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Statewide Search for Highly Active Associative N-Fixation Systems[1]

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ABSTRACT

A search was conducted in 57 of Florida's 67 counties for highly active, associative (grass-bacteria) N_2-fixing sites. Two hundred sixty-four soil-grass cores were analyzed for nitrogenase activity by the acetylene reduction method (AR). Cores that were collected by Extension Service personnel, shipped to Gainesville and analyzed after standardization in the greenhouse gave results similar to cores that were taken and analyzed directly.

Four sites had cores which evolved over 1000 nM ethylene core^{-1}hr^{-1}, which is very high for grass-root-soil cores. Nineteen sites had cores with over 500 nM core^{-1}hr^{-1} and 34 cores had over 300 nM core^{-1}hr^{-1}. *Paspalum notatum* Flügge was the most prevalent grass species in the highly active cores, and *Cynodon dactylon* (L.) Pers. was the next most prevalent.

Soil moisture and zinc content were positively correlated and calcium content was negatively correlated with AR activity. Elevated soil moisture was required for high AR, but not all cores with elevated soil moisture were active.

Acetylene reduction was positively correlated with CO_2 concentration and negatively correlated with O_2 concentration in the cores at the end of incubation. These active sites are being evaluated further to determine the necessary requirements and best organisms for active associative N_2-fixation.

Additional Index Words: Acetylene reduction, Nitrogenase activity, Soil moisture, Soil-grass cores, Soil minerals, Zinc.

INTRODUCTION

Associative N_2-fixation has attracted considerable research attention in recent years, much of which has been reviewed by van Berkum and Bohlool (20). This research has led to the conclusion that these systems fix low, but often agronomically important, amounts

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of nitrogen (2, 3, 5, 6, 7, 8, 9, 19). In field inoculation studies we were able to increase yields 20 to 40% for grasses that had been fertilized lightly with nitrogen and inoculated with *Azospirillum brasilense* Krieg (4, 12, 13, 14). Other studies in various parts of the world have corroborated these results (10, 11, 16, 17). Yield responses to inoculation have been consistent in Israel (10, 11) while ours have been variable (12, 13, 14).

Although inoculation has increased dry matter yields, we have been unable to establish the inoculated *Azospirillum* in our soils or rhizospheres. Our data (unpublished) indicate that the inoculated strains do not become established in field plots, and we have not found a correlation between inoculation and subsequent acetylene reduction (AR) rates (1, 17). Other data from natural field cores have shown relatively high background levels of AR activity (our unpublished results). This indicates that more effective, better-adapted strains of N_2-fixing bacteria may be present in Florida soils. If these better-adapted strains can be identified and isolated, they then can be used as inoculum.

The results from a systematic search for natural, highly active N_2-fixing sites in Florida are reported here. In addition to serving as a source for adapted N_2-fixing bacteria, the highly active sites were compared with those of low activity for the purpose of identifying environmental or other conditions necessary for high activity.

METHODOLOGY

We were mainly interested in measuring nitrogenase activity of sites where plants (especially grasses) were growing better than expected based on past nitrogen application. We wanted to avoid sites that had been fertilized recently or regularly with nitrogen and also nitrogen enriched areas such as old corral sites or animal bed grounds. We wanted to sample a broad geographic area including diverse soil types and plant species. To provide the needed local expertise in locating and sampling the desired sites over this large area, we requested assistance from personnel of the Florida Extension Service.

We assembled a sampling kit and distributed it to each county to facilitate sampling. It consisted of: 1) instructions for identifying the desired sites and for taking soil-plant cores, 2) plastic soil core tubes made by cutting 11 cm plastic pipe (PVC) into 15 cm lengths and sharpening one end for easier soil penetration, 3) data sheets for describing site and plant species information, and 4) packing materials for shipping the cores back to Gainesville.

Soil-plant cores were returned to Gainesville where they were standardized in the greenhouse for growth and soil moisture. River gravel was placed on the surface of the cores to control blue-green algae growth.

The acetylene reduction (AR) assays used to estimate the nitrogenase activity of the cores were conducted two ways. The greenhouse standardized cores were incubated overnight in plexiglass chambers, 16 cm dia x 51 cm tall, in the light (280 $\mu Em^{-2}sec^{-1}$) at 30°C. Before incubation, oxygen content was reduced to 5% by flushing with argon or helium and 10% acetylene was added. Ethylene evolution was measured by gas chromatography (GC). The cores where AR was measured directly were taken in steel tubes 8.5 or 11 cm dia by 17.7 cm long that were sealed by rubber caps and septum stoppers. (Data were adjusted to a standard 11 cm dia core size). The cores were incubated overnight after adding 10% acetylene. Gas samples were removed after incubation for immediate GC analysis or were stored in Vacutainers® for later analysis.

Soil samples were taken from the core at the end of the AR assay and oven-dried to determine moisture content. Soil analyses for P, K, Ca, Fe, Mg, Mn, Mo, Zn, and pH were conducted by the IFAS Soil Science Laboratories.

Estimates of core respiration were presumed by analyzing CO_2 and O_2 after core incubation. This was done by GC (thermal conductivity detector) using a Poropak N column to separate CO_2 and a Molecular Sieve 5A for O_2 and N_2. Nitrogen served as an internal standard.

Statistical analyses were conducted using SAS (Statistical Analysis System). AR values were transformed using log transformation to stabilize the variances in the statistical analyses.

RESULTS AND DISCUSSION

Two hundred sixty-four cores were analyzed for AR from 57 of Florida's 67 counties, giving us data from a broad area of diverse soils and plant species. Only 2 to 10 cores were taken per county in this initial study.

AR activities over 1000 nM ethylene evolved $core^{-1}hr^{-1}$, which is very active for grass-soil cores, were obtained from four of the sites sampled. These sites were in Clay (1501 nM), Madison (1227 nM), Putnam (1094 nM) and Union (1159 nM) counties. Extrapolations made solely to help the reader better understand the magnitude of these AR activities, gave quantities of fixed nitrogen in the range of 20 to 30 kg ha^{-1} $season^{-1}$. These extrapolations were based on a ratio of 4 moles of ethylene to 1 mole of nitrogen and a 135-day season. Since the AR assay is a short term assay and several of the necessary assumptions have not been verified we do not imply this much nitrogen was actually fixed.

Table 1 summarizes the counties producing highly to moderately active cores (above 500 nM ethylene $core^{-1}hr^{-1}$). Acetylene reduction activities of over 500 nM were measured in 7.2% of the sites, over 400 nM in 9.1% of the sites, and over 300 nM in 12.9% of the sites. The proportion of highly active cores was about the same whether the cores were sent in and standardized in the greenhouse or measured directly.

Figure 1 shows the counties that were sampled and those with AR activities over 500 nM $core^{-1}hr^{-1}$. These most active cores came primarily from the northern portion of the state. More intensive sampling was done there, but we believe this may not fully account for the greater number of highly active cores assayed there.

Table 2 summarizes the grass species with AR activities over 300 nM $core^{-1}hr^{-1}$. Of the four cores over 1000 nM $core^{-1}hr^{-1}$, three were *Paspalum notatum* Flügge. Forty-seven percent of cores over 500 nM and 41% of those over 300 nM $core^{-1}hr^{-1}$ were *P. notatum*. This species was found in 37% of all cores sampled and was widely distributed in all counties and over all soil types. The second most prevalent species with high

TABLE 1.—SUMMARY SHOWING COUNTIES SUBMITTING GRASS-SOIL CORES WITH AR ACTIVITIES OF 500 nM CORE^{-1}HR^{-1} OR HIGHER.

County	Total no. cores	No. of cores with AR activity above: 500 nM core^{-1}hr^{-1}	400 nM core^{-1}hr^{-1}	300 nM core^{-1}hr^{-1}
Alachua	8	1	2	3
Bradford	6	1	1	3
Charlotte	4	0	0	1
Clay	4	3	3	3
Dade	7	0	2	2
Dixie	2	1	1	1
Gadsden	9	0	1	3
Glades	4	1	1	1
Hernando	4	0	0	1
Jefferson	4	3	3	4
Leon	4	1	1	1
Madison	5	1	1	1
Manatee	4	1	1	1
Marion	4	1	1	1
Martin	4	0	0	1
Okaloosa	1	0	1	1
Putnam	3	1	1	1
St. Lucie	6	0	0	1
Sumter	6	1	1	1
Union	6	1	1	1
Unlisted*	108			
Total	204	19	26	34
% of Total		7.2	9.1	12.9

* Twenty-eight counties produced cores whose AR activities were below 300 nM core^{-1}hr^{-1}.

activity was *Cynodon dactylon* (L.) Pers. The list includes 15 species of both improved and native types.

Occasionally a core would have many times higher activity than the mean of the experiment. To check the consistency of the AR data, we resampled 11 of the sites. Six high activity (three above 1000 nM core^{-1}hr^{-1} and three above 500 nM core^{-1}hr^{-1}), one moderate activity (above 300 nM) and four inactive sites (below 50 nM) were selected for replicated resampling. These AR data are presented in Table 3. The inactive sites remained inactive. The activities of the highly active sites were generally lower in the resampled data, at least partly due to drier soil and the lateness of the season (after September). With the exception of the Union county Coastal bermudagrass site which was severely stressed because of close grazing, the highly active cores remained active, (range 210-1057 nM core^{-1}hr^{-1}) indicating a trend for the active sites to remain active.

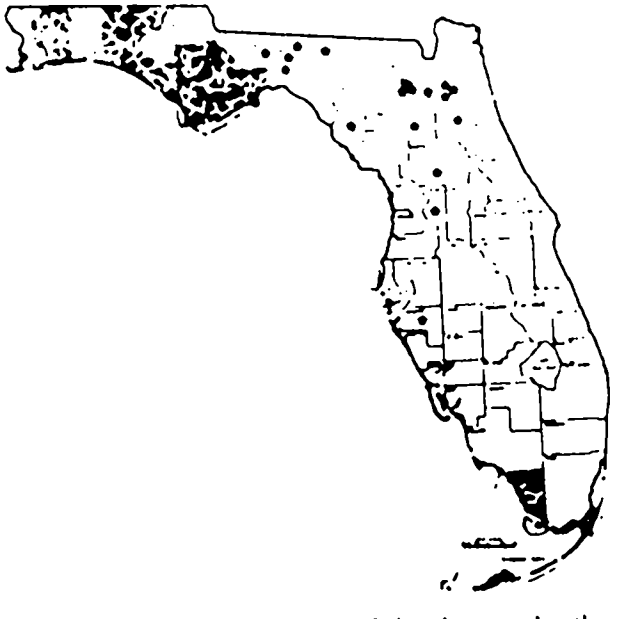

Fig. 1.—Map showing counties sampled and county locations of the 19 highly active (AR) sites. Shaded counties were not sampled. Stars indicate counties producing highly active cores (above 500 nM ethylene core^{-1}hr^{-1}).

A comparison of soil factors of active and inactive sites is shown in Table 4. Stepwise multiple regression analyses of these factors and AR values indicate significant effects for soil moisture ($p = 0.001$), Ca concentration ($p = 0.009$) and Zn concentration ($p = 0.039$). Soil moisture appeared to be an important factor ($r = 0.70$, $p = 0.0001$), with high soil moisture being necessary for high activity (also see Figure 2). High Ca was associated with low AR in these data, but in other studies where Ca content was much lower the reverse was true. Here, the Ca data was biased by some high Ca roadside samples. Both Zn and Mn appear to be below plant deficiency levels in the low activity sites. Both Mn and Fe were significantly correlated with AR ($r = 0.35$, $p = 0.0015$ and $r = 0.34$, $p = 0.0015$, respectively), but this was not indicated in the regression analysis.

Figure 2 shows the effect of soil moisture on acetylene reduction. As soil moisture increased, AR generally increased. In all cases high AR was associated with high soil moisture.

Mean CO_2 and O_2 concentrations in the cores were measured at the end of AR incubation periods for both high and low activity sites (Table 5). During incubation, carbon dioxide accumulation was higher (14.3%) in the containers from the active sites than in those from in the inactive sites (10.0%). Oxygen was reduced from ambient to 2.5% in the active site cores and to 5.1% in the inactive site cores. The increase in CO_2 and decrease in O_2 may be a presumption of different respiratory activities in the respective cores. Simple correlations with AR were positively significant for CO_2 ($r = 0.49$) and negatively for O_2 ($r = -0.47$).

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TABLE 2.—SUMMARY OF GRASS SPECIES AND NUMBER OF CORES WITH AR ACTIVITIES OVER 500 nM ETHYLENE EVOLVED CORE^{-1}HR^{-1}.

AR Activities					
Over 1000 nM		Over 500 nM		Over 500 nM	
No. Cores	Species	No. Cores	Species	No. Cores	Species
3	*Paspalum notatum*	9	*P. notatum*	14	*P. notatum*
1	*Cynodon dactylon*	3	*C. dactylon*	4	*C. dactylon*
4	Total	2	*Heteropogon* sp.	2	*Heteropogon* sp.
		1	*Eriochloa ophiuroides*	2	*Paspalum urvillei*
		1	*Imperata cylindrica*	2	*Panicum* sp.
		1	*Panicum repens*	2	*Stenotaphrum secundatum*
		1	*Panicum* sp.	1	*E. ophiuroides*
		1	Unknown	1	*I. cylindrica*
		19	Total	1	*Sporobolus poiretii*
				1	*Panicum hemitomon*
				1	*Panicum repens*
				1	*Hemarthria altissima*
				1	*Pennisetum americanum*
				1	Unknown
				34	Total

TABLE 3.—ACETYLENE REDUCTION CONSISTENCY OF ACTIVE AND INACTIVE SITES UPON SUBSEQUENT ANALYSIS.

AR Activity	Site County	Grass species	Initial Assay	Subsequent Assay*
			(nM ethylene core^{-1}hr^{-1})	(nM ethylene core^{-1}hr^{-1})
High	Madison	Pensacola bahiagrass	1226	1057 (12)
	Putnam	Pensacola bahiagrass	1094	210 (4)
	Union	Broad-leaf bahiagrass	659	759 (6)
		Coastal bermudagrass	1088	74** (12)
	Manatee	Cogan's grass	629	465 (12)
	Bradford	Centipede grass	522	511 (6)
Moderate	Levy	St. Augustine grass	508	59 (8)
Low	Madison	Pensacola bahiagrass	55	55 (12)
	Taylor	Centipede grass	1	56 (12)
	Lake	Pampas grass	7	59 (6)
	Baker	Bermudagrass	25	52 (8)

*The number of cores sampled is shown in parentheses.
**The Coastal bermudagrass had been cut and was being closely grazed at second sampling date.

CONCLUSIONS

Highly active, associative N_2-fixing systems, as measured by AR, were found at various locations in Florida. All of the highly active cores came from moist sites. We believe that high moisture enhances AR activity by reducing O_2 in addition to the moisture effect *per se*. Oxygen can inactivate nitrogenase, and with the exception of *Azotobacter* sp., associative N_2-fixing organisms have no effective O_2 protection mechanisms. Excessive soil O_2 can reduce N_2-fixing activity.

TABLE 4.—MEAN SOIL FACTOR VALUES FOR ACTIVE AND INACTIVE SITES AND THEIR RELATION TO AR BY MULTIPLE REGRESSION.

Factor (Units)	Acetylene Reduction Activity Group	
	High	Low
AR (nM Core^{-1}hr^{-1})	597	35
pH (pH units)	6.1	6.5
Ca (ppm)*	882	1518
Mg (ppm)	57	72
K (ppm)	21	25
P (ppm)	50	35
Fe (ppm)	45	11
Mn (ppm)	6.2	3.7
Zn (ppm)*	2.4	0.9
Moisture (percent)**	14.0	7.5

*Significant 0.05 and **at the 0.01 P level calculated by SAS Stepwise Multiple Regression Procedure.

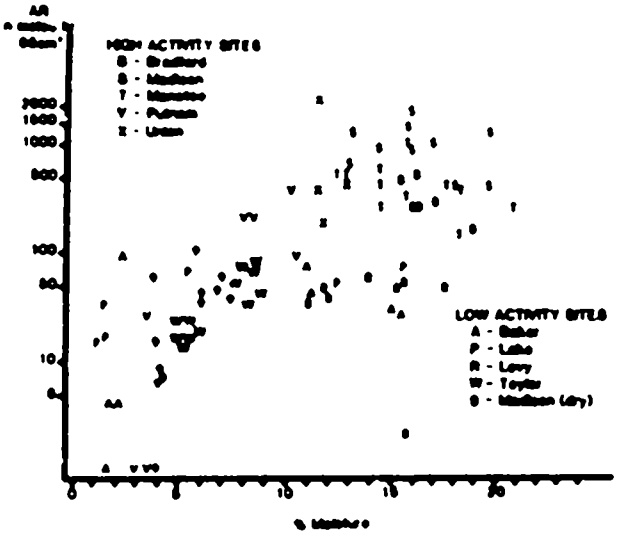

Fig. 2.—Acetylene reduction activity in nM hr^{-1}core^{-1} (95 cm^2 soil surface area) plotted versus percent soil moisture.

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TABLE 5 – MEAN CO_2 AND O_2 VALUES OF CORES FROM ACTIVE AND INACTIVE SITES AS ESTIMATES OF CORE RESPIRATION DURING INCUBATION.

Variable	Low Activity sites	High Activity sites
Acetylene Reduction*	35	59.7
% CO_2	10.0	14.3
% O_2	5.1	2.5

*Acetylene reduction in nM ethylene evolved core^{-1}hr^{-1}.

No particular grass species appeared to be highly superior in supporting AR (N_2-fixation). *Paspalum notatum* was the most prevalent species with high activity. At locations showing moderate to high activity, *P. notatum* was more frequent than expected, indicating a possible species effect. However, we believe that site is more important than species in activity. Species on wet sites generally were more active than on dry sites.

We are studying the 19 highly active sites further to determine necessary soil and microbiological factors necessary for high AR (N_2-fixing) activity.

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