American-Eurasian J. Agric. & Environ. Sci., 18 (2): 89-95, 2018
ISSN 1818-6769

DOI: 10.5829/idosi.aejaes.2018.89.95

Plant Growth Promoting Rhizobacteria (PGPR) Protect Date Palm (*Phoenix dactylifera* L.) Plantlets from Fungal Attack During Acclimatization Stage

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Abstract: Plantlets of date palm face many difficulties during acclimatization stage; the most serious difficulties are fungal infections leading to heavy losses. The main aim of this study is using plant growth promoting rhizobacteria (PGPR) to limit fungal growth leading to the increased growth of plantlets of date palm cv. Malacabe (received from *in vitro* propagation) during acclimatization stage. A greenhouse experiment was carried out to use *Azotobacter chroococcum, Azospirillum lipoferum* and *Bacillus polymyxa*, either individually or a mixture (as a soil drench). The resistance of plantlet and survival percentages significantly increased. Moreover, the content of indol, chlorophyll a, b and carotenoids in the leaves and total count of bacteria also increased in both seasons with the superiority of a mixture bacteria. From the previous result, it could be recommended that using a mixture of plant growth promoting rhizobacteria (PGPR) such as *Azotobacter chroococcum*, *Azospirillum lipoferum* and *Bacillus polymyxa* to increase the plant resistance against fungi especially in the early stages in greenhouse during acclimatization stage of date palm plantlets cv. Malacabe.

Key words: *Phoenix dactylifera* · Date palm, *Azotobacter* · *Azospirillum* · *Bacillus and* Acclimatization stage

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is one of the most economically important plants in arid and hot regions, especially in the Middle East and southern Mediterranean countries. Plantlets of date palm are exposed to infection by many pathogenic fungi at all stages of growth resulting in a significant loss and farmers often rely heavily on the use of synthetic fungicides to control these plant diseases. Some researchers have focused their efforts to develop alternative agents to synthetic chemicals for controlling plant diseases, referred to as biological control using microbial antagonists. Many microbial antagonists have been reported to possess antagonistic activities against plant fungal pathogens, such as *Pseudomonas fluorescens*, *Agrobacterium radiobacter*, *Bacillus subtilis*, *B. cereus*, *B. amyloliquefaciens*, *Trichoderma virens, Burkholderia cepacia, Saccharomyces* spp. And *Gliocadium* spp. Antagonistic bacteria commonly inhabit soil which can be used as biological control agents for the management of soil borne diseases of various crops [1]. Many soils it secretes potent enzymes which destroy other cells by digesting their cell walls and degrade the cellular materials. For example, *Aspergillus* has an antagonistic effect on *Penicillium* and *Cladosporium, Trichoderma* has an effect on *Actinomycetes*, *Pseudomonas* show antagonism toward *Cladosporium*. Fungal strains are growing best in absence of bacteria and most severely affected by bacterial presence. Additionally, the antagonism between bacteria and fungi is connected to competition for substrate, but this competition can be drastically altered if fungi are given an opportunity to establish before inoculation with bacteria [2]. Plant growth-promoting rhizobacteria (PGPR) are the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms like phosphate solubilization, siderophore production, biological nitrogen fixation, production of 1-Aminocyclopropane-1-carboxylate deaminase (ACC), quorum sensing (QS) signal

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interference and inhibition of biofilm formation, phytohormone production, exhibiting antifungal activity, production of volatile organic compounds (VOCs), induction of systemic resistance, interference with pathogen toxin production. Indirect effects of stimulating plant growth are based on the reduction or complete elimination of the harmful effect of phytopathogenic organisms, The pathogen control by PGPR action involves competition with the ecological niche and nutrients, while bacteria produce antibiotics and lysis enzymes and secondary metabolites to combat pathogens [3, 4]. Ibatsam *et al.* [5] evaluated the antagonistic activity of some bacteria isolated from soil. Bacteria are able to synthesis a wide range of secondary metabolites with fungicidal capabilities. The antagonistic potential of five soil bacterial strains (*E. coli, Bacillus fortis, B. faragiris, Pseudomonas fluorescence and P. malophilia*) was assessed *in vitro* against *Alternaria alternate*, *A. citri, Aspergillus aculatus, A. japonicus* and *Dreshlera biseptata*. Results indicated that tested bacterial species exhibited a varying degree of antagonistic potential against all pathogenic fungi. *Pseudomonas melophilia* showed maximum inhibitory potential against all tested fungi with reduction of up to 60 % of fungal colony diameter. *Pseudomonas fluorescence* and *Bacillus fortis* exhibited almost similar biocontrol potential against three pathogenic fungi viz. *Aspergillus aculatus, A. japonicus* and *Dreshlera biseptata*. On other hand, *Escherichia coli* showed least effective biocontrol prospective against *A. citri, Aspergillus aculatus* and *Dreshlera biseptata*. A significantly high number of fungal diseases have an influence on crop plants throughout the year when a farmer fails to take proper preventative measures. Plant disease control therefore has become heavily dependent on fungicides to combat the wide variety of fungal diseases. Plant protection is an important area, which needs attention due to hazardous inputs of chemicals control [6]. Therefore, the search for new antimicrobial agents is a field of utmost importance in disease management. A large body of information has been accumulated regarding antagonism between bacteria and fungi and its possible role in the biological control of plant pathogenic fungi [7]. Production of antimicrobial compounds seems to be a general phenomenon for most bacteria. A broad-spectrum of classical antibiotics, metabolic byproducts, lysozyme and several types of protein exotoxins and bacteriocins have been reported in bacteria [8]. These biological substances are remarkable in diversity and natural abundance since some substances are restricted to some bacterial groups while others are extensively produced [9]. Antifungal metabolites produced by bacteria like *Pseudomonas* spp. *Bacillus* spp., have been investigated for their antifungal properties [10]. This study determines the antagonistic effects of some soil bacteria against some phytopathogenic fungi *in vitro*.

MATERIALS AND METHODS

A trial was conducted under greenhouse conditions at Central Laboratory of Date palm Researches and Development, Giza, Egypt during the two successive seasons 2016-2017(six month in each season) on date palm (*Phoenix dactylifera*) plantlets cv. Malacabe. The main purpose of this research is to use plant growth promoting rhizobacteria (PGPR) such as *Azotobacter chroococcum*, *Azospirillum lipoferum* and *Bacillus polymyxa* either individually or a mixture (as a soil drench) to increase the plant resistance of date palm plantlets cv. Malacabe against fungi especially in the early stages in greenhouse during acclimatization stage.

Plant Material: Date palm plantlets cv. Malacabe received from tissue culture technique was used average was 10-12 cm height, 1 -2 leaves / plantlets and 2-3 main roots/ plantlets with average 5-7 cm. Plantlets were first thoroughly rinsed with tap water to remove any medium residues, then immersed in a portion of diluted fungicide Topsin 1gm /liter for 5 mint for the disinfection of superficial and rinsed again with tap water. Plantlets were then transferred individually (each in 5 x18cm dimensions plastic pot) filled with (peat moss + perlit) at 3:1 ratio by volume.

Source of Bacterial Strains: The Representative bacterial strains used throughout this study were kindly obtained from the Culture Collection of Microbiological Resource Center, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The selected strains were purified by streaking on agar plates containing the selective medium for each bacterium type as the following:

- Nitrogen - deficient medium of Ashby for *Azotobacter* [11].
- Semi - solid malate medium for Azospirillium [12].
- Bacillus medium [13].

Maintenance of the selected strains was carried out by sub-culturing on several selective agar media as slants periodically, which were then kept in refrigerator at (4°C). Strains were separately grown in liquid medium for the

preparation batch cultures by inoculating 10 mL of 10^5-10^6 cells mL^{-1} in selective culture media and incubated in a rotary shaker at 100 rpm and temperature was controlled at 30°C to reach population density of $>10^7$ cfu mL^{-1} (Colony forming/ unit). PH was adjusted to 7. 0.

The Following Treatments Were Used:

- Control treatment (irrigation with tap water).
- 30 cm / l *Azotobacter chroococcum.*
- 30 cm / l *Azospirillum lipoferum.*
- 30 cm/ l *Bacillus polymyxa.*
- 30 cm/ l (*Azotobacter chroococcum* + *Bacillus polymyxa* + *Azospirillum lipoferum*)

All treatments applied with irrigation water weekly for 12 months. Three Plantlets were planted in each treatment with 3 replicates per each.

At the end of each season (November) the following parameters were assessed:

Vegetative Growth:

- Plant height (cm), number of leaves /plantlet and leaf width (cm).
- Survival percentages.

Chemical Composition: In fresh leaf samples total indole content (mg/100gfw) was determined according to Larsen *et al*. [14] and Photosynthetic pigments chlorophyll a,b and carotenoids (mg/gm F.w) were determined according to Moran [15].

Microbial Data: Date palm rhizosphere soil was also sampled at in the end of each season to determined the soil biological activity [16].

Statistical Analysis: The data were then tabulated and SAS program [17] was used for statistical analysis whereas Duncan's Multiple Range Test [18] was employed to verify the differences among means of various treatments.

RESULTS AND DISCUSSION

Vegetative Growth: The ability of plant growth-promoting rhizobacteria (PGPR) to enhance the plant growth was tested during this study.

Data in Table (1) revealed that plant height (cm), number of leaves/plant, leaf width (cm), significantly increased in most cases of both seasons as a result of treatment with various PGPR, with the superiority of the mixture of *Azotobacter chroococcum*+ A*zospirillum lipoferum*+ *Bacillus polymyxa* treatment which gave the highest significant values of plant height (20.5 and 28.5 cm), number of leaves (5 and 6) and leaf width (1.00 and 1.2cm) in the first and second seasons respectively.

On the other hand, the control recorded the lowest values of plant height (14 and 17.5 cm), number of leaves (3 and 4) and leaf width (0.50 and 0.6 cm) during the first and second seasons respectively. These results are in agreement with Burkett-Cadena *et al*. [19] and Cawoy *et al*. [20] who revealed that plant growth promoting rhizobacteria (PGPR) which synthesize growth substances enhance plant growth and inhibit plytopathogenic growth by secreting inhibitors also help in nutrient uptake and produce some biochemical substances such as protein, amino acids. Moreover, they help to increase nutrient availability and restore soil fertility for better crop response. Also, Darwish [21] and Hala *et al.* [22] revealed that application of *Azospirillum brasilense* might play a significant role in improving the growth response of date palm by producing good quality planting stock, these plants may perform better growth, survival and more fruit production due to IAA production, the inoculated microorganisms in the rhizosphere enhance soil aggregation by production polysaccharides and improved the water availability to plant during dry periods. Moreover, Fuentes and Caballero (2005) reported that inoculation with a mixture of *Azotobacter chroococcum*, *Azospirillum lipoferum* and *Bacillus polymyxa (*PGPR) maximized the counts and the activity of microorganisms

Table 1: Effect of PGPR on vegetative growth of date palm plantlets during acclimatization stage

	Plant height (cm)		No. of leaves/plant		Leaf width (cm)	
Treatments	First season	Second season	First season	Second season	First season	Second season
Control (tap water)	14 d	17.5 d	3 c	4 c	0.50 c	0.6 c
30cm/l *Azotobacter chroococcum*	17.5 bc	25.5 ab	4 b	5 b	0.70 ab	0.9 ab
30cm/l *Azospirillum lipoferum.*	17.5 bc	22.5 c	4 b	5 B	0.60 ab	0.8 b
30cm/l *Bacillus polymyxa*	18.4 b	24.4 b	4b	5 b	0.70 ab	0.8 b
30cm/l Mixture of all	20.5a	28.5 a	5a	6 a	1.00a	1.2 a

Means within a column or row having the same letters are not significantly different according to Duncan's Multiple Range Test at 5% level.

Table 2: Effect of PGPR on survival percentage of date palm plantlets during acclimatization stage.

	Survival (%)	
Treatments	First season	Second season
Control (tap water)	20 c	33 c
30cm/l *Azotobacter chroococcum*	65 ab	65 b
30cm/l *Azospirillum lipoferum.*	60 b	65 b
30cm/l *Bacillus polymyxa*	60 b	65 b
30cm/l Mixture of all	70 a	75 a

Means within a column or row having the same letters are not significantly different according to Duncan's Multiple Range Test at 5% level

Table 3: Effect of PGPR on indol content of date palm plantlets during acclimatization stage

	Indol content (mg / 100gm f.w)	
Treatments	First season	Second season
Control (tap water)	2.1 c	2.0 c
30cm/l *Azotobacter chroococcum*	3.9 b	3.5 b
30cm/l *Azospirillum lipoferum.*	3.6 b	3.2 b
30cm/l *Bacillus polymyxa*	3.5 b	3.3 b
30cm/l Mixture of all	5.6 a	5.0 a

Means within a column or row having the same letters are not significantly different according to Duncan's Multiple Range Test at 5% level

in soil rhizosphere. The diazotroph inoculants only appeared less effective than the mixture of co-inoculation with PGPR [24].

Survival Percentage: Data in Table (2) revealed that the mixture of *Azotobacter, Azospirillum* and *Bacillus* exhibited statistically the highest survival percentage (70 and 75 %) during first and second seasons respectively. On the contrary, there was no significant difference between both *Azospirillum* (60 and 65 %) or *Bacillus* (60 and 65%) in the first and second seasons respectively. On the other hand control treatment recorded the lowest values (20 and 33%) in the first and second seasons respectively.

The present results are in harmony with those of Pierik *et al*. [25] who mentioned that, the processes of plant growth, phytohormones, production of indole-3-acetic acid (IAA), ethylene, cytokinins and gibberellins, play an important role in adventitious root formation, root hair growth and root elongation of plant. These hormones can be synthesized by the plant themselves and also by their associated microorganisms. Furthermore, plant-associated bacteria can influence the hormonal balance of the plant. Ethylene is an important example to show that the balance is most important for the effect of hormones: at low levels, it can promote plant growth in several plant species including *Arabidopsis thaliana*, while it is normally considered as an inhibitor for plant growth and known as a senescence hormone. Interestingly, bacteria are able to reduce the ethylene level.

Chemical Composition

Indol Content: Data in Table (4) showed that, the high significant values of indoles in the leaves resulted from plant treated with mixture of *Azotobacter, Azospirillum* and *Bacillus* (5.6 and 5.0 mg/ gm F.W.) in the first and second seasons respectively. On the other hand, control treatment gave the lowest value (2.1and 2. 0 mg / 100gm f.w) in the first and second seasons respectively. While there were no significant difference between *Azotobacter, Azospirillum* and *Bacillus* in the first and second seasons.

These results are in agreement with those of Tiwari [26] who proved that *Azospirillum*, *Azotobacter*, *Klebsilla* and *Pseudomonas* sp. produced highly significant indole content. Lobna *et al.* [27, 28] on date palm plantlet revealed high significant values of indoles in the leaves resulted from plant treated with 15 gm/ plantlet biogien and 15 gm /plantlet nitrobien. Also, the highest significant values of indoles in the leaves of date palm cv. Malacabe plantlet resulted from mixture of *Anabaena oryzae* and *Nostoccm uscorum*.

Chlorophyll a, b and Carotenoids Contents in the Leaves: Results presented in Table (4) showed the effect of PGPR on chlorophyll a, b and Carotenoids contents of plantlet leaves. The highest value of chlorophyll a (1.73 and 1.77 mg/gfw), chlorophyll b (1.08 and 1.09 mg/gfw) and carotenoids (0.58 and 0.64 mg/gfw) were recorded in the leaves of plantlets treated with the mixture of *Azotobacter chroococcum*, *Azospirillum lipoferum* and *Bacillus polymyxa*) in both seasons respectively. But there was no significant difference between *Azospirillum lipoferum* or *Bacillus polymyxa* treatment in chlorophyll a, b and Carotenoids contents of plantlet leaves recorded in both seasons.

On the other hand control treatment recorded the lowest values of chlorophyll a (0.53 and 0.52 mg/gfw), chlorophyll b (0.27 and 0.29) and Carotenoids (0.24 and 0.27) in both seasons respectively.

Microbial Data: Results presented in Table (5) showed that, the highest counts were attributed to the mixture of *Azotobacter*, *Azospirillum and Bacillus* the corresponding high values were $5X10^7$and $6X10^7$cfu/g dry soil in the first and second seasons respectively. While *Azospirillum* showed $4X10^7$and $4X10^7$cfu/g dry soil during first and second seasons respectively.

Table 4: Effect of PGPR on chlorophyll a, b and carotenoids (mg/g fresh weight) of date palm plantlets during acclimatization stage.

	Chlorophyll a (mg/gf.w)		Chlorophyll b (mg/gf.w)		Carotenoids (mg/gf.w)	
Treatments	First season	Second season	First season	Second season	First season	Second season
Control (tap water)	0.53 c	0.52 c	0.27 d	0.29 d	0.24 d	0.27d
30cm/l *Azotobacter chroococcum*	1.15 b	1.17 b	0.74 c	0.75 c	0.48 c	0.47 c
30cm/l *Azospirillum lipoferum.*	1.15 b	1.19 b	0.85 b	0.86 b	0.52 b	0.53 b
30cm/l *Bacillus polymyxa*	1.12 b	1.17 b	0.83 b	0.85 b	0.52 b	0.55 b
30cm/l Mixture of all	1.73 a	1.77 a	1.08 a	1.09 a	0.58 a	0.64 a

Means within a column or row having the same letters are not significantly different according to Duncan's Multiple Range Test at 5% level.

Table 5: Effect of inoculation with PGPR on total counts of bacteria.

	Total counts ($X10^7$/ cfu/g DW)	
Treatment	First season	Second season
Control (tap water)	2	2
30cm/l *Azotobacter chroococcum*	3	3
30cm/l *Azospirillum lipoferum.*	4	4
30cm/l *Bacillus polymyxa*	3	3
30cm/l Mixture of all	5	6

Moreover *Azotobacter* and *Bacillus* recorded the least values (3 $X10^7$and 3 $X10^7$ & $X10^7$ and 3 $X10^7$ cfu/g dry soil) after first and second seasons respectively. On the other hand, the lowest values were recorded by control treatment ($2X10^7$ and $2X10^7$cfu/g dry soil) after first and second season, respectively. This can be explained by the ability of PGPR to increase microbial counts in soil. These results are in agreement with those of Pondey *et al*. [29]; Abotaleb *et al*. [30] and Mahmoud *et al*. [31] whom reported that inoculation with diazotrophic bacteria had an activation effect on the population of rhizospheric microorganism and increased their numbers by more than 50% at the end of the experiment compared with the numbers recorded before planting. EL-Kassas [32] reported that inoculation with the nitrogen fixing *Azospirillm brsilense* increased the soil *Azospirillm and* other microbial population including fungi actinomycetes and *Azotobacter* and consequently increased both the dehydrogenase activity and CO2 evolution. Ferjani *et al*. [33] and Yaish *et al*. [34] reported that the positive relationship was found between inoculation with PGPRs and date palm root system as well as rhizospheric microorganisms.

CONCLUSION

From the previous results, it could be recommended that using mixture of plant growth promoting *rhizobacteria* (PGPR) such as *Azotobacter chroococcum*, *Azospirillum lipoferum* and *Bacillus polymyxa*to increase the plant resistance against fungi especially in the early stages in green house during acclimatization stage of date palm plantlets cv. Malacabe.

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