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Resistance to Abiotic Stress and Effectiveness of Native Rhizobia on Bambara Groundnut [*Vigna subterranea* (L.) Verdc.] in Benin

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Abstract

Bambara groundnut [*Vigna subterranea* (L.) Verdc.], as a legume, can establish relationships with nitrogen-fixing bacteria such as Rhizobium. However, Rhizobium efficacy is not always optimal due to the lack or poor efficient strains in the soil. This study aimed to evaluate symbiotic efficiency of endogenous Rhizobia nodulating Bambara groundnut and their resistance to abiotic conditions. Root nodules were randomly sampled from three agroecological zones across the country, surface sterilized, ground and paste plated on YEMA media. After 24 hours, the bacterial colonies were purified. The pure cultures were further characterized using morphological and biochemical methods and their resistance to antibiotics and heavy metals was evaluated. Lastly, the symbiotic efficiency of the isolates was assessed through a greenhouse experiment. A total of eighty-five presumptive strains were isolated from Bambara groundnut roots nodules obtained from the farms. The physiological characterization of the isolated showed a decrease in isolates growth when NaCl concentration was more than 7%. In addition, 47% of the isolates were tolerant to a temperature of 40˚C. Most of the isolates were highly resistant to Erythromycin in all its concentration levels and to Kanamycin, Spectinomycin, Neomycin and Ampicillin at 10 μg·mL^{-1}. Most of them showed resistance to Cu and Zn at 10 μg·mL^{-1}. Results of the effectiveness test on two Bambara groundnut varieties yielded dry shoot matter varying from 3.33 g·plant^{-1} to 7.21 g·plant^{-1} for variety 1 and from 4.38 g·plant^{-1}

to 8.38 g·plant^{-1} on variety 2. N uptake ranged between 0.09 g·plant^{-1} and 0.29 g·plant^{-1} for variety 1 and between 0.12 and 0.29 g·plant^{-1} for variety 2. The isolates yielding higher shoot dry weight and N uptake were LMSEM312, LMSEM338, LMSEM307, LMSEM351 for variety 1 and LMSEM338, LMSEM309, LMSEM307 for variety 2. The isolates showing better performance can be used to develop bio-fertilizer for sustainable Bambara groundnut production in Benin.

Keywords

Indigenous Rhizobia, Nitrogen Fixation, Biodiversity, Bambara Groundnut, West Africa

1. Introduction

Bambara groundnut [*Vigna subterranea* (L.) Verdc.] is one of the indigenous legumes with high nutritional value, grown primarily in smallholder farming systems in Sub-Saharan Africa (SSA) [1]. According to [2], the Bambara groundnut has a high potential of solving food insecurity, malnutrition, and poverty menace in Sub-Saharan Africa. In west Africa, it is an efficient crop in boosting the food system and pastoral systems and land-use sustainability [3]. In most countries in SSA, where food systems depend primarily on high input demanding cereal crops such as maize, rice, sorghum and millet, the improvement in neglected legumes production like Bambara groundnut is necessary for diet diversification and food security. Bambara groundnut has nutritional and nutraceutical benefits, including high-quality and low-cost dietary proteins [4] [5]. Indeed, Bambara groundnut seeds are rich in protein (18% - 24%), oil (4% - 12%) and carbohydrates (51% - 71%) and contain approximately 32% essential amino acids and 67.28% non-essential amino acids per 100 g [6]. As a nitrogen fixing legume, Bambara groundnut improves soil fertility through biological nitrogen fixation (BNF) as well as others soil characteristics such as organic carbon, cations exchange capacity and soil water holding capacity [7]. Moreover, Bambara groundnut is regarded as a "climate change ready crop" due to its high tolerance to drought and its adaptability to arid and semi-arid conditions [8] [9]. Also, it can grow under marginal land conditions where peanut and soybean cannot be cultivated [10]. Bambara groundnut is one of the legumes crops that can develop a diversity of interaction with Gram-negative soil rhizobacteria for biological nitrogen fixation and improve soil fertility and productivity [11] [12]. BNF represents the most renewable and sustainable nitrogen source in agricultural systems, especially in smallholder farming systems where access to mineral fertilizer is still limited and costly. Bambara groundnut can fix approximately 4 to 200 kg N ha^{-1} through a symbiotic relationship with soil bacteria "rhizobia" [13]. Rhizobia is a group of symbiotic endophytic bacteria which fix the atmospheric nitrogen and make it available to the host legume, the subsequent crops

and inter-crop in the cropping system [14] [15].

A successful BNF depends on the availability of highly effective population of rhizobia and favourable abiotic conditions such as soil and climatic characteristics [16]. In previous studies by [17] and [18], Bambara groundnut inoculation with Bradyrhizobium strains significantly increased grain yield and symbiotic N fixation, but their efficacy is not always optimal due to poor efficiency of introduced symbiotic microorganisms. Other previous study reported a failure of BNF or commercial rhizobia related to the incompatibility with agro-environmental conditions or the competition with native rhizobia or other soil microorganisms [19]. Similarly, the native rhizobia population may be present in a low population or are not efficient in N fixation [20]. One way of overcoming these constraints is by applying a significant amount of selected competitive native microorganisms as an inoculant to the crop seedling. According to [21], native rhizobia isolates are effective because they can establish interactions with other soil microorganisms available in the ecosystems and adapt to local edaphic conditions. Native rhizobia strains have been demonstrated to be effective BNF in different legumes species such as soybean, groundnut, and cowpea grown in different agroecosystems in SSA [22] [23] [24]. Despite its importance in smallholders' farming systems, there is little information on the biodiversity of rhizobia nodulating Bambara groundnut in African agrosystems, except for a few studies showing Bambara groundnut nodulation by species in the Bradyrhizobium genus [25] [26]. Previous studies on Bambara groundnut nodulation conducted in west Africa revealed a significant difference in the diversity of native rhizobia isolates [27]. Furthermore, apart from the significant genetic diversity of the isolated strains [28], some strains are more efficient or more competitive than others [27]. In Benin, the crop is still neglected and underused [29] [30]. So far, no research has been conducted on inoculation of Bambara groundnut and the biodiversity of the rhizobia nodulating this crop. Bambara groundnut is still predominantly cultivated extensively at small scale in marginal agricultural systems with low input, resulting in low yield, approximately 650 $kg{\cdot}ha^{-1}$ [31]. Studies were predominantly focused on herbaceous legumes such as peanut and soybean [22] [24] [32]. Therefore, it is imperative to study the biodiversity of indigenous native microsymbionts nodulating Bambara groundnut. So, this study aimed to evaluate symbiotic efficiency of endogenous Rhizobia nodulating Bambara groundnut and their resistance to abiotic conditions.

2. Material and Method

2.1. Study Area

This study was carried out in three major agroecological zones (AEZ) in Benin (**Figure 1**) during the Bambara groundnut cropping season in 2015 and 2016. These were namely AEZ 5 (Cotton zone of central Benin, AEZ 4 (West Atacora zone) and AEZ 3 (Food crop zone of northern Benin). The agroecological zones are located within the Sudano-Guinean and the Sudanian phytogeographical

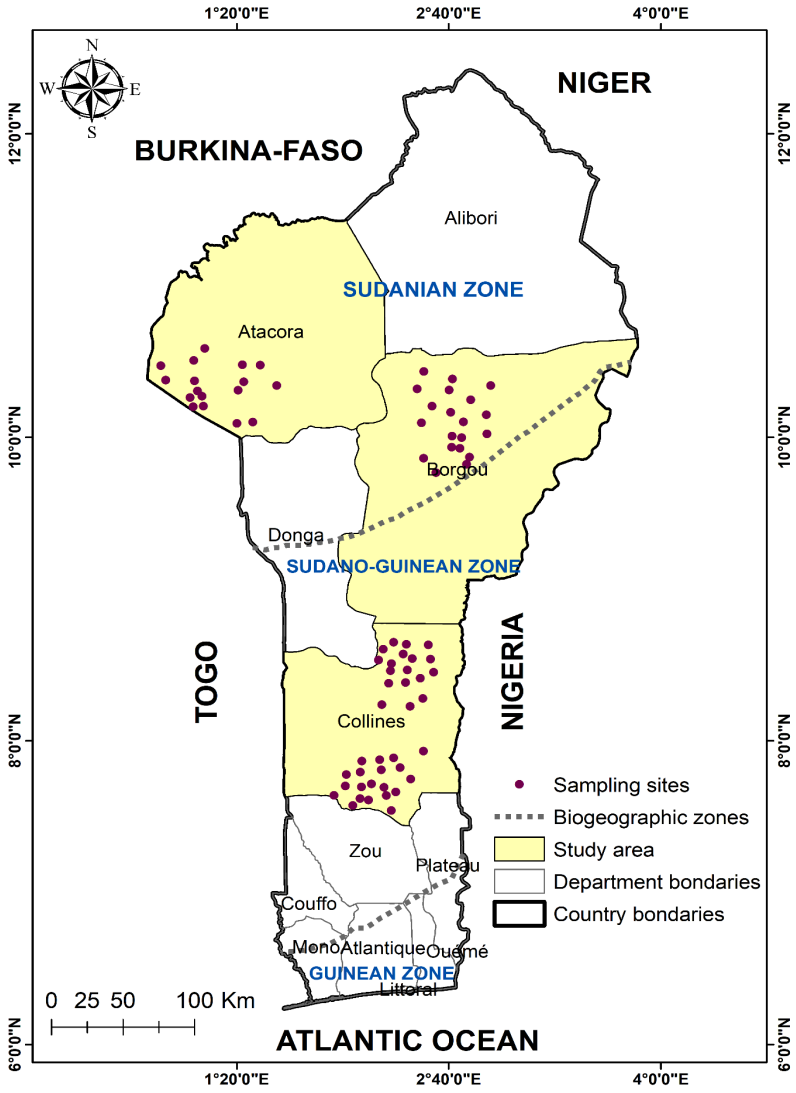

Figure 1. Map showing the prospected agro-ecological zones.

zone of Benin [33]. These regions are characterized by a humidity gradients south-north owing to their climatic and edaphic characteristics [34]. The Sudano-Guinean zone is a transitional ecological zone that extends between the Guinean zone and the Sudanian zone. This zone experienced a trend of unimodal climatic patterns with an annual rainfall ranging from 1100 to 1300 mm and an average temperature ranging from 25˚C to 34˚C. The farming systems are dominated by diverse crops, including cereals (*Zea mays*, *Orizay sp.* and *Sorghum bicolor*), legumes (*Glycine max*, *Vigna unguiculata*, *Vigna subterranean*, *Arachis hypogea*, *Cajanus cajan* and *Macrotyloma geocarpum*) and roots and tubers (*Dioscorea spp.* and *Manihot esculenta*). The main soil types in this zone are Acrisol and Livisol, which are characterized by relatively low pH (around 5.5 - 6), poor clay type (Kaolinite), low soil organic carbon content, low cation exchange capacity, deep profile, moderate water holding capacity and medium to low soil fertility level [35]. In contrast, the Sudanian zone is located in the

northern region of Benin and experiences a primary unimodal climatic pattern characterized by one rainy season with annual average rainfall ranging from 900 mm to 1100 mm and average annual temperature varying from 21˚C to 40˚C. The farming systems are mainly cereal based generally in intercropping and rotation with legumes such as *Vigna unguiculata*, *Vigna subterranea*, *Glycine max* and *Arachis hypogea*. The dominant soil type in this zone is Luvisol with medium to low soil fertility that is caused by poor clay type and low soil organic carbon content. Despite their low soil fertility, the selected AEZ are suitable for Bambara groundnut and have environmental conditions favourable for intensive proliferation of a wide range of microorganisms, including rhizobia [36] [37]. In Benin, Bambara groundnut remains a neglected and underused crop [31] and characterized by low yield [29]. Bambara groundnut is grown on 0.65% of the cultivated land in Benin for an annual production of 11,251 tons in 2011 [38]. The crop is grown in the Sudanian and the Sudano-guinean zones and is not produced in the Guinean zone because of opposing cultural beliefs and the climatic conditions which are not favourable [31]. The Bambara groundnut production is high on light-textured and well-drained soils. However, heavy texture soils with high humidity affect the roots and make the pods go rotten.

2.2. Bioprospecting in Farmer's Field for Collecting Bambara Groundnut Nodules

2.2.1. Bambara Groundnut Sampling

Local agricultural officers in the twelve department of Benin were preliminary consulted and Bambara groundnut production zones were identified and explored prior to bioprospection. In each of the target zones (AEZ3, AEZ 4 and AEZ 5), 30 farmer's fields were randomly selected. Well-developed nodules were collected from ten Bambara groundnut plants. The zigzag sampling method was used to randomly sample the nodules from the Bambara groundnuts in each field. The sampled nodules were put in test tube containing desiccant material (silica gel) and transported to the laboratory for rhizobia isolation.

2.2.2. Isolation, Growing and Multiplication of Rhizobial Strains

The rhizobia bacteria were isolated using the protocols developed by [39] and [40]. The nodules were put in a Petri dish and immersed in sterilized distilled water to rehydrate for four hours. Nodules were surface sterilized by putting the nodules in 70% (v/v) ethanol for one minute using sterile forceps, followed by three minutes in 3% (v/v) sodium hypochlorite solution. Afterwards, they were carefully rinsed in six changes of sterile distilled water. After rinsing, the nodules were crushed aseptically, and one drop of sterile distilled water was added. Thereafter, a loop full of the crushed nodule was streaked across the Petri dishes containing Yeast Extract Mannitol Agar media supplemented with 0.0025% (w/v) Congo red. The components of YEMA ($g{\cdot}L^{-1}$) were: K_2HPO_4 (0.5), $MgSO_4$ (0.2), NaCl (0.1), Mannitol, Yeast extract, Agar (15). The inoculated Petri dishes were incubated at 28˚C for 2 to 7 days but checked every 24 hours to observe the

growth of the rhizobia and contaminant strains. The obtained isolates were purified by subculturing to obtain pure cultures. Pure isolates were then preserved on YEMA slants containing 0.3% $CaCO_3$ and stored at 4˚C for short-term storage as described by Muletta and Assefa [41] and in glycerol (50% v/v) at −80˚C for long-term storage as indicated by [42]. Then, the pure cultures were subjected to presumptive tests such as gram reaction and Bromothymol blue (BBT) test to detect acid/alkaline production.

2.3. Physiological Characterization of Bambara Groundnut-Nodulating Rhizobia

2.3.1. pH, Salt and Temperature Tolerance

In order to test the ability of the presumptive rhizobia to grow in acid and alkaline media, the isolates were grown on YEMA media and the pH was adjusted to 4.5, 5, 5.5, 6,5, 7.5, 8, 9 and 10 by using sterile HCl or NaOH [43]. Presumptive Rhizobium strains were grown on YEMA medium with varying NaCl concentrations ranging from 0% to 9% to test their resistance to different salt concentrations [43]. The effect of temperature on the isolates was performed with varying degrees of temperatures: 4˚C, 28˚C, 35˚C, 37˚C, 40˚C and 45˚C [44].

2.3.2. Antibiotic and Heavy Metal Resistance of the Isolates

Antibiotic resistance of the isolates was determined using the methods developed by [44]. Selected antibiotics ($\mu g \cdot mL^{-1}$) were filter-sterilized (0.2 µm) and added to YEMA culture medium. These include Erythromycin, Kanamycin, Spectinomycin, Neomycin and Ampicillin at a concentration of 10 $\mu g \cdot mL^{-1}$, 25 $\mu g \cdot mL^{1}$, and 50 $\mu g \cdot mL^{-1}$. Heavy metal resistance was assessed as explained by [45] and [46] with the following heavy metals and concentration: Cu (10, 50, 100 $\mu g \cdot mL^{-1}$); Zn (10, 30, 50 $\mu g \cdot mL^{-1}$); Al (100, 200, 500 $\mu g \cdot mL^{-1}$); Pb (100, 200, 500 $\mu g \cdot mL^{-1}$).

2.4. Screening of Presumptive Rhizobia for Their Symbiotic Efficiency

2.4.1. Authentication of Isolated Rhizobia Strains

The isolates were tested for their ability to nodulate Bambara groundnut in axenic conditions. Experiments were conducted in the greenhouse of the Laboratory of Soil Microbiology and Microbial Ecology of the University of Abomey-Calavi in Benin. The experimental design was a completely randomized design (CRD) with three replication composed of 57 treatments (55 indigenous isolates, non-inoculated plants without mineral Nitrogen (N) and non-inoculated plant with mineral N). The rhizobia isolates were cultivated seven days by inoculating them into 50 mL of YEMA broth as described by [39]. The broth was sealed in 200 mL conical flasks and incubated at 28˚C on a rotary shaker. N-deficient soil was used as substratum and was sampled from the beach near the Atlantic Ocean in Cotonou [11]. The soil was washed with laboratory water, air-dried and autoclaved at 121˚C for one hour. One Bambara groundnut variety

named *Azigokouiwéwékpè* [30] was used as the test crop. Seeds were surface sterilized by immersion in 3% sodium hypochlorite solution for ten minutes and then rinsed with five changes of sterile water. The seeds were pre-germinated into water-agar plates (7%) and incubated at 28˚C for 72 hours until radicles were about 1 cm long. After germination, two seedlings were planted in 250 ml pots containing 200 g of washed and autoclaved soil. At planting, 1 mL of the Rhizobium culture with a density of 10^9 viable rhizobia was dispensed around the pre-germinated seedlings following the experimental design. Seedlings were thinned to one plant per pot seven days after planting. For the mineral nitrogen control, KNO_3 (0.05%) was applied [47]. Every two days, Bambara groundnut plants were watered with 25 ml of a sterile Jensen's nutrient solution (N-free nutrient solution) containing per litre: 0.2 g K_2HPO_4; 0.1 g $FeCl_3{\cdot}6H_2O$; 0.2 g NaCl; 1 g $CaHPO_4$; 2.86 mg H_3BO_3; 0.2 g $MgSO_4{\cdot}7H_2O$; 2.03 mg $MnSO_4{\cdot}4H_2O$; 0.08 mg $CuSO_4{\cdot}5H_2O$; 0.09 mg $Na_2MoO_4{\cdot}H_2O$; and 0.22 g $ZnSO_4{\cdot}7H_2O$.

After eight weeks, nodulation was observed by careful recovery of roots. Shoots were harvested, oven-dried at 60˚C until constant weight and weighed. Nodulation scoring was assessed based on 0 to 5 ranking [48]. The leaf colour (greenness of plants) was qualitatively scored by comparing the plant colour of non-inoculated plant with mineral N and non-inoculated plants without mineral N, which they were assigned the scores of 1 and 3, with 2 being given for a colour in between the controls [49].

2.4.2. Effectiveness of Best Presumptive Rhizobia Strains on Two Bambara Groundnut Varieties

The twenty best performing isolates from the authentication test were selected and used to test their performance on two Bambara groundnut varieties named *Azigokouiwéwé and Azigokouiwéwékpè* [30]. The test soil was collected at "Ferme d'application et de production de la Faculté des Sciences Agronomiques, Université d'Abomey-Calavi" and was air-dried and sieved. This experiment used a similar approach as described above. The experimental units consisted three-litre plastic containers 2 kg of sterilized soil and arranged as a Completely Randomized Design with four replications. Two factors were considered: nitrogen (N) sources with 22 levels (the 20 Rhizobia strains, control with N and control without N) and variety with two levels (*Azigokouiwéwé*: V1; *Azigokouiwéwékpè*: V2). Three pre-germinated seedlings were planted per pot and thinned to one plant per pot seven days after planting. For the mineral nitrogen control, KNO_3 (0.05%) was applied [50]. Pots were watered every two days. After two months, plants were carefully uprooted, nodules observed, shoots, roots and nodules recovered, oven-dried at 70˚C to constant weight and plant biomass recorded.

2.5. Data Analysis

Data were analysed using descriptive and inference statistical methods. After testing normality and variances equality conditions, data recorded in the green-

house experiment were submitted to a two-way Analysis Of Variance (ANOVA) using General Linear Models (GLM) procedure in Statistical Analysis System (SAS) software 9.2 version. The main effects of variety and nitrogen sources were tested and the Least Significant Difference (LSD) test was used for mean separation at 5% threshold.

3. Results

3.1. Isolation, Growing and Multiplication of Rhizobia Strains

Fifty-five root presumptive rhizobia isolates (10 from the AEZ 3, 20 from AEZ 4 and 25 from AEZ 5) were isolated from Bambara groundnut nodules (Table 1). Results of presumptive tests showed that all strains were Gram-negative (Gram-) and fast growers (colonies visible after two to three days of incubation). These isolates showed the capacity to acidify the culture medium after one to three days of incubation at 28˚C. Bromothymol blue (BBT) test Reaction showed that 100% of the isolates were acid producers.

Table 1. Origins and characteristics of presumptive isolates.

Strains	Origins			Growing	BTB reaction	Gram reaction
	AEZ	Township	Villages			
LMSEM301		N'dali	Warikpa	Fast	Yellow	Gram-
LMSEM302		N'dali	Bankou-guéou	Fast	Yellow	Gram-
LMSEM303		N'dali	Tamarou	Fast	Yellow	Gram-
LMSEM304		N'dali	Tamarou	Fast	Yellow	Gram-
LMSEM 305		Bembèrèkè	Pedaro	Fast	Yellow	Gram-
LMSEM306	AEZ 3	Bembèrèkè	Gando	Fast	Yellow	Gram-
LMSEM307		Bembèrèkè	Bembereke centre	Fast	Yellow	Gram-
LMSEM308		Bembèrèkè	Pedaro	Fast	Yellow	Gram-
LMSEM309		Bembèrèkè	Gando	Fast	Yellow	Gram-
LMSEM310		Bembèrèkè	Pedaro	Fast	Yellow	Gram-
LMSEM311		Bembèrèkè	Pedaro	Fast	Yellow	Gram-
LMSEM312		Natitingou	Natitingou	Fast	Yellow	Gram-
LMSEM313		Natitingou	Perma	Fast	Yellow	Gram-
LMSEM314		Natitingou	Natitingou	Fast	Yellow	Gram-
LMSEM315	AEZ 4	Natitingou	Kouba	Fast	Yellow	Gram-
LMSEM316		Natitingou	Perma	Fast	Yellow	Gram-
LMSEM317		Natitingou	Kouba	Fast	Yellow	Gram-
LMSEM318		Natitingou	Natitingou	Fast	Yellow	Gram-
LMSEM319		Natitingou	Natitingou	Fast	Yellow	Gram-

Continued

LMSEM320	Natitingou	Kouba	Fast	Yellow	Gram-
LMSEM321	Natitingou	Perma	Fast	Yellow	Gram-
LMSEM322	Natitingou	Perma	Fast	Yellow	Gram-
LMSEM323	Natitingou	Perma	Fast	Yellow	Gram-
LMSEM324	Natitingou	Natitingou	Fast	Yellow	Gram-
LMSEM325	Boukoumbé	Koumandogou	Fast	Yellow	Gram-
LMSEM326	Boukoumbé	Tassayota	Fast	Yellow	Gram-
LMSEM327	Boukoumbé	Tassayota	Fast	Yellow	Gram-
LMSEM328	Boukoumbé	Koumandogou	Fast	Yellow	Gram-
LMSEM329	Boukoumbé	Koumangou	Fast	Yellow	Gram-
LMSEM330	Boukoumbé	Koumangou	Fast	Yellow	Gram-
LMSEM331	Boukoumbé	Koumandogou	Fast	Yellow	Gram-
LMSEM332	Ouèssè	Odougba	Fast	Yellow	Gram-
LMSEM333	Ouèssè	Wokpa	Fast	Yellow	Gram-
LMSEM334	Ouèssè	Odougba	Fast	Yellow	Gram-
LMSEM335	Ouèssè	Odougba	Fast	Yellow	Gram-
LMSEM336	Ouèssè	Wokpa	Fast	Yellow	Gram-
LMSEM337	Ouèssè	Dokoundoho	Fast	Yellow	Gram-
LMSEM338	Ouèssè	Odougba	Fast	Yellow	Gram-
LMSEM339	Ouèssè	Wokpa	Fast	Yellow	Gram-
LMSEM340	Ouèssè	Wokpa	Fast	Yellow	Gram-
LMSEM341	Ouèssè	Odougba	Fast	Yellow	Gram-
LMSEM342	Ouèssè	Wokpa	Fast	Yellow	Gram-
LMSEM343	Ouèssè	Dokoundoho	Fast	Yellow	Gram-
LMSEM344	AEZ 5 Ouèssè	Wokpa	Fast	Yellow	Gram-
LMSEM345	Ouèssè	Dokoundoho	Fast	Yellow	Gram-
LMSEM346	Ouèssè	Dokoundoho	Fast	Yellow	Gram-
LMSEM347	Ouèssè	Odougba	Fast	Yellow	Gram-
LMSEM348	Dassa	Essekpa	Fast	Yellow	Gram-
LMSEM349	Dassa	Minifi	Fast	Yellow	Gram-
LMSEM350	Dassa	Moudja	Fast	Yellow	Gram-
LMSEM351	Dassa	Moudja	Fast	Yellow	Gram-
LMSEM352	Dassa	Moudja	Fast	Yellow	Gram-
LMSEM353	Dassa	Essekpa	Fast	Yellow	Gram-
LMSEM354	Dassa	Moudja	Fast	Yellow	Gram-
LMSEM355	Dassa	Minifi	Fast	Yellow	Gram-

3.2. Physiological Characterization of Bambara Groundnut-Nodulating Rhizobia

3.2.1. pH Tolerance

All isolates showed a wide range of pH tolerance (Figure 2). All of them grew well at a pH ranging from 6.5 to 7.5. In addition, 42% of the isolates were acid-tolerant exhibiting a marked tolerance at pH 4.5. Also, most of the isolates were tolerant to alkaline pH than acidic pH.

3.2.2. Salt Tolerance

Presumptive test of rhizobia isolates showed significant difference in the Bambra groundnut isolates reaction to various NaCl concentrations (Figure 3). All the isolates grew at a concentration of 0% NaCl. Majority of isolates reported growth on media with varying NaCl concentration ranging from 1% - 7%. This growth decreased with the increase of NaCl concentration. A decrease of isolates growth was observed when NaCl concentration exceeded 7% and. Out of the total isolates only 9% tolerated NaCl concentration of 8% while 5% survived in 9% NaCl concentration.

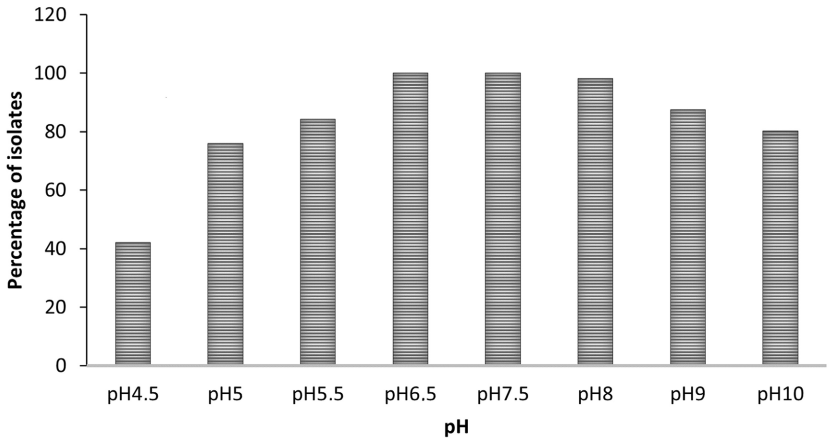

Figure 2. Tolerance of Bambara groundnut strains to different pH levels.

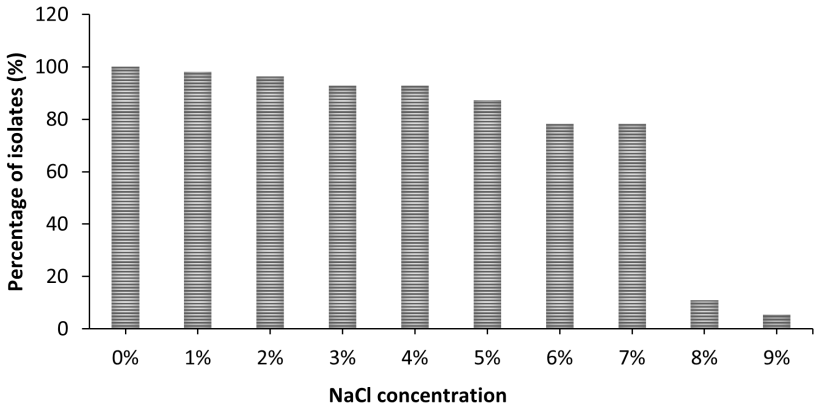

Figure 3. Tolerance of Bambara groundnut strains to different NaCl concentration.

3.2.3. Temperature Tolerance

Generally, in the presumptive all the rhizobia isolates exhibited growth at 28˚C and 35˚C (Figure 4). Most of them also survived at 37˚C. However, none of the isolates survived at 4˚C. Also, 47% of them showed tolerance to a temperature of 40˚C while only 9% could grow at 45˚C.

3.2.4. Antibiotic and Heavy Metal Resistance of the Isolates

Most of the isolates were highly resistant to Erythromycin in all its concentration levels and resistant to Kanamycin, Spectinomycin, Neomycin and Ampicillin at 10 µg·mL^{-1} (Table 2). Besides, 22% of the isolates reported growth in 50 µg·mL^{-1} of Kanamycin, Spectinomycin (18%), Neomycin (37%) and Ampicillin (23%).

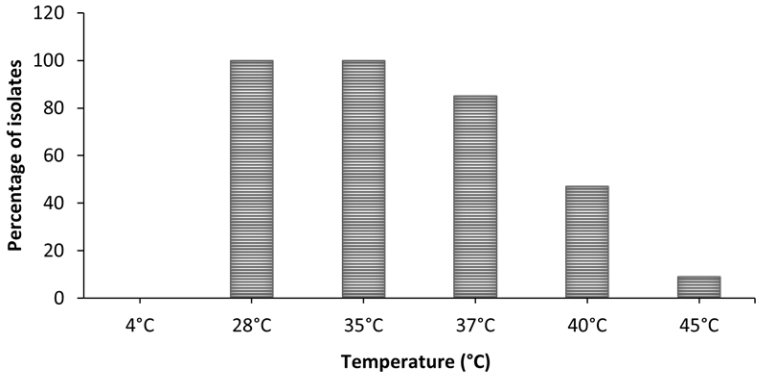

Figure 4. Tolerance of Bambara groundnut strains to different temperatures.

Table 2. Antibiotic and heavy metal resistance of the isolates.

Antibiotics	Proportion of resistant isolates		
	10 µg·mL^{-1}	25 µg·mL^{-1}	50 µg·mL^{-1}
Erythromycin	92	92	88
Kanamycin	55	33	22
Spectinomycin	70	35	18
Neomycin	93	72	37
Ampicillin	85	36	23
Heavy metals	Proportion of resistant isolates		
Cu	10 µg·mL^{-1}	50 µg·mL^{-1}	100 µg·mL^{-1}
	80	45	25
Zn	10 µg·mL^{-1}	30 µg·mL^{-1}	50 µg·mL^{-1}
	100	85	56
Al	100 µg·mL^{-1}	200 µg·mL^{-1}	500 µg·mL^{-1}
	100	100	95
Pb	100 µg·mL^{-1}	200 µg·mL^{-1}	500 µg·mL^{-1}
	100	96	84

On heavy metal resistance, most of the isolated grew well in the presence of Al and Pb in all concentrations. Most of the isolates showed resistant to Cu and Zn at 10 μg·mL^{-1} while only 56% and 25% were resistant to Cu and Zn at a concentration of 50 μg·mL^{-1} and 100 μg·mL^{-1} respectively.

3.3. Screening of Presumptive Rhizobia for Symbiotic Efficiency

3.3.1. Authentication of Isolated Rhizobia Strains

The authentication test revealed that Bambara groundnut nodulated all the presumptive rhizobia isolates. A significant difference was observed between nodule score of plants inoculated with different isolates and non-inoculated controls at $p < 0.001$. The scores for leaf colour showed that plants inoculated with the isolates LMSEM312, LMSEM338, LMSEM318, LMSEM339, LMSEM317 and LMSEM347 improved the plant green colour than the uninoculated without nitrogen control but lower than plus N control. The highest average nodules scores were reported on isolates: LMSEM312 (4.2), LMSEM338 (4.2), LMSEM318 (3.8), LMSEM339 (3.7), LMSEM317 (3.6) and LMSEM347 (3.2).

3.3.2. Effectiveness of Best Presumptive Rhizobia Isolates on Two Bambara Groundnut Varieties

Indigenous rhizobia isolated from Benin soils nodulated the two Bambara groundnut varieties V1 and V2. Low nodulation was observed in plants on nitrogen application (**Figure 5**). There was significantly different in nodules induced by different isolates at $p < 0.001$ with nodules ranging from 38 (control + N) to 120 (strain LMSEM312) on variety V1 and from 29 (control + N) to 124 (strain LMSEM338) on variety V2. Isolates LMSEM312 and LMSEM338 produced the highest nodules number on V1 and V2 varieties. Moreover, there was significant difference in nodule dry weight ($p < 0.01$). The nodule dry weight ranged from 11 mg·plant^{-1} (control + N) to 120.24 mg·plant^{-1} on variety V1 and from 15 mg·plant^{-1} to 124.33 mg·plant^{-1} on variety 2.

Most (above 80%) of the isolates significantly increased Bambara groundnut plant height at ($p < 1‰$) compared to the control without nitrogen. Plant height ranged from 10.87 cm and 10.9 cm (control without nitrogen) to 19.47 (LMSEM312) cm and 20.27 (control + nitrogen) on variety 1 and variety 2 respectively. The highest plant height was reported in plants inoculated with isolates LMSEM339, LMSEM317, LMSEM347, LMSEM318, LMSEM309 on variety 1 and isolates LMSEM 338, LMSEM309, LMSEM335, LMSEM 347, LMSEM339, on variety 2.

A higher number of isolates showed positive effect at $p < 5\%$ and increased number of Bambara groundnut leaves. The leave number ranged from 93 (control without N) to 169 (LMSEM312) on variety 1 and from 113 (control without N) to 169 (control + N) on variety 2 (**Table 3**). The other best isolates for this parameter were LMSEM351, LMSEM338, LMSEM352, LMSEM307, LMSEM309 on variety 1 and LMSEM338, LMSEM326, LMSEM307, LMSEM317, LMSEM335 on variety 2.

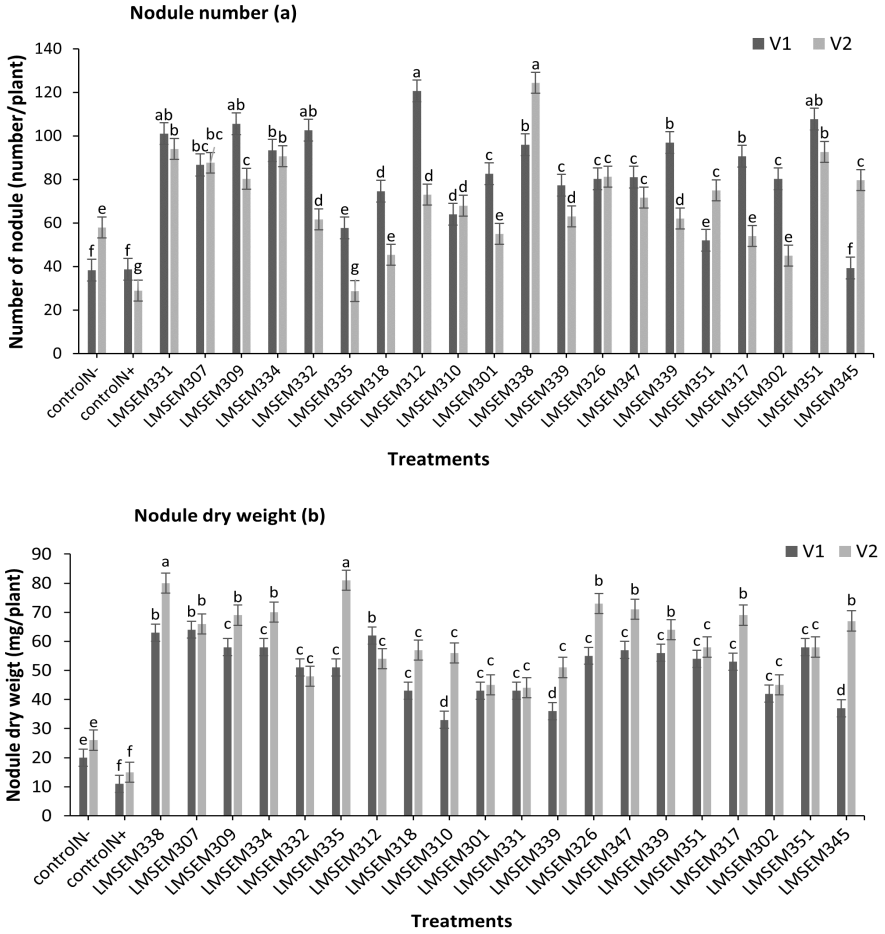

Figure 5. Effect of strains on nodulation of Bambara groundnut: Nodule number (a); Nodule dry weight (b). Bars followed by a same letter in the same graph are not significantly different at $p < 0.05$ according to Student Newman-Keuls test.

Table 3. Effect of isolate on height, number of leaves and root dry weight of Bambara groundnut.

Treatments	Height (cm)		Number of leaves (number·plant^{-1})		Root dry weight (g·plant^{-1})	
	Variety 1	Variety 2	Variety 1	Variety 2	Variety 1	Variety 2
ControlN−	10.87e	10.90e	68.00f	93.00 e	1.13d	1.58d
ControlN+	19.40a	20.27a	143.00b	169.00a	2.75a	2.82a
LMSEM338	18.00a	19.03a	154.00b	162.00a	2.70a	2.70a
LMSEM307	16.20b	15.90b	148.00b	156.00ab	2.47ab	2.60ab
LMSEM309	16.60b	18.20ab	147.00b	147.67b	2.45ab	2.45b
LMSEM334	16.43b	16.17b	129.00c	144.00b	2.15b	2.40b
LMSEM332	16.07b	16.33b	131.33c	108.00d	2.18b	1.80c
LMSEM335	15.50b	18.70ab	145.33b	151.00ab	2.42ab	2.52b
LMSEM312	19.47a	17.17ab	169.33a	148.00b	2.80a	2.47bc
LMSEM318	15.03b	15.23c	102.00d	115.00d	1.70c	1.92c
LMSEM310	13.43d	13.60d	103.00d	108.00d	1.72c	1.80c

Continued

LMSEM301	15.07b	14.17c	95.00e	106.00d	1.58c	1.77c
LMSEM331	14.23c	13.53d	97.00e	113.00d	1.62c	2.07bc
LMSEM340	12.93d	13.60d	93.00e	115.00d	1.55d	2.10bc
LMSEM326	17.13ab	16.83b	113.00d	157.00ab	1.88bc	2.32bc
LMSEM347	17.50ab	17.87ab	130.00c	147.33b	2.17b	2.63b
LMSEM339	18.83a	17.40ab	115.67d	150.00ab	1.93b	2.48bc
LMSEM352	16.43b	15.57b	155.33b	132.33c	2.58a	2.47bc
LMSEM317	17.83ab	17.03ab	126.33c	156.67ab	2.10b	1.63c
LMSEM302	15.33b	12.70d	94.33e	97.00e	2.02b	1.58c
LMSEM351	16.33b	14.17c	165.33a	123.00cd	2.58a	2.05bc
LMSEM345	12.73d	14.67c	107.33e	148.00b	1.78c	2.47bc
Levels of significance	***	***	***	***	**	**
CV	2.1	2.8	13.8	14.4	0.24	0.6

Means followed by a same letter in the same column are not significantly different at $p < 0.05$ according to LSD test. ns: not significant ($p > 5\%$); **: Significant ($p < 1\%$); ***: Significant ($p < 1‰$).

The effect of different isolates on root dry weight of Bambara groundnut were significant at $p < 1\%$ (Table 3). The best values obtained for root dry weight were 2.80 g·plant^{-1} (LMSEM312) on variety 1 and 2.82 g·plant^{-1} (control with N) on variety 2. The lowest values were observed on control without N (1.13 and 1.58 g·plant^{-1} on variety 1 and variety 2). Root dry weight was also higher in isolates LMSEM338, LMSEM351, LMSEM307, LMSEM309 on variety 1 and LMSEM338, LMSEM307, LMSEM339, LMSEM312, 347 on variety 2.

3.3.3. Effect of Different Isolate Strains on Shoot Dry Matter and N Uptake of Bambara Groundnut

The effect of different isolates on shoot dry matter was significant at $p < 5\%$ (Table 4). The shoot dry matter ranged from 3.33 g·plant^{-1} (Control without N) to 7.21 g·plant^{-1} (LMSEM312) on variety 1 and from 4.38 g·plant^{-1} (Control without N) to 8.38 g·plant^{-1} (Control with N) on variety 2. Other strains also indicated high shoot dry matter. There were LMSEM338, LMSEM307, LMSEM351, LMSEM347, LMSEM352 on variety 1 and LMSEM338, LMSEM335, LMSEM326, LMSEM347, LMSEM334 on variety 2.

There was significant difference between isolate on Bambara groundnut N uptake strains ($p < 1\%$). The N uptake ranged from 0.09 (Control without N) and 0.29 (LMSEM312) on variety 1 and between 0.12 (Control without N) and 0.29 g·plant^{-1} (Control with N) on variety 2 (Table 4). Therefore, in a descending order of the isolate N uptake was as follow: LMSEM312 > LMSEM338 > LMSEM307 > LMSEM351 > LMSEM309 > LMSEM334 with variety 1 and LMSEM312 > LMSEM338 > LMSEM309 > LMSEM307 > LMSEM334 > LMSEM326 with variety 2.

Table 4. Effect of isolate on shoot dry weight and N uptake of Bambara groundnut.

Treatments	Shoot dry weight (g·plant^{-1})		N uptake (g·plant^{-1})	
	Variety 1	Variety 2	Variety 1	Variety 2
ControlN−	3.33c	4.38 d	0.09e	0.12d
ControlN+	6.79a	8.38a	0.23a	0.29a
LMSEM338	6.38a	8.05a	0.22a	0.28a
LMSEM307	6.37a	6.58b	0.22a	0.23b
LMSEM309	5.81ab	6.92b	0.20b	0.24b
LMSEM334	5.79ab	7.01ab	0.19c	0.23b
LMSEM332	5.09b	4.83d	0.17cd	0.16c
LMSEM335	5.09b	8.08a	0.18c	0.28a
LMSEM312	7.21a	5.42b	0.29a	0.19c
LMSEM318	4.31bc	5.69b	0.13d	0.17c
LMSEM310	3.73c	5.61b	0.11d	0.17c
LMSEM301	4.29bc	4.45d	0.13d	0.13d
LMSEM331	4.28bc	6.73b	0.13d	0.21b
LMSEM340	3.57c	5.05c	0.10d	0.14d
LMSEM326	5.48b	7.33ab	0.18c	0.23b
LMSEM347	5.72ab	7.06ab	0.19c	0.23b
LMSEM339	5.58b	6.41b	0.18c	0.20b
LMSEM352	5.40b	5.77b	0.19c	0.21b
LMSEM317	5.33b	6.92b	0.17cd	0.23b
LMSEM302	4.27bc	5.10c	0.13d	0.15d
LMSEM351	5.84ab	5.80c	0.21b	0.21b
LMSEM345	4.18bc	4.55d	0.12d	0.13d
P	*	*	**	**
CV	2.01	1.98	0.07	0.09

Means followed by a same letter in the same column are not significantly different at $p < 0.05$ according to LSD test. ns: not significant ($p > 5\%$); *: significant ($p < 5\%$) **: Significant ($p < 1\%$).

4. Discussion

The presumptive rhizobia isolates tested in this study were Gram-negative. They were fast-growing strains (colonies visible after two to three days of incubation) and showed an ability to acidify the culture medium after one to three days of incubation at 28˚C. The ability to change the pH within 24 hours distinguishes fast-growing bacteria from slow-growing bacteria. The fast-growing strains have the ability to modify the colour of BBT culture medium to yellow, while the slow-growing bacteria show an alkaline reaction that changes the medium colour into blue, as indicated by [41] and [51]. This is consistent with previous

studies in Cameroon on Bambara groundnut by [52]. The fast-growing characteristic of these isolates allows the isolates to survive in the soil when they are used in the field as Bambara groundnut inoculant. The morpho-cultural characteristics (Gram- and fast-growing ability) confirmed the standard morpho-cultural characteristics of Rhizobium species as reported by [39] and [40].

All isolates survived at pH ranging from 6.5 to 7.5, confirming optimum pH for the growth of legumes nodulating bacteria. Similar results were observed on chickpeas [53] and peanut [54]. The results also showed that 42% of the isolates were acid-tolerant and most of them were tolerant to alkaline pH more than acidic pH. Therefore, these acidic and alkaline tolerant isolates could be potentially used as inoculants in highly alkaline or acidic conditions. The majority of the presumptive rhizobia isolates survived on NaCl concentrations between 1% and 7%. However, a decrease in isolates growth was observed when NaCl concentration exceeded 7%. In this study, only 9% of the isolates survived in 8% NaCl, while 5% survived in 9% NaCl. Previously, it has been reported that salinity inhibits biological nitrogen fixation by increasing inhibition of oxygen diffusion in the nodules with consequent blocking of nitrogenase activity [55]. Generally, all the presumptive rhizobia exhibited growth at 28˚C and 35˚C. In addition, some of the isolates reported growth at 37˚C, while 47% of the isolates showed tolerance to a temperature of 40˚C. However, 9% of the isolates showed growth à 45˚C. Nevertheless, none of the isolates survived at 4˚C. Temperature adaptation and heat stress in rhizobia have been widely studied, showing that nodulating legume bacteria are mesophilic and grow at different temperatures ranging from 28˚C to 37˚C, with 28˚C as an optimum temperature [54]. The high temperature optima of these isolates may be beneficial for their application in temperature stressed conditions, especially in arid and semi-arid areas where climatic conditions are unstable.

Most of the isolates were highly resistant to Erythromycin in all concentrations, while resistance to Kanamycin, Spectinomycin, Neomycin and Ampicillin was at 10 $\mu g \cdot mL^{-1}$. In addition, 22% of the isolates survived in 50 $\mu g \cdot mL^{-1}$ of Kanamycin, Spectinomycin (18%), Neomycin (37%) and Ampicillin (23%). Antibiotic resistance is a positive and valuable selection marker used to select symbiotically effective legume nodulating bacteria [55]. Besides, the rhizosphere is composed of large populations of antibiotic-producing microorganisms that affect rhizobia isolates [56]. Therefore, antibiotic-resistant isolates of this study will be able to compete with antagonist soil microorganisms producing those antibiotics.

On heavy metal resistance, most isolates survived in different concentrations of Al and Pb. Also, some isolates were resistant to Cu and Zn at 10 $\mu g \cdot mL^{-1}$. However, 25% of the isolates were resistant to Cu at 100 $\mu g \cdot mL^{-1}$, while 56 % of the isolates were resistant to Zn at a concentration of 50 $\mu g \cdot mL^{-1}$. The heavy metal tolerant rhizobia isolates are potential isolates that can be utilized in heavy metal bioremediation. In heavy metal contaminated sites, after the successful establishment of rhizobial symbiosis with the host plant, the heavy metals tend to

be accumulated in the nodules [57] [58]. This could be an alternative and cheap way to eliminate heavy metals from soil and enhance plant and soil health with nitrogen fixation and other plant growth-promoting pathways in any contaminated soil. This can significantly contribute to food and nutritional security [48].

Rhizobia isolates' effectiveness is improved by screening a wide range of rhizobia isolates and acclimatizing these isolates to specific soil environments. The screening test results in this study showed significant variations in nodulation growth parameters, dry matter yield, and nitrogen uptake. The authentication (nodulation test) revealed that all the isolates in this study induced nodules in the roots of Bambara groundnut. These isolates could be confirmed as rhizobia due to their ability to induce nodules on Bambara groundnut root [59]. However, some isolates were more efficient in symbiotic efficiency than others. The highest nodules scores were recorded with LMSEM312, LMSEM338, LMSEM318, LMSEM339, LMSEM317 and LMSEM347. These isolates improved the green colour than the nitrogen un-inoculated control but were lower than the + N control. Some isolates induced higher shoot dry weight than the control + N treatment, confirming that a small amount of N-fertilizer application did not benefit the legumes plants [60]. These findings agree with [49], who reported that N-fertilizer application did not improve the soybean yield in South Kivu soils in DR Congo.

The effectiveness of best presumptive rhizobia isolates tested on two Bambara groundnut varieties revealed that indigenous rhizobia isolated from Benin soils nodulated the two Bambara groundnut varieties; variety 1 (Azigokouiwéwé) and variety 2 (Azigokouiwéwékpè). However, low nodulation was observed in plants where nitrogen was applied. This agrees with [61] and [62] who showed that nitrogen application negatively affects nodulation and results to low nodule and biomass production. Increasing nitrogen in soil decreases nodule formation and the amount of biomass ascribed to existing nodules [63]. In the biological process, increasing the soil nitrogen increases the plant's ability to obtain maximum nitrogen needs through direct soil nitrogen uptake, which is cheaper than nitrogen fixation [64] [65]. Most of the isolates tested significantly promoted growth and yield parameters of the two Bambara groundnut varieties. Also, there was a significant interaction between rhizobial isolates and the tested varieties. This may be due to the host specificity between some rhizobial isolates and the two Bambara groundnut varieties, as demonstrated by [49].

5. Conclusion

This study aimed to evaluate symbiotic efficiency of endogenous Rhizobia nodulating Bambara groundnut and their resistance to abiotic conditions. A bio-prospecting carried out in the different agro-ecological zones of Benin has shown that Bambara groundnut can naturally form nodules on its roots, a sign of an association with bacteria of the genus Rhizobium. Fifty-five root presumptive rhizobia isolates were isolated from Bambara groundnut nodules. These iso-

lates were tolerant to different environmental stresses such as acidity and alkalinity, temperature, heavy metals and antibiotics. In addition, some had higher symbiotic efficiency than others, which resulted in higher nodulation, dry biomass yield and nitrogen uptake. Several indigenous rhizobia isolates, including LMSEM312, LMSEM338, LMSEM307, LMSEM351, LMSEM309, LMSEM334 on variety 1 and LMSEM338, LMSEM309, LMSEM307, LMSEM326 and LMSEM334 demonstrated high symbiotic efficiency. Field evaluation across several agro-ecological zones in small holder farmers are necessary in the development and authentication of commercial inoculants.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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