The sections were cut with a diamond knife and the grids containing sections were stained with 2% uranyl acetate then with lead citrate and examined with JEM-100 CX JEOL, electron microscope (Japan) in Faculty of Science, Zagazig University, Egypt.

Extraction, purification and identification of antiviral substance (s) produced by *P. viridilivida* strain ZUFS. For production of sufficient amounts of the antiviral substance (s) *P. viridilivida* was cultured in 40 Erlenmeyer flasks, 250 mL capacity, each containing 100 mL of soil extract liquid medium pH 7, then inoculated, were incubated at 37°C for 3 days. At the expiry of the incubation period, 12 organic solvents namely: benzene, ethyl alcohol, ethyl acetate, acetone, methanol, pentanol, hexane, cyclohexan, chloroform, diethyl ether, petroleum ether and butanol (v/v), were tested for their extractability of the antiviral compounds from broth. The extract was obtained by separating funnel 3 times. The organic phase collected evaporated under reduced pressure by using evaporator (rotary). The evaporation was continued until least amount obtained (5 mL). The extraction were spotted on thin layer chromatography (TLC) silica gel plates using butanol: acetic acid: water (2: 1: 1, v: v: v) as the solvent developing system, then examined under UV lamp and two spots blue and green were appeared. The R_f value for each spot was determined. Each spot was separately scratched out, combined and eluted in chloroform and filtered. The elute was concentrated till dryness by vacuum rotary evaporation. The dry film of each spot dissolved in 1 mL methanol, then the elute completed to 5 mL by distilled water. The antiviral test was carried out as previously mentioned after mixing the eluted compounds with sap containing ZYMV (v: v) and the % of viral inhibition was calculated. The concentration of virus was detected in treated host cucumber plants by ELISA technique. The identification of the two purified antiviral compounds produced by *P. viridilivida* strain ZUFS was carried out by IR, UV, NMR, mass spectrum and elemental analysis (C, H, O, N & S) in the Microanalytical center of Cairo University, Egypt.

RESULTS AND DISCUSSION

Stability of ZYMV-Sh_1 Isolate. The thermal inactivation point, dilution end point and longevity *in vitro* of ZYMV were 55 – 60 C, 10^{-3} and 24 h, respectively as shown in (Tables I, II & III). Similar results were obtained by Sayed *et al*. (2003), who reported that the thermal inactivation point of tomato spotted wilt virus (TSWV) was 47°C, the dilution end point 1 - 8 x 10^{-3} and the virus was completely inactivated after incubation for 5 - 20 h at room temperature.

The detection of the virus by ELISA technique in mechanically inoculated cucumber plants showed mosaic, blistering, malformation and crinkle symptoms (Fig. 1c) confirmed that the virus is ZYMV.

Identification of bacterial isolate. The bacterial isolate was identified by Biolog Microplate System as *Pseudomonas viridilivida*. We designated this bacterium as *P. viridilivida* strain ZUFS.

Screening of antiphytoviral activities of *P. viridilivida* strain ZUFS. The bacterial culture (3 days age) was mixed with sap containing virus (*in vitro*) and mechanically inoculated into cotyledonary leaves and first leave of cucumber (100 µL/l). The strain ZUFS showed 90% viral inhibition and improve the growth of the treated plants. The viral control plants had stunt, mosaic, yellowing, blistering of the leaves and malformation symptoms. Our results were in accordance with that obtained by Kegler *et al*. (1993), where the *Bacillus subtilis* inhibited the infection process when mixed with cucumber mosaic virus (CMV), tobacco mosaic virus (TMV), tobacco rattle tobravirus (TRV) and *prunus ringspotilarvirus* (PNRV).

The treatment of cucumber seeds with bacterial pellets not only inhibit the virus infectivity but also improve the plant growth as in (Table IV & Fig. 2) Also the bacterium increase the number of root hairs (Transmission electron microscopy indicated the presence of *P. viridilivida* in the root tissue of cucumber plants (its seeds treated with this bacteria) (Fig. 3) This result indicate that this bacterium is

Table I. Effect of different temperatures on ZYMV-infectivity

Temperatures	Thermal inactivation point of ZYMV						
Symptoms	30°C+	40°C+	50°C+	60°C-	70°C-	80°C-	90°C-

+ = ZYMV-symptoms appeared on cucumber leaves
- = ZYMV-symptoms did not appear on cucumber leaves

Table II. Effect of different dilutions on ZYMV-infectivity

Dilutions	Dilution of ZYMV sap				
Symptoms	10^{-1}+	10^{-2}+	10^{-3}+	10^{-4}-	10^{-5}-

+ = ZYMV-symptoms appeared on cucumber leaves
- = ZYMV-symptoms did not appear on cucumber leaves

Table III. Longevity of ZYMV *in vitro*

Time	Longevity in vitro at room temperature					
Symptoms	0 time+	3 h+	6 h+	12 h+	24 h+	48 h-

+ = ZYMV-symptoms appeared on cucumber leaves
- = ZYMV-symptoms did not appear on cucumber leaves

Table IV. Effect of *P. viridilivida* strain ZUFS on the inefectivity of ZYMV in seed treatment

Treatment M.C.	Seed treatment	Viral control	Healthy control
% of inhibition	100	0	All are healthy
Number of leaves	5.30 ± 0.19	2.60 ± 0.11	5.25 ± 0.19
Shoot length	26.80 ± 0.62	11.30 ± 0.12	20.50 ± 0.40
Fresh weight	5.75 ± 0.16	1.30 ± 0.01	4.00 ± 0.16

M.C. = morphological character

Table V. ELISA detection of ZYMV in cucumber leaves in seed and soil Experiments

Treatment	ELISA values
Positive control of ZYMV	1.618
Negative control of ZYMV	0.665
Healthy cucumber leaves	0.799
Diseased cucumber leaves	1.884
Seed treatment	0.805
Soil treatment	1.231

plant growth promoting bacterium and capable of entering root tissue. Metabolic exchange between the plant and the bacteria could explain growth promoting effect in inoculated plants. These results agree with Araugo *et al*. (1994 & 1995), who observed that *P. fluorescens* BR-5 had good survival and colonize maize root in soil microsome. Also, Raupach *et al*. (1996) reported that seed treatments with PGPR strains *P. fluorescens* 89 -B27 and *Serratia marcescens* 90 - 166 protect cucumber against CMV under greenhouse condition. Similar results obtained by DileepKumar (1998), who explained that *Fluorescent pseudomonads* RB8, RBP3 and RBW2 inhibit the fungal and bacterial plant pathogens. Moreover, Meena *et al*. (2001) revealed that seed treatment of powder formulation of *P. fluorescens* strain pf1 effectively reduced groundnut root rot from 88.8 to 33.3% and also increase pod yield in all field trials. Botlho *et al*. (1998) reported that inoculation with *P. fluorescens* BR-5 had a significant effect on maize growth and detected inside the maize root cells. The ELISA value in (Table V) indicate that ZYMV has very low concentration (0.805) in the cucumber leaves its seeds

Fig. 1. (a) Naturally infected zucchini fruit (b) healthy fruit

(c) mechanically infected cucumber leaf

(d) healthy leaves

Fig. 2. (a) Healthy plant, (b) plant grown from treated Seeds and (c) diseased plant

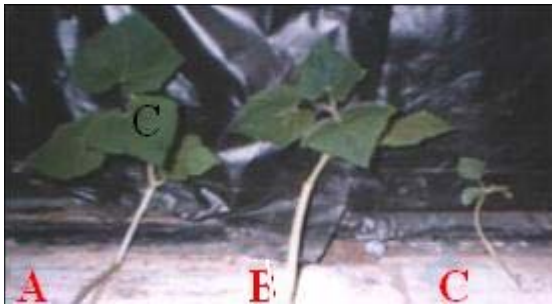


Fig. 3. Bacterial cell inside the root tissue of treated Plant (transmission electron microscope)

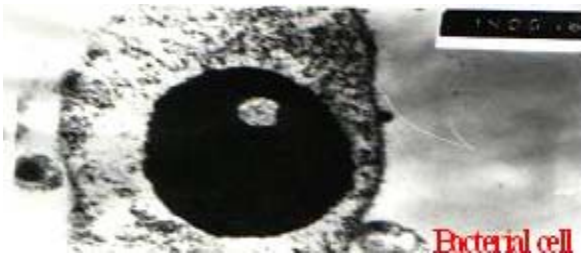
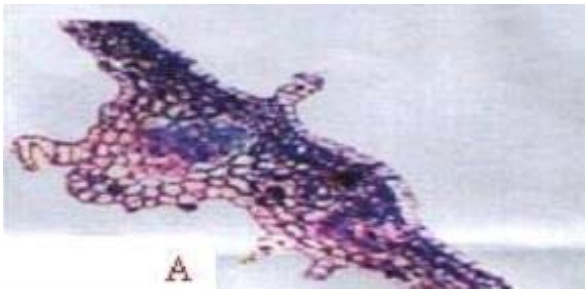
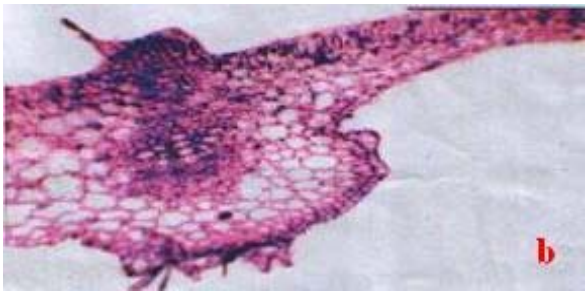


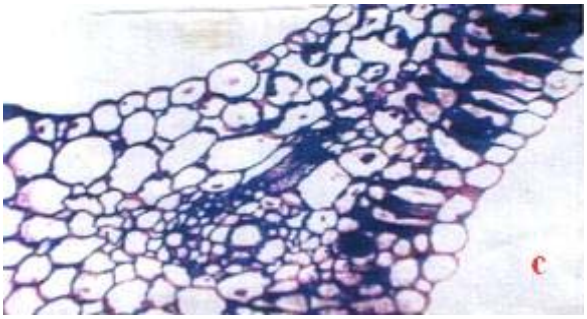
Fig. 4. (a). Histology of infected leaf



(b). Histology of treated leaf



(c). Histology of healthy leaf



treated with *P. viridilivida* in comparison with 1.884 in viral control.

The treatment of nature and sterile soil with culture of *P. viridilivida* inhibit ZYMV by 70 and 89.3%, respectively (Table VI). The ELISA value of cucumber leaves cultivated in treated soil with *P. viridilivida* was high (1.231) near to the positive ZYMV control (1.884) as showen in (Table V) Similar results obtained by Maurhofer *et al.* (1994), who reported that *P. fluorescens* strain CHA0, when applied on soil induced resistance in tobacco against TNV. Also, soil application of powder formulation of *P. fluorescens* strain BF1 reduced groundnut root rot (Meena *et al.* 2001). Soil application of banana with *P. fluorescens* BF10 reduced the wilt incidence of banana caused by *Fusarium oxysporum* f.sp. cubense by 50% compared with the control (Thangavelu *et al*., 2001). The *Pseudomonas viridilivida* is an antagonistic bacterium against rice sheath blight caused by *Rhizoctonia solani* in Japanica rice cv. Bing 96 - 42 (Yu *et al*., 2002).

Our results on *Pseudomonas viridilivida* strain ZUFS showed that this strain inhibit zucchini yellow mosaic virus (ZYMV), inhibit the phytipathogenic fungi, stimulate the growth of cucumber plants and root colonizer. Therefore it is PGPR.

Ultra Investigation of Treated and Virus-infected Cucumber Plants

Histopathology. A marked histological feature is that the mesophyll of the infected leaves not differentiated into palisade and spongy parenchyma. Cells are usually isodiametric or cuboidal or rounded in shape and are rather completely packed since the intercellular spaces gradually disappear (Fig. 4a).

The epidermal cells ruptured, while in healthy or treated leaves not. The chloroplasts present in few numbers. Meanwhile the mesophyll of treated leaves differentiated in to palisade and spongy tissue. The cells have intercellular spaces, the parenchyma cells is polyhedral. Upper and lower epidermis are healthy without any rupture. There is no abnormality in phloem and xylem tissue (Fig. 4b).

The healthy leaf has not any ruptured in epidermal cells and the mesophyll differentiated in to palisade and spongy tissue with intercellular spaces (Fig. 4c).

These results agree with Galal (1995), who found that large vesicle in parenchyma cells in lupine leaves naturally infected with CMV. Also, Sayed *et al.* (2003) illustrate that the mesophyll in the green areas of the mottled *Physalis peruviana* infected with TSWV was differentiated in to one layer of palisade cells and spongy tissue.

Cytopathology. Electron microscopic examination of ZYMV-diseased mesophyll revealed that the intercellular spaces were very small. The cell wall of infected cells may be thinner than the healthy cells. There is degeneration and reduction in the chloroplasts. The chloroplasts are deformed and swollen with reduced lamellar system (Fig. 5a).

The ultra-structure of cucumber leaves infected with ZYMV and treated with *P. viridilivida* showed that there is a gradual recovery in the cell organelles (Fig. 5b).

These recoveries are represented by the reduction of vesicles in the chloroplasts and the presence of regular distribution of grana and stroma lamellae. The thickness of the cell wall reoccurred. The intercellular spaces between cells increase. Formation of large vacules in cytoplasm

Table VI. Effect of soil treatment by liquid culture of *P. viridilivida* strain ZUFS on the inefectivity of ZYMV

Treatment M.C.	Sterile soil + bacteria	Sterile soil no bacteria	Sterile soil healthy plant	Natural soil + bacteria	Natural soil no bacteria	Natural soil healthy plant
% of inhibition	89.30	0	All healthy	70	0	All healthy
Number of leaves	4.20 ± 0.25	2.40 ± 0.22	3.50 ± 0.16	4.50 ± 0.16	2.90 ± 0.17	3.70 ± 0.15
Shoot length	29.40 ± 1.40	19.30 ± 1.00	30.40 ± 1.09	32.60 ± 1.02	19.70 ± 0.74	32.90 ± 0.45
Fresh weight	3.00 ± 0.16	1.90 ± 0.17	2.80 ± 0.15	3.60 ± 0.11	1.80 ± 0.80	3.50 ± 0.02

M.C. = morphological character

Fig. 5. The chloroplast of (a) infected cell, (b) treated cell and (c) healthy cell.

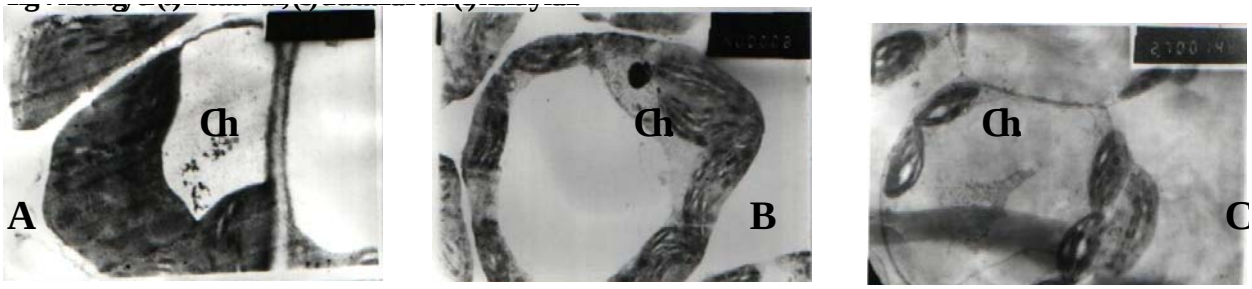
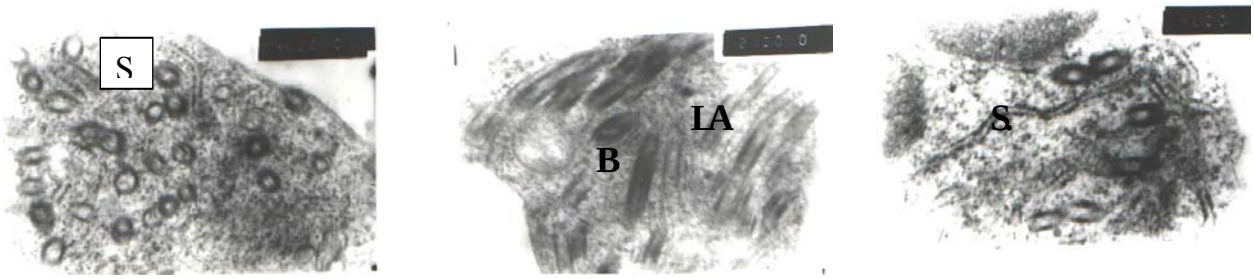


Fig.6 the inclusion bodies of zucchini yellow mosaic virus. [P.W.=pinwheels, B.= bundle, S.= scroll & V.P.= virus particle].



appear. Nucleus mitochondria appeared in a normal size and shape with no rupture. The chloroplasts have starch grains like healthy one. The size, shape and arrangement of chloroplasts is normal and similar to the healthy chloroplasts (Fig. 5c).

These results similar to that obtained by Sayed *et al* (2003), who reported that the chloroplasts of TSWV-infected *Physalis peruviana* is reduced in number, swollen, deformed and degenerate. Also, Zechmann *et al*. (2003) showed that the number of chloroplasts of ZYMV-infected Styrian pumpkin leaves reduced and its internal structure deformed in comparison with control plants.

Inclusion bodies. The electron micrographs of ultra-thin sections of ZYMV infected leaves showed inducing of cytoplasmic inclusions as scrolls, bundles and laminated aggregates (Fig. 6).

These results agree with Abdel-Ghaffar *et al*. (1998) and Zechmann *et al*. (2003), who reported that the presence of cytoplasmic inclusion bodies scrolls, pin-wheels in squash leaves infected by an Egyptian isolate of ZYMV and in Styrian pumpkin leaves infected by ZYMV, respectively. The inclusion bodies characterized our virus isolate as a member of potyvirus subdivision 1 according to classification of Edwardson (1974). Cylindrical inclusions (CI) are formed by the virus CI-protein, which accumulate within the cytoplasm of infected cells (Edwardson & Christie, 1996; Lopez *et al*., 2001). They are involved in virus replication, protein synthesis and short distance movement of the virus from cell to cell (Rodrigues *et al*.,

Fig. 7. TLC of two purified antiviral substances

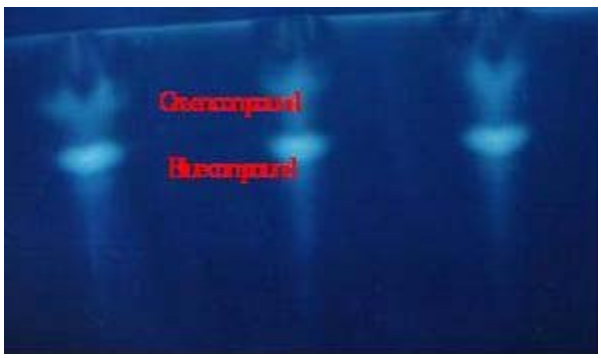


Fig.8 (A & B) mass spectrum; (C&D) NMR of the blue and green antiviral compound respectively.

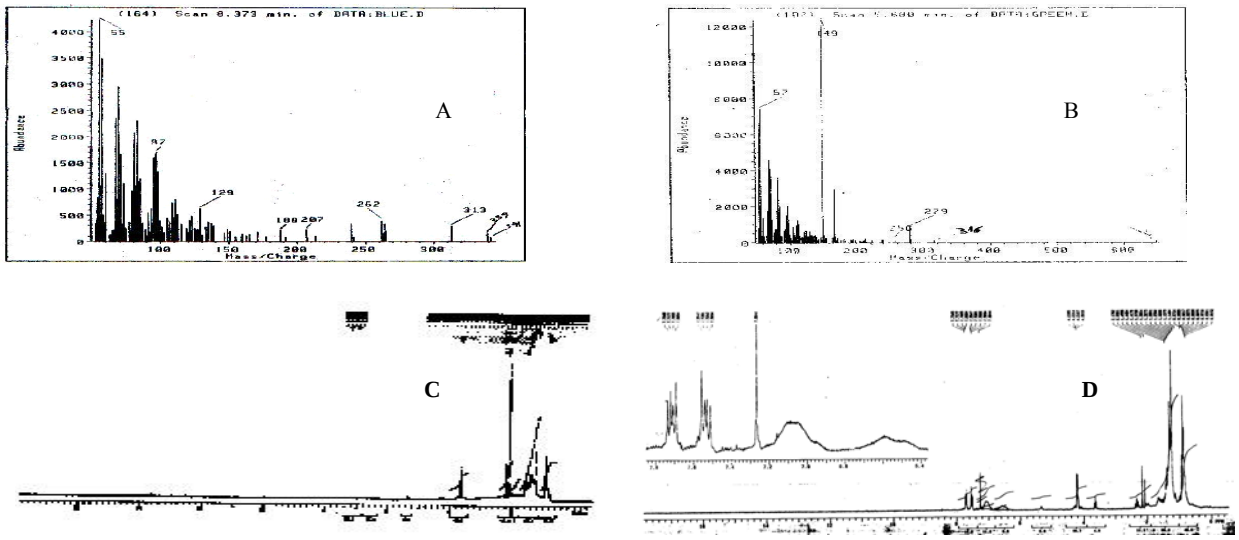
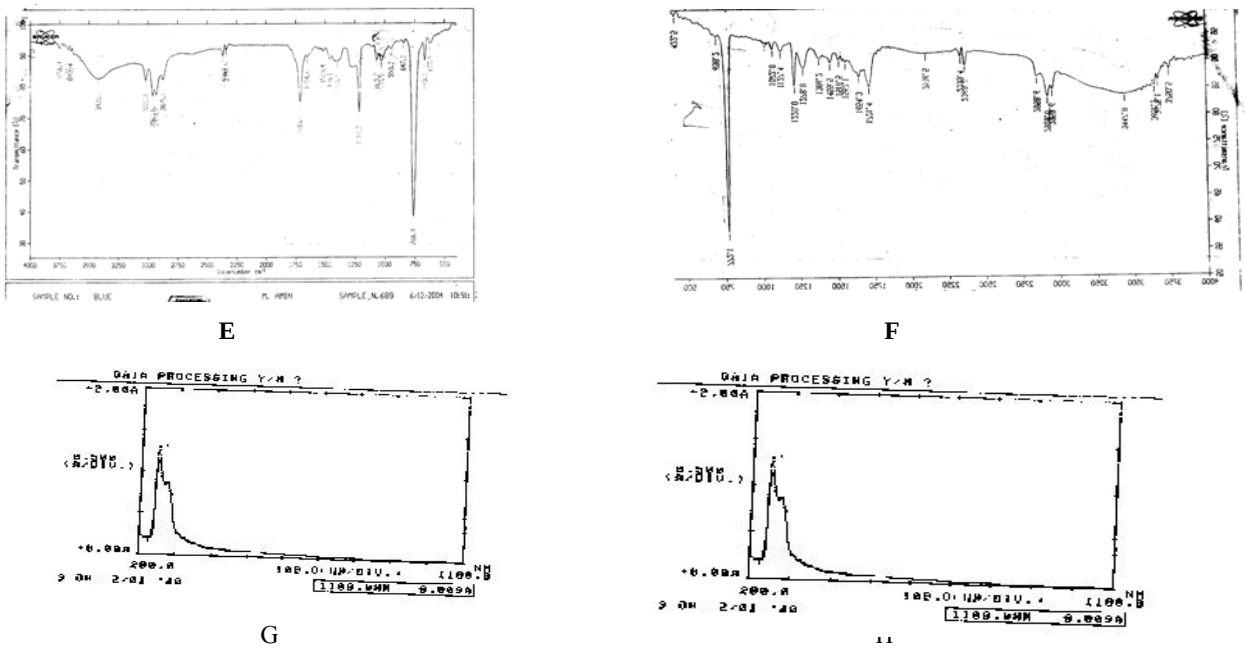


Fig.8 (E&F) IR; (G&H) UV of blue and green antiviral compounds respectively.



1997; Roberts *et al*., 1998; Heinlein, 2002).

Characterization of the antiviral substance (s) produced by *Pseudomonas viridilivida* strain ZUFS. The results of extractability of the antiviral substances revealed that benzene, ethyl acetate, pentanol, hexan, cyclohexan, chloroform, petroleum ether and butyle alcohol were capable of extracting the substances from the culture of *Pseudomonas viridilivida*, while absolute ethyl alcohol, acetone, methanol and diethyl ether could not extract them. The extract was evaporated in rotary evaporation under vacuum to dryness. The dried extract was dissolved in the least amount of chloroform, loaded on to silica gel plates and then developed in n-butanol: acetic acid: water (2: 1: 1). After convenient development, the plates were air dried and the blue and green spots were determined under TLC apparatus (Fig. 7).

The R_f of the blue and green compounds at were 0.80 and 0.93, respectively. The spots were separately scratched out, combined singly and eluted in methanol and filtered. The elute was concentrated by evaporation under vacuum to about 2 mL. The chloroform, petroleum ether and butanol were the most efficient solvent for extraction of the two antiviral substances. Similar results obtained by Ezzat *et al*. (2001), who used the chloroform

Table VII. Antiviral activity of different eluted spots of chloroform extract of *P. viridilivida* against ZYMV and on morphological characters of cucumber plants

Treatment	% of inhibition	Number of leaves	Shoot length (cm)	Fresh weight (g)
Healthy(general control)	All healthy	5.20 ± 0.16	30.0 ± 0.50	5.10 ± 0.25
Viral control	0.0	2.80 ± 0.10	16.2 ± 0.60	1.60 ± 0.08
Blue compound	100	5.00 ± 0.16	35.0 ± 0.84	5.20 ± 0.19
Green compound	100	5.10 ± 0.09	35.0 ± 0.70	4.80 ± 0.14
Blue & green compound	83	4.90 ± 0.20	28.0 ± 0.74	4.20 ± 0.19

Table VIII. Detection of ZYMV in cucumber leaves treated with blue and green compounds by ELISA technique

Treatment	ELISA reading
Positive control of ZYMV	1.618
Negative control of ZYMV	0.665
Healthy cucumber plants	0.697
ZYMV in cucumber leaves	1.884
Cucumber leaves treated by blue compound	0.975
Cucumber leaves treated by green compound	0.928
Cucumber leaves treated by two compounds	1.10

+ petroleum ether (1: 1 v/v) to extract antimicrobial substances. Also, (Nassar, 1998) reported that the chloroform was efficient solvent for the extraction of many antivirals. Also, this method similar to that used by Honda *et al*. (2001) and Uneo *et al*. (2002) for purification of different antibiotics. Moreover, purification techniques were carried out by many investigator using thin layer chromatography (TLC) and different solvent system (Nassar, 1998; Taro *et al*., 1998). Antifungal compounds of *T. koningu* (tk), *T. lignorum* (ti) and *T. pseudokoningu* (tpk), were purified by thin layer chromatography (TLC) (Amer & Singh, 2002). The characteristic of the purified antiviral compounds showed that they were colorless with no characteristic odor. According to the described chemical assignments obtained from the mass spectrum, NMR, IR, UV (Fig. 8 a, b, c, d, e, f, g & h) and elemental analysis, the proposed molecular formula were formula $C_{19}H_{35}O_4N$ and $C_{17}H_{34}O_4N$ and the molecular weights were 341 and 316 kDa for blue and green compounds, respectively.

Antiviral activity of the purified compounds. The antiviral test was carried out after mixing the eluted compounds with sap containing ZYMV in equal volums (V: V). Data in (Table VII) showed that the blue and green compounds give 100% inhibition against ZYMV and the number of leaves and fresh weight were similar to healthy plants. Also, these compounds increase the shoot length of treated plants more than healthy one. The mixture of the two compounds give 83% inhibition to ZYMV. The cucumber plants treated with two compounds together give number of leaves 4.9, shoot length 28 cm and fresh weight 4.2 g similar to healthy plant. The ZYMV within leaves of cucumber treated plants was detected by ELISA technique. The results in (Table VIII) showed that the extract of cucumber leaves treated by blue compound give 0.975 ELISA value compared to 1.884 in viral control. The green compound give 0.928 i.e. inhibit the virus from 1.884 to 0.928 near the negative ZYMV control. Similar results obtained by Maurhofer *et al*. (1994), who reported that the *P. fluorescens* strain CHA0 produces siderophore pyoverdine, salicylic acid, indoleacetate and several toxic metabolites for example HCN, 2, 4 diacetylphloroglucinol and bioluteorin. Marek *et al*. (2000) revealed that the plant growth promoting *P. fluorescent* strain 267 isolated from soil, produced pseudobactin A, 7-sulfonic acid derivatives of pseudobactin A and several B group vitamins. Yu-xuefang *et al*. (2002) reported that the *Pseudomonas viridilivida* is an antagonistic bacterium against rice sheath blight caused by *Rhizoctonia solani* in Japanica rice cv. Bing 96 - 42. Several trials were made to seek for antiviral substances of microbial origin (Zdorovenk *et al*. 1983). Fakhouri *et al*. (2001) purified a novel antibiotic N-mercapto-4-formylcarbostyril, from *P. fluorescens* strain G 308. This antibiotic is effective against many phytopathogenic microorganism *in vitro.*

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