Applied Soil Ecology 46 (2010) 54–58

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Increased plant uptake of nitrogen from ^{15}N-depleted fertilizer using plant growth-promoting rhizobacteria

A.O. Adesemoye[a,*], H.A. Torbert[b], J.W. Kloepper[a]

[a] Department of Entomology & Plant Pathology, 209 Life Science Building, Auburn University, Auburn, AL 36849, USA
[b] USDA Agricultural Research Services, National Soil Dynamics Laboratory, 411 South Donahue Drive, Auburn, AL 36849, USA

ARTICLE INFO

Article history:
Received 1 January 2010
Received in revised form 16 June 2010
Accepted 17 June 2010

Keywords:
Plant growth-promoting rhizobacteria
Plant–microbe interaction
^{15}N isotope
Plant nutrient management
Sustainable agriculture

ABSTRACT

Harmful environmental effects resulting from fertilizer use have spurred research into integrated nutrient management strategies which can include the use of specific micro-organisms to enhance nutrient use efficiency by plants. Some strains of plant growth-promoting rhizobacteria (PGPR) have been reported to enhance nutrient uptake by plants, but no studies with PGPR have used ^{15}N isotope techniques to prove that the increased N in plant tissues came from the N applied as fertilizer. The current study was conducted to demonstrate that a model PGPR system can enhance plant uptake of fertilizer N applied to the soil using different rates of ^{15}N-depleted ammonium sulfate. The experiments were conducted in the greenhouse with tomato using a mixture of PGPR strains *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4. Results showed that PGPR together with reduced amounts of fertilizer promoted tomato growth compared to fertilizer without PGPR. In addition, atom% ^{15}N per gram of plant tissue decreased as the amount of fertilizer increased, and PGPR inoculation resulted in a further decrease of the atom% ^{15}N values. The atom% ^{15}N abundance in plants that received 80% fertilizer plus PGPR was 0.1146, which was significantly lower than 0.1441 for plants that received 80% fertilizer without PGPR and statistically equivalent to 0.1184 for plants that received 100% fertilizer without PGPR. The results demonstrate that increased plant uptake of N applied in fertilizer could be achieved with PGPR as indicated by the differences in ^{15}N uptake. Strains of PGPR that lead to increased nutrient uptake by plants should be evaluated further as components in integrated nutrient management systems.

1. Introduction

Natural sources of nitrogen (N) such as precipitation and fixation by bacteria symbionts of legumes are not enough to maximize crop productivity; thus, chemical fertilizers have been used for a long period of time to increase N availability and plant uptake (Frink et al., 1999). In recent years, the rate of N fertilizer application has increased tremendously, a trend which is expected to continue (Adesemoye and Kloepper, 2009). One major concern is that large fractions of the applied N (often half or more) are lost from agricultural systems as N_2, trace gases, and nitrate leaching. Thus, increasing N fertilizer use portends grave environmental consequences that are usually long-term and are seen as significant drivers of global change (Vitousek et al., 1997; Diaz and Rosenberg, 2008). These consequences include negative effects on microbial diversity, species composition, and functioning of many terrestrial and aquatic ecosystems (Vitousek et al., 1997). Nitrogen loading in the Mississippi River and the Gulf of Mexico is a practical example (Turner and Rabalais, 1995). A recent review (Adesemoye and Kloepper, 2009) detailed the impacts of excessive N use.

For agricultural production to keep pace with the growing global population, the use of chemical fertilizers will continue. Therefore to achieve sustainability, proper management techniques must be designed and implemented against the pollution potential of fertilizers. Some states in the U.S. are implementing legislation targeted at the reduction of agro-environmental pollution and protection of groundwater quality (Sharpley et al., 2003). One possible solution to this agro-environmental problem is integrated nutrient management systems (Adesemoye et al., 2008) that supplement chemical fertilizers with biofertilizers.

Among biofertilizers are plant growth-promoting rhizobacteria (PGPR), which are free-living rhizosphere bacteria that promote plant growth (Kloepper et al., 1989) by a variety of mechanisms (Vessey, 2003; Glick et al., 2007). The specific mechanism involved in PGPR-elicited enhanced nutrient uptake was recently proposed by Adesemoye et al. (2009). It was proposed that PGPR promoted the growth of the plant and increased the root surface area or the general root architecture, better roots then released higher amounts of C in root exudates, the increase prompted more microbial activity and the cycle of events made more N available for

* Corresponding author. Present address: Department of Plant Pathology & Microbiology, 232, Fawcett Laboratories, University of California, Riverside, CA 92521, USA. Tel.: +1 951 827 3499.
E-mail address: adeseao@ucr.edu (A.O. Adesemoye).

0929-1393/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.apsoil.2010.06.010

plants' uptake. The 2 PGPR strains *B. amyloliquefaciens* IN937a and *B. pumilus* T4 (the same strain used in this study) were not N fixers based tests with JNFb medium. The relation of plant growth promotion by PGPR and plant nutrient uptake is still an ongoing investigation. Hernandez and Chailloux (2004) and Adesemoye et al. (2008) are two examples among some other studies, which showed that PGPR-elicited plant growth promotion resulted in enhanced N uptake by plant roots. In these studies, it was suggested that the increase in plant N content might result from increased fertilizer N utilization efficiency. If this suggestion were correct, then PGPR should be useful in integrated nutrient management systems (Adesemoye et al., 2008). However, the increased N content in plant tissues could result from increased overall uptake of soil N without changing the uptake of N applied in fertilizers. In order to confirm or reject the hypothesis that PGPR can increase fertilizer use efficiency, experiments are needed in which the N applied in fertilizer can be traced in a way to determine its content in plant tissues.

Isotope tracer techniques have been used in studying the different parts of the N cycle including estimating crop N uptake from inorganic fertilizers (Hauck and Bremner, 1976; Saoud et al., 1992; Torbert et al., 1992; Hood et al., 1999; Bronson et al., 2000). An underlying basis of using isotopic techniques for monitoring plant uptake of N is that the chemical identities of the isotopes ^{15}N and ^{14}N are maintained in the plant, and plants cannot differentiate between them (Hauck et al., 1994). Isotopes ^{15}N and ^{14}N can be differentiated with exchange or distillation columns (Hauck et al., 1994; Mulvaney et al., 1997). The objective of this study was to use ^{15}N isotope techniques to test the hypothesis that PGPR will enhance plant uptake of N from ^{15}N-labeled fertilizer.

2. Materials and methods

2.1. Bacterial inoculation, test plant, and growth conditions

The experimental model system used for this study was tomato (*Solanum lycopersicum*) cultivar Juliet (Park Seed, Greenwood, SC) grown in pots containing a 1:3 mixture of field:sand soil. The sand soil was builders' sand obtained for general use in the greenhouse. This soil mixture was analyzed for nutrient content using Mehlich 1 (double acid) extraction method (Mehlich, 1953). For the analysis, 5 g of sieved air-dried soil was added to a 150 ml extraction flask, followed by 25 ml of Mehlich 1 extracting solution (0.05 M H_2SO_4 + 0.05 M HCl) and then shaken for 5 min on a reciprocating shaker (Barnstead/Thermolyne, Dubuque, IO) at 180 oscillations per min. It was centrifuged, filtered through Whatman no. 2 filter paper, and analyzed using inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Varian, Victoria, Australia). Further details on this method can be seen in Adesemoye et al. (2008). The soil mixture consisted of 0.19% C, 0% N (value of N was below the detectable limit of equipment), 7.3 $\mu g\,g^{-1}$ P, 8.8 $\mu g\,g^{-1}$ K, 15.4 $\mu g\,g^{-1}$ Ca and 3.2 $\mu g\,g^{-1}$ Mg. Inoculants included two plant growth-promoting rhizobacteria (PGPR) strains, *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4, which were used as a mixture. The PGPR strains were obtained from the culture collection of the Department of Entomology and Plant Pathology, Auburn University, Auburn, AL, USA and used as spore preparations. The spore suspensions of the two PGPR strains were diluted and the concentration was adjusted to log 5 cfu/ml and used for inoculation.

The pots used in this study were 10-cm diameter with a volume capacity of 754.4 ml. WeedEX landscaping cloth (Dalen Products Inc., Knoxville, TN) was placed in each pot to prevent soil from washing out through the holes under the pots, especially during watering. The pots were filled with the mixed soil and one seed per pot was planted. At planting, the soil inside the pot was dug up slightly, one seed was put into the hole, and 1 ml of the bacterial suspension was applied onto the seed before covering with soil. A follow-up drench inoculation was done at 1 week after planting by applying 1 ml of PGPR onto every pot around the base of each germinating plant.

2.2. Fertilizer solution of ^{15}N

We prepared a hydroponic solution with slight modifications of Hoagland solution (Maynard and Hochmuth, 2007) to produce an N-free solution (Hershley, 1994). The constituents of the fertilizer solution were 10 ml of 0.05 M monocalcium phosphate ($Ca[HPO_4]_2$), 20 ml of 0.1 M calcium sulfate dihydrate ($CaSO_4{\cdot}2H_2O$), 5 ml of 0.5 M potassium sulfate (K_2SO_4), 2 ml of 1 M magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), 2 ml of Fe-EDTA, and 1 ml of micronutrient stock. All Chemicals listed above were obtained from Sigma–Aldrich, St. Louis, MO, USA. One liter of 100% N solution was prepared by using 1 M of ^{15}N-depleted ammonium sulfate ($^{15}NH_4)_2SO_4$ (ISOTEC™, Miamisburg, OH) as the only N source. The amount of ^{15}N was varied by changing the volume of $^{15}(NH_4)_2SO_4$ as detailed below. The background atom% ^{15}N abundance in the $^{15}(NH_4)_2SO_4$ was 0.01%.

2.3. Experimental design

The studies were conducted in the greenhouse at the Plant Science Research Center, Auburn University, Alabama, where temperature was maintained at 21–25 °C. The experiment was a 2×3 factorial in a randomized complete block (RCB). The factor 2 was with or without PGPR inoculation while the factor 3 was three rates of fertilizer solution containing −210, 168, 157.5 mg L^{-1} N in water (i.e., ppm N) and was labeled as 100, 80, and 75% N rates, respectively. Each fertilizer treatment had 10 replicates. The six treatments (i.e., 2×3 factorial) reported here were chosen based on experience after a series of assays with ^{14}N and ^{15}N testing different N rates from 50 to 100% with or without PGPR. The 100% N contained 210 mg L^{-1} N, from which other percentages were calculated, as previously explained (Adesemoye et al., 2009). Preliminary findings on ^{15}N was similar to those shown for ^{14}N in that rates of 70% or lower did not show consistent results (Adesemoye et al., 2009); thus, the reason for using 75% or greater rates in this study. The amount of N in solution was varied by changing only the content of $(^{15}NH_4)_2SO_4$. In the study, 25 ml of nutrient solution was applied per pot twice per week for each treatment during the period of plant growth. Each pot was watered twice daily with 25 ml of water, except that watering was skipped on the days that fertilizer solution was applied. The volume (25 ml) was chosen for the hydroponic solution and water after trying different volumes with the intent of avoiding leaching. The plants were allowed to grow for 4 weeks before destructive sampling.

2.4. Plant sample preparation and isotope analysis

Plants were removed carefully from pots, roots were washed in slow-running water (to remove adhering soil), laid on paper towels to drain, blotted, and fresh weights were recorded. Samples were dried at 70 °C for 7 days, dry weights were recorded and whole plants (root and shoot) were prepared for nutrient analysis. Samples were analyzed for total N and ^{15}N contents with the help of Dr. Richard Mulvaney at the Department of Natural Resources and Environmental Sciences, University of Illinois, Urbana-Champaign. Estimating the impact of ^{15}N uptake correctly requires that a portion of the tracer be recovered in a chemically defined state. The samples were digested according to standard protocols (Mulvaney, 1993; Bremner, 1996), and diffusion of digest was done using the Mason-Jar diffusion method (Mulvaney et al., 1997). An automated Rittenberg apparatus-mass spectrometer (ARA-MS) which

utilizes the Rittenberg techniques for N isotope analysis was used (Mulvaney and Liu, 1991).

2.5. Calculation of ^{15}N uptake and statistical analysis

The amount of ^{15}N material to apply based on the atom% abundance of the $(^{15}NH_4)2SO_4$, ^{15}N recovery, and differences in ^{15}N uptake were calculated using standard methods (Hauck and Bremner, 1976; Cabrera and Kissel, 1989; Zhou et al., 1998; Bronson et al., 2000; Wanek and Arndt, 2002). Analyses were done with the Statistical Analysis System 9.1 (SAS Institute, Cary, NC). Data were analyzed using GLM procedure, and Fisher's protected LSD was used to separate treatment differences after testing the normal distribution assumption. The Glimmix procedure was used to plot diffograms (mean–mean scatter plot) and to evaluate treatment effects and their interactions (Littell et al., 2006). Statistical significance was considered at $\alpha = 0.05$ unless otherwise stated.

3. Results

3.1. Total nitrogen in tomato tissues

It was observed that the amount of N in whole plant tissues in the 100% N treatment was significantly greater than the 80% N treat-

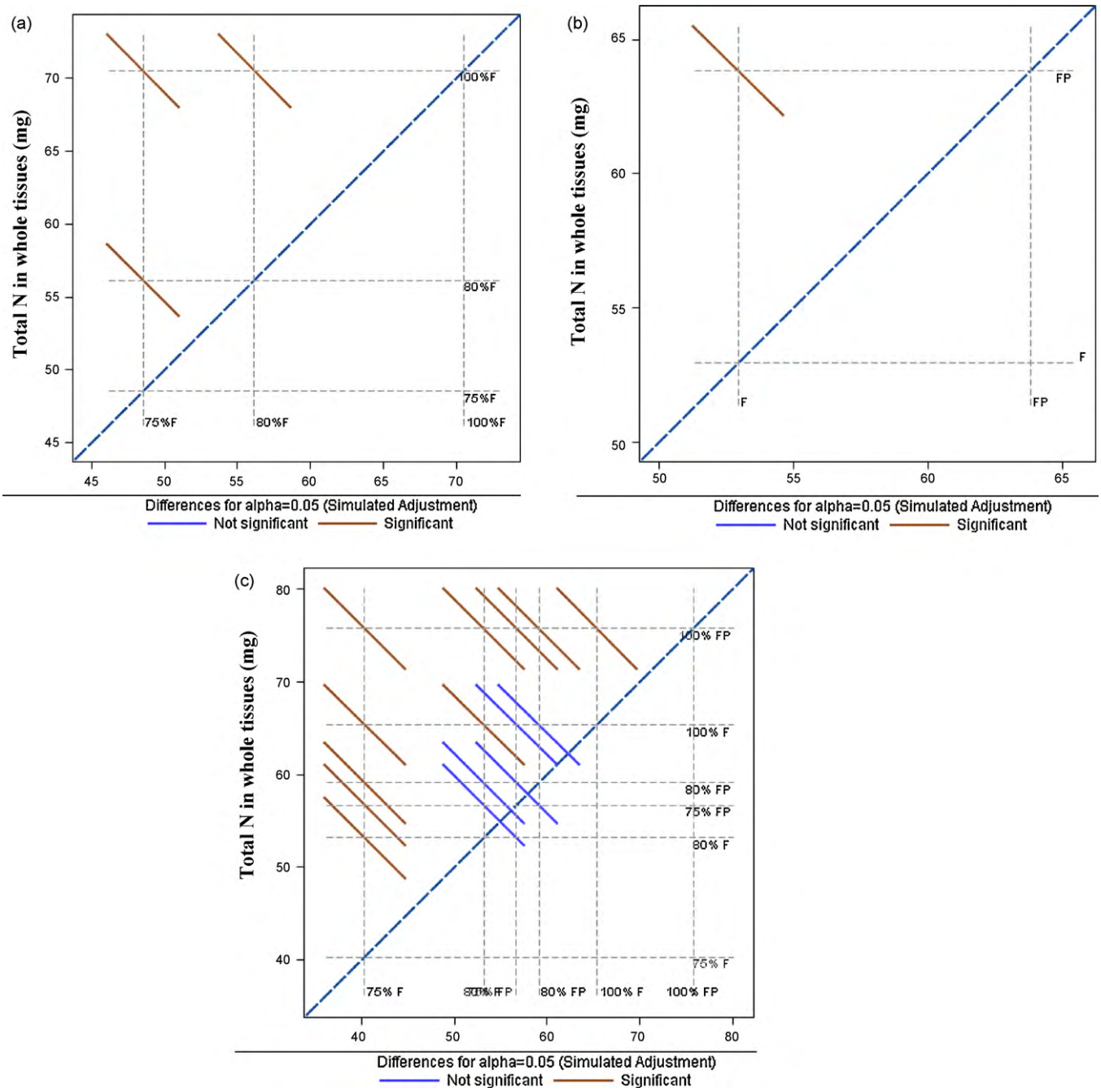

Fig. 1. Diffogram (mean–mean scatter plot) comparing treatment effects on total amount of N in whole plant tissues: (A) is fertilizer effect; (B) is bacterial inoculation effect; and (C) is interaction effect of fertilizer and bacterial inoculation. All (%) reflected percentage of N content; F is fertilizer without PGPR; and FP is fertilizer with PGPR mixture. The 45° reference line indicates whether two least-square means are different at 0.05 significant levels. The thick lines drawn at the intersection of grid lines corresponds to $(1-\alpha) \times 100\%$ confidence interval of the difference of the two least-square means in each comparison. Any thick line that crosses the 45° reference line implies no significant difference for that comparison.

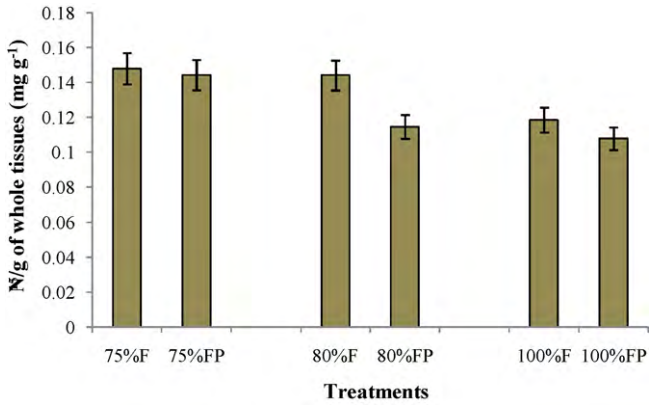

Fig. 2. Atom% ^{15}N per gram of whole tomato tissues. All (%) reflected percentage of N content; 75% F is 75% level of fertilizer without PGPR; 75% FP is 75% level of fertilizer with the PGPR mixture; and similar explanation for 80 and 100%. The 100% fertilizer contained 210 mg L^{-1} N, from which other percentages were calculated. Standard errors (at $p = 0.1$) are shown as whisker hairs on graphs. The fertilizer material used was depleted in ^{15}N isotope with a content of 0.01%.

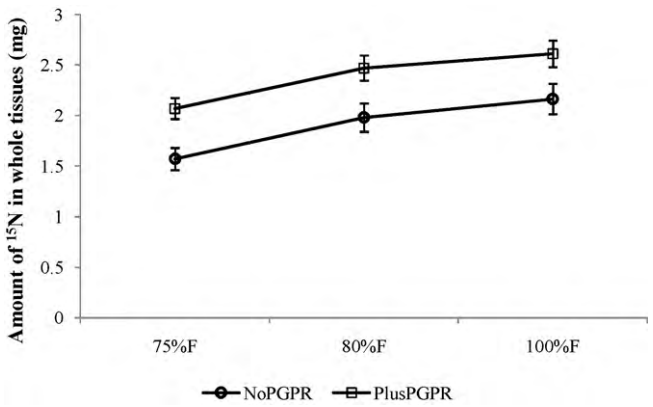

Fig. 3. Total amount of ^{15}N contained in whole tomato tissues. All (%) reflected percentage of N content, F is fertilizer only; NoPGPR is fertilizer without PGPR; and PlusPGPR is fertilizer with PGPR mixture. Standard errors are shown as whisker hairs on graphs. The 100% fertilizer contained 210 mg L^{-1} N, from which other percentages were calculated.

ment which was in turn greater than the 75% treatment (Fig. 1A). The PGPR-inoculated plants contained significantly more N in their whole tissues than the non-inoculated (Fig. 1B). There was a significant interaction effect between PGPR treatment and fertilizer level. The addition of PGPR to each N fertilizer level produced plants with greater biomass containing more total N than treatments without PGPR inoculation (Fig. 1C). Interestingly, the total amount of N in plants that received 75% N rate with PGPR was not significantly different from plants that received 100% N without PGPR, as shown in the comparison in Fig. 1C. Ten (i.e., those lines that did not cross the diagonal) of the 15 comparisons were significantly different (Fig. 1C).

3.2. Effect of plant growth-promoting rhizobacteria on changes in ^{15}N

Generally, the amount of ^{15}N per gram of tissue decreased as the amount of applied total N taken up increased. However, the total amount per plant increased with PGPR inoculation owing to increase in dry biomass. Inoculation with PGPR led to a further decrease in atom% ^{15}N per gram and higher changes in ^{15}N compared to a corresponding non-PGPR treated (Fig. 2). Specifically, the atom% ^{15}N per gram in plants that received 80% N rate plus PGPR was 0.1146, which was significantly ($\alpha = 0.1$) lower than 0.1441 for plants that received 80% N rate without PGPR, and equivalent to 0.1184 for plants that received full N (100%) without PGPR (Fig. 2). Based on total amount of ^{15}N contained in whole plant tissues, there was no significant difference between plants that received 100% N without PGPR treatment and those that received 75 or 80% N plus PGPR treatments. However, plants that received 100% N plus PGPR contained significantly more ^{15}N than those with 100% N without PGPR (Fig. 3).

4. Discussion and conclusion

The overall results presented here support our hypothesis that treatment with PGPR can increase plant uptake of N from fertilizer. Specifically, the mixture of PGPR strains *Bacillus amyloliquefaciens* strain IN937a and *B. pumilus* strain T4 caused a significant increase in uptake of N in tomato as indicated by the differences in ^{15}N and total N (Figs. 1–3). Using ^{15}N-depleted fertilizer, it became clear that the source of N taken up by the plant was the applied N fertilizer and not simply 'residual' soil N. The concentration of ^{15}N per gram of plant tissue decreased as applied ^{15}N fertilizer level increased, indicating better plant uptake of the applied ^{15}N. As more of the labeled N entered the plants, the concentration of ^{15}N in plant tissues shifted toward the 0.01 atom% ^{15}N abundance contained in the applied ^{15}N-labeled fertilizer ($[^{15}NH_4]_2SO_4$) and away from the 0.3363 atom% ^{15}N natural abundance. With PGPR inoculation, the concentration of ^{15}N further decreased (Fig. 2). This tracking of movement of N from the applied fertilizer into plant tissue agrees with other studies where ^{15}N-depleted fertilizer was reported to be a reliable tool to trace the movement of N (Hauck and Bremner, 1976; Ditsch et al., 1992). Ditsch et al. (1992) used ^{15}N-depleted ammonium sulfate ($[^{15}NH_4]_2SO_4$) in a corn-winter rye crop rotation and reported a decrease in atom% ^{15}N concentrations in rye tissue as N rate increased from zero to 336 kg N ha^{-1}.

Based on the current ^{15}N study and a previous study with ^{14}N (Adesemoye et al., 2009), it appears that the 75% rate was a threshold below which the PGPR-fertilizer interaction could not produce consistent nutrient uptake comparable to non-inoculated full fertilizer rates. It should be noted that in this study that the 100% fertilizer contained 210 mg L^{-1} N based on the recommendations for tomato in the southeast U.S., and other percentages were calculated from this. This result also agrees with the work of Hernandez and Chailloux (2004) and Dell and Rice (2005). While selected biofertilizers can improve the use efficiency of chemical fertilizers by plants, thereby allowing reduced rates of fertilizers; it is unlikely that fertilizer use can be reduced beyond 75% of recommended inorganic fertilizer rates.

In the literature on fertilizer uptake, there is an ongoing discussion about the best methods for estimating fertilizer use efficiency in plants. One common method is the "difference method" which calculates the difference in N uptake between fertilized and non-fertilized treatments. While the difference method provides useful data, studies with fertilizer materials depleted or enriched in isotope ^{15}N offer more precision. This is because the "difference method" may be obfuscated by stimulation of N mineralization in the presence of fertilizer (i.e., added N interaction, ANI, or priming effect) (Ditsch et al., 1992; Bronson et al., 2000). Changes in plant tissues can be accurately detected with ^{15}N isotope, although it should be emphasized that the accuracy is not achieved just by the use of ^{15}N but is directly related to how precisely are calculated the amount of labeled material added to the experimental system and the relative deviation or difference from the natural atmospheric ratio of ^{15}N:^{14}N (Edwards and Hauck, 1974; Cabrera and Kissel, 1989; Yoneyama et al., 2001).

In conclusion, PGPR have the potential to enhance fertilizer use efficiency which should have applications in regulating landscape-

scale nitrogen fluxes in the future. The benefits of this finding can be viewed from two perspectives (Adesemoye and Kloepper, 2009). First, PGPR-elicited enhanced uptake of N could lead to less use of fertilizer while still maintaining the expected high crop productivity. Second, use of PGPR could help reduce leaching of N during the growing season and reduce residual N in soils after harvest. This assertion can be explained from this standard equation: Soil N (i.e., 'residual soil N') = initial soil N + fertilizer N–N uptake by plants. It follows that if an integrated system can be developed where fertilizer N and N uptake by plants can be almost equal; then, 'residual soil N' will be approximately equal to initial soil N. Therefore, in this scenario, almost no N would be left in the soil to pollute terrestrial and aquatic ecosystems. PGPR and other biofertilizers should be further evaluated as components of integrated nutrient management systems.

References

Adesemoye, A.O., Kloepper, J.W., 2009. Plant-microbes interactions in enhanced fertilizer use efficiency. Appl. Microbiol. Biotechnol. 85, 1–12.

Adesemoye, A.O., Torbert, H.A., Kloepper, J.W., 2009. Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microb. Ecol. 58, 921–929.

Adesemoye, A.O., Torbert, H.A., Kloepper, J.W., 2008. Enhanced plant nutrient use efficiency with PGPR and AMF in an integrated nutrient management system. Can. J. Microbiol. 54, 876–886.

Bremner, J.M., 1996. Nitrogen-total. In: Sparks, D.L., et al. (Eds.), Methods of soil analysis. Part 3. Book Ser. 5. SSSA, Madison, WI, pp. 1085–1121.

Bronson, K.F., Hussain, F., Pasuquin, E., Ladha, J.K., 2000. Use of ^{15}N-labeled soil in measuring nitrogen fertilizer recovery efficiency in transplanted rice. Soil Sci. Soc. Am. J. 64, 235–239.

Cabrera, M.L., Kissel, D.E., 1989. Review and simplification of calculations in ^{15}N tracer studies. Fertil. Res. 20, 11–15.

Dell, C.J., Rice, C.W., 2005. Short-term competition for ammonium and nitrate in tallgrass prairie. Soil Sci. Soc. Am. J. 69, 371–377.

Diaz, R.J., Rosenberg, R., 2008. Spreading dead zones and consequences for marine ecosystems. Science 321, 926–929.

Ditsch, D.C., Alley, M.M., Kelley, K.R., Lei, Y.Z., 1992. Detectability of ^{15}N-depleted fertilizer N in soil and plant tissue during a corn–rye crop rotation. Fertil. Res. 31, 355–362.

Edwards, A.P., Hauck, R.D., 1974. Nitrogen-15-depleted versus nitrogen-15-enriched ammonium sulfate as tracers in nitrogen uptake studies. Soil Sci. Soc. Am. Proc. 38, 765–767.

Frink, C.R., Waggoner, P.E., Ausubel, J.H., 1999. Nitrogen fertilizer: retrospect and prospect. Proc. Natl. Acad. Sci. U.S.A. 96, 1175–1180.

Glick, B.R., Todorovic, B., Czarny, J., Cheng, Z., Duan, J., McConkey, B., 2007. Promotion of plant growth by bacterial ACC deaminase. Crit. Rev. Plant Sci. 26, 227–242.

Hauck, R.D., Bremner, J.M., 1976. Use of tracers for soil and fertilizer nitrogen research. Adv. Agron. 28, 219–266.

Hauck, R.D., Meisinger, J.J., Mulvaney, R.L., 1994. Practical considerations in the use of nitrogen tracers in agricultural and environmental research. In: Weaver, R.W., et al. (Eds.), Methods of Soil Analysis, Part 2. Book Ser. 5. SSSA, Madison, WI, pp. 907–950.

Hernandez, M.I., Chailloux, M., 2004. Las micorrizas arbusculares y las bacterias rizosfericas como alternativa a la nutricion mineral del tomate. Cultivos Tropicales 25, 5–12.

Hershley, D.R., 1994. Solution culture hydroponics: history and inexpensive equipment. Am. Biol. Teacher 56, 111–118.

Hood, R.C., N'Goran, K.N., Aigner, M., Hardarson, G., 1999. A comparison of direct and indirect ^{15}N isotope techniques for estimating crop N uptake from organic residues. Plant Soil 208, 259–270.

Kloepper, J.W., Lifshitz, R., Zablotowicz, R.M., 1989. Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol. 7, 39–44.

Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D., Schabenberger, O., 2006. SAS® for Mixed Models, 2nd ed. SAS Institute Inc., Cary, North Carolina, pp. 21–41.

Maynard, D.N., Hochmuth, G.J., 2007. Knott's Handbook for Vegetable Growers, 5th ed. John Wiley & Sons Inc., Hoboken, NJ, pp. 68, 93.

Mehlich, A., 1953. Determinations of P, Ca, Mg, K, Na, and NH_4 by North Carolina soil testing laboratories. North Carolina State University, Raleigh, North Carolina, USA.

Mulvaney, R.L., 1993. Mass spectrometry. In: Knowles, R., Blackburn, T.H. (Eds.), Nitrogen Isotope Techniques. Academic Press, San Diego, CA, pp. 11–57.

Mulvaney, R.L., Khan, S.A., Stevens, W.B., Mulvaney, C.S., 1997. Improved diffusion methods for determination of inorganic nitrogen in soil extracts and water. Biol. Fertil. Soils 24, 413–420.

Mulvaney, R.L., Liu, Y.P., 1991. Refinement and evaluation of an automated mass spectrometer for nitrogen isotope analysis by the Rittenberg technique. J. Automatic Chem. 13, 273–280.

Saoud, A.A., Cleemput, O.V., Hofman, G., 1992. Uptake and balance of labeled fertilizer nitrogen by potatoes. Fertil. Res. 31, 351–353.

Sharpley, A.N., Weld, J.L., Beegle, D.B., Kleiman, P.J.A., Gburek, W.J., Moore Jr., P.A., Mullins, G., 2003. Development of phosphorus indices for nutrient management planning strategies in the United States. J. Soil Water Conserv. 58, 137–152.

Torbert, H.A., Mulvaney, R.L., Vanden Heuvel, R.M., Hoeft, R.G., 1992. Soil type and moisture regime effects on fertilizer efficiency calculation methods in a nitrogen-15 tracer study. Agron. J. 84, 66–70.

Turner, R.E., Rabalais, N.N., 1995. Changes in Mississippi River water quality this century. Bioscience 41, 140–147.

Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571–586.

Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., Tilman, D.G., 1997. Human alteration of the global nitrogen cycle: sources and consequences. Ecol. Appl. 7, 737–750.

Wanek, W., Arndt, S.K., 2002. Difference in δ^{15}N signatures between nodulated roots and shoots of soybean is indicative of the contribution of symbiotic N_2 fixation to plant N. J. Exp. Bot. 53, 1109–1118.

Yoneyama, T., Matsumaru, T., Usui, K., Engelaar, W.M.H.G., 2001. Discrimination of nitrogen isotopes during absorption of ammonium and nitrate at different nitrogen concentrations by rice (*Oryza sativa* L.) plants. Plant Cell Environ. 24, 133–139.

Zhou, X., Leibovitch, S., MacKenzie, A.F., Madramootoo, C.A., Dutilleul, P., Smith, D.L., 1998. Confined microplot size for nitrogen-15 uptake by corn plants in a corn intercrop system. Agron. J. 90, 155–161.