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Leverage of biofertilizers on the plantlets of date palm (*Phoenix dactylifera*) cv Malacabe in the acclimatization stage

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ABSTRACT

The acclimatization stage of the palm trees is considered the most important period in the plant life. In this work, some microorganisms had pivotal role in boosting the performance of biological functions of plant. A mixture of bacteria and cyanobacteria with 50 and 75 % of recommended dose of nitrogen improved the morphological properties of plant and enhanced the excretion of biochemical compounds. The mixture of bacteria and cyanobacteria was the best treatment followed by the mixture of bacteria over control in all tested parameters at 75 % of N for 16 months for all the tested parameters. Morphological parameters such as plant height significantly increased by 43 % in case of mixture of bacteria and cyanobacteria at 75 % N after 16 months over control while for leaf width, the increase reached to the double compared to control. Indole content increased by 93 % over control. Chlorophyll (a) and carotenoid contents increased by 1.8 and 1.6 time over control due to the mixture of bacteria and cyanobacteria at 75 % N after 16 months, respectively. The activity of dehydrogenase enhanced and increased 5.7 time compared to control. Unlikely, nitogenase activity recorded the highest rate at 50 % N after 16 months due to the mixture of both bacteria and cyanobacteria. All biological treatments exerted no significant difference between 50% and 75 % N either after 8 or 16 months for the most morphological and physiological parameters except cyanobacterial treatment.

Key words: acclimatization, bacteria, cyanobacteria, biofertilizers, inole acetic acid, chlorophyll, carotenoids, nitrogenase activity, dehydrogenase activity.

Introduction

Phoenix dactylifera L. belonging to family *Palmaceae* is one of the common and popular fruit grown in tropical and subtropical regions in the world. In Egypt, palm trees cover a large area extends from Aswan to North Delta passing through the Oasis of Siwa, Bahariya, Farafra, Kharga and Dakhla. Factors affecting the successful production of date palm include length of plantlets, number of leaves, strength of root system, humidity conditions, and composition of the soil (Abul- Soad and Mushtaque, 2014). Efficacy of the protocol of date palm micropropagation referred to the acclimatization phase deemed the most critical period till transfer to the open field. The most important obstacles confronting date palm plantlets through the first acclimatization stage is sluggishness vegetative growth with low survival rate (Abd El-Galeil *et al*., 2017). Microorganisms had vital function in enhancing plant growth through producing amino acids, growth regulators such as indole acetic acid and gibberellin, vitamins, antifungal, antibacterial and exopolysaccharides which improve the growth and productivity (Lugtenberg and Kamilova, 2009). Plant growth promoter rhizobacteria (PGPR) such as *Azotobacter chroococcum*, *Azospirillum lipoferum* and *Paenebacillus polymyxa*, individually or in combination maximized the counts and the activity of microorganisms in rhizosphere region and convert nutritionally important elements from unavailable to available form through biological processes (Vessey, 2003). Furthermore, PGPR reigned some mechanisms could encourage the plant outgrowth through nitrogen fixation, phosphate solubilization, siderophore production, inhibition of biofilm formation, production of volatile organic compounds (VOCs), induction of systemic

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resistance, promoting beneficial plant-microbe symbioses and interference with pathogen toxin production (Bhattacharyya and Jha, 2012). Fuentes and Caballera-mellado (2005) reported high microbial counts and activity occurred by inoculation with the mixture of *Azotobacter chroococcum*, *Azospirillum lipoferum* and *Paenebacillus polymyxa*. Cyanobacteria acted as potent biofertilizer because of enhancing all the morphological and biochemical criteria of plants such as proteins, carbohydrates, total nitrogen contents, lowering the chemical nitrogen fertilizers up to 50%, boosting nutrient uptake and limiting the growth weeds (Jagannath *et al*., 2002 and Ojaghloo *et al*, 2007). Abd El-Galeil and Abd El Aal (2016) stated manipulation of cyanobacteria as biofertilizers promoted growth and increased survival percentage in acclimization stage of date palm. Moreover, nonassociative bacteria such as cyanobacteria and *Azospirillum brasilense* played a considerable role in enhancing the growth of date palm and good quality of yield (Farrag *et al*., 2011). Hence, cyanobacteria and bacteria were recommended as biofertilizers substitution for chemical fertilizers to avert the high cost and environmental problems.

The aim of this work was to promote the outgrowth of plantlet of date and hearten the acclimatization phase through utilize microbial activity.

Materials and Methods

Microorganisms used

:

Azospirillum lipoferum was grown on nitrogen deficient semi solid malate medium (Dobereiner *et al*., 1976). *Azotobacter chroococcum* was cultured on modified Ashby medium (Abd El Malek and Ishac, 1968). *Paenebacillus polymyxa* was grown according to Hino and Wilson (1958). Cyanobacterial strains (*Nostoc muscorum* and *Anabaena oryzae*) were propagated on BG11 liquid medium of Rippika *et al*. (1979).

All bacterial and cyanobacterial strains were obtained from Department of Agricultural Microbiology; Soils, Water and Environment Research Institute; Agricultural Research Centre.

Preparation of agriculture

Six month old healthy of date palm (*Phoenix dactylifera*, cv Malacabe) was obtained from tissue culture laboratory with average 20 -25 cm height and 3- 4 leaves/plantlet. The plantlets were cultured in 25 diameter plastic pots (one plantlet/pot) and filled with mixture of peat moss and sand (1:2 volume/ volume). The additives were applied every month from March to November for two seasons. All treatments had 3 replicates and every replicate contains 3 plantlets.

Experimental layout

This experiment was implemented under greenhouse conditions at Central Laboratory of Date Palm Research and Development ,Giza, Egypt for two successive seasons (2015 and 2016) to investigate the influence of either mixture of bacteria (*Azospirillum lipoferum, Azotobacter chroococcum* and *Paenebacillus polymyxa*) or mixture of cyanobacteria (*Nostoc muscorum and Anabaena oryzae*) and the combination between the mixture of bacteria and cyanobacteria in the presence of two levels of ammonium nitrate as nitrogen source (50 and 75 % of recommended dose).

The experimental layout was complete randomize design and the data were subjected to analyses of variance at 5% significance according to Steal and Torrie (1983).

Treatments

1- Control (recommended dose of mineral fertilizers) (T1).
2- Mixture of *Nostoc muscorum*+ *Anabaena oryzae* + 50% of N (T2).
3- Mixture of *Nostoc muscorum*+ *Anabaena oryzae* +75% N (T3).
4- Mixture of *Azotobacter chroococcum* + *Azospirillum lipoferum* + *Paenebacillus polymyxa* + 50% N (T4).

5- Mixture of *Azotobacter chroococcum* + *Azospirillum lipoferum* + *Paenebacillus polymyxa* + 75% N (T5).
6- Mixture of *Nostoc muscorum*+ *Anabaena oryzae* + *Azotobacter chroococcum* + *Azospirillum lipoferum* + *Paenebacillus polymyxa* + 50 % N (T6).
7- Mixture of *Nostoc muscorum*+ *Anabaena oryzae* + *Azotobacter chroococcum* + *Azospirillum lipoferum* + *Paenebacillus polymyxa* + 75 % N (T7).

Plant samples were taken after 8 and 16 months to determine shoot length, leaf width and total amount of leaves/plant and other chemical determinations.

Carotenoids and chlorophyll (a) and (b) were determined according to Saric *et al*. (1976) while total indole was determined according to Larsen *et al*. (1962). Soil biological activity such as dehydrogenase and nitrogenase activity were determined according to Skujins (1976) and Dilowarth (1970), respectively.

Statistical analyses:

Analysis of Variance (ANOVA) and least significant difference (LSD) were calculated according to Steal and Torrie (1983).

Results and Discussion

Table (1) demonstrated the effectiveness of cyanobacteria and bacteria at 50 and 75 % of nitrogen on morphological quality of date palm throughout acclimatization stage. The mixture of *Nostoc muscorum*+ *Anabaena oryzae* at 50 % N did not have distinct effect during acclimatization period compared to control, while the same treatment at 75 % N exerted significant effect for the morphological measurements during the trial period. Both of plant height and leaf width were affected by the mixture of cyanobacteria at 75 % N after 8 or 16 months. Plant height increased by 8.3 and 9.4 cm after 8 and 16 months compared to control respectively, while leaf width increased by 0.21 and 0.32 cm. The treatment of bacteria was more supportive than cyanobactria treatment on all morphological parameters either after 8 or 16 months. Plant height increased from 47.0 to 56.33 cm by 9.33 cm in the case of mixture of *Azotobacter chroococcum* + *Azospirillum lipoferum* + *Paenebacillus polymyxa* at 50% N after 8 months while the same treatment at 75 % increased from 47.0 to 58.47 cm by 11.5 cm. After 16 months, the mixture of bacteria treatment significantly affected the plant height compared to control while no significant difference between 50 and 75 % N was noticed. Leaf width was also affected significantly by bacterial treatment either at the different two levels of N or at the different two periods.

Table 1: Efficacy of biofertilizers on morphological properties of date palm plants throughout acclimatization stage.

Treatment	After 8 months			After 16 months		
	Means of leaves numbers	Plant height (cm)	Leaf width (cm)	Means of leaves numbers	Plant height (cm)	Leaf width (cm)
T1	5.330	47.000	1.017	6.670	57.130	1.217
T2	4.360	48.000	0.983	5.000	60.8 30	1.250
T3	5.370	55.270	1.224	6.000	66.5 30	1.533
T4	6.0 70	56.330	1.483	7.370	74.230	1.831
T5	6.330	58.470	1.499	7.680	74.670	1.842
T6	7.330	64.370	2.033	8.330	80.700	2.446
T7	7.600	66.000	2.050	8.670	81.700	2.503
L.S.D at 5%	0.768	2.479	0.110	0.736	3.987	0.121

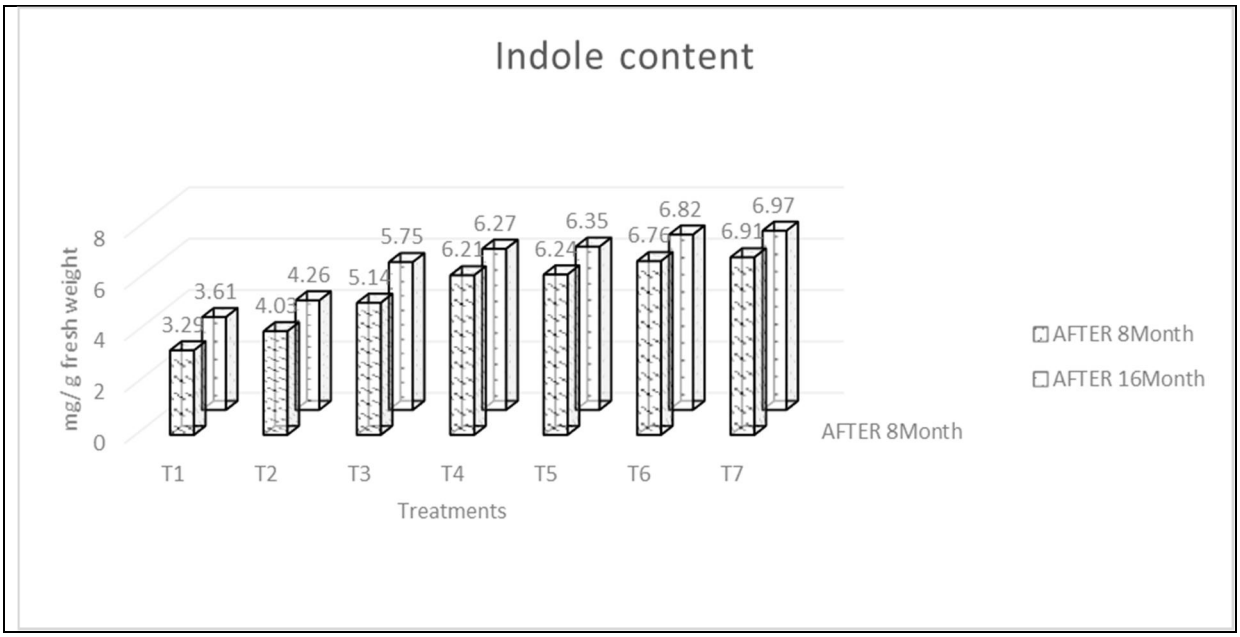
Leaf width increased by 0.47 and 0.48 cm at 50 and 75 % N after 8 months respectively, while it increased by 0.61 and 0.63 cm after 16 months over control, respectively. The promising treatment was the mixture of bacteria and cyanobacteria either at the two different levels of nitrogen or at the two experimental periods. The mean of leaf numbers increased by 37.5 and 42.6 % at 50 and 75 % N

after 8 months respectively, while after 16 months, the mean of leaf numbers increased by 24.89 and 30 % over control, respectively. The increase rates of plant height after 16 months for two levels of nitrogen were higher than after 8 months. The increase rates after 8 months were 37 and 40 % for 50 and 75 % N respectively, and was 41.0 and 43.0 % after 16 months for the same levels of N. Plant width after 8 and 16 months reached to nearly the double of plant width of tested levels of nitrogen over control. All biological treatments exerted no significant difference between 50% and 75 % N either after 8 or 16 months for all morphological parameters except cyanobacterial treatment.

Enhancing of growth and morphological properties of date palm as a result of biological treatments was ascertained to excrete growth promoting regulators such as gibberellin and auxin, vitamins, amino acids, polypeptides, antibacterial and antifungal substances (Lugtenberg and Kamilova, 2009).

Fig (1) revealed the role of bioagents on excretion of indole acetic acid throughout date palm acclimatization. The results showed obvious variations among cyanobacterial and bacterial treatments for indole acetic acid production. About 20% increase in indole acetic acid was excreted by cyanobacteria at 50 % N after 8 or 16 months, while the increase elevated to above 55 % at 75 % N after 8 or 16 months over control. The bacterial treatment at 50 % and 75% N after 8 months raised the excretion of indole acetic acid to almost 90 % than control while at the same tested levels of nitrogen, the excretion increased by nearly 75 % after 16 months than control. The increase ratio of indole acetic acid excretion for the mixture of all microbes at 50 and 75 % N after 8 months ranged between 105 and 110 % respectively whilst after 16 months at the same pervious condition ranged between 88 and 93 %, respectively.

Dilfuza (2011) reported that the auxin level is usually higher in the rhizosphere, where high percentage of rhizosphere bacteria is likely to synthesize auxin as secondary metabolites because of the rich supplies of root exudates. The author also stated that the production of auxin (IAA), has been recognized as an important factor in direct plant-growth-promoting abilities of rhizosphere bacteria and they stimulate proliferation of lateral roots that increase nutrient absorbing surfaces and results in better assimilation of water and nutrients from the soil.

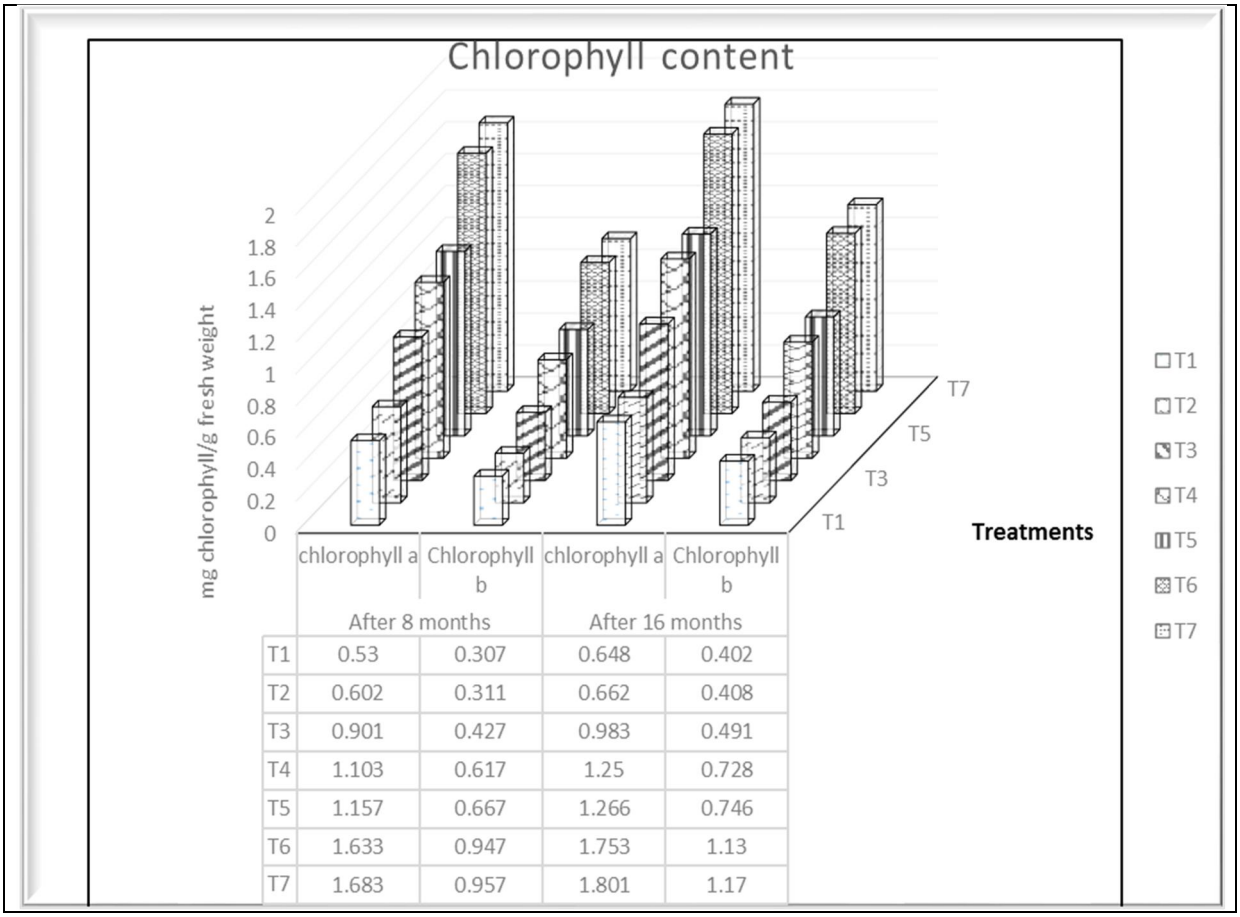


LSD after 8 months = 0.38 and LSD after 16 months = 0.39

Fig 1: Influence of bioagents on indole excretion through date palm acclimatization experiment.

Fig. (2) indicated that highest values in chlorophyll (a) and (b) excretion were in the mixture of all tested microbes while the least values were in cyanobaterial treatment at either 50 or 75% N after the both tested periods of cultivation. The mixture of bacteria and cyanobacteria increased chlorophyll (a) by 2.1-2.2 times over control at 50 and 75 % N after 8 months, respectively. No differences among

the values of chlorophyll (a) were noticed at 50 and 75 % N after 16 months of cultivation where the increases reached to 1.8 times over control. Free-living nitrogen-fixing bacteria eg *Azotobacter chroococcum* and *Azospirillum lipoferum*, were found to have not only the ability to fix nitrogen but also the ability to release phytohormones similar to gibberellic acid and indole acetic acid, which could stimulate plant growth, absorption of nutrients, and photosynthesis (Essam, 2013). Covindjee and Shevela (2011) stated that most cyanobacteria contain carotenoids, chlorophyll (a), phycocyanin and allophycocyanin as pigments for photosynthesis.



	After 8 months		After 16 months	
	chlorophyll a	Chlorophyll b	chlorophyll a	Chlorophyll b
T1	0.53	0.307	0.648	0.402
T2	0.602	0.311	0.662	0.408
T3	0.901	0.427	0.983	0.491
T4	1.103	0.617	1.25	0.728
T5	1.157	0.667	1.266	0.746
T6	1.633	0.947	1.753	1.13
T7	1.683	0.957	1.801	1.17

LSD after 8 months= 0.095 for chlorophyll (a) and 0.076 for chlorophyll (b)
LSD after 16 months= 0.01 for chlorophyll (a) and 0.079 for chlorophyll (b)

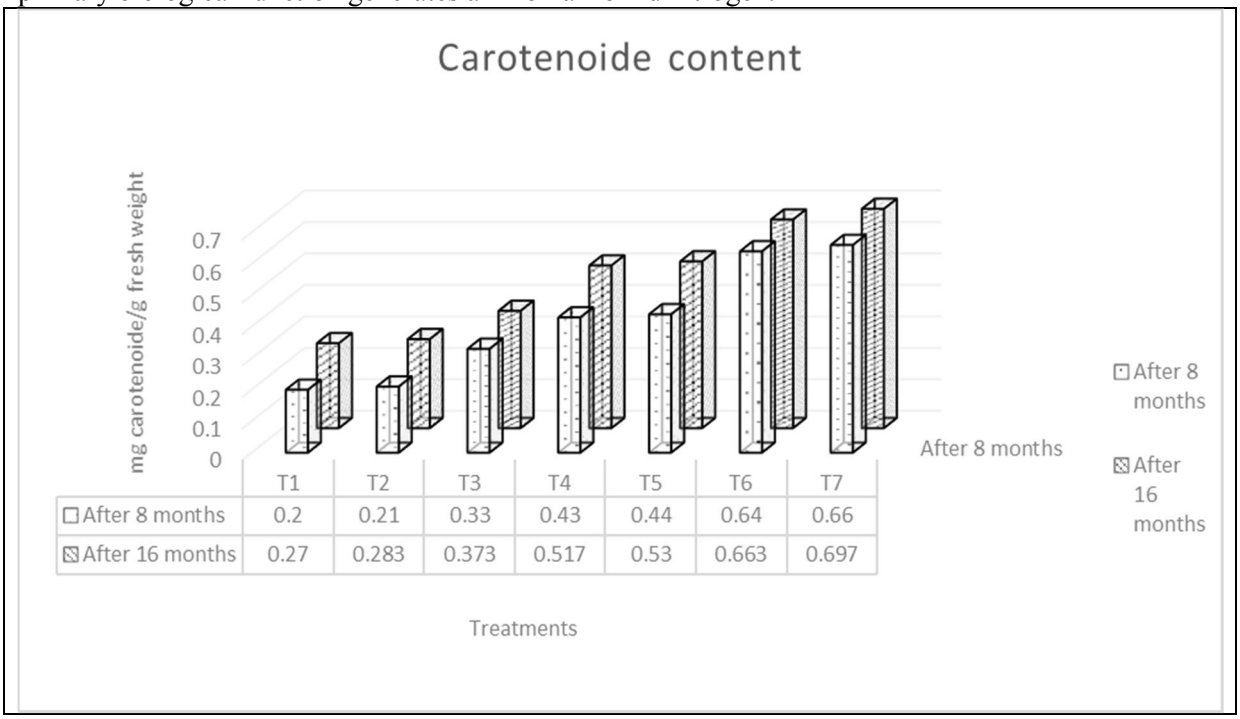
Fig. 2: Effectiveness of microbial additives on chlorophyll in acclimatization of date palm at two levels of nitrogen after 8 and 16 months.

Fig. (3) showed the role of microbial treatments on carotenoid contents after 8 and 16 months of date palm's acclimatization. The mixture of all tested microbes was more preferable treatment among the other treatments. The increases of carotenoid content reached the maximum rate at 50 and 75 % N after 8 months of cultivation. The increases were 2.2 – 2.3 times over control respectively, while the increases at 50 and 75 % N after 16 months were 1.45-1.58 times over control, respectively.

Fig. (4) revealed the effectiveness of cyanobacterial and bacterial strains and the mixture of them on nitrogenase activity during the acclimatization period of date palm. Nitrogenase activity after 16 months was higher than after 8 months while at 50 % N, it was the best compared to 75% N for all microbial treatments. The mixture of all microbes was superior followed by bacterial treatment. The maximum activities of niterogenase were 164.63 and 167.1 µmole C_2H_4/g soil/h at 50 % N after 8 and 16 months of cultivation while for bacterial treatment, the activities of nitrogenase were 149.37 and 153.8 µmole C_2H_4/g soil/h at the same level of nitrogen and the same period, respectively.

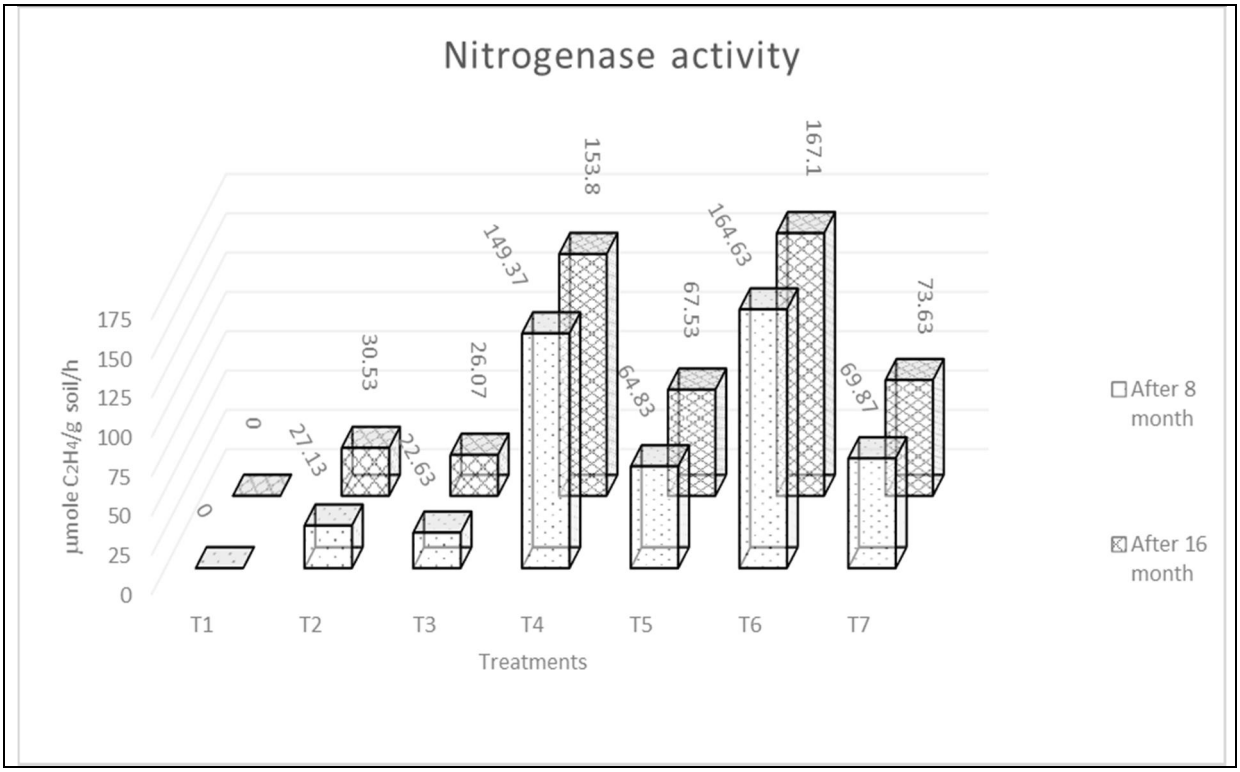
Microorganisms like cyanobacteria, *Azotobacters*, *Azospirillum* etc. are the natural nitrogen fixers. Natural process of Nitrogen fixation is accomplished by complex but important enzyme system known as nitrogenase. This important biological fixation of nitrogen into ammonia makes it

accessible for synthesis of nucleotide, DNA, RNA, amino acid and protein. Nitrogenase enzyme's primary biological function generates ammonia from dinitrogen.



LSD after 8 months = 0.05 and LSD after 16 months = 0.052

Fig. 3: Effect of microbial treatments on carotenoid contents in acclimatization period of date palm.

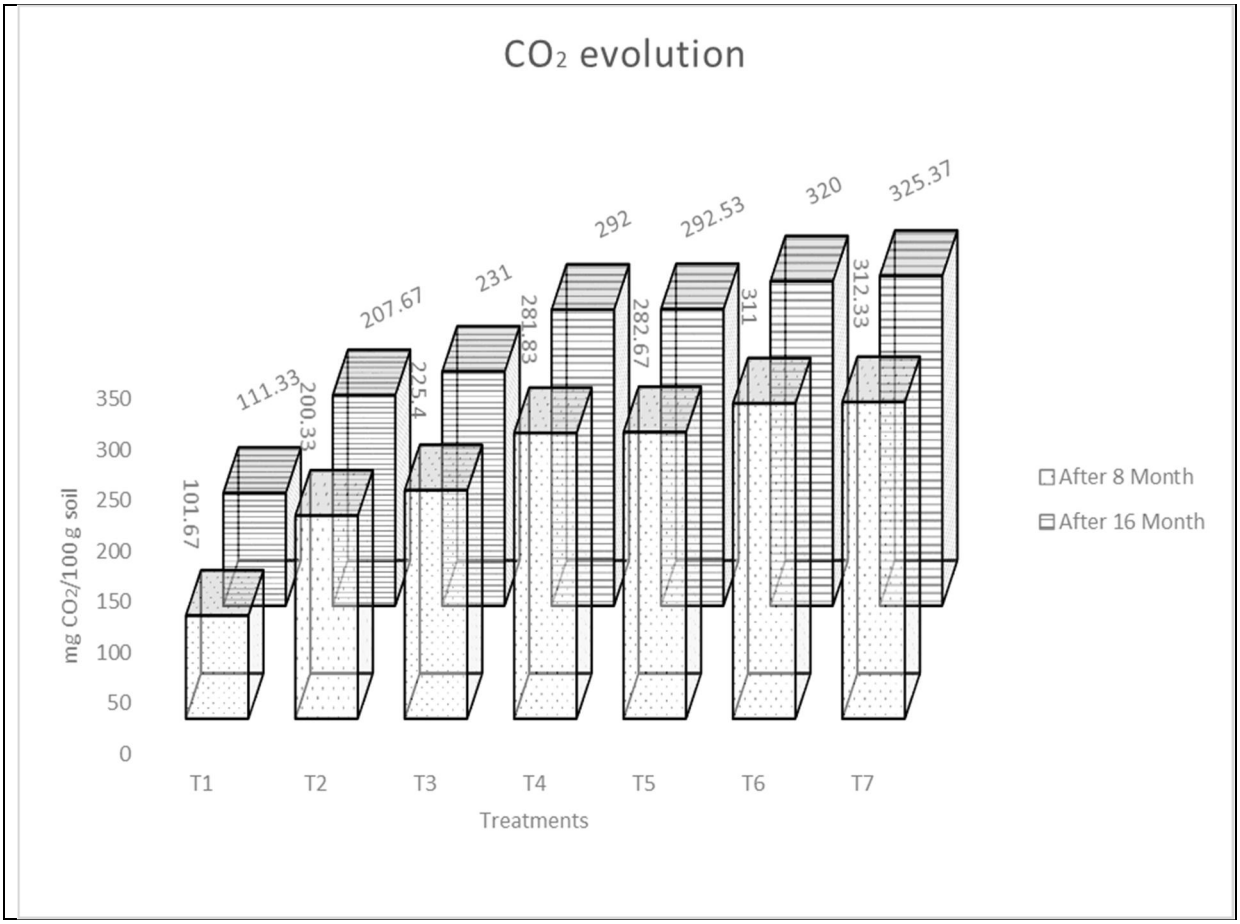


LSD after 8 months = 6.053 and LSD after 16 months = 5.774

Fig. 4: Influence of microbial treatments on nitrogenase activity through acclimatization stage.

This activity is essential for all organisms because it sequesters atmospheric nitrogen gas in a biologically accessible form. Artificial agricultural fertilizer production is now the largest source of fixed nitrogen in the Earth's ecosystem (Dighe *et al*., 2010).

Fig. (5) demonstrated the role of microorganisms which enhance the respiration rate throughout acclimatization of date palm stage. All biological treatments encouraged of CO_2 evolution compared to control. Results obtained after 16 months of cultivation were more preferable than after 8 months either at 50 % or at 75 % N. The mixture of all tested microorganisms or bacterial treatments were superior than cyanobacterial treatment after 8 or 16 months of cultivation. The increase of CO_2 evolution reached to about two folds at either 50 or 75 % N after 16 months compared to control.

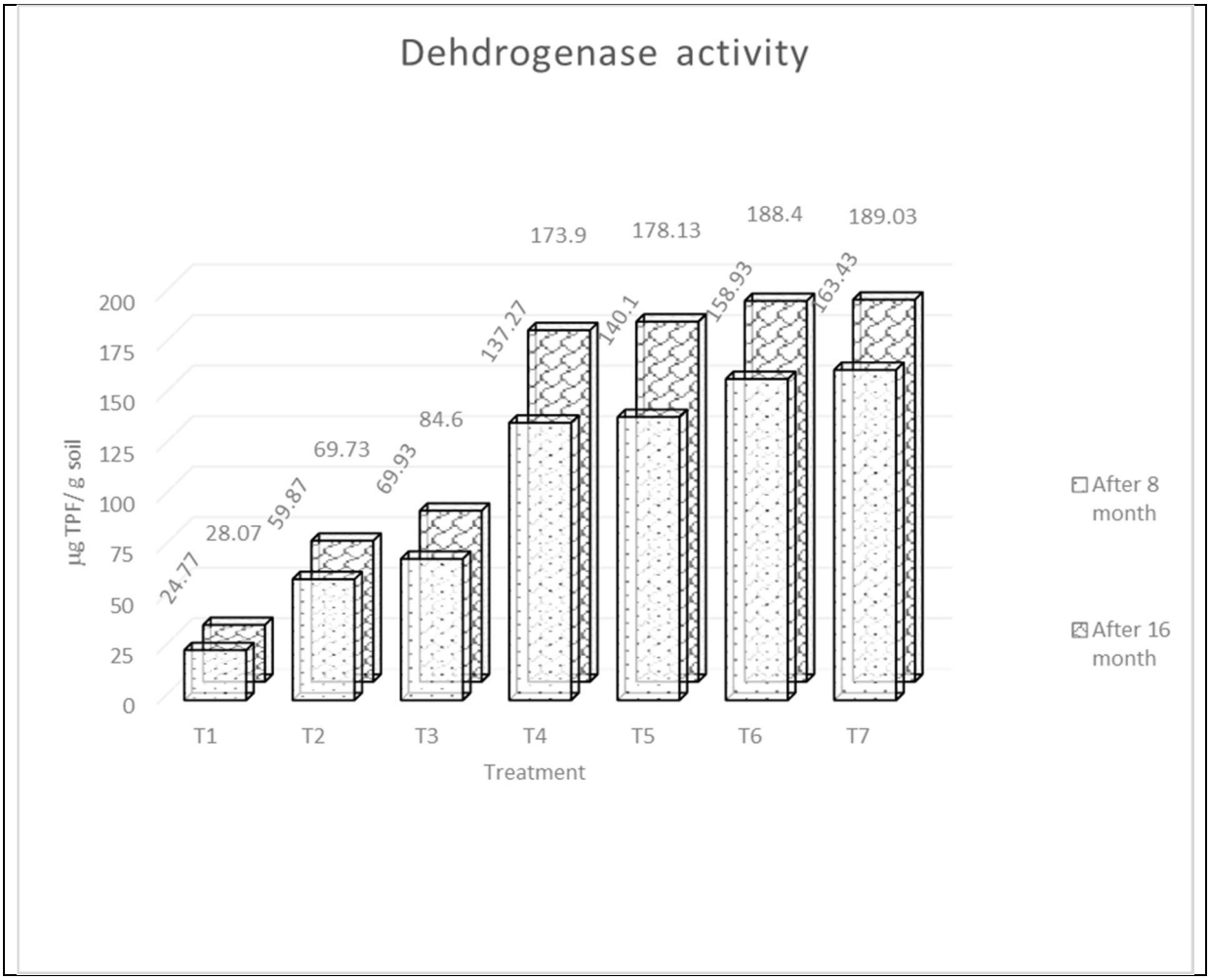


LSD after 8 months = 5.761 and LSD after 8 months = 6.083

Fig. 5: The role of microorganisms in enhancing CO_2 evolution during acclimatization stage of date palm at 50 or 75 % N after 8 or 16 months of cultivation.

Fig. (6) showed the dehydrogenase activity through acclimatization period resulted from microbial treatments. The mixture of all tested microbes was the best treatment in dehydrogenase activity followed by bacterial treatment. The cyanobacterial treatment exerted the lowest effect on dehydrogenase activity. Dehydrogenase activity after 16 months was better than after 8 months and also at 75 % N was more preferable than at 50 % N for cyanobacterial treatment. Dehydrogenase activity reached to 189.03 µg TPF/soil with mixture of all tested microbes at 75 % N after 16 months versus 28.07 µg TPF/ soil for control in the same condition.

EL- Kassas (2002) reported that inoculation with the nitrogen fixing *Azospirillum* to wheat increased the soil Azospirilla and other microbial population including fungi, actinomycetes and *Azotobacter*, and consequently increased both the dehydrogenase activity and CO_2 evolution, which are considered as index for biological activity and soil fertility. De Caire *et al* (2000) studied the identification changes in the activity of soil extracellular enzymes; b-glucosidase, phosphomonoesterase, arylsulphatase, protease and urease and the intracellular dehydrogenase following the addition of cyanobacterial exopolysaccharide and biomass to soil.



LSD after 8 months = 5.128 and LSD after 16 months = 5.452

Fig. 6: Effect of microbial treatments on dehydrogenase activity through acclimatization stage.

Conclusion

Bacteria and cyanobacteria applied in that research had a vital role in encourage and enhancing the growth qualitatively and quantitavely. The combination of some bacteria and cyanobacteria was the promising treatment for all experimental parameters. All biological treatments exerted no significant difference between 50% and 75 % N either after 8 or 16 months for all morphological and physiological parameters except cyanobacterial treatments which showed that production of indole acetic acid, chlorophyll and carotenoid contents, CO_2 evolution and dehydrogenase activity in addition to all morphological parameters was more superior at 75 % N than at 50 % N either after 8 or 16 months.

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