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COMBINED MICROBIAL INOCULATION AS A PROMISING APPROACH TO ENHANCE PROMISCUOUS SOYBEAN NODULATION AND NITROGEN CONTENT IN SUDAN SAVANNA

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ABSTRACT

Applications of microbial inoculants and reduced amount of inorganic fertilizers could lead to low-input agriculture and sustain smallholders' crops production. In this study, the effect of promiscuous soybean inoculation with combined microbial inoculants was evaluated during harmattan season under furrow irrigation. Rhizobial inoculants and urea on one hand, and fungal inoculants and triple superphosphate (TSP) on the other hand, were considered as nitrogen (N) and phosphorus (P) sources, respectively. The soil was sandy loam and slightly alkaline. Significant effect from rhizobial inoculants was observed on nodule dry weight. As well, the interaction between N and P source had significant effect on %Ndfa. The interaction between 1495MAR and TSP induced the highest %Ndfa. The dual inoculation of 1495MAR and Rhizatech induced relatively high shoot N content. This study showed TGx soybean responded to rhizobial inoculation in Nigeria Sudan savanna. It showed that biofertilizers could effectively increase soybean yield under furrow irrigation. It also suggested that microbial inoculants could perform during harmattan season. Furthermore, the study showed that selective interactions occur between rhizobial strains and fungal inoculants for soybean development.

Keywords: Soybean, *Rhizobium*, *Trichoderma harzianum*, Arbuscular mycorrhizal fungi, Harmattan, Irrigation.

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Contribution/ Originality

This study is one of very few studies which have investigated the effects of commercial rhizobial and fungal inoculants on promiscuous soybean during harmattan dry season in Sudan savanna.

1. INTRODUCTION

Soybean is an important crop in the world: it has larger amount of protein and calcium than other pulse and various health benefits and it serves as cash crop. In Nigeria soybean cultivation is being increasing because of its numerous potentials (Soyinfo, 2009). Soybean is produced mainly in Nigeria's savanna where soils have poor fertility due to nutrients deficiencies and lack of beneficial soil management (Manyong *et al.*, 2001). Sustain soybean production and increase its yield in those agroecological zones with low-input could constitutes a clue to improve smallholders gains and ensure somewhat food security.

Soils harbor an immense diversity of rhizospheric microorganisms which influence nutrient pools in the soil. The interactions between the roots system of the plant and soil microorganisms result sometime in increased plant growth. These plant-growth promoting soil microorganisms are ubiquitous and mainly selected for their potential in biological nitrogen fixation (BNF), phosphorus solubilization, and biocontrol of soil-borne diseases and pathogens. The symbioses between soil bacteria from Rhizobiaceae Family and soybean plant can provide up to 94% of the crop's nitrogen requirement (Peoples and Craswell, 1992; Hungria *et al.*, 2006).

Soybean could also benefit from other symbioses with soil fungi such as AMF and *Trichoderma* that have the ability to increase plant nutrient uptake and resistance to certain biotic and abiotic stresses. *Trichoderma* spp., an antagonistic fungus well-known as biocontrol agent against several soil borne plant pathogens (Harman *et al.*, 2004; Perazzolli *et al.*, 2011), is also involved in plant growth and yield enhancing (Rudresh *et al.*, 2005; Verma *et al.*, 2007; John *et al.*, 2010). Equally, arbuscular mycorrhizal fungi (AMF), a worldwide occurring plant-fungi association, colonize roots of soybean, increase functional soil volume and promote plant growth through improvement of plant nutrient uptake, especially phosphorus (Marschner and Dell, 1994; Ortas, 2010); Farzaneh *et al.* (2009). This makes AMF an important soil microbe which could be used in tropical soil to alleviate P deficiency stress on crops (Cardoso and Kuyper, 2006) and improve BNF effectiveness (Giller, 2001).

Consequently, applied soil microbiology in agriculture leads to the selection and inoculation of effective beneficial soil microbes to improve soil fertility, growth, health and yield of different crop species. In addition, the tendencies of using mixt microbial inoculants are increasing and give satisfying results. For example, combination of effective soil microorganisms to inoculate soybean resulted in an increased growth, nodulation and BNF activity (Antunes *et al.*, 2006; Bisht *et al.*, 2009). Some of those microorganisms of interest for sustainable agriculture are selected, commercially produced by agribusiness companies and are available on the market (Thuita *et al.*, 2012). As living microorganisms, their activities could be limited by some soil nutrients deficiency (Giller and Cadisch, 1995), host-plant weakness or environment specific factors (Zahran, 1999).

In Nigeria, like in many African countries, *Bradyrhizobium japonicum* populations which could nodulate the "US-type" soybean that was predominantly cultivated were not endemic to African soils. Therefore, this soybean variety needs to be inoculated with specific and effective strains of *Bradyrhizobium japonicum* to nodulate (Osunde *et al.*, 2003). The non-abundance of commercial *B. japonicum* inoculants and nitrogenous fertilizers in the 1970s and 1980s led to the option of breeding promiscuous cultivars in the International Institute of Tropical Agriculture (IITA) to avoid the need to inoculate (Abaidoo *et al.*, 2000; Tefera *et al.*, 2010). Soybean breeders in IITA developed new soybean genotypes with reduced nodulation specificity for Africa, known as Tropical Glycine cross (TGx). As result, different varieties with different maturity lengths were released to farmers in Nigeria and other African countries to replace local or imported soybean varieties; 82% of the farmers used improved varieties in Nigeria. Promiscuous TGx soybean which nodulate freely with *Bradyrhizobium* spp. populations indigenous to African soils might then constitute an obstacle to inoculation, especially when *Bradyrhizobium* spp. strains present in commercial inoculants are usually selected based on their specificity to non-promiscuous soybean cultivated mostly out of Africa.

Considering the economic and environmental importance of the use of rhizobial inoculants (Alves *et al.*, 2003) and the benefits that could provide their co-inoculation with other soil microorganisms in sustaining agriculture in sub-Saharan Africa, the reported experiment was undertaken during harmattan season in Nigeria Sudan savanna (1) to measure the response of promiscuous TGx soybean to rhizobial commercial inoculants, and (2) assess the influence of P fertilizer and fungal inoculants on promiscuous soybean inoculated with rhizobia.

2. MATERIALS AND METHODS

2.1. Experimental Site and Soil Properties

The trial was undertaken during the dry season from January to March 2011, at the Irrigation Research Station of the Institute for Agricultural Research, Ahmadu Bello University, Zaria, Nigeria. Kadawa is situated in Kano State of Nigeria and the agro-ecological zone is determined as Sudan savanna on latitude 11°39'N and 08° 02'E and altitude 500 m above sea level. The irrigation is done by flooding. Fifteen samples of soil (0–15 cm) per replication have been taken randomly and bulked to form a composite sample. A sub-sample of each composite was processed to be analyzed for general characteristics determination in the Analytical Service Laboratory of the IITA (1982). The chemical and physical parameters of the plots at the beginning of the experiment are shown in Table 1. The soils of the three blocks were slightly alkaline with pH ranging from 7.5 to 7.6. The soil phosphorus levels ranged from 27.38 to 35.02 g kg^{-1} of soil. The organic carbon ranged from 6.40 to 6.70 g kg^{-1}. The soil texture was determined as sandy loam.

2.2. Application of Microbial Inoculants and Inorganic Fertilizers

Four rhizobial (generic denomination including *Rhizobium* and *Bradyrhizobium* strains) and two fungal inoculants were the microbial based products used in the experiment. The origin and characteristics of each microbial inoculant are shown in Table 2. The rhizobial based inoculants and Eco-T were peat-based products. The seeds were coated with the rhizobial inoculants and Eco-

T just prior to the sowing. The rate of application of the laboratory produced rhizobial inoculants was 10 g per 1 kg of seeds. Legumefix and Eco-T were used following the recommendation from the different companies, respectively. The arbuscular mycorrhizal fungal inoculant was applied as follows: 50 mg of Rhizatech was mixed with 500 mg of soil and applied in the sowing furrow before the seeds were placed on top.

Table-1. Soils chemical and physical properties of the blocks at planting

Properties	Units	Plot 1	Plot 2	Plot 3
pH (H_2O) 1:1		7.60	7.60	7.50
OC	g kg^{-1}	6.70	6.60	6.40
N	g kg^{-1}	0.92	0.88	0.81
Mehlich P	g kg^{-1}	35.02	29.24	27.38
Zn	µg g^{-1}	9.73	6.11	6.81
Cu	µg g^{-1}	2.27	2.11	2.17
Mn	µg g^{-1}	52.70	44.31	49.91
Fe	µg g^{-1}	125.25	122.63	111.83
Ca	$Cmol_c$ kg^{-1}	7.46	6.49	6.27
Mg	$Cmol_c$ kg^{-1}	1.41	1.13	1.45
K	$Cmol_c$ kg^{-1}	0.53	0.47	0.71
Na	$Cmol_c$ kg^{-1}	0.15	0.17	0.16
Exch. Acidity	$Cmol_c$ kg^{-1}	0.00	0.00	0.00
ECEC	$Cmol_c$ kg^{-1}	9.55	8.25	8.59
Sand	g kg^{-1}	650	650	650
Silt	g kg^{-1}	230	230	230
Clay	g kg^{-1}	120	120	120

Table-2. Origin and active agents of the microbial products

Products	Origins	Types	Active microorganisms
1495MAR	Marondera SPRI, Zimbabwe	Laboratory	*Bradyrhizobium* sp.
RACA6	IITA, Ibadan (Nigeria)	Laboratory	*Bradyrhizobium* sp.
TSBF560	TSBF, Coast Kenya	Laboratory	*Rhizobium*
Legumefix	Legume Technology Ltd., UK	Commercial	*Rhizobium*/*Bradyrhizobium*
Rhizatech	Dudutech Ltd., Kenya	Commercial	Spores and mycelia fragments of AMF
Eco-T	Plant Health Products (Pty) LTD, South Africa	Commercial	*Trichoderma harzianum* strain Rfai KRL AG2

Inorganic nitrogen (N) was applied as urea (46-0-0), phosphorus (P) as triple superphosphate (46% P_2O_5) and potassium (K) as muriate of potash (60% K_2O). The inorganic fertilizers were applied by banding the sowing rows. Phosphorus and potassium were applied, once at planting, at rate of 33 kg P ha^{-1} and 29 kg K ha^{-1}, respectively. Potassium was applied basically to the entire experimental plots. Two rates of nitrogen (40 and 80 kg N ha^{-1}) were split applied: half at planting and half at 3 weeks after planting. The experiment was then set out in a randomized complete block design with 2 factors. Factor 1 was constituted of one control (no nitrogen, no rhizobial inoculant), four N_2-fixers rhizobial inoculants and two rates of inorganic N fertilizer. Factor 2 comprised one control (no fungal inoculant, no P applied), inorganic P fertilizer and two fungal inoculants. Each experimental treatment was replicated three times.

The promiscuous early-maturing (85-99 days) rust resistant soybean variety TGx 1835-10E was used as test crop (Tefera, 2011). The experimental plot measured 3 m x 3 m with 4 rows and 0.75 m inter-rows. The plots were separated from one another by one non-planted row as buffer. To minimize any contamination between the plots, the irrigation was done plot by plot.

2.3. Data Collection

The plants sampling was done at 8 weeks after planting (R2 - R3 growth stage). The plant shoot was dried for 72 hours at 60 °C to determine the shoot dry matter accumulated by plant. The stem and leaves were then ground and sieved separately to pass 0.4 mm sieve. One volume of leave and two volumes of stem were mixed and analyzed for N content. Nodule dry weight was taken to assess the nodulation intensity (infectivity of the inoculant) while the ureides method was used to evaluate the proportion of plant N derived from atmosphere through BNF (%Ndfa) for each rhizobia inoculant (Herridge *et al.*, 2008). Soybean stems were oven dried at 60 °C for 24 hours and ground to pass through 1 mm sieve (Herridge, 1982; Herridge and Peoples, 1990). Samples were extracted with 25 ml boiling water for 2 min (Herridge, 1982). The extracts were filtered, made up to 50 ml with distilled and stored at - 15°C to be analyzed later for ureides content.

The dependency of soybean on mycorrhizal inoculation was measured using the formula proposed by Plenchette *et al.* (1983) to assess mycorrhizal inoculation effect under unsterilized soil. The data collected were submitted to 2-way factorial analysis in SAS using GLM procedure to determine the significance of the main factors and their interaction effects (Statistical Analysis System Institute Inc., 2009). Factor 1 was called source of N and Factor 2 was referred mainly as P sources. Multiple means comparison was conducted with the least significant difference (LSD) option. The means from the interaction between N and P sources were plotted with GenStat Discovery Edition 4.

3. RESULTS

3.1. Shoot Dry Weight

The shoot dry weight was not significantly influenced by rhizobial inoculants. The application of 40 kg N ha^{-1} favored soybean shoot dry weight production more than other treatments. Among rhizobial inoculants, the plants inoculated with TSBF560 had a relatively higher mean of shoot dry weight with 5.28 g plant^{-1}than other rhizobial treatments (Table 3). Plants inoculated with Rhizatech produced more shoot and nodule dry weight than those that received P fertilizer. The mycorrhizal dependency of TGx 1835-10E was evaluated to 19 % for shoot dry weight. The interaction between rhizobial and fungal inoculants did not have significant effect on shoot dry weight. The interaction between 1495MAR and Eco-T reduced the shoot dry weight compared to the single inoculation of Eco-T (Figure 1). The effect of Rhizatech in interaction with 1495MAR (1495MAR + Rhizatech) favored shoot dry weight production more than did the combined application of 1495MAR and inorganic P fertilizer (1495MAR + P). As well, co-inoculation of

Eco-T and TSBF560 or RACA 6, were more beneficial in shoot dry weight production than the effect of the application of P fertilizer on TSBF560 and RACA 6.

Table-3. Main factors effects on soybean shoot and nodule dry weight, %Ndfa and shoot N content

Factors	Shoot dry weight (g plant^{-1})	Nodule dry weight (mg plant^{-1})	%Ndfa	Shoot N content (%)
Factor 1				
Control	4.75	52.64	75.22	2.22
1495MAR	4.79	93.11	78.03	2.49
Legumefix	5.04	101.66	78.15	2.24
TSBF560	5.28	55.35	62.57	2.08
RACA6	4.87	154.68	75.11	2.25
80 N	6.03	32.03	55.48	2.13
40 N	6.37	47.37	66.29	2.07
$LSD_{(0.05)}$	1.6734	54.616	16.764	0.4159
Factor 2				
Control	4.35	46.51	77.53	2.30
Rhizatech	5.35	106.37	76.25	2.29
Eco-T	4.71	70.94	71.45	2.19
P fertilizer	5.33	85.69	68.83	2.22
$LSD_{(0.05)}$	1.447	53.763	14.496	0.3596

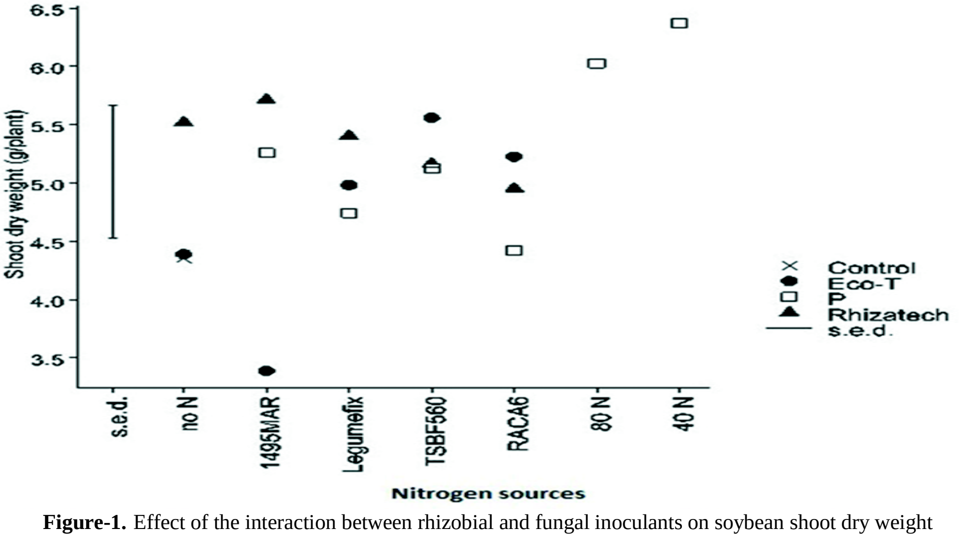

Figure-1. Effect of the interaction between rhizobial and fungal inoculants on soybean shoot dry weight

3.2. Nodule Dry Weight

TGx soybean significantly (P = 0.0003) responded to rhizobial inoculation. The plants inoculated with RACA 6 had the highest nodules dry weight with a mean of 154.64 g plant^{-1}. It was followed by plants inoculated with Legumefix which produced 101.66 g plant^{-1}. Plants inoculated with Rhizatech produced nodule dry weight than P fertilizer, even though its effect was not significant. The mycorrhizal dependency of TGx 1835-10E was evaluated to 56 % for nodules dry weight production. Rhizatech single effect favored soybean nodulation with 78.54 mg plant^{-1} compared to the uninoculated control which produced 51.30 mg plant^{-1}.The combined application of rhizobial and fungal did have significant effect on nodule dray weight. The combined application of RACA6 and P fertilizer induced nodule dry weight production of 165.57 mg plant^{-1}. The distribution of the nodules dry weight as influenced by the interaction between the experimental factors is shown in Figure 2. Rhizobial mycorrhizal dependency for soybean nodulation varied among the rhizobial inoculants. The co-inoculation of Rhizatech with 1495MAR induced insignificantly higher nodule dry weight than when 1495MAR and P fertilizer are applied together. In contrast, RACA6 profusely nodulated soybean when P fertilizer was applied.

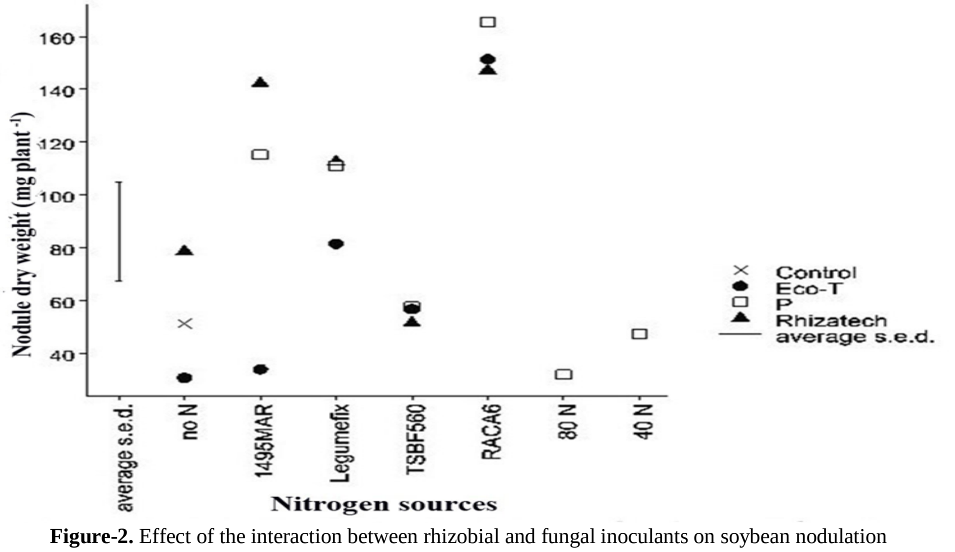

Figure-2. Effect of the interaction between rhizobial and fungal inoculants on soybean nodulation

3.3. Estimation of Nitrogen Derived From Atmosphere (%Ndfa)

The different factors did not have significant effect on soybean %Ndfa. Legumefix and 1495MAR had positive effect on soybean while all treatment applied in Factor 2 depressed %Ndfa compared to plant that did not received P and fungal inoculants. The interaction between rhizobial and fungal inoculants led to significant effect (P = 0.02) on soybean N fixation (%Ndfa). The interaction between P fertilizer and 1495MAR gave the highest %Ndfa with 92.24 %. In nitrogen fixation, rhizobial inoculants showed varied behavior to P and fungal application (Figure 3). Legumefix and TSBF560 co-inoculated respectively with either Rhizatech or Eco-T were more effective in nitrogen fixation than when P fertilizer was applied to those rhizobial inoculants.

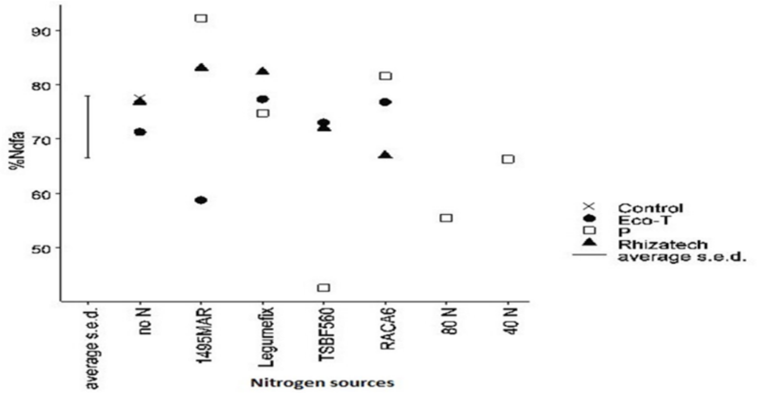

Figure-3. Effect of the interaction between rhizobial and fungal inoculants on soybean on nitrogen fixation

3.4. Shoot Nitrogen Content

The application of microbial products and their interaction did not have significant effect on shoot N content. Increase shoot N content was therefore recorded from the inoculation of 1495MAR, RACA 6 and Legumefix with 2.49, 2.25, and 2.24 %, respectively (Table 3). The shoot N content was not significantly influenced by the interaction between the Factors. The interaction between 1495MAR and P fertilizer (1495MAR + P), and between 1495MAR and Rhizatech increased the shoot N content compared to the combined application of N and P. The effect of interaction on shoot N content is shown in Figure 4. Positive and highly significant relationship ($r = 0.77$; $p < 0.0001$) was found between %Ndfa and shoot N content.

4. DISCUSSION

Soybean, like other legume, had two sources of nitrogen: N derived from BNF and mineral N of soil. High amounts of N fertilizer usually lead to increases in leaf mass, and total dry weight may be higher than plants relying on BNF (Alves *et al.*, 2003). This study is in agreement with that observation. However, 40 kg N ha^{-1} slightly increased shoot dry weight more than 80 kg N ha^{-1}. With the flooded irrigation system which could increase N leaching and decrease mineral N uptake, N fertilizer applied at 40 kg ha^{-1} could have been used as ‘‘starter N’’ by the plant (Alves *et al.*, 2003). This could then allow soybean to partially benefit from BNF.

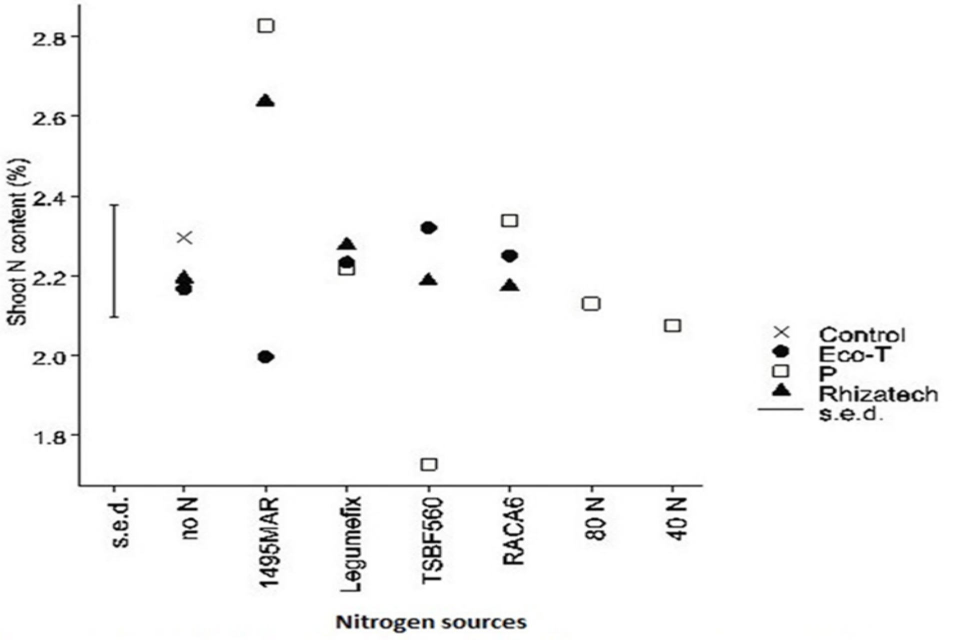

Figure-4. Effect of the interaction between rhizobial and fungal inoculants on soybean shoot N content

The nodulation of the control treatment showed the presence of infective indigenous rhizobial strains in the soil. However, this poor nodulation could be related to a low rhizobial population in the soil as shown the study conducted by Sanginga *et al.* (1996) and Thuita *et al.* (2012) in Nigerian and Kenyan soils, respectively. The number and effectiveness of indigenous rhizobia in soil have a direct influence on the response of an introduced legume to artificial inoculation

(Trotman and Weaver, 1986). It is relevant that soybeans require approximately 1 x 10^3 rhizobia g^{-1} of soil for maximal numbers of nodules on seedling tap roots (Weaver and Frederick, 1974). The significant difference observed in the nodules dry weight among rhizobial inoculants also confirmed the host-specificity factor in soybean nodulation as shown the study of Shutsrirung *et al.* (2002). It has been also demonstrated by Barcellos *et al.* (2009) that genetic-related efficiency exists among soybean *Bradyrhizobium* population. Despite TGx 1835-10E is a promiscuous soybean variety, there was a discrepancy between rhizobial strains in its nodulation. Therefore, promiscuous soybean could require highly efficient rhizobia strains to nodulate well and increase shoot dry weight. These results are in agreement with those recorded by Sanginga *et al.* (1996) in moist savanna. These authors reported that promiscuous soybean N requirement can be met only when efficient rhizobia are available in sufficient number.

The study on the %Ndfa revealed that Legumefix and 1495MAR were superior strains in N_2-fixation, either in single or combined inoculation. As well, shoot N content of plant inoculated with Legumefix and 1495MAR were improved. These observations are, somewhat by extension, in line with the one of Thuita *et al.* (2012) who concluded that Legumefix and 1495MAR are suitable rhizobial inoculants for promiscuous TGx 1740-2F soybean. Despite Legumefix did not produced the highest nodules dry weight, it induced the maximum %Ndfa. Thus, Legumefix had the highest potential of N_2-fixation during harmattan season. Furthermore, the less performance of RACA 6, which produced the highest nodule dry weight, could be related to the soil conditions. The variations of the temperature during harmattan season constitute a source of variation of rhizobia N_2-fixation activities. In the tropics, the soil temperature sometimes exceeds 40 °C. During harmattan season, this situation is worsened by very high diurnal and very cold nocturnal temperatures.

Under soil containing Mehlich-3 extractable P values varying from 29.24 to 35.02 g kg^{-1}, fungal inoculants were able to increase soybean shoot and nodules dry weight as did inorganic P applied at 75 kg P_2O_5 ha^{-1} (no significant difference was shown between P application and fungal inoculation). This behavior of fungal inoculants in nutrient transportation is a significance of the tripartite symbiosis (soybean, rhizobium and fungus). The reliance of TGx soybean on mycorrhizal fungi inoculation for nodulation was higher than the effect of P fertilizer on it. This also was shown in the shoot dry weight of soybean co-inoculated with 1495MAR and Legumefix, respectively. The tripartite symbiosis could help to increase the conspicuous role of BNF in soybean production as observed by Wang *et al.* (2011); Co-inoculation with rhizobia and AM fungi had synergistic effects on soybean nodulation, especially under low P and low N conditions. There observation supports our findings in which the tripartite interaction induced positive effect on soybean shoot N content which is a significant indicator for legume grain yield. In soybean, 80 – 90% of N of vegetative tissues is remobilized for seed N demand (Schiltz *et al.*, 2005). The increased shoot N content from tripartite symbiosis could be supported by AMF and *T. harzianum* P and other nutrient transportation. Applied in two splits, N fertilizer did not increase the soybean shoot N content in the experimental conditions. This observation could be explained by the leaching of N below the root zone due to drainage. Moreover, the study suggested that rhizobial strain differed in their interactional behavior with fungal inoculants. The significance of the tripartite soybean-*Rhizobium*-

fungus interaction has appeared like depending on rhizobial-fungus specificity. The benefit from the tripartite interaction was depending on the nature of the interaction between the symbionts. These results are somehow in agreement with those of Xavier and Germida (2003), who reported that selective interactions occur between AMF and *Rhizobium leguminosarum* bv. Viceae.

In conclusion, the present study indicates that promiscuous TGx soybean responded to rhizobial inoculation during harmattan season. It showed that benefits from soybean biofertilization could be exploited for growth and yield increase under furrow irrigation. Furthermore, this study suggested that some microbial strains could well-perform during harmattan hard-dry season. Since, the co-inoculation also showed some specificity between rhizobial and *Trichoderma* or AMF inoculants, it could be suggested that further investigation should be conducted to select synergistic microbial interaction for promiscuous soybean inoculation.

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