INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18F–130/2019/21–1–77–84
DOI: 10.17957/IJAB/15.0865
http://www.fspublishers.org

Full Length Article

Isolation of Plant Growth Promoting Rhizobacteria and Selection of Microbial Organic Fertilizer Carriers

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Abstract

Plant growth promoting rhizobacteria in microbial fertilizer can not only produce plant hormones to improve the growth of roots and shoots, but also accelerate the release of nutrients in traditional organic fertilizer. Using mixture of plant growth promoting rhizobacteria and traditional organic fertilizer is considered an economical and environment-friendly approach. In this research, a multifunctional composite microbial organic fertilizer was found and tried to solve the problems of soil caused by long-term use of chemical fertilizer and change the irregular release of nutrients in traditional organic fertilizer. Using active microbial concentration and the release of nutrients as indicators to study the effect of the microbial organic fertilizer application on soil fertility, this study indicated that the quantity of active bacteria increased more greatly by turf application than other traditional organic fertilizer carriers. However, using mixed chicken manure (CH) and corn straw (CO) as carriers had same active microbial concentration compared with turf. It indicated that this carrier can be used as an alternative carrier to turf as non-renewable resources. The present findings showed that the CH + CO carrier had the optimal conditions with 30% water content, 30°C temperature, and 10^8 CFU mL^{-1} (CFU = Colony-Forming Units) initial cell concentration. The application of the microbial fertilizer was conducive to improving the rhizospheric microecological environment, improving crop yield and quality and alleviating the problems associated with long-term use of chemical fertilizer. © 2019 Friends Science Publishers

Keywords: Microbial fertilizer; Carrier; Inoculation; Optimization

Introduction

In modern agricultural production, long-term and large-scale application of fertilizers causes deterioration of the physical and chemical properties of soil (Wang and Xing, 1998), decrease of organic matter content (Chen, 1984), loss of nutrients, decline of agricultural crop quality, and other issues (Lichtenberg and Shapiro, 1997). Application of organic fertilizer can significantly decrease soil bulk density and increase its porous structure, soil texture structure and soil fertility (Xu, 2004; Zhang *et al.*, 2009). However, the content of the quick-acting nutrients in organic fertilizer is low, and a variety of environmental factors restrict the decomposition and release of slow-acting nutrients. Moreover, the fertilizer efficiency is difficult to predict and control (Shen *et al.*, 2007). For example, after applying organic fertilizer to tobacco, the nitrogen release period of inorganic nitrogen in soil is not consistent with the nitrogen requirement of flue-cured tobacco. When the amount of organic fertilizer surpasses the requirement, tobacco plants show a slow growth in the resettling stage as well as a delay in long-term and mature stages, which seriously affects the yield and quality of flue-cured tobacco (Cao, 1991). The activities of soil microorganisms play a decisive role in the conversion, supply, and bioavailability of soil nitrogen (Wang *et al.*, 2002). If sufficient organic nitrogen decomposition bacteria are present in the soil with organic fertilizer, nutrients manure can be released at the right time. In addition, microorganisms cannot guarantee survival and have no impact on the environment when there are microorganisms alone. Several functional microorganisms can be prepared into microbial fertilizer, which release nutrients in organic fertilizer and assure the survival functional bacteria (Wu *et al.*, 2009; Li *et al.*, 2010; Cao *et al.*, 2011; Ding *et al.*, 2012).

Microbial fertilizer can not only increase yield, but also improve the utilization rate of fertilizers, and improve the quality of agricultural products. This has played an

To cite this paper: Ding, M., N. Shang, Y. Huang, L. Liu, W. Fan, L. Peng, Y. Zhang, J. Zhou and Z. Zhou, 2019. Isolation of plant growth promoting rhizobacteria and selection of microbial organic fertilizer carriers. *Intl. J. Agric. Biol*., 21: 77–84

active role in the development of green agriculture (Cheng, 2000; Shen *et al.*, 2007; Saleem *et al.*, 2015; Ziane *et al.*, 2017). Plant growth promoting rhizobacteria (PGPR) inhabiting the rhizosphere capable of producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase can promote the growth of plant and the development in the early stages (Saleem *et al*., 2015). Auxins are a group of compounds having an indole ring, which have a positive effect on plants by stimulating seed germination, root initiation, cell elongation, and seedling growth (El-Tarabily, 2008). Direct promotion induced by plant growth promoting rhizobacteria (PGPR) inhabiting the rhizosphere is capable of producing 1- aminocyclopropane-1-carboxylate (ACC) deaminase, indol-3-acetic acid (IAA), gibberellic acid (GA3), cytokinin, Iron carrier (Glick *et al*., 1999), abscisic acid (Cohen *et al*., 2008) and soluble phosphate (De-Bashan and Bashan, 2004). ACC deaminase, gibberellic acid (GA3), cytokinin, and Iron carrier (Glick *et al.,* 1999; Saleem *et al*., 2015), can promote the growth of plant and the development in the early stages. Plants receive phosphorus in the form of phosphate, which is sometimes present in nature in an insoluble form that is unusable by plants. Microorganisms that possess phosphate solubilising capabilities provide phosphorus in a soluble form, as required by plants (Nutaratat *et al*., 2014). The quality control of microbial fertilizer has always been the most difficult problem, with the active microorganism and carrier representing the two key factors. Carrier materials must provide a suitable environment for the survival and release of microorganisms (Li *et al.*, 1999).

The carriers of microbial fertilizer in the market mainly include turf, diatomaceous earth, vermiculite, glauconite, fly ash, and mushroom bran. Among these, turf is the most common (Georgakopoulos *et al.*, 2002). However, extraction of turf, as mineral resources, can cause great environmental damage (Zhang *et al.*, 2016). Ideal carrier should have orderly arrangement of pores, large specific surface area, and strong adsorption characteristics. At the same time, a carrier must be conducive to the survival and function of functional bacteria, should be easy to produce, and harmless to crops and soil (Zhang, 2006). Livestock and poultry droppings as a carrier have a wide range of sources, low cost, high fineness and good adsorption performance, and are rich in nitrogen, phosphorus, potassium and organic matter (Li *et al.*, 2012). However, livestock and poultry excrement C/N ratio is generally low. Studies have shown that the optimal C/N ratio is 25:1 (corn straw: cattle manure 6:4), allowing bacterial and fungal growth and reproduction in the fermentation process (Geng, 2014).

Therefore, in the present study we used the mixture of isolated plant growth promoting rhizobacteria with different carriers to identify the feasibility and optimal conditions of composting for functional microbial carriers. Moreover, we also optimized key factors such as water content, temperature, and initial cell concentration.

Materials and Methods

Microbes and Experimental Materials

This experiment was conducted in the laboratory of Xikang River in Jietou Town, Yunnan Province, in 2017. *Naxibacter* sp. A-6 (GeneBank Accession No. MH718835), *Stenotrophomonas* sp. A-8 (GeneBank Accession No. MH718840), *Bacillus pumilus* A-9 (GeneBank Accession No. MH717445), *Geobacillus stearothermophilus* A-11 (GeneBank Accession No. MH717377), *B. stratosphericus* A-12 (GeneBank Accession No. MH718808), *Cellulosimicrobium cellulans* A-17 (GeneBank Accession No. MH718838), *B. altitudinis* A-19 (GeneBank Accession No. MH718834) and *B. megaterium* A-36 (GeneBank Accession No. MH720217) were isolated from soil and identified as high-efficiency strains of microbial agents for the decomposition of soil organic nitrogen (Ding *et al*., 2017). Nutrient broth medium (pH 7.0-7.2) was composed of 3.0 g/L beef extract, 5.0 g/L peptone and 15.0 g/L NaCl, 1000 mL water. Stephenson medium was composed of 2.0 g/L $(NH_4)_2SO_4$, 0.75 g/L K_2HPO_4, 0.03 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $MnSO_4{\cdot}4H_2O$, 5.0 g/L $CaCO_3$, 0.25 g/L NaH_2PO_4, 1000 mL water. Cattle manure (CA), chicken manure (CH), pig manure (PI), corn straw (CO) and turf as carries were grinded in 0.5 mm size and sterilizated twice (Table 1).

16S rRNA Gene Sequencing and Analysis

To characterize PGPR, the 16S rRNA genes were amplified using primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (AGAGTTTGATCCTGGCTCAG) and were sequenced by BioSune Biotechnology (China). For the phylogenetic analysis, the 16S rRNA sequences of the related strains were retrieved from the GenBank database, followed by the alignment by CLUSTAL_X 2.0 program (Larkin *et al*., 2007). The neighbour-joining method was used to construct the phylogenetic tree by using the MEGA 6 software (Tamura *et al*., 2011).

Growth Promoting Experiment

Naxibacter sp. A-6, *Stenotrophomonas* spp. A-8, *Cellulosimicrobium cellulans* A-17, were grown in Stephenson medium at 30°C for 7 d and in a shaker with a speed of 150 rpm. *B. pumilus* A-9, *G. stearothermophilus* A-11, *B. stratosphericus* A-12, *B. altitudinis* A-19 and *B. megaterium* A-36 were grown in nutrient broth medium at 30°C for 48 h and in a shaker with a speed of 180 rpm. After fermentation, the two fermentation fluids were mixed into a 1:1 ratio to form a fertilizer–effective bacterial agent. The number of bacteria in the mixture was about 10^9 CFU mL^{-1} by flat colony counting method.

The ACC deaminase activity was determined by estimating a-ketobutyrate production using the method of Saleh and Glick (2001). One unit of enzyme activity was defined as producing a mole of alpha-f-ketobutyrat per

minute. Phosphate solubilization was measured by the methods of Vyas *et al.* (2007). The phosphate solubilising ability of bacterium agent was tested using insoluble tricalcium phosphate [$Ca_3(PO_4)_2$] as sole P source in Pikovskaya's medium. The intensity of yellow color was read on spectrophotometer at 430 nm and the amount of P-solubilized was extrapolated from the standard curve. Iron carrier content was determined by the method of Schwyn and Neilands (1987). An aliquot of solution of bacterium agent was mixed with CAS assay solution. After reaching equilibrium, the absorbance was measured at 630 nm to determine the content of iron carrier. The content of indoleacetic acid (IAA), gibberellin (GA_3) and abscisic acid (ABA) were determined by HPLC system (Agilent 1200 Series, equipped with a 150 mm length reversed phase C-18 analytical column, ZORBAX SB-C18, USA) (Sgroy *et al*., 2009). Bacterium agent was centrifuged at 8,000 rpm for 20 min at 4°C, and supernatants were acidified at pH 2.5 with acetic acid solution (1% v/v). Each sample was partitioned four times with the same volume of acetic acid-saturated ethyl acetate (1%, v/v). Acidic ethyl acetate was evaporated to dryness at 36°C. The dried sample was diluted in 100 μL acetic acid/acetonitrile/water (1:15:85) for IAA determination, acetic acid/methanol/water (1:30:70) for ABA determination, methanol/water (30:70) for GA3, 6-Furfurylaminopurine (6-KT) and 6-Benzylaminopurine (6-BA). Data from experiments were analyzed by analysis of variance (ANOVA) followed by post hoc Tuckey test $p < 0.05$.

Pot Experiments

The pot experiments were evaluated for microorganisms ability to release the nutrients of different organic fertilizer to enhance crop growth and yield under the field conditions. For the experiment we used two controls and six treatments with microorganisms initial cell concentration applied at three levels. The controls and treatments were given in the Table 4. Data of main nutrient content in the soil were recorded at different stages.

Analysis of Carriers

The corn straw was mixed with cattle manure, chicken manure and pig manure in a ratio of 1:4 as mixed carriers, respectively. 10 g of cattle manure (CA), chicken manure (CH), pig manure (PI), corn straw (CO), turf (turf, TU) and cattle manure (CA) + corn straw (CO), chicken manure (CH) + corn straw (CO), pig manure (PI) + corn straw (CO) were mixed with 50 mL compound bacterium agent at 30°C, respectively. The quantity of active bacteria was measured at 12, 24, 48 and 72 h. Each experiment was carried out in triplicate.

To evaluate the effect of water content, temperature, and initial cell concentration on content of active bacteria in mixed carrier, the assays were performed under different water concentrations (20, 30 and 40%), temperature (20, 30 and 40ºC) and initial cell concentration (10^6, 10^7 and 10^8 CFU mL^{-1}). Samples were collected at 1, 3, 5 and 7 day. The number of bacteria in the sample was calculated by flat colony counting method.

Statistical Analysis

Data statistical analyses were performed by Excel 2007 (Microsoft Corporation, USA) and SPSS 18.0 data processing system (IBM, USA). Significant differences between treatments were analyzed by a one-way analysis of variance (ANOVA). Comparisons of means between different treatments were performed using Duncan's studentized range test. The significance level was set to 0.05.

Results

16S rRNA Gene Sequencing and Analysis

Among 15 strains were isolated from soil and 8 strains were identified as high-efficiency strains for the decomposition of soil organic nitrogen. Gram stain showed a distribution of 75% positive and 25% negative. As shown in Table 2, 16S rDNA sequence analysis indicates that strain A-6 has homology with *Naxibacter* spp., A-8 was identified as *Stenotrophomonas* spp., A-11 was identified as *G.* spp., A-17 was identified as *Cellulosimicrobium* spp. and A-9, A-11, A-12, A-19 and A-36 were identified as *Bacillus* sp. Phylogenetic tree constructed using the partial 16S rDNA sequences of the plant growth promoting rhizobacteria and representative bacteria of related taxa is shown in Fig. 1.

Growth Promoting Experiments

The fermentation products of microbial organic fertilizer have plant hormones such as Indoleacetic-3-Acid (IAA), abscisic acid (ABA), Gibberellin Acid (GA3), ACC deaminase, 6-Furfurylaminopurine (6-KT), and 6-Benzylaminopurine (6-BA) which can be found in the fermentation products (Table 3). The content of plant hormones was determined by standard curves: IAA: 7.90 mg L^{-1}, ABA: 0.01367 mg L^{-1}, GA_3: 2.33 mg L^{-1}, ACC deaminase: 0.65 mg U^{-1}, 6-KT: 1.52 mg L^{-1}, 6-BA: 0.89 mg L^{-1}. It was indicated that those PGPR could be as growth supporting agents for plants.

Pot Experiments

The results from the soil physical and chemical properties were showed in Fig. 2. The ammonium nitrogen content of microorganisms addition in the process of corn straw decomposed (3680, 3709, 4026 mg/kg) was higher than control treatment (3526 mg/kg) and increased with the increase of the dosage of the microorganisms.

Table 1: Basic properties of the decomposed compost carrier

Name	Total Nitrogen%	Total Phosphorus%	Total Potassium%	Organic Carbon%	Carbon/Nitrogen
Cattle manure (CA)	2.29	0.35	0.66	52.77	23.04
Chicken manure (CH)	3.02	1.42	0.64	52.21	17.29
Pig manure (PI)	2.99	2.71	2.70	38.21	12.78
Corn straw (CO)	0.92	0.27	0.68	61.74	67.11
Turf (TU)	1.71	0.15	1.44	64.6	37.78

Table 2: Identification of plant growth promoting rhizobacteria based on 16S rDNA sequence

Strain	Identified as	GeneBank Accession	Query coverage (%)	Homology (%)
A-6	*Naxibacter* spp.	MH718835	99	98
A-8	*Stenotrophomonas* spp.	MH718840	99	100
A-9	*Bacillus pumilus*	MH717445	100	100
A-11	*Geobacillus stearothermophilus*	MH717377	100	100
A-12	*Bacillus stratosphericus*	MH718808	100	100
A-17	*Cellulosimicrobium cellulans*	MH718838	100	99
A-19	*Bacillus altitudinis*	MH718834	100	100
A-36	*Bacillus megaterium*	MH720217	100	100

Table 3: Plant growth index of the compound bacterial agent

Name	Content
IAA	7.90 mg L^{-1}
GA	2.33 mg L^{-1}
ABA	0.56 mg L^{-1}
Soluble phosphorus	8.6 mg L^{-1}
6-Furfurylaminopurine	1.52 mg L^{-1}
6-Benzylaminopurine	0.89 mg L^{-1}
ACC deaminase	0.65 mg U^{-1}

Table 4: Effect of microorganisms on release of ammonium nitrogen in organic fertilizer

Treatments	Microorganisms addition (mL)	Corn straw (kg)	chicken manure (kg)
1	0	2	0
2	2	2	0
3	10	2	0
4	20	2	0
5	0	0	2
6	2	0	2
7	10	0	2
8	20	0	2

The ammonium nitrogen content of microorganisms addition in the process of chicken manure decomposed (4823, 5014 mg/kg) was significantly higher than control treatment (4731 mg/kg) after 30 days. From the above results, applicated plant growth promoting rhizobacteria in organic fertilizer could tolerate variety of environmental factors restricts and accelerate the decomposition and release of nutrients.

Analysis of Carriers

The result showed that the quantity of active bacteria in each carrier increased as time went on (Fig. 2). There was no significant difference in the quantity of active bacteria in each carrier within 12 h. However, the quantity of active bacteria in TU carrier was significantly higher than that of other carries within 24 h. The quantity of active bacteria in the TU carrier was 23.46% higher than that in CA, 23.46% higher than CH, 11.11% higher than CO (Fig. 4), and 25.00% higher than PI within 72h. It was indicated that TU was a better carrier than CA, CH, and PI.

Effects of mixed carrier, water content, temperature, and initial cell concentration on quantity of active bacteria were also investigated. The growth rate of the active bacteria in TU was higher than that of the other carriers within 24 h (Fig. 3). However, the quantity of active bacteria in TU and CH+CO and PI+CO was no significant difference after 24 h, but significantly 12.68% higher than CA+CO and 11.11% higher than CO. The results displayed in Fig. 5 showed that the proper water content was 30%. The quantity of active bacteria in chicken manure CH+CO was significantly higher than that of other experimental treatments within 10 days. Fig. 6 showed that the quantity of active bacteria in CH + CO at 30°C was significantly higher than that of other experimental treatments within 3 days. High initial cell concentration was faster colonized in CH +CO than other experimental treatments within 10 days.

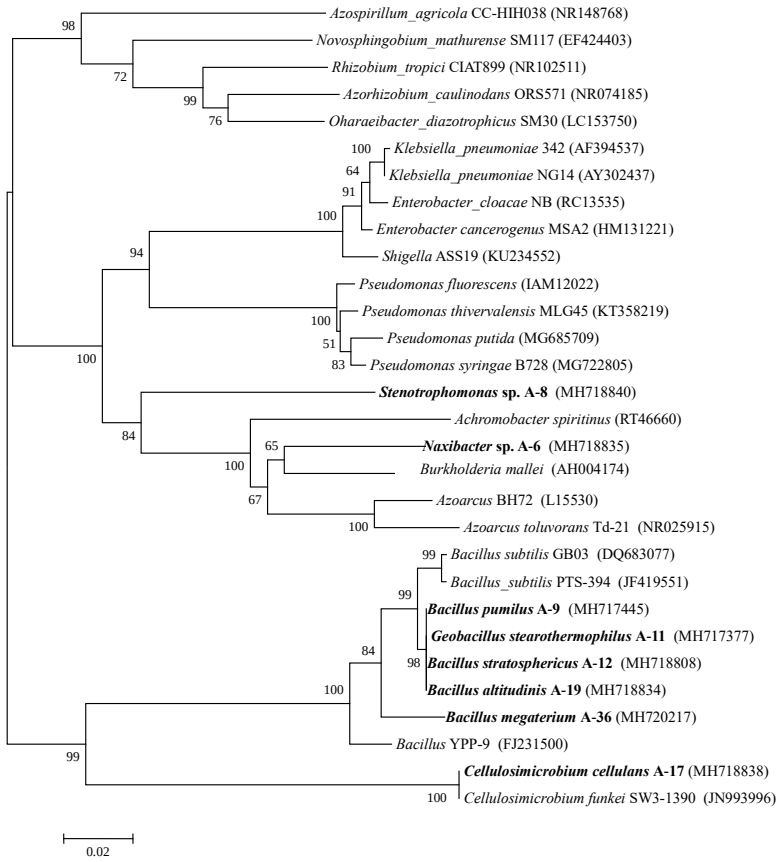

Fig. 1: Phylogenetic tree constructed using the partial 16S rDNA sequences of the plant growth promoting rhizobacteria and representative bacteria of related taxa. Bootstrap values were expressed as percentages of 1000 replications, and only bootstrap values above 50% are shown

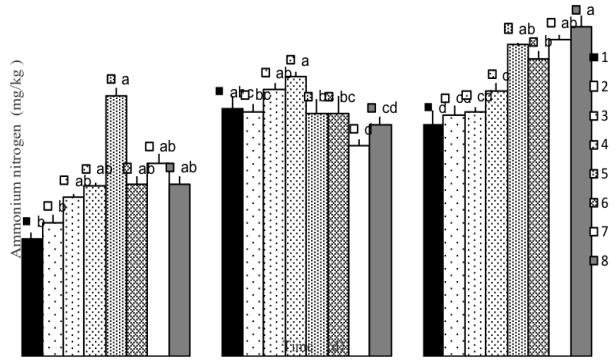

Fig. 2: Effects of adding microorganisms on ammonium nitrogen content of corn straw and chicken manure. 1. Control 1; 2. 2 mL microorganisms + CO; 3. 10 mL microorganisms + CO; 4. 20 mL microorganisms + CO; 5. Control 2; 6. 2 mL microorganisms + CH; 7. 10 mL microorganisms + CH; 8. 20 mL microorganisms + CH

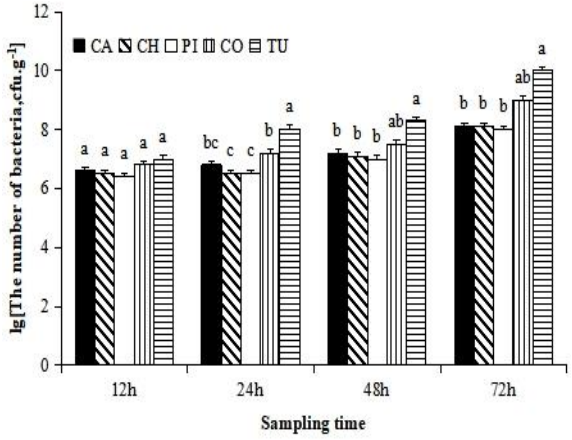

Fig. 3: Changes of active bacteria quantity in single carrier

From the above results, the mixed carrier CH+CO (80% chicken manure + 20% corn straw) was a suitable alternative to turf as a functional bacterial carrier (Fig. 7). The optimal conditions were 30% water content, 30°C temperature, and 10^8 CFU mL^{-1} initial cell concentration.

Discussion

Harmful quantities of fertilizers were applied annually in terrestrial agrosystems as they were not absorbed effectively (Baligar *et al*., 2001). It is very important to optimize the

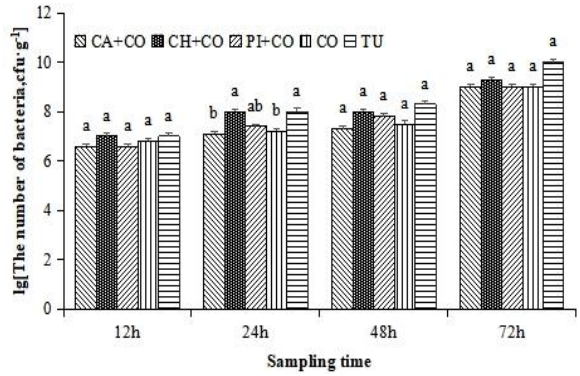

Fig. 4: Changes of active bacteria quantity in mixed carriers

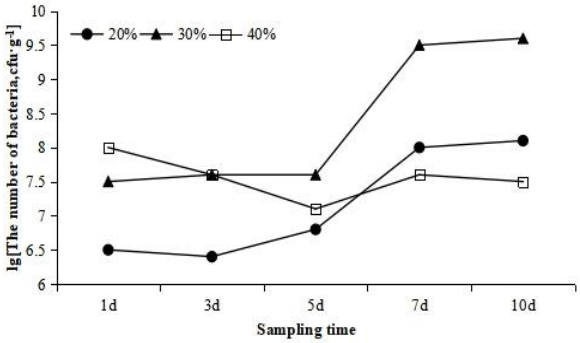

Fig. 5: Effect of water content on the active bacteria quantity in the CH+CO mixed carrier

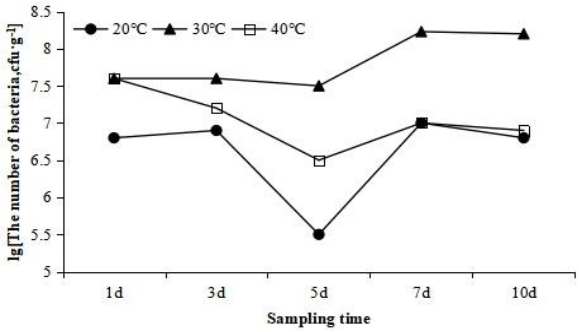

Fig. 6: Effect of temperature on the quantity of active bacteria in the CH+CO mixed carrier

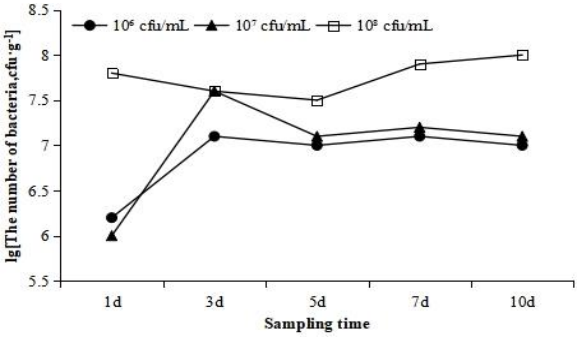

Fig. 7: Effect of initial cell concentration on the quantity of active bacteria in the CH+CO mixed carrier

efficiency of fertilizer use in agricultural production (White and Brown, 2010). In this context, microbial organic fertilizer as an economical and environment-friendly approach was an essential component to consider. In this research, a multifunctional composite microbial organic fertilizer was found and tried to solve the problems of soil caused by long-term use of chemical fertilizer and change the irregular release of nutrients in traditional organic fertilizer.

In the present study, eight rhizobacteria were isolated from soil and identified as high-efficiency strains of microbial agents for the decomposition of soil organic nitrogen. Gram stain showed a distribution of 75% positive and 25% negative. Genotypic identification by 16S rDNA sequencing showed that strain A-6 has homology with *Naxibacter* spp., A-8 was identified as *Stenotrophomonas* spp., A-11 was identified as *Geobacillus* spp., A-17 was identified as *Cellulosimicrobium* spp. and A-9, A-11, A-12, A-19 and A-36 were identified as *Bacillus* spp. Production of phytohormones such as IAA, ACC Deaminase, GA3 and ABA, was proved in the microbial organic fertilizer. The content of plant hormones was produced 7.90 mg L^{-1} IAA, 0.01367 mg L^{-1} ABA, 2.33 mg L^{-1} GA_3, 0.65 mg U^{-1}ACC deaminase, 1.52 mg L^{-1} 6-Kt and 0.89 mg L^{-1} 6-BA. Authors found that production of phytohormones by PGPR was higher than Sgroy *et al.* (2009) reported. It is widely known that PGPR hydrolyze ethylene precursor ACC, resulting in increased root growth (Chen *et al*., 2013). Nadeem *et al*. (2006) and Hameeda *et al*. (2008) also reported increase in nutrient uptake by microbial inoculation under stressful conditions. These strains also possessed phosphate solubilization ability which may have helped the plants to get extra nutrients. Naveed *et al*. (2014) also reported the increase in antioxidant enzymes activity under abiotic stress in wheat by bacterial inoculation. It is highly likely that above production of these strains might have helped the plants to tolerate their pathogens thus resulting in better growth. Therefore, the microbial organic fertilizer application could provide a good soil environment for plant growth.

Comparison of the effects of compost, turf, and mixed compost as functional microbial carriers indicated that the quantity of active bacteria in the vector increases with the time of culture (Fig. 2 and 3). Since carriers can provide certain nutrient elements and easily degrade organic matter, microorganisms could be able to use the abundant carbon and nitrogen in compost to grow and reproduce (Zhou, 1999). The quantity of active bacteria of CA (1.26×10^8 CFU g^{-1}), CH (1.25×10^8 CFU g^{-1}), PI (1.02×10^8 CFU g^{-1}) and CO (9.64×10^8 CFU g^{-1}) in a single vector was significantly lower than that of TU (9.97×10^9 CFU g^{-1}), which depended on the good ventilation of grass charcoal, and the content of nutrients such as C/N was suitable for colonization of bacteria.

However, turf is a non-renewable resource, so that ideal carrier should be found to replace turf. Animal manure mixed with corn straw can create a microbial carrier with suitable nutrient coordination and good ventilation to form a microecological environment (Cai *et al*., 2003). Laird *et al.* (2010) also showed that the straw biochar application could provide a good soil environment for plant growth. Further results showed that the quantity of active bacteria on CH+CO (2.00×10^9 CFU g^{-1}) was equal to TU carriers (9.97×10^9 CFU g^{-1}) with time going on (Fig. 3), indicating that the CH+CO carrier could be an alternative for turf as a good soil conditioner. Water content, temperature, and initial cell concentration in CH+CO carrier were the key factors affecting the quantity of active bacteria in the carrier. Fig. 4 showed that 30% water content of carrier was the most suitable for bacteria survival. In contrast, 20% water content of carrier was too low to sustain bacteria growth. Carrier with 40% water content which filled surface and pores resulted poor ventilation in the carrier and a rapid decrease in the quantity of active bacteria. The results showed that the biggest amount of active bacteria was at 30°C (Fig. 5). Compared to 20°C and 40°C, lower temperature would slow down the growth of bacteria, and too high temperature would accelerate the aging of bacteria (Kong *et al*., 2014). The optimal initial cell concentration was 108 CFU mL^{-1}, when bacteria could colonize on carrier rapidly.

The current study showed that microorganisms of the microbial organic fertilizer have ability for the decomposition of soil organic nitrogen and producing plant hormones. Hu *et al*. (2010) also reported that the yield and P acquisition of wheat plants grown with a long-term NPK application were clearly limited but that they could be significantly increased by inoculation with the AMF *Glomus caledonium*. The quantity of active bacteria after CH+CO application was significantly higher than that of the control treatment. Zhang *et al*. (2016) also reported that corn straw is a good soil conditioner in tobacco field, which can improve tobacco growth and nutrients adsorption at appropriate level through both directly and indirectly effect. Taken together, these results suggested that the rational combination of microorganisms and CH+CO organic fertilizer can be advantageous to crop production and reduce the use of chemical fertilizers in large quantities. However, since we did not evaluate the bacterial community that actually colonized the root system, more specific experiments are needed to better address the relationship between crop production and the microbial organic fertilizer.

Conclusion

We report the potential for using the rational combination of microorganisms and chicken manure + corn straw as the microbial organic fertilizer to enhance yield. This treatment can be widely applied as an environmentally friendly biotechnology in a more sustainable farming system because it can improve the rhizospheric microecology of continuously cropped soil, reduces the use of chemical fertilizers in large quantities.

Acknowledgements

This study was supported by the project of development and application of in situ nitrogen metabolism fertilizer (700197172210) and China National Tobacco Corp Yunnan Science and Technology (2015YN22).

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(Received 25 June 2018; Accepted 23 August 2018)