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A COMPARATIVE STUDY ON NITROGEN FIXATION IN PULSES

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ABSTRACT

In recent years, our understanding of biological nitrogen fixation has been bolstered by a diverse array of scientific techniques. Still, the origin and extant distribution of nitrogen fixation has been perplexing from a phylogenetic perspective, largely because of factors that confound molecular phylogeny such as sequence divergence, paralogy, and horizontal gene transfer. Here, we make use of 110 publicly available complete genome sequences to understand how the core components of nitrogenase, including NifH, NifD, NifK, NifE and NifN proteins, have evolved. These genes are universal in nitrogen fixing organisms- typically found within highly conserved operons-and overall, have remarkably congruent phylogenetic histories. Additional clues to the early origins of this system are available from two distinct clades of nitrogenaseparalogs; a grou composed of genes essential to photosynthetic pigment biosynthesis and a group of uncharacterized genes present in methanogens and in some photosynthetic bacteria. We explore the complex genetic history of the nitrogenase family, which is replete with gene duplication, recruitment, fusion and horizontal gene transfer and discuss these events in light of the hypothesized presence of nitrogenase in the last common ancestor of modern organisms, as well as the additional possibility that nitrogen fixation might have evolved later, perhaps in methanogenicarchaea and was subsequently transferred into the bacterial domain.

KEYWORDS: - NifH, NifD, NifK, NifE and NifN.

INTRODUCTION

Pulses are important because of their ability to fix atmospheric nitrogen through symbiosis with rhizobia. In the tropics where the majority of the population obtains its living from the land, legumes are likely to increase in importance. Pulses form a major component of tropical agrosystems (Norman, 1982; Rachie, 1977; and Okigbo, 1977) and can provide cash income to the farming community. Food legumes provide large quantities of good quality dietary protein to the population and legumes also help to maintain a reasonable level of soil fertility. Cropping systems involving monoculture of non-leguminous plants cause a decline of yields and depletion of soil nitrogen. This decrease in productivity in the past, especially in tropical Africa, has been alleviated by shifting cultivation or more recently by the use of inorganic fertilizers. As the population increases, the resulting pressure on the land has made shifting cultivation untenable. Recent increases in prices of synthetic fertilizer have also made it difficult for small farmers to use inorganic nitrogen for crop production. Consequently, biological nitrogen fixations become the only alternative source of nitrogen for crop production. If biological nitrogen fixation by legumes is to become a sustained reliable source of nitrogen for crop production, certain questions have to be answered. How much nitrogen do various legumes fix? How much residual nitrogen do legumes supply to the cropping systems? However, these questions and many others cannot be answered without a reliable method to estimate biological nitrogen fixation. Reliable estimates of biological nitrogen fixation will allow selection of superior N_2-fixing legume species. Gibson et al. (1977) for instance, indicated that biological nitrogen fixation (BNF) can be improved by: (1) growing better cultivars adapted to specific environments, (2) inoculation with the most effective and competitive, (2) inoculation with the most effective and competitive strains of Rhizobia, and (3) the application of management practices designed to minimize the impact of nutritional and environment limitations. Literature suggests that there are several methods that can be used to estimate field N^2-fixation. The ^{15}N isotope dilution and the difference methods are among the most widely used for estimating field nitrogen fixation by legumes. There are advantages and disadvantages associated with each method. The advantage of the difference method is that, it is inexpensive, simple and does not require special techniques and equipment which are needed for the ^{15}N isotope dilution method. The ^{15}N isotope dilution method which was first described by Mac Aulife et al. (1958) has been used recently by many workers (Fiedler et al. 1972; Fried et al., 1977; Vose et al., 1981; Rennie et al., 1982; Rennie and Kemp, 1983 a,b; Rennie et al., 1984; Rennie and Dubetz, 1984) to estimate field N_2-fixation by various legumes. The advantage of the ^{15}N isotope dilution method is that it

makes it possible to separate N taken up by the plant from fertilizer and soil from that fixed in the plant. Many workers have described the ^{15}N isotope dilution method as the most reliable measure of N_2-fixation (Gibson et al., 1977; Amarger et al., 1979; Larue and Patterson, 1982). The accuracy of either method depends on the type of reference crop used. The best reference crop should be closely related to the test plant. This can be an inoculated plant, a non-nodulating isoline, or a cereal such as cron. An assumption in all cases is that the test plant and control both have the same root systems exploring the same volume of soil. In soils where native rhizobia do not nodulate the test plant, the ideal reference crop is the uninoculated test plant. Where the native rhizobia nodulate the test plant however, the appropriate reference crop is not readily apparent. It is also not clear whether there is agreement between the ^{15}N isotope dilution and the difference methods. Although there are many problems associated with the measurement of the gross amount of N fixed by a legume over the whole period of its growth, the residual nitrogen contribution to the soil-plant system can be determined through a series of measurement including the portion of N derived from mineralization, the residual N that was taken up by the plant, and the portion that remains in the soil. The objectives of this study were to: (1) determine the relationship between the ^{15}N isotope dilution and the difference methods, (2) investigate the field inoculation response of field-grown legumes, (3) quantify the amount of nitrogen fixed by each species using the two methods, (4) determine the best reference crop for N fixation estimates in cowpea and peanut, and (5) determine the residual nitrogen contribution to a subsequent corn crop.

MATERIALS AND METHOD

The seeds for each cultivar were inoculated immediately before planting with appropriate peat-based Rhizobium strains obtained from the NIFTAL Rhizobium Collection. The strains were: TAL 1000, TAL 169, TAL 182, and TAL 102 for peanut, cowpea, bush bean, and soybean respectively. Seeds for each plot were treated with 3 ml gum Arabic solution (40g L^{-1} H_2O) then a peat based inoculants applied to the seeds at the time rate of 10 g per 100 g seed, and then pelleted with 6 g of calcium carbonate. Seeds were planted in four rows 5m long and 65cm apart. The spacing resulted in plant population of $3x10^5$, $1.05x10^5$, $1x10^5$, and $0.8x10^5$ plants ha^{-1} for soybean, peanut, cowpea, bush bean, and corn respectively. Uninoculated plots were planted first in order to avoid cross contamination between plots. Subsequent field operations such as weeding were cautiously done to avoid transfer of rhizobia from inoculated plots to uninoculated plots. Plants were thinned to one plant per hill 12 days after germination. Lasso, a premergence herbicide was applied at a rate of 9 ml m^{-2} at

the time of planting. Thiodan, a foliar insecticide, was applied at a rate of 3g m^{-2} 3 days after emergence. Plots were subsequently sprayed with appropriate chemicals to control insects. Cowpea plots were replanted 7 days after emergence because of the damage by chemicals.

Fertilizer and ^{15}N Application: All plots received a blanket fertilizer application of potassium as K_2SO_4, phosphorus as triple super phosphate, magnesium as Mg $SO_4.7H_2O$, zinc as $ZnSO_4$, molybdenum as Na_2MO_3. H_2O and boron as H_2BO_3 at rates of 250, 400, 67, 15, 1 and 5 kg ha^{-1}, respectively. Lime was applied at ten Mg ha^{-1} as $CaCO_3$ and dolomite n a ration of 60:40 three weeks before planting. A solution of ^{15}N-labelled $(NH_4)_2SO_4$, about 4 atom % ^{15}N, was prepared by dissolving 17.401 g of enriched material and 363.53 g of ordinary $(NH_4)_2SO_4$ which were equivalent to 10 kg N ha^{-1}, in 40 liters of deionized water. The solution was then made up to 80 liters by adding more deionized water. Two liters were sprayed on the ^{15}N micro plot which was 2.6 m^2 of ^{15}N. The reminder of the plots received the equivalent of 10 kg N ha^{-1} as ordinary ammonium sulfate. All plots were sprinkler-irrigated soon after planting. Subsequent moisture supply was maintained at 0.1 bars with the aid of tensiometers.

Determination of Indigenous Rhizobin in Kuiaha Soil: Soil samples were taken from the uninoculated plots 11 days after planting. Samples were composited and a subsample of 50 g of dry soil was taken and mixed with 450 ml of sterile water and shaken vigorously for 10 minutes. A series of dilutions ranging from 10^{-1} to 10^{-6} were made by adding 1 ml of suspension into 9 ml sterile water and repeated 5 times. About 100 seeds of each species were surface sterilized and pre germinated in sterile vermiculite. Well germinated seeds of similar size and radical length were selected and transferred aseptically to growth pouches. Seedlings in growth pouches received 30-40 ml of B&D plant nutrient solution. There were 30 pouches to count dilution, for 10^{-1} to 10^{6} in quadruplicate plus one control pouch following each group of four. Plants were inoculated by pipetting 1ml of each dilution (form 10^{-1} to 10^{-6}) to each one of the four replicate in each set starting from the highest dilution and proceeding down the series with the same pipette. For every species, the number of nodules for each dilution was recorded and the most probable number (MPN) determined 21 days after inoculation.

Sampling and Nitrogen Determination: Plant samples for fresh and dry weight were taken at 30, 60, and 80 days after emergence (DAE) from sample rows of the main plots. Samples for ^{15}N subplots. Samples for nodule count, nodule dry weight, and plant dry matter yield were taken at 30 DAE, from border rows to N_2-fixing cultivars. All plants sampled were

composited for total N analysis. No attempts were made to collect abscised leaves and petioles. N_2 fixation estimates for all sampling dates were based on the total above ground plant parts. Plant samples were oven dried to a constant weight at 70^0C, ground to pass a 1-mm screen, and then sub sampled for N analysis.

Total N was determined on all shoots by digesting 250mg of oven dried samples (70^0C) in 7ml of concentrated sulfuric/salicylic acid mixture with sodium sulfate and selenium as a salt/catalyst mixture added, to raise the boiling temperature of the digestion mixture. Alkaline phenol was used for color detection. The analysis was done by the Agricultural Diagnostic Services Center, Agronomy and Soil Science Department, University of Hawaii. For ^{15}N determination, 100mg of plant sample were mixed in a digestion tube with 3 ml of salicylic acid in concentrated sulfuric acid with 5g of sodium thiosulfate and allowed to react overnight. Hydrogen peroxide (5ml) and 10 g of a salt mixture consisting of K_2SO_4, $CuSO_4$, and metallic selenium were added to the digestion tubes and the mixture heated for 3 hours in a digestion block. The temperature was increased gradually from 150 to 350 C. The clear digest was mixed with 20 ml of 13N NaOH, steam-distilled, and the ammonia collected in 15 ml of 0.02N H_2SO_4. The distilling apparatus was cleaned between samples by distilling 20 ml of ethanol through it. The collected distillate was evaporated to 1 ml for ^{15}N determination at the Las Alamos Scientific laboratory. Calculation for atom% ^{15}N excess was based on the natural abundance of 0.369 atom % ^{15}N for Kuiaha Soils. The atom % excess refers to the difference between the relative amounts of ^{14}N and ^{15}N in a given material and that of the natural abundance. The natural abundance refers to the relative amounts of ^{14}N and ^{15}N of samples in nature. Both the amount and percent N fixed for each cultivar were determined using the total nitrogen difference method and ^{15}N isotope dilution method. The difference method was based on the difference in total N between the N_2-fixing legume (Nfl) and the reference crop (RC).

Evaluation of Reference Crops: Reference crops or non-fixing control plants are essential when using the ^{15}N isotope dilution method to estimate the nitrogen derived from fixation (Ndfa). The control plants are also used to determine the contribution of soil nitrogen and/or fertilizer N to the N yield of the fixing plant (FP). Although there are several possible non-fixing controls, theoretically, the best control is the fixing system itself in non-fixing mode (Rennie et al., 1984). Thus, in soils where no indigenous rhizobia exist, the uninoculated nodulating cultivar would be an excellent control. In case where indigenous rhizobia exist,

and a number of species are being tested, Rennie (1984) reported that a non-legume such as corn can be used. Under such conditions however, the non-fixing control must assimilate its N from the soil and fertilizer N pool so that maximum N uptake is reached at about the same time after emergence as the fixing plant. This means they should have identical ^{15}N: ^{14}N ratios, but total N does not have to be identical to the legume species (Rennie and Kemp, 1984). The authors also reported that it is crucial that both the fixing and non-fixing controls have similar rooting patterns.

^{15}N Dilution Method: The effect of reference crops on N_2- fixation estimates in field-grown legumes using the ^{15}N isotope dilution method is shown in Table 2. Consistently higher estimates of fixation were obtained with soybean than with corn in all legumes, but the differences in estimates were not significant. It should be borne in mind that where N fixation estimates were negative, a minimum value of 0.1 was included for statistical analysis. In order to compare bush bean (which matured in 60 days) with other reference crops, it was necessary to compare all reference crops with the mean values for 30 and 60 days after emergence. No significant differences were found between the reference crops. However, soybean again gave the highest estimates followed by bush bean while corn gave the lowest estimates for all species. Although there were no significant differences between the reference crops for the N fixation estimates, the low estimates given by corn confirm that N assimilation by corn at 30 and 60 DAE was different from that of soybean and bush bean.

Harvest Date by Reference Crop Interaction by the ^{15}N Method: The use of soybean as a reference crop yielded consistently higher estimates of N fixed in inoculated cowpea than the use of corn at all harvest dates. Cowpea appeared to lose N at 80 days after emergence according to the estimates obtained by using soybean as a reference crop, but gained N when corn was used as a reference crop. Since both inoculated cowpea and corn were physiologically mature at the final harvest and soybean was still accumulating nitrogen, it is possible that estimates made using soybean at 80 days after emergence, may have been due to the differential N uptake by soybean and cowpea. Nevertheless, these estimates, suggest that inoculated cowpea lost more than 10 kg N ha^{-1}. In the case of uninoculated cowpea, there was no significant harvest date by reference crop interaction. However, the use of soybean as a reference crop gave consistently higher estimates than the use of corn. Similarly, there was no significant harvest date by reference crop interaction for N_2-fixation estimates in both inoculated and uninoculated peanut and in inoculated soybean. These results indicate that

when ^{15}N isotope dilution method was used to estimate N fixed in field-grown legumes, the use of soybean as a reference crop resulted in non-significant higher estimates than when corn was used as a reference crop.

Difference Method: With the difference method, the use of soybean as a reference crop yielded significantly higher estimates of nitrogen fixed in uninoculated cowpea than when corn was used. There were so significant differences between the estimates of N fixation in the other species using soybean and corn. When the nitrogen fixation estimates, obtained using all there reference crops, were averaged over the 30 and 60 days and compared, the use of soybean as a reference crop gave significantly higher estimates in all species than the use of corn as a reference crop. The use of soybean as reference crop gave significantly higher estimates in inoculated bush bean than when bush bean was used as a reference crop. Estimates obtained when bush bean was used as a reference crop were significantly different from the estimates obtained when corn was used in most species except inoculated soybean and bush bean. The use of soybean as a reference crop resulted in higher estimates for all species followed by bush bean, while the use of corn always gave the lowest estimates. These results indicate that at 30 and 60 days after emergence, the estimates obtained when soybean and corn were used as reference crops, were significantly different in all species. Averaged over 30, 60, and 80 days after emergence however, the estimates obtained using soybean and corn as reference crops, were significantly different in uninoculated cowpea.

Harvest Date by Reference Crop Interaction by the Different Method: When the difference method was used to estimate the amount of N fixed in various legumes with soybean and corn as reference crops, significant harvest date by reference crop interaction were observed. For inoculated cowpea, there was a significant interaction between harvest date and reference crop. At 60 DAE, the use of soybean as a reference gave significantly higher estimates in inoculated cowpea than the use of corn. At 30 days after emergences, the use of soybean as a reference crop gave non-significant higher estimates than the use of corn. Estimates obtained using corn as a reference crop at 80 DAE was higher than those with soybean but were not significantly different. In the case of uninoculated cowpea, a highly significant harvest date by reference crop interaction ($P<0.01$) was observed. The use of soybean as a reference crop gave significantly higher estimates than the use of corn at 60 days after emergence. The estimates at 30 and 80 DAE using soybean as a reference crop were non-significantly higher than the use of corn. Significant differences between estimates

using soybean and corn as reference crops at 60 DAE were attributed to large differences in N uptake between cowpea and corn at 60 days after emergence. There was no significant harvest date by reference crop interaction for inoculated peanut ($P < .09$). However, the use of soybean as a reference crop gave a significantly higher estimate than the use of corn at 60 DAE. At 80 DAE, the use of corn gave a non-significantly higher estimate than the use of soybean. Higher estimate obtained with corn as a reference crop at 80 DAE. May have been due to the differential N uptake by corn and peanut since peanut was still accumulating N while corn was mature. Moreover, the total N yield by corn at 80 DAE was lower than a 60 DAE, suggesting that corn must have lost N between 60 and 80 DAE. Such a loss of total N by corn explains why N fixation in inoculated peanut may have been overestimated. For uninoculated peanut, the use of soybean as a reference crop gave significantly higher estimates that the use of corn as a reference crop at 60 DAE (Figure 14). At 30 and 80 DAE, the estimates using both soybean and corn as reference crops were not significantly different although the use of soybean as a reference crop gave higher estimate than they use of corn. There was no significant harvest date by reference crop interaction for the N_2-fixation estimates in inoculated soybean (Figure 15). However, the use of soybean as a reference crop gave consistently higher estimates than the use of corn at all dates. At 80 DAE, estimates by corn and soybean were close probably because corn lost N at 80 DAE and the amount of N fixed by inoculated soybean may have been overestimated. These results indicate that on the average, the N fixation estimates obtained by using soybean as a reference crop were higher than those obtained when bush bean and corn were used as reference crops. The N uptake pattern for the soybean reference crop was similar to that of inoculated peanut, uninoculated peanut and inoculated soybean. The N uptake pattern of corn was similar to that of the inoculated cowpea, but different from those of other legumes. The harvest elate by reference crop interactions which were observed with the difference method might have been caused by the large differences in N uptake pattern between corn and the legumes. The results are also in agreement with those of Witty 1983), who found that the ideal legume-control combination should have similar rooting patterns and similar N uptake profiles, specifically the same crop growth constant and the same time to half maximum N content.

Nitrogen Fixation Estimates by the ^{15}N Method: The two parameters evaluated in the N_2-fixation estimates by the ^{15}N method were harvest date and the reference crops. As displayed in table 6, inoculated cowpea attained maximum N content between 60 and 80 days after emergence (DAE). There were no significant differences between the estimates at 60 and 80

DAE. However, estimates at 30 days were significantly different from estimates at 60 and 80 DAE. Nitrogen fixation estimates in uninoculated cowpea at 60 and 80 were not significantly different. Maximum N content in uninoculated cowpea was at 80 DAE. However, estimates at 30 DAE were significantly different from estimates at 60 and 80 DAE. While N fixation estimates in inoculated cowpea showed a decline at 80 DAE, N fixation estimates in uninoculated cowpea showed an increase. It appears from these results that the N fixation period in inoculated cowpea was shorter than that in uninoculated cowpea probably due to different rhizobia strains. N_2-fixation estimates in inoculated peanut at 80 DAE were significantly different from estimates at 30 and 60 DAE which were also significantly different from each other. Similarly, N fixation estimates in uninoculated peanut at 80 DAE were significantly different from estimates at 60 and 30 DAE which also were significantly different from each other. Although the N fixation estimates in inoculated peanut were not significantly different from the estimates in uninoculated peanut at each harvest date, the estimates in inoculated peanut were higher than those in uninoculated peanut at 60 and 80 DAE. N fixation estimates in inoculated soybean at 80 DAE were significantly different from estimates at 60 and 30 DAE which were also significantly different from each other. In the case of bush bean, N fixation estimates at 30 and 60 DAE were not significantly different. These results indicate that N fixation estimates in inoculated peanut, uninoculated peanut and inoculated soybean followed the same pattern, increasing from lowest at 30 DAE to highest at 80 DAE. This is probably because peanut and soybean were long duration crops compared to bush bean and cowpea which were short-duration crops. Within species however, only cowpea attained maximum nitrogen fixation at different harvest dates probably as a result of inoculation with exotic or native Rhizobium strains in the inoculated and uninoculated cowpea, respectively.

N_2-Fixation Estimates by the Difference Method: The difference method has been reported to be less accurate than the ^{15}N isotope dilution method by many workers (Rennie et al. 1984., Patterson 1982., Vasilas et al. 1984). The N fixation estimates calculated using the difference method was based on the total N balance between a N_2 fixing (F1) and a non-fixing system (nFs). Thus, N_2-fixed=N yield (Nfl)- N yield (RC)..(1) The effect of harvest date on the mean of nitrogen fixation estimates using soybean, bushbean and corn as reference crops in field-grown legumes calculated using the difference method. N fixation estimates in inoculated cowpea at 30 DAE were significantly different from each other. Maximum N fixation in inoculated cowpea occurred between 60 and 80 DAE. At 80 DAE, N

fixation estimates in inoculated cowpea were less than the estimates at 60 DAE, indicating that inoculated cowpea lost N. N fixation estimates in uninoculated cowpea at 30 DAE were significantly different from estimates at 60 and 80 DAE which were not significantly different from each other. At 80 DAE, N fixation estimates in uninoculated cowpea were still increasing. N fixation estimates at 30 DAE in inoculated and uninoculated peanut were significantly different from estimates at 60 and 80 DAE which were significantly different from each other. N fixation estimates at 80 DAE in both inoculated and uninoculated peanut were still increasing. Similarly, N fixation estimates at 30 DAE in inoculated soybean were significantly different from estimates at 60 and 80 DAE which were significantly different from each other. At 30 and 60 DAE, N fixation estimates in inoculated bush bean were not significantly different. These results indicate that N fixation estimates in a short-duration crop such as bush bean were not significantly different at 30 and 60 DAE. For an intermediate-duration crop such as cowpea, N fixation estimates at 30 DAE were significantly different from estimates at 60 and 80 DAE which were in turn not significantly different from each other. In long-duration crops such as peanut and soybean, N fixation estimates at 30, 60, and 80 DAE were significantly different from each other.

Comparison of the Methods: The parameter which was used in the evaluation of the two methods was the amount of nitrogen fixed by all the inoculated legumes at three harvest dates using the three reference crops. The relationship of the estimates for the amount of nitrogen fixed at 30 days after emergence using soybean as a reference crop is displayed. The correlation between the difference and the ^{15}N isotope dilution methods was very low (r=0.15). When bush bean was used as a reference crop, the correlation was also low (r=0.38) as displayed. When corn was used as a reference crop, the relationship between the estimates by the difference and ^{15}N methods was negative (r=-17). These results indicate that there was no agreement between the two methods using soybean. Bush bean and corn reference crops at 30 DAE. At 60 days after emergence however, the correlation (r=0.82**) for the relationship of the estimates by the difference and the ^{15}N isotope dilution method using soybean as a reference crop was high and significant. Similarly, the correlation (r=0.77**) for the relationship between the estimates by the difference and the ^{15}N isotope dilution methods using bush bean was high and significant. When corn was used as a reference crop, the correlation (r=0.11) for the relationship between the difference and the ^{15}N isotope dilution methods was very low (Figure 21). At 80 DAE, the correlations (r=0.91** and r=0.69**) for the relationship between the estimates by the difference and the ^{15}N dilution methods using

soybean and corn, respectively, were high. At 30 DAE, the coefficients of variation of the estimates by the difference method using soybean, bush bean, and corn were 110.3, 141.7, and 234.9 respectively. At 60 DAE, the coefficients of variation (31.7, 43.1, and 111.7) of the estimates by the difference method using soybean, bush bean, and corn as a reference crop respectively were lower than those obtained at 30 DAE. However, the coefficient of variation of the estimates by the difference method using corn as a reference crop at 60 DAE was still very high compared to those obtained when soybean and bush bean were used as reference crops. At 80 DAE, bush bean was already mature and the coefficients of variation (28.3 and 49.8) of the estimates by the difference method using soybean and corn respectively were lower than those obtained at 60 DAE. The lowest coefficient of variation (28.3) of the estimates by the difference method was obtained when soybean was used as a reference crop at 80 DAE. This explains why the best agreement (r=0.91**) between the estimates by the difference and the ^{15}N isotope dilution methods was obtained at 80 DAE using soybean as a reference crop. These results indicate that agreement between the estimates by the difference and ^{15}N isotope dilution methods was possible depending on (1) the time of harvest, (2) the type of reference crop used, and (3) the coefficient of variation of the estimates by the difference method. Thus, at 30 DAE, there was no agreement between the estimates by the difference and the ^{15}N isotope dilution methods. At 60 DAE however, there was agreement between the two methods when soybean and bush bean were used as reference crops, but not when corn was used as a reference crop. At 80 DAE, the best agreement between the estimates by the difference and the ^{15}N isotope dilution methods was obtained with soybean as the reference crop rather than corn. There was agreement between the estimates by the two methods whenever the coefficients of variation of the estimates by the difference method were less than 100%. These results are in agreement with those of Talbott et al. (1982). They found close agreement between the ^{15}N and the difference methods with correlations ranging from r=0.89 to r=0.92 in two sets of experiments. The authors however, found poor agreement between the two methods (r=0.38) when the percent of total nitrogen fixed (%Ndfa) was used as the parameter for the evaluation. Rennie (1984), working with phaseolus cultivars also obtained good agreement between the ^{15}N and difference methods most of the time when the soil n values were low with only isolated instances of good agreement when soil N values were high. Vasilas et al., (1984) also found excellent agreement between N fixation estimates of the ^{15}N and the difference methods estimates when soil N conditions permitted proper development of the control plants, but did not depress N_2 fixation.

RESULTS AND DISCUSSION

As most of the tropical soils are limited in their ability to sustain continuous crop production due to low nitrogen and the cost of inorganic fertilizer nitrogen increased, biological nitrogen fixation became the only alternative cheap source of nitrogen for crop production. Information on the residual N contribution to the cropping system by cowpea, peanut, soybean and bush bean can be used by farmers to improve their crop production practices.

Land Preparation: The experiment was planted approximately one year after the first experiment. Sweet corn (U.H # 9) cv. "Super sweet" was grown on plots that had either been left fallow, grown cowpea, peanut, soybean, and bush bean inoculated or uninoculated, or sweet corn in the previously described experiment. Weeds were kept to a minimum after harvesting the first experiment. A rotovator was used to plow the plots before planting.

Experimental Design: The experiment had been installed in a randomized complete block design with 12 treatment replicated four times in the first experiment. Plots consisted of 4 rows 5 meters long and 65 cm apart.

Treatment Design: The treatments were as described in Experiment I plus three plots which had been left fallow. Urea was applied to all fallow plots at 0, 50 and 100 kg N ha^{-1}; these amendments represent treatments 10, 11 and 12.

Planting and Management: Sweet corn (U.H # 9) cv. "Super sweet" was planted in all plots at 20 cm between hills and 65 cm between rows, giving a plant population of approximately 76,000 plants ha^{-1}. Rows in each plot were run approximately along the rows of the previous experiment with equal width. Furadan was applied at the time of planting at a rate of 3g/linear meter in furrow rows together with seeds to control stem borers. Drip irrigation lines were laid along the corn rows and plots were irrigated to field capacity every time the top two inches of the soils were dry. Nitrogen in the form of Urea was applied to the fallow plots at 0, 50 and 100 kg N ha^{-1} in two doses, 1/3 at planting and 2/3 at 40 days after planting.

Harvest and Data Collection: Plant samples for fresh and dry weight were harvested from three meters of the inner rows of each plot 50 days after planting. Subsamples were taken, fresh weight recorded and then subsamples were oven dried at 70^0 and dry weight recorded. Samples were then ground to pass a 1 mm screen, and sub sampled for N analysis. Total shoot N was determined on above-ground plant parts. Nitrogen was determined by the

Agricultural Diagnostic Services Center, Agronomy and Soil Science Department, University of Hawaii. Analysis of variance and the Waller test were used to compare treatment means of total N yield.

N Yield: Fallow + 100 kg N ha^{-1} gave the highest N yield and was significantly different from other treatments. Inoculated cowpea gave the second highest N yield followed by Fallow +50 kg N ha^{-1} (II) but the two treatments were not significantly different from each other, and were not significantly different from the corn, uninoculated soybean, inoculated soybean, uninoculated cowpea, and Fallow + 0 kg N ha^{-1}. They were, however, significantly different from plots which grew inoculated bush bean, inoculated peanut, uninoculated bush bean, and uninoculated peanut. Since most of the N contributed by the corn plot came from mineralized soil nitrogen and these contributed more N than the Fallow + 0 kg 14 ha^{-1}, it appears that mineralization of soil N in plots that previously grew corn was higher than in fallow plots. However, total N yield by corn in Experiment I at 60 days after emergence was 106.4 kg N ha^{-1} while total N yield of corn at 50 days after emergence was 59.15kg N ha^{-1} in Experiment II. It is not clear whether the N that was lost by both corn and inoculated cowpea in Experiment I was responsible for the relatively high residual N contribution to the subsequent crop in Experiment II. These findings emphasize the difficulties involved in quantifying the residual nitrogen from the BNF to the cropping systems. The major conclusion drawn from a field experiment to evaluate the measurement of nitrogen fixation by field-grown legumes is given below.

- Soil N was sufficiently high that it suppressed the inoculation response of bush bean, an early maturing legume, but not of soybean, a late maturing legume, indicating that soil N level affects the inoculation response by field-grown legumes with varying rhizobial requirements.

- There was no relationship between nodulation indices and dry matter yield at 35 days after emergence.

- There was no significant difference in ^{15}N uptake between the reference crops, soybean, bush bean and corn.

- There were no significant differences between N fixation estimates using soybean and corn as reference crops with the ^{15}N isotope dilution method.

- Nitrogen fixation estimates using soybean as the reference crop were significantly higher than the estimates using corn as the reference crop by the difference method for inoculated cowpea, uninoculated cowpea and uninoculated peanut. However, N fixation estimates using soybean as a reference crop were not significantly different from the estimates using corn as a reference crop for inoculated peanut and soybean.

- The use of bush bean as a reference crop was only suitable for inoculated bush bean since it matured before the other species.

- There was no agreement between the ^{15}N isotope dilution and the difference methods in the estimates of the amount of N fixed at 30 days after emergence with any of the three reference crops.

- There was agreement between the estimates by the ^{15}N and the difference method at 60 days after emergence using soybean and bush bean as reference crops and at 80 days using soybean and corn as reference crops.

- The best agreement between the two methods was obtained at 80 DAE using soybean as a reference crop.

- The coefficient of variation for the N fixation estimates by the difference method was lowest with soybean and highest with corn as reference crops at all three harvest dates.

- It is difficult to estimate residual N contributed by field-grown legumes without measuring the portion of the soil N that was mineralized during the period between the legume and the subsequent crop.

In recent years, our understanding of biological nitrogen fixation has been bolstered by a diverse array of scientific techniques. Still, the origin and extant distribution of nitrogen fixation has been perplexing from a phylogenetic perspective, largely because of factors that confound molecular phylogeny such as sequence divergence, paralogy, and horizontal gene transfer. Here, we make use of 110 publicly available complete genome sequences to understand how the core components of nitrogenase, including NifH, NifD, NifK, NifE and NifN proteins, have evolved. These genes are universal in nitrogen fixing organisms-typically found within highly conserved operons-and, overall, have remarkably congruent phylogenetic histories. Additional clues to the early origins of this system are available from

two distinct clades of nitrogenaseparalogs; a grou composed of genes essential to photosynthetic pigment biosynthesis and a group of uncharacterized genes present in methanogens and in some photosynthetic bacteria. We explore the complex genetic history of the nitrogenase family, which is replete with gene duplication, recruitment, fusion and horizontal gene transfer and discuss these events in light of the hypothesized presence of nitrogenase in the last common ancestor of modern organisms, as well as the additional possibility that nitrogen fixation might have evolved later, perhaps in methanogenicarchaea, and was subsequently transferred into the bacterial domain. Bacteroid differentiation was examined in developing and mature alfalfa nodules elicited by wild-type or Fix-mutant strains of Rhizobium meliloti. Ultra structural studies of wild-type nodules distinguished five steps in bacteroid differentiation (types 1 to 5), each being restricted to a well-difined histological region of the nodule. Correlative studies between nodule development, bacteriod differentiation and acetylene reduction showed that nitrogenase activity was always associated with the differentiation of the distal zone III of the nodule. In this region, the invaded cells were filled with heterogeneous type 4 bacteroids, the cytoplasm of which displayed an alternation of areas enriched with ribosomes or with DNA fibrils. Cytological studies of complementary halves of transversally sectioned mature nodules confirmed that type 4 bacteroids were always observed in the half of the nodule expressing nitrogenase activity, while the presence of type 5 bacteroids could never be correlated with acctylene reduction. Bacteria with a transposon Tn5 insertion in pSym fix genes elicited the development of Fix- nodules in which bacteroids could not develop into the last two ultrastructural types. The use of mutant strains deleted of DNA fragments bearing functional reiterated psym fix genes and complemented with recombinant plasmids, each carrying one of these fragments, strengthened the correlation between the occurrence of type 4 bactoriods and acetylene reduction. A new nomenclature is proposed to distinguish the histological areas in alfalfa nodules which accound for and are correlated with the multiple stages of bacteroid development.

CONCLUSION

Ancient farmers would not have known about nitrogen fixing bacteria but they did know that growing pulses yielded good food and helped other crops grow. In fact of the eight Neolithic founder crops- the first plants domesticated by man- four were pulses. Like our ancient ancestors, modern farmer’s plant pulses to in effect their other crops. I just did this myself in the community garden plot where I grow vegetables. The whole garden here is regularly

rototilled at both the beginning and end of the season. Unfortunately while this looks neat tidy and ready to plant, it really does a number on the soil life. The bean plant roots did not have a single nodule. While you will still get a crop from the seed and sow both soil and crop are improved if the right nitrogen fixing bacteria are present. As a conclusion it has been observed that Soya bean, Pisum sativum and beans fix more atmospheric free nitrogen into nitrates and nitrite compounds, because they have nodules attached with their roots in which nitrogen fixing bacteria like rhizobium, Nostoe, Eubacteria are present, economically the family is second only to the grasses in importance. Pulses provide valuable and nutritive foods because the food stored for the in the seed is rich in protein. These food and forage pulses are cheap among the plants used as green manure. Nitrogen fixing bacteria dwelling in nodules of the roots of most pulses fix free nitrogen from the air into the nitrogenous compounds needed by all forms of life for building proteins. They are used in agricultural practice. The pulse family also provides gums and resins. Timber, medicines, perfume oils, vegetable oils and other commercial items such as fibers and insecticides. Pulses are economically and commercially important for food, beverages and forage. Pulses are rich source of protein. We predict the anthropogenic N-fixation rate will increase by about 60% by the year 2020, primarily due to increased fertilizer use occur in Asia, which by 2020 will account for over half of the global anthropogenic N fixation. This is a very important work, dealing very fully with all the more important tropical crops and giving shorter descriptions of many others of lesser importance, such as fruits and vegetables. It is founded on a wide survey of the literature and includes a large number of references, but it also embodies much direct study of the living plants.

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