Effect of Chitinase Producing Bacteria and Humate on Growth, Productivity and Root Knot Nematode Control of Flame Seedless Grapevines.

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Abstract: This study was conducted for two successive seasons (2013 and 2014) in a private vineyard located at 64 Km of Cairo-Alexandria desert road to study the effect of chitinase producing bacteria and humate on growth, productivity and root knot nematode control of Flame Seedless grapevines. The chosen vines were ten-years-old, grown in a sandy loam soil, spaced at 2 X 2.75 meters apart and irrigated by the drip irrigation system, trained to bilateral cordon with spur pruning, and trellised by the "Y" shape system. The vines were pruned during the last week of January with bud load of (60 buds/vine). The chitinase producing bacteria strains were isolated from soil of the same farm 10 to 15 cm depth from the rhizospheric zone of grape plants and enriched in a minimal medium containing chitin as a sole source of carbon. The screening of chitinase producing isolates was performed by spot inoculating each of the isolates at the center of Colloidal Chitin Agar (CCA) plates containing colloidal chitin 0.5% w/v. The three isolates which showed the most clear zone were considered as the potential chitinase producing strain and then grown in chitin broth to determine chitinase enzyme activity. Then identified by Bio-log Technique as *Bacillus subtilis* Bs12, *Bacillus subtilis* Bs14 and *Pseudomonas fluorescens,* these strains were used in the experimented field with humate supported by macro-elements NPK (10: 10:10) (HA1) or micro-elements (Fe 1%, Mn 0.5%, Mg 1%) (HA2). Bacterial inoculants and humate were soil drench applied at 10 L/ fed either individually or in combination among them at three application dates: the 1st date (after bud burst), the 2nd date (after shattering) and the 3rd date (4 weeks after shattering). The results showed that, the inoculation of *Pseudomonas fluorescens* + Humate (HA1) significantly were the best results in comparison with the other treatments and control in both seasons. However, it reduced in nematode no. in soil and roots, which it reflected later in increasing the yield and its components and achieve the best physical characteristics of bunches including bunch weight, length and width, as well as improving the physical characteristics of the berries, i.e. (berry weight, size and dimensions) and chemical characteristics of the berries, including T.S.S. (%), total acidity (%), TSS /acid ratio and total anthocyanin, in addition enhancement of some vegetative attributes i.e. (shoot length and number of leaves) and leaf content of total chlorophyll and mineral content including NPK (%). The economical study indicated that bio inoculation with *Pseudomonas fluorescens* bacteria accompanied with Humate supported by macro-elements (HA1) gave the highest net income as compared to the control of Flame Seedless grapevines.
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1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important and favorable fruit crops in Egypt, it is considered the second fruit crop after citrus. The planted area reach about 188,543 feddan producing 1,378,815 tons (**Ministry of Agriculture statistics, 2013).** The production of grapes affected by several problems such as nematodes **(El-Hady *et al.*, 2015).**

Nematodes populations in the field could be suppressed and maintain at levels below the economic threshold by using nematicide application, planting resistant crop varieties, fallowing, intercropping, heat treatment, use of biological agents, soil amendments and all cultural practices that are inhibitory to nematode development and reproduction **(Daramola *et al.*, 2015).** *Meloidogyne incognita* is a major plant-parasitic root-knot nematode species, which affects the final yields, production and quality of many annual and perennial crops **(Ruiz *et al.*, 2014).** Chitinases, used in agriculture, are known to carry out functions as biological control agents against root-knot nematode **(Zaghloul *et al.*, 2015).** Chitinases from micro-organisms are a potential weapon for the management of root-knot nematodes, because the nematode eggshell cuticle is contain of a chitin layer **(Ashoub and Amara, 2010);** which can be degraded by chitinases. Moreover, the eggshell cuticle is an infection site for microorganisms, which are applied for biological control of nematodes. *Pseudomonas fluorescens* is antagonistic to plant pathogens by

producing antibiotics and cell wall degrading enzymes, such as b-1, 3 glucanase, chitinase and protease **(Yong and Kil, 2015).** Moreover, the chitinolytic bacteria, *Bacillus subtilis* suppress nematodes **(Mokbel and Alharbi, 2014).**

Many occurring compounds are known to possess nematicidal activity such as organic acids. Organic acids released during the decomposition of raw organic materials are one of many factors contributing to reduce nematodes population **(McBride *et al.*, 2000),** but little is known about the direct effects of low-molecular-weight organic acids for nematode control **(El-Sherif *et al.*, 2015).** Humic and Fulvic acids have been early recorded to have appositive effect against plant parasitic nematodes (**El-Mougy *et al.*, 2013**). Humate as an organic substances are among the means available to achieve sustainability in agricultural production. They play a vital role because of their beneficial effects on physical, chemical and biological characteristics of soil **(Afifi, 2010).** Humate can be added to the soil for improving the crop yield. A benefit of humate due to their ability to complex metal ions and form aqueous complexes with micronutrients and also may form an enzymatically active complex, which can be carry on reactions that are usually assigned to the metabolic activity of living microorganisms **(Stevenson, 1994).** Many studies reported that humate preparations succeeded to increase the uptake of mineral elements **(Mackowiak *et al.*, 2001),** to promote the root length **(Canellas *et al.*, 2002),** and to increase the fresh and dry weights of crop plants (**Chen *et al.*, 2004**).

Concerning the effects of humic acid on *Vitis vinifera* the available literature is scarce, which generally investigated the effects of commercial humic acid products on table grape (**Colapietra 2000, Sánchez-Sánchez *et al.*, 2006; Ferrara *et al.*, 2007; Ferrara and Brunetti, 2010 and Aydin, 2011**) and grapevines rootstocks **(Zachariakis *et al.*, 2001).** Humic acid has positive effects in increasing the productivity of fruit crops due to the conversion of unavailable minerals into soluble forms that plants can use , improving plant nutrition by stimulating the absorption of mineral elements through the roots , stimulating root growth thus enabling better uptake of nutrients, helps keeping inorganic fertilizers in the root zone this improve growth, chlorophyll content enhanced photosynthesis and increased tissue concentrations of N, K and increase yield and improve fruit quality**(Li-Nan *et al* ., 1999; Silva *et al.*, 1999 ; Guo *et al* ., 2000 ; Zhu and Zhu, 2000; Hussien *et al.*, 2005 and Omar, 2005).**

The aim of this investigation was to study the possibility of using organic products and biological agents to suppress root-knot nematode and their effect of growth, productivity and fruit quality of Flame Seedless grapevines.

2. Material and Methods

This study was conducted for two successive seasons (2013 and 2014) in a private vineyard located at 64 Km of Cairo-Alexandria desert road to study the effect of chitinase producing bacteria and humate on growth, productivity and root knot nematode control of Flame Seedless grapevines. The chosen vines were ten-years-old, grown in a sandy loam soil (Table, 1), spaced at 2 X 2.75 meters apart and irrigated by the drip irrigation system, trained to bilateral cordon with spur pruning, and trellised by the "Y" shape system. The vines were pruned during the last week of January with bud load of (60 buds/vine).

Table (1): Chemical and physical characteristics of soil samples for the experimental soil

Mechanical analysis.										
Clay %		Silt %			fine sand course %				Texture grade	
27.3		2.4			70.3				Sandy loam	
Chemical analysis.										
pH 1:2.5	EC Mmos/cm	SP	Anions (m mol/ L)				Cations (m mol/ L)			
			CO_3^{2--}	$HC0_3^-$	Cl^-	$SO4^{2-}$	Ca^{++}	Mg^{++}	Na^+	k^+
7.75	1.64	32.0	--	3.13	53.9	31.42	34.0	23.6	29.5	1.35
Concentration (mg/ kg soil) of available macro and micro elements in soil.										
N	K	P		Cu		Fe	Mn		Zn	
97.44	431.2	21.06		2.36		14.00	3.36		19.26	
No. nematodes (larva/ 200 g soil) 650										

Microorganisms:

Collection of Soil Samples and Screening for chitin degraders.

Soil samples were collected from 10 to 15 cm depth from the rhizospheric zone of grape plants in plastic bags from the same private vineyard. The soil samples were enriched in a minimal medium containing chitin as a sole source of carbon and nitrogen 5 grams of each soil samples were enriched in 100 ml of Minimal Salts Medium (MSM) (Chitin

powder 1g, KH_2PO_4 0.03g, K_2HPO_4 0.07g, $MgSO_4$ 0.05g, $FeSO_4.7\ H_2O$ 0.001g, $MnCl_2.4H_2O$ 0.001g $ZnSO_4.7H_2O$ 0.001g in 100 ml of deionised water) (Jholapara *et al.,* 2013). The enrichment was carried out under room temperature with shaking conditions (150 rpm). Viable bacterial counts were performed to assess for the type of microflora observed and select the colonies for further screening test. The colonies which were persistently detected in viable count studies were selected for further screening. The screening of chitinase producing isolates was performed by spot inoculating each of the isolates at the center of plates of Colloidal Chitin Agar (CCA) medium containing colloidal chitin 0.5% w/v (Jholapara *et al.,* 2013). The plates were incubated at room temperature for 5 days. The plates were observed for a zone of clearance around the colony (Dingel *et al.,* 2013). The isolate which showed the most clear zone was considered as the potential chitinase producing strain and was selected for further studies. The active selected isolates producing chitinase enzyme were identified as *Bacillus subtilis* Bs12, *Bacillus subtilis* Bs 14 and *Pseudomonas fluorescens*using Bio-log Technique at Plant Pathology Research Institute, ARC, Ministry of Agriculture, Giza, Egypt.

Preparation of Colloidal Chitin.

Colloidal chitin was prepared from commercial chitin by the method of **Roberts and Selitrennikoff (1988),** with few modifications adopted as follows: To five grams of powdered chitin 50 ml of concentrated HCl was added slowly. The mixture was left standing for 6 hours. The mixture then was added to 250 ml of ice-cold ethanol with continuous stirring. The pH of the solution was adjusted to 7 and the precipitate was collected by centrifugation at 8000 rpm for 30 minutes at 4°C. The collected colloidal chitin was dried at room temperature and later stored at 4°C for further use.

Chitinase Assay

Chitinase was assayed using colloidal chitin as a substrate. The strain was grown in chitin broth with continuous shaking at (150 rpm) at 30°C for five days. The cell suspension was centrifuged at 8,000 rpm for 20 minutes at 4°C to obtain cell free supernatant which was used for chitinase assay. One ml of the crude enzyme solution was added to 1% of substrate solution in acetate buffer (20 mm, pH 4.6), and the solution was incubated at 50°C for 30 minutes. After centrifugation, the amount of reducing sugars in the cell free supernatant was determined by the method of **Imoto and Yagishita (1971**). The activity was calculated from the standard curve plotted using known concentrations of N-acetylglucosamine. One unit of enzyme activity was expressed as the amount of enzyme required to liberate one µg NAGA/ml/min. under the assay conditions.

Preparation of bacterial inoculants.

Conical flasks (250 ml) containing 100 ml of King's broth medium (King *et al*., 1954) for *Pseudomonas fluorescens* and Nutrient broth medium (**Difco, 1985**) for *Bacillus subtilis* were sterilized at 121°C for 15 min were used as growth medium. The flasks were inoculated with a loop- full of the tested strain then incubated at 28-30°C on rotary shaker (150 rpm) for 2 days. Bacterial inoculants (10^9 CFU/ml) were added to soil at rate of 10 L/fed. at three application dates: the 1st date (after bud burst), the 2nd date (after shattering) and the 3rd date (4 weeks after shattering).

Humate:

The chemical analysis of used liquid humate shown in table (2). It produced by Arctech com. Humate added to soil at rate of 3 L/fed. at three application dates: the 1st date (after bud burst), the 2nd date (after shattering) and the 3rd date (4 weeks after shattering).

Table (2): The chemical analysis of used liquid humate.

Component	Humate macro-elements NPK (HA1)	Humate micro-elements (HA2)
Humic acid (%)	3	3
Organic matter	73.24	73.24
Organic carbon (%)	42.48	42.48
pH	6.0	8.10
N %	10	2.0
P %	10	0.35
K %	10	2.5
Fe mg/L	900	10000
Mn mg/L	90	5000
Mg mg/L	90	10000

Bacillus subtilis Bs12, *Bacillus subtilis* Bs14, *Pseudomonas fluorescens* Pf and humate supported by macro-elements NPK (10: 10:10) (HA1) or micro-elements (Fe 1%, Mn 0.5%, Mg 1%) (HA2) were soil drench applied individually or in combination with bacteria. One hundred and forty four uniform vines were chosen. Each four vines acted as a replicate and each three replicates were treated by one of the experiment treatments**.**

Twelve treatments were applied as follows:

1.	*Bacillus subtilis* Bs12	7.	*Bacillus subtilis* Bs12 +(HA2)
2.	*Bacillus subtilis* Bs14	8.	*Bacillus subtilis* Bs14 +(HA1)
3.	*Pseudomonas fluorescens* Pf	9.	*Bacillus subtilis* Bs14 +(HA2)
4.	Humate NPK (HA1)	10.	*Pseudomonas fluorescens* Pf+(HA1)
5.	Humate micro-elements (HA2)	11.	*Pseudomonas fluorescens* Pf+(HA2)
6.	*Bacillus subtilis* Bs12 +(HA1)	12.	Control (Untreated vines).

The following parameters were adopted to evaluate the tested treatments:-

1. **Soil bacterial activity:**

Samples of soil were taken from the rhizospheric zone of grape plants at two dates: the 1st date, after bud burst and the 2nd date, after shattering to record population dynamics of total bacterial, *Pseudomonas* sp count, dehydrogenase and chitinase activities.

The total bacterial count and *Pseudomonas* sp count were determined by the plate count method according to **Reinhold *et al.* (1985)** using Nutrient ager medium for total bacterial count and King's agar medium for *Pseudomonas* sp. count **(Difco, 1985);** Dehydrogenase and chitinase activities in rhizosphere were determined according to **Skujins (1976)** and **Rodriguez-Kabana *et al.* (1983)** respectively.

2. **Exmination of nematodes:**

Samples of soil were taken from the rhizospheric zone of grape plants at two dates: the 1st date, after bud burst and the 2nd date, after shattering to record the reduction on number of juveniles in soil and roots according to **Norton (1978)**.

Representative random samples of six bunches/vine were harvested at maturity when TSS reached about 16-17% according to **Tourky *et al.* (1995).**

3. **Yield and physical characteristics of bunches:**

Yield/vine (kg) was determined as number of bunches/vine X average bunch weight (g). In addition, average bunch weight (g), bunch length and width (cm) were determined.

4. **Physical characteristics of berries:**

Average berry weight (g), average berry size (cm^3) and average berry dimensions (length and diameter) (cm) were determined.

5. **Chemical characteristics of berries:**

Total soluble solids in berry juice (T.S.S.) (%) by hand refractometer and total titratable acidity as tartaric acid (%) (AOAC 1985). Hence TSS /acid ratio and total anthocyanin of the berry skin (mg/100g fresh weight) according to **Husia *et al.*, (1965)** were calculated.

6. **Some characteristics of vegetative growth**

At growth cessation, the following morphological and chemical determinations were carried out on three fruitful shoots / the considered vine:

1- Average shoot length (cm)

2- Average number of leaves.

7. **Leaf content of total chlorophyll and mineral content**

1-Leaf content of total chlorophyll (SPAD)

Samples of leaves were taken at full bloom and it's were measured by using nondestructive Minolta chlorophyll meter SPAD 502 of the apical 5th and the 6th leaves **(Wood *et al.*, 1992).**

2- Leaf mineral content

Leaves opposite to the clusters were collected then dried to estimate nitrogen, phosphorus and potassium percentages according to **Bremner and Mulvaney (1982), Olsen and Sommers (1982) and Jackson (1970),** respectively**.**

- **Statistical analysis:**

The complete randomized block design was adopted for the experiment. The statistical analysis of the present data was carried out according to **Snedecor and Cochran (1980).** Averages were compared using the new L.S.D. values at 5% level.

3. Results

Isolation and Screening of Chitinolytic Bacteria

A total of 20 bacterial isolates were isolated from the soil samples. The isolates were enriched in MSM medium supplemented with 1% chitin powder. On the basis of screening on colloidal chitin agar plates, three isolates were demonstrated as chitinolytic potential which produced a more zone of clearance. To evaluate bacteria producing chitinase; the three isolates were grown in chitin broth medium for five days. The cell suspension was centrifuged (150 rpm). The supernatant was use for chitinase assay; Table (3) shows the quantitative of chitinase enzyme producing by bacteria. The active isolates producing chitinase enzyme were identified by Bio-log Technique as *Bacillus subtilis* Bs12, *Bacillus subtilis* Bs14 and *Pseudomonas fluorescens;* then used in the field experiment.

Table (3): Chitinase enzyme producing by selected bacteria.

Strains	Chitinase activity (µg NAGA/ml/min).
Bacillus subtilis Bs12	786.3
Bacillus subtilis Bs14	896.4
Pseudomonas fluorescens Pf	1065.8

Soil bacterial activity:

Regarding to data in Table (4) the total bacterial count was increased in all treatment. The high increase was observed in the combination of bacteria with humate (HA1). The most increase was observed in the treatment of *Pseudomonas fluorescens* plus humate (HA1) which recorded (154 and 183 CFU×10^7/ g dry soil) at the 1stdate (after bud burst) and the 2nd date (after shattering) at 2013. Also the season 2014 showed the same trended. On the other hand, *Pseudomonas* sp. count was increased in all treatment especial with the treatment of *Pseudomonas fluorescens* individual or with humate, but the combination of *Pseudomonas fluorescens* and humate (HA1) gave the highest increased which recorded 156 and 182 CFU×10^6/ g dry soil at the 1stand 2nd date at

2013; and 157 and 186 CFU×10^6/ g dry soil at the 1st and 2nd date of sample taking at 2014, respectively. Moreover, dehydrogenase activity was significantly increased after bacterial inoculation and humate addition espial in combination treatment as shown with *Pseudomonas fluorescens* and humate (HA1) which recorded 68.33 and 82.33µg TPF / g dry soil / day after the 1st and 2nd date at 2013, And the same trend at the season 2014 which recorded 65.00 and 79.33µg TPF / g dry soil / day after the 1st and 2nd date respectively. On the other hand, chitinase activity was significantly increased in most treatments especially in the treatment with *Pseudomonas fluorescens* and *Bacillus subtilis* individually or in combined with humate (HA1), the most increased was observed in treatment with *Pseudomonas fluorescens* plus humate (HA1) which recorded 7.30 and 8.53µg NAGA/g dry soil/h the 1st and 2nd date at 2013; and 6.90 and 8.77 µg NAGA/g dry soil/h the 1st and 2nd date at 2014, respectively.

Table (4): Effect of *Bacillus subtilis*, *Pseudomonas fluorescens* and humate on soil microbial counts of Flame Seedless grapevines at 2013 and 2014 seasons.

Characteristics	Total bacterial count (CFU×10^7/ g dry soil)				*Pseudomonas* sp. count (CFU×10^6/ g dry soil)			
	2013		2014		2013		2014	
Treatments	1st date	2nd date	1st date	2nd date	1st date	2nd date	1st date	2nd date
Bacillus subtilis Bs12	76	89	74	91	51	63	49	62
Bacillus subtilis Bs14	75	91	76	93	54	65	56	68
Pseudomonas fluorescens (Pf)	85	105	89	109	134	152	132	149
Humate 1 (HA1)	112	138	115	142	112	138	116	143
Humate 2 (HA2)	108	126	113	129	108	123	112	127
Bs12 + (HA1)	126	149	128	156	87	96	85	97
Bs12 + (HA2)	123	151	126	153	84	92	86	93
Bs14 + (HA1)	131	167	134	169	121	138	123	141
Bs14 + (HA2)	112	136	116	138	119	131	117	136
Pf+ (HA1)	154	183	157	191	156	182	157	186
Pf + (HA2)	141	179	144	181	143	161	146	163
Control (Untreated vines)	43	57	46	59	38	47	41	49

Table (5): Effect of *Bacillus subtilis*, *Pseudomonas fluorescens* and humate on soil enzymes activities of Flame Seedless grapevines at 2013 and 2014 seasons.

Characteristics	Dehydrogenase activity (µg TPF/g dry soil/ day)				Chitinase activity (µg NAGA/g dry soil/h).			
	2013		2014		2013		2014	
Treatments	1st date	2nd date	1st date	2nd date	1st date	2nd date	1st date	2nd date
Bacillus subtilis Bs12	42.67	51.00	44.00	51.67	5.10	6.10	5.23	6.33
Bacillus subtilis Bs14	44.00	58.33	44.33	61.00	5.20	6.90	5.30	7.20
Pseudomonas fluorescens (Pf)	46.67	60.00	49.33	61.67	5.90	7.13	6.07	7.30
Humate 1 (HA1)	55.67	65.33	54.00	66.33	3.87	4.27	4.10	4.40
Humate 2 (HA2)	51.33	61.33	52.00	62.33	3.63	4.03	3.93	4.47
Bs12 + (HA1)	60.33	71.00	61.67	75.33	6.13	7.53	6.40	8.00
Bs12 + (HA2)	55.67	69.00	69.00	75.33	5.47	7.10	6.63	8.10
Bs14 + (HA1)	65.33	76.33	70.67	85.33	6.40	7.90	7.53	8.57
Bs14 + (HA2)	58.33	75.33	57.33	71.00	5.83	7.47	5.67	7.40
Pf+ (HA1)	68.33	82.33	65.00	79.33	7.30	8.53	6.90	8.77
Pf + (HA2)	63.67	77.33	61.67	76.00	6.70	8.10	6.10	8.40
Control (Untreated vines)	20.67	28.67	22.00	32.00	3.63	4.17	3.80	4.07
new L.S.D. at (0.05) =	4.31	4.98	4.62	5.03	0.49	0.44	0.46	0.51

Reduction of nematode larva in soil and roots.

As shown in Table (6) there was a reduction of nematode larva population density in soil and infected roots in all treatments, the most reduction of nematodes population in soil was estimated in the treatment of *Pseudomonas fluorescens* plus humate (HA1) which recorded 76 and 95 % at 1st and 2nd date, respectively at 2013, and the same trend was

observed in the season 2014. Also there was a clearly reduction in nematode roots infection in all treatment especially in the treatment *Pseudomonas fluorescens* plus humate (HA1) which recorded 87 and 98 % at 1st and 2nd date of at 2013, and the same trend was observed in the season 2014.

Table (6): Effect of *Bacillus subtilis*, *Pseudomonas fluorescens* and humate on reduction of nematode larva in soil and roots (%) of Flame Seedless grapevines in 2013 and 2014 seasons.

Characteristics	Reduction of nematode larva in soil (%)				Reduction of nematode larva in roots (%)			
	2013		2014		2013		2014	
Treatments	1st date	2nd date	1st date	2nd date	1st date	2nd date	1st date	2nd date
Bacillus subtilis Bs12	51	69	48	67	65	78	63	81
Bacillus subtilis Bs14	48	71	51	74	59	82	61	79
Pseudomonas fluorescens (Pf)	65	83	67	81	76	89	81	91
Humate 1 (HA1)	34	49	36	51	54	67	51	72
Humate 2 (HA2)	29	38	31	42	43	59	46	61
Bs12 + (HA1)	63	76	65	74	73	82	74	83
Bs12 + (HA2)	58	71	61	73	67	73	64	75
Bs14 + (HA1)	68	81	72	83	78	89	81	91
Bs14 + (HA2)	54	79	57	81	71	84	73	86
Pf+ (HA1)	76	95	81	94	87	98	86	97
Pf + (HA2)	73	89	75	91	81	93	83	96
Control (untreated)	-	-	-	-	-	-	-	-

Yield and physical characteristics of bunches:

Table (7) showed that all treatment caused a significantly increase in yield and physical characteristics of bunch in comparison to control in both seasons. The most increase of yield was observed in the treatment of *Pseudomonas fluorescens* plus humate (HA1), followed by the inoculation of *Pseudomonas fluorescens* combined with humate (HA2), while, the lowest values were shown with control in both seasons. The beneficial effect of application treatments on the yield could be ascribed mainly to the increase in bunch weight in the first season and the increase of number of bunches /vine beside the increase in bunch weight in the second season. With respect to bunch weight, it is positively affected by the conducted treatments in a similar manner to that of yield per vine. As for bunch dimensions, bunch length and width were influenced by all treatments; the treatment of *Pseudomonas fluorescens* plus humate (HA1), followed by the treatment of *Pseudomonas fluorescens* combined with humate (HA2), whereas, the lowest values were obtained with control in both seasons.

Table (7): Effect of *Bacillus subtilis*, *Pseudomonas fluorescens* and humate on yield and bunch physical characteristics of Flame Seedless grapevines in 2013 and 2014 seasons.

Characteristics	Yield/vine (kg)		No. of bunches		Average bunch weight (g)		Average bunch length (cm)		Average bunch width (cm)	
Treatments	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
Bacillus subtilis Bs12	12.4	12.1	20.9	21.1	591.7	571.3	21.6	20.8	15.2	14.8
Bacillus subtilis Bs14	12.8	13.4	20.7	22.4	620.1	598.7	22.6	21.8	15.9	15.6
Pseudomonas fluorescens (Pf)	13.8	14.5	21.3	23.2	647.4	625.1	23.6	22.7	16.6	16.2
Humate 1 (HA1)	12.1	11.8	20.6	20.9	586.1	565.9	21.4	20.6	15.0	14.7
Humate 2 (HA2)	12.0	11.5	20.8	20.7	574.6	554.8	21.0	20.2	14.7	14.4
Bs12 + (HA1)	12.6	12.9	20.7	22.0	607.1	586.2	22.2	21.3	15.6	15.2
Bs12 + (HA2)	12.5	12.5	21.0	21.7	596.5	575.9	21.8	20.9	15.3	15.0
Bs14 + (HA1)	13.5	14.4	20.9	23.1	643.9	621.7	23.5	22.6	16.5	16.2
Bs14 + (HA2)	13.1	13.8	21.0	22.8	624.7	603.2	22.8	21.9	16.0	15.7
Pf+ (HA1)	14.8	15.4	21.8	23.5	679.8	656.4	24.8	23.9	17.4	17.1
Pf + (HA2)	14.5	15.0	21.7	23.3	668.3	645.3	24.4	23.5	17.1	16.8
Control (Untreated vines)	11.7	11.1	20.8	20.5	561.0	541.7	20.5	19.7	14.4	14.1
new L.S.D. at (0.05) =	0.2	0.3	N.S.	0.2	11.3	10.9	0.4	0.3	0.3	0.2

Physical characteristics of berries:

Data presented in Table (8) showed that physical characteristics of berries i.e. berry weight, size, length and diameter significantly increased by all treatments containing bacterial inoculants combined with humate. The highest values of those

parameters were obtained from the treatment of *Pseudomonas fluorescens* plus humate (HA1), followed by the treatment of *Pseudomonas fluorescens* combined with humate (HA2), while, the lowest values were shown with control in both seasons.

Chemical characteristics of berries:

As shown in table (9) all bacterial inoculants and humate treatments succeeded to improve berry chemical characteristics; i.e. TSS, Acidity, TSS/acid ratio and anthocyanin content of berry skin. The treatment of *Pseudomonas fluorescens* plus humate (HA1) resulted in significantly the highest values of TSS percentage, TSS/acid ratio and anthocyanin content of berry skin and the lowest values of acidity of the berry juice, followed by the treatment of *Pseudomonas fluorescens* combined with humate (HA2). On the other hand, the lowest values of TSS percentage, TSS/acid ratio and anthocyanin content of berry skin and the highest values of acidity of the juice were recorded with control in both seasons.

Table (8): Effect of *Bacillus subtilis*, *Pseudomonas fluorescens* and humate on physical properties of berries of Flame Seedless grapevines at 2013 and 2014 seasons.

Characteristics	Average berry weight (g) berry weight (g)		Average berry size (cm^3)		Average berry length (cm)		Average berry diameter (cm) berry diameter (cm)	
Treatments	2013	2014	2013	2014	2013	2014	2013	2014
Bacillus subtilis Bs12	3.61	3.49	3.08	2.98	1.66	1.63	1.58	1.56
Bacillus subtilis Bs14	3.77	3.65	3.21	3.12	1.71	1.69	1.62	1.61
Pseudomonas fluorescens (Pf)	3.93	3.80	3.35	3.26	1.79	1.75	1.69	1.66
Humate 1 (HA1)	3.57	3.46	3.05	2.96	1.64	1.62	1.56	1.55
Humate 2 (HA2)	3.51	3.39	2.99	2.92	1.63	1.60	1.56	1.54
Bs12 + (HA1)	3.69	3.56	3.14	3.07	1.69	1.66	1.60	1.58
Bs12 + (HA2)	3.63	3.51	3.10	3.01	1.68	1.64	1.59	1.56
Bs14 + (HA1)	3.91	3.78	3.33	3.24	1.76	1.73	1.66	1.64
Bs14 + (HA2)	3.80	3.69	3.25	3.16	1.74	1.72	1.64	1.64
Pf+ (HA1)	4.11	3.99	3.52	3.41	1.85	1.82	1.74	1.72
Pf + (HA2)	4.05	3.92	3.46	3.36	1.82	1.80	1.72	1.69
Control (Untreated vines)	3.43	3.32	2.92	2.84	1.61	1.58	1.54	1.52
new L.S.D. at (0.05) =	0.06	0.05	0.05	0.04	0.02	0.01	0.02	0.01

Table (9): Effect of *Bacillus subtilis*, *Pseudomonas fluorescens* and humate on chemical properties of berries of Flame Seedless grapevines at 2013 and 2014 seasons.

Characteristics	TSS (%)		Acidity (%)		TSS/acid ratio		Anthocyanin (mg/100g F.W.)	
Treatments	2013	2014	2013	2014	2013	2014	2013	2014
Bacillus subtilis Bs12	16.9	16.7	0.63	0.62	26.8	26.9	38.2	36.8
Bacillus subtilis Bs14	17.3	16.9	0.61	0.62	28.4	27.3	38.6	37.2
Pseudomonas fluorescens (Pf)	17.5	17.3	0.58	0.60	30.2	28.8	39.1	37.7
Humate 1 (HA1)	16.7	16.6	0.64	0.66	26.1	25.2	38.1	36.6
Humate 2 (HA2)	16.6	16.4	0.65	0.66	25.5	24.8	37.9	36.5
Bs12 + (HA1)	17.1	16.8	0.62	0.63	27.6	26.7	38.5	37.0
Bs12 + (HA2)	17.0	16.7	0.63	0.64	27.0	26.1	38.3	36.9
Bs14 + (HA1)	17.4	17.2	0.59	0.61	29.5	28.2	38.9	37.4
Bs14 + (HA2)	17.3	17.0	0.60	0.61	28.8	27.9	38.7	37.3
Pf+ (HA1)	17.9	17.5	0.55	0.57	32.5	30.7	39.7	38.3
Pf + (HA2)	17.6	17.3	0.58	0.59	30.3	29.3	39.2	37.9
Control (Untreated vines)	16.5	16.2	0.66	0.68	25.0	23.8	37.7	36.4
new L.S.D. at (0.05) =	0.2	0.1	0.02	0.01	1.7	1.3	0.4	0.3

Some vegetative growth parameters:

Data presented in Table (10) showed that shoot length and number of leaves significantly increased by all bacterial treatments combined with humate. The highest values of those parameters were obtained from the treatment of *Pseudomonas fluorescens* plus humate (HA1), followed by the treatment of *Pseudomonas fluorescens* combined with humate (HA2), while, the lowest values were shown with control in both seasons.

Table (10): Effect of *Bacillus subtilis*, *Pseudomonas fluorescens* and humate on shoot length and number of leaves of Flame Seedless grapevines at 2013 and 2014 seasons.

Characteristics	Shoot length (cm)		Number of leaves	
Treatments	2013	2014	2013	2014
Bacillus subtilis Bs12	138.9	143.5	28.9	29.7
Bacillus subtilis Bs14	140.6	151.8	30.5	30.1
Pseudomonas fluorescens (Pf)	148.7	161.5	32.5	31.8
Humate 1 (HA1)	129.7	139.6	28.1	27.7
Humate 2 (HA2)	128.1	136.7	27.5	27.4
Bs12 + (HA1)	137.5	147.6	29.7	29.4
Bs12 + (HA2)	134.6	144.4	29.0	28.8
Bs14 + (HA1)	145.4	157.8	31.7	31.1
Bs14 + (HA2)	143.7	154.3	31.0	30.7
Pf+ (HA1)	158.3	174.2	35.0	33.9
Pf + (HA2)	151.2	162.4	32.6	32.3
Control (Untreated vines)	122.9	133.8	26.9	26.3
new L.S.D. at (0.05) =	6.9	7.4	1.7	1.3

Leaf content of total chlorophyll and mineral content

As shown in table (11) showed that all bacterial inoculants and humate treatments increased leaf content of total chlorophyll and mineral content including: nitrogen, phosphorus and potassium. The treatment of *Pseudomonas fluorescens* plus humate (HA1) resulted in significantly the highest values of those parameters, followed by the treatment of *Pseudomonas fluorescens* combined with humate (HA2). On the other hand, the lowest values were shown with control in both seasons.

Table (11): Effect of *Bacillus subtilis*, *Pseudomonas fluorescens* and humate on leaf content of total chlorophyll and mineral content of Flame Seedless grapevines at 2013 and 2014 seasons.

Characteristics	Total chlorophyll (SPAD)		N (%)		P (%)		K (%)	
Treatments	2013	2014	2013	2014	2013	2014	2013	2014
Bacillus subtilis Bs12	41.5	38.7	1.42	1.46	0.34	0.30	1.26	1.29
Bacillus subtilis Bs14	41.9	40.9	1.50	1.48	0.34	0.32	1.34	1.31
Pseudomonas fluorescens (Pf)	44.4	43.5	1.59	1.56	0.36	0.34	1.42	1.38
Humate 1 (HA1)	38.7	37.6	1.38	1.36	0.32	0.30	1.23	1.21
Humate 2 (HA2)	38.2	36.8	1.35	1.35	0.31	0.29	1.20	1.19
Bs12 + (HA1)	41.0	39.8	1.46	1.45	0.33	0.31	1.30	1.28
Bs12 + (HA2)	40.2	38.9	1.42	1.42	0.33	0.31	1.27	1.25
Bs14 + (HA1)	43.4	42.5	1.56	1.53	0.35	0.33	1.39	1.35
Bs14 + (HA2)	42.9	41.6	1.52	1.51	0.35	0.33	1.36	1.34
Pf+ (HA1)	47.2	46.9	1.72	1.67	0.39	0.37	1.53	1.47
Pf + (HA2)	45.1	43.8	1.60	1.59	0.37	0.34	1.44	1.39
Control (Untreated vines)	36.7	36.1	1.32	1.29	0.30	0.28	1.17	1.14
new L.S.D. at (0.05) =	1.9	2.3	0.07	0.04	0.01	0.02	0.04	0.03

Economical justification of the recommended treatments (*Pseudomonas fluorescens* + Humate HA 1) compared with control in both seasons:

It can be shown from the data presented in Table (12) that bio inoculation with *Pseudomonas fluorescens* at 10 L/fed accompanied with Humate (HA1) at 3 L/fed gave the maximum net profit compared with the control in both seasons. The very slight raise in the cost of *Pseudomonas fluorescens* in combined with Humate (HA1) over control. Hence, it can be anticipated that the added cost of establishment will be offset by an increase in vine productivity.

Table (12): Economical justification of the recommended treatments *Pseudomonas fluorescens* + Humate (HA1) compared with control in both seasons.				
Per Feddan	2013 season		2014 season	
	Pf + HA 1	Control	Pf+HA 1	Control
***Pseudomonas fluorescens* (Pf) (L) at three dates**	30	---	30	---
HA 1 (L) at three dates	9	---	9	---
Price of *Pseudomonas fluorescens* (Pf) (L.E.) at three dates	300	---	300	---
Price of HA 1 (L.E.) at three dates	162	---	162	---
Labour cost (L.E.)	200	---	200	---
Cost of cultural practices (L.E.)	11000	11000	11500	11500
Total cost (L.E.)	11662	11000	12162	11500
Yield (Kg)	11317	8911	11779	8480
Kg (L.E.)	3.00	3.00	3.00	3.00
Yield (L.E.)	33950.4	26732.2	35338.2	25440.2
The net profit (L.E.)	22288.4	15732.2	23176.2	13940.2

4. Discussion

Pseudomonas fluorescens and *Bacillus subtilis* have a great role as a PGPR and effectiveness for controlling *Meloidogyne* sp under filed conditions of Flame Seedless grapevines. The increase of total bacterial count and *Pseudomonas fluorescens* count in the rhizosphere of grape vines proved that inoculation with *Pseudomonas fluorescens* and *Bacillus subtilis* increased the soil microbial population **(Botha, 2011).** Concerning the activity of dehydrogenase activity, data cleared a close correlation between activity of dehydrogenase activity and microbial population **(Tolba *et al.*, 2010).** Moreover, chitinase activity was significant increased, it may attributed to soil inoculation with *Pseudomonas* sp. and *Bacillus* sp. **(Sharma *et al.*, 2011 and Zaghloul *et al.*,2015).** On the other hand, humate induced metabolic activity of living microorganisms **(Stevenson, 1994).**

Pseudomonas fluorescens and *Bacillus subtilis* are important as antagonists of soil pathogens such as *Meloidogyne* sp. The reduction in No. of nematode larva in soil and roots could be attributed to *Pseudomonas fluorescens* is antagonistic to soil pathogens by producing antibiotics and cell wall degrading enzymes, such as b-1,3-glucanase, chitinase and protease **(Yong and Kil, 2015).** On the other hand, *Bacillus* spp. was found to be an effective agent in biological control of several parasite nematode, suppression of pathogens by strain *Bacillus* spp. is attributed in part to chitinase, chitosanase, β-glucanase, glucanases and proteases production and by secretion of a number of metabolites including antibiotics, volatile compound HCN and siderophores capable of acting against nematodes **(Abdel-Aziz, 2013).** These results were agreements with **Ruiz *et al.* (2014)** who suggested that the cell-free culture filtrate of *Bacillus subtilis* might be able to contain toxic metabolites against J2 *M. incognita* nematode. Although, **Zaghloul *et al.* (2015)** reported that *Pseudomonas fluorescens* and *Bacillus subtilis* caused high mortality percentage against root-knot nematodes (J2) of *Meloidogyne incognita*. Moreover, these bacteria succeeded to record maximum hydrolysis zone values of gelatinase, protease and chitinase. **Nour and Tolba (2015)** reported that microbial inoculation of cowpea plants with *Pseudomonas fluorescens* + *Bacillus subtilis* significantly enhanced total bacterial count , soil enzymes activities, plant growth and yield and caused reduction on number of *Meloidogyne* sp larva in soil and roots.

On the other hand, Humic acid is a suspension, based on potassium humate, which can be applied successfully in many areas of plant production as a plant growth stimulant or soil conditioner for enhancing natural resistance against plant diseases and pests **(Scheuerell and Mahaffee, 2006)**which consequently increase yield of plant. Humic acid application consistently enhanced antioxidants such as á-tocopherol, â-carotene, superoxide dismutases, and ascorbic acid concentrations in plant **(Sun *et al.*, 2004);** these antioxidants may play a role in the regulation of plant development, flowering and chilling of disease resistance **(Ziadi *et al.*, 2001).** Amino acids have a chelating effect on micronutrient when applied, that make the absorption and transportation of micronutrients inside the plant is easier due to its effect on cell membrane permeability **(El-Ghamry *et al.*, 2009).** Some of these micronutrients play roles in plant resistance by regulating the levels of auxin in plant tissues by activating the auxin oxidase system **(Marschner *et al.*, 1997)** it appears to be required in synthesis of intermediates in the metabolic pathway, through tryptophan to auxin **(Chen *et al.*, 2004).** Consequently, auxin lead to increase of total phenol, calcium content and activity of catechol oxidase, these materials protect plants against pathogen stress **(Chowdhury, 2003).** In addition, humic acid affects

the nematods fertility and consequently its fecundity **(Kesba and El-Beltagi, 2012).**

The great role of *Pseudomonas fluorescens* and *Bacillus subtilis* as a PGPR was clear in improvement the plant growth, nutrients up take and increased yield and its contents in Flame Seedless grapevines. The enhancement of plant emergence by *Pseudomonas fluorescens* may be attributed to the secretion of some substances on the plants that may activate the biological process and accelerate the emergence. Promoting of plant growth by the bioagents could be resulted from facilitating uptake of nutrients by roots. It was reported that PGPR promote plant growth directly through nitrogen fixation, phosphorus solubilization and production of phytohormones like auxin ,cytokinin, ethylene, indole-3- acetic acid and gibberellic acid, and indirectly by suppressing soil borne pathogens **(Rakib *et al.*, 2013)** . On other hand, *Bacillus* sp were also found to be a good producer for siderophore and indole acetic acid, secretion of indole acetic acid promotes roots to grow directly and stimulating plant cell elongation, plant fortification, and healthy **(Abdel-Aziz, 2013).** These findings were agreements with **Morsy *et al.* (2010)** who reported that *Pseudomonas fluorescens* played a clear role as a PGPR in tomato planted in soil infested with *Meloidogyne* sp., And with **Nour and Tolba (2015)** who found that microbial inoculation of cowpea plants with *Pseudomonas fluorescens* + *Bacillus subtilis* significantly enhanced plant growth, NPK content of plant and seeds and yield. Also, **Ahemad and Khan (2012)** who found that *Pseudomonas* sp. significantly increased plant dry weight, nodules numbers, total chlorophyll content, leghaemoglobin, root N, shoot N, root P, shoot P, seed yield and seed protein of Greengram (*Vigna radiata* (L.) wilczek).

Plant growth-stimulating effect of humate is associated with increased nutrient uptake **(Nardi *et al.*, 2009)**. The statement about the effect of humate acid on plant growth by **Vaughan and Mc Donald (1976),** is that humate affect the ion exchange of plant nutrients that are useful in microbial activity by increasing conversions directly as well as indirectly as a result of the stimulating plant growth hormones. According to **Lobartini *et al.* (1997),** humate in nutrition of the plants plays an important role directly and indirectly. In the full bloom period of humate application, berry weight, titratable acidity and maturity index values of Italy grape cultivar increased significantly **(Ferrara and Brunetti, 2010).** Several studies have reported the positive effect of humate on crop yield **(Vaccaro *et al.*, 2015**) and on root and shoot development **(Canellas and Olivares, 2014).** In addition, leaf chlorophyll content **(Vaughan and Malcom 1985),** and the activities of enzymes involved in several physiological pathways, such as nitrogen assimilation **(Vaccaro *et al.*, 2009)** and energy metabolism **(Ferretti *et al.*, 1991).**

Regarding the effects of commercial HA on products on table grape are supported by many researches, these findings were agreement with the findings **Brownell *et al.*, (1987)** reported that humic acid application slightly increased yield of various wine grapes. Also, **Wang *et al.*, (1991)** stated that using organic and chemical fertilizers increased shoot length and leaf area of grapes. In this respect, **Colapietra (2000)** found that the increase in berry size were observed in Italia table grape after the treatment of soil three times with organic fertilizer containing humic acid. Moreover, **Harhash and Abdelnasser (2000)** reported that organic application increased total chlorophyll content in leaves of Flame Seedless grapevines. Also, **Zachariakis *et al.*,(2001**) found that leaf chlorophyll content increased by humic acid application in 41B and 110 Richter grapevine rootstocks. In addition to, **El-Shenawy and Fayed (2005a)** reported that shoot length increased by using organic manure and chemical fertilizer on Crimson Seedless in the third season. Also, **El-Shenawy and Fayed (2005b)** reported that humic acid with organic fertilizers increased yield and chemical properties of berries including total soluble solids and total soluble solids/acid ratio and decreased the total acidity, as well as enhancing the percentages of nitrogen, phosphorus and potassium in the leaves of Crimson Seedless grapevines significantly than organic fertilizer alone. In This respect, **Omar (2005)** found that mineral fertilization and its combinations with compost and/or humic acid significantly increased yield and chemical properties of berries including total soluble solids and total soluble solids/acid ratio and decreased the total acidity of Thompson Seedless grapevines. Also, **Omar and Abd El-All (2005)** recorded that applying humic acid at 9 or 12 liter/ feddan divided into four equal doses added in February, April, May and June increased leaf area, yield and physical properties of berries including berry weight and size, as well as enhancing the percentage of potassium in the leaves of Superior grapevines. In addition to, **Ali *et al.*, (2006)** mentioned that leaf area, yield, chemical properties of berries including total soluble solids and total soluble solids/acid ratio and the percentages of nitrogen, phosphorus and potassium in the leaves increased by applying 15cm/vine of humic acid with mineral N and K sources on Flame Seedless and Superior Seedless grapevines. Also, **Belal (2006)** found that using different doses from organic fertilizers significantly increased total chlorophyll content and the percentages of nitrogen, phosphorus and potassium in the leaves of Thompson Seedless grapevines. Moreover, **Saleh *et al.*, (2006)** showed that adding

biofertilizer with humic acid significantly increased yield and decreased the total acidity of Thompson Seedless grapevines. Also, **Abd El- Monem *et al.* (2008**) reported that humic acid and bio-fertilizer increased yield, fruit quality (berry weight and size) and the percentage of nitrogen in the leaves, while, the percentages of phosphorus and potassium were not affected in Thompson seedless grapevine. In addition to, **Ferrara and Brunetti (2010)** found that the humic acid at four different times: pre-bloom, full-bloom, fruit set and veraison induced a significant increase of physical properties of berries including berry weight and size and chemical properties of berries including total soluble solids and total soluble solids/acid ratio and decreased the total acidity as well as enhancement the total chlorophyll content in the leaves of Italia grapevines. Also, **Rizk-Alla and Tolba (2010)** reported that humic acid application increased growth, root density and distribution, yield and quality of Black Monukka grapevines by enhanced the growth characters namely total leaf area/vine, shoot diameter and coefficient of wood ripening, total chlorophyll, NPK% of the leaves and total carbohydrates of the canes. In addition to, the treated vines produced the higher fibrous root fresh weight, larger number and longest fibrous root. Also, it gave high yield and best its components namely physical and chemical characteristics of bunches and berries. Moreover, **Aydin (2011)** reported that humic acid application increased grape yield and quality of cultivars such as berry weight, berry red and blue color intensity values of Horoz Karasi grape variety.

Conclusion

From the previous results of this investigation, it could be concluded that microbial inoculation of grape with *Pseudomonas fluorescens* and *Bacillus subtilis* in combined with humate significantly enhanced total bacterial count, soil enzymes activities, plant growth, nutrient uptake, yield and its components and caused reduction on population density of *Meloidogyne* sp larva in soil and roots.

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