FERTILIZER-NITROGEN AND COVER-CROP ROTATION EFFECTS ON INBRED MAIZE (*ZEA MAYS L.*) YIELD AND SOIL QUALITY

by

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ABSTRACT

This thesis evaluates the effects of fertilizer-nitrogen (N) and cowpea (*Vigna unguiculata* (L.) Walp.) cover crop (CC) rotation on inbred maize production, microbial community structure and activity and soil quality. The study was conducted in field plots established in the municipality of Guayama, Puerto Rico. The cropping systems evaluated were a maize-cowpea cover crop rotation vs a typical practice of maize fallow (FA), each with five fertilizer-N levels. The maize planting sequence was an initial spring 2014 planting, followed by a winter 2014-2015 planting and a final winter 2015-2016 planting. The cowpea cover crop was planted in the summers of 2014, 2015 and 2016. The inbred maize lines were SSH65VH in 2013-2014 and 2014-2015 and SLM15VH in 2015-2016. Mean maize plant densities ranged from 59,391 to 69,182 plants/ha for the cropping seasons. The soil plant analysis development (SPAD-502) chlorophyll meter® (leaf greenness), NDVI (normalized difference vegetation index measured with GreenSeeker®) and plant height measurements were used as indicators of crop N status. Fertilizer-N applications and CC rotation significantly influenced ($p<0.05$) leaf greenness and plant height. Fertilizer-N rates significantly influenced maize seed yield. Nutrient use efficiency (NUE) was measured as partial factor productivity (PFP), agronomic efficiency (AE), partial nutrient balance (PNB), apparent recovery utilization (RE), internal utilization efficiency (IE) and physiological efficiency (PE). Optimal NUE values were reached for PFP, AE and RE at 90 kg N/ha (2013-2015) and 50 kg N/ha (2015-2016). Non-linear regression models indicated fertilizer-N requirements of 143 kg N/ha at a seed yield of 6,917 kg/ha for the 2013-2014 season, 156 kg N/ha with a 4,579 kg/ha seed yield for the 2014-2015 season and 36 kg N/ha at a yield of 2,535 kg/ha for the 2015-2016 season. Fertilizer-N applications decreased soil pH with increasing N rates. The soil management assessment framework (SMAF) soil quality index (SQI) score was higher for cover crop treatment

than fallow. The lowest mean SQI (%) score was obtained for the biological and biochemical category with 43. This indicates that more management practices that improve soil biology can be implemented. Fatty acid methyl esters (FAME's) were used to assess microbial community composition and soil enzyme activity was used to assess microbial community activity. The microbial community was influenced by CC rotation but not by fertilizer-N applications. Bacterial relative abundance for CC rotation was 15.6% greater than FA rotation. Enzyme activity was also influenced solely by CC rotation. Results from a combined enzyme activity assay (β-glucosidase, β-glucosaminidase and acid phosphatase) showed higher total enzyme abundance in CC treatment when compared to FA. Overall fertilizer-N rates affected plant height, SPAD-502, seed yield, immediately available N (0-30 cm), potentially leached N (30-90 cm) and soil pH at varying seasons. Cover crop rotation during different seasons affected all three agronomic indicators (SPAD-502, NDVI and plant height), soil pH, potentially mineralizable N, immediately available N, and microbial community structure and activity.

RESUMEN

Esta tesis describe los efectos de fertilizante-nitrogenado (N) y rotación con la cobertora caupí (*Vigna unguiculata* (L.) Walp.) (CC) sobre la producción en líneas de maíz endogámicas, la estructura y actividad de la comunidad microbiana y la calidad del suelo. Esto se evaluó en un experimento en el municipio de Guayama, Puerto Rico. Los sistemas de cultivo evaluados fueron un monocultivo de maíz versus una rotación de cultivo de maíz-caupí, con cinco niveles de fertilizante nitrogenado. La secuencia de siembra fue una siembra inicial de maíz en la primavera del 2014, siembra de cobertura en el verano del 2014, maíz en el invierno del 2014-2015, caupí en el verano del 2015, maíz en el invierno del 2015-2016 y cobertura en el verano del 2016. Las líneas de maíz utilizadas fueron SSH65VH en el 2013-2014 y 2014-2015 y SLM15VH en el 2015-2016. La densidad de plantas vario entre 59,391 y 69,182 plantas/ha para los años de estudio. El *SPAD-502 chlorophyll meter*® (verdor de las hojas), *NDVI (GreenSeeker®)* y la altura de la planta se utilizaron como indicadores del estatus de N en el cultivo. Las aplicaciones de fertilizante-N y la rotación de CC influyeron significativamente ($p < 0.05$) el verdor de las hojas y la altura de la planta. Las tasas de fertilizante-N influenciaron significativamente el rendimiento de grano del maíz. La eficiencia de uso de nutrientes se alcanzó a 90 kg N/ha (2013-2015) y 50 kg N/ha (2015-2016). Los modelos de regresión no lineal indicaron valores requeridos de fertilizante nitrogenado de 143 kg N/ha con un rendimiento de grano de 6,917 kg/ha para la temporada 2013-2014, 156 kg N/ha con un rendimiento de grano de 4,579 kg/ha para la temporada 2014-2015 y para la temporada 2015-2016 36 kg N/ha con un rendimiento de grano de 2,535 kg/ ha. Se desarrolló un índice de calidad de suelo basado en valores calculados por el sistema de evaluación de manejo de suelo. Las aplicaciones de fertilizante-N disminuyó el pH del suelo al aumentar las tasas de aplicación de fertilizante nitrogenado. El índice de calidad de suelo fue más alto con el tratamiento

de cobertora que con barbecho (FA). La media total del índice de calidad de suelo (%) fue de 58. La media más baja del índice de calidad de suelo (%) fue para la categoría biológica y bioquímica con 43. Esto indica que se puede implementar otras prácticas de manejo que mejoren la biología del suelo. Los ésteres metílicos de ácidos grasos se utilizaron para evaluar la composición de la comunidad microbiana. La actividad de las enzimas del suelo se usaron para evaluar la actividad de la comunidad microbiana. La comunidad microbiana fue influenciada por la rotación pero no por las aplicaciones de fertilizante-N. La abundancia relativa bacteriana con cobertora fue 15.6% mayor que la de FA. La actividad microbiana también fue afectada por CC. Los resultados de los ensayos enzimáticos combinados determinaron una mayor abundancia de enzimas en el tratamiento de CC en comparación con barbecho. El fertilizante-N afecto la altura de la planta, SPAD-502, el rendimiento de grano, el N inmediatamente disponible (0-30 cm), el N potencialmente lixiviado (30-90 cm) y el pH del suelo. La rotación con cobertura afectó a los tres indicadores agronómicos (SPAD-502, NDVI y altura de planta), el pH del suelo, N potencialmente mineralizable, N inmediatamente disponible y la estructura y actividad de la comunidad microbiana.

DEDICATION

To my parents, Ada L. Vilches Ortega and Carlos R. Vilches, for always believing in me and never losing faith. To my sister Carla for constantly being my support system and to my brother Carlos Juan for never failing to make me laugh. To all my friends throughout the years that had a part in this journey for always being patient with me and having my best interests at heart, especially Milexa, Fernando, Paloma, Luis and Leyda.

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INTRODUCTION

The continuous world population increase coupled with increased environmental consciousness has prompted a need for farming systems that are sustainable and that can ensure global food security (Garibaldi et al., 2017). Around 94% of the worldwide cereal consumption comes from wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.). These three cereal crops provide about 50% of the total food calories required by humans (Ranum et al., 2014). Of these, maize is the largest produced globally in terms of quantity (FAOSTAT, 2014). This crop is used in a wide range of products such as animal feed, biofuel, protein, oil and food starch.

A major concern in maize cropping systems is appropriate nutrient management, especially nitrogen (N) fertilization. This concern arises from the difficulty of being able to precisely predict soil N availability to plants. Therefore a tendency to apply fertilizer-N rates exceeding crop N requirements has been observed (Ju et al., 2009). Nitrogen is an essential nutrient and usually the most limiting because of large plant demands and ecosystem losses (Kant et al., 2011). Crops may utilize 30% to 60% of the fertilizer-N applied because of the multiple N transformation processes and because ammonium and nitrate are mobile compounds in the soil (Raun and Johnson, 1999). In the case of urea fertilizers, losses of N are generally larger with increasing soil pH, temperature and surface residue (Hargrove et al., 1961).

Over the last three decades about seven agro-technology companies have been established in Puerto Rico. These industries have focused on incrementing hybrid maize seed production. Hybrid maize lines are produced from breeding inbred lines in order to gain material with higher yield, tolerance to diseases, pests and drought (Poehlman and Sleper, 1995). Inbred maize lines have lower seed yields and in efforts to improve yields producers may apply N in excess of the

crop requirements (Sotomayor-Ramírez et al., 2012). One of the most important practices in inbred maize cropping systems is nutrient management, especially N fertilization. In order to establish an adequate nutrient management plan, predictors and analysis of crop N status should be used. Agronomic indicators such as reflectance sensors and chlorophyll meters can help to predict crop N status. Plant tissue and soil N analysis can also be used to determine N use efficiency and potential losses in the system.

A way to interpret the effect that nutrient management practices have on a soil is through the use of tools that assess soil quality. Soil quality can be defined as "the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, maintain the quality of air and water environments, and promote plant, animal, and human health" (Drijber et al., 2000). The soil management assessment framework (SMAF) as applied to specific agricultural systems provides an index of soil quality (Andrews and Carroll, 2001). The SMAF was designed to be applied to any crop, soil type, climate and management situation. Overall it has three basic steps: indicator selection, indicator representation, and integration into an index (Andrews et al., 2004). With this tool the user can make a quantitative assessment of the effects of multiple practices on soil functions.

There is a need to assess soil quality because improper soil management can affect soil function. Microorganisms are crucial in soil quality and soil functions due to their participation in nutrient cycling, organic matter dynamics and soil decomposition processes (Acosta-Martínez et al., 2008). Microorganisms make up the biological processes that transform N between its organic and inorganic (NH_4^+ and NO_3^-) plant available phase (Paul and Clark, 1996).

This thesis is divided into four parts. The first chapter describes agronomical indicators as crop N status predictors. The second chapter examines crop response to fertilizer-N and cover crop

rotation. The third chapter describes SMAF soil quality index results as affected by site management practices. Lastly, the final chapter examines soil microbial community composition and function with regards to cover crop rotation and fertilizer-N applications. The overall objective of this thesis is to provide information that will lead to improved nitrogen management in inbred maize agricultural systems of the tropics.

CHAPTER 1

AGRONOMIC MEASUREMENTS AS INDICATORS OF CROP N STATUS

INTRODUCTION

Nitrogen (N) is one of the essential elements in developmental physiological plant processes. Nitrogen plays a crucial role in chlorophyll production and in the crop photosynthetic capacity (Rostami et al., 2008). Nitrogen is a component of proteins that regulate plant-growth, aids in delaying senescence, also helps produce protective chemical components that fight against plant diseases and pests. In maize N plays a pivotal role in vegetative, kernel and ear development (Earl and Tollenaar, 1997; Rostami et al., 2008). Plants absorb N from soil as the inorganic forms of nitrate-N (NO_3^-) and ammonium-N (NH_4^+) but there is usually a limited soil N supply (Mullen, 2011). In response to these N limitations farmers usually increase their fertilizer-N rates which may lead to overfertilization. Excess N can then contribute to environmental pollution, groundwater contamination and global warming through processes like nitrate leaching, volatilization and soil denitrification (Havlin et al., 2005b; Zebarth et al., 2009). Therefore, efficient N management is needed in order to decrease these environmental impacts. Optimized fertilization strategies can be established through the use of various tools and methods (Meisinger et al., 2008).

In order to predict optimal N fertilizer rates and achieve successful N management a synchronization between crop N requirements and N supply is needed. Crop N status determination can be achieved through methods such as leaf tissue analysis (Kjeldahl-digestion, Dumas-combustion) but these are destructive and time consuming. There are non-destructive tools for N status determination. Crop canopy reflectance and leaf pigments like polyphenols and chlorophyll are often used as N status indicators (Demotes-Mainard et al., 2008; Muñoz Huerta et

al., 2013). Tools such as leaf chlorophyll meters, digital image processing and reflectance sensors can help estimate crop N status (Rostami et al., 2008; Tremblay et al., 2011).

Chlorophyll is responsible for leaf greenness and previous studies have shown a correlation between leaf greenness and plant N status (Piekkielek and Fox, 1992; Tremblay et al., 2011). The SPAD-502 (Soil Plant Analysis Development)® chlorophyll meter is commonly used as an in-season crop N diagnostic tool. When using a SPAD-502 chlorophyll meter an enclosed leaf section is exposed to a red (640 nm) and an infrared (940 nm) light. The instrument sensors capture the filtered light and the transmission difference between the two wavelengths indicates the chlorophyll leaf content. A drawback of chlorophyll meters is that measurements can be affected by chlorophyll level saturation and plant deficiencies in nutrients other than N (Villeneuve et al., 2002; Westerveld et al., 2004).

Active reflectance sensors such as the hand-held GreenSeeker® (NTech Industries Inc., Ukiah, CA, USA) have their own light source. The GreenSeeker generates two wavelengths, a red (Red) [650 ± 10 nm full width half magnitude (FWHM)] and a near-infrared (NIR) (770 ± nm FWHM) bands (Freeman et al., 2007). The instrument's sensor measures the emitted light in the determined crop area which is then quantified as a normalized difference vegetation index (NDVI) (Shanahan et al., 2008). The sensor computes the NDVI as:

$$\mathrm{NDVI} = \frac{\mathrm{NIR} - \mathrm{Red}}{\mathrm{NIR} + \mathrm{Red}}$$

were NIR= near-infrared. NDVI calculations by reflectance sensors have been shown to reach a saturation point at maximum biomass or plant height (Shaver et al., 2011; Muharam et al., 2014). Plant height is a plant growth indicator that in combination with reflectance sensors has been used to estimate crop N status and biomass yield (Muharam et al., 2014).

The objective of this chapter was to assess the efficacy of plant height, plant health and chlorophyll content as agronomic indicators of crop N status.

MATERIALS AND METHODS

Location

The experiment was located in the municipality of Guayama, Puerto Rico in a farm owned by Dow AgroSciences. Since 2018 Dow Agrosciences was acquired by DuPont Pioneer and renamed Corteva Agriscience. The company produced maize and soybean seeds. Their maize growing season was from September to April. The soil where the experiment was located is Güamaní (fine-loamy over sandy or sandy-skeletal, mixed, superactive, isohyperthermic Torrifluventic Haplustepts). The project was conducted for 3 years. The study area was an abandoned grassland before it was tilled and planted with maize. Cumulative precipitation during maize growth period was 157, 173 and 196 millimeters for the 2013-2014, 2014-2015 and 2015-2016 seasons, respectively (Figure 1). Cumulative precipitation during the cover crop growth was 551, 376 and 274 millimeters for the 2013-2014, 2014-2015 and 2015-2016 seasons, respectively (Figure 1). Supplemental irrigation to the maize cropping was provided with drip irrigation according to the company's established practices.

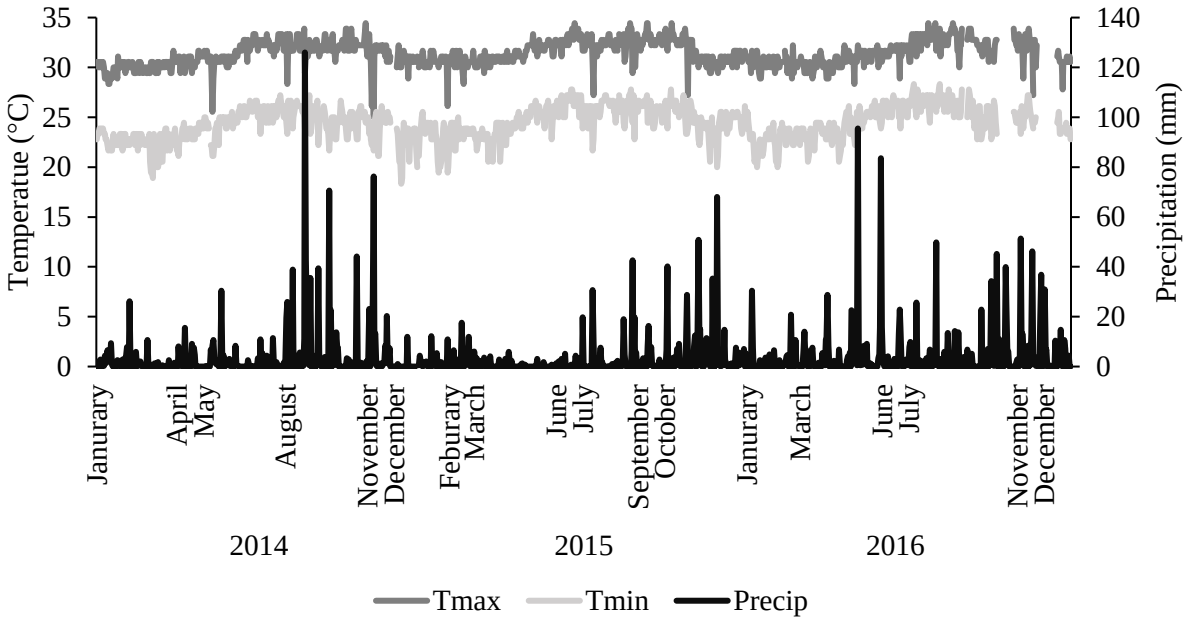


Figure 1. Precipitation (mm), max temperature (Tmax) and minimum temperature (Tmin) for all experimental years. Weather date acquired from www.ncdc.noaa.gov/cdo-web/datatools/findstation. Weather station used was Guayama 2 E, US. Latitude: 17.9783. Longitude: -66.0874. Elevation: 72 ft.

Experimental design

The experimental design for the 2013-2014 season was a randomized complete block design with four replicates. There was no cover crop rotation at this time. There were 20 mainplots measuring 9 x 37 m (30 x 120 ft) and each of these plots contained 12 rows of planted maize. Maize was planted on 14 April 2014 and harvested 16 July 2014. After this, the first cover crop rotation was established. The cover crop was planted on 8 August 2014 and incorporated 24 October 2014. For the 2014-2015 and 2015-2016 seasons, the design was a strip-plot in a randomized complete block design with four replicates (Figure 2). The mainplot was the five fertilizer-N levels and the strip was the effect of the cowpea (*Vigna unguiculata* (L.) Walp.) cover crop rotation. The mainplots were 9 x 37 m (30 x 120 ft) and the subplots were 9 x 18 m (30 x 60 ft). Each plot had 12 rows of maize when planted. The total experimental area was 1.65 acres (0.67 ha) for all years. For 2014-2015 season, maize was planted on 10 December 2014 and harvested on 23 April 2015. The cover crop was planted on 21 May 2015, but a second planting had to be

done on 9 June 2015 due to large patches of cowpea cover that did not germinate. Cowpea was incorporated on 2 November 2015. Maize was planted on 21 December 2015, harvested 21 March 2016 and cowpea cover crop was planted on 15 June 2016 and incorporated 8 September 2016 for the 2015-2016 trial period. The inbred maize line SSH65VH was used for 2013-2014 and 2014-2015. In 2015-2016 line SLM15VH was planted. Both of these endogamic maize lines were used as female lines for commercial hybrid maize production. Both of them are tolerant to insects of the order Lepidoptera and resistant to Glyphosate.

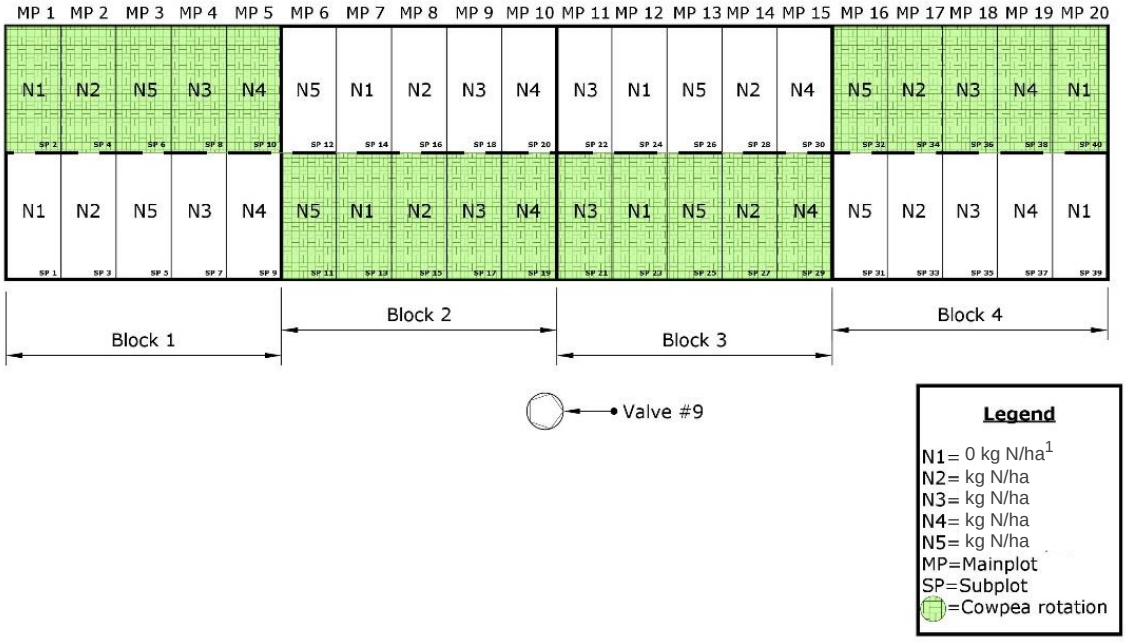


Figure 2. Experimental arrangement for the 2014-2015 and 2015-2016 seasons. 1) The N1 value was the same for both 2014-2015 and 2015-2016. Corresponding fertilizer-N values used for N2-N5 at both trial periods are mentioned in the treatments section of this chapter.

Treatments

The five fertilizer-N levels for both the 2013-2014 and 2014-2015 seasons were: 0, 90, 135, 180 and 225 kg N/ha respectively. The five fertilizer-N levels for 2015-2016 were: 0, 50, 100, 150 and 200 kg N/ha respectively. The fertilizer-N sources were ammonium sulfate and urea at an urea-N:NH_4-N ratio of 3:1 (2013-2014 and 2014-2015) and 1:1 (2015-2016). The fertilizer-N

applications were split, one at planting and one several days after planting (DAP) (Table 1). The side-dressing or fertilization between rows was applied manually. Nitrogen applications were not applied to the control treatments (0 kg N/ha). At planting for all three seasons, all treatments received an application of 56, 93, and 25 kg/ha of P_2O_5, K_2O, and ME (minor elements), respectively. These were applied in the forms of triple superphosphate, potassium chloride and Granusol Five-Star-Mix® in order to ensure that the only limiting nutrient for the maize was N. During maize growth, drip irrigation was provided twice a week depending on the climate conditions. Scouting and pest control was done by Mycogen Seeds employees and applications were done following company practices. Iron clay cowpea (*Vigna unguiculata* (L.) Walp.) seeds were ordered from Johnny Seeds® for all years. The cover crop was sprayed with glyphosate and incorporated during flowering (between 60 to 90 DAP). Afterwards the field was tilled weekly until the next maize planting. No weed or pest control was used during cowpea growth.

Table 1. Fertilizer-N rates applied in Guayama experiment from 2013-2016.

	Fertilizer-N applications[1]					
	2013-2014		2014-2015		2015-2016	
N-level[2]	1st[3]	2nd[4]	1st	2nd	1st	2nd
----------kg/ha----------						
N2	68	22	68	22	30	20
N3	68	67	68	67	30	70
N4	68	112	68	112	30	120
N5	68	157	68	157	30	170

1- Fertilizer-N rates were done in two split applications.
2- 2013-2014 and 2014-2015 total fertilizer-N levels were 90, 135, 180 and 225 kg N/ha for N2, N3, N4 and N5 respectively. 2015-2016 N2, N3, N4 and N5 total fertilizer-N levels were 50, 100, 150 and 200 kg N/ha respectively.
3- The first fertilizer-N application was done at planting.
4- The second application was done at 36 DAP, 34 DAP and 38 DAP for the 2013-2014, 2014-2015 and 2015-2016 seasons, respectively.

Field Measurements

Chlorophyll content

The SPAD-502 (Konica Minolta, Tokyo, Japan) was used to determine leaf N greenness. Twenty plants were selected and measured at 29, 37, 44 and 49 DAP, at 31, 38, 45, 52 and 59 DAP and at 35, 42, 49 and 56 DAP for the 2013-2014, 2014-2015 and 2015-2016 seasons, respectively. The SPAD-502 measurement was taken from the youngest leaf with a fully expanded collar. As described by Mengel (2008), the measurement was made halfway between the leaf margin and the midrib and halfway between the leaf tip and the leaf collar every time.

Plant health

The GreenSeeker handheld crop sensor (NuTech Industris, Inc., Ukiah, Ca) was used to determine plant greenness which is an indirect indicator of plant health and N sufficiency. The measurement was taken while walking parallel to the plants and at a height of 80 cm to 120 cm (31 to 47 inches) above the crop canopy in a 12 m (40 ft) segment. Measurements were done 3 m (10 ft) in from the border of the plots and at a pace of 1 m/s. The GreenSeeker provides the data through its sensor that emits red and infrared light which are quantified as normalized difference vegetation index (NDVI) values that range from 0.00 to 0.99. These values are unitless (Mengel, 2008). Greenseeker measurements were taken the same days as SPAD-502 measurements for all years.

Plant height

Plant height was measured the same days as SPAD-502 and Greenseeker for all years. Ten plants were measured in one row of each subplot for the 2014-2015 and 2015-2016 seasons and in one row of each main plot for the 2013-2014 season. The measurement was taken from the base of the trunk to the top of the plant while fully extending the leaves.

Data analysis

Statistical analysis was done using the software InfoStat (2014®). An ANOVA with a randomized complete block design arrangement was done for all agronomic indicator 2013-2014 data. This ANOVA consisted of two factors: nitrogen level (N-level) and replicate. The agronomic indicator data for the periods of 2014-2015 and 2015-2016 had a strip-plot in a randomized complete block design ANOVA arrangement with the factors of N-level, rotation and replicate. A Shapiro-Wilks test was used to verify data normalization and Levene for data homogeneity. Significant differences in the ANOVA analysis was determined using the means separation LSD Fisher test with an α of 0.05.

RESULTS AND DISCUSSION

All three agronomic indicators were not significantly different ($p>0.05$) for the interaction of fertilizer-N level x cover crop rotation for the two seasons (Table 2). Therefore only the results pertaining to the principal effects of rotation and fertilizer-N level will be discussed.

Table 2. Summarized ANOVA for the agronomic indicators in Guayama experiment from 2013 to 2016.

Agronomic Indicator	2013-2014	----------2014-2015----------			----------2015-2016----------		
	Fertilizer-N	Fertilizer-N	Cover crop	Fertilizer-N x Cover crop	Fertilizer-N	Cover crop	Fertilizer-N x Cover crop
Plant Height 1[1]	0.0145	0.0735	0.2030	0.2591	0.0094	0.0051	0.7563
Plant Height 2	0.0043	0.0233	0.7417	0.6135	0.0910	0.0017	0.5346
Plant Height 3	0.0224	0.0036	0.1988	0.5464	0.2256	0.0250	0.4530
Plant Height 4	0.1199	0.0047	0.1932	0.6167	0.8812	0.2055	0.6158
NDVI 1[1]	0.8774	0.0668	0.2227	0.3212	0.5539	0.4385	0.7126
NDVI 2	0.4508	0.7171	0.4638	0.9479	0.0633	0.0310	0.1581
NDVI 3	0.2123	0.8609	0.0177	0.5339	0.9146	0.7508	0.0866
NDVI 4	0.5102	0.8342	0.0139	0.5617	0.0934	0.9054	0.8934
NDVI 5	--------[2]	0.9202	0.0565	0.3385	--------[2]	--------[2]	--------[2]
SPAD 1[1]	0.5854	0.0359	0.7237	0.5020	0.0454	0.0455	0.2457
SPAD 2	0.1537	0.1765	0.0006	0.3891	0.0957	0.0061	0.5437
SPAD 3	0.0901	0.0324	0.1410	0.7516	0.8311	0.0307	0.4636
SPAD 4	0.6752	0.0323	0.1419	0.7452	0.0945	0.2609	0.0731
SPAD 5	--------[2]	0.0015	0.1533	0.9864	--------[2]	--------[2]	--------[2]

1- Plant height measurements, SPAD and NDVI (GreenSeeker) readings in 2013-2014 were taken at 29, 37, 44, and 49 DAP. In 2014-2015 plant height was taken at 38, 45, 52 and 59 DAP, while SPAD and NDVI were measured at 31, 38, 45, 52 and 59 DAP. Plant height, SPAD and NDVI for the 2015-2016 season were taken at 35, 42, 49, and 56 DAP.

2- NDVI and SPAD data was not collected at a 5th date for the 2013-2014 and 2015-2016 seasons.

In 2013-2014 season, N-levels significantly affected plant height at 29, 37 and 44 DAP ($p<0.05$) (Table 3). Plant height has been previously observed to be affected by N treatments (Sotomayor-Ramírez et al., 2012; Muharam et al., 2014; Rivera-Zayas, 2015). A difference between the control treatment 0 kg N/ha and all other N-level treatments at 29, 37 and 44 DAP for the corresponding date was observed. The plant height measurements for 29, 37 and 44 DAP at 0

kg N/ha were 79.3 cm, 112.1 cm and 146.4 cm respectively. There was no significant difference for N treatments 90 kg N/ha and increasing fertilizer-N levels at 29, 37 and 44 DAP. The fertilizer-N levels did not affect SPAD-502 or NDVI. The means for SPAD-502 measurements for all fertilizer-N levels were 43.7, 43.7, 48.9 and 51.6 and means for NDVI were 0.66, 0.69, 0.74 and 0.74, at 29, 37, 44 and 49 DAP, respectively. Plant height increased in each fertilizer-N treatment as the season progressed, but it was not affected by increasing N-levels for each individual date. In general no significant differences were observed for plant height at N-levels greater than 90 kg N/ha. Rivera-Zayas (2015) reported no significant differences in plant height with N values greater than 68 kg N/ha.

Table 3. Effects of fertilizer-N on plant agronomic indicators of nutrient status in Guayama experiment in 2013-2014.

Agronomic indicator		Fertilizer Treatment (2013-2014) 0	90	135	180	225	Means
		----------kg/ha----------					
Plant Height (cm)	29 DAP	79.3a[1]	93.9b*	93.9b	94.5b	92.6b	--------[4]
	37DAP	112.1a	125.6b*	128.1b	128.4b	126.3b	--------[4]
	44DAP	146.4a	159.9b*	162.8b	162.3b	159.1b	--------[4]
	49DAP	164.7ns[2]	178.5	178.7	178.6	174.6	175.0
SPAD-502	29 DAP	42.4ns	44.9	43.7	43.5	44.0	43.7
	37DAP	43.2ns	44.2	42.8	42.2	46.1	43.7
	44DAP	46.6ns	49.2	54.5	47.2	46.9	48.9
	49DAP	49.5ns	51.9	57.5	48.2	50.8	51.6
NDVI (GreenSeeker)	29 DAP	0.66ns	0.67	0.67	0.67	0.63	0.66
	37DAP	0.68ns	0.69	0.70	0.70	0.70	0.69
	44DAP	0.75ns	0.73	0.73	0.74	0.74	0.74
	49DAP	0.74ns	0.72	0.75	0.74	0.75	0.74

1- Means for each agronomic indicator with different letters within a DAP are significantly different as determined with Fisher's LSD test ($P<0.05$).
2- ns denotes that treatments were not significant at $P>0.05$.
3- * no significant differences from this value onwards.
4- Means were not calculated for significantly different data ($p<0.05$).

Plant height and SPAD-502 for the 2014-2015 season were influenced by fertilizer-N levels at varying DAP (Table 4). Sotomayor et al. (2012) and Rivera-Zayas (2015) also reported a

significant difference for plant height and SPAD-502 for endogamic maize lines in Puerto Rico. Plant height was significantly affected by fertilizer-N at 45, 52 and 59 DAP and for SPAD-502 at 31, 45, 52 and 59 DAP. Sotomayor et al. (2012) reported mean SPAD-502 values of 51, 53 and 54 at 40, 54 and 70 DAP, respectively. Rivera-Zayas (2015) reported SPAD-502 values of 39, 46, 43 and 47 at 33, 42, 52 and 62 DAP, respectively. Plant heights at 45, 52 and 59 DAP were not significant for fertilizer-N rates greater than 135 kg N/ha. SPAD-502 values for almost all N-levels increased as the season progressed. NDVI measurements were not influenced by the N-level treatments for this trial period. Mean NDVI values averaged across fertilizer-N levels for 31, 38, 45, 52 and 59 DAP were 0.61, 0.72, 0.73, 0.73 and 0.69, respectively.

Table 4. Effects of fertilizer-N on plant agronomic indicators of nutrient status in Guayama experiment in 2014-2015.

		Fertilizer Treatment (2014-2015)					
Agronomic indicator		0	90	135	180	225	Means
		----------kg/ha----------					
Plant Height (cm)	38DAP	135.0ns[1]	144.5	136.3	144.0	137.1	139.4
	45DAP	163.4a[2]	165.4ab	172.6c*	172.4c	170.4bc	--------[4]
	52DAP	161.1ab	158.5a	170.3c*	169.3c	167.0bc	--------[4]
	59DAP	162.5ab	159.0a	170.5c*	170.5c	168.6bc	--------[4]
SPAD-502	31DAP	37.4a	38.8ab	39.5abc*	40.7bc	41.9c	--------[4]
	38DAP	40.9ns	43.41	44.7	43.8	43.6	43.3
	45DAP	42.4a	46.1b*	47.2b	46.4b	47.9b	--------[4]
	52DAP	42.4a	46.1b*	47.2b	46.4b	47.9b	--------[4]
	59DAP	43a	47.1bc	45.6b*	49.1c	48.4c	--------[4]
NDVI (GreenSeeker)	31DAP	0.62ns	0.56	0.63	0.64	0.60	0.61
	38DAP	0.71ns	0.72	0.72	0.73	0.73	0.72
	45DAP	0.73ns	0.73	0.72	0.74	0.74	0.73
	52DAP	0.73ns	0.73	0.72	0.74	0.74	0.73
	59DAP	0.69ns	0.70	0.68	0.70	0.68	0.69

1- ns denotes that treatments were not significant at $P>0.05$.
2- Means for each agronomic indicator with different letters within a DAP are significantly different as determined with Fisher's LSD test ($P<0.05$).
3- * no significant differences from this value onwards.
4- Means were not calculated for significantly different data ($p<0.05$).

In the 2015-2016 season plant height was affected by fertilizer-N at a rate of 50 kg N/ha at 35 DAP and was different than the control N treatment (Table 5), with a value of 119.3 cm. Plant heights reported for all dates within each N-level gradually increased as the growing season advanced. Fertilizer-N rates of 100, 150 and 200 kg N/ha had lower plant heights than the N rate of 50 kg N/ha at 35, 42, 49 and 56 DAP. SPAD-502 measurements were affected by fertilizer-N at 35 DAP with similar values in fertilizer-N rates above 50 kg N/ha. SPAD-502 values decreased as the season progressed. Tajul et al. (2013) also observed a decrease in SPAD-502 values with increasing plant age. All NDVI values were not significant and had means of 0.68, 0.73, 0.71 and 0.61 for 35, 42, 49 and 56 DAP, respectively. NDVI was not affected by fertilizer-N for the three seasons. This could be attributed to the GreenSeeker sometimes being sensitive when NDVI values are close to saturation levels. Tremblay et al. (2009) concluded that the GreenSeeker hand-held sensor can be used to estimate crop N requirements before the V5 (Vn=maize vegetative stage (V) with n^{th} leaf collar visible) growth stage. In another study by Shaver et al. (2011) the GreenSeeker was able to determine N variability at the V12 and V14 growth stages. They also reported that the GreenSeeker could reach saturation levels earlier in the maize growing season. The authors suggest not to use the GreenSeeker at later crop growth stages due to greater plant biomass. The GreenSeeker data in our study could be coinciding with what Tremblay et al. (2009) found seeing as NDVI values from V5 (25-35 DAP) and onwards were not significantly different.

Table 5. Effects of fertilizer-N on plant agronomic indicators of nutrient status in Guayama experiment in 2015-2016.

Agronomic indicator		Fertilizer Treatment (2015-2016) 0	50	100	150	200	Means
		----------kg/ha----------					
Plant Height (cm)	35DAP	108.5a[1]	119.3b*	115.3b	118.6b	119.1b	--------[4]
	42DAP	139.3ns[2]	149.0	143.5	147.5	147.9	145.4
	49DAP	160.6ns	170.6	165.4	166.3	167.3	166.0
	56DAP	175.6ns	179.1	176.8	177.4	175.8	176.9
SPAD-502	35DAP	41.1a	44.3bc	42.7ab	43.9abc*	45.8c	--------[4]
	42DAP	35.5ns	38.6	37.1	38.8	41.1	38.2
	49DAP	36.7ns	37.5	38.2	38.3	39.4	38.0
	56DAP	35.1ns	36.7	34.0	36.2	41.9	36.8
NDVI (GreenSeeker)	35DAP	0.67ns	0.67	0.68	0.69	0.69	0.68
	42DAP	0.71ns	0.73	0.74	0.73	0.75	0.73
	49DAP	0.70ns	0.7	0.71	0.71	0.71	0.71
	56DAP	0.61ns	0.61	0.63	0.58	0.63	0.61

1- Means for each agronomic indicator with different letters within a DAP are significantly different as determined with Fisher's LSD test ($P<0.05$).
2- ns denotes that treatments were not significant at $P>0.05$.
3- * no significant differences from this value onwards.
4- Means were not calculated for significantly different data ($p<0.05$).

Overall in both the 2014-2015 and 2015-2016 seasons, almost all agronomic indicator observations had higher values with CC rotation than FA treatment. The cowpea cover crop (CC) rotation had a greater effect in the 2015-2016 season than the 2014-2015 season for most of the agronomic indicator measurements (Table 6). Rivera-Zayas (2015) also reported a CC rotation effect on all three agronomic indicators ($p<0.05$). Espinosa-Irizarry (2016) observed that the cowpea CC significantly affected plant height and SPAD-502. In the 2014-2015 season, there was a rotation effect for NDVI at 45 and 52 DAP with values of 0.74 and 0.72 for CC and FA, respectively. Rivera-Zayas (2015) reported NDVI values in inbred maize with CC rotation of 0.51, 0.60 and 0.63, and for FA of 0.60, 0.66 and 0.67 at 33, 42 and 52 DAP, respectively. SPAD-502 was significantly different at 38 DAP between CC and FA with values of 43.8 and 42.7, respectively. In the 2014-2015 season plant height was not significantly different. Plant height

means were 139, 169, 165 and 166 cm at 38, 45, 52 and 59 DAP, respectively. In the 2015-2016 season plant height was significant at 35, 42 and 49 DAP with CC values of 119.1, 149 and 170.1 cm and FA treatment values of 113.3 , 141.9 and 162 cm, respectively. Espinosa-Irizarry (2016) reported plant heights, for a 2011 study, of 101.4 and 158.4 cm for CC and 105.7 and 155.7 cm for FA at 43 and 63 DAP, respectively. In the 2012 study a plant height of 97.6 cm with CC rotation and 92.6 cm for FA treatment at 37 DAP was reported (Espinosa-Irizarry, 2016). Rivera-Zayas (2015) found significant differences in plant height with values of 108 cm for CC rotation and 120 cm for FA at 42 DAP. In the 2015-2016 season NDVI was significantly different at 42 DAP with values of 0.74 for CC and 0.72 for FA. In the 2015-2016 season SPAD-502 was significant at 35, 42 and 49 DAP with CC values of 45.5, 39.8 and 40.1 and for FA of 41.6, 36.7 and 36, respectively. Rivera-Zayas (2015) reported SPAD-502 values in inbred maize of 37 with CC and 40 for FA at 33 DAP. Espinosa-Irizarry (2016) reported SPAD-502 CC rotation values of 42.90 and 47.42 and for FA of 41.06 and 46.21 at 30 and 63 DAP, respectively. The higher plant height and SPAD-502 values for both the 2014-2015 and 2015-2016 seasons with CC could be due to the greater amount of immediately available N (0-30cm) found in the soils with cowpea rotation (Appendix 5). Legumes are known to increase N inputs through N_2 fixation (Havlin et al., 2005a; Kaspar and Singer, 2011)

Table 6. Effects of cover crop rotation on plant agronomic indicators of nutrient status in Guayama experiment in 2014-2015 and 2015-2016.

Agronomic Indicator	CC[4]	FA	Means
----------2014-2015----------			
Plant Height 1[1]	141.5ns[2]	137.3	139.4
Plant Height 2	169.1ns	168.6	168.8
Plant Height 3	166.0ns	164.5	165.2
Plant Height 4	167.1ns	165.4	166.2
NDVI 1	0.61ns	0.60	0.61
NDVI 2	0.73ns	0.72	0.73
NDVI 3	0.74b	0.72a	--------[5]
NDVI 4	0.74b	0.72a	--------[5]
NDVI 5	0.70ns	0.68	0.69
SPAD 1	39.8ns	39.5	39.7
SPAD 2	43.8b	42.7a	--------[5]
SPAD 3	47.0ns	45.0	46.0
SPAD 4	47.0ns	45.0	46.0
SPAD 5	48.0ns	45.3	46.6
----------2015-2016----------			
Plant Height 1[1]	119.1b[3]	113.3a	--------[5]
Plant Height 2	149.0b	141.9a	--------[5]
Plant Height 3	170.1b	162.0a	--------[5]
Plant Height 4	179.4ns	174.5	176.9
NDVI 1	0.69ns	0.67	0.68
NDVI 2	0.74b	0.72a	--------[5]
NDVI 3	0.71ns	0.70	0.71
NDVI 4	0.61ns	0.61	0.61
NDVI 5	--------[6]	--------[6]	--------[5]
SPAD 1	45.5b	41.6a	--------[5]
SPAD 2	39.8b	36.7a	--------[5]
SPAD 3	40.1b	36.0a	--------[5]
SPAD 4	37.9ns	35.7	36.8
SPAD 5	--------[6]	--------[6]	--------[5]

1- Plant height was recorded at 38, 45, 52 and 59 DAP for 2014-2015 respectively. NDVI (GreenSeeker) and SPAD-502 values for 2014-2015 were taken at 31, 38, 45, 52 and 59 DAP respectively. All three indicator measurements were determined at 35, 42, 49 and 56 DAP for 2015-2016 respectively.

2- ns denotes that treatments were not significant at $P>0.05$.

3- Means for each agronomic indicator with different letters within a DAP are significantly different as determined with Fisher's LSD test ($P<0.05$).

4- Rotation factor, CC=cover crop and FA=fallow.

5- Means were not calculated for significantly different data ($p<0.05$).

6- NDVI (GreenSeeker) and SPAD data was not collected a 5th time for the 2015-2016 season.

CONCLUSIONS

The results of this study reveal that some of the agronomic indicators were affected by fertilizer-N level rates and by the cover crop rotation. There was no significant fertilizer-N effect for SPAD-502 or NDVI in the 2013-2014 season and no differences for NDVI in the periods of 2014-2015 and 2015-2016. Overall SPAD-502 and plant height could be accepted as adequate agronomic indicators of crop N status until the V5 (25-35 DAP) maize growth stages. In this case, the hand-held GreenSeeker was not an effective crop N status predictor which could be due to earlier saturation levels. Plant height and SPAD-502 were higher as a result of cover cropping for both 2014-2015 and 2015-2016. Further experiments should be conducted taking into consideration different reflectance sensors other than the GreenSeeker in order to determine which one would work more efficiently for an inbred maize line crop production under the local conditions and soil type.

CHAPTER 2

EFFECTS OF FERTILIZER-N RATES AND COVER CROP ROTATION ON INBRED MAIZE YIELD

INTRODUCTION

Nitrogen (N) is the most limiting nutrient in non-legume cropping systems (Havlin et al., 2005b; Mullen, 2011). Nitrogen deficiency can lead to excess nitrogen applications which negatively impact the environment by contributing to global warming through nitrous oxide emissions, eutrophication of inland surface water and marine waters and contamination of ground water resources (Meisinger et al., 2008). Therefore, effective nitrogen management is essential in order to achieve a balance between yields, nitrogen supplementation and nitrogen losses (Andraski and Bundy, 2002; Mullen, 2011). Establishing a relationship between crop yields and fertilizer-N applications will aid in improving N management and N use efficiency (Ma et al., 2005).

Studies in Puerto Rico on maize response to fertilizer-N applications have had varying results regarding the optimum level of fertilizer-N needed for maximum crop yields. In an experiment done in a Santa Isabel clay (Fine, smectitic, isohyperthermic Typic Haplusterts) which evaluated the effect of three N-levels (0, 67 and 135 kg N/ha) on Mayorbela corn it was observed that plots that were continuously irrigated with applications of 135 kg N/ha out-yielded the non-irrigated treatment and that overall nitrogen applications increased yields (Vazquez, 1961). An experiment done by Capo (1967) utilized a fertilizer-yield equation with data from various experiments to estimate the optimum fertilizer application levels in Mayorbela. The estimated optimum N applications were 247, 22, 853 and 1,190 kg N/ha for the locations Isabela, Gurabo, Lajas and Rio Piedras, respectively (Capo, 1967). In a study with seven cultivars of corn on a Coto soil (Very-fine, kaolinitic, isohyperthermic Typic Eutrustox), fertilizer-N to reach maximum

yields was 67 kg N/ha (Feliciano et al., 1979). Another study with a hybrid in Oxisols and Ultisols also found that fertilizer-N at 67 kg N/ha produced near maximum yields (Fox et al., 1974). In a hybrid maize experiment conducted in Sabana Grande PR with five N-levels (0, 60, 120, 180 and 240 kg N/ha) optimum yield was obtained with fertilizer-N at 120 kg N/ha with a plant density of 40,000 plants/ha (Quiles et al., 1988). An inbred maize experiment done in 2012 reported a seed yield of 2,726 kg/ha and 1,447 kg/ha, with applications of 84 kg N/ha y 112 kg N/ha, respectively (Sotomayor-Ramírez et al., 2012). A study done on an inbred line in Santa Isabel had a maximum seed yield of 2,918 kg/ha with an application of 68 kg N/ha (Rivera-Zayas, 2015).

Legume cover crops in rotation is a conservation method that can maintain or increase soil N and reduce losses in a rotation system (Havlin et al., 2005a; Snapp et al., 2005). Cover crops (CC) are living ground covers that are planted after the production crop and killed before the main crop is planted again (Hartwig and Ammon, 2002). They may provide multiple benefits such as increased soil organic carbon, improved soil water infiltration, protecting against soil erosion, improving soil physical properties, providing weed control, providing a habitat for beneficial insects, reduce pathogens, reduce N and P losses and promote nutrient recycling (Havlin et al., 2005a; Snapp et al., 2005; Kaspar and Singer 2011). It has been suggested that bare fallow could be replaced with cover-crops to reduce N leaching and improve nitrogen use efficiency in certain systems (Dinnes et al., 2002).

Legumes can be used as cover crops because of their ability to fix soil N. After the legume cover-crop is incorporated into the soil, there may be more N available for the successive production crop (Havlin et al., 2005b, Kaspar and Singer 2011). Depending on the type of cover and its management it has been assessed that between 30 to 40 kg N is transferred to the soil after the legume cover crop is incorporated (Peoples et al., 2009). Studies have demonstrated yield

increases in a maize-cover-crop rotation systems (Rao and Mathuva, 2000; Gabriel and Quemada, 2011; Mupangwa et al., 2012; Sotomayor-Ramírez et al., 2012). An experiment conducted in Kenya had a 17 and 24% higher maize yield with short-duration legume (cowpea; *Vigna unguiculata* (L.) Walp.) and long duration legume (pigeon pea; *Cajanus cajan* (L.) Millsp.), respectively, with the maize-legume rotation as compared to continuous maize (Rao and Mathuva, 2000). Gabriel and Quemada (2011) observed greater maize yield and biomass in vetch (*Vicia villosa* L.) treatment in rotation versus bare fallow. The results showed that the maize planted after vetch suffered less from water limitation than the maize in the fallow treatment (Gabriel and Quemada, 2011).

Due to these discrepancies in N-fertilizer requirement results further research is needed in the island in order to establish the minimum application of fertilizer-N necessary to reach maximum inbred maize yields for specific soil types and soil management practices. In this chapter, the objective was to evaluate inbred maize-line response to fertilizer-N levels and cover-crop rotation in an Inceptisol in order to establish a maximum N application rate for the inbred maize-lines used.

MATERIALS AND METHODS

Study site

The experiment was located in the municipality of Guayama, Puerto Rico. Experimental design details and treatments were previously described in Chapter 1. Soil and plant N data and maize harvest data was collected for all three experimental years, 2013-2014, 2014-2015 and 2015-2016.

Soil N analysis

In the 2013-2014 season soil samples (pre-plant) were taken before maize planting for all 20 mainplots. Soil samples for the 2014-2015 season (pre-plant) and the 2015-2016 season (pre-plant) were taken after the cover-crop had been incorporated into the soil but before each maize planting, for each subplot, to determine plant available N. In the 2015-2016 season (post-harvest) soil samples were taken after last maize harvest for each subplot. Sampling was done with a bucket auger. A soil sample was taken at depths of 0-15 cm, 15-30 cm, 30-60 cm and 60-100 cm per sub-plot. Soil samples were left to air dry and then sieved (as reported by Rivera-Zayas, 2015). Afterwards they were sent to AgSource Laboratory (Lincoln, NE, www.agsourcelaboratories.com) for a 1M KCL extractable NH_4^+-N and NO_3-N analysis.

Stover N analysis

Plant vegetative material was gathered during maize harvest to quantify the biomass and N concentration in the plants. Plant biomass was taken from a 3 linear meter segment in each mainplot for the 2013-2014 season and for each subplot in the 2014-2015 season. For the 2015-2016 season two rows were selected from each subplot and the sampling segment was 6 meters. The plants were cut at approximately 5 centimeters off the ground and the fresh weight was recorded for each year. A sub sample of 3 plants was cut into smaller pieces and weighed. To

determine plant water content samples were oven dried at 65°C for 48h. The dry samples were then ground and analyzed for N concentration (as reported by Rivera-Zayas, 2015).

Maize yield and N analysis in season

Maize was harvested from a 6 meter segment for both the 2013-2014 season for all mainplots and the 2014-2015 season for all subplots. In the 2015-2016 season two rows in each subplot were used for maize harvest. The number of maize ears collected in each row was counted and weighed. The ears were oven dried in an industrial oven until they reached a 15.5% moisture. Afterwards the ears were thrashed in order to separate the seed from the cob. These seeds were then weighed and analyzed for moisture. A 100 g subsample for 2013-2014, 200 g for 2014-2015 and 250 g for 2015-2016 was used to determine the number of seeds. Seeds were also analyzed for N concentration (as reported by Rivera-Zayas, 2015).

Indicator leaf N analysis

Indicator leaf samples were taken near 49 DAP. The last completely developed leaf was collected from 10 plants from a previously selected row for each mainplot (2013-2014) or subplot (2014-2015 and 2015-2016). These samples were weighed, oven dried (55°C) and ground for N analysis (as reported by Rivera-Zayas, 2015).

Cowpea biomass and N analysis

For cover-crop biomass determination a 1 m^2 quadrant was randomly placed twice in each subplot with cowpea. The plants inside the 1 m^2 quadrant were cut at ground level and weighed. A portion of the plant material was oven dried at 65°C and re-weighed to determine plant moisture (as reported by Rivera-Zayas, 2015). Dry biomass samples were ground and sent for total N analysis at the Agsource Soil and Forage Laboratory.

Nutrient use efficiency

Nutrient use efficiency was calculated as specified by Fixen et al. (2015). The partial factor productivity (PFP) was calculated as: grain yield/fertilizer-N application. Agronomic efficiency (AE) was calculated as: (grain yield with nutrient application - grain yield with no nutrient application)/fertilizer-N applied. The partial nutrient balance (PNB) was calculated as: grain N-uptake/fertilizer rate applied. The apparent recovery utilization (RE) was calculated as: (crop N-uptake with nutrient applied - crop N-uptake no nutrient applied)/fertilizer-N applied. The internal utilization efficiency (IE) was calculated as: grain yield/crop N-uptake. The physiological efficiency (PE) was calculated as: (grain yield with nutrient application - grain yield with no nutrient application)/(crop N-uptake with nutrient applied - crop N-uptake no nutrient applied).

Partial Nitrogen budget

The aboveground nitrogen budget was constructed using fertilizer-N applications, stover N-uptake, cowpea cover crop N-uptake and immediately available N (samples consisting of a 0-30 cm depth) as N inputs. Used nitrogen outputs were seed N-uptake and potentially leached N (samples taken at depths ranging from 30-90 cm). Total aboveground N budget was calculated as the sum of all N inputs per season minus the sum of all N outputs per season. Unaccounted N for each N-level by season was calculated as N-level nitrogen inputs minus N-level nitrogen outputs. (adapted from Prasad and Hochmuth, 2013)

Statistical Analysis

The data was analyzed using InfoStat (2014®) statistical software. An ANOVA analysis was done for cowpea cover crop, maize harvest and soils data and significant differences were determined by LSD Fisher test with a $p<0.05$. The 2013-2014 season was a randomized complete block design with an ANOVA of two factors, replicate and N level application. The 2014-2015

and 2015-2016 seasons were a strip-plot in a randomized complete block design, the ANOVA had three factors: replicate, N level application and rotation. The data was verified for normality and homogeneity using Shapiro-Wilks and Levene tests. Non-linear regression models were done to determine various relationships in the system and crop nutrient requirements. The model with the lowest MSE (mean squared error), AIC (akaike's information criterion) and BIC (Bayesian information criterion) was selected as the most representative of the data. The critical leaf N value (CV) for the square-root model ($a+bx+cx^{0.5}$) was calculated as $cv= c^2/4b^2$ and for the quadratic model ($a+bx+cx^2$) as $cv= -2/bc$.

RESULTS AND DISCUSSION

Fertilizer-N rates applied to inbred maize did not affect cover crop biomass and N-uptake for any of the three experimental periods. This may be due to the capacity of the legume to fix nitrogen. Nodules were observed in CC roots (Appendix 4). Rivera-Zayas (2015) reported that fertilizer-N did not affect cowpea biomass and N-uptake. Fertilizer-N applications of over 25 kg N/ha have been reported to have no effect on cowpea biomass (Hasan et al., 2010). The mean biomass for the 2013-2014, 2014-2015 and 2015-2016 seasons were 5,201, 2,595 and 2,154 kg/ha, respectively. Cowpea biomass means of 2,593 and 1,262 kg/ha (Rivera-Zayas, 2015) and 4,255 and 2,200 kg/ha (Espinosa-Irizarry, 2016) have been previously reported for Puerto Rico. Cover crop N-uptake means were 125.6, 51.0 and 58.5 kg/ha for the 2013-2014, 2014-2015 and 2015-2016 seasons, respectively (Table 7). The higher mean cover crop N-uptake for 2013-2014 could be due to the greater precipitation at the time (Figure 1). Espinosa-Irizarry (2016) reported cover crop N-uptake values of 101 and 96 kg N/ha.

In the 2013-2014 season the fertilizer-N applications affected maize seed N-uptake (p-value=0.0381), seed yield (p=0.0076) and seed yield at 15.5% (p=0.0024) (Table 8). There was no cover crop effect for the 2014-2015 season but fertilizer-N affected various parameters. The lack of cover crop effect contrasts with the findings of Sotomayor et al. (2012) which observed an effect of cover crop rotation on seed yield. In the 2015-2016 season, fertilizer-N applications only had an effect on plant density with the control treatment being significantly different than the other N rates. The cover crop rotation influenced indicator leaf N concentration only. There was no fertilizer-N x cover crop interaction for the 2014-2015 and 2015-2016 seasons.

Table 7. Cover-crop biomass yield and N-uptake during summer plantings for all years as affected by N treatments.

N-level	Biomass dry matter	N-uptake
----------kg/ha----------		
----------Summer 2014----------		
0	4,854ns[1]	121.9ns
90	5,151	113.2
135	5,264	121.5
180	5,132	128.5
225	5,602	143.1
Means	5,201	125.6
----------Summer 2015----------		
0	2,097ns	37.5ns
90	2,895	57.2
135	2,555	47.1
180	2,976	58.8
225	2,450	54.4
Means	2,595	51.0
----------Summer 2016----------		
0	2,235ns	56.6ns
50	2,166	57.9
100	2,157	54.8
150	2,038	55.8
200	2,176	67.3
Means	2,154	58.5

1- ns denotes that fertilizer-N treatments were not significant ($p>0.05$).

Table 8. Summary of ANOVA p-value results for Guayama experiment from 2013-2016.

	2013-2014	----------2014-2015----------			----------2015-2016----------		
Measurements	Fertilizer-N (N)	Fertilizer-N (N)	Cover crop (CC)	N x CC[1]	Fertilizer-N (N)	Cover crop (CC)	N x CC[1]
Indicator leaf N concentration (%N)	0.2408	0.0039	0.5575	0.8970	0.5344	0.0256	0.4785
Seed N concentration	0.6357	0.0091	0.7627	0.4237	0.3785	0.3765	0.4944
Stover N concentration	0.8949	0.0554	0.3582	0.5880	0.5997	0.3618	0.1457
Stover N-uptake	0.8241	0.3012	0.1643	0.4869	0.2054	0.0779	0.1872
Seed N-uptake	0.0381	0.0012	0.3619	0.9079	0.2314	0.4776	0.5444
Biomass N-uptake[2]	0.4728	0.0148	0.1786	0.3922	0.2079	0.0695	0.2397
Stover	0.7336	0.5732	0.1292	0.2557	0.0955	0.0742	0.8478
Seed yield	0.0076	0.0161	0.4420	0.9286	0.5569	0.642	0.6916
Biomass[3]	0.4694	0.1875	0.1637	0.2713	0.1526	0.2026	0.9041
Seed Yield 15.5%	0.0024	0.0176	0.4409	0.9174	0.5525	0.6409	0.6874
Harvest index	0.8276	0.5629	0.8861	0.7051	0.6242	0.8561	0.5267
Plant density	0.0666	0.3813	0.7187	0.5159	0.0390	0.2062	0.1803
Number of seeds	0.0658	0.1557	0.5512	0.8869	0.2911	0.6025	0.5381
Ear density	--------[4]	0.5106	0.8126	0.5788	0.1274	0.9200	0.3411

1- Interaction between fertilizer-N (N) and cover crop (CC) rotation.
2- Biomass N-uptake was calculated by adding stover and seed N-uptake.
3- Biomass was calculated by adding seed yield dry weight and stover dry weight.
4- Data not measured for this time.

In the 2013-2014 season seed yield for the 0 kg N/ha was significantly lower than all other N levels (Table 9). Seed yield tended to increase with increasing fertilizer N levels until 180 kg N/ha, which had the highest seed yield at 7,234 kg/ha. Rivera-Zayas (2015) reported a 62 kg N/ha optimal N rate with a seed yield of 2,770 kg N/ha. Espinosa-Irizarry (2016) found no significant differences with N rates from 60 to 160 kg N/ha. The optimal N rate application of 180 kg N/ha for 2013-2014 was over that range. Therefore, the determined optimal N rate does not coincide with Espinosa-Irizarry (2016) findings. Maximum seed N-uptake of 124 kg N/ha was obtained with a fertilizer-N rate of 180 kg N/ha. The mean harvest index was 0.49. Espinosa-Irizarry (2016)

reported for 2011 trial a harvest index of 0.44. Stover was not found to be significantly different for all three experimental periods. This contradicts with Sotomayor et al. (2012) which reported significant fertilizer-N effect on plant stover yields.

Table 9. Maize production measurements for 2013-2014 as affected by N level applications

	Fertilizer Treatment (2013-2014)					
Measurements	0	90	135	180	225	Means
	----------kg/ha----------					
Seed yield[4]	6,168a[1]	6,746b	7,034bc	7,234c	6,656b	--------[3]
Stover	7,758ns[2]	8,490	9,608	8,255	7,356	8,293
Biomass	14,912ns	16,150	17,700	16,570	15,018	16,070
Seed N-uptake	103.5a	110.4ab	119.9bc	123.9c	116.3bc	--------[3]
Stover N-uptake	77.4ns	92.0	103.3	90.9	77.9	88.3
Biomass N-uptake	180.9ns	202.4	223.2	214.9	194.2	203.1
Harvest index	0.49ns	0.48	0.47	0.51	0.52	0.49
Number of seeds	2.94E+07ns	3.39E+07	3.48E+07	3.45E+07	3.28E+07	3.31E+07
Indicator leaf N concentration (%N)	2.33ns	2.56	2.56	2.57	2.75	2.55

1- Measurements means with different letters within N-levels are significantly different at $p<0.05$.
2- ns denotes that N application rates were not significant at $p>0.05$.
3- Means were not calculated for measurements that had significant differences ($p<0.05$).
4- Seed yield at a seed moisture of 15.5%.

In the 2014-2015 season seed yield was significantly higher with a fertilizer-N rate of 90 kg N/ha, with no significant difference among the applied fertilizer-N levels, with the highest yield of 4,708 kg/ha (Table 10). This is consistent with Espinosa-Irizarry's (2016) 60 to 160 kg N/ha inbred maize line application range. The highest seed N-uptake of 76.3 kg N/ha was obtained with a fertilizer-N application of 225 kg N/ha. Biomass N-uptake at 180 kg N/ha was the highest with

111.9 kg N/ha. Indicator leaf N concentration for the N control treatment was significantly different from the other four N application rates (Table 10). The mean harvest index was 0.59.

Table 10. Maize production measurements for 2014-2015 as affected by N level applications.

Measurements	Fertilizer Treatment (2014-2015) 0	90	135	180	225	Means
	----------kg/ha----------					
Seed Yield[4]	3,693a[1]	4,708b	4,166ab	4,695b	4,663b	--------[3]
Stover	3,336ns[2]	3,404	3,139	3,977	3,314	3,434
Biomass	7,444ns	8,633	7,745	9,209	8,511	8,309
Seed N-uptake	50.5a	69.0bc	62.3b	74.1c	76.3c	--------[3]
Stover N-uptake	26.7ns	28.1	27.1	37.8	35.3	31.0
Biomass N-uptake	77.2a	97.1ab	89.4a	111.9b	111.6b	--------[3]
Harvest index	0.56ns	0.6	0.59	0.58	0.61	0.59
Number of seeds	1.01E+07ns	1.23E+07	1.01E+07	1.06E+07	1.18E+07	1.10E+07
Indicator leaf N concentration (%N)	2.50a	2.76b	2.87b	2.81b	2.96b	--------[3]

1- Measurements means with different letters within N-levels are significantly different at $p<0.05$.
2- ns denotes that N application rates were not significant at $p>0.05$.
3- Means were not calculated for measurements that had significant differences ($p<0.05$).
4- Seed yield at a seed moisture of 15.5%.

The seed yield for the 2015-2016 season was not significantly different by fertilizer-N level or cover crop rotation. The mean seed yield was 2,510 kg/ha (Table 11). The 2015-2016 mean harvest index was 0.24 which coincides with inbred maize harvest index values reported by Sotomayor et al. (2012) of 0.26 and 0.21, Rivera-Zayas (2015) with 0.24 and Espinosa-Irizarry (2016) 2012 trial with 0.28.

Table 11. Maize production measurements for 2015-2016 as affected by N level applications.

Measurements	Fertilizer Treatment (2015-2016) 0	50	100	150	200	Means
	----------kg/ha----------					
Seed Yield[2]	2,357ns[1]	2,555	2,395	2,485	2,758	2,510
Stover	7,518ns	8,421	7,311	8,650	8,393	8,059
Biomass	9,924ns	11,020	9,750	11,189	11,204	10,617
Seed N-uptake	39.0ns	44.0	41.8	44.3	49.6	43.7
Stover N-uptake	89.9ns	112.5	97.1	122.1	109.3	106.2
Biomass N-uptake	128.9ns	156.5	138.8	166.4	158.9	149.9
Harvest index	0.24ns	0.23	0.24	0.23	0.25	0.24
Number of seeds	1.43E+07ns	1.75E+07	1.56E+07	1.54E+07	1.75E+07	1.61E+07
Indicator leaf N concentration (%N)	1.68ns	1.54	1.92	2.06	1.71	1.78

1- ns denotes that N application rates were not significant at $p>0.05$.
2- Seed yield at a seed moisture of 15.5%.

The indicator leaf N concentration for 2013-2014 had a critical value of 2.56% with yields of 6,944 kg/ha (Figure 3). In the 2014-2015 season the indicator leaf N concentration critical value was 3.13% with a seed yield of 4,779 kg/ha (Figure 4). In the 2015-2016 season there was no significant correlation between indicator leaf N and seed yield. Optimal indicator leaf concentration levels found for 2013-2014 and 2014-2015 fall into reported sufficiency ranges of 2.5-3.5% (Campbell, 2001). Rivera-Zayas (2015) reported an optimal leaf N value of 2.5% at a seed yield of 2,660 kg/ha. Sotomayor et al. (2012) reported optimum leaf N concentration values of 2.14 and 3.31%, for two separate growing seasons.

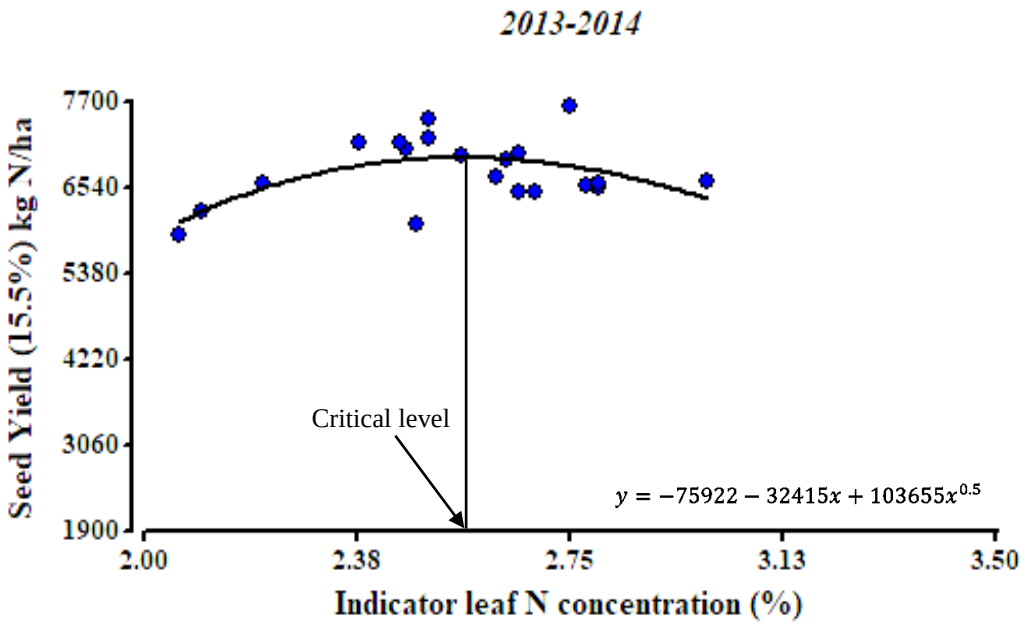


Figure 3. Relationship between indicator leaf N concentration and yield for the 2013-2014 season. The data was fit to the square-root model.

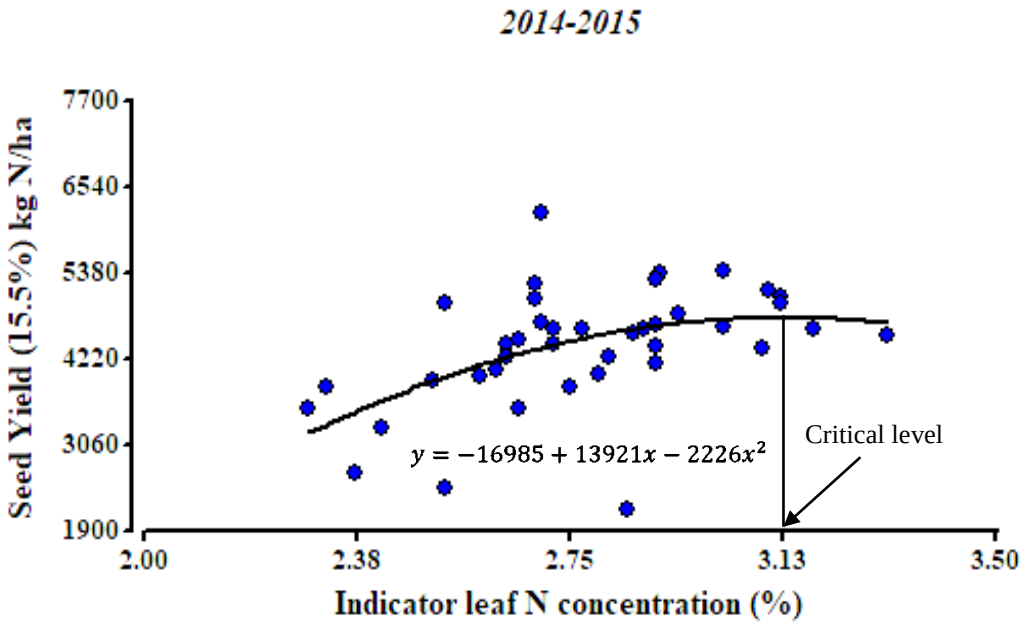


Figure 4. Relationship between indicator leaf N concentration and yield for the 2014-2015 season. The data was fit to the quadratic model.

Fertilizer-N applications, rotation, and fertilizer-N x rotation had no significant effects for immediately available soil N (0-30cm depth), potentially leached soil N (30-90cm depth) and total inorganic N (immediately available N + potentially leached N) for the 2013-2014 and 2015-2016 seasons (Table 12). The means for the 2013-2014 season for immediately available soil N, potentially leached soil N and total inorganic N were 85, 48.4 and 133.4 kg N/ha, respectively. The means for the 2015-2016 season were 66.4, 64.7 and 131.1 kg N/ha for immediately available soil N, potentially leached soil N and total inorganic soil N, respectively. The potentially leached soil N for the 2014-2015 season was significantly affected by fertilizer-N rates. Means for immediately available soil N and total inorganic soil N for the 2014-2015 season were 59 and 114.6 kg N/ha, respectively. In the 2014-2015 season, apart from the N2 value for potentially leached N, there was a tendency to increase with increasing N applications (Table 12). This suggests that there may be excess soil N. The 2016 post-harvest immediately available N was affected by fertilizer-N and cover crop rotation. Immediately available N for CC rotation was 67.43 kg N/ha and for FA was 47.87 kg N/ha.

Table 12. Mean soil inorganic N by fertilizer-N levels for 2013-2016.

N-level	Immediately available (0-30 cm)	Potentially leached (30-90 cm)	Total inorganic N
----------kg N/ha----------			
----------2013-2014----------			
N1[1]	84.4	52.9	137.3
N2	79.0	46.9	125.9
N3	91.7	51.2	142.9
N4	94.4	43.8	138.2
N5	75.4	47.2	122.6
Means	85.0	48.4	133.4
----------2014-2015----------			
N1	49.6	46.9ab[2]	96.5
N2	54.0	44.4a	98.4
N3	58.2	48.3ab	106.4
N4	67.2	64.6bc	131.8
N5	66.1	73.8c	139.9
Means	59.0	-------[4]	114.6
----------2015-2016----------			
N1	61.1	65.2	126.2
N2	55.8	53.7	109.5
N3	50.8	54.2	105.0
N4	81.2	69.1	150.3
N5	83.1	81.2	164.3
Means	66.4	64.7	131.1
----------2016 Post-Harvest[3]----------			
N1	45.9a	29.8	75.7
N2	55.6ab	41.2	96.9
N3	54.6ab	34.8	89.5
N4	63.6b	31.2	94.8
N5	68.5b	60.9	129.3
Means	-------[4]	39.6	97.2

1- 2013-2014 and 2014-2015 fertilizer-N levels were 0, 90, 135, 180 and 225 kg N/ha for N1, N2, N3, N4 and N5 respectively. 2015-2016 N1, N2, N3, N4 and N5 fertilizer-N levels were 0, 50, 100, 150 and 200 kg N/ha respectively.

2- Measurements means with different letters within N-levels by year are significantly different at $p<0.05$.

3- 2015-2016 post-harvest soil samples.

4- Means were not calculated for measurements that had significant differences ($p<0.05$).

The data between immediately available N and seed yield was fit to a linear-plateau model (Figures 5-7). The crop nutrient requirement (CNR) was 143 kg N/ha for a 6,917 kg/ha seed yield

(Figure 5), 156 kg N/ha with a 4,579 kg/ha seed yield (Figure 6) and 36.25 kg N/ha at a seed yield of 2,535 kg/ha (Figure 7) for the 2013-2014, 2014-2015 and 2015-2016 seasons, respectively.

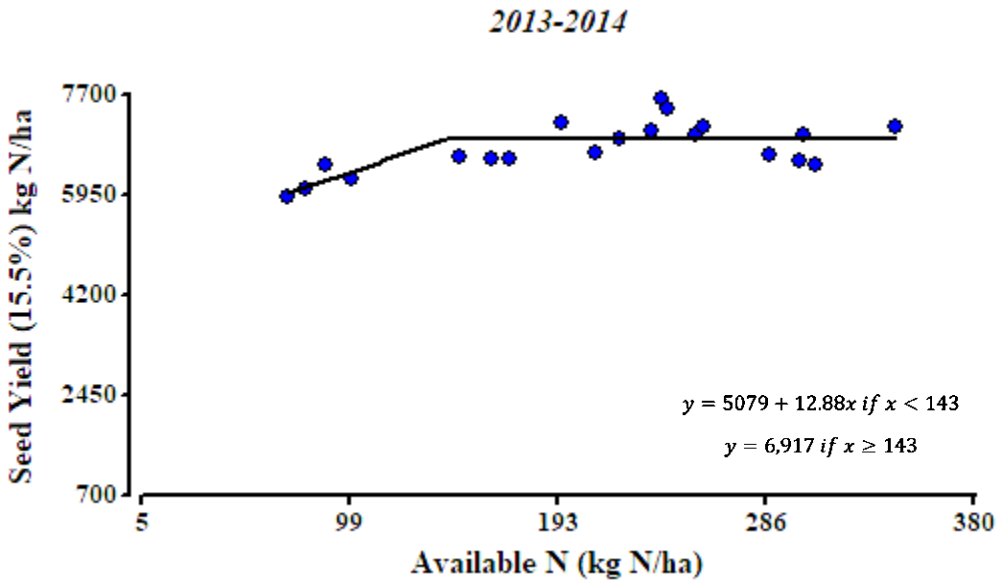


Figure 5. Linear plateau regression model, for the 2013-2014 season, used to determine crop nutrient requirement (CNR). Available soil N was calculated by the ∑ of applied amounts of fertilizer-N levels plus immediately available N (0-30 cm).

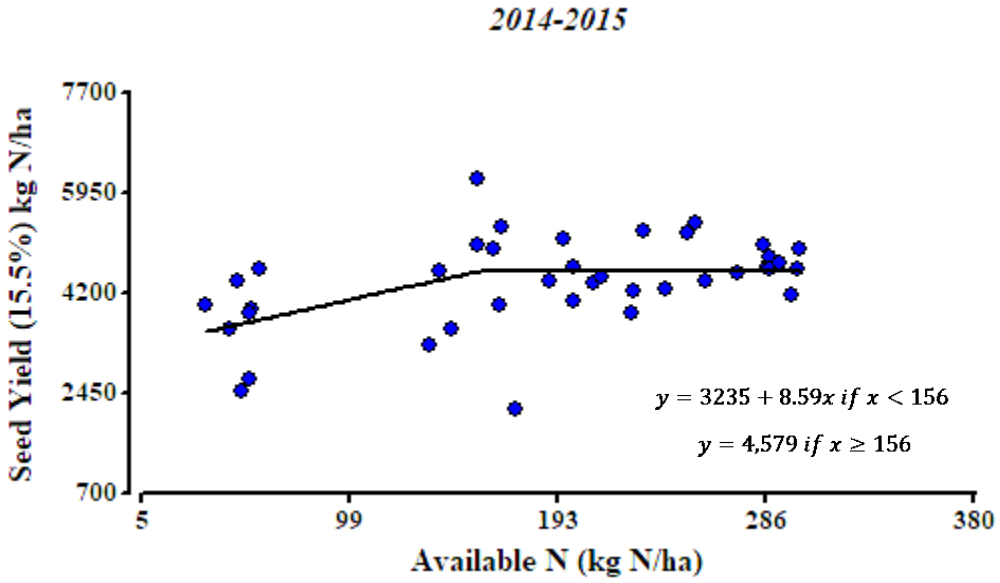


Figure 6. Linear plateau regression model, for the 2014-2015 season, used to determine crop nutrient requirement (CNR). Available soil N was calculated by the ∑ of applied amounts of fertilizer-N levels plus immediately available N (0-30 cm).

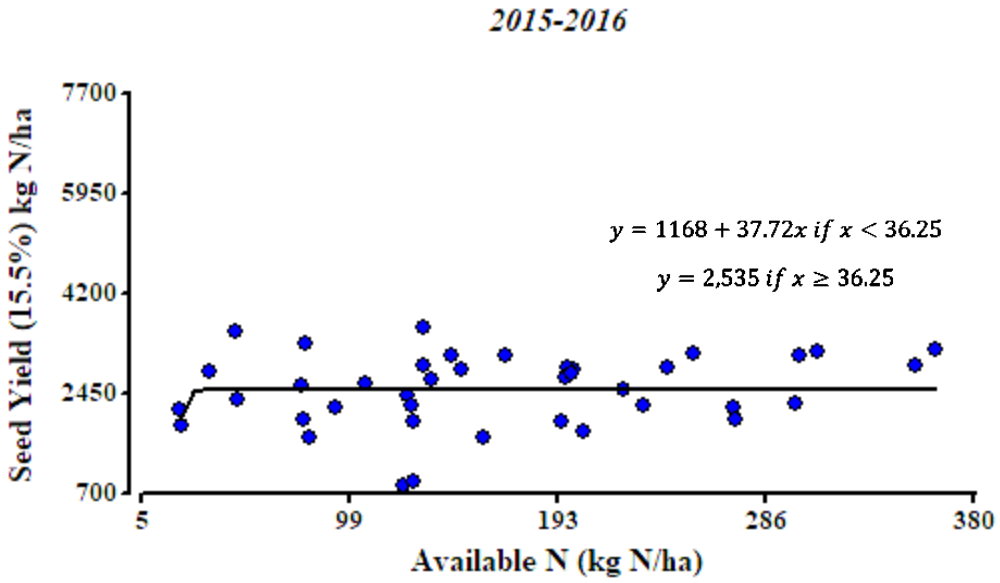


Figure 7. Linear plateau regression model, for the 2015-2016 season, used to determine crop nutrient requirement (CNR). Available soil N was calculated by the ∑ of applied amounts of fertilizer-N levels plus immediately available N (0-30 cm).

The total aboveground N budget had unaccounted N values of 239, 1,369, 761 and 966 kg N/ha for the complete 2013-2014, 2014-2015, 2015-2016 seasons and 2016 post-harvest samples, respectively (Figure 8). Unaccounted N for each individual N-level across all seasons had mostly positive values which indicates an N surplus, N inputs were greater than outputs. For the 2014-2015, 2015-2016 seasons and the 2016 post-harvest samples the control treatment (0 kg N/ha) was the closest in achieving N input-output stability, meaning when the difference between N inputs and N outputs equals zero. In the 2013-2014 season the fertilizer-N rate of 90 kg N/ha was the closest to input-output balance. In all seasons the major component of total N input for non-control N treatments was mostly fertilizer-N applications. In the 2013-2014 and 2014-2015 seasons the major N output was seed N-uptake. For the 2015-2016 season the major N output was potentially mineralizable N (30-90 cm). The higher cowpea cover crop N-uptake for the 2014-2015 season could be due to the higher precipitation during cowpea establishment at the time (Figure 1). Seed N-uptake was also greater for 2013-2014, this could be attributed to it being the initial maize

planting and that the experiment location had been an abandoned grassland beforehand. Afterwards seed N-uptake was always less than the initial maize planting and cover crop N-uptake was also lower after the first cowpea planting.

In this study the frequently used aboveground N budget approach (Ketterings et al., 2003; Liu et al., 2003; Shapiro et al., 2003; Ju et al., 2006) was done instead of the whole-crop approach because of the short experimental period (3 years). The whole-crop approach is not commonly used to establish N recommendations (Meisinger et al., 2008). In this case, nitrogen in roots was not quantified. Chevalier and Schrader (1977), in a study of NO_3^- absorption of maize plant parts, reported that root N content was 18.4% to 25 % in inbred and 18.3% to 30.3% in hybrid, of the total plant N uptake. As we did not include root N content in the budget, plant N-uptake and hence plant N contribution to soil may be underestimated.

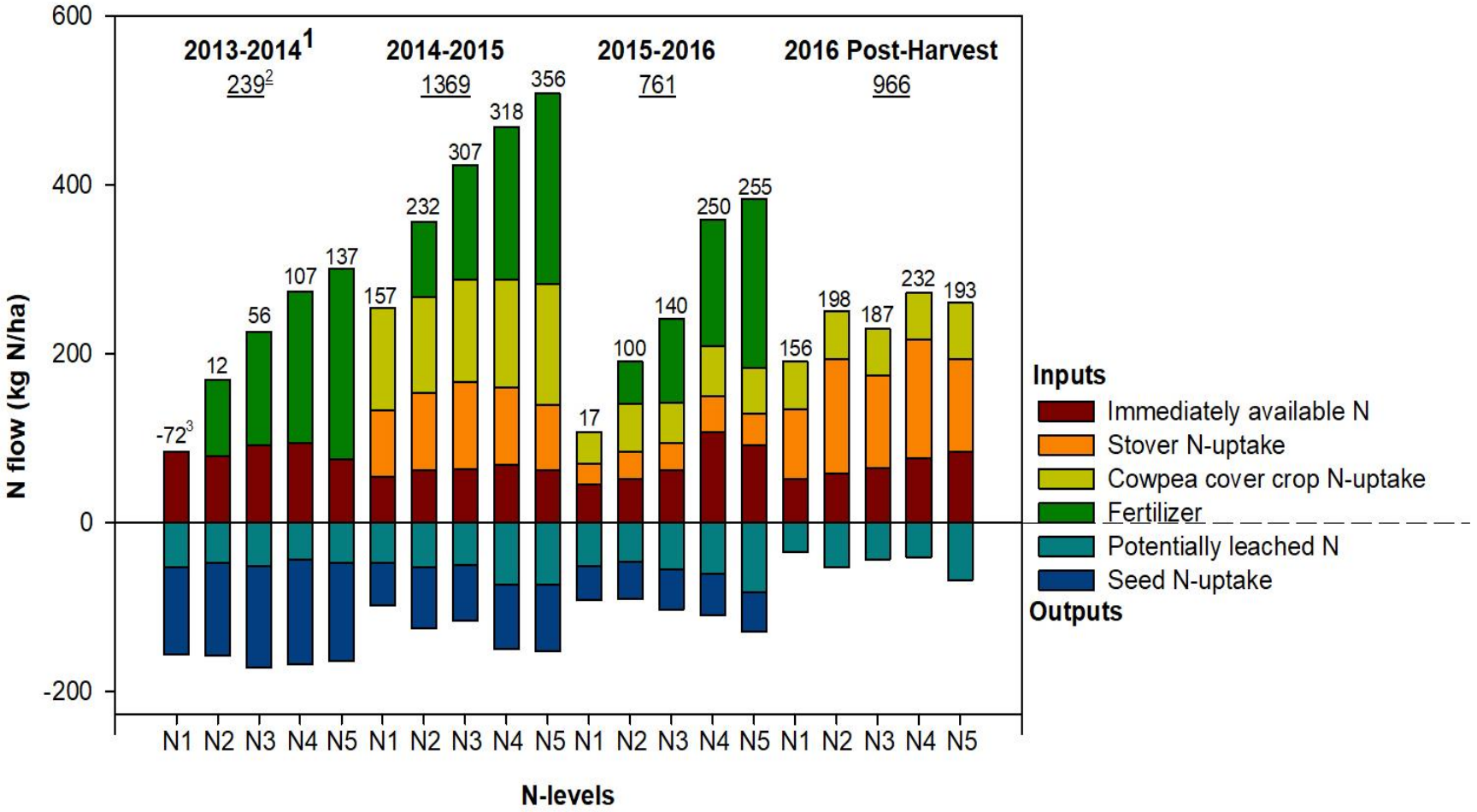


Figure 8. Nitrogen flow and budget of fertilizer-N levels for all seasons of Guayama experiment for the cowpea rotation treatment. N inputs consisted of applied fertilizer-N rates (N1, N2, N3, N4 and N5) at 0, 90, 135, 180 and 225 kg N/ha for 2013-2014 and 2015-2016 seasons. The 2015-2016 season had N applications of 0, 50, 100, 150 and 200 kg N/ha for N1, N2, N3, N4 and N5, respectively. Cowpea cover crop N-uptake from the previous summer, stover N-uptake from the previous season and initial immediately available N (0-30 cm depth) were used as soil N inputs. Soil N outputs were seed N-uptake and potentially leached N (30-90 cm depth). 1) Is the sample season. 2) Total aboveground N budget calculation for unaccounted N for a complete experimental season. Total Unaccounted N= ∑All Inputs for the complete season-∑All Outputs for complete season (kg N/ha). 3) Calculation of unaccounted N for each individual N-level by season. Unaccounted N= ∑Inputs-∑Outputs (kg N/ha).

To determine the nutrient use efficiency (NUE) various terms were calculated (Table 13). In general for the 2013-2014 and 2014-2015 seasons PFP, AE and PNB decreased with increasing fertilizer-N rates. For the 2015-2016 season PFP and PNB values also declined with increasing fertilizer-N rates. In the 2013-2014 season, the optimal PFP was 74.96 at 90 kg N/ha, for the 2014-2015 season it was 52.31 at 90 kg N/ha and in the 2015-2016 season it was 51.10 at 50 kg N/ha. PFP values of 59.1 (33 kg N/ha), 17.5 (84 kg N/ha) and 24.4 (112 kg N/ha) have been reported for inbred maize lines in Puerto Rico (Sotomayor-Ramírez et al., 2012; Rivera-Zayas, 2015). An optimal range of 40-90 PFP values have been reported for cereal crops (Fixen et al., 2015). Optimal AE values were 6.42 (2013-2014), 11.27 (2014-2015) and 3.95 (2015-2016) for 90, 90 and 50 kg N/ha respectively. Rivera-Zayas (2015) reported an optimal AE of 28.3 at 33 kg /ha. Fixen et al. (2015) determined optimal AE values within a range of 15-30. In the case of this study, none of the experimental trial periods had AE's in that range. Fixen et al. (2015) also reported RE in ranges of 40-65%, the only reported RE in this range was 55% at 50 kg N/ha for 2015-2016. Lower RE values could be implying that nutrients are accumulating in the soil instead of being taken up by the crop. Overall optimal PFP, AE and RE values for inbred maize in this experiment were achieved at 90 kg N/ha (2013-2015) and 50 kg N/ha (2015-2016).

Table 13. Nutrient use efficiency (NUE) calculations for 2013-2016 Guayama experiment.

N-level	PFP[1]	AE[2]	PNB[3]	RE[4]	IE[5]	PE[6]
			2013-2014			
0	----------					
90	74.96	6.42	0.02	0.24	33.33	26.82
135	52.10	6.41	0.01	0.31	31.51	20.43
180	40.19	5.92	0.01	0.19	33.67	31.35
225	29.58	2.17	0.01	0.06	34.27	36.55
			2014-2015			
0	----------					
90	52.31	11.27	0.01	0.22	48.48	50.88
135	30.86	3.50	0.01	0.09	46.61	38.71
180	26.08	5.57	0.01	0.19	41.96	28.85
225	20.72	4.31	0.01	0.15	41.79	28.18
			2015-2016			
0	----------					
50	51.10	3.95	0.03	0.55	16.33	7.16
100	23.95	0.37	0.02	0.10	17.25	3.72
150	16.57	0.85	0.01	0.25	14.93	3.41
200	13.79	2.00	0.01	0.15	17.36	13.34

1- PFP=grain yield/ fertilizer application
2- AE= (grain yield $_{\text{nutrient application}}$-grain yield $_{\text{no nutrient application}}$)/fertilizer-N applied
3- PNB=grain N-uptake/fertilizer rate applied
4- RE=(crop N-uptake $_{\text{with nutrient applied}}$-crop N-uptake $_{\text{no nutrient applied}}$)/fertilizer-N
5- IE=grain yield/crop N-uptake
6- PE=(grain yield $_{\text{nutrient application}}$-grain yield $_{\text{no nutrient application}}$)/(crop N-uptake $_{\text{with nutrient applied}}$-crop N-uptake $_{\text{no nutrient applied}}$)

CONCLUSIONS

This study demonstrated that fertilizer-N applications affected inbred maize growth and production, especially seed yield. Cover crop rotation treatment did not affect seed yield. With regards to the aboveground N budget, nitrogen was near balance at 90 kg N/ha for the 2013-2014 season and at 0 kg N/ha for the 2014-2015 and 2015-2016 seasons. The partial N budget reported that N inputs were mostly greater than N outputs, which could suggest that there is excess N in the system. Optimal nutrient use efficiency levels were reached with 90 kg N/ha for the 2013-2014 and 2014-2015 seasons and 50 kg N/ha for the 2015-2016 season. Overall the ANOVA analysis and NUE efficiency calculations suggest that the optimal N application rate for the inbred maize-line SSH65VH was 90 kg N/ha and 50 kg N/ha for the inbred maize-line SLM15VH in an Inseptisol.

CHAPTER 3

APPLYING SMAF SOIL QUALITY INDICATOR TO FERTILIZER-N AND COVER CROP ROTATION MANAGEMENT PRACTICES

INTRODUCTION

Soil quality assessments are necessary and useful as a guide or management tool for farmers, and as a measure of a soils sustainability (Doran and Parkin, 1994). Different biological, physical and chemical properties of soil can be used to assess a soil's quality and health (Doran, 2002) by evaluating the potential changes in different soil ecosystem functions as affected by soil management practices (Doran and Parkin, 1996; Andrews et al., 2004). The importance of implementing a periodic soil quality assessment lies in the need to be able to know the direction of the condition of the soil in response to management decisions because soil degradation is still a persistent threat due to erroneous land management (Karlen et al., 2008).

The Soil Management Assessment Framework (SMAF) is a tool created by Susan Andrews and Douglas Karlen with various collaborators to evaluate the impact of management practices on selected chemical, physical, and biological indicators that affect soil functions such as nutrient cycling, water relations, physical stability and support, filtering and buffering, resistance and resilience, and biodiversity and habitat (Karlen et al., 2001; Andrews et al., 2002a, 2004). SMAF involves three steps: indicator selection, indicator interpretation, and integration into an index value (Andrews et al., 2004). The 2014 version of SMAF includes specific soil quality indicators: aggregate stability, available water capacity, bulk density, β-Glucosidase activity, electrical conductivity, soil test potassium, microbial biomass carbon, pH, potentially mineralizable N, soil test P, sodium adsorption ratio, soil organic carbon and water filled pore space (Table 14).

Table 14. Possible SMAF soil quality indicators and their important functions in soils.

Soil Quality Indicator	Functions
Aggregate stability	Is a function of the relative amount of sand, slit and clay-sized particles and is influenced by residue management and tillage
Available water capacity	Affects available water for crops and biological activities
Bulk density	Influences plant root development
β-Glucosidase	Enzyme activity involved in cellulose degradation, provides energy for microorganisms, affects organic matter decomposition
Electrical conductivity	A measurement of soluble nutrients and soil salinity
Soil K	Measurement of essential nutrient availability
Microbial biomass C	Measurement of the biological activity and nutrient cycling processes
Soil pH	Affects soil nutrient availability, microorganisms, ammonium and nitrate processes and soil nutrient toxicities and deficiencies
Potentially mineralizable N	Measurement of nutrient availability, interaction between crop productivity and microbial activity
Soil P	Indicator of crop response and environmental threat
Sodium adsorption ratio	Useful for arid and semi-arid regions with salt build up
Soil organic C	Affects plant growth, provides nutrients for soil microorganisms
Water filled pore space	Affects microbial activities, crop production and root respiration

Table constructed from information gathered from Andrews et al. (2004), Karlen et al. (2008), and Wienhold et al. (2009).

In order to develop the SMAF soil quality index (SQI) the creators had to first define the site-specific soil management goals. These management goals took into consideration the broader spectrum of the agroecosystem and focused on sustainability rather than only crop yields. Once the soil functions were chosen a minimum data set (MDS) of soil quality indicators was obtained, analyzed and validated as established by Andrews and Carroll (2001).

The MDS variables were transformed through the use of nonlinear scoring curves (Karlen and Stott, 1994; Andrews et al., 2002b). The general curve shapes were: upper asymptotic curve (more is better), lower asymptotic curve (less is better) and Gaussian function (mid-point optima)

(Karlen and Stott, 1994; Andrews et al., 2002b). The SQI value was obtained by summing the scores of the indicators and dividing them by the total amount of indicators used and then multiplying this result by 10 or 100 to provide index values in a 1-10 scale or a 1-100 scale. This value is the general assessment of the soils quality. It is understood that the higher the SQI score the greater the soil quality (Andrews and Carroll, 2001).

Two experiments in California showed greater soil quality index (SQI) scores under organic management systems when compared to other systems including the conventional management (Andrews et al., 2002a, b). The indicators used were soil organic matter, total nitrogen, electrical conductivity, soil Ca, Na, S, K, Zn and P, pH and the sodium absorption ratio. In another study, 50 fields were selected and within these, three well-developed and one stressed crop canopy area was examined per field in the Iowa River South Fork Watershed. More SQI indicators were used than in California which included bulk density, pH, electrical conductivity, extractable P and K, organic C, microbial biomass C, potentially mineralizable N, water filled pore space, aggregate stability and β-glucosidase activity. The Iowa study showed a higher overall mean (86%) SQI score for well-developed crop canopy systems than the stressed crop canopy (82%). Soil organic C and β-glucosidase activity had the lowest SQI scores and according to the study it could indicate that the soils biological capacity is being compromised (Stott et al., 2011).

Previous studies help to demonstrate SMAF's adaptability and effectiveness in evaluating diverse types of management practices and serving to pin point areas that need improvement or change in order to have a healthier and more productive system. Assessment tools like SMAF could help to increase the sustainability of agricultural management choices in Puerto Rico. Therefore, the objective of this chapter was to apply the SMAF soil quality model to assess the effects of fertilizer-N applications and cowpea cover crop rotation.

MATERIALS AND METHODS

Study site and field measurements

The SMAF SQI was applied to the experiment described in Chapters 1 and 2 at the end of the 2015-2016 season. The location, fertilizer-N treatments and cover crop rotation were described in detail for 2015-2016 in Chapter 1. All soil samples used for the selected indicators were taken at a depth of 0-15cm before maize harvest near 90 DAP. Ten subsamples from each subplot were homogenized to form a composite sample.

SMAF soil quality index (SQI)

The soil quality indicators selected were: microbial biomass carbon (MBC), soil pH (pH), microaggregate stability (AGG), potentially mineralizable N (PMN), β-glucosidase (BG), soil phosphorus (P) and soil potassium (K). The additive SQI values were calculated as:

$$additive\ SQI = \frac{MBC + pH + AGG + PMN + BG + P + K}{7}$$

were MBC, pH, AGG, PMN, BG, P and K are the scores obtained from each of the parameters and 7 is the number of total indicator values used. Soil physical SQI was calculated as AGG/1, chemical SQI was pH/1, biological and biochemical SQI was equal to (MBC+BG+PMN)/3 and nutrients SQI was (P+K)/2. SQI values were multiplied by 100 in order to use a percentage scale of 1 to 100.

Microbial biomass carbon (MBC) and nitrogen (MBN)

Soils were sampled to a depth of 0-15 cm using a soil recovery probe (1.27 cm x 102 cm). Samples were stored in the freezer until they were ready to be analyzed. The samples were analyzed using the chloroform fumigation extraction method (Vance et al., 1987) . Three samples of 15 g of moist soil were weighed, two of these samples were fumigated and the third was the

non-fumigated control. Fumigated samples were placed in a dessicator with the chloroform and kept in the dark for 24hrs. After the 24hr fumigation the chloroform was removed from the soil by using a vacuum pump. Seventy-five mL of 0.5M K_2SO_4 was added to each sample and shaken for an hour and afterwards centrifuged at 1,800 rpm for one minute. Samples were then filtered through a Whatman No. 42 filter. The filtered extraction solvent was diluted with deionized water at a 1:1 ratio (10 mL each) and placed in a CN analyzer (Shimadzu Model TOC-V/$_{CPH}$-TN). To determine soil moisture a 10 g sample was placed in an oven at 105°C for 24 hrs. Biomass C was calculated as:

$$Biomass\ C = \frac{(Cf - Cuf)}{K_{ec}}$$

were Cf is the carbon in the fumigated extract, Cuf is the C in the unfumigated extract and K_{ec} is the quantity of microbial C extracted from the soil. The K_{ec} used for biomass C was 0.45 (Wu et al., 1990).

Soil pH

Soil pH was measured in the Soil and Water laboratory at the Finca Alzamora, UPRM. Samples were air dried and read with a pH meter using the deionized water procedure (Thomas, 1996). Ten grams of air dried soil were placed in a 50 mL beaker and 10 mL of deionized water was added to the soil. The suspension was put in the shaker for an hour and left standing for two hours. The pH probe was submerged in the supernatant and the samples were read with a pH meter.

Microaggregate stability (AGG)

A modified version of the Methods of Soil Analysis SSSA wet-sieving method was used (Nimmo and Perkins, 2002). Air dried soil samples were sieved through a #4 (4.75 mm) and #10

(2 mm) sieve. Soil retained in the #10 sieve was used for the analysis. Two samples of 30 g of soil were measured. One was used to determine soil moisture, it was placed in an oven at 105°C for 8 hours. The other soil sample was placed on a filter paper (12-15 cm diameter) and put on the #10 sieve. This sieve was then put on top of a #20 (850 µm) sieve and placed in the wet sieving system. Water was added to the system until it reached the filter paper, the soil was allowed to moisten for 10 minutes. Afterwards more water was added until one inch of water covered the filter paper that was then carefully removed. The soil was in the wet sieving system for 30 minutes at a ratio of 35 cycles per minute. The samples were then put in the oven to dry at 105°C for 8hrs and weighed. Aggregate stability was calculated as:

$$\%\ Aggregate\ Stability = \frac{(A+B)}{C} x\ 100$$

were A was the soil dry weight in sieve #10, B was the soil dry weight in sieve #20 and C was the dry weight of the initial 30g soil sample.

Potentially mineralizable N (PMN)

Soil samples were collected for each subplot at a 15 cm depth and analyzed using the soil incubation procedure for 28 days (Stanford and Smith, 1987; Cabrera and Kissel, 1988). Air dried soils were sieved through a 6-mm sieve. The incubation vessels consisted of 0.30 g of celite filter and a 3 cm filter that was placed in a 60 mL syringe and weighed. Afterwards 40 g of soil were packed into the syringe and the total weight measured. Inorganic N extraction was done using 80 mL $CaCl_2$ 0.01M. The leaching system ran for 3 hours. The extracted volume of the leaching solution was measured. Twenty-five mL of the leaching solution was then put into a 25 mL bottle with 2 drops of sulfuric acid for sample preservation. The leaching solution samples were stored by freezing until analysis. Afterwards 10 mL of N free solution containing concentrations of 340

$CaSO_4$ mg/L, 120 $MgSO_4$ mg/L, 15.04 K_2SO_4 mg/L and 2.19 KH_2 PO_4 /L were added and run in the leaching system for 30 minutes to replace exchangeable ions. The syringes were then weighed and put into the suction system for 4 hours. Applied suction was -0.33 kPa in order to leave the soils close to field capacity. Finally before being placed in the incubator (40°C) syringes were weighed. The process was repeated at 14 days and 28 days. The reported PMN data in this chapter is the cumulative amount of N mineralized at each time period during the 28 day incubation plus the initial N.

β-glucosidase (BG) activity

This assay measured *p*-nitrophenol released by β-glucosidase through colorimetric determination (Tabatabai, 1994). A 0.5g soil sample was be placed in an Erlenmeyer flask with 4 mL of MUB (modified universal buffer pH 6) and 1 mL of PNG (*p*-nitrophenyl-β-D-glucoside) substrate to start the reaction. The flasks were then placed with a stopper in an incubator at 37°C for 1 hour. Afterwards the stopper was removed and 1 mL of 0.5M $CaCl_2$ and 4 mL of THAM (tris(hydroxymethyl) aminomethane) buffer were used to stop the reaction. The intensity of the yellow color was measured with a colorimeter (Evolution 60S Spectrophotometer). This procedure was done in the USDA-ARS (United States Department of Agriculture-Agricultural Resource Service), Cropping Systems Research Laboratory in Lubbock, TX.

Soil extractable P (P) and soil exchangeable K (K)

Soils were sampled for each subplot at a 0-15 cm depth. Soil samples were left to air dry and then sieved. Samples were sent to AgSource Laboratory (Lincoln, NE) for soil extractable P (Kuo, 1996) and soil exchangeable K (Helmke and Sparks, 1996) analysis.

Statistical Analysis

The SMAF SQI indicator data was analyzed using the 2014 SMAF model which was acquired from Diane Stott, USDA-NRCS (United States Department of Agriculture-Natural Resource Conservation Service, personal communication, 2015). Soil quality indicator data was analyzed using InfoStat (2014®) software. An ANOVA was run for a strip-plot completely randomized block design with factors of: fertilizer-N, rotation and fertilizer-N x rotation interaction. Significant differences in the ANOVA analysis were determined by using the LSD Fisher test ($p<0.05$).

RESULTS AND DISCUSSION

Individual response of soil quality indicators

During this 3-year study, there was no significant interaction for fertilizer-N and rotation treatments for the soil quality indicators evaluated ($p>0.05$) (Table 15). The most important treatment effects on the SQI indicators were found in soil pH ($p<0.05$) due to the fertilizer-N treatment (Table 16) and in PMN and pH due to the cover crop rotation ($p<0.05$) (Table 17). Soil pH values decreased as fertilizer-N rates increased as: 7.21, 7.03, 6.98, 6.88 and 6.83 for 0, 50, 100, 150 and 200 kg/ha N-levels, respectively (Table 16). This tendency has been observed in previous studies (Ritchey et al., 2015; Tian and Niu, 2015; Tyler et al., 2018) due to an increase in nitrification (e.g., NH_4^+ to NO_3^-) (Ritchey et al., 2015). Mean values for indicators that were not significantly different (AG, MBC, BG, P, K and PMN) were 19.9, 308.2, 36.4, 9.6, 125.6 and 4.2 (Table 16).

Table 15. Summarized ANOVA for soil quality indicators for the Guayama experiment in the 2015-2016 season.

Indicator[1]	Fertilizer-N	Cover crop	Fertilizer-N x Cover crop
AG	0.3423	0.5873	0.3182
MBC	0.5058	0.2134	0.2157
BG	0.1057	0.0858	0.4021
P	0.9583	0.6330	0.8390
K	0.1667	0.1914	0.6981
pH	0.0004*[2]	0.0313*	0.2313
PMN	0.9300	0.0346*	0.4562

1- Soil quality indicators: microaggregate stability (AG), microbial biomass carbon (MBC), β-glucosidase activity (BG), soil extractable P (P), soil exchangeable K (K), soil pH (pH) and potentially mineralizable N (PMN).

2- * indicates p-values of less than 0.05.

In this study, the use of a legume as a cover crop demonstrated benefits in N cycling as indicated by an increase in PMN, which was almost three times greater (6.1 vs. 2.3 mg N/kg) under CC than FA treatments. Similar increases in PMN (72% greater) were reported by Mcdaniel and Grandy (2016) in a twelve year rotation study. This can also explain a slight decrease in soil pH under CC (6.9) compared to FA (7.1) due to the increase in N found in the system with the legume cover crop (Ritchey et al., 2015). Mcdaniel and Grandy (2016) also reported a 28 to 112% increase in MBC due to cover crops. All other indicators had no differences with regards to rotation (Table 17).

Table 16. Effects of fertilizer-N treatment on selected SQI indicators during 2015-2016.

N-level	AG[1]	MBC[1]	BG[1]	P[1]	K[1]	pH[1]	PMN[1]
kg/ha	%	(mg/kg soil)	(mg PNkg^{-1} soil h^{-1})	mg/kg	mg/kg		mg/kg
0	16.8ns[2]	339.2ns	36.0ns	9.3ns	120.1ns	7.2d[3]	4.7ns
50	16.8	252.9	37.7	9.1	130.3	7.0c	3.8
100	23.2	358.9	36.7	9.1	116.6	7.0bc	4.6
150	21.7	303.7	39.3	9.6	121.6	6.9ab	3.9
200	20.9	286.6	32.4	10.6	139.1	6.8a	4.2
Means	19.9	308.2	36.4	9.6	125.5	--------[4]	4.2

1- Soil quality indicators: microaggregate stability (AG), microbial biomass carbon (MBC), β-glucosidase activity (BG), soil extractable P (P), soil exchangeable K (K), soil pH (pH) and potentially mineralizable N (PMN).
2- ns denotes that fertilizer-N rates were not significant at $p>0.05$.
3- Measurements with different letters within N-levels are significantly different at $p<0.05$.
4- Means were not calculated for significantly different data ($p<0.05$).

Table 17. Cowpea cover crop rotation effect on SQI indicators during 2015-2016.

Rotation	AG[1] %	MBC[1] (mg/kg soil)	BG[1] (mg PNkg^{-1} soil h^{-1})	P[1] mg/kg	K[1] mg/kg	pH[1]	PMN[1] mg/kg soil
CC	19.1ns[2]	325.2ns	40.3ns	10.1ns	143.2ns	6.9a[3]	6.1b
FA	20.6	291.2	32.5	9.0	107.9	7.1b	2.3a
Means	19.9	308.2	36.4	9.6	125.6	--------[4]	--------[4]

1- Soil quality indicators: microaggregate stability (AG), microbial biomass carbon (MBC), β-glucosidase activity (BG), soil extractable P (P), soil exchangeable K (K), soil pH (pH) and potentially mineralizable N (PMN).
2- ns denotes that fertilizer-N rates were not significant at $p>0.05$.
3- Measurements with different letters within N-levels are significantly different at $p<0.05$.
4- Means were not calculated for significantly different data ($p<0.05$).

Overall response of SMAF according to SQI scores

The SMAF SQI scores were calculated for each treatment (N-level by rotation) (Table 18). The overall average soil quality index score was 58% for this soil. The mean SQI for CC rotation was 63% and 54% for FA. The additive SQI scores ranged from 53% for FA (for 0, 50 and 100 kg N/ha treatment) to 67% under the CC rotation (for 100 kg N/ha treatment). The mean categorical SQI's were 63%, 94%, 43% and 61% for the physical, chemical, biological and biochemical, and nutrient categories, respectively. The high chemical SQI score (94%) could suggest that crop production was not limited by the soil chemical indicators.

Overall, the averaged additive SQI of 58% indicates that there is room for soil health improvement (Table 18). Among the different categorical indicators, the soil biological and biochemical properties had the lowest SQI score (43%), making it the only category with a mean SQI score below 50%. Mbuthia et al., (2015) found that although long term tillage, fertilizer-N and cover crop affected the soil microbial community and activity, lower SQI scores were also found for total organic carbon and β-glucosidase activity. They concluded that better management practices should be incorporated in order to increase soil N and C storage. In the current study with this soil in Puerto Rico, practices that can improve the SQI scores could be the reduction of tillage

intensity, which has been reported to increase soil biological activity as it allows for the establishment of varied microhabitats (Kladivko, 2001). Also, the use of diverse crops or cover crops can help to stimulate microbial activity and diversity because there is more food quantity and quality for the microorganisms. The addition of organic materials through compost, crop residues and manure can also influence microbial food availability (Kladivko and Clapperton, 2011). Addition of organic matter, through a combination of reduced tillage, crop rotations and organic materials can effectively benefit soil physical properties by providing better soil structure, which in turn will increase soil moisture and soil conditions for microbial activity and mobility (Kladivko and Clapperton, 2011).

Table 18. 2015-2016 SMAF soil quality index scores for N-level and rotation.

Treatment		Additive[2]			Physical[3]			Chemical[4]			Biological & Biochemical[5]			Nutrients[6]		
N-level	Rotation	Sum of scores	Number of indicators	SQI	Sum of scores	Number of indicators	SQI	Sum of scores	Number of indicators	SQI	Sum of scores	Number of indicators	SQI	Sum of scores	Number of indicators	SQI
N1[1]	CC	4.40	7	63	0.58	1	58	0.92	1	92	1.74	3	58	1.17	2	58
N1	FA	3.72	7	53	0.55	1	55	0.88	1	88	1.07	3	36	1.22	2	61
N2	CC	4.03	7	58	0.60	1	60	0.95	1	95	1.24	3	41	1.23	2	62
N2	FA	3.72	7	53	0.53	1	53	0.92	1	92	1.09	3	36	1.18	2	59
N3	CC	4.69	7	67	0.74	1	74	0.96	1	96	1.73	3	58	1.26	2	63
N3	FA	3.73	7	53	0.67	1	67	0.93	1	93	1.07	3	36	1.07	2	54
N4	CC	4.44	7	63	0.60	1	60	0.98	1	98	1.47	3	49	1.38	2	69
N4	FA	3.77	7	54	0.74	1	74	0.94	1	94	1.06	3	35	1.04	2	52
N5	CC	4.37	7	62	0.56	1	56	0.99	1	99	1.34	3	45	1.48	2	74
N5	FA	3.94	7	56	0.74	1	74	0.94	1	94	1.09	3	36	1.17	2	59
Means[7]				58			63			94			43			61

1- N1= 0 kg N/ha, N2= 50 kg N/ha, N3= 100 kg N/ha, N4= 150 kg N/ha and N5= 200 kg N/ha
2- Additive SQI= ((AGG+BD+pH+MBC+BG+PMN+P+K)/7)*100
3- Physical SQI= ((AGG)/1)*100
4- Chemical SQI= ((pH)/1)*100
5- Biological and Biochemical SQI= ((MBC+BG+PMN)/3)*100
6- Nutrients SQI= ((P+K)/2)*100
7- Percentage means of SQI scores

CONCLUSIONS

The fertilizer-N levels only significantly affected soil pH and the cover crop rotation affected both soil pH and PMN. The use of cowpea cover crop rotation appears to be beneficial in increasing the microbial community and N mineralization in soils, but it may take more than three years for this rotation to impact the soil quality/health. The SMAF SQI scores were able to distinguish differences between N-levels when comparing CC rotation vs fallow. The overall low SQI scores, including the biological and biochemical indicators (43%), suggest the application of more practices that incorporate organic matter are needed including reduced tillage, lower pesticide usage, greater crop diversity and/or cover crop rotations to detect further benefits in this soil agroecosystem.

CHAPTER 4

MICROBIAL COMMUNITY COMPOSITION AND FUNCTION IN REGARDS TO FERTILIZER-N AND COVER CROP ROTATION

INTRODUCTION

Management induced changes in soil quality/health can be detected earlier via the response of the soil microbial community (Doran and Parkin, 1994; Doran and Zeiss, 2000), due to their key role in soil processes that can modify the soil chemical and physical properties (Spedding et al., 2004). The microbial component is important in essential soil functions and properties including nutrient cycling, soil structure and stability, protection against agrochemicals, pollutants and waste, increasing water availability and in suppressing pathogens and weed growth (Wall et al., 2004; Falkowski et al., 2008; Kowalchuk et al., 2008; Pritchard 2011; Lehman et al., 2015). Changes in the microbial community can be assessed in terms of size, composition and activity.

Microbial biomass C or N can be determined via chloroform-fumigation-incubation/extraction methods (Jenkinson and Powison, 1976) providing an estimation of the microbial community size, the most labile C pool in soil. In addition to information of the microbial community size, fatty acid methyl ester analysis (FAME's) can provide information of changes in community composition by using markers for specific groups of bacteria (i15:0, a15:0, i17:0, a17:0, cy17:0, cy19:0), actinomycetes (10Me16:0, 10Me17:0, 10Me18:0), saprophytic fungi (18:2.6c) and arbuscular mycorrhizal fungi (AMF) (16:1.5c) populations (Zelles, 1999; Willers et al., 2015) (Fatty acid nomenclature descriptions in Appendix 12). Changes in fungal populations can be interpreted as beneficial to soil quality/health as they are able to increase C sequestration in soil and they also are able to physically promote aggregation increasing soil structure and stability, helping to prevent erosion, increasing soil water retention and nutrient uptake (Rillig,

2004; Cano et al., 2018) . Changes in the community size and composition can lead to changes in nutrient cycling and SOM dynamics via the enzymatic capacity of soil. Microbial community activity can be assessed through the measurement of soil enzyme activities. Enzymes such as β-glucosidase, β-glucosaminidase and acid phosphatase are used as indices of C, C and N, and P cycling, respectively (Mbuthia et al., 2015; Acosta-Martínez et al., 2018). The enzyme β-glucosidase is involved in cellulose degradation and glucose production. The enzyme β-glucosaminidase is involved in chitin degradation and helps produce amino sugars. Glucose and amino sugars are energy sources for microorganisms. Acid phosphatase is predominant in acid soils and is involved in the production of plant available phosphates.

Microbial communities and their enzymatic activities are among the most sensitive indicators of soil quality/health due to their rapid response to changes in soil management practices and their close relationship to soil biological properties (Acosta-Martínez et al., 2018). A study that examined the effects of crop rotation on soil C and N showed that having a crop rotation increased microbial biomass C by 21% and microbial biomass N by 26% (McDaniel et al., 2014). Mbuthia et al. 2015 showed that in long term studies, no-tillage practices increased mycorrhizae, actinomycetes and gram positive bacteria FAME markers by 5%, 6% and 17%, respectively under four fertilizer-N levels in an Alfisol. Similarly, FAME markers for bacteria and fungi were more abundant in pasture soils than under vegetable (tomatoes (*Solanum lycopersicum*), sweet peppers (*Capsicum annum*) and watermelon (*Citrullus lanatus*)) production in a Mollisol under a long-term management history in Puerto Rico (Acosta-Martínez et al., 2008). The study also showed lower microbial biomass C and N under vegetable production when compared with the pasture and mango trees (*Mangifera indica*) (Acosta-Martínez et al., 2008). Similarly, another study in

long-term sites showed greater microbial biomass C and N in pasture sites when compared to forest and agriculture sites for a Vertisols in the Lajas Valley, PR (Sotomayor-Ramírez et al., 2009).

Studies on long-term managed sites showed subsequent increases in the enzymatic potential of the soil following the changes of the microbial community size and composition. For example, a study conducted in the Central Valley of California on 13 organically managed tomato fields, showed greater C cycling enzyme activities with increasing inorganic N availability (Bowles et al., 2014). Similar to greater microbial biomass, the activities of β-glucosidase and β-glucosaminidase (C and N cycling enzymes) were greater under pasture than forest or agricultural sites for the Lajas Valley of Puerto Rico (Sotomayor et al. 2009).

In this study, evaluation of microbial community size, composition and activity may provide other insights into the effects of cover-crops and fertilizer-N for this tropical soil scenario. The first objective of this chapter was to evaluate the effects of fertilizer-N levels and cover-crops on the soil microbial component. The secondary objective was to validate a new combined enzyme assay procedure to determine several enzyme activities simultaneously in the same soil sample to obtain an index of biogeochemical cycling (Acosta-Martínez et al., 2018). This combined assay has not been used before in Puerto Rico and could become a more suitable and economical option to compare management practices in our soils because it can save soil, resources and reduce waste generated with the assay.

MATERIALS AND METHODS

Study site

A detailed description of the location, experimental design and treatments was described in Chapter 1 of this document. Procedures to determine microbial community composition and function were only done for the 2015-2016 season. All soils samples analyzed were taken at a 0-15 cm depth. Soil samples for FAME profile, β-glucosidase (BG), microbial biomass C (MBC) and microbial biomass N (MBN) were collected near 90 DAP, before maize harvest. Two soil sampling dates were done for the combined enzyme assay procedure, the first sample was collected after maize harvest (28 March 2016) and the second after cowpea cover crop incorporation (20 December 2016).

Chloroform Fumigation Incubation Method: Microbial Biomass C and N

The procedures used to analyze MBC and MBN were described in detail in the previous chapter. Biomass N was expressed as:

$$Biomas\ N = \frac{(Nf - Nuf)}{K_{en}}$$

were Nf is the total N from the fumigated sample, Nuf is the total N from the unfumigated soil sample and K_{en} is the extracted inorganic N and organic microbial N from the soil. The K_{en} used was 0.54 (Jenkinson, 1988).

EL-FAME Method: Microbial Community Composition

Microbial community structure was characterized according to FAME profiling with the Ester-linked Fatty Acid Methyl Esters (EL-FAME) extraction method by Schutter and Dick (2000). Soil samples were stored and kept frozen until analysis. Soils were thawed and three grams of soil were placed in a test tube and 15 mL of 0.2 M KOH was added. The sample were then

vortexed for 10 seconds and then heated at 37°C in a water bath for 1 hour. In this step, the ester-linked fatty acids are released and methylated (Schutter and Dick, 2000). During the incubation period, samples were vortexed for 5 seconds at 15 minute intervals. Afterwards the samples were cooled at ambient temperature for 5 minutes. Three milliliters of 0.1 M acetic acid was added to neutralize the pH of the samples. Samples were vortexed for 5 seconds and then left at ambient temperature for 5 minutes. Three milliliters of hexane were added and the tubes inverted 5 times and centrifuged at 2200 rpm for 8 minutes to separate the organic phase. The top organic phase was transferred to a Teflon lined test tube. These tubes were then placed in the concentrator at 37°C until they were completely dry. Afterwards 100 µL of Standard Redissolve solution (1:1 hexane:methyl-tert butyl ether) was added and vortexed. Samples were analyzed through gas chromatography. FAMEs used as microbial biomarkers were selected according to previous research (Zelles, 1999).

Enzyme Activity Assays: β-Glucosidase and Combined Enzyme Activity Assay

The protocol for determination of β-glucosidase activity was described in the previous chapter. A new combined enzyme activity assay was used for the simultaneous determination of β-glucosidase, β-glucosaminidase and acid phosphatase activities. The combined enzyme activity assay was preformed using 0.5 g of air dried soil and incubated with MUB pH 6.0 and THAM pH 12.0 buffer according to the specifications and procedure established by Acosta-Martinez et al. (2018).

Data Analysis

The data was subjected to statistical analysis using InfoStat (2014®) statistical software. The experimental design was a strip-plot in a randomized complete block with three factors: fertilizer-N, CC rotation and the interaction between fertilizer-N x rotation. A Shapiro-Wilks test

was used to verify data normalization and Levene data homogeneity. Significant differences were established with an α of 0.05. A principal component analysis (PCA) was used for the FAME data with InfoStat (2014®). The PCA consisted of the N-level and cover crop rotation, five Gram positive (G+) bacterial biomarkers (i15:0, a15:0, i16:0, i17:0 and a17:0); two Gram negative (G-) bacterial biomarkers (cy17:0 and cy19:0); two actinomycetes (10Me17:0 and 10Me18:0), and saprophytic (18:1ω9c) and mycorrhizal (16:1ω5c) fungal biomarkers. Linear regressions done using InfoStat (2014®) were used to identify correlations between various measurements.

RESULTS AND DISCUSSION

Early shifts in microbial community size and structure

Within this 3-year study, the microbial biomass carbon (MBC) was not affected by fertilizer-N, cover crop rotation or fertilizer-N x cover crop. The mean MBC was 308 mg/kg of soil (Table 19). Microbial biomass nitrogen (MBN) was affected by cover crop rotation ($p<0.05$) (Table 19). The MBN in the CC rotation was 17 mg/kg of soil and 14 mg/kg of soil for FA. The MBN for cover crop rotation was 20.3% greater than FA treatment. McDaniel et al. (2014) reported a MBN increase of 26% with crop rotation.

Table 19. Summarized ANOVA for microbial biomass C and N, combined enzyme activity assay and β-Glucosidase for Guayama experiment 2015-2016.

Treatment[1]	Microbial biomass carbon (MBC) (mg/kg of soil)	Microbial biomass nitrogen (MBN) (mg/kg of soil)	After maize harvest (AMH) (mg PNkg^{-1} soil h^{-1})	After Cover crop incorporation (ACCI) (mg PNkg^{-1} soil h^{-1})	β-Glucosidase (BG) (mg PNkg^{-1} soil h^{-1})
N1	339.2	18.7	207.0	166.9	36.0
N2	252.9	16.3	205.5	186.0	37.7
N3	358.9	15.0	218.6	174.8	36.7
N4	303.7	12.0	226.9	200.7	39.3
N5	286.6	15.8	193.9	166.0	32.4
Means	308.2	15.5	210.4	178.9	36.4
CC	325.2	17.0 b[2]	228.1 b	221.4 b	40.3
FA	291.2	14.1 a	192.7 a	136.4 a	32.5
ANOVA table ($p<0.05$)					
Fertilizer-N (N)	0.5058	0.0722	0.2792	0.2336	0.1057
Rotation (R)	0.2134	0.0166*[3]	0.0196*	0.0223*	0.0858
N x R	0.2157	0.7517	0.9917	0.2928	0.4021

1- N1= 0 kg N/ha, N2= 50 kg N/ha, N3= 100 kg N/ha, N4= 150 kg N/ha and N5= 200 kg N/ha. CC= cover crop and FA= fallow

2- Only means with significant difference at $p<0.05$ determined by Fisher's LSD test were denoted by letters

3- * indicates p-values of less than 0.05.

Fatty acid methyl ester profiles have been reported as sensitive indicators of shifts in soil microbial community composition due to changes in management practices (Schutter and Dick, 2000; Cotton et al., 2013; Mbuthia et al., 2015). Although non-significant the sum of total FAME's revealed a greater community size under CC rotation (43.62%) compared to FA (38.85%) which coincides with microbial biomass C being 11.6% greater under CC. Even at this early stage of the study, the sum of biomarkers for Gram positive (G+) bacteria (i15:0, a15:0, i16:0, i17:0 and a17:0), Gram negative (G-) bacteria (cy17:0 and cy19:0), actinomycetes (10Me17:0 and 10Me18:0) and saprophytic fungi (18:1ω9c) were more abundant under the CC rotation. The total bacterial abundance was 15.6% greater for CC than FA (Table 20). This finding shows how early these trends can be observed in soil bacterial populations as Mbuthia et al. (2015) also observed a greater relative abundance in G+ and total bacteria for plots with cover crop (vetch cover > wheat cover > no cover) in a long-term study (31 years).

In our study the only statistically significant difference found in the soil microbial community structure was a lower mycorrhizal fungal marker (16:1ω5c) under the cover crop treatment than FA ($p<0.05$) (Table 20). The study by Mbuthia et al. (2015) also observed lower mycorrhizal activity in a legume cover crop system relative to other systems. Mycorrhizal colonization has been reported to decrease with increased nutrient availability (Wang et al., 2009; Guo et al., 2018). Even in soils under long-term management, it is possible that the FAME analysis reveals more the climatic and/or seasonal variability effects in soil conditions than the actual management impacts. For example, the soil conditions under FA could have been drier than under CC, and thus, more conducive for mycorrhiza populations to explore the less favorable conditions. Mycorrhiza are known to promote drought tolerance in plants (Paul and Clark, 1996; Augé, 2001;

Cano et al., 2018). However, more samplings could be needed to verify the response of mycorrhiza to cover crops over time due to the minimal difference reported between the FA and CC treatments.

Table 20. FAME profiles of microbial community composition by fertilizer-N rates and cover crop rotation.

	Bacteria (nmolg^{-1} soil)			Fungi (nmolg^{-1} soil)				
Treatment[1]	G+	G-	Actinomycetes	Total bacteria[2]	Saprophytic	Mycorrhiza	F:B[3]	Sum of total FAME[5]
N1	23.74	5.40	3.64	32.78	7.92	2.95	0.25	43.65
N2	24.02	5.55	3.75	33.32	7.58	2.83	0.23	43.73
N3	21.67	4.92	3.41	30.00	7.09	2.48	0.24	39.57
N4	22.32	5.14	3.59	31.06	7.37	2.42	0.24	40.84
N5	20.85	4.88	3.36	29.08	6.85	2.44	0.24	38.38
CC	24.15	5.54	3.82	33.51	7.62	2.49a[4]	0.23	43.62
FA	20.89	4.81	3.29	28.99	7.10	2.76b	0.25	38.85
ANOVA table ($p<0.05$)								
Fertilizer-N (N)	0.7776	0.7827	0.8958	0.7952	0.6715	0.5908	0.5419	
Rotation (R)	0.0967	0.1789	0.1717	0.1169	0.4785	0.0013*[6]	0.1458	
N x R	0.3150	0.2919	0.3301	0.3086	0.3386	0.3578	0.1108	

1- N1= 0 kg N/ha, N2= 50 kg N/ha, N3= 100 kg N/ha, N4= 150 kg N/ha and N5= 200 kg N/ha. CC= cover crop and FA= fallow
2- Sum of G+, G- and actinomycetes
3- F:B= saprophytic fungi/total bacteria
4- Only means with significant difference at $p<0.05$ determined by Fisher's LSD test were denoted by letters
5- Sum of total FAME= total bacteria + saprophytic + mycorrhiza.
6- * indicates p-values of less than 0.05.

The PCA of FAME profiles included all the biomarkers used for G+ and G- bacteria, biomarkers for actinomycetes, saprophytic fungi and mycorrhizal fungi (Figure 10). A differentiation on the microbial community composition is noticeable for the rotation treatment. The first principal component (CP 1) explained 86.1% of variability and differentiated between CC and FA rotation treatment. Principal component 2 (CP 2) explained 10.0% of variability. Microbial communities with CC rotation had a higher relative abundance of all biomarkers used for G+ (i15:0, a15:0, i16:0, i17:0 and a17:0), G- (cy17:0 and cy19:0), actinomycetes (10Me17:0

and 10Me18:0) and saprophytic fungi (18:1ω9c). The only biomarker not differentiated by CC was 16:1ω5c (mycorrhizal fungai). The PCA demonstrated no differences based on fertilizer-N treatments.

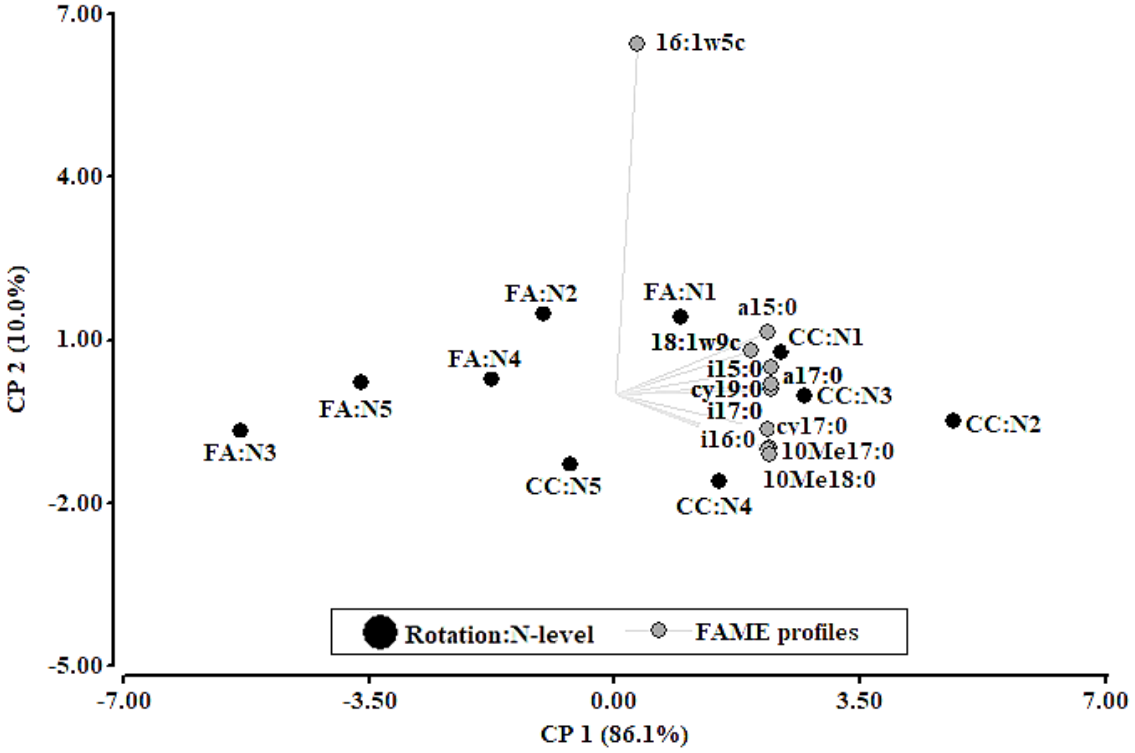


Figure 9. Microbial community structure according to FAME markers in soils from the 0-15 cm depth layer as influenced by fertilizer-N and cover crop rotation treatments. The bacterial biomarkers identified include five Gram positive (G+) bacteria (i15:0, a15:0, i16:0, i17:0 and a17:0); two Gram negative (G-) bacteria (cy17:0 and cy19:0); two actinomycetes (10Me17:0 and 10Me18:0). The fungal biomarkers used represented saprophytic (18:1ω9c) and mycorrhizal (16:1ω5c) populations. N1, N2, N3, N4 and N5 represents 0, 50, 100, 150 and 200 kg N/ha, respectively. Cover crop rotation is CC and no cover crop or fallow is FA.

The relative abundance for the combined enzyme assay of β-glucosidase, β-glucosaminidase and acid phosphatase was determined for each fertilizer-N level after 2015-2016 maize harvest (AMH) and after 2015-2016 cover crop incorporation (ACCI) (Table 19). At both sampling times, the CC rotation tended to have a higher enzymatic activity than the FA treatments, although there was no significant differences by fertilizer-N and fertilizer-N x rotation. The cover crop rotation affected both AMH and ACCI enzyme abundance. Relative enzyme abundance was

higher for samples taken after maize harvest than those taken after cover crop incorporation. This could be due to the one month of continuous tillage after the cowpea cover crop was incorporated. Tillage has been previously shown to decrease microbial activity (Roldán et al., 2005; Mbuthia et al., 2015b; Nivelle et al., 2016). The β-glucosidase activity was not significantly influenced ($P>0.05$) by fertilizer-N, rotation or fertilizer-N x rotation. Although non-significant, β-glucosidase activity was still numerically higher with CC rotation than FA treatment (Table 19). The mean value of β-glucosidase found in this soil was 36.40 mg PN kg^{-1} soil h^{-1}.

Combined enzymatic assay approach for Guamani soil in Puerto Rico

There has been limited soil enzymatic work done in Puerto Rico soils. Most of this work has been done by Sotomayor and Acosta-Martínez. Sotomayor-Ramírez et al. (2009) in a study on Vertisols in the Lajas Valley of Puerto Rico (0-15 cm depth) reported values of β-glucosidase activity of 40 mg PN kg^{-1} soil h^{-1} in an agricultural soil under Solanacea (eggplant, sweet pepper and tomato), Cucurbitacea (squash) and Fabaceae (bean) production. Another study in Puerto Rico comparing enzyme activities under diverse land uses in three soil orders (Oxisols, Ultisols and Inceptisols) in the Rio Grande de Arecibo watershed found values of β-glucosidase activity (0-15 cm) of ~20 mg PN kg^{-1} soil h^{-1} for Inceptisols and lower than 20 mg PN kg^{-1} soil h^{-1} when the soils were under agricultural practices (Acosta-Martínez et al., 2007).

The use of a combined assay approach for multiple enzyme activities demonstrated that it was more effective in distinguishing among the systems (CC vs FA) implemented three years ago than the single enzyme assay (Table 19). The benefit of using a combined assay approach is that a single value can be used to compare across regions and countries due to management, climate and/or land-use by determining several enzyme activities simultaneously. The enzymes in this combined assay have been recently suggested by soil health and conservation initiatives (Acosta-

Martínez et al., 2018). It is still suggested to continue evaluation of enzyme activities individually as this can provide information of a specific reaction and process of interest. For example, β-glucosidase activity is used to evaluate cellulose degradation which is involved in glucose production. Glucose is an energy source for microorganisms and plants. The enzyme, β-glucosaminidase is involved in chitin degradation which produces amino sugars and is important in C and N cycling. The enzyme, acid phosphatase produces plant available phosphates (Acosta-Martínez and Waldrip, 2014; Acosta-Martínez et al., 2018; Cano et al., 2018). These three previously mentioned enzymes were simultaneously evaluated in the combined enzyme activity assay for this study. There was a significant difference by cover crop rotation for both combined assay samplings. In both cases enzyme activity was higher for CC rotation than FA by 18% for samples taken after maize harvest and by 62% for samples taken after CC incorporation (Table 19).

Linear regression analysis were done to evaluate if any associations existed between a new combined enzyme activity assay procedure (Acosta-Martinez et al., 2018) and individually measured β-glucosidase activity. Only linear regressions with a p-value ≤ 0.05 were reported. Combined assay results for samples taken after cover crop incorporation had a significant positive correlation with β-glucosidase activity for both CC rotation and FA treatment. The linear regression for the ACCI with CC rotation compared to β-glucosidase activity had an r of 0.33 and a p-value of 0.0082 (Figure 10). The ACCI with FA treatment had an r of 0.50 and p-value of 0.0005 (Figure 10). The after maize harvest combined assay abundance was significantly correlated with β-glucosidase for FA treatment. The AMH for FA treatment had an r of 0.52 with a p-value of 0.0004 (Figure 10). Overall the new combined enzyme assay procedure did correlate with BG activity.

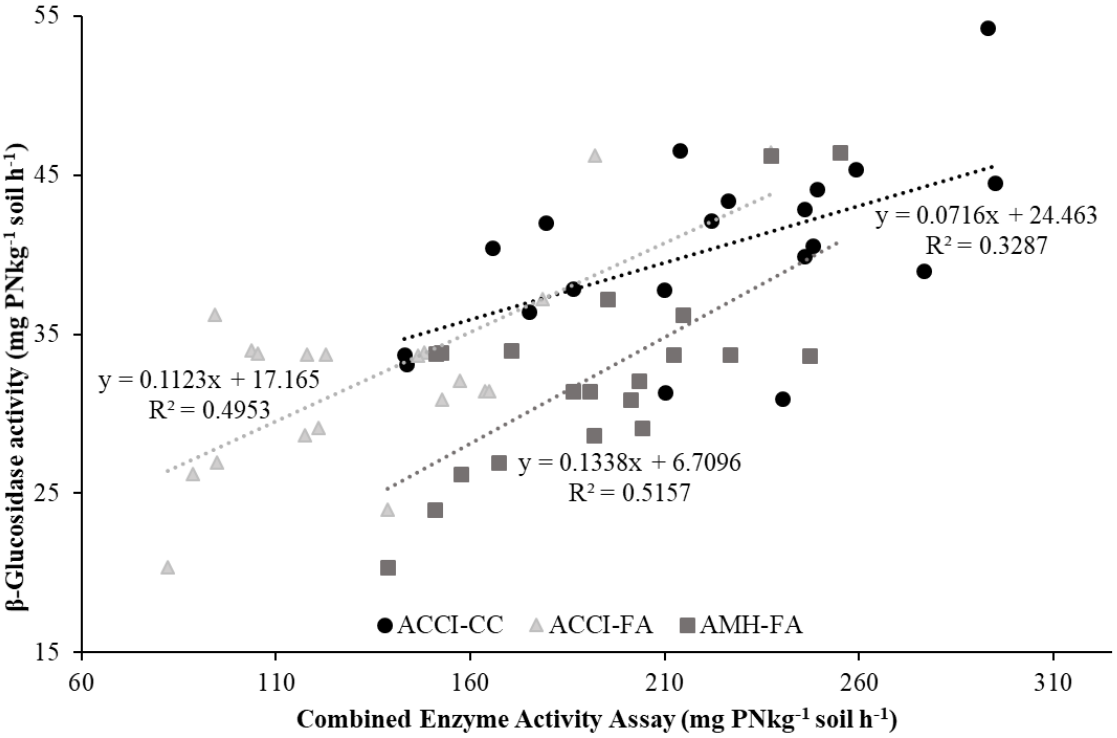


Figure 10. Linear regression correlations of results for β-glucosidase activity vs combined enzyme activity assay. The combined enzyme activity assay consisted of the simultaneous determination of β-glucosidase, β-glucosaminidase and acid phosphatase activities. Combined enzyme activity assay samples were taken after cover crop incorporation (ACCI) for CC rotation and FA treatment, and after maize harvest (AMH) for FA.

CONCLUSIONS

This study found early changes in the soil microbial community size, composition and activity as affected by the incorporation of a cowpea legume cover crop into a maize system. Although MBC and BG did not have significant differences with regards to rotation treatment, their values were higher with cover crop. The FAME profile results differed for mycorrhizal fungi in the cowpea cover crop rotation compared to fallow. Microbial biomass nitrogen was also significantly affected by the CC rotation. A new combined assay to determine multiple enzyme activities in a soil sample was used, which demonstrated distinctions due to the incorporation of the cowpea cover crop while β-glucosidase activity as an individual assay did not. This approach has the potential to be used in other soils in order to establish biogeochemical cycling indexes to better guide soil management selections in Puerto Rico.

REFERENCES

Acosta-Martínez, V., D. Acosta-Mercado, D. Sotomayor-Ramírez, and L. Cruz-Rodríguez. 2008. Microbial communities and enzymatic activities under different management in semiarid soils. Appl. Soil Ecol. 38(3): 249–260.

Acosta-Martinez, V., A. Cano, and J. Johnson. 2018. Simultaneous determination of multiple soil enzyme activities for soil health-biogeochemical indices. Appl. Soil Ecol. 126: 121–128.

Acosta-Martínez, V., Cruz, L., Sotomayor-Ramírez, D., Pérez-Alegría, L., 2007. Enzyme activities as affected by soil properties and land use in a tropical watershed. Applied Soil Ecology. 35, 35–45.

Acosta-Martínez, V., Waldrip, H.M., 2014. Soil enzyme activities as affected by manure types, application rates, and management practices. p. 99-122 *In* Applied Manure and Nutrient Chemistry for Sustainable Agriculture and Environment. Springer.

Andraski, T.W., and L.G. Bundy. 2002. Using the pre-sidedress soil nitrate test and organic nitrogen crediting to improve corn nitrogen recommendations. Agron. J. 94:1411–1418.

Andrews, S.S., and C.. Carroll. 2001. Designing a Soil Quality Assessment Tool for Sustainable. Ecol. Appl. 11(6): 1573–1585.

Andrews, S.S., D.L. Karlen, and C.A. Cambardella. 2004. The Soil Management Assessment Framework. Soil Sci. Soc. Am. J. 68(6): 1945–1962.

Andrews, S.S., D.L. Karlen, and J.P. Mitchell. 2002a. A comparison of soil quality indexing methods for vegetable production systems in Northern California. Agric. Ecosyst. Environ. 90(1): 25–45.

Andrews, S.S., J.P. Mitchell, R. Mancinelli, D.L. Karlen, T.K. Hartz, W.R. Horwath, G.S. Pettygrove, K.M. Scow, and D.S. Munk. 2002b. On-Farm Assessment of Soil Quality in California ' s Central Valley. 94:12–23.

Augé, R.M., 2001. Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11: 3–42.

Bowles, T.M., V. Acosta-Martínez, F. Calderón, and L.E. Jackson. 2014. Soil enzyme activities, microbial communities, and carbon and nitrogen availability in organic agroecosystems across an intensively-managed agricultural landscape. Soil Biol. Biochem. 68: 252–262.

Cabrera, M.L., and D.E. Kissel. 1988. Division S-4-Soil Fertility and Plant Nutrition Evaluation of a Method to Predict Nitrogen Mineralized from Soil Organic Matter Under Field Conditions. 1031(87): 1027–1031.

Campbell, C.R. 2001. Reference sufficiency ranges for plant analysis in the southern region of the United States.

Cano, A., Núñez, A., Acosta-Martinez, V., Schipanski, M., Ghimire, R., Rice, C., West, C., 2018. Current knowledge and future research directions to link soil health and water conservation in the Ogallala Aquifer region. Geoderma. 328: 109–118.

Capo, B.G. 1967. Additional evidence on the applicability of the new fertilizer-yield relation. J. Agric. Univ. Puerto Rico 51(2): 97–119.

Chevalier, P., and L.E. Schrader. 1977. Genotypic Differences in Nitrate Absorption and Partitioning of N Among Plant Parts in Maize. Crop Science. 17: 897–901.

Cotton, J., Acosta-Martinez, V., Moore-Kucera, J., Burow, G., 2013. Early changes due to sorghum biofuel cropping systems in soil microbial communities and metabolic functioning. Biol. Fertil. Soils 49: 403–413.

Demotes-Mainard, S., R. Boumaza, S. Meyer, and Z.G. Cerovic. 2008. Indicators of nitrogen status for ornamental woody plants based on optical measurements of leaf epidermal polyphenol and chlorophyll contents. Sci. Hortic. (Amsterdam). 115(4): 377–385.

Dinnes, D.L., D.L. Karlen, D.B. Jaynes, T.C. Kaspar, J.L. Hatfield, T.S. Colvin, and C.A. Cambardella. 2002. Nitrogen management strategies to reduce nitrate leaching in tile-drained midwestern soils. Agron. J. 94(1): 153–171.

Doran, J.W. 2002. Soil health and global sustainability: Translating science into practice. Agric. Ecosyst. Environ. 88(2): 119–127.

Doran, J.W., and T.B. Parkin. 1994. Defining and Assessing Soil Quality. p. 3–21. *In* Soil Science Society of America.

Doran, J.W., and T.B. Parkin. 1996. Quantitative indicators of soil quality: a minimum data set. p. 25–37. *In* Methods for Assessing Soil Quality. Soil Science Society of America.

Doran, J.W., and M.R. Zeiss. 2000. Soil health and sustainability: managing the biotic component of soil quality. Appl. Soil Ecol. 15(1): 3–11.

Drijber, R.A., J.W. Doran, A.M. Parkhurst, and D.J. Lyon. 2000. Changes in soil microbial community structure with tillage under long-term wheat-fallow management. Soil Biol. Biochem. 32(10): 1419–1430.

Earl, H. J., and M. Tollenaar. 1997. Maize Leaf Absorptance of Photosynthetically Active Radiation and Its Estimation Using a Chlorophyll Meter. Crop Sci. 37:436-440.

Espinosa-Irizarry, J. 2016. Efecto de la fertilización nitrogenada y cobertora sobre el rendimiento de una linea endogámica de maíz.

Falkowski, P.G., T. Fenchel, and E.F. DeLong. 2008. The microbial engines that drive earth's biogeochemical cycles. Science 320:1034-1039.

FAOSTAT. 2014. Food and Agriculture Organization of the United Nations. FAOSTAT Stat. database.

Feliciano, J.B., M.A. Lugo-Lopez, and T.W. Scott. 1979. Influence of cultivars, N levels and time of N application on plant characters, leaf, composition and yield of corn grown on an oxisol. J. Agric. Univ. Puerto Rico LXIII(3): 273–280.

Fixen, P., F. Brentrup, T. Bruulsema, F. Garcia, R. Norton, and S. Zingore. 2015. Nutrient/fertilizer use efficiency: measurement, current situation and trends. p. 1–30. *In* Drechsel, P., Heffer, P., Magen, H., Mikkelsen, R., Wichelns, D. (eds.), Managing Water

and Fertilizer for Sustainable Agricultural Intensification. International Fertilizer Industry Association (IFA), International Water Management Institute (IWMI), International Plant Nutrition Institute (IPNI), and International Potash Institute (IPI).

Fox, R.H., H. Talleyrand, and D.R. Bouldin. 1974. Nitrogen Fertilization of Corn and Sorghum Grown in Oxisols and Ultisols in Puerto Rico. Agron. J. 66(4): 534–540.

Freeman, K.W., K. Girma, D.B. Arnall, R.W. Mullen, K.L. Martin, R.K. Teal, and W.R. Raun. 2007. By-Plant Prediction of Corn Forage Biomass and Nitrogen Uptake at Various Growth Stages Using Remote Sensing and Plant Height. 99: 530–536.

Gabriel, J.L., and M. Quemada. 2011. Replacing bare fallow with cover crops in a maize cropping system: Yield, N uptake and fertiliser fate. Eur. J. Agron. 34(3): 133–143.

Garibaldi, L.A., B. Gemmill-Herren, R. D'Annolfo, B.E. Graeub, S.A. Cunningham, and T.D. Breeze. 2017. Farming Approaches for Greater Biodiversity, Livelihoods, and Food Security. Trends Ecol. Evol. 32(1): 68–80.

Guo, J., P. Luo, X. Han, J. Yang, and D. Li. 2018. Influence of Long-term Fertilization on AM Fungi Community Structures in a Brown Soil. p. 1-5 *In* IOP Conf. Series: Earth and Environmental Sciences. IOP Publishing.

Hargrove, W.L., D.E. Kissel, and L.B. Fenn. 1961. Field Measurements of Ammonia Volatilization from Surface Applications of Ammonium Salts to a Calcareous Soil. 69: 473-476.

Hartwig, N.L., and H.U. Ammon. 2002. Cover crops and living mulches. Weed Sci. 50(6): 688–699.

Hasan, M.R., M.A. Akbar, Z.H. Khandaker, and M.M. Rahman. 2010. Effect of nitrogen fertilizer on yield contributing character, biomass yield and nutritive value of cowpea forage. Bang. J. Anim. Sci 39(1–2): 83–88.

Havlin, J.L., Tisdale, S.L., Beaton, J.D., Nelson, W.L., 2005a. Agricultural Productivity and Environmental Quality. p. 447–502. *In* Soil Fertility and Fertilizers. 7th ed. PHI Learning Private Limited, New Delhi.

Havlin, J.L., S.L. Tisdale, J.D. Beaton, and W.L. Nelson. 2005b. Nitrogen. p. 97–159. *In* Soil Fertility and Fertilizers. 7th ed. PHI Learning Private Limited, New Delhi.

Helmke, P.A., Sparks, D.L., 1996. Lithium, Sodium, Potassium, Rubidium, and Cesium. p. 551–574. *In* Methods of Soil Analysis. Part 3. Chemical Methods. SSSA, Madison, WI.

INFOSTAT. 2014. Grupo INFOSTAT, FCA, Universidad Nacional de Córdoba, Argentina.

Jenkinson, D.S. 1988. Determination of microbial biomass carbon and nitrogen in soil. p. 368-386. *In* Advances in Nitrogen Cycling in Agricultural Ecosystems. CAB International/Marcel Dekker, Wallingford, UK/New York.

Jenkinson, D.S., and D.S. Powison. 1976. The effects of biocidal treatments on metabolism in soil-V. A. method for measuring soil biomass. Soil Biol. Biochem. 8: 209–213.

Ju, X.T., Kou, C.L., Zhang, F.S., Christie, P., 2006. Nitrogen balance and groundwater nitrate contamination : Comparison among three intensive cropping systems on the North China Plain. Environmental Pollution 143: 117–125.

Ju, T., G. Xing, X. Chen, and S. Zhang. 2009. Reducing environmental risk by improving N management in intensive Chinese agricultural systems. Proc. Natl. Acad. Sci. 106(19): 8077–8077.

Kant, S., Y. Bi, and S.J. Rothstein. 2011. Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. J. Exp. Bot. 62(4): 1499–509.

Karlen, D.L., S.S. Andrews, and J.W. Doran. 2001. Soil quality: Current concepts and applications. Adv. Agron. 74: 1–40.

Karlen, D.L., S.S. Andrews, B.J. Wienhold, and T.M. Zobeck. 2008. Soil Quality Assessment : Past , Present and Future. J. Integr. Biosci. 6(1): 3–14.

Karlen, D.L., and D.E. Stott. 1994. A Framework for Evaluating Physical and Chemical Indicators of Soil Quality. p. 53–72. *In* Defining Soil Quality for a Sustainable Environment. Soil Science Society of America, Madison, WI.

Kaspar, T.C., and J.W Singer. 2011. The Use of Cover Crops to Manage Soil. p. 321–337. *In* Hatfield, J.L., Sauer, T.J. (Eds.), Soil Management: Building a Stable Base for Agriculture. American Society of Agronomy and Soil Science Society of America, Madison, WI.

Ketterings, Q.M., S.D. Klausner, and K.J. Czymmek. 2003. Nitrogen guidelines for field crops in New York. Dep. Crop and Soil Sci. Ext. Ser. E03–16, second release. Dep. Crop and Soil Sci., Cornell Univ., Ithaca, NY.

Kladivko, E.J. 2001. Tillage systems and soil ecology. Soil Tillage Res. 61(1–2): 61–76.

Kladivko, E.J., and M.J. Clapperton. 2011. Soil Biology. p. 145–160. *In* Hatfield, J.L., Sauer, T.J. (eds.), Soil Managment: Building a Stable Base for Agriculture. American Society of Agronomy and Soil Science Society of America, Madison, WI.

Kowalchuk, G.A., S.E. Jones, and L.L. Blackall. 2008. Microbes orchestrate life on earth. ISME Journal 2:795-796.

Kuo, S., 1996. Phosphorus. p. 869–919. *In* Methods of Soil Analysis. Part 3. Chemical Methods. SSSA, Madison, WI.

Liu, X., Ju, X., Zhang, F., Pan, J., Christie, P., 2003. Nitrogen dynamics and budgets in a winter wheat – maize cropping system in the North China Plain. Field Crops Research 83: 111–124.

Lehman, R.M., Acosta-Martinez, V., Buyer, J.S., Cambardella, C.A., Collins, H.P., Ducey, T.F., Halvorson, J.J., Jin, V.L., Johnson, J.M.F., Kremer, R.J., Lundgren, J.G., Manter, D.K., Maul, J.E., Smith, J.L., Stott, D.E., 2015. Soil biology for resilient, healthy soil. J. Soil Water Conserv. 70: 12–18.

Ma, B. L., K. D. Subedi, and C. Costa. 2005. Comparison of Crop-Based Indicators with Soil Nitrate Test for Corn Nitrogen Requirement. Agron. J. 97:462-471.

Mbuthia, L.W., V. Acosta-Martínez, J. DeBruyn, S. Schaeffer, D. Tyler, E. Odoi, M. Mpheshea, F. Walker, and N. Eash. 2015. Long term tillage, cover crop, and fertilization effects on microbial community structure, activity: Implications for soil quality. Soil Biol. Biochem. 89: 24–34.

Mcdaniel, M.D., and A.S. Grandy. 2016. Soil microbial biomass and function are altered by 12 years of crop rotation. SOIL Discuss 2: 583–599.

McDaniel, M.D., L.K. Tiemann, and A.S. Grandy. 2014. Does agricultural crop diversity enhance soil microbial biomass and organic matter dynamics? A meta-analysis. Ecol. Appl. 24(3): 560–570.

Meisinger, J.J., J.S. Schepers, and W.R. Raun. 2008. Crop Nitrogen Requirement and Fertilization. p. 563–612. *In* Schepers, J.S., Raun, W.R. (eds.), Nitrogen in Agricultural Systems, Agronomy Monographs 49. ASA, CSSA, SSSA, Madison, WI.

Mengel, D. 2008. Enhancing Nitrogen Use Efficiency in Irrigated Corn Using Sensor Technology. p. 8-1 - 8-5. *In* Fertilizing for Irrigated Corn- Guide to Best Management Practices.

Muharam, F.M., K.F. Bronson, S.J. Maas, and G.L. Ritchie. 2014. Inter-relationships of cotton plant height, canopy width, ground cover and plant nitrogen status indicators. F. Crop. Res. 169(3): 58–69.

Mullen, R.W. 2011. Nutrient Cycling in Soils: Nitrogen. p. 67–78. *In* Hatfield, J.L., Sauer, T.J. (eds.), Soil Managment: Building a Stable Base for Agriculture. American Society of Agronomy and Soil Science Society of America, Madison, WI.

Muñoz Huerta, R.F., R.G. Guevara Gonzalez, L.M. Contreras Medina, I. Torres Pacheco, J. Prado Olivarez, and R. V. Ocampo Velazquez. 2013. A Review of Methods for Sensing the Nitrogen Status in Plants: Advantages, Disadvantages and Recent Advances. Sensors 13: 10823–10843.

Mupangwa, W., S. Twomlow, and S. Walker. 2012. Reduced tillage, mulching and rotational effects on maize (Zea mays L.), cowpea (Vigna unguiculata (Walp) L.) and sorghum (Sorghum bicolor L. (Moench)) yields under semi-arid conditions. F. Crop. Res. 132: 139–148.

Nimmo, J.R., and K.. Perkins. 2002. Aggregate stability and size distribution. p. 317–328. *In* Methods of soil analysis, Part 4-Physical methods. Soil Science Society of America. Madison, WI.

Nivelle, E., J. Verzeaux, H. Habbib, Y. Kuzyakov, G. Decocq, D. Roger, J. Lacoux, J. Duclercq, F. Spicher, J.-E. Nava-Saucedo, M. Catterou, F. Dubois, and T. Tetu. 2016. Functional response of soil microbial communities to tillage, cover crops and nitrogen fertilization. Appl. Soil Ecol. 108: 147–155.

Paul, E.A., and F.E Clark. 1996. Soil microbiology and biochemistry. Academic Press.

Peoples, M.B., J. Brockwell, D.F. Herridge, I.J. Rochester, B.J.R. Alves, S. Urquiaga, R.M. Boddey, F.D. Dakora, S. Bhattarai, S.L. Maskey, C. Sampet, B. Rerkasem, D.F. Khan, H. Hauggaard-Nielsen, and E.S. Jensen. 2009. The contributions of nitrogen-fixing crop

legumes to the productivity of agricultural systems. Symbiosis 48(1–3): 1–17.

Piekkielek, W.P., and R.H. Fox. 1992. Use of a Chlorophyll Meter to Predict Sidedress Nitrogen. Agron. J. 84: 59-65.

Poehlman, J.M., and D.A. Sleper. 1995. Applications: Field Crops Utilizing Hybrid Breeding Procedures. p. 321-344. *In* Breeding Field Crops. Iowa State Press, Iowa.

Prasad, R., Hochmuth, G., 2013. How to Calculate a Partial Nitrogen Mass Budget for Potato. University of Florida/Institute of Food and Agricultural Sciences Extension, Gainesville.

Pritchard, S.G. 2011. Soil organisms and global climate change. Plant Pathology 60:82-89

Quiles, A., A. Sotomayor-Rios, and S. Torres-Cardona. 1988. Corn responses to N applications and population desnsities at two locations in Puerto Rico. J. Agric. Univ. Puerto Rico 72(1): 127–139.

Ranum, P., J.P. Peña-Rosas, and M.N. Garcia-Casal. 2014. Global maize production, utilization, and consumption. Ann. N. Y. Acad. Sci. 1312(1): 105–112.

Rao, M.R., and M.N. Mathuva. 2000. Legumes for improving maize yields and income in semi-arid Kenya. Agric. Ecosyst. Environ. 78(2): 123–137.

Raun, W.R., and G.V. Johnson. 1999. Improving nitrogen use efficiency for cereal production. Agron. J. 91: 357–363.

Rillig, M.C., 2004. Arbuscular mycorrhizae and terrestrial ecosystem processes. Ecol. Lett. 7: 740–754.

Ritchey, E.L., D.D. Tyler, M.E. Essington, M.D. Mullen, and A.M. Saxton. 2015. Nitrogen rate, cover crop, and tillage practice alter soil chemical properties. Agron. J. 107(4): 1259–1268.

Rivera-Zayas, J. 2015. Manejo de fertilizante nitrogenado para la optimización del rendimiento de una línea pura de maíz (Zea mays L.).

Roldán, A., J.R. Salinas-García, M.M. Alguacil, and F. Caravaca. 2005. Changes in soil enzyme activity, fertility, aggregation and C sequestration mediated by conservation tillage practices and water regime in a maize field. Appl. Soil Ecol. 30(1): 11–20.

Rostami, M., A. Reza Kppcheki, M. Nassiri Mahallati, and M. Kafi. 2008. Evaluation of chlorophyll meter (SPAD) data for prediction of nitrogen status in corn (Zea mays L .). Am. J. Agric. Environ. Sci. 3(1): 79–85.

Schutter, M.E., and R.P. Dick. 2000. Comparison of Fatty Acid Methyl Ester (FAME) Methods for Characterizing Microbial Communities. 1668(11590): 1659–1668.

Shanahan, J.F., N.R. Kitchen, W.R. Raun, and J.S. Schepers. 2008. Responsive in-season nitrogen management for cereals. Comput. Electron. Agric. 61(1): 51–62.

Shapiro, C.A., R.B. Ferguson, G.W. Hergert, A.R. Dobermann, and C.S. Wortmann. 2003. Fertilizer suggestions for corn. Coop. Ext. NebGuide G74–174-A. Coop. Ext. Inst. Agr. and Nat. Resources, Univ. Nebraska, Lincoln.

Shaver, T.M., R. Khosla, and D.G. Westfall. 2011. Evaluation of two crop canopy sensors for

nitrogen variability determination in irrigated maize. Precis. Agric. 12(6): 892–904.

Snapp, S.S., S.M. Swinton, R. Labarta, D. Mutch, J.R. Black, R. Leep, J. Nyiraneza, K. O 'neil, W.K. Kellogg, and B. Stn. 2005. Evaluating Cover Crops for Benefits, Costs and Performance within Cropping System Niches of Crop and impact of foregoing a cash crop, some farmers express Michigan and New York producers are experimenting. Agron. J. 97: 322–332.

Sotomayor-Ramírez, D., Y. Espinoza, and V. Acosta-Martínez. 2009. Land use effects on microbial biomass C, β-glucosidase and β-glucosaminidase activities, and availability, storage, and age of organic C in soil. Biol. Fertil. Soils 45(5): 487–497.

Sotomayor-Ramírez, D., R. Huckaba, R. Barnes, and J. Espinosa. 2012. Inbred maize response to cover crop and fertilizaer-nitrogen. J. Agric. Univ. Puerto Rico 96(1–2): 37–55.

Spedding, T.A., C. Hamel, G.R. Mehuys, and C.A. Madramootoo. 2004. Soil microbial dynamics in maize-growing soil under different tillage and residue management systems. Soil Biol. Biochem. 36(3): 499–512.

Stanford, G., and S.J. Smith. 1987. Nitrogen mineralization potentials of soils. Soil Sci. Soc. Amer. Proc. 36: 465–472.

Stott, D.E., C.A. Cambardella, M.D. Tomer, D.L. Karlen, and R. Wolf. 2011. A Soil Quality Assessment within the Iowa River South Fork Watershed. Soil Sci. Soc. Am. J. 75(6): 2271.

Tabatabai, M.A. 1994. Soil Enzymes. p. 775–833. *In* Methods of Soil Analysis: Part 2. Microbiological and Biochemical Properties. Soil Science Society of America, Madison, WI.

Tajul, M.I., M.M. Alam, S.M.M. Hossain, K. Naher, M.Y. Rafii, and M.A. Latif. 2013. Influence of plant population and nitrogen-fertilizer at various levels on growth and growth efficiency of maize. Sci. World J. 1–9.

Thomas, G.W. 1996. Soil pH and soil acidity. p. 475–490. *In* Methods of Soil Analysis. Part 3 - Chemical Methods. Soil Science Society of America, Madison, WI.

Tian, D., and S. Niu. 2015. A global analysis of soil acidification caused by nitrogen addition. Environ. Res. Lett. 10(2).

Tremblay, N., E. Fallon, and N. Ziadi. 2011. Sensing of Crop Nitrogen Status: Opportunities, Tools, Limitations, and Supporting Information Requirements. 21: 274-281.

Tremblay, N., Z. Wang, B.-L. Ma, C. Belec, and P. Vigneault. 2009. A comparison of crop data measured by two commercial sensors for variable-rate nitrogen application. Precis. Agric. 10(2): 145–161.

Tyler, D., E.L. Ritchey, M.E. Essington, M.D. Mullen, and A.M. Saxton. 2018. Nitrogen, Cover Crop, Tillage and Lime Effects On Soil Acidity In Cotton Production Systems. 107-126.

Vance, E.D., P.C. Brookes, and D.S. Jenkinson. 1987. An extraction method for measuring microbial biomass C. Soil Biol. Biochem. 19: 703-707.

Vazquez, R. 1961. Effects of irrigation at different growth stages and of nitrogen levels on corn

yields in Lajas Valley, PR. J. Agric. Univ. Puerto Rico 45(2): 85–105.

Villeneuve, S., J. Coulombe, C. Bélec, and N. Tremblay. 2002. A Comparison Of Sap Nitrate Test And Chlorophyll Meter For Nitrogen Status Diagnosis In Broccoli (*Brassica oleracea* L. spp. italica). Acta Hortic. 571: 171–177.

Wall, D.H., R.D. Bardgett, A.P. Covich, and P.V.R. Snelgrove. 2004. The need for understanding how biodiversity and ecosystem functioning affect ecosystem service in soils and sediments. p. 1-12. *In* Sustaining Biodiversity and Ecosystem Services in Soils and Sediments, ed. D.H. Wall. Washington, DC: Island Press.

Wang, M.Y., W.H. Wang, S.T. Liu, M. Li, and R.J. Liu. 2009. Influence of Long-Term Fixed Fertilization on Diversity of Arbuscular Mycorrhizal Fungi. Pedosphere 19(5): 663–672.

Westerveld, S.M., A.W. Mckeown, C.D. Scott-Dupree, and M.R. Mcdonald. 2004. Assessment of Chlorophyll and Nitrate Meters as Field Tissue Nitrogen Tests for Cabbage, Onions, and Carrots. Horttechnology 14(2): 179–188.

Wienhold, B.J., D.L. Karlen, S.S. Andrews, and D.E. Stott. 2009. Protocol for indicator scoring in the soil management assessment framework (SMAF). Renew. Agric. Food Syst. 24(04): 260–266.

Willers, C., Jansen van Rensburg, P.J., Claassens, S., 2015. Microbial signature lipid biomarker analysis–an approach that is still preferred, even amid various method modifications. J. Appl. Microbiol. 118: 1251–1263.

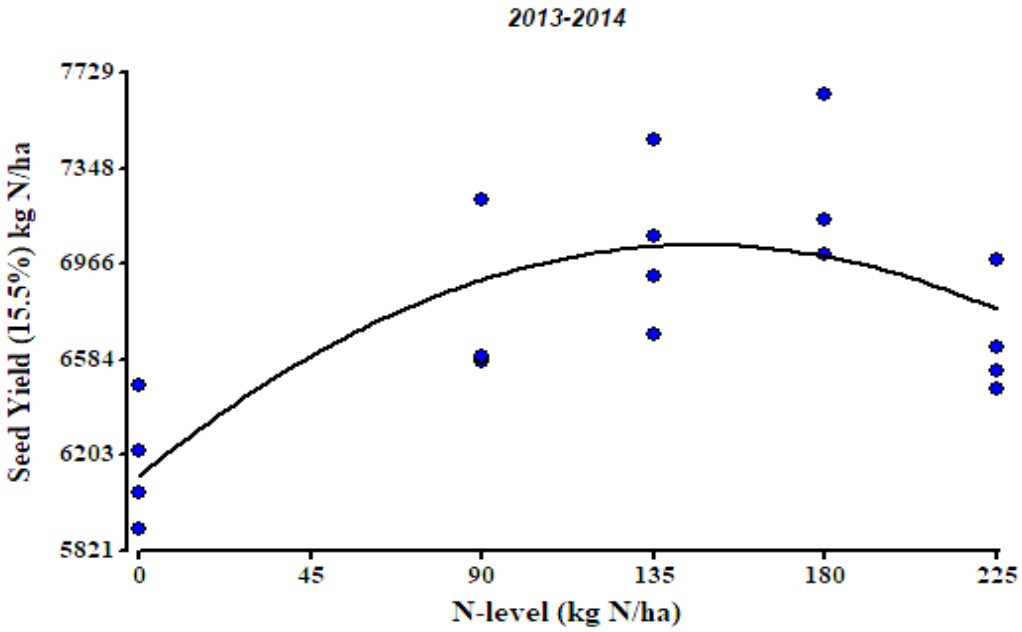
Wu, J., R.G. Joergensen. B. Pommerening, R. Chaussod, and P.C. Brookes. 1990. Measurement of soil microbial biomass C by fumigation extraction-an automated procedure. Soil Bio. Biochem. 22: 1167-1169.

Zebarth, B.J., C.F. Drury, N. Tremblay, and A.N. Cambouris. 2009. Opportunities for improved fertilizer nitrogen management in production of arable crops in eastern Canada: A review. Can. J. Soil Sci. 89(2): 113–132.

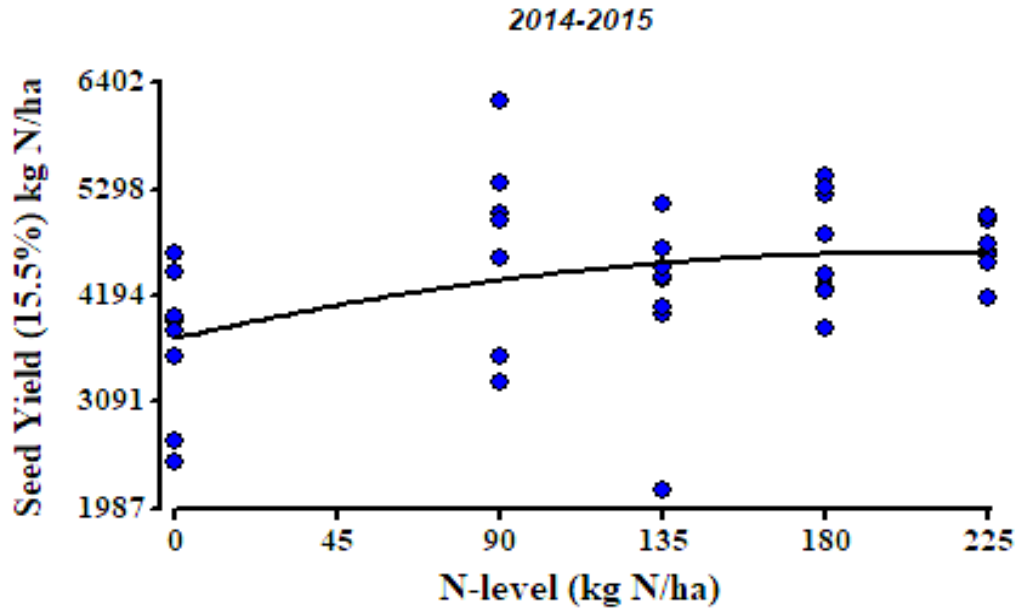
Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. Biol. Fertil. Soils 29(2): 111–129.

APPENDICES

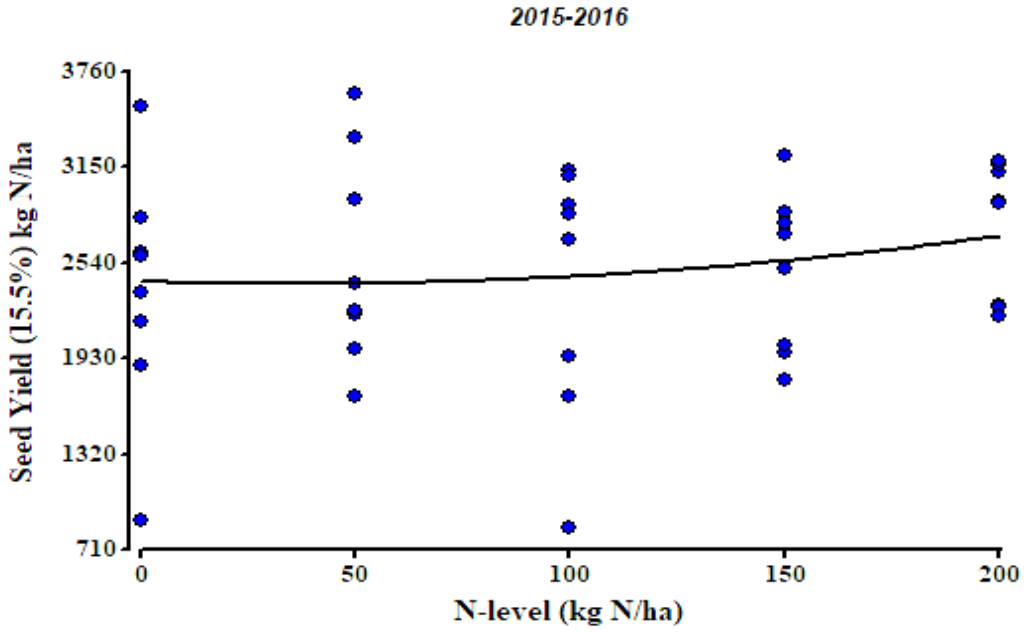
Appendix 1. Relationship between fertilizer-N rates and yield for the 2013-2014 season. The data was fit to a quadratic model.



Appendix 2. Relationship between fertilizer-N rates and yield for the 2014-2015 season. The data was fit to a quadratic model.



Appendix 3. Relationship between fertilizer-N rates and yield for the 2015-2016 season. The data was fit to a quadratic model.



Appendix 4. Photo of cowpea cover crop root nodules for the 2014-2015 season.

Appendix 5. Mean 2013-2016 soil inorganic N before maize planting (pre-plant) and after cover crop incorporation (post-harvest) in Guayama experiment.

	Pre-plant (2013-2014)			Post-harvest (2013-2014) or pre-plant (2014-2015)			Post-harvest (2014-2015) or pre-plant (2015-2016)			Post-harvest (2015-2016)		
Fertilizer-N	Immediately available	Potentially leached	Total profile N	Immediately available	Potentially leached	Total profile N	Immediately available	Potentially leached	Total profile N	Immediately available	Potentially leached	Total profile N
	0 to 30 cm	30 to 90 cm	0 to 90 cm	0 to 30 cm	30 to 90 cm	0 to 90 cm	0 to 30 cm	30 to 90 cm	0 to 90 cm	0 to 30 cm	30 to 90 cm	0 to 90 cm
	kg N/ha											
Fallow												
N1[1]	84.44	52.86	137.3	44.1	46.07	90.17	76.19	78.98	155.17	39.94	25.11	65.05
N2	78.98	46.88	125.86	46.27	36.66	82.92	59.34	61.36	120.69	52.4	29.16	81.56
N3	91.68	51.24	142.92	53.11	46.98	100.09	38.93	53.26	92.19	44.4	26.63	71.03
N4	94.37	43.84	138.21	65.46	56.2	121.66	54.98	78.37	133.35	50.28	21.57	71.84
N5	75.43	47.19	122.62	70.17	73.91	144.08	74.27	79.59	153.85	52.35	54.27	106.62
Means	84.98	48.40	133.38	55.82	51.96	107.78	60.74	70.31	131.05	47.87	31.35	79.22
Covercrop												
N1	ND [2]			55.18	47.69	102.87	45.97	51.34	97.3	51.94	34.43	86.37
N2				61.66	52.15	113.81	52.25	46.07	98.32	58.88	53.26	112.14
N3				63.23	49.51	112.74	62.57	55.19	117.76	64.85	43.03	107.88
N4				69	72.9	141.9	107.48	59.74	167.22	76.95	40.81	117.76
N5				62.02	73.71	135.73	91.84	82.83	174.66	84.55	67.44	151.98
Means				62.22	59.19	121.41	72.02	59.03	131.05	67.43	47.79	115.23

1- 2013-2014 and 2014-2015 fertilizer-N levels were 0, 90, 135, 180 and 225 kg N/ha for N1, N2, N3, N4 and N5 respectively. 2015-2016 N1, N2, N3, N4 and N5 fertilizer-N levels were 0, 50, 100, 150 and 200 kg N/ha respectively.

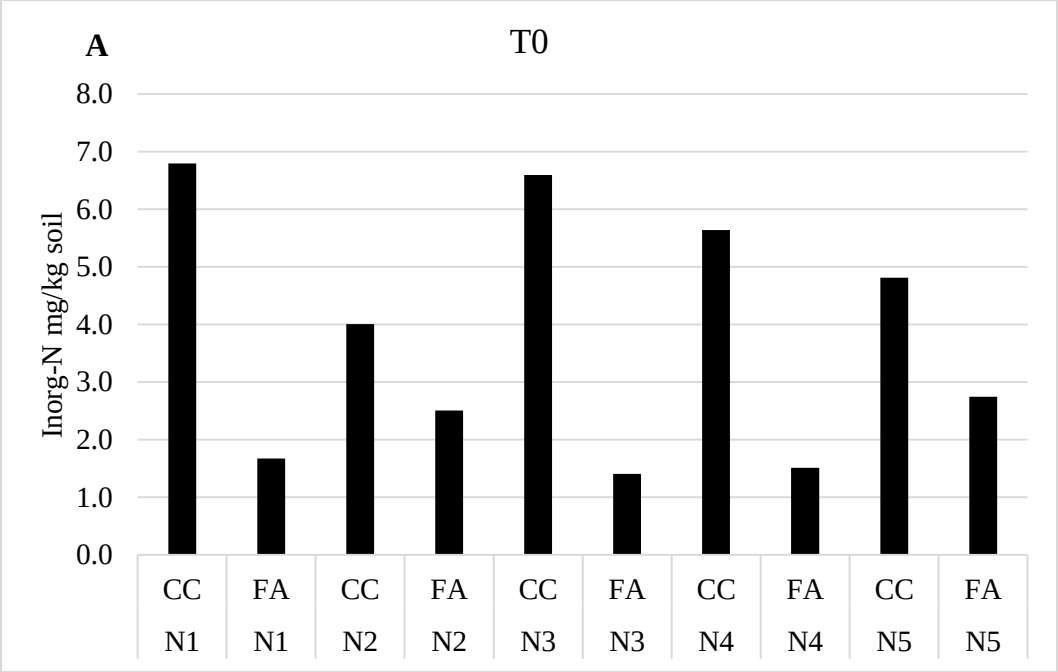
2- ND= no data, cover crop rotation treatment was not present at the time.

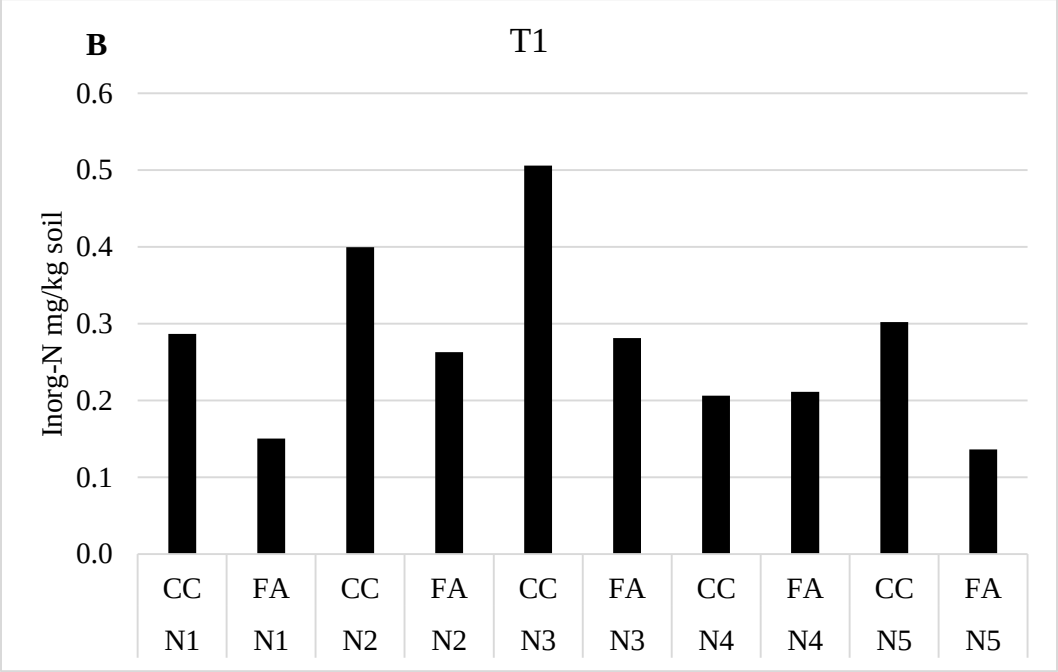
Appendix 6. Photo of maize cob for the 2015-2016 season.

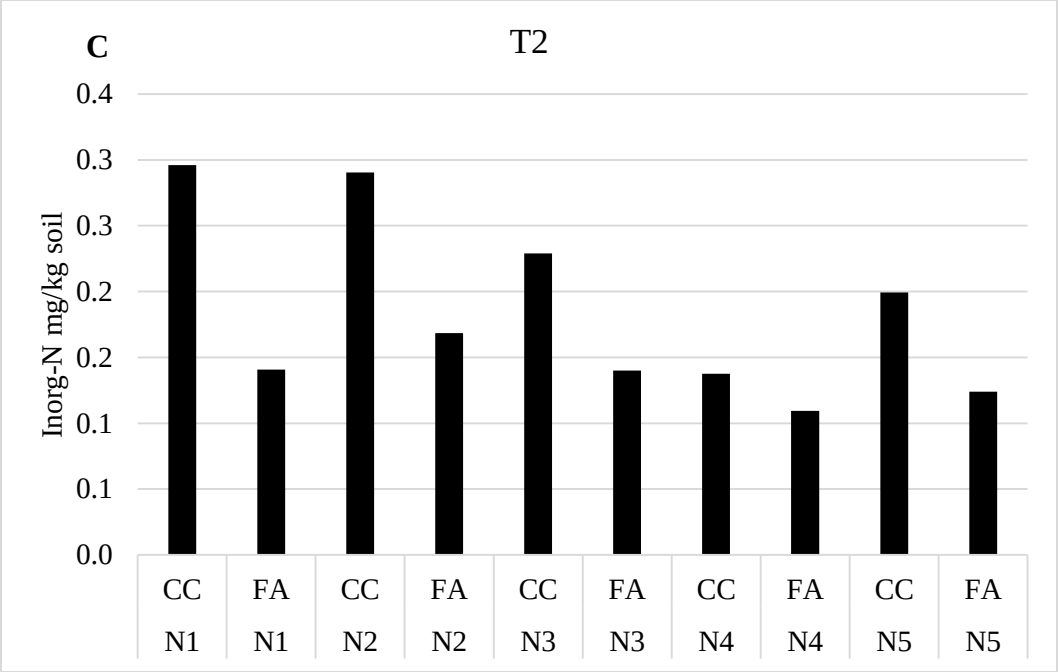
Appendix 7. Photo of cowpea cover crop planting for the 2015-2016 season.

Appendix 8. Photo of inbred maize for the 2015-2016 season.

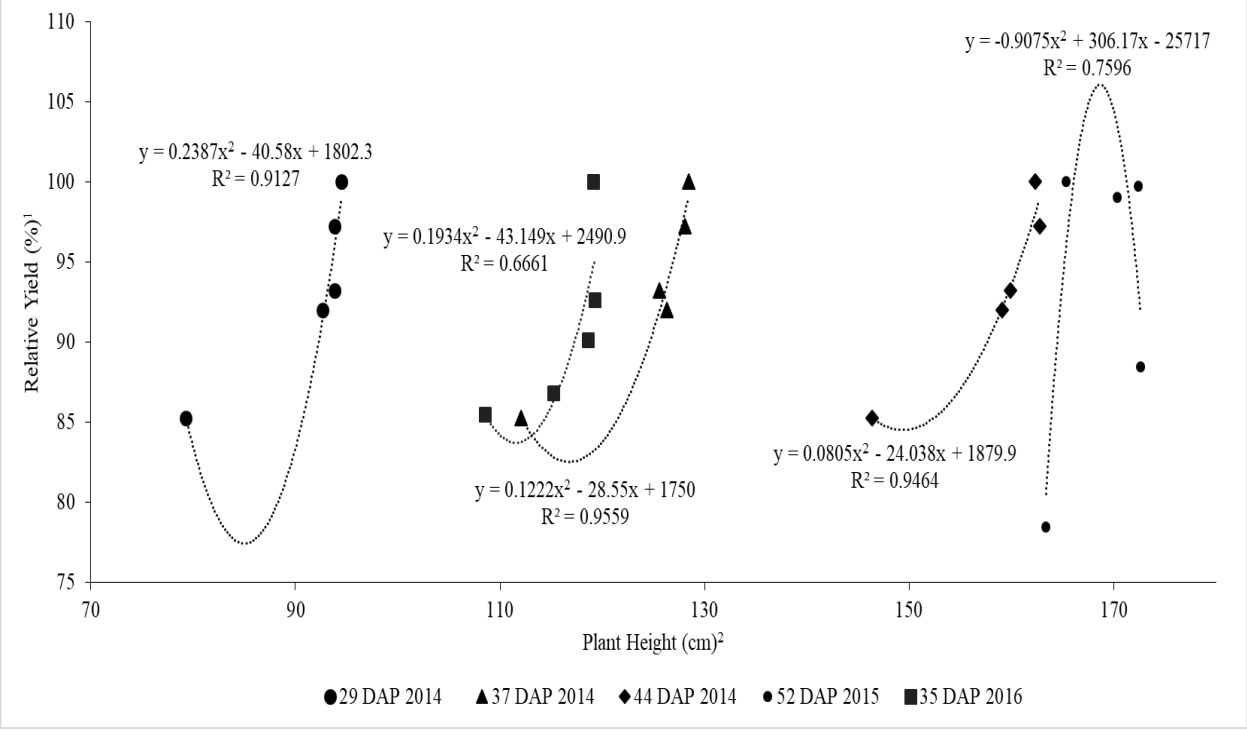
Appendix 9. Averaged potentially mineralizable N for N-level by rotation for Guayama experiment. A) Inorganic N at initial extraction. B) Inorganic N at 14 days. C) Inorganic N at 28 days.





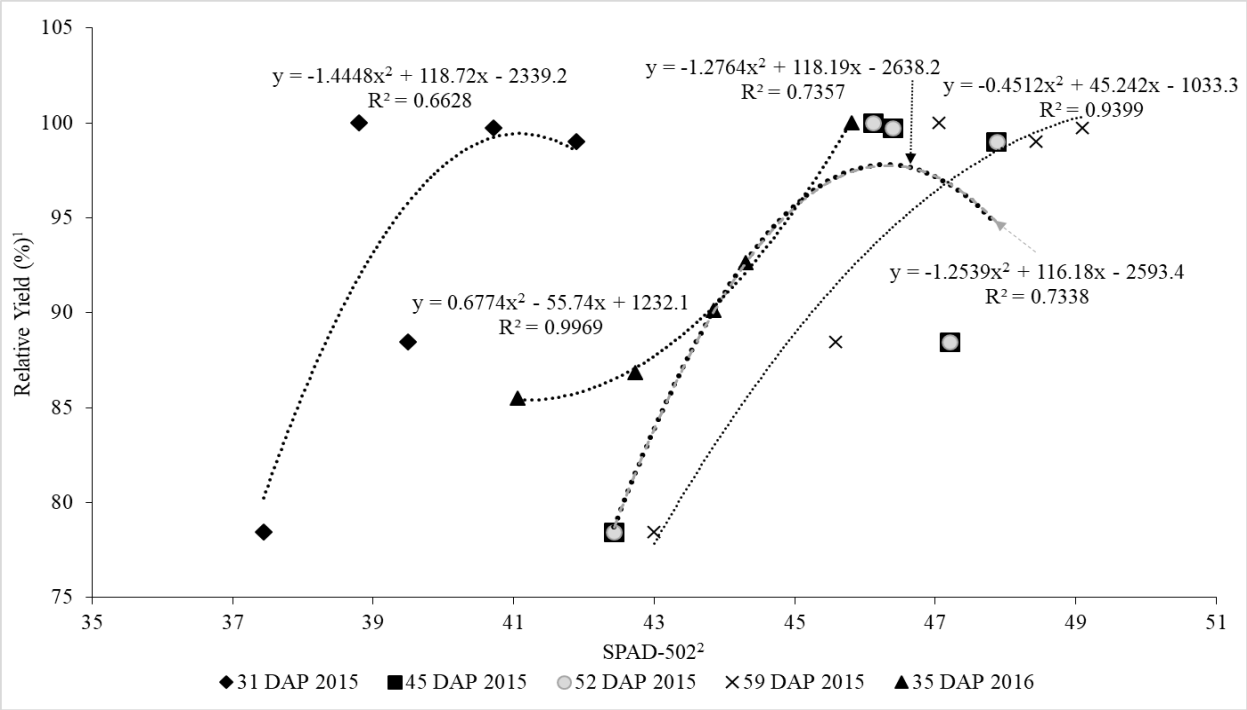


Appendix 10. Relationship between relative seed yield and plant height for Guayama experiment.



1- Relative yield=seed yield per N-level/maximum seed yield per N-level*100
2- Only significantly different plant heights with an R^2 greater than 0.5 are shown.

Appendix 11. Relationship between relative seed yield and SPAD-502 for Guayama experiment.



1- Relative yield=seed yield per N-level/maximum seed yield per N-level*100
2- Only significantly different SPAD measurements with an R^2 greater than 0.5 are shown.

Appendix 12. Fatty acid nomenclature descriptions

Fatty Acid	Description	Example	Name[1]
Saturated	No C-C doubles bonds	$CH_3(CH_2)_{14}COOH$	16:0
Monounsaturated	Only one C-C double bond	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	16:1ω7
Polyunsaturated	Two or more C-C double bonds	$CH_3(CH_2)_5CH=CHCH_2CH=CH(CH_2)_6COOH$	18:2ω7
Iso	methyl branching on second C from the methyl end	CH_3-$C(CH_2)_{12}COOH$ | CH_3	i16:0
Anteiso	methyl branching on third C from the methyl end	$CH_3CH_2CH(CH_2)_{11}COOH$ | CH_3	a16:0

1) In fatty acid nomenclature the number of carbons (C) is followed by a colon (:) and then by the number of unsaturation's (C-C double bonds). The number of C atoms between the methyl end and the terminal double bond is preceded by ω. Iso methyl branching is represented as i and anteiso methyl branching is represented as a. A cy in the name (e.g., cy17:0) indicates a cyclopropane ring and Me (e.g., 10Me16:0) follows the location of the methyl branching. Table adapted from Paul and Clark (1996).