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IMPROVED QUALITY OF VIRAL INFECTED GRAPE PLANTS CULTIVATED IN SOIL INOCULATED WITH RHIZOPHERIC MICROORGANISMS

[119]

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ABSTRACT

A considerable rhizopheric bacteria and mycorrhizae collectively known as plant growth promoting microorganisms (PGPM) have ability to induce acquire resistance in plant against pathogens and to provide benefits to their hosts. Grapevine viruses cause reducing yield and shortening the life span of infected plants in the vineyard. The current study aims to improve quality of *Grape fan leaf virus* (GFLV) infected grape plants via the soil inoculation with PGPM. Pot experiments were conducted under greenhouse during two seasons 2014/15 and 2015/16 in a Virology Greenhouse, Microbial. Dep. Fac. Agric., Ain Shams Univ. Cairo, Egypt. Grape cv. Flam grafted with GFLV infected stick and cultivated in inoculated soil with rhizopheric PGPB and mycorrhiza (VAM). GFLV was detected in infected leaves by DAS-ELISA. Plant growth parameters and chemical immune acquired resistance were assessment in GFLV infected grape cv. flam. The results were clearly indicated that PGPM inoculation in soil improved of plant growth in the second season (2015/16) compared with first season (2014/2015). PGPM (*Bacillus* sp., *Pseudomonas* sp. and *Serratia* sp. isolates and VAM) improved quality of GFLV infected grape plants via increased plant growth parameters (leave number, stem diameter, plant length, and phosphorus and potassium components in leaves). PGPM induced acquire resistance in plant against GFLV; it was found that, significant increase of proline and SA contents in GFLV infected grape leaves compared as healthy ones. The results revealed that chlorophyll a; b and carotenoids were significant decreased while inoculated PGPM in soil showed significant increase compared with healthy control ones. Expressed proteins and resistance enzymes (POD and PPO) of antiviral proteins were significant increase in PGPM application of GFLV infected grape growth related no inoculated PGPM ones**.** So that the current study recommended that the combination among VAM and PGPB soil inoculation improved quality of GFLV infected grape plant under greenhouse conditions.

INTRODUCTION

Grapevine is affected by 55 virus species belonging to 20 different genera being recorded (**Elbeaino, et al 2010 and Martelli, et al 2012**). Grapevine fan leaf virus (GFLV) is one of the most destructive and can cause severe losses by substantially reducing yield, affecting fruit quality, and shortening the life span of infected plants in the vineyard (**Aballay, et al 2012 and Gottula, et al 2013**). The major problem if grapes are already infected with GFLV, it's too late to do anything about this tragic disease, can avoid this disease by using some Plant Growth-Promoting Bacteria (PGPB) with or without Mycorrhizal treatment. Mycorrhizal fungi are obligating symbiotic of plants that colonize the root cortex and develop an extrametrical mycelium which helps the plant acquire water and mineral nutrients from the soil. Mycorrhizal are generally able to tolerate pathogens viz. viruses, soil-borne plant pathogens , reparation the root damage and photosynthetic drain by pathogens (**Li, et al 2007 and Ortas, 2008**). PGPR stimulate plant growth through one or more mech-

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anisms, either directly by supplying plant to phytohormones, phosphate solubilization; nitrogen fixation and siderophores production or indirectly protecting plant from phyto pathogens through antagonistic mechanisms or generating induced systemic resistance (ISR) in host plants. Induced resistance is a physiological "state of enhanced defensive capacity" elicited by plant growth promoting rhizobacteria (PGPR) (**Akram and Anjum., 2011; Zamioudis and Pieterse., 2012**). ISR has been successfully used for plant protection under both green house and field conditions for longer times (**Yang et al 2011**). The current study aimed to evaluate the soil inoculation with mycorrhizae (VAM) and three plant growth-promoting bacteria (PGPB) on growth of GFLV infected grape plant. GFLV was detected in infected by DAS-ELISA. Plant growth parameters and chemical immune acquired resistance were assessment in GFLV infected grape cv. flam.

MATERIALS AND METHODS

This study was conducted during two successive seasons 2014/2015 and 2015/2016 in a virology greenhouse, Department of Microbiology, Fac. of Agric., Ain Shams Univ., Cairo, Egypt. **Certified healthy Grape plants** that exhibited virus symptomless were obtained from special farm Horticulture Dept., Fac. of Agric., Ain Shams Univ., Cairo, Egypt. Depending on serological detection, GFLV inoculated into plant by stick grafting.

VAM inoculums: (*Glomus mosseae* and *G. intraradies*) were obtained from bio-fertilization Unit., Fac. of Agric., Ain Shams Univ., Cairo, Egypt. It was used at rate of 250 spores/5 Kg soil and infected root. The inoculums was distributed as a thin layer below the surface of the soil, pre-transplanting. The spores count in soil experiment and root infection by wet sieving method **(Gredemann and Nicolson, 1963).**

Plant Growth-Promoting Bacteria (PGPB): Soil samples were collected as deep as 10-15cm from cultivated grape plants in spring season from Fac. of Agric., Ain Shams Univ., Cairo, Egypt. The broth nutrient media were serially diluted in sterile 0.85 % NaCl solution and at 10^{-1} to 10^{-6} were placed on nutrient agar plate medium. Plates were incubated at 28-35^{o}C for 48 h. Each different colony was isolated on yeast extract glucose agar.

Identification of the isolated bacteria was made based on morphological cultural and biochemical identifications according to the Bergey's Manual of Systematic Bacteriology **(Krieg and Holt, 1984)**.

Phosphate solubilization was quantitative assayed according to (**Parmar and Sindhu, 2013)**. The selected bacteria isolates were cultivated in 100 ml of Pikovskaya broth medium and incubated at 28 ^{o}C in shaking incubator at 200 rpm for 7 days. Every 24 h 1.5 ml culture medium was centrifuged at 1000 rpm for 10 min. The amount of soluble phosphate was measured in the supernatant by atomic absorption spectrometer (AA-700 AAS with flame air C_2H_2) at 830 nm wave length.

The PGPB inoculums was prepared by mixing selected bacteria isolates ($5.5x10^{12}$ cfu/ml) and distributed as a thin layer below the surface of the soil, pre-transplanting.

Experimental design: The soil texture was clay with pH of 7.38 and EC of 3.5. The experimental design was complete random design with five replicates. Each replicate consisted of five treatments which were (control healthy), (control GFLV infected plant), (inoculated PGPB in soil), (inoculated VAM in soil), (inoculated PGPB+VAM in soil), (GFLV + VAM), (VAM + PGPB) and (GFLV+PGPB + VAM). Recommended irrigation, fertilization and pest control programs for the grape were applied. (Agricultural Monstry 2010).

Determinations

GFLV was detected in inoculated grape plants by their external symptoms, disease severity and double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) according to (**Clark and Adam, 1977)**. ELISA Kits were provided by Sanofi Sante Animal Paris, France (Virus & Phytoplasma Dept.; Plant Disease Inst.; A.R.C., Giza, Egypt).

Randomly ten plants were collected from each pot to determine number of leaf, diameter (cm) of stem, and plant length (cm). Root infection (%) and spore numbers of VAM were estimated by the method described by **(Li, et al 2007)**.

Macro-elements: (phosphorus, potassium, carbon and nitrogen) and micro-elements (iron, zinc and manganese) were determined according to the method described in **A.O.A.C. (2005).**

Photosynthetic pigments: Chlorophyll-A, B and Carotenoids were estimated in the fresh foliage leaves. One gram of fresh leaf was extracted by grinding with 10 ml of 80% acetone. The mixture was then centrifuged for 5 min, at 3000 rpm. The supernatant was used for spectrophotometric determination according to method **(Lichtenthaler., 1987).**

Proline content: 0.2 gm of leaf samples were homogenized in 5 mL of 3% (w/v) sulfosalicylic acid and centrifuged at 3000 rpm at 4°C for 10 min. The supernatants were used for proline estimation according to the method described by **(Bates, et al 1973)** at 520 nm using UV-spectrophotometer (Labomed, inc.23)**.**

Salicylic acid (sa): Determination of salicylic acid in plant tissues by (HPLC). SA from leaves extracted with 9:1 (*v*/*v*) methanol–chloroform was derivatives by use of bis (trimethylsilyl) trifluoroacetamide (BSTFA) under the optimum reaction conditions (120°C, 60 min). Quantitative analysis by GC–MS was performed in selected ion monitoring (SIM) mode using an internal standard. Procedures for sample preparation and reaction conditions were optimized. Analysis was completed within 2 h. A sensitivity of 10 mg g^{-1} fresh weight and a relative standard deviation less than 5.0% for SA in leaves were achieved **(Chunhui, et al 2003).**

Enzymes activities: The plant materials used for estimation of enzymes were 2 gm of the terminal buds homogenized with 10 ml of phosphate buffer PH 6.8 (0.1M), then centrifuged at 2°C for 20 min at 20000 rpm in a refrigerated centrifuge. The clear supernatant (containing the enzymes) was taken as the enzymes source **(Mukherjee and Choudhuri, 1983).**

Peroxidase (POX) activity: was assayed by measuring the inhibition of the auto-oxidation of pyrogallol using a method described by **(Bergmeyer, et al 1974)** at 470 nm wave length using UV-spectrophotometer (Labomed, inc.23).

Polyphenol oxidase (PPO) activity: was assayed by measuring the inhibition of the auto-oxidation catechol using a method described by (**Matta and Diamond,1963).** The absorbance was measured at 495 nm wave length using UV-spectrophotometer (Labomed, inc.23).

Statistical analysis: Obtained data were statistically analyzed according to the analysis of variance as described by **(Waller and Duncan, 1969).**

RESULTS

The results were clearly indicated that PGPM soil inoculation improved of grape plant growth in the second season 2015 / 16 compared with first season 2014 /15. These results were recorded in the second season.

The isolated three Bacteria were identified morphologicaly, physiologicaly and biochemical characteristics as shown in **Table (1)** according to Bergey's manual of determinative bacteriology. These results were confirmed by PCR test as *Bacillus* sp., *Pseudomonas* sp. and *Serratia* sp. Selection and Screening of best bacterial isolates occurred according to its production to Hydrogen Cyanide (HCN), Indol -3-acetic acid(IAA),and Phosphate solubilization (P) as *Ps. fluorescence, B. megatherium* and *S. marcescens***.**

Virological assessments

Disease severity and external symptoms: GFLV was diagnosed by the symptoms of vein banding, crinkle, deformation and fan leaf **(Fig.1)**. GFLV was detected using DAS-ELISA. The disease severity of GFLV and with or without VAM and PGPB listed in **Table (2),** which shows that treatment of VAM and PGPB reduced the disease severity of virus corresponding to virus only.

Serological confirmation of GFLV in leaves and roots were carried out using DAS-ELISA. VAM or / and PGPB were reduced the Virus concentration, Corresponding to virus only in Grape vein leaves. On the other hand due to dominated the virus from root.

VAM frequency: The results in **Table (4)** showed the variation of grape plants responsibility to VAM while treated with and without PGPB. The highest values of VAM frequency and relative intensity were recorded in the root of the plant treated with VAM. The lower values of infected VAM frequency were recorded in the non-treated grape plants (control). This indicates that, increased formation of VAM structures in the roots. As well as VAM spore count and infection percentage in grape roots showed similar trend as clear from **Table (4)**.

Table 1. Morphological, physiological and biochemical characteristic of the bacterial inoculum

Morphological, Physiological and Charecteristics		*Bacillus* sp.	*Pseudomonas* sp.	*Serratia* sp.
Morphological characters	**Pigmentation**	**---**	Florescent green	Red
	O_2 requirements	Aerobic	Obligate aerobic	Aerobic
Microscopic examination	**Gram reaction**	**+**	**-**	-
	Cell shape	Rod	Rod	Rod
	Sporulation	**+**	**-**	-
	Capsule	**-**	**-**	-
	Motailty	**+**	**+**	+
Biochemical characteristic	**Methyl red**	**-**	**-**	-
	VP	**+**	**-**	+
	Citrate	**+**	**+**	+
	H_2S	**+**	**+**	-
	Catalase	**+**	**+**	+
	Oxidase	**+**	**+**	-
	Urease	**+**	**+**	-
	Lipase	**+**	**+**	+
	Coagulase	**+**	**+**	-
	Gelatin lequification	**+**	**+**	-
	Starch hydrolysis	**+**	**-**	-
Assimilation of carbon source	**Glucose**	**+**	**+**	+
	Lactose	**+**	**+**	-
	Maltose	**+**	**+**	-
	Sucrose	**+**	**+**	+
	Cellulose	**-**	**-**	+
	Sorbitol	**-**	**+**	+
	Histidine	**-**	**+**	-

Fig. 1. Photo plate showing viral symptoms on grape plants infected with GFLV

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Table 2. Disease severity of infecting treated grape plants grown under greenhouse condition

Treatments	Vein banding	Deformation	Crinkle	Fan leaf	*D.S%
GFLV	8**	9*	7*	8*	50.6
PGPB+GFLV	5	6	4	3	26.3
VAM+GFLV	4	5	5	3	25.6
VAM+PGPB+GFLV	3	4	3	2	18.1

**Number of infected plant D.S % *Disease severity

Table 3. Serological confirmation of GFLV in leaves and roots grown soil inoculated with VAM and PGPB using DAS-ELISA

Plant treatments	ELISA value at 405 nm			
	Leaves		Roots	
	ELISA value	Result	ELISA value	Result
Healthy	0.223	-	0.185	-
Infected plant with GFLV	0.735	+	0.445	+
GFLV Infected plant treated with PGPB	0.349	±	0.236	-
GFLV Infected plant treated with VAM	0.315	±	0.228	-
GFLV Infected plant treated with PGPB and VAM	0.376	±	0.266	-

10 inoculate plants (leaves and roots)
OD +ve control = 0.825
OD -ve control = 0.208

Table 4. Spore count and root infection level percentage of VAM infecting grown in grape plants grown soil inoculated with VAM and BGPB under greenhouse condition

Treatments	Count of VAM	
	Spore count (in soil)	VAM root infection (%)
Control	100	9.2
VAM	495.2	18.5
VAM+PGPB	480.5	16.25
VAM+GFLV	325	9.5
VAM+PGPB+GFLV	433. 5	14.75
L.S.D 1%	75	3.25

Effect of VAM, and PGPB on growth characteristics: The impacts of VAM, and PGPB on growth of grape plants were significantly under greenhouse conditions. The results clearly indicated that VAM inoculation increased significantly diameter of stem plant; leaf number of plant in the second season. As the respect to the interaction effect of plant diameter results indicate that all used plants stimulants plus VAM showed the highest values in two tested seasons. As for., plants treated with VAM or PGPB alone showed the highest value in addition to the companions of PGPB plus VAM. Additionally, data in **Table (5)** emphasized that VAM inoculation with grape plants treated with PGPB had positive significant effect on most of plant parameters, whereas number of leaf per plant were increased significantly compared with control ones.

Effect of VAM+PGPB on macro-elements in soil: VAM inoculation, associated with PGPB on grape rhizosphere. It was clearly from results in **Table (6)** that there were significant differences in N, P and K contents in plants treated with VAM only and PGPB. Also, data indicate that soil inoculated with VAM + PGPB gave the highest Phosphate (P) content but those treated with VAM + PGPB showed the highest Potassium (K) content than the other treatments.

Chemical immune acquired resistance: Proline, SA and resistance enzymes (POD and PPO) were significant increased in GFLV infected grape cv. flam cultivated in inoculated PGPB,VAM and PGPM soil compared with GFLV infected ones **(Table, 7&8)** with significant differences. Salyslic acid content was 785.5, 925.2, 952.7, 775, in GFLV, GFLV + PGPB, GFLV + VAM, GFLV + PGPB + VAM plants treated with VAM only and PGPB respectively. Also, data indicate that plants infected with GFLV gave the highest proline content (49.15), those plants inoculated with VAM + PGPB showed the increased content than the other treatments.

Expressed resistance enzymes of antiviral proteins were significant increase in GFLV infected grape plants cultivated in PGPB and VAM inoculated soil compared with healthy control ones (**Table, 8**). The results revealed that, PPO and POD were significantly increased in GFLV infected grape plants Followed with cultivated in soil inoculated with PGPM (**Table, 8**).

The results revealed that, chlorophyll a; b and carotenoids were significantly decreased in GFLV infected grape plants compared with healthy ones. On the contrary, GFLV infected grape plants cultivated in PGPM inoculated soil showed significant increase in chlorophyll a ; b and carotenoid compared with healthy control ones **(Table, 9)**.

Table 5. Impact of VAM and PGPB on grape growth characteristics

Treatment	Dry Weight (g)	Number of leaves plant	Stem Diameter (cm)	Plant Length (cm)
Control	14.7	45	0.5	50
GFLV	12.6	35	0.4	45
PGPB	20.15	57	0.7	58
PGPB+GFLV	18.75	52	0.6	52
VAM	19.01	58	0.7	59
VAM+GFLV	19.25	50	0.6	54
VAM+PGPB	21.35	65	0.7	62
VAM+PGPB+GFLV	18.95	60	0.8	75
L.S.D 1%	1.5	8	0.2	5

Table 6. Macro-elements in grape rhizosphere under different treatments

Treatment	Macro-elements content		
	N %	P mg/l	K mg/l
Control	1.0	4.63	1.69
GFLV	0.8	4.23	1.59
PGPB	1.5	6.78	5.78
PGPB+GFLV	2.0	5.63	4.69
VAM	2.02	7.18	7.85
VAM+GFLV	1.88	6.04	5.34
VAM+PGPB	1.98	7.04	6.34
VAM+PGPB+GFLV	1.98	7.04	6.34
L.S.D 1%	0.05	0.45	0.55

Table 7. Effect of PGPM on chemical indicators for systemic resistances in infected grape vein plants Proline and Salsylic acid

Treatment	Salyslic acid(Mg/g f.w)	Proline (Mg/g f.w)
Control	575.25	43.08
GFLV	785.5	49.15
PGPB	725.5	45.53
GFLV+PGPB	925.2	46.72
VAM	650.25	45.25
GFLV+VAM	952.7	48.75
VAM+PGPB	752.3	42.75
GFLV+PGPB+VAM	775	46.72
L.S.D 1%	25.4	2.3

Table 8. Effect of PGPM on chemical indicators for systemic resistances in infected grape vein plants enzymes Peroxidase (POD), Polyphenol oxidase (PPO)

Treatment	PPO (Unit/mg protein)	POD (Unit/mg protein)
Control	4380	0.057
GFLV	5655	0.143
PGPB	6865	0.075
GFLV+PGPB	5315	0.044
VAM	4257	0.125
GFLV+VAM	5275	0.030
VAM+PGPB	6425	0.072
GFLV+PGPB+VAM	5235	0.052
L.S.D 1%	145	0.02

Table 9. Effect PGPR on Chemical indicators for systemic resistances in infected grape vein plants Chlorophyll (A, B) and Carotenoids

Treatment	Chlorophyll A Mg/g fw	Chlorophyll B Mg/g fw	Carotenoids Mg/g fw
Control	12.86	13.34	7.92
GFLV	10.24	12.67	7.25
PGPB	14.55	14.74	8.28
GFLV+PGPB	15.01	15.25	9.21
VAM	14.75	15.0	8.50
GFLV+VAM	15.21	14.95	9.5
VAM+PGPB	14.62	14.34	8.34
GFLV+PGPB+VAM	14.99	14.87	8.99
L.S.D 1%	0.75	0.8	0.7

DISCUSSION

The results were clearly indicated that, PGPM inoculation in soil improved of plant growth as well as GFLV infected grape plants in the season (2015/2016). Plant growth promoting Bacteria (*Ps. fluorescence, B. megatherium* and *S. marcescens)* (or PGPB) were isolated from grape plant rhizosphere on nutrient agar media .The isolated PGPB were identified based on morphological, cultural and biochemical according to Bergey's Manual systemic Bacteriology **(Krieg and Holt, 1984)**. As well as Phosphate solublization **(Parmar and Sindhu, 2013**). The plant growth -promoting bacteria belong to a beneficial and heterogeneous group of microorganisms that can be found in the rhizosphere, on the root surface or associated to it, and are capable of enhancing the growth of plants and protecting them from disease and abiotic stresses **(Dimkpa, et al 2009 and Grover, et al 2011).** PGPR are good inoculant candidates, because they colonize roots and create a favorable environment for development and function **(Bacon and Hinton, 2006).** The results were recorded at 2016 season. PGPM (*Bacillus* sp., *Pseudomonas* sp. and *Serratia* sp. isolates and VAM) improved quality of GFLV infected grape plants via increased plant growth (leave number, stem diameter, plant length, Phosphorus and potassium in leaves). To achieve maximum benefits in terms of fertilizer savings and better growth, the PGPR-based inoculation technology should be utilized along with appropriate levels of fertilization. Moreover, the use of efficient inoculants can be considered an important strategy for sustainable management and for reducing environmental problems by decreasing the use of chemical fertilizers **(Alves, et al 2004; Adesemoye, et al 2009; Hungria, et al 2013)**.

Plant roots react to different environmental conditions through the secretion of a wide range of compounds which interfere with the plant-bacteria interaction, being considered an important factor in the efficiency of the inoculants **(Cai, et al 2009, 2012.** The mechanisms by which bacteria can influence plant growth differ among species and strains, so typically there is no single mechanism for promoting plant growth. Many bacteria promote plant growth at various stages of the host plant life cycle through different mechanisms. The influence of bacteria in the rhizosphere of plants is largely due to the production of auxinphytohormones **(Spaepen, et al 2007).** Several bacterial species can produce indolic compounds (ICs) such as the auxinphytohormone indole-3-acetic acid (IAA), which present great physiological relevance for bacteria-plant interactions, varying from pathogenesis to phytostimulation **(Spaepen, et al 2007).** PGPM induced acquire resistance in plant against GFLV; it was found that, significant increase of proline and SA contents in GFLV infected grape leaves than healthy ones. The results revealed that chlorophyll a; b and carotenoids were significant decreased while inoculated PGPM in soil showed significant increase compared with healthy control ones. Expressed proteins and resistance enzymes (POD and PPO) of antiviral proteins were significant increase in PGPM application of GFLV infect-

ed grape growth related no inoculated PGPM ones. In addition to the utilization of IAA, strain 1290 also produced IAA in culture medium supplemented with L-tryptophan. In co-inoculation experiments in radish (*Raphanus sativus* L.) roots, this strain minimized the negative effects of high IAA concentrations produced by the pathogenic bacteria *Rahnella aquaticus* and *P. syringae*. In this context, microorganisms that catabolize IAA might also positively affect the growth of plants and prevent pathogen attack **(Leveau and Lindow, 2005).**

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