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Inoculation of sugar mill by-products compost with N_2-fixing bacteria

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Abstract

Inoculation of sugar mill by-products compost with N_2-fixing bacteria may improve its quality by increasing total N and available P. Compost was inoculated with *Azotobacter vinelandii* (ATCC 478), *Beijerinckia derxii* (ATCC 49361), and *Azospirillum* sp. TS8, each alone and all three together. Numbers of all N_2-fixing bacteria in compost declined from an initial population of 5×10^5 cells g^{-1} during incubation. The population of *Azotobacter* declined to approximately 2×10^2 cells g^{-1} and the population of *Beijerinckia* and *Azospirillum* declined to approximately 9×10^3 and 3.5×10^4 cells g^{-1} respectively, at day 50. Inoculation with N_2-fixing bacteria increased acetylene reduction, total N by 6–16% and available P by 25–30% in comparison to the uninoculated control. Increasing the N content and P availability of compost increases its value and there may be additional benefit from providing N_2 fixing bacteria.

Introduction

Fertilization of crops using chemical fertilizers has become a common practice for efficient crop production. But the low purchasing power of small and marginal farmers in developing countries greatly limits availability of fertilizers. Farmers need alternative technologies to supply nutrients to crops. Using more local organic materials from agro-industrial by-products as nutrient sources may help. Composting is considered to be one of the most suitable ways of converting organic wastes into products that are beneficial for plant growth (Stantiford, 1987). Compost inoculated with N_2-fixing bacteria, which can fix atmospheric nitrogen, solubilize phosphorous and stimulate plant growth by biosynthesis of plant growth promoting substances (plant growth promoting rhizobacteria, PGPR) may be particularly useful (Dobbelaere et al., 2003).

One under utilized source of organic material is the sugar cane industry. Global sugar production from sugar cane releases large amounts of sugar mill by-products as filter cake and bagasse. A quality compost can be developed from co-composting filter cake and bagasse but an increase in N content and P availability would increase the value. The N content and P availability may be enhanced by addition of N_2-fixing bacteria as occurred for soil (Kumar and Narula, 1999; Kumar and Singh, 2001). Enhanced uptake of nutrient minerals by plants that had been grown in soil inoculated with *Azospirillum* spp. was the reason for increased plant growth (Barton et al., 1986; Bashan and Holguin, 1997; Bashan and Levanony, 1990; Lin et al., 1983). *Azospirillum* inoculation may lead to a reduction in nitrogen fertilizer applications. Thus, considering the possible large saving of fertilizer (up to 50%) (Okon and Labandera-Gonzalez, 1994) advances in knowledge of the bacterial inoculants ecology may promote sustainable agriculture, with low environmental impact.

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Although diazotrophs may be naturally present in compost the thermophilic stage of decomposition or ammonium concentrations occurring early during the composting process may greatly reduce their populations (De Bertoldi et al., 1983). Inoculation with diazotrophs after the thermophilic phase of composting may increase survival and provide a more active population that possesses higher N_2-fixing potential (Zlotnikov et al., 1997). Keeling et al. (1995) has shown that certain waste-derived composts could provide a favorable environment for diazotrophs during the composting process. However, there are little data on survival of N_2-fixing bacteria and PGPR in compost and their impact on total N and P availability.

The objective of our investigation was to determine the effect of inoculating sugar mill by-product compost with N_2-fixing bacteria on numbers of N_2-fixing bacteria, total N and available P.

Materials and methods

Compost was prepared from sugar mill by-products, filter cake and bagasse collected from Mitr Kasetr Industry Co., Ltd. Tamaka, Kanchanab uri province, Thailand. Both by-products were air-dried to the moisture content of approximately 10%. Compost was prepared by mixing filter cake and bagasse at 2:1 by weight, increasing the water content to 60% by weight, and mixing at 3–5 d intervals for a period of 40 d. The compost was contained in concrete cylinders with the tops open. Moisture content was maintained by adding water as needed. Temperature in the compost increased to approximately 60 °C by 10 d but reduced to 35 °C after 20 d. After composting to the early maturation phase (40 d), the compost was air-dried to a moisture content of 10%. Characteristics of the compost at this time were 7.30 pH, 1.23 dS m^{-1} EC, 390 g kg^{-1} OM, 18.5 g kg^{-1} total N, 12.0 g kg^{-1} total P, 235 mg kg^{-1} Bray II available P, 3835 mg kg^{-1} exchangeable K, 93.6 g kg^{-1} total Ca and 18.5 g kg^{-1} total Mg. The compost was moistened with distilled water to a final moisture content of 60% and 3 kg was placed into 10 L plastic tanks.

N_2-fixing bacteria from three genera were used, *Azotobacter vinelandii* (ATCC 478), *Beijerinckia derxii* (ATCC 49361), and *Azospirillum* sp. TS8 which was isolated from sugar cane in Thailand. Cultural media were used according to the methods of Döbereiner, (1980); *Azotobacter vinelandii* (ATCC 478) was cultured in LG medium composed of; glucose 10 g, K_2HPO_4 0.05 g, KH_2PO_4 0.15 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $CaCl_2$ 0.02 g, $FeCl_3$ 0.01 g, $Na_2MoO_4 \cdot 2H_2O$ 0.002 g, distilled water 1000 mL, and with the pH adjusted to 6.8 with 1 N NaOH.

Beijerinckia derxii (ATCC 49361) was grown in NB broth medium composed of; glucose 20 g, KH2PO4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeCl_3 \cdot 6H_2O$ 0.01 g, $Na_2MoO_4 \cdot 2H_2O$ 0.02 g, distilled water 1000 mL and adjusted pH 5 with 1 N NaOH.

Azospirillum sp. TS8 was grown in a NFb medium containing; malic acid 5 g, K_2HPO_4 0.1 g, KH_2PO_4 0.4 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 0.1 g, $CaCl_2 \cdot 2H_2O$ 0.02 g, $FeCl_3 \cdot 6H_2O$ 0.1 g, $NaMoO_4 \cdot 2H_2O$ 0.002 g, bromthymol blue (0.5% in ethanol) 2 mL, KOH 4 g, biotin 10 mg, 1000 mL of distilled water and with the pH adjusted to 6.8 with 1 N KOH.

For determination of acetylene reduction activity on pure cultures and for inoculation of compost, bacteria were cultured in 2000 mL flasks containing 1000 mL of liquid medium specific for each culture. The liquid was inoculated to achieve a starter density of approximately 1×10^8 cells - min^{-1} of each genus. The cultures were incubated to the late log phase, which was 2, 3 and 7 d for *Azospirillum*, *Azotobacter* and *Beijerinckia*, respectively. The bacterial cells were centrifuged and the inoculum adjusted to a concentration of approximately 1×10^9 cell mL^{-1} by using the nitrogen free NFb mineral solution medium without P.

Acetylene reduction activity of *Beijerinckia derxii* and *Azotobacter vinelandii* cultures were determined by inoculating 100 μL of the cell suspensions of each isolate into 20 mL of their respective N-free medium in 125 mL flasks. The cultures were shaken at 120 rpm at 30 °C for 15 d for *Beijerinckia* and 7 d for *Azotobacter*, respectively due to their different growth rates. The headspace volume in the cultural flasks was replaced with 10% (V/V) of C2H2 and incubation was at 30 °C on a shaker for 1 h. The C_2H_4 gas production was measured with a gas chromatograph equipped with a hydrogen flame ionization detector (FID) and a 1 m stainless steel column packed with Poropak N.

Results were expressed as μmoles of C_2H_4 produced per 1×10^9 cells per hour.

For *Azospirillum*, 50 μL of a cell suspension was used for a starter to inoculate into 5 mL of nitrogen free NFb semi-solid medium in 20 mL test tubes. The cultures were incubated at 30 °C for 2 d. After which the cap on the test tubes were closed with a serum stopper cap and acetylene reduction activity was measured. The headspace volume in the culture tube was adjusted to contain 10% (V/V) of C_2H_2 and incubation was at 30 °C for 1 h. The C_2H_4 gas production was measured with a gas chromatograph equipped with a hydrogen FID, and a 1 m stainless steel column packed with Poropak N. Results were expressed as μmoles of C_2H_4 produced per 1×10^9 cells per hour.

Inoculation of compost was achieved by mixing 1 mL of diazotrophic liquid inoculum with 5 mL of 0.85% NaCl and adding it to 3 kg of compost. Compost was mixed as thoroughly as possible during and after inoculation. The uninoculated control received 6 mL of 0.85% NaCl which was mixed into the compost as thoroughly as possible. Five treatments were used with three replications:

T1; Uninoculated compost.
T2; *A. vinelandii* ATCC 478.
T3; *B. derxii* ATCC 49361.
T4; *Azospirillum* sp.TS8.
T5; Mixed inoculation (1 mL of each culture).

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

To determine population sizes of 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 *Beijerinckia* 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_2 fixing potential of bacteria in compost (Weaver and Danso, 1994). Compost having a moist weight of 15 g was placed into a 250 mL Erlenmeyer flasks and incubated with 10% C_2H_2 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 (Hewlett Packard HP 5890 series II, CA, USA). After analysis the results were expressed on a dry weight basis of compost.

At 0, 10, 20, 30, 40 and 50 d organic carbon and nitrogen content, were analyzed on samples dried at 65 °C for 72 h, ground and sieved to 0.2 m. The organic matter (OM) content of the compost was determined by weight loss on ignition at 430 °C for 24 h. Total organic carbon was calculated from OM content (Navarro et al., 1993).

The N concentration was determined with a CN analyzer (NC-900; Sumitomo, Osaka, Japan) according to the manufacture's protocol. The pH was determined on a compost to water ratio of 1:5 by weight. Available P was determined colorimetrically by the method of John (1970).

Loss of OM, gain in total-N (N_T) and gain in available P (P) were calculated from the initial (X_1) and final (X_2) ash contents and the initial (N_1 or P_1) and final (N_2 or P_2) total-N and available P concentrations according to the following equations (Paredes et al., 1996).

$$\text{OM loss (\% of initial value)} = 100 - 100(X_1(100 - X_2))/(X_2(100 - X_1))$$

$$N_T \text{ gain (\% of initial value)} = 100(X_1N_2)/(X_2N_1) - 100$$

$$\text{Available P gain (\% of initial value)} = 100(X_1P_2)/(X_2P_1) - 100$$

Results and discussion

The number of N_2-fixing bacteria in inoculated compost declined during incubation (Figure 1). No N_2-fixing bacteria were detected in uninoculated compost at any sampling time. Survival of *Azospirillum* was better than for the other bacteria when inoculated alone or with *B. derxii* and *A. vinelandii* (Figure 1). There appeared to be no influence of mixed or single inoculation on survival of any genera. The numbers of *Azospirillum* and *Beijerinckia* strongly declined by 10 d but the numbers of *Azotobacter* continued to

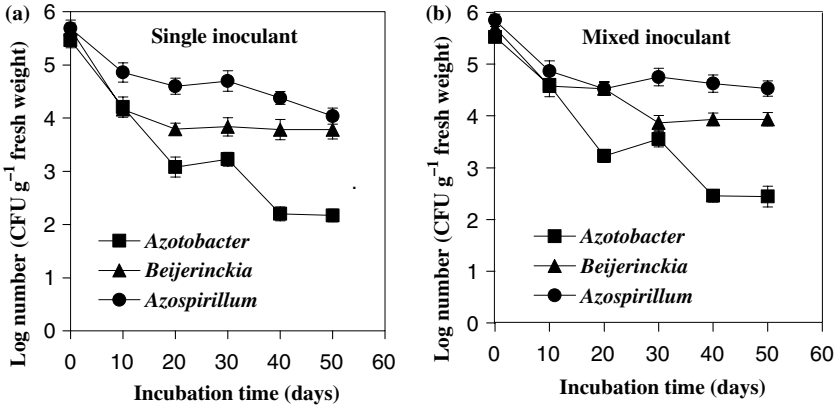

Figure 1. Numbers of N_2-fixing bacteria following inoculation of compost. (a); single inoculations, (b); mixed inoculation. Error bars represent the standard deviation.

decline for 40 d. The numbers of *Azospirillum* and *Beijerinckia* surviving at 50 d were approximately 9×10^3 and 3.5×10^4 cells g^{-1} respectively and were in the range of the number in soil or on plant roots. The number of N_2-fixing bacteria reported for soil ranges from 10^2 to -10^6 cells g^{-1} dry weight and the number for plant roots ranges from 10^2 to -10^6 cells g^{-1} fresh weight of root (Baldani et al., 1992; Barraquio et al., 1997; Kirchhof et al., 1997; Weber et al., 1999). The impact 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.

Acetylene reduction activity of the pure cultures were 0.12 ± 0.02, 6.3 ± 0.17, and 0.33 ± 0.07 μmole per billion cells h^{-1} for *Azospirillum, Beijerinckia*, and *Azotobacter*, respectively. Even though *Beijerinckia* had much higher acetylene reduction activity in pure culture than *Azospirillum*, in compost *Azospirillum* was significantly higher than *Beijerinckia* at 50 d (Figure 2). The higher activity may have been at least partially due to its better survival characteristic in compost (Figure 1). Acetylene reduction activity in compost at day 10 increased due to all inoculation treatments (Figure 2). Acetylene reduction activity remained high for compost inoculated with *Azospirillum* and *Beijerinckia* alone and the mixed inoculation treatment but declined with time for *Azotobacter* inoculation alone. The decline matched with the poor survival of *Azotobacter* (Figure 1). 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 and Gibson, 1989; Dorothy, 1993). Acetylene reduction of compost from a mixture of leaves from several tree species, measured

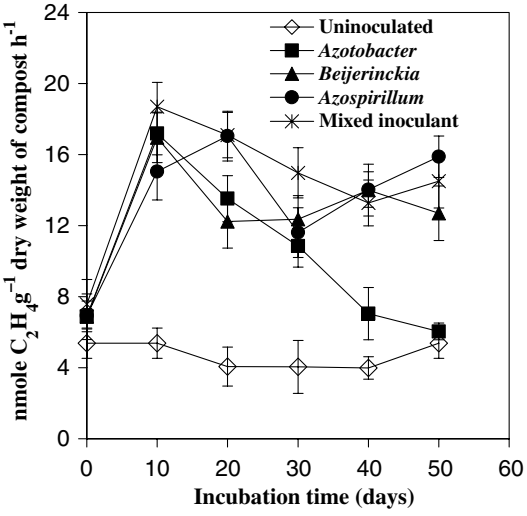

Figure 2. Acetylene reduction activity of compost following inoculation with N_2-fixing bacteria singly and together. Error bars represent the standard deviation.

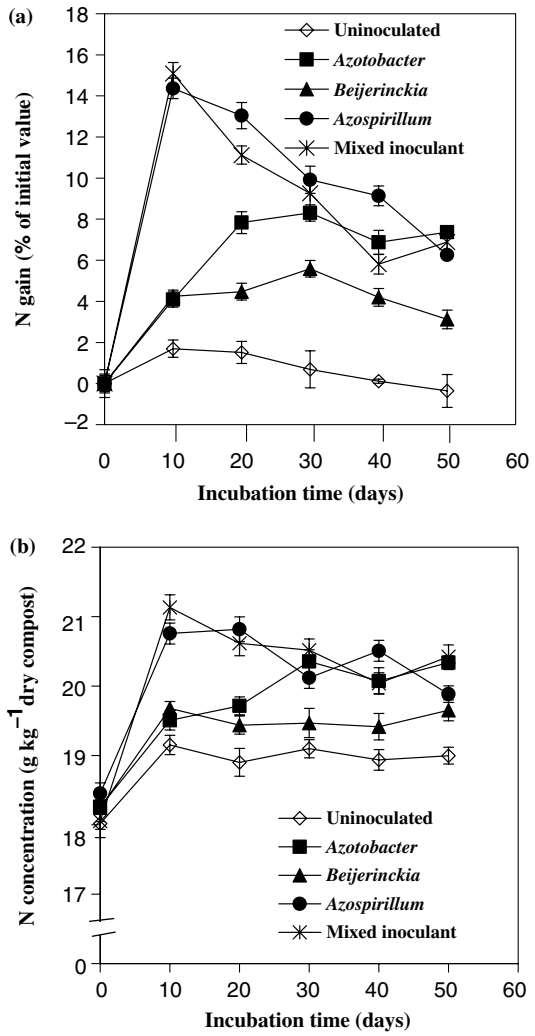

Figure 3. Overall gain in N content of compost from N_2 fixation (a) and N concentration of compost (b) with time following inoculation of 40 d-old compost with N_2-fixing bacteria singly and together. Error bars represent the standard deviation.

at week 6 of composting was 26 nmole g^{-1} h^{-1} but at week 10 it had declined 1.75 nmole g^{-1} h^{-1} (Schwintzer et al., 2002). Acetylene reduction activity, of olive mill wastewater sludge co-composted with maize straw was highest at week 7 of composting and was extrapolated to be 2.42 nmole N fixed g^{-1} h^{-1} (Paredes et al., 2002). The acetylene reduction activity taking place in our sugar mill-waste compost indicates that it is as well suited for supporting N_2-fixation as compost made from other plant materials.

Besides the acetylene reduction activity, other evidence for N_2-fixation was the gain in N that occurred during incubation (Figure 3a). Inoculation of compost with *Azospirillum* alone and together with *Azotobacter* and *Beijerinckia* resulted in the most N accumulation. At 50 d 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 d (Figure 3a). The decrease was not likely 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 same composting materials, much of the N was present as NH_4^+ and NO_3^- at this time of incubation (Meunchang et al., 2004). Therefore, some N could be lost by denitrification in anaerobic microsites and by NH_3 volatilization. The latter does not seem too likely since the pH of most treatments was near neutrality (Figure 4).

The N concentration of the compost increased for the first 10 d of the experiment (Figure 3b). Besides the contribution of N_2-fixation in adding N to the system much of the increase in N concentration in our compost was due to decomposition of the OM (Figure 5) as indicated by weight loss. The weight loss of dry matter ranged between approximately 6 and 14% depending on the treatment. *Beijerinckia* and *Azospirillum* alone and in mixed inoculation resulted in more

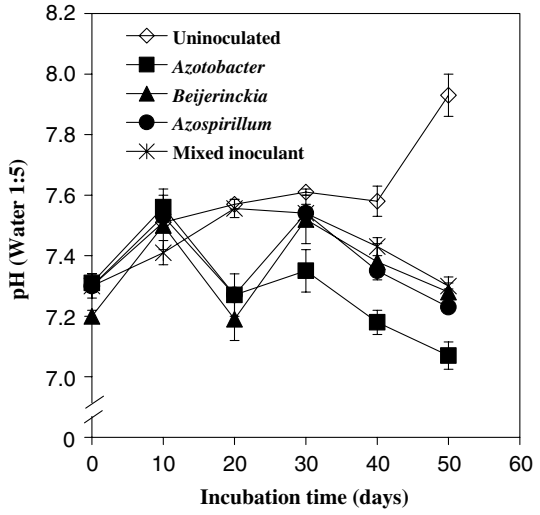

Figure 4. pH of compost following inoculation with N_2-fixing bacteria singly and together. Error bars represent the standard deviation.

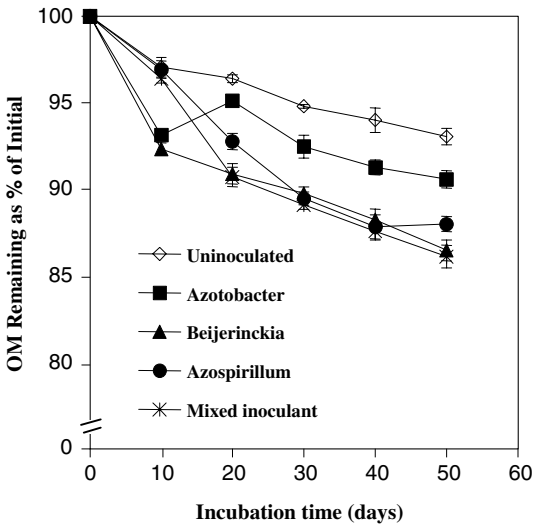

Figure 5. Organic matter (OM) loss following inoculation of 40 d-old compost with N_2-fixing bacteria singly and together. Error bars represent the standard deviation.

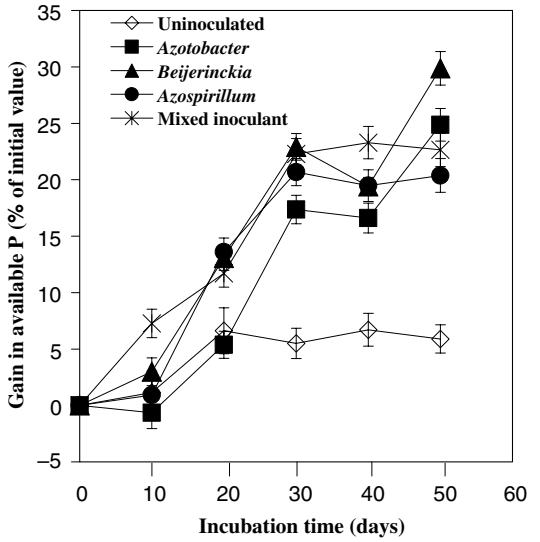

Figure 6. Overall gain in available P content of compost with time following inoculation of 40 d-old compost with N_2-fixing bacteria singly and together. Error bars represent the standard deviation.

OM loss than inoculation with *Azotobacter* (Figure 5).

Phosphorous availability in the compost was significantly increased as a result of inoculation with N_2-fixing bacteria (Figure 6). Single inoculations and mixed inoculations with *Azotobacter*, *Beijerinckia* and *Azospirillum* all increased available P by 25 to 30% by 50 d (Figure 6). The mechanism may have been from reduced pH (Figure 4). It may also have been due to an increase in phosphatase from N_2-fixing bacteria that mineralizes organic P (Dobbelaere et al., 2003).

Conclusion

Inoculation of compost with *A. vinelandii*, *B. derxii*, *Azospirillum* sp. singly and together enhanced N accumulation by 6–16% and availability P by 25–30%. *Azospirillum* sp. TS8 or *B. derxii* may be the best choices as a compost inoculant because they had excellent survival along with increasing total N and making P more available.

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References

Baldani V L D, Baldani J I, Oliveira F and Döbereiner J 1992 Identification and ecology of *Herbaspirillum seropedicae* and the closely related *Pseudomonas rubrisubalbicans*. Symbiosis 13, 65–73.

Barraquio W L, Revilla L and Ladha J K 1997 Isolation of endophytic diazotrophic bacteria from wetland rice. Plant Soil 194, 15–24.

Barton L L, Johnson G V and Orbock Miller S 1986 The effect of *Azospirillum brasilense* on iron adsorption and translocation by sorghum. J. Plant Nutr. 9, 556–565.

Bashan Y and Holguin G 1997 *Azospirillum*–plant relationships: environmental and physiological advances (1990–1996). Can. J. Microbiol. 43, 103–121.

Bashan Y and Levanomy H 1990 Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture. Can. J. Microbiol. 36, 591–607.

De Bertoldi M, Vallini G and Para A 1983 The biology of composting: a review. Waste Manage. Res. 1, 157–176.

Dobbelaere S, Vanderleyden J, and Okon Y 2003 Plant growth promoting effects of diazotrophs in the rhizosphere. Crit. Rev. Pl. Sci. 22(2), 107–149.

Döbereiner J 1980 Forages grasses and grain crops. *In* Methods for Evaluating Biological Nitrogen Fixation. Ed. F J Bergersen. pp. 535–555. John New York: Wiley & Sons Ltd.

Dorothy M H 1993 Inoculation of wheat straw to enhance lignocellulose breakdown and associated nitrogenase activity. Soil Biol. Biochem. 25(4), 419–429.

Dorothy M H and Gibson A H 1989 Nitrogenase activity by diazotrophs grown on a range of agricultural plant residues. Soil Biol. Biochem. 21(8), 1037–1043.

John M K 1970 Colorimetric determination of phosphorus in soil and plant materials with ascorbic acid. Soil Sci. 109, 214–220.

Keeling A A, Griffiths B S, Ritz K and Myers M 1995 Effects of compost stability on plant growth, soil microbiological parameters and nitrogen availability in media containing mixed garden waste compost. Bioresource Technol. 54, 279–284.

Kirchhof G, Reis V M, Baldani J I, Eckert B, Dobereiner J and Hartmann A 1997 Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. Plant Soil 194, 45–55.

Kumar V and Narula N 1999 Solubilization of inorganic phosphates and growth emergence of wheat as affected by *Azotobacter chroococcum*. Biol. Fert. Soils 28, 301–305.

Kumar V and Singh K P 2001 Enriching vermicompost by nitrogen fixing and phosphate solubilizing bacteria. Bioresource Technol. 76, 173–175.

Lin W, Okon Y and Hardy R W F 1983 Enhanced mineral uptake by *Zea mays* and *sorghum bicolor* roots inoculated with *A. brasilense*. Appl. Environ. Microbiol. 45, 1775–1779.

Navarro A F, Cegarra J, Roig A, and Garcia D 1993 Relationships between organic matter and carbon contents of organic waste. Bioresource Technol. 44, 203–207.

Okon Y and Labandera-Gonzalez C A 1994 Agronomic application of *Azospirillum*: an evaluation of 20 years worldwide inoculation. Soil Biol. Biochem. 26, 1591–1601.

Paredes C, Bernal M P, Cegarra J, Roig A and Novarro A F 1996 Nitrogen transformations during the composting of different organic wastes. *In* Progress in Nitrogen Cycling Studies. Eds. Van Cleemput, O Hofman, and G Vermoesen. pp. 121–125. Dordrecht: Kluwer Academic Publishers.

Paredes C, Bernal M P, Cegarra J and Roig A 2002 Bio-degradation of olive mill wastewater sludge by its co-composting with agricultural waste. Bioresource Technol. 85, 1–8.

Schwintzer C R, Tjepkema J D and Seekins B 2002 Nitrogenase activity in composting horse bedding and leaves. Plant Soil 242, 277–282.

Meunchang S, Panichsakpatana S and Weaver R W 2004 Co-composting of filter cake and bagasse; by-products from a sugar mill. Bioresource Tech. (In press).

Stantiford E I 1987 Resent developments in composting. *In* Compost, Production, Quality and Use. Eds. M Debertoldi, M L Ferranti, P Hermite and F Zucconi, pp. 52–62. London: Elsevier.

Weaver RW and Danso KA 1994 Dinitrogen fixation. *In* Methods of Soil Analysis, part 2 Microbial and Biochemical Properties. Eds. R W Weaver, J S Angle and P S Bottomley. pp. 1019–1043. Madison, WI. USA: Soil Sci. Soc. Am., Inc.

Weber O B, Baldani V L D, Teixeira K R S, Kirchhof G, Baldani J I and Dobereiner J 1999 Isolation and characterization of diazotrophic bacteria from banana and pineapple plants. Plant Soil 210, 103–113.

Zlotnikov A K, Glagoleva O B and Umarov M M 1997 Relationship between nitrogenase activity, stability and the ratio of components in mixed cultures of diazotrophic bacteria. Microbiology 66, 676–680.

Zuberer D 1994 Recovery and enumeration of viable bacteria. *In* Methods of Soil Analysis, part 2 Microbial and Biochemical Properties. Eds. R W Weaver, J S Angle and P S Bottomley, pp. 118–144. Madison, WI. USA: Soil Sci. Soc. Am., Inc.

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