

## Inoculation of Grass-cyclingcompost with N<sub>2</sub>-fixing bacteria

Merfat Taher Ben Mahmoud<sup>1</sup>, Eman Ali Mohmmed Ferjani<sup>2</sup>, Marwa F. Elalem<sup>3</sup>

<sup>1,2,3</sup> Faculty of Agriculture, Department of Microbiology Soil and Bio Fertilization /  
University of Tripoli

<sup>1</sup> dr.mbenmahmoud@yahoo.com, <sup>2</sup> emanferjani@yahoo.com

### Abstract

*Inoculation of grass-cyclingcompost with N<sub>2</sub>-fixing bacteria may improve its quality by increasing total N and available P. Compost was inoculated with Azotobacter, Burkholderia and Azospirillum sp. each alone and all three together. Numbers of all N<sub>2</sub>-fixing bacteria in compost declined from an initial population of (5 x 10<sup>5</sup> cells/ g) during incubation. The population of Azotobacter declined to approximately (2 x 10<sup>2</sup> cells/g) and the population of Burkholderia and Azospirillum declined to approximately (9 x 10<sup>3</sup> and 4x 10<sup>4</sup> cells/ g) respectively, at day 40. Inoculation with N<sub>2</sub>-fixing bacteria increased acetylene reduction, total N by 5–15% and available P by 20–30% in comparison with the uninoculated control. Increasing the N content and P availability of compost increases its value and there may be additional benefits from providing N<sub>2</sub>-fixing bacteria.*

**Keywords:** Inoculation, N<sub>2</sub>-fixing bacteria, Compost.

## Introduction

Compost is an organic matter that has been decomposed and recycled as fertilizer and soil amendment. Additionally, composting is considered to be one of the most suitable ways of converting organic wastes into products that are beneficial for plant growth and is suggested as one of the environmentally and friendly alternative method for the disposal of solid organic wastes as it leads to the minimization, stabilization, and utilization of organic waste. Nowadays, a natural decomposition technology is widely used to deal with the organic waste due to the large accumulation of the agricultural waste, animal husbandry excrement and urban garbage, which has become a serious environmental issue (Hinrichs, 2005) and the effectiveness of using more local organic materials from agro-industrial by-products as nutrient sources (Aghsaghali *et al.*, 2008).

Composting is natural processes by which micro-organisms decompose organic matter into simpler nutrients and convert the organic wastes into mature compost. For example, aerobic composting, where de-composition takes place in the presence of oxygen, is the quickest way to produce high quality compost and widely accepted way of stabilizing organic wastes and converting them into usable and valuable products (Chen *et al.*, 1996).

What's more, a number of benefits are gained from the compost: the use of inorganic fertilizers is avoided, carbon is bounded to soil and composting of N-rich wastes can be efficiently decreased N losses during the composting process (Eklind, 2000).

Further, the concentration of inorganic nitrogen (N) in compostable material is one of the most important factors in determining agronomic value because most of the N found in a compost mixture is in an organic form. If N availability is low, microbial activity during composting will be slow. In contrast, if there is excess N, it is often lost from the natural system by denitrification to N<sub>2</sub> and N<sub>2</sub>O or by leaching of nitrate (Trenkel, 1997) or by deep drainage in winter, when plants growth and nitrogen uptake are slow due to low temperatures and low solar radiation (Turner *et al.*, 1989).

It is worth adding that there are a number of important factors which affect the growth and activity of microbes in composting; namely, C/N ratio, moisture content and pH value, which heavily affects the microorganism growth in compost. In general, the effective composting needs an optimal rate of moisture content between 50% and 70% (Liang, 2003), and pH value between 6 and 9 (Nakasakii, 1993). That is to say, the final product of compost needs to have a

suitable pH for crop production, be stable with respect to microbial activity, and be free of phytotoxic substances (Wu *et al.*, 2000).

The inoculation of composting with diazotrophs may increase the survival and provision of a more active population that possesses higher N<sub>2</sub>-fixing potential (Zlotnikov *et al.*, 1997). Keeling *et al.* (1995) have shown that certain waste-derived composts could provide a favorable environment for diazotrophs during the composting process. However, there is still little data on the survival of N<sub>2</sub>-fixing bacteria and PGPR in compost and their impact on total N and P availability.

Therefore, the aim of this composting trial was to evaluate the effect of inoculating grass cycling compost with N<sub>2</sub>-fixing bacteria on the numbers of N<sub>2</sub>-fixing bacteria, organic matter OM, total N and available P.

## Materials and methods

### Composting

The compost was prepared from grass-cycling by mixing green and brown materials (freshly cut grass was considered 'green'), balanced out by the addition of some brown material, like dry leaves, branches, twigs, its water content was increased to 60% by weight, and mixed at 3–5 days intervals for a period of 50 days.

The compost was kept in concrete cylinders with the tops open and the moisture content was maintained by adding water as needed. Then, the temperature in the compost was increased to approximately 60°C by 10 days but reduced to 35°C after 20 days. After composting to the early maturation phase (40 days), the compost was air-dried to a moisture content of 10%. At this time, the characteristics of the compost were 7.3 pH, 1.25 dS/m EC, 400 g/kg OM, 16.5 g/kg, total N, 13.0 g/kg, total P 235 mg/kg, available P, 4035 mg/kg, exchangeable K, 89.8 g/kg total Ca and 18.5 g/kg total Mg. Consequently, the compost was moistened with distilled water to a final moisture content of 60% and 3 kg was placed into 10 L plastic tanks (Meunchang, 2005).

### Bacterial Isolation

N<sub>2</sub>-fixing bacteria from three genera were used :*Azotobacter*, *Burkholderia* and *Azospirillum* sp, which were isolated from Wadi Elrabie (East of Tripoli, Libya) soil. In addition, cultural media were used according to the methods of (Döbereiner, 1980, Ben Mahmud, 2016).More specifically, *Azotobacter* was cultured in LG medium with the pH adjusted to 6.8,*Burkholderia* was grown in Burk's medium and adjusted pH 7.5 and *Azospirillum* sp was grown in a NFb medium and with the pH adjusted to 6.8.

### **Bacterial Culturing and Inoculum preparation**

To specify the acetylene reduction activity on pure cultures and for the inoculation of the compost, the bacteria were cultured in 2000 mL flasks containing 1000 mL of liquid medium specific for each culture. Then, the liquid was inoculated to achieve a starter density of approximately  $1 \times 10^8$  cells/ min of each genus. After that, the cultures were incubated to the late log phase, which was 2, 3 and 8 days for *Azospirillum*, *Azotobacter* and *Burkholderia*, respectively. The bacterial cells were then centrifuged and the inoculum was adjusted to a concentration of approximately  $1 \times 10^8$  cell/ mL by using the nitrogen free biotin (NFb) mineral solution medium without P (Meunchang, 2005).

### **Determining Acetylene Reduction Activity (ARA)**

Acetylene reduction activity of *Burkholderia* and *Azotobacter* cultures were determined by inoculating 100  $\mu$ L of the cell suspensions of each isolate into 20 mL of their respective N-free medium in 125 mL test tubes. The cultures were shaken at 120 rpm at 30°C for 15 days for *Burkholderia* and 7 days for *Azotobacter*, respectively due to their different growth rates. For *Azospirillum*, 50  $\mu$ L of a cell suspension was used as a starter to inoculate into 5 mL of NFb semi-solid medium in 20 mL test tubes, and then the cultures of *Azospirillum* were incubated at 30°C for 2 days (Myoungsuet *al.*, 2005 and Ben Mahmoud, 2008).

The cultural tubes of each were injected with 10% (V/V) of acetylene (C<sub>2</sub>H<sub>2</sub> 99.99%, Linde Specialty Gas) and were incubated at 30°C on a shaker for 1 h, after which the cap on the test tubes were closed with a serum stopper cap and acetylene reduction activity was measured with a Varian Aerograph (series 1400) gas chromatograph prepared with a hydrogen flame ionization detector (FID) and a 1m stainless steel column packed with Poropak N. Then, the results were expressed as  $1 \mu$ mol of C<sub>2</sub>H<sub>4</sub> produced per  $1 \times 10^8$  cells per hour (Ben Mahmoud, 2008).

## Compost Inoculation

The inoculation of compost was achieved by adding a mixture of 1 mL of diastrophic liquid inoculum and 5 mL of 0.85% NaCl to 3 Kg of compost and the inoculated compost was mixed thoroughly during and after inoculation. However, the uninoculated compost was mixed by only 6 mL of 0.85% NaCl (Meunchang, 2005).

Five treatments were used with three replications:

T<sub>1</sub>: Uninoculated compost. T<sub>2</sub>: *Azotobacter*. T<sub>3</sub>: *Burkholderia*. T<sub>4</sub>: *Azospirillum* sp. T<sub>5</sub>: Mixed inoculation (1 mL of each culture).

At 0, 10, 20, 30, 40, and 50 days, the composts were thoroughly mixed and approximately 250 g of compost was collected from each replication after mixing for the following analyses.

## Measuring Bacterial Growth in Compost

To determine the population of the bacteria in compost, 10 g of compost from each replication were diluted with 90 mL of sterile nitrogen free NFB mineral solution medium without KOH; *Azotobacter* and *Burkholderia* were measured by making serial dilutions and spread plating on N-free LG and N-free NB media (Döbereiner, 1980), respectively. *Azospirillum* was measured by most probable number (MPN) techniques according to (Zuberer, 1994) in a selective nitrogen free NFB semi-solid medium (Döbereiner, 1980). At 0, 10, 20, 30, 40, and 50 d, the Acetylene Reduction Assay (ARA) was used to measure N<sub>2</sub> fixing potential of bacteria in compost (Weaver *et al.*, 1994). Compost having a moist weight of 15 g was placed into a 250 mL flask and incubated with 10% C<sub>2</sub>H<sub>2</sub> for 1 h. Ethylene production was measured with a gas chromatograph equipped with a hydrogen FID, and a 1 m stainless steel column packed with Poropak N. After analysis, the results were expressed on a dry weight basis of compost. At 0, 10, 20, 30, 40 and 50 days, the pH was determined on a water extract from compost using a compost to water ratio of 1:5 by weight, organic carbon and nitrogen content, were analyzed on samples dried at 65°C for 72 h, ground and sieved to 0.2 m.

## Measuring Organic Matter, N and P

The organic matter (OM) content of the compost was determined by weight loss on ignition at 430°C for 24 h and the total organic carbon was calculated from OM content (Navarro *et al.*, 1993). The available P was determined colorimetrically by the method of Iatrouet *et al.*, (2014). The loss of OM, total of N loss (NT) and the available (P) were calculated from the ash content

at the beginning ( $X_1$ ) and the ash content at any particular time during composting ( $X_2$ ) and the initial of nitrogen or phosphor concentrations ( $N_1$  or  $P_1$ ) and nitrogen or phosphor concentrations at any particular time during composting ( $N_2$  or  $P_2$ ) according to the following equations (Paredes *et al.*, 1996).

- OM loss (%) =  $100 - 100(X_1(100 - X_2)) / (X_2(100 - X_1))$
- NT loss (%) =  $100 (X_1N_2) / (X_2N_1) - 100$
- Available P (%) =  $100 (X_1P_2) / (X_2P_1) - 100$

## Results and discussion

### Measuring Bacterial Growth in Compost

The number of  $N_2$ -fixing bacteria in inoculated compost declined during incubation (Fig 1), however, in uninoculated compost, there were no numbers of  $N_2$ -fixing bacteria detected at any sampling time. The survival of *Azospirillum* was also more significant than the other bacteria when inoculated alone or with *Burkholderia* and *Azotobacter* (Fig 1). In addition, it has been shown that there is no influence of the mixed or single inoculation on the survival of any genera. Further, the numbers of *Azospirillum* and *Burkholderia* strongly decreased by 10 days but the numbers of *Azotobacter* continued to decrease for 40 days and the numbers of *Azospirillum* and *Burkholderia* surviving at 50 days were approximately  $9 \times 10^3$  and  $3.5 \times 10^4$  cells/ g respectively and were in the range of the number in soil. What's more, the number of  $N_2$ -fixing bacteria showed for soil ranges from  $10^2$  to  $10^6$  cells/g dry weight and the number for plant roots ranges from  $10^2$  to  $10^6$  cells/ g fresh weight of root (Barraquio *et al.*, 1997, Kirchhof *et al.*, 1997, Weber *et al.*, 1999). It was also clear that the effect of the addition of compost to soil with respect to increasing populations of  $N_2$ -fixing bacteria would depend on how much compost was added, the population already in the soil and how well the introduced bacteria survived or proliferated. Clearly, the numbers in the compost would not directly increase soil populations if they were already relatively high in the soil.

### Determining Acetylene Reduction Activity

Acetylene reduction activity of the pure cultures were  $0.13 \pm 0.03$ ,  $7.3 \pm 0.15$ , and  $0.35 \pm 0.09$  Imole per billion cells/ h for *Azospirillum*, *Burkholderia*, and *Azotobacter* respectively. Even though *Burkholderia* had much higher acetylene reduction activity in pure culture than *Azospirillum*, compost *Azospirillum* was significantly higher than *Burkholderia* at 50

days(Fig2).More clearly, the higher activity may have been ,at least, partially due to its better survival characteristic in compost (Fig 1);acetylene reduction activity in compost at day 10 also increased due to all inoculation treatments (Fig 2);acetylene reduction activity remained high for compost inoculated with *Azospirillum* and *Burkholderia* alone and the mixed inoculation treatment but declined with time for *Azotobacter* inoculation alone. This decline matched with the poor survival of *Azotobacter* (Fig1). Other researchers have also demonstrated that *Azospirillum* inoculated into decomposing plant residues increased acetylene reduction activity. The level of acetylene reduction activity in our compost was comparable to that in *Azospirillum* inoculated wheat straw and rice hull residues (Dorothy, 1993).

Acetylene reduction of compost from a mixture of leaves from several tree species, measured at week 6 of composting was 26 nmole/ g.h but at week 10 it had declined 1.75 nmole/ g.h (Schwintzer *et al.*, 2002). Acetylene reduction activity, of olive mill wastewater sludge cocomposted with maize straw was highest at week 7 of composting and was extrapolated to be 2.42 nmole N fixed/ g.h (Paredes *et al.*, 2002). The acetylene reduction activity taking place in our grasscycling compost indicates that it is as well suited for supporting N<sub>2</sub>-fixation as compost made from other plant materials.

### Measuring Organic Matter, N and P in compost

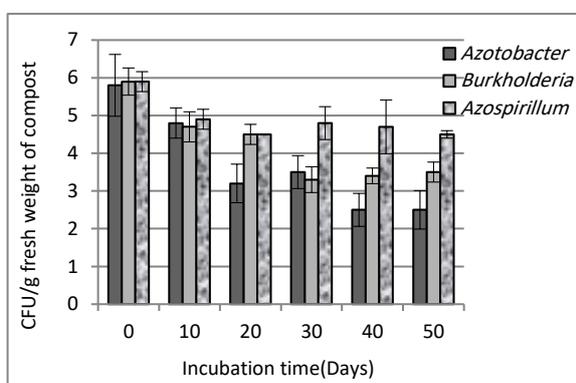
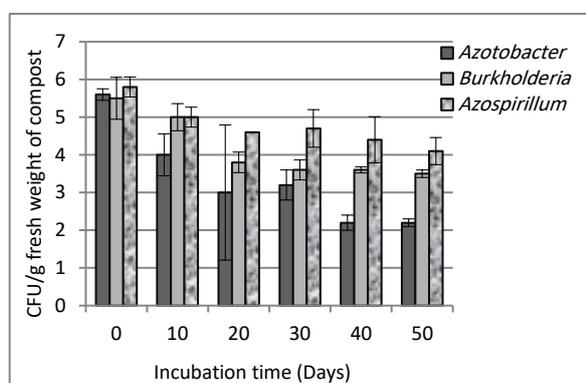
Besides the acetylene reduction activity, the other evidence for N<sub>2</sub>-fixation was the total N that occurred during incubation (Fig 3a). Specifically speaking,the inoculation of compost with *Azospirillum* alone and mixed with *Azotobacter* and *Burkholderia* resulted in the most N accumulation. At 50 days the total increase of N was modest and amounted to approximately 7% depending on the treatment. For some treatments, there was a decrease in N content beginning at 10 days(Fig 3a). That decrease was not probably due to leaching since the containers were closed at the bottom and water was not added in excess of that needed to restore the water content of the compost. In another study, using the different composting materials, much of the N was present as NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> at this time of incubation (Meunchanget *et al.*, 2004). Therefore, some N could be lost by denitrification in anaerobic microsites and by NH<sub>3</sub> volatilization. The latter does not seem too likely since the pH of most treatments was near neutrality (Table 1). The N concentration of the compost increased for the first 10 days of the experiment (Fig 3b).

The contribution of N<sub>2</sub>-fixation in adding N to the systemmuch of the increase in N concentration in our compost was due to the decomposition of the OM (Fig 4) as indicated by

weight loss. The weight loss of dry matter ranged between approximately 5 and 15% depending on the treatment. *Burkholderia* and *Azospirillum* alone and in mixed inoculation resulted in more available P by 20 to 30% by 50 d (Fig 5). The mechanism may have been from reduced pH (Table 1). It may also have been due to an increase in phosphates from N<sub>2</sub>-fixing bacteria that mineralizes organic P (Dobbelaere *et al.*, 2003).

### Statistical Analysis

Data from this experiment were statistically analysed using Minitab14 software (Version 13.20, Minitab 2004). After checking data for normality, three-way analysis of variance was performed and the significance of differences between the means was determined using Tukey's family error test (standard deviation) at a probability of P = 0.05.



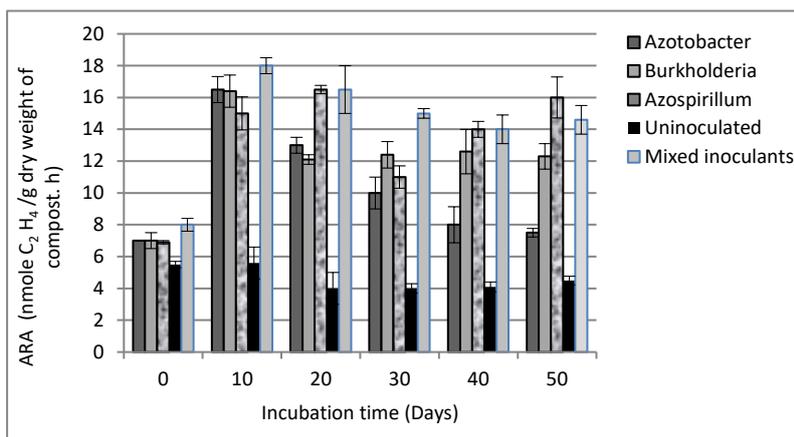
Error bars represent the standard deviation

Figure (1a) single inoculations

Figure (1b) Mixed inoculations

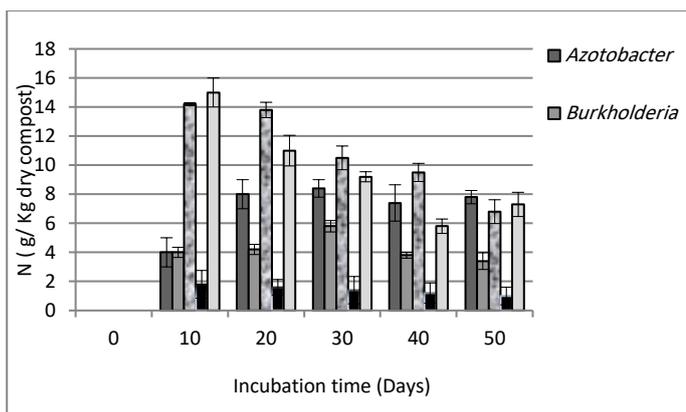
Figure 1. Numbers of N<sub>2</sub>-fixing bacteria(CFU/g fresh weight) following inoculation of compost.

(a) single inoculations (b)Mixed inoculations



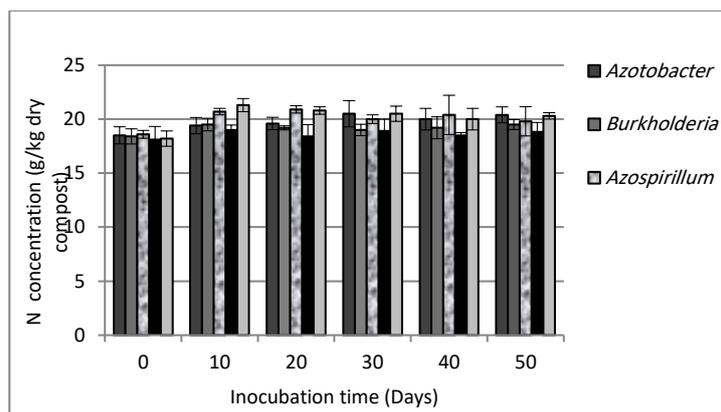
Error bars represent the standard deviation

**Figure 2. Acetylene reduction activity(ARA) of compost following inoculation with N<sub>2</sub>-fixing bacteria singly and mixed inoculants (nmole C<sub>2</sub> H<sub>4</sub> /g dry weight of compost. h)**



Error bars represent the standard deviation

**Figure (3a) Gain in N content of compost**



Error bars represent the standard deviation

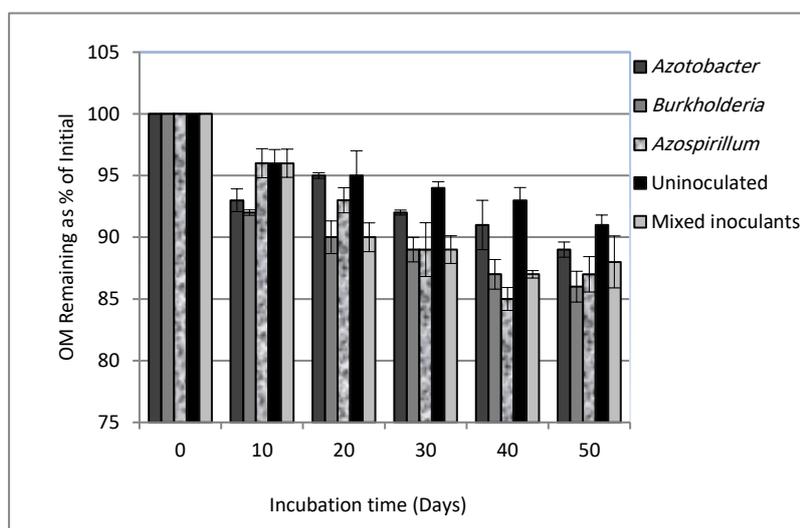
**Figure (3b) N concentration of compost**

**Figure 3. Overall gain in N content of compost from N<sub>2</sub> fixation (a) and N concentration of compost (b) with time following inoculation of 40 days old compost with N<sub>2</sub>-fixing bacteria singly and together.**

**Table 1. pH of compost following inoculation with N<sub>2</sub>-fixing bacteria single and mixed**

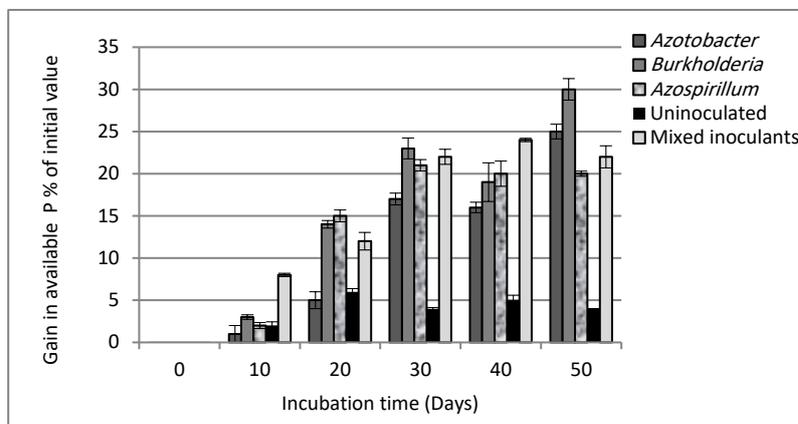
Incubation time (days)	<i>Azotobacter</i>	<i>Burkholderia</i>	<i>Azospirillum</i>	Uninoculated	Mixed inoculants
0	7.3±0.1	7.15±0.1	7.3±0.2	7.3±0.04	7.3±0.0
10	7.6±0.1	7.48±0.04	7.5±0.1	7.4±0.1	7.5±0.7
20	7.2±0.03	7.18±0.03	7.15±0.3	7.5±0.1	7.5±0.3
30	7.3±0.1	7.5±0.1	7.5±0.2	7.6±0.2	7.6±0.1
40	7.2±0.2	7.3±0.2	7.3±0.1	7.58±0.2	7.58±0.1
50	7.5±0.1	7.25±0.1	7.26±0.1	7.9±0.3	7.2±0.2

Numbers with ± represent the standard deviation



Error bars represent the standard deviation

**Figure 4. Organic matter (OM) loss following inoculation of 40 days old compost with N<sub>2</sub>-fixing bacteria single and mixed inoculants d-old compost with N<sub>2</sub>-fixing bacteria singly and mixed inoculants**



Error bars represent the standard deviation

**Figure 5. P% content of compost with time following inoculation of 40 days old compost with N<sub>2</sub>-fixing bacteria singly and mixed inoculants**

## Conclusion

In conclusion, this study has shown that the inoculation of compost with *Azotobacter*, *Burkholderia*, *Azospirillum* sp singly and mixed enhanced N accumulation by 5–15% and the availability of P by 20–30%. *Azospirillum* or *Burkholderia* sp may be the greatest choices as compost inoculants because they had excellent survival along with increasing total N and making P more available.

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## تلقیح مخلفات نباتية متحللة (Grass-cycling compost) ببكتيريا المثبتة للنيتروجين الجوي

ميرفت الطاهر رمضان بن محمود<sup>1</sup>، إيمان علي محمد الفرجاني<sup>2</sup>، مروة فكري احمد العالم<sup>3</sup>

<sup>1,2,3</sup> قسم الأحياء الدقيقة التربة، كلية الزراعة، جامعة طرابلس-ليبيا

<sup>1</sup> dr.mbenmahmoud@yahoo.com, <sup>2</sup> emanferjani@yahoo.com

### الملخص

تم دراسة تأثير تلقیح مخلفات نباتية متحللة بالبكتيريا المثبتة للنيتروجين الجوي، وقد تبين من النتائج أن لها دور إيجابياً في تحسين نوعية السماد، عن طريق زيادة نسبة النيتروجين الكلي "N" والفسفور المتيسر "P" في السماد. وتم تلقیح المخلفات النباتية ببكتيريا *Azotobacter*، *Burkholderia* و *Azospirillum* وكل بكتيريا منفصلة عن الأخرى والثلاثة معاً، وأظهرت النتائج انخفاض أعداد البكتيريا المثبتة "N<sub>2</sub>" في السماد خلال فترة الحضانة عن أعدادها في الأيام الأولى من التجربة بمقدار (5×10<sup>5</sup> خلية/جم)، حيث انخفض أعداد بكتيريا *Azotobacter* إلى حوالي (2×10<sup>2</sup> خلية/جم) والبكتيريا *Burkholderia* و *Azospirillum* إلى ما يقرب من (9×10<sup>3</sup> و 4×10<sup>4</sup> خلية / جم) على التوالي خلال 40 يوماً من الحضانة. وكذلك تبين نتيجة التلقیح بالبكتيريا المثبتة للنيتروجين الجوي ارتفاع كل من "N" الكلي بنسبة 5-15% و "P" المتيسر بنسبة 20-30% بالمقارنة مع معاملة الشاهد *uninoculated*. ارتفاع نسبة كل من "N" و "P" في السماد يرفع من قيمته السمادية، ويزيد من أهمية هذه البكتيريا زارعيًا.

**الكلمات الدالة:** التلقیح، بكتيريا المثبتة للنيتروجين، مخلفات نباتية متحللة.