



Full Length Article

Mycorrhiza-*Rhizobium-Vigna subterranea* Dual Symbiosis: Impact of Microbial Symbionts for Growth and Sustainable Yield Improvement

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ABSTRACT

Improvement of soil fertility and promotion of sustainable agriculture in Adamawa region is feasible if under-utilized crops such as bambara groundnut (*Vigna subterranea*) could be inoculated by *Rhizobium* and mycorrhiza strains in the field. An experiment was conducted in a completely randomized block design with five treatments; each of which was repeated thrice: control, *Rhizobium*, mycorrhiza, *Rhizobium*+mycorrhiza and chemical fertilizer. *V. subterranea* seeds used had a very high emerging capacity of between 92–100%. The soil organic matter was reduced by 20 to 30% after harvest, indicating their utilization for plant growth. Compared to un-inoculated plants, *Rhizobium* and mycorrhiza significantly ($p \leq 0.0007$) increased nodulation and plant biomass at 30 and 60 DAP. The number of root nodules was reduced as from 50 days after planting, but increased in their dry weight, as an indication of their effectiveness to nodulation. The seed yield at harvest of *Rhizobium*+mycorrhiza treatment was boosted to three folds than that of control. Based on these results, it is suggested to prescribe the sustainable improvement of bambara groundnut production through *Rhizobium*+mycorrhiza inoculation. © 2012 Friends Science Publishers

Key Words: *Vigna subterranean*; *Rhizobium*; Mycorrhiza; Nodulation; Plant growth; Sustainable production

INTRODUCTION

In most of the Sub-Sahara African countries, increased population growth leads to hunger, the main cause of malnutrition and food insecurity. This food insecurity relies on low crop productivity, limited by low soil fertility which is accelerated by low soil cation exchange capacity and organic matter, high soil acidity and/or bad cultural practices (Bado, 2002; Yao *et al.*, 2005). All these constraints create disequilibrium between the available quantity of crops produced and the population needs on one hand, and the environment on the other, suggesting that this population is not eating or living safely (Cooke, 1998). In an effort to enhance crop production, most of our tropical countries rely on chemical fertilizers, which have potentially revealed to be a pollutant to human life and environment (Margni *et al.*, 2002), in addition to their high cost, as the result of lack of own available chemical fertilizer manufacturers. The lack of food rich in proteins in the world and particularly in developing countries has urged researchers to seek for alternative source of proteins to complete their diet (Ahmed & Abdallah, 2010). Hence, the food insecurity, the environmental pollution and the soil fertility problems need to be solved if promotion of

biological and sustainable agriculture is to be accomplished through measures such as improved fallow, intercropping, agroforestry or biofertilizers. Among these strategies, biofertilizers have been reported to increase yield of several crops in Cameroon (Megueni *et al.*, 2006; Ngakou, 2007; Ngakou *et al.*, 2007a, 2008, 2011). Unlike other crop legumes, very little is known about *Rhizobium*-mycorrhiza-*Vigna subterranea* interactions. Bambara groundnut belongs to the under-utilized grain legumes that possess high crude protein content between 22 and 37% (Adeparusi, 2001; Fasoyiro *et al.*, 2006). It is widely grown in Nigeria and in other African countries like Ghana, Cameroon, Ivory Coast and Togo (Klu *et al.*, 2001). Bambara groundnut is the third most important legume after groundnut (*Arachis hypogaea*) and cowpea (*Vigna unguiculata*) (Howell *et al.*, 1994). Hence, there is a need to improve the productivity of this food security crop known to grow on low soil fertility, where it can withstand drought and low rainfall (Berchie *et al.*, 2010). Therefore, we hypothesize that *Rhizobium*-mycorrhiza dual symbiosis could alleviate the host plant nutrient requirements, thus resulting to sustainable yield improvement of this valuable crop, so as to substitute chemical fertilizers.

MATERIALS AND METHODS

Study area: The field trials were located at Dang-Ngaoundéré in the Adamawa region of Cameroon (7° 24.61'N 13° 34.24'E, at 1155.8 m elevation). The climate is of the soudano-guinea type (Yonkeu, 1993), with the rainy and dry seasons of the same length and extending from middle march to middle september and middle september to middle march respectively. The soil is brown-red and is developed on a basaltic rock, with a mean polyedric structure. The minimum temperature ranges from 5 to 7°C and maximum from 30 to 35°C, while the average humidity varies from 37.7 to 81% (Mope Simo, 1997).

Biological material: Bambara groundnut seeds (*V. subterranea* (L.) Verdc.) of Mendeo variety (Fig. 1) was provided by the Institute of Research and Agricultural Development (IRAD) Wakwa, Ngaoundere. The growing cycle varies from 90 to 105 days at maturity, from which flowers are yellow in colour, while harvested seeds are grey with stripes.

Rhizobium and mycorrhiza inoculants: Nitrogen fixing bacteria of the genus *Rhizobium*, specific for Bambara groundnut (Ngakou *et al.*, 2009), was multiplied in the microbiology of IRAD Ngaoundere (Fig. 2a). Mycorrhizal inoculant was of the genus *Glomus*, provided by the Biotechnology Centre of the University of Yaoundé I (Fig. 2b).

Experimental field, experimental design and treatments: The experiment was conducted on 110 m² surface area after manual clearance with cutlasses, and ploughing at 30 cm depth with hoes, in order to remove all the roots of the natural occurring weed *Imperata cylindrica*. A fence made of small woods was made around the field to protect plots from the devastating effect of cattle, usually abandoned to feed in natural environment in the region. The experiment was carried out in a Randomized Complete Block Design (RCBD) with five treatments, each of which was repeated three times. The five treatments were: Plot with seeds coated with *Rhizobium* at sowing (Rhi); plot with seeds inoculated with mycorrhiza (Myc); plot with seeds coated with *Rhizobium*, and inoculated with mycorrhiza at sowing (RhMc); plot with seeds uncoated with *Rhizobium* and not inoculated with mycorrhiza at sowing (Ctr or negative control); plot with seeds uncoated with *Rhizobium*, not inoculated with mycorrhiza, but where seedlings have received chemical fertilizers (NPKS) thrice in three months after sowing (CFr).

Seed inoculation and sowing: Sowing was performed on May 22nd 2010. Control plots were sown first to avoid contamination with bio-inoculants. *V. subterranea* seeds were inoculated as described by Ngakou (2007). Then, small holes were dug in each experimental unit to be inoculated. An experimental unit was a 4.5 m², consisting of 4 rows planted at one seed/hole, spaced at 25 × 30 cm within and between rows respectively. Plots were separated one another by 0.50 m. For dual inoculated plots

Fig. 1: *Vigna subterranea* seeds of Mendeo variety used in the study



Fig. 2: *Rhizobium* (a) and Mycorrhiza (b) inoculants



inoculation was performed as described by Ngakou *et al.* (2007b).

Weeding and chemical fertilizers application: Weeding was performed monthly, thrice during the growing cycle of the crop. During growth, each plant was protected against wind or devastators by accumulating soil under the stems. The chemical fertilizers NPKS (MgO) with the formula 14:24:14:0.5:3 was used, and provided at a rate of 5 g around the plant.

Soil analysis: Experimental soils were randomly sampled before sowing and after harvest on the whole study area at between 10 and 15 cm depth along the medians and diagonals. After homogenization, three replicates of 1 kg soils were sub-sampled from each group. These soils were physico-chemically analyzed in the Laboratory of Soil and Plant Analysis (LASEP) of ITRAD (Research Institute for Agronomic Development) of N'Djamena, Tchad, following AFNOR (1982) method.

Evaluation of date to seeds emergency: Emergence of seedlings was assessed by counting and recording the number of plot holes from which a plantlet has emerged from the second to the fourteen day after sowing.

Assessment of nodulation, plant biomass, fructification and seed maturation: Determination of nodulation period of *V. subterranea* was performed as from the 10th day after planting on 5 randomly selected plants per treatment. Each plant was dipped in water to remove all the soil in roots and enable visualization of nodules (Ngakou *et al.*, 2009). The number and dry weight of root nodules were determined at 30 and 60 days after planting on 10 randomly selected plants per treatments. Nodules from individual plants were air dried for 15 days and weighed on a Sartorius balance at 0.001 g sensibility. Nodules efficiency was estimated, then, the coloration of leghemoglobin within the nodule, which gives an indication on the nitrogen fixation level, was observed and recorded (Ngakou *et al.*, 2009).

The fructification period was estimated by randomly removing 6 plants per treatment after every 7 days as from 40 days after planting. The biomass of plants was also determined at 30 and 60 DAP on 10 plants per treatment, air dried for 30 days and weighed on a Gilbertini balance at 0.01 g sensibility.

Assessment of yield components: Bambara groundnut pods were harvested from 20 randomly selected plants per plot, labelled counted and weighed separately. Then, the total pod weight was calculated as the sum of seed weight of individual plant from the same plot. The number of seeds per pod was determined by counting the seed from 10 randomly selected pods from 20 plants per plot. Seeds from individual plants were sun dried for 30 days and weighed individually. The 100 seed dry weight was evaluated in triplicate by counting and weighing 100 seeds per treatments. Seed yield expressed in kg/ha was obtained by multiplying the seed weight from a 4.5 m² plot by 10000 m².

Statistical analysis: Data collected were subjected to analysis of variance (ANOVA) using the Stat Graphics plus software program. Treatment means were compared using the Least Significance Difference (LSD) at $p < 0.0001$. Correlations between variables were determined using the statistical package for social science (SPSS).

RESULTS

Physico-chemical properties of the experimental soil: The chemical composition of the experimental area before

planting date as summarized in Table I indicates that this soil was a loamy sand type, poor in organic matter and mineral nutrients. After harvest, the soil organic matter composition was considerably reduced by 20–30%, with slight increase or decrease in pH depending on the treatment. The soil pH estimated at 5.7 was slightly acidic. Organic matter has the ability to moderate major changes in the soil pH. Organic matter has been reported to buffer the soil against major swings in pH by either taking up or releasing H⁺ into the soil solution, making the concentration of soil solution H⁺ more constant (Cooperband, 2002). The organic matter left in soil after crop harvesting does have an essential importance in preservation and reproduction of soil fertility in crop rotation. As reported by Martin *et al.* (1990), the positive effect of crop legumes such as *V. subterranea* on the soil organic matter content did not show during the first growth year, but by the second growth year. In fact, crop residues including roots, chaff, stems and leaves which are left after harvesting are considered as the prime source of organic matter replenishment and this is more noticeable only after decomposition of the crop legumes that is made available to the next cash crop season (Ilumäe *et al.*, 2009). The low organic matter of our soil samples after harvest was obvious, since sampling was carried out before crop residues decomposition.

Growth Parameters

Seed emergency: *V. subterranea* seeds started emerging from the soil 7 days after planting in all the 5 treatments. Germination was complete in the field 12 DAP, thus 5 days after the first emergency. The germination rates were 100%, 98%, 95% and 92.5%, respectively for treatments control, mycorrhiza, *Rhizobium*+mycorrhiza and *Rhizobium*.

Plant phenology: *V. subterranea*, variety Mendeo, used in this study, had a growing cycle of between 90–105 days. The first flowering (flowers of yellow colour) appeared at 38 DAP for control and *Rhizobium*+mycorrhiza co-inoculated treatments, at 39 and 40 DAP respectively for *Rhizobium* and mycorrhiza treatments. The date to 50% flowering varied between 43 DAP (treatments Myc & RhMc) and 48 DAP for treatment CFr (Fig. 3). Treatment CFr significantly delayed the 50% flowering date compared to other treatments. Pods setting started at 49 DAP for treatment RhMc and 52 DAP for treatment CFr, but no significant difference was observed between treatments as far as this parameter is concerned.

Nodule number, weight and nodulation efficiency: The analysis of variance indicates that nodule number varies with time from one treatment to another, with *Rhizobium*+mycorrhiza significantly ($p = 0.0008$) stimulating formation of more root nodules at 30 DAP than the un-inoculated plants (Table II). The number of nodules at 60 DAP was reduced compared to that of 30 DAP. As far as the efficiency of nodules is concerned, root nodules of inoculated treatments (Rhi, Myc, RhMc) were significantly ($p = 0.0001$) more effective in fixing biological nitrogen than those of the control treatment, suggesting that although

Table I: Physico-chemical properties of studied soil before sowing and after harvest

Soil properties before sowing			
Granulometry	Organic matter	Chemical components	Exchangeable cations
Clay (23%)	C (1.69%)	pH (5.7)	Ca (3.9 meq/100 g soil)
Limon (6.5%)	OM (2.12%)	Conductivity (100.8 μ s)	Mg (1.18 meq/100 g soil)
Lg (21.18%)	Total N (0.038%)	CEC (7.91 meq/100 g soil)	K (0.25 meq/100 g soil)
Sf (26.95%)	Total P (0.023%)	Saturation rate (69.65%)	Na (8.0 meq/100 g soil)
Sg (22.32%)	C/N ratio (43.97)		Others(5.51 meq/100 g soil)
Soil properties after harvest			
Treatments	Parameters		
	C (%)	OM (%)	pH
Ctr	0.06c	0.11d	5.66a
Rhi	0.04a	0.07b	5.57a
Myc	0.036a	0.06a	5.87b
RhMc	0.046b	0.08c	5.60a
CfR	0.04a	0.07b	5.57a

Values of the column for a variable followed by the same letter are not significantly different at the level of probability considered
RhMc = *Rhizobium*+mycorrhiza; Rhi = *Rhizobium*; Myc = Mycorrhiza; CfR = Chemical fertilizers; Ctr = Negative control; Lg: Bid size limon; Sf: Small size sand; Sg: Big size sand

Table II: Number and efficiency of nodules at 30 and 60 DAP

Treatments	Nodule Plant ⁻¹		Nodules weight (mg plant ⁻¹)		Nodule efficiency (%)	
	30 DAP	60 DAP	30 DAP	60 DAP	30 DAP	60 DAP
Ctr	110a	39a	43.93a	33.06a	28.44a	38.77a
Rhi	117a	40a	80.13bc	83.0b	75.24b	99.50b
Myc	124a	48ab	99.0c	136.6c	71.94b	99.81
RhMc	172b	64bc	88.13c	120.46bc	83.09b	99.23b
CfR	105a	74c	56.8ab	123.8b	32.23a	99.34b
LSD	31.33	24.66	36.2	53.6	39.70	60.73
P value	0.0008	0.0006	0.001	< 0.0001	< 0.0001	< 0.0001

Values of the column for a variable followed by the same letter are not significantly different at the level of probability considered
DAP = Days After Planting; RhMc = *Rhizobium*+mycorrhiza; Rhi = *Rhizobium*; Myc = Mycorrhiza; CfR = Chemical fertilizers; Ctr = Negative control

Table III: Pod and seed yield as influenced by different treatments

Yield parameters	Treatments						LSD	P value
	Ctr	Rhi	Myc	RhMc	CfR			
Pod number (plant ⁻¹)	26a	34b	32b	43b	24a		5.73	0.0001
Pod weight (g plant ⁻¹)	122.01a	195.2b	181b	185.3b	136a		44.17	0.0001
Seed number (plant ⁻¹)	22a	29b	28b	28b	27b		5.28	0.0001
Seed weight (g plant ⁻¹)	18.01a	19a	18.1a	21.03b	19.4b		4.47	0.0002
100 Seeds dry weight (g)	58.02a	87.67b	83.3ab	98.76b	76.1ab		29.65	0.007
Seed yield (kg/ha)	524.25a	863.6b	867.3b	912.1b	902.2b		339.5	0.0006

Values of the column for a variable followed by the same letter are not significantly different at the level of probability considered
RhMc = *Rhizobium*+mycorrhiza; Rhi = *Rhizobium*; Myc = Mycorrhiza; CfR = Chemical fertilizers; Ctr = Negative control

native microbial symbionts can nodulate the host plant, these nodules are not always efficient, thus cannot efficiently fix biological nitrogen or improve plant nutrients uptake.

Mycorrhiza, *Rhizobium* and Mycorrhiza+*Rhizobium* significantly ($p = 0.0014$) doubled the nodules weight of inoculated compared to un-inoculated plants at 30 and 60 DAP. Despite the high number of nodules in control plants, their nodule weight remained low, and this can be related to their low nodulation efficiency.

Plant biomass: Plant biomass was assessed at 30, 60 and 105 DAP. Results on Fig. 4 indicate that treatments mycorrhiza and chemical fertilizers highly and significantly ($p = 0.007$) increased the *V. subterranea* biomass at 30 DAP compared to negative control treatment. However, no significant difference was observed between treatments *Rhizobium* and *Rhizobium*+mycorrhiza for this parameter.

At this stage, inoculation effect seems to be not yet well expressed.

At 60 DAP the plant biomass was consistently greater than that of 30 DAP. The biomass of control plants was significantly ($p = 0.005$) lower than that of other treatments. There was a positive and significant correlation ($r = 0.37$; $p = 0.01$) between the biomass and nodulation, suggesting that the plant biomass is closely dependent on nodulation. In other words, the most efficient are the root nodules, the higher is the nitrogen fixing ability of the host plant, and the greater is the plant biomass.

At harvest (105 DAP), *Rhizobium*, mycorrhiza and the chemical fertilizers consistently ($p < 0.0001$) enhanced the plant biomass more than the control. However, no significant difference was observed between the two biofertilizers and the chemical fertilizers, suggesting that the former can substitute the later in the alleviation of

Fig. 3: Dates to 50% flowering and pods setting

RhMc = *Rhizobium*+mycorrhiza; Rhi = *Rhizobium*; Myc = Mycorrhiza; CFr = Chemical fertilizers; Ctr = Negative control
 Bars of the same parameter with values of the same letter are not significantly different at the level of probability considered

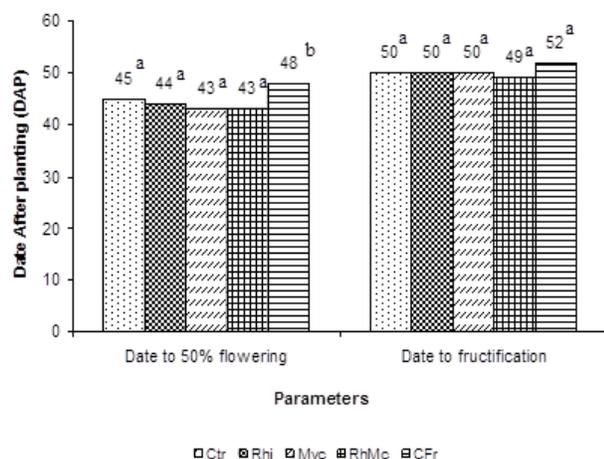
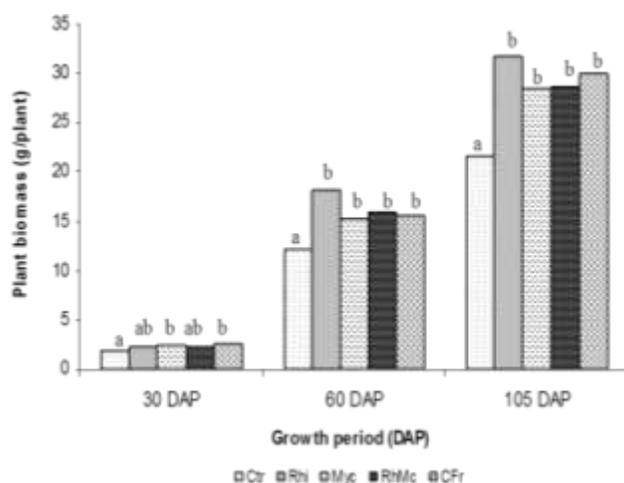


Fig. 4: Plant biomass at different DAP as affected treatments

RhMc = *Rhizobium*+mycorrhiza; Rhi = *Rhizobium*; Myc = Mycorrhiza; CFr = Chemical fertilizers; Ctr = Negative control
 Bars of the same DAP with the same letter are not significantly different at the level of probability considered



environmental pollution risks strategy.

Yield attributes at harvest: Inoculation of *V. subterranea* plants with *Rhizobium* and/or mycorrhiza significantly ($p < 0.0001$) enhanced the number and weight of pods at harvest as compared to the negative control and chemical fertilizer treatments (Table III). The lowest number of pods per plant was produced by controlled plant (26), while the highest accounted for by *Rhizobium*+mycorrhiza inoculated plant (43). The number of pods per plant was significantly ($p < 0.0001$) proportional to the plant biomass.

The number of seeds per plant varied from 22 in uninoculated to 29 in *Rhizobium* inoculated plants, and was consistently ($p < 0.0001$) lower than in other treatments. The

low number of seeds compared to pods per plant can be justified by the fact that some pods apparently well formed had immature or aborted seeds.

The seed weight varied from 18.01 g in uninoculated to 21.03 g in *Rhizobium*+mycorrhiza dual inoculated plants. Inoculation of *V. subterranea* seeds at sowing or application of chemical fertilizers significantly ($p = 0.002$) improved the 100 seed weight compared to the negative control. The lowest seed yield of *V. subterranea* was obtained in uninoculated treatment (524.05 kg/ha), while the highest was that of *Rhizobium*+mycorrhiza treatment (912.15) kg/ha.

DISCUSSION

V. subterranea has been reported to have a best development at between pH 5–6.5 (Yao *et al.*, 2005), in which falls the 5.7 pH of our soil samples. The reduction of organic matter in soil after harvest was similar to report by Lovehand and Webb (2003), in agricultural soil of temperate regions. This reduction in organic matter highlights the fact that the host plant is using it for its growth. The soil acidity attributed to treatments Rhi, RhMc and CFr could be assigned to liberation of non exchangeable cations that were bound to organic matter colloids. In contrast, the little increased pH toward the neutrality in treatment RhMc might suggest the fixation of hydronium ions to colloids following the transformation of non-available to assimilable phosphorus by the host plant (Ndéye, 2002; Nwaga *et al.*, 2003).

After sowing, germination occurred 7 days later in all the treatments. This was within the range of 7–15 days (Swanevelder, 1998), or 7–10 days (Yao *et al.*, 2005) period reported as necessary for germination of bambara groundnut. However, *V. subterranea* seeds in this study were sown 24 h after a heavy rain that might have acted in favour of this seed germination. The reduction of germination rate in inoculated treatments could be attributed to devastating insects such as ants that were found to be attracted by powder milk (Nido) used to adhere *Rhizobium* on seeds (Ngakou, 2007), or to gray worms attack as reported by Brink *et al.* (2006).

A recent report by Brink *et al.* (2006) indicated that flowering in bambara occurs within 30–55 days after sowing. This observation and our findings are different from those made by Ocran *et al.* (1998) that bambara groundnut flowers within 43 days, while Berchie *et al.* (2010) revealed date to 50% flowering at between 39 and 41 DAP. These results suggest that dates to 50% flowering for bambara groundnut significantly vary with the seed variety and the growing area.

Nodule formation on un-inoculated plant root suggests that the experimental soil harbours native strains of *Rhizobium* and mycorrhiza, although they are less competitive than the introduced ones (Ngakou, 2007), but also demonstrates the specific interaction between these microbial symbionts and the host plant. The decrease root

nodule number after flowering at 60 DAP was justified by their deterioration with time after nitrogen fixation. For *V. unguiculata*, roots nodule destruction was reported to start as from 45 DAP (Ngakou, 2007), and at 50 DAP for *V. subterranea* (Ngakou *et al.*, 2009). Roots nodules from un-inoculated plants were not efficient, thus unable to fix biological nitrogen, similar to other findings observed in field grown *V. unguiculata* (Ngakou *et al.*, 2007b). Increased nodule weight after microbial inoculation agreed with other reported results in Senegal on crop legumes (Diagne, 1992). At 60 DAP the increased nodule weight by chemical fertilizer was similar to increment of root nodules after amendment of legumes with 4.8 g/m² of fertilizers (Ndéye, 2002).

The enhanced biomass of inoculated plants has been attributed to increased nutrient uptake in co-inoculated plants by biofertilizers (Smith & Read, 1997; Ngakou *et al.*, 2007a, 2008), or by dissolution and uptake of nutrients provided by chemical fertilizers (Kanabo & Gilkes, 1987). The dependence of biomass on nodulation was the result of a correlation between these two growing parameters, as previously reported by Ngakou *et al.* (2009), from pot experiment on four main grain legumes in Ngaoundere.

As regards yield parameters, low number of seeds/pod compared to that of pods/plant was attributed to seed abortion, reported to occur in *V. subterranea* under severe water stress (Vorasoot *et al.*, 2003). The 100 seed weight of *V. subterranea* was lower than the average seed weight of 28.89 g/plant previously reported (Begemann & Mushonga, 1995), but was also greater than 25.2g and 48.33–55.64 g/100 seeds obtained respectively by Ofori *et al.* (2009), Yao *et al.* (2005) for the same crop, although it was lower than 72–86 g/plant reported by Karikari (2003) in some Bambara groundnut land races. The 100 seed weight may thus vary from one plant species to another, and for the same plant species from one variety to another, and with the growing conditions.

The seed yield obtained from this research was closer to 300–800 kg/ha reported by Brink *et al.* (2006), but was higher than 130–470 kg/ha revealed by Begemann and Mushonga (1995). Higher yields in several inoculated plants have been reported to be attributed to improvement of nutrient uptake by several host plants (Megueni *et al.*, 2006; Ngakou *et al.*, 2006; Ngakou *et al.*, 2007a, 2008).

In conclusion, *Rhizobium*+mycorrhiza improved nodule number and nodulation efficiency by 64 and 80%, the seed weight by 52%, the plant biomass by 30%, as compared to un-inoculated treatment. Nodulation positively stimulated biological nitrogen fixation, and was correlated with the pod and seed yield. This type of fertilization could be recommended to boost the impact of these biofertilizers on the crop productivity and alleviate the long cooking properties of *V. subterranea* seeds. Further research efforts could be undertaken to improve and promote it as a food security crop, especially for drought stress areas such as those of the far northern Cameroon. Moreover, improved

soil properties such as water infiltration, water storage and particle aggregation attributed to crop legume residues decomposition after harvest could be evaluated for the benefit of the next cash crops.

Acknowledgement: The authors wish to acknowledge the Institute for Research and Agricultural Development (IRAD) Wakwa for providing the *V. subterranea* seeds.

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(Received 06 August 2012; Accepted 14 September 2012)