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Growth Promotion of Date Palm Plantlets *Ex vitro* by Inoculation of Rizosphere Bacteria

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Abstract: This study was aimed to use phytohoromones produced by some strains like (*Azospirillum brasilense, Bacillus megaterium* and *Klebsiella pneumoniae)* as a protocol in date palm (*Phoenix dactylifera* L.) cv. Bartomouda to enhance *ex vitro* plants during adaptation stage. Plantlets which acclimatized at the greenhouse (six month) were subjected with three bacteria to evaluate the useful effect of plant growth promoting were produced by these different bacteria on growth parameters of the plantlets and chemical contents. Three treatments were used 10, 20 and 30 cm/l with irrigation water with 2.5 g/l NPK two times at the week and two periods were used (six month for each), control treatment (no bacteria) was used. Results showed that plant height (cm), number of leaves/plant, root length (cm), number of roots/plant, indole (mg/g fw) and chlorophyll (mg/g fw) were most enhanced with the three types of bacteria at the three levels 10, 20 and 30 cm/l. The highest significant results were occurred by *Klebsiella pneumoniae* consecutive by *Bacillus megaterium* and *Azospirillum brasilense* compared to control treatment. Little effect of the level 10 cm/l, meanwhile the level of 30 cm/l has left the greatest results of growth parameters in the two seasons. Greatest contents of leaves were found in indole IAA (mg/g fw) and chlorophylls a and b with three types of bacteria and varies concentrations, highly content have been produced by the level of 30 cm/l. The present study has clearly shown that the application of *Azospirillum brasilense,Bacillus megaterium* and *Klebsiella pneumoniae* might play a significant role in improving the growth response of date palm thereby producing good quality planting stock. These plants may perform better growth, survival and more fruits production.

Key words: Date palm · Azospirillum brasilense · Bacillus megaterium and Klebsiella pneumoniae · Indole · Chlorophyll

INTRODUCION

Biofertilizers are the formulations of living microorganisms, which are able to fix atmospheric nitrogen in the available form to plants, either by living freely in the soil or being associated symbiotically with plants [1]. They are capable of mobilizing nutritive elements from non-usable form to usable form through biological processes [2].Biological nitrogen fixation is carried out by both symbiotic and free living bacteria and blue green algae.

Azospirillum promoted epidermal cell differentiation in root hairs that increased the number of potential sites for *Bradyrhizobial* infection [3], as a result more nodules were developed [4]. Gutierrez-Manero *el al.* [5] reported that *Bacillus licheniformis* and *B. pumilus* were active in auxin-like (IAA-1) compounds production. The isolates produced 1.736 and 1.790 mg IAA-1/L culture growth medium, respectively. The results were particularly in agreement with Sedik [6] who identified isolates as members of genera *Klebsilla* sp., *Azospirillum, Azotobacte, Enterobacter* and *Pseudomonas.* Dong *et al.* [7] revealed that culture medium for *Bacillus megaterium* contained IAA and GA3. Addition of tryptophan (IAA-precursor) increased growth regulator concentration. Neelam *et al.* [8] identified different isolated bacteria as *Bacillus* and *Pseudomonas* which can produce IAA in addition, it has the ability to solubilize of phosphate. Sivaprasad *et al.* [9] noticed that *Azospirillum spp*. associated with rice were isolated and tested for its efficiency and found these isolates produced IAA *in vitro*, at concentrations from 30 to 55 mg/100mL.

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In the same purpose, Tensingh Baliah *et al.* [10] also found that incorporation of *Azospirillum* strains in the soil resulted in increasing the soil content of GA3 to 850% over control. Bacterial strains (diazotrophs) like *Azospirillum brasilense, Bacillus megaterium* and *Klebsiella pneumoniae* instead artificial fertilization as a protocol in date palm adaptation. Many phytohormones like auxins, cytokinins, gibberellins (GA3) and abscisic acid (ABA) or their derivatives can be produced by microorganisms. Numerous studies have shown the synthesis of plant growth regulators (PGRs) by bacteria in soil and play big role in biological activities which markedly enhanced by microbial interactions in the rhizosphere of plants [11]. The present investigation aimed to use bacterial strains as a production of many growth regulators to stimulate and development of date palm plantlets after acclimatization stage.

MATERIALS AND METHODS

This experiment was occurred in the greenhouse of the Central Laboratory of Date Palm Researches and Development, Agricultural Research Center (ARC), Giza, Egypt throughout the period from 2010-2011 to investigate the effects of bacterial strains (diazotrophs) like *Azospirillum brasilense, Bacillus megaterium* and *Klebsiella pneumoniae* instead artificial fertilization as a protocol in date palm adaptation.

Source of Bacterial Strains: Representative bacterial strains used throughout this study were: *Klebsiella pneumoniae* and *Azospirillium brasilense* which were obtained from a previous study carried out in the Environmental Studies and Research Unit (ESRU) Department of Agricultural Microbiology, Faculty of Agriculture, Cairo University, Giza by Farrag [12]. The other strain *Bacillus megaterium* was obtained from the Culture Collection of Microbiological Resource Center, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Purification and Maintenance of the Isolates: The selected strains were purified by streaking on agar plates containing the selective medium:

- Nitrogen fixation deficient medium (NFDM) for *Klebsiella* [13]
- Semi-solid malate medium for *Azospirillium* [14]
- *Bacillus* medium [15]

These selective media were surface inoculated with single strain and incubated at 28±2°C for 48hr to 7 days (5-7 times or more) until pure colonies were obtained.

Characteristics of colonies for each tested strains of every culture medium developed on agar plates were carefully examined, which has different colors. Maintenance of the selected strains was carried out by sub-culturing on several selective agar media as slants, which were then kept in refrigerator at (4°C). Strains were separately grown in liquid medium for preparation of 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 of 30ºC to reach to the population density of $>10^7$ cfu mL^{-1}(colony forming/ unit) pH was adjusted to 7.0. Growth and population of tested strains were followed by measuring optical density (OD) in spectrophotometer at 580 nm.

Plant Material: Plants of date palm *Phoenix dactylifera* cv. Bartomouda which derived from tissue culture, these plants derived by somatic embryogenesis for 12-16 months, plantlets cultured on rooting media ¾ MS [16] media supplemented with 0.05mg/l BA+ 0.1mg/l NAA+ 6g/l agar+ 30g/l sugar) for two months. Then, the plantlets were cultured on ¼ MS media supplemented with 0.1mg/l NAA for pre-acclimization stage for 3-6weeks. Plantlets which at length 10-15cm, contained 2-3leaves and 5-6 roots/plant sterilized with fungicide for 10minutes, then washing with water then cultured on pots containing a mixture of peat moss+ perlite at 2:1and putting under tunnels at 90% humidity for three months. After 6 months these plants which described (2-4 leaves, 10-15cm for shoot length and 3-5 roots/plantlet) were subjected with *Azospirillum brasilense, Bacillus megaterium* and *Klebsiella pneumoniae* supernatants at three concentration (10, 20 and 30cm/l) for each one with irrigation water two times in the week, three replicates were used at three plantlets for each one. This experiment was carried out for two seasons 6 months for one season. Also, all treatments were used as solution with irrigation water and 2.5g/l NPK. After the end of this experiment the following estimation were take:

- Plant height (cm)
- Number of leaves/plant
- Root length (cm)
- Root numbers/plant
- Total indoles6- Chlorophyll a, b and carotene

Total indole(mg/l): as described by Salim *et al.* [17] and the concentration was calculated as mg indole acetic acid/100 g fresh weight.

Chlorophyll:

Cl.A (mg/g) = 9.784 E 660 - 0.99 E 640
Cl. B (mg/g) = 21.426 E 640 - 4.65 E 660
Carotene (mg/g) = 4.695 E 440 - 0.268 (a + b)

RESULTS AND DISCUSSION

Figure 1 showed the effect of the different bacterial supernatant on growth parameters of date palm plants at the first and the second seasons.

Data in Table 1 showed the effects of bacterial supernatants (*Azospirillium brasilense*, *Bacillus megaterium* and *Klebsiella pneumonia*) on plant height (cm) and number of leaves/plantlet of date palm at two seasons.

Plant Height: Data analysis at two seasons showed that plant height significantly increased by increased the concentrations of bacterial supernatant from zero to 30mg/l. It was noticed that the highest concentration of three bacteria supernatants produced the highest plant length in both seasons. *Klebsiella pneumonia* supernatant at 30mg/l gave the highest plant length in both seasons (18.4 and 23.6 cm in the 1st and 2nd seasons, respectively), compared with other used concentrations. Respecting to the effect of substances, Bartomouda plant height was significantly affected by bacteria supernatants as compared with control in both seasons.

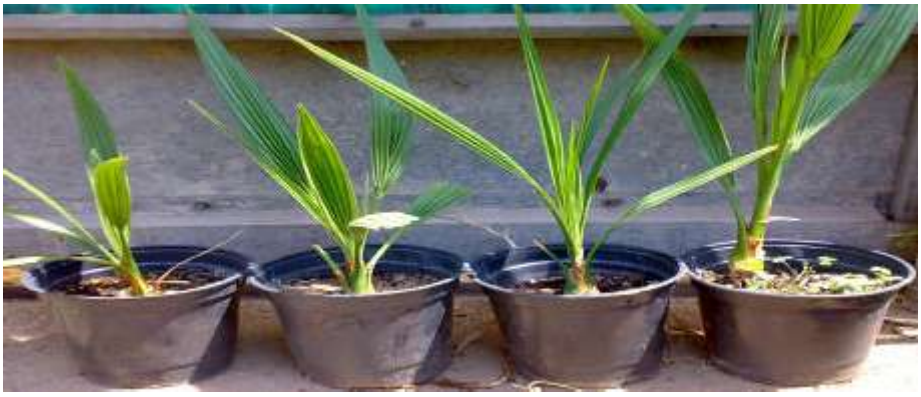
However, significant differences could be detected between *Azospirillium brasilense* (18.3cm at the 1st and 19.2cm at 2nd seasons) and *Bacillus megaterium* (18.4cm at the 1st and 21.7 cm at 2nd seasons) supernatants, also significant differences detected with *Klebsiella pneumonia* supernatant (20.2cm at the 1st and 30.2cm at 2nd seasons).

The interaction between the types of bacteria supernatants and their concentrations indicated that 30mg/l of *Klebsiella pneumonia* supernatant in two seasons gave the highest Bartomouda plant height (22.0cm at the 1st and 33.5 cm at 2nd seasons) comparing with other interactions.

Number of Leaves: Data in Table 1 cleared that the increasing of concentration of bacteria supernatants significant production in number of leaves/plant as the highest production was obtained by using 30mg/l which gave 3.4 and 3.7leaves/plant in the 1st and 2nd seasons, respectively.

Concerning the types of bacteria, data showed that using *Klebsiella pneumonia* supernatant was the most effective in the number of leaves/plant which gave (3.6 at the 1st and 3.9leaves/plant at 2nd seasons) followed by *Bacillus megaterium* supernatant gave (3.2 at the 1st and 3.5leaves/plant at 2nd seasons) with significant differences in between. The lowest values were produced by the control treatment (no bacteria supernatant used) as 2.2 and 2.3 leaves/plant in the 1st and 2nd seasons, respectively.

The interaction between types of bacteria supernatants and their concentrations, observed that the highest number of leaves/plant (4.4 at the 1st and 4.8leaves/plant at 2nd seasons) obtained as the result of using *Klebsiella pneumonia* supernatant. However, control treatment produced the lowest number of leaves/plant which gave (2.2 at the 1st and 2.3leaves/plant at 2nd seasons). Several studies have been performed with most focusing on the effect of different types of bacteria



Control Bacillus megaterium Azospirillum brasilens Klebsiella pneumoniae

Fig. 1: Effect of different bacterial supernatant on growth parameters of date palm plants at the 1st and 2nd seasons

Table 1: Effect of different bacterial supernatant on plant height (cm) and number of leaves/plant of date palm plants at the 1st and 2nd seasons

	Plant height (cm)							
	First season				Second season			
Bacterial strains	10	20	30	Mean	10	20	30	Mean
Control	12.5	11.4	11.9	11.9	13.1	11.9	12.5	12.5
Azospirillium brasilense	16.3	18.9	19.7	18.3	16.8	19.5	21.2	19.2
Bacillus megaterium	16.4	18.7	20.0	18.4	17.1	20.9	27.1	21.7
Klebsiella pneumoniae	18.7	19.9	22.0	20.2	27.7	29.3	33.5	30.2
Mean	16.0	17.2	18.4		18.7	20.4	23.6	
L.S.D	A= 0.5	B= 0.6	AB=1.1		A= 0.9	B= 0.8	AB=1.5	
	Leaves number/plant							
	First season				Second season			
Control	2.2	2.2	2.3	2.2	2.3	2.4	2.3	2.3
Azospirillium brasilense	2.6	2.8	3.2	2.9	2.9	3.2	3.7	3.3
Bacillus megaterium	2.8	3.3	3.6	3.2	3.0	3.4	4.0	3.5
Klebsiella pneumoniae	3.0	3.5	4.4	3.6	3.1	3.8	4.8	3.9
Mean	2.7	3.0	3.4		2.8	3.2	3.7	
L.S.D	A= 0.1	B=0.1	AB=0.2		A=0.2	B=0.3	AB=0.5	

Table 2: Effect of different bacterial supernatant on root length (cm) and roots number/plant of date palm plants at the 1st and 2nd seasons

	Root length (cm)							
	First season				Second season			
Bacterial strains	10	20	30	Mean	10	20	30	Mean
Control	6.1	6.4	6.8	6.4	6.6	6.8	7.1	6.8
Azospirillium brasilense	11.5	13.0	13.3	12.6	12.1	13.6	14.1	13.3
Bacillus megaterium	11.7	13.0	13.5	12.8	12.3	13.5	14.1	13.3
Klebsiella pneumoniae	13.1	14.1	15.6	14.3	13.5	14.6	16.2	14.8
Mean	10.6	11.6	12.3		11.1	12.1	12.9	
L.S.D	A= 0.3	B= 0.3	AB=0.5		A= 0.3	B=0.3	AB=0.6	
	Roots number/plant							
	First season				Second season			
Control	3.7	3.8	4.0	3.8	4.1	4.2	4.4	4.3
Azospirillium brasilense	3.8	6.0	6.5	5.4	4.2	6.5	6.9	5.9
Bacillus megaterium	4.8	6.4	7.1	6.1	5.1	6.6	7.5	6.4
Klebsiella pneumoniae	5.3	7.0	7.9	6.7	5.6	7.3	8.3	7.1
Mean	4.4	5.8	6.4		4.8	6.1	6.8	
L.S.D	A=0.4	B=0.5	AB=0.8		A=0.4	B=0.5	AB=0.8	

on the growth parameters, Sheng [18] on cotton stated that Bacillus edaphicus NBT strain was increased plant height, Medina *et al.* [19] on *Alpinia purpurata*, El-Barougy *et al.* [20] on soybean and Mafia *et al.* [21] on *Eucalyptus globules*. They found that plant height and number of leaves were increased with *Azospirillum brasilense, Azotobacter chroococcum* and *Bacillus megaterium* and subtilis, recently Sandeep *et al.* [22] on *Amaranthus gangetcus*, proved that *Azotobacter chroococcum* was raised the plant height, root length and leaves number.

Root Length: Data presented in Table 2 indicated that the increasing in bacteria supernatants levels significantly increased root length (cm), as the highest values of root length resulted from 30mg/l which gave (12.3 and 12.9 cm) in the 1st and 2nd seasons, respectively, followed by 20mg/l which gave (11.6 and 12.1cm) with significant differences in between.

The highest stimulation effect occurred by using *Klebsiella pneumonia* supernatant (14.3cm at the 1st and 14.8 at 2nd seasons) followed by *Bacillus megaterium* supernatant (12.8 cm at the 1st and 13.3cm at 2nd seasons)

with significant differences in between followed by *Azospirillium brasilense* supernatant (12.6 cm at the 1st and 13.2cm at 2nd seasons). The interaction between type of bacteria supernatants and their concentrations indicated that 30mg/l of *Klebsiella pneumonia* supernatant in two seasons gave the highest significant Bartomouda root length (15.6 cm at the 1st and 16.2cm at 2nd seasons) comparing with other interactions.

Respecting to the effect of type of bacteria, data cleared that *Klebsiella pneumonia* supernatant was the most effective in number of roots/plant (6.7 roots/plant at the 1st season and 7.1 roots/plant at 2nd seasons) followed *Bacillus megaterium* supernatant (6.1 roots/plant at the 1st and 6.4 roots/plant at 2nd seasons) with significant differences in between, followed by *Azospirillium brasilense* supernatant (5.4 roots/plant at the 1st and 5.9 roots/plant at 2nd seasons) and finally control treatment (3.8 roots/plant at the 1st and 4.3 roots/plant at 2nd seasons) with significant differences among them. Regarding to the concentration, it was clearly noticed that the highest concentration 30mg/l of three bacteria supernatants produced the highest Bartomouda roots number in both seasons (6.4 roots/plant at the 1st season and 6.8 roots/plant at the 2nd season). Whereas, the highest significant results of number of roots was (7.9 roots/plant at the 1st season and 8.3 roots/plant at 2nd seasons) obtained as the result of using *Klebsiella pneumonia* supernatant at 30 cm/l. The observed promotion in roots of plants in this study could be attributed to the cumulative effects of those types of bacteria. Similar results were obtained by Shishido *et al.* [23] on *Pinus contorta* showed that roots length and numbers were increment with *Bacillus polymxa*, Wahyudi *et al.* [24] on soybean stated that *Bacillus sp* were increased root length and numbers.

Total Indoles (mg/g fw): Data in Table 3 illustrated that the positive effect of three types of bacteria and levels on the total indoles mg/g for two seasons. Clearly shown that total indole was varies with the different types of bacteria, largest significant content of total indoles were occurred by treatment of *Klebsiella pneumonia* supernatant, consecutive by Bacillus and Azospireillum at the 1st season, meanwhile, at the second season the gradual increasing of indoles from Klebsiella followed by Azospirellum and Bacillus, significant differences were found. To regard the levels of three types of bacteria, the level 30 mg/l have left the highest significant values of indoles, while the other levels 10 and 20 mg/l were hadn't significant differences among them at the 1st season. At the 2nd season the significant differences were found between levels 30 and 10 mg/l. Highly interaction occurred at 30 mg/l of Klebsiella at the 1st, on the other hand great significant interaction was found at the level 30 mg/l and three types of bacteria at the 2nd season. In this respect Bent *et al.* [25] on *pinus contorta*, Mirza *et al.* [26] on sugarcane *in vitro*, they found that *Klebsiella oxytoca* increased IAA.

Total Chlorophylls a and b: Total chlorophyll a and b content (Table 4) was found to be great in the plant leaves with different three types of bacteria and three levels 10, 20 and 30 mg/l. the increasing in bacteria supernatants levels significant gradually rising contents in chlorophyll a and b at the 1st and 2nd seasons, respectively. The highest values were found by the level 30 mg/l at two seasons respectively.

For the types of bacteria, *Klebsiella* supernatant had the greatest significant results of chlorophyll a and b respectively at the 1st and 2nd seasons sequenced by other types of bacteria. Meenakshisundaram *et al.* [27]

Table 3: Effect of different bacterial supernatant on indoles (mg/g f w) of date palm plants at the 1st and 2nd seasons

	Indoles (mg/g f w)							
	First season				Second season			
	10	20	30	Mean	10	20	30	Mean
Control	1.1	1.0	1.0	1.0	1.1	1.0	1.1	1.0
Azospirillum	2.6	2.7	3.3	2.9	2.6	4.3	4.4	3.8
Bacillus	2.9	2.9	2.8	2.9	3.1	3.5	3.0	3.2
Klebsiella	2.9	3.3	4.5	3.6	3.2	3.7	5.3	4.1
Mean	2.4	2.4	2.9		2.5	3.1	3.5	
L.S.D.	A= 0.5	B= 0.5	AB=0.9		A= 0.5	B=0.6	AB=1.1	

Table 4: Effect of different bacterial supernatant on chlorophyll a and b of date palm plants at the 1st and 2nd seasons

	Chlorophyll a (mg/g f w)							
	First season				Second season			
	10	20	30	Mean	10	20	30	Mean
Control	0.7	0.7	0.8	0.7	0.7	0.7	0.6	0.7
Azospirillum	1.3	1.4	2.2	1.6	1.3	1.5	2.1	1.6
Bacillus	1.4	1.7	2.4	1.8	1.4	1.8	2.3	1.8
Klebsiella	1.6	2.1	2.3	2.0	1.8	2.2	3.2	2.4
Mean	1.3	1.5	1.9		1.3	1.6	2.1	
L.S.D.	A= 0.1	B= 0.2	AB=0.3	A= 0.1	B=0.1	AB=0.2		
	Chlorophyll b(mg/g f w)							
	First season				Second season			
Control	0.2	0.2	0.3	0.2	0.3	0.2	0.2	0.2
Azospirillum	0.1	0.8	0.9	0.6	0.1	1.1	1.9	1.0
Bacillus	0.7	1.1	0.2	0.7	0.9	1.3	0.1	0.8
Klebsiella	0.5	0.4	2.0	1.0	0.7	0.7	2.1	1.2
Mean	0.4	0.6	0.9		0.5	0.8	1.1	
L.S.D.	A=0.1	B=0.1	AB=0.2		A=0.3	B=0.3	AB=0.5	

on *Delonix regia*, Ravikumar *et al.* [28] on *Jatrova curcas* they stated that *Azospirellum* strain and *azotobacter* were increasing chlorophyll content.

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