Microbial Bio-Fertilization Approaches to Improve Yield and Quality of Washington Navel Orange and Reducing the Survival of Nematode in the Soil

Abdelaal Shamseldin[1]; Mohamed H. El-Sheikh[2]; H. S. A. Hassan[*3] and Kabeil S.S.[4]
[1]Environmental Biotechnology Department, [2]Horticulture Department at Arid lands Cultivation and Development Institute (ARADI); Mubarak City for Scientific Research and Technology applications, New Borg-Elarab, Alexandria, Egypt, [*3] Pomolgy Department, National Research center, Cairo, Dokki-Egypt. [4]Protien Research Department at Genetic Engineering and Biotechnology Research Institute (GEBRI)
Hsasm2000@yahoo.com

Abstract: To test the ability of microbial strains *Pseudomonas flourescence* strain 843 and *Azospirillum brasilense* strain W24 to improve Washington navel orange fruit quality and to control the persistence of nematode in the soil, strains were applied one time monthly during the period of experiment to trees at two levels 300 ml and 500 ml per tree with 10^{-8} cells ml^{-1}. Bio-fertilizer inoculation with strain *Pseudomonas fluorescence* strain 843 growth promoting rhizobacteria was significantly improve fruit quality as well as increased fruit yield, fruit weight, fruit length, TSS and juice volumes, while inoculation with strain *Azospirillum brasilence* strain W24 increase but not significantly improve fruit quantity and quality of Washington navel orange. Commonly, three types of nematode were detected in the roots including *Tylenchulus Spp*, saprophytic nematode and *Pratylenchulus* while the dominant species was *Tylenchulus semipenetrans*. Generally there is a reduction in the number of nematode with the two examined strains while the addition of *Pseudomonas f.* strain 843 was successfully greater to inhibit the growth of nematode than *Azospirillum b.* strain W24 suggesting that this strain can be use as a bio-fertilizer for promoting citrus growth and bio-control for reducing the distribution and propagation of nematode associated with citrus. Enhancement and maintenance of soil fertility and conservation of the soil's health through bio-fertilizer applications will be a vital role and occupy significant concern for many of researcher in the future as a unique key for sustainable agriculture in developing countries. [Journal of American Science. 2010;6(12):264-271]. (ISSN: 1545-1003).

Key words: Citrus, Bio-fertilizers, *Azospirillum brasilence*, *Pseudomonas fluorescence*, *Tylenchulus semipenetrans* and biological control.

1. Introduction

Citrus (*Citrus spp.*) is one of the most important fruit crops grown in many tropical and subtropical countries. At the moment there is a bout 1.5 million hectares of citrus fruits cultivated for commercial scale in the world yielded nearly 40 million metric tons of oranges, lemons, limes, etc (Anonymous, 2008).

In Egypt, citrus has great attention due to its importance for local consumption or as a main source for foreign currencies by exportation to the European country. The area of citrus cultivated in Egypt was increased rapidly with the reclamation of new desert lands reaches about 35.59 hectare (Anonymous, 2008).

According to the annual report of the ministry of Agriculture in Egypt (2004), the production rate of citrus is mainly low as affected by different factors such as the nitrogen fertilization and soil born diseases like nematode. Nitrogen fertilizer is playing an important role for producing orange fruits due to its accumulative harmful as nitrate or nitrite in fruit juice (Montasser *et al.,* 2003). Thus is giving a special importance for optimizing the nitrogen requirements of citrus through different strategies. One of those strategies is the use of bio-fertilizers. The use of bio-fertilizers in enhancing plant growth and yield has gained momentum in recent years because of higher cost and hazardous effects of chemical fertilizers. Organic and/or bio-fertilizers improved vegetative growth, nutritional status and reduced the residuals of nitrate and nitrite in banana and grape fruits (Gomaa, 1995). Farag, (2006) and Saleh and Ahmed (1988) they noted that organic fertilizers and humic acid significantly decreased nitrogen as nitrate and nitrite content and improved yield and fruit quality of treated vines. Aseri *et al* (2008) found that the use of bio-fertilizers gave a significant improvement of fruits of pomegranate in India as well as enhancing the rhizosphere microbial activity and concentration of various nutrients. Ashokan *et al* (2000) reported a significant enhancement of banana plants as a result of dual inoculation of Arbscular mycorrhizal fungi (AMF) and Azotobacter.

Plant diseases caused by soil borne plant pathogens such as nematode (Duncan, 2005; Abd-

Elgawad *et al*., 2010) are considered major problems in agricultural production throughout the world, reducing yield and quality of crops. The nematode of the citric fruits probably infests more than 50% of the citrus production areas. The losses attributable to this nematode are estimated in the entire world at approximately 10% (Duncan, 2005 and Duncan *et al.*, 1995). There are several species of nematode that known to attack citrus including *Tylenchulus semipenetrans, Belonolaimus longicaudatus, Pratylenchus coffeae* and *P. brachyurus* (Duncan, 2005; Duncan *et al.*, 1995). Controlling of nematode diseases on citrus depends mainly on chemical applications mean while, these chemical substances are always undesirable due to their high cost and their hazard potentials to the environment. Therefore the view of scientists are directed to use bio-control instead of the chemical forms of fungicides or mineral fertilizers that can be a beneficial way to control soil borne plant pathogens and produce the natural clear fruits free from mineral residues (Russo and Berlyn 1990). In this respect, application of bio-fertilizers for controlling soil borne pathogenic fungi has been applied to several plants (Aaltan *et al*., 2003 and Siddiqui *et al*., 2009).

Consequently, the present investigation was outlined to study the effect of inoculation of citrus trees with *Azospirillum brasilense* and *Pseudomonas fluorescence* as growth promoting substances and bio-control on the yield and quantity of Washington navel orange as well as inhibiting the survival of nematode in the soil which causes considerable losses in citrus production in Egypt.

2. Materials and Methods:

2.1 Bacterial strains and growth

Strains of *Azospirillum brasilense* W24 and *Pseudomonas fluorescence* 843 DZM were kindly dedicated by D. Werner, Philipps University of Marburg, Germany. Strains of *Azospirillum* were grown on Doberiner medium but strains of *Pseudomonas* were grown on nutrient broth medium. Strains were grown in liquid medium on a rotary shaker at 30 °C and 120 rpm and the optical density of culture adjusted to 1 at 550 nm, then the culture were added to the trees once per month at a rate of 300 ml or 500 ml per tree.

2.2 Field Experiment:

Experiments were conducted in a silty clay soil in the middle of Delta Nile Valley characterized by high pH (8.9) and salinity level (3.28 mmohs cm^{-1}). The study was carried out in a private orchard at Al–Menoufia Governorate during 2008 and 2009 season on 10 years old Washington Navel orange trees, budded on sour orange (*C. ourantium* L.) rootstock, spaced at 5x5m. Completely block randomize design with four replicates was used for each treatment.

2.3 Collection of samples:

During spring and summer of 2008 and 2009 planted sites representing different habitat types in reclaimed and old parts of El-Menoufia governorate surveyed for the presence of entomopathogenic nematodes (EPN) using a design similar to Mrácek and Webster (1993) samples were taken with a hand trowel to a 20 cm depth and a volume of 1000 cc (cubic mete) per soil sample was collected. Samples were transported to the laboratory in sealed and labeled polyethylene bags within an ice chest.

2.4 Extraction of nematode from the soil and microscopic examination

250 g of the collected soil washed with tape water to remove the residues of roots then precipitated. The precipitate sieved through sieve of 325 mesh three times, then the water removed and the suspension containing nematode transferred to 50 ml beaker. The beaker agitated well then left for one minute for precipitating and 1 ml taken from the suspension and accounted using the 10x lenses of light microscopy and the number of nematode population accounted using this equation.

Number of nematode 250 ml= number of cells per ml x volume of the suspension

2.5 Determination of (N; P and K):

Methods for analyses and determination of (N; P and K):

To determine leaf mineral content, forty leaves were collected in the late of August in each season from tagged non-fruiting and non-flushing spring growth cycle (Jones and Embleton, 1960). Leaf samples were washed with tap water then distilled water several times, dried at 70°C, grinded and digested with percholoric acid to estimate N, P and K contents as percent refers to dry weight according the methods described by Cottenie *et al*. (1982).

2.6 Determination of horticulture aspects:

Fruit quality

At maturity stage, 10 representative fruits were taken from each tree and both the physical and chemical characteristics were determined.

Physical characteristics

Average fruit weight (g) fruit volume (cm3), fruit peel thickness (cm) and fruit firmness (by means Manges Taglor Pressure Tester) were measured. The

fruit length and diameter (cm) were measured by a vernier calliper and the fruit shape index (length/diameter ratio) was calculated.

Rate of fruit weight increase = Fruit weight of inoculated plant- fruit weight of un-inoculated plant÷fruit weight of uninoculated plantx100.

Reduction rate of nematode= Population of nematode in inoculated plant-population of nematode in un-inoculated plant ÷population of nematode in uinoculatedx100.

Chemical characteristics

Juice volume, total soluble solids, acidity content and vitamin C (mg/100 ml juice) were calculated adopting the standard procedures (A. O. A. C., 1990). The total soluble solids (TSS/acid ratio) for each sample were estimated.

Statistical Analysis:

The means and standard deviations of four replicates were estimated and an analysis of variance was carried out using the ANOVA procedure with SPSS software while the comparison of mean effects was based on least significant difference (LSD) multiple-comparison tests. Significant differences were considered at $P < 0.05$.

3. Results and Discussion:

3.1 Effect of bio-fertilizer inoculation on quantity and quality of Washington navel orange fruits

Production of horticultural crops has taken significant increase in the last decade due to development of innovative technologies including integrated nutrient management practices involving bio-fertilizers aiming to reduce the cost of agricultural process and the reduction of environmental pollution that happened due to the extravagant of fertilizers use. These bio-fertilizers include different kinds of microorganisms such as phosphate-solubilizing bacteria (PSBs), symbiotic (*Rhizobium*; *Bradyrhizobium* etc) and non-symbiotic (*Pseudomonas*; *Azospirillum* and *Azotobacter*) N_2-fixing bacteria and arbuscular mycorrhizal fungi (AMF). AMF and *Azospirillum* were found to enhance the growth and production of various vegetable crops (Ghazi, 2006 and Paramaguru *et al*., 1993) while *Azospirillum* and *Azotobacter* were found to increase significantly the production of some fruit plants such as banana and sweet orange (Jeeva *et al*., 1988; Tiwary et al., 1999 and Singh and Sharma, 1993), Besides improving the microbiological activity in the rhizosphere (Kohler *et al*., 2007). Therefore we aimed in our research to study the effect of biofertilizer application on the production of Washington navel orange tree to encourage the clean or green agriculture in order to help small farmers in developing countries such as Egypt to depend on the biofertilizer as a main source to increase the soil fertility in their farming system and to confront the over increase in synthetic fertilizer prizes. Results in Table (1) show the effect of inoculation with bio-fertilizers on fruit characteristics including yield, fruit weight, fruit volume and fruit length. It is clearly shown that inoculation with 500 ml $tree^{-1}$ of strain *Pseudomonas fluorescens* 843 resulted in significant increase of the number of fruit $tree^{-1}$ in the two seasons as well as increasing the yield. The increasing rate in the number of fruits $tree^{-1}$ was 12.3% (231.48-206.18÷206.18x100) over the un-inoculated trees (control) in the first season while the increasing rate was 8.7% in the second season. The fruit weight also increased with the inoculation of this strain by a rate of 49.6% where the increase of fruit weight was 42.9% in the second season. Our results are not only in agreement with those obtained by Abd El-Migeed *et al* (2007) who reported that inoculation of Washington navel orange trees with *Azospirillum lipoferum* as a source of bio-fertilizer improved average fruit weight, and also agree with those published by Aseri *et al*. (2008) who noted that combined inoculation with *Azotobacter chroococcum* and *Glomus mosseae* (AMF) increased the microbial activity in the rhizosphere and yield of Pomegranate.

Bio-fertilizer inoculation with strain of *Pseudomonas fluorescens* had enhanced fruit weight, fruit volume and fruit length by a rate of (33.25% and 31.6%), (4.1% and 9.9%) and (6.2% and 11.7%) for the first and second season on respectively. However non significant results were not observed in fruit diameter and fruit shape index except in fruit diameter in the first season (2008). Results in Table 2 summarize the effect of microbial inoculation on the total soluble salts, acidity, juice volume and vitamin C. As noted from the previous results that strain *Pseudomonas fluorescens* was superior for promoting the TSS, juice volumes in the whole duration of experiment while this strain promoted significantly vitamin C in the second season only. There is no significant results were observed as a result of microbial biofertilization on the fruit shape index (Table 1) and peel thickness (Table 2) as reported by Bassal (2009) who stated that the use of rootstocks were not significantly increase of both the peel thickness and fruit shape. The positive effect of inoculation with strain *Pseudomonas fluorescens* on the quality and quantity of Navel orange may be due synthesis of phytohormones (Xie *et al*., 1986), reduction of membrane potentials of the roots (Bashan and Levanony 1991), synthesis of some enzymes that modulate the level of plant hormones (Glick *et al*. 1998) and solubilizing of inorganic

phosphate (Krasilnikov, 1961). On the other hand, strain *Azospirillum brasilense* was less effective than *Pseudomonas* strain for increasing the fruit quantity (Table 1) and fruit quality (Table 2) although this strain was repeatedly reported as a PGBR in many different publications. The little effect of *Azospirillum brasilense* strain was reported by *Burdman et al. (1997)* who found that high titer (10^{-8} cfu ml^{-1}) like in our case of this strain reduced the shoot and root weight of common bean when it is dual inoculated with *Rhizobium* and *Azospirillum* strain. Perotti and Pidello (1999) stated that the inoculation with *Azospirillum brasilense* strain reduces the activity of urease in the soil and this led to decrease the available nitrogen.

Table (1) Effect of microbial biofertilizers On yield, fruit weight ,fruit volume, fruit length ,fruit diameter and fruit shape index of Washington navel orange trees

Measurement / Treatment	yield				fruit weigh (gm)		fruit volume (cm3)		fruit length (cm)		fruit diameter (cm)		fruit shape index	
	No. of fruit / tree		fruit weigh/tree (kg)		2008	2009	2008	2009	2008	2009	2008	2009	2008	2009
	2008	2009	2008	2009										
Control	206.18	250.30	30.83	36.11	149.50	144.20	208.15	192.63	7.15	6.82	7.08	6.86	1.01	0.99
Azospirillium b. strain W24 (300 ml/tree)	193.05	242.11	28.36	37.40	146.90	154.47	205.47	231.61	7.26	7.85	7.19	7.59	1.01	1.03
Azospirillium b. strain W24 (500 ml/tree)	201.59	238.61	31.10	40.13	154.27	168.18	196.02	200.30	7.02	7.30	7.00	7.22	1.00	1.01
Pseudomonas f. 843 (300 ml/ tree)	210.08	220.43	37.62	43.10	179.07	195.52	215.60	219.08	7.50	7.66	7.35	7.49	1.02	1.02
Pseudomonas f. strain 843 (500 ml tree)	231.48	272.05	46.12	51.62	199.23	189.74	217.10	211.83	7.59	7.73	7.41	7.58	1.02	1.02
L.S.D. at 0.05	18.64	15.18	6.35	8.90	17.03	14.65	8.15	11.40	0.23	0.18	0.09	N.S	N.S	N.S

Azospirillium b.: Azospirillium brasilensce; Pseudomonas f.: Pseudomonas fluorescens strains were dedicated from laboratory of Werner, Philipps University of Marburg, Germany.

Table (2) Effect of microbial biofertilizers on fruit quality of Washington navel orange trees.

Measurement / Treatmen	Peel thickness		TSS %		Acidity		TSS/ Acid %		Juice Volume		Vitamin C (mg /100 ml juice)	
	2008	2009	2008	2009	2008	2009	2008	2009	2008	2009	2008	2009
Control	0.39	0.40	12.80	12.50	1.16	1.19	11.03	10.50	43.20	42.91	41.63	43.08
Azospirillium b. strain W24 (300 ml/tree)	0.38	0.41	12.34	12.59	1.23	1.15	10.03	10.94	43.00	43.58	40.15	43.27
Azospirillium b. strain W24 (500 ml/tree)	0.38	0.40	12.08	12.62	1.30	1.06	9.29	11.90	44.25	45.30	42.60	45.40
Pseudomonas f. 843 (300 ml/ tree)	0.41	0.42	12.87	12.53	1.11	1.10	11.59	11.39	46.18	47.11	42.82	42.85
Pseudomonas f. strain 843 (500 ml tree)	0.42	0.43	12.90	13.02	1.13	1.08	11.41	12.05	46.93	47.15	42.56	46.11
L.S.D. at 0.05	N.S	N.S	0.41	0.32	0.09	N.S	0.93	0.78	1.20	1.53	N.S	1.83

3.2 Effect of bio-fertilizer inoculation on NPK leaf contents of Washington navel orange

Results presented in Table (3) explain the effect of microbial bio-fertilizer on percentage of macro-elements (NPK) in leaves. Both the two examined strains gave significant increase in nitrogen percent, by a rate of (13.7% mean) with strain W24 while this rate was (6.8% mean) with strain 843 in the first year. In the second year the increasing rate was 39.6% and 26.7% with the examined strains on respectively. Strain of *Azospirillum brasillence* gave high nitrogen percent than the pseudomonas strain because this strain is a free living nitrogen fixing bacteria (Perotti, and Pidello, 1999). The P and K contents were also increased due to the application of bio-fertilizer with the two examined strains compared by control.

3.3 Effect of bio-fertilizer inoculation on the reduction of nematode associated with Washington navel orange

Plant-parasitic nematodes cause serious crop losses worldwide and are among the most important agricultural pests (Koenning *et al*., 1999). The management of nematodes is more difficult than that of other pests because nematodes mostly inhabit the soil and usually attack the underground parts of the plants (Stirling, 1991). Although chemical nematocides are effective, easy to apply, and show rapid effects, they have begun to disappear from the market in some developed countries owing to concerns about public health and environmental safety (Schneider *et al*., 2003). The search for novel, environmentally friendly alternatives with which to manage plant-parasitic nematode populations has therefore become increasingly important. The nematode of the citric fruits probably infests more than 50% of the citrus production areas leading to a rate of 10% losses approximately in the entire world (Duncan, 2005 and Duncan *et al.*, 1995). Consequently, one of our main targets was to test the possibility of these strains to reduce citrus infected nematode in the field. Results in Table (4) revealed that there were number of nematode types detected in the roots of Navel orange including *Tylenchulus Spp*, saprophytic nematode and *Tylenchulus semipenetrans* while the dominant species was *Tylenchulus semipenetrans*, therefore results show the reduction in population numbers of *Tylenchulus semipenetrans* nematode as a result of bio-fertilizer inoculation. Generally, the number of infected roots with nematode was increased at low temperature in the winter while it reduced in the summer months due to hot weather. The inoculation with *Pseudomonas fluorescens* strain 843 (500 ml tree^{-1}) did significantly reduce the number of infected roots with nematode after two months by a rate of 56.6% than the control while the reduction rate was 45.5% with the inoculation doze (300 ml tree $^{-1}$). The retardation of the beneficial effect of this strain to reduce the nematode may be due to the time that needed until the strain adapted and multiply in the soil. Several investigators (Shanmugan *et al*. 2002; Aalten *et al*., 2003; Siddiqui *et al*., 2009) have been used strains belong to this species as a bio-control for large numbers of soil borne diseases including nematode. The reduction in nematode infections due to the inoculation with *Pseudomonas fluorescens* strain 843 may be due to decreasing or preventing the deleterious effect of pathogenic microorganisms by produce antibiotics (Sivan and Chet 1992; Mel'nikova *et al*. 2002) or siderophores (Leong 1986), different species of *Pseudomonas* produce *N*-acylhomoserine lactones which is involved in the cell-density dependent control of secondary metabolite and virulence gene expression (Lau *et al*., 2000) or by regulating nematode behavior (Sikora and Hoffmann-Hergarten, 1993), interfering with plant–nematode recognition (Oostendorp and Sikora, 1990), competing for essential nutrients (Oostendorp and Sikora, 1990), promoting plant growth (El-Nagdi and Youssef, 2004), inducing systemic resistance (Hasky-Günther *et al*., 1998) and directly antagonizing by producing of toxins, enzymes and other metabolic products (Siddiqui and Mahmood, 1999). Other authors were reported to produce hydrogen cyanid that kill the eggs of nematode (Aalten *et al*, 2003) or protease enzyme that responsible for inhibiting cell walls (Siddiqui et al, 2005). The work in the future will focus on studying the mode of action of *Pseudomonas fluorescens* strain 843 that helps it to enhance the growth of fruits or decreasing the number of nematode in the soil. This is the first field trial that give promising results for reducing the nematode so that many numbers of farmers ask us to supply them with strains but we need to do the experiments in other area like the north and south of Delta to evaluate this strains correctly before going to the large scale of field application to increase the confidence between researchers and farmers that was missing in the past.

4. Conclusion:

Under Egyptian soil conditions the inoculation of Washington navel orange with *Pseudomonas flourescence* strain 843 was not only highly effective to increase the production of Washington navel orange as well as improving the quality of fruits but also inhibited the survival of nematode in the soil concluding that this strain can be used as bio-fertilizer and bio-control of pathogenic nematode infected citrus trees.

Table (3) Nitrogen, Phosphrus and potassium contents of orange leaves at the end of experiment

First season 2008			
	NPK		
Treatments	**%N**	**%P**	**%K**
Control	1.17	0.11	1.27
Azospirillium b. strain W24 (300 ml/tree)	1.35	0.17	1.45
Azospirillium b. strain W24 (400 ml/tree)	1.30	0.16	1.49
Pseudomonas f. strain 843 (300 ml/tree)	1.27	0.13	1.35
Pseudomonas f. strain 843 (500 ml tree)	1.23	0.15	1.40
L. S. D. 0.05	**0.08**	**0.02**	**0.11**
Second season 2009			
Treatment			
	NPK		
	%N	**%P**	**%K**
Control	1.01	0.13	1.05
Azospirillium b. strain W24 (300 ml/tree)	1.40	0.15	1.50
Azospirillium b. strain W24 (500 ml/tree)	1.42	0.17	1.46
Pseudomonas f. 843 (300 ml/ tree)	1.26	0.13	1.38
Pseudomonas f. 843 (500 ml/ tree)	1.30	0.17	1.33
L.S.D 0.05	**0.12**	**0.03**	**0.24**

Table (4) Number of nematode cells in the soil of experiment for four months

First season					
	Time of sampling				
Treatments	**15/1/2008**	**15/2/2008**	**15/3/2008**	**15/4/2008**	**15/5/2008**
Control	55.3	66.3	78.3	25	35.3
Azospirillium b. strain W24 (200 ml/tree)	67.3	65.7	61.7	25.3	39.3
Azospirillium b. strain W24 (300 ml/tree)	56.7	70.3	65.3	19.3	38
Pseudomonas f. strain 843 (200 ml/tree)	57.6	63.0	42.7	19	16
Pseudomonas f. strain 843 (300 ml tree)	61.7	58.0	34.0	19.7	13.3
L. S. D. 0.05	**N. S.**	**N. S.**	**40**	**4.6**	**9**
Second season					
Treatment	**15/1/2009**	**15/2/2009**	**15/3/2009**	**15/4/2009**	**15/5/2009**
Control	64.0	59.5	56.0	24.3	40.0
Azospirillium b. strain W24 (200 ml/tree)	60.7	67.7	61.7	19.0	30.7
Azospirillium b. strain W24 (300 ml/tree)	55.3	52.3	48.7	12.3	36.3
Pseudomonas f. 843 (200 ml/ tree)	45.0	32.0	26.0	16.3	12.3
Pseudomonas f. 843 (300 ml/ tree)	38.0	29.3	19.7	13.7	8.0
L.S.D 0.05	**15.2**	**24**	**23**	**N. S.**	**18**

Corresponding author
H. S. A. Hassan
Pomolgy Department, National Research center, Cairo, Dokki-Egypt.
Hsans2000@yahoo.com

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