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Phenotypic and Symbiotic Diversity of Rhizobia Isolated from Root Nodules of Soybean [*Glycine max* (L.) Merrill] in Côte d'Ivoire

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ABSTRACT

Soybean [*Glycine max* (L.) Merrill] is a legume known for nitrogen fixation interacting with efficient *Bradyrhizobium* strains. In Côte d'Ivoire, the *Bradyrhizobium* inoculums used in soybean production is a foreign strain, which requires the search for indigenous strains adapted to Ivorian soils. The present study aimed to isolate and select rhizobial bacteria from soybeans grown on soils sampled from six localities in the central-western region of Côte d'Ivoire. Thirty bacteria were obtained from the nodules of plants grown on soils of two localities that had previously received soybean cultivation (Gonate North and South). These isolates were coded as RSC and identified as being able to genus *Bradyrhizobium* (slow growth) and *Sinorhizobium* (intermediate growth) depending on cultural and morphological characteristics. Except for RSC 327 and RSC 330, all isolates were authenticated and were able to nodulate the host plant in controlled culture. The isolates RSC 309, RSC 310, RSC 312, RSC 323, RSC 324, RSC 325 and RSC 326 were efficient and significantly ($P < 5\%$) increased the number and weight of the nodules, the height and plant biomass. Moreover, these same isolates also showed a high tolerance to salt (NaCl) added in YEM media at a concentration ranging from 4 to 5%. Based on the symbiotic and physiological characteristics, isolates RSC 309, RSC 310, RSC 312, RSC 323, RSC 324 and RSC 325 could be recommended as the native soybean inoculums under soil and climatic conditions of Côte d'Ivoire.

Introduction

Nitrogen is an essential nutrient for plant growth and development. Much of this nitrogen is provided to cropping systems in the form of industrially produced nitrogen fertilizers. However, using of fertilizers has become detrimental for soil fertility and led to worldwide ecological problems as well as affects the human health (Vitousek, 1997; Yusuf *et al.,* 2009). Thus, maintaining and increasing soil fertility through Biological Nitrogen Fixation (BNF) are the major agricultural priorities in many parts of countries development. BNF is the cheapest and ecologic way in which micro-organisms interacting with leguminous plants such as soybean [*Glycine max* (L.) Merrill] fix aerobic nitrogen (Franche *et al.*, 2009).

Soybean is a legume cultivated for seeds which are highly rich in protein (40 %) and oil (20 %) (Nyabyenda, 2005; Cahuzac-Picaud, 2010). Many leguminous crops provide some protein, but soybean is the only available crop that provides an inexpensive and high quality source of protein comparable to meat, poultry and eggs. It also provides useful crop residues for animal feed or left in the field to decompose, thereby increasing the organic matter content of the soil (Mahamood *et al.*, 2009). On a global scale, the average yearly amount of nitrogen fixed by *Bradyrhizobium japonicum* is about 450 kg nitrogen ha^{-1} in a soybean crop, representing about 90 % of the nitrogen requirement (Giller, 2001; Zablotowicz and Reddy, 2004). Maintaining this significant nitrogen input can be important for economically sustainable soybean yields, especially in soils containing low available soil nitrogen (Klubeck *et al.,* 1988; Zablotowicz and Reddy, 2004). This capacity of soybean to fix nitrogen reduces the need to supply crops with synthetic nitrogen fertilizers.

In Côte d'Ivoire, attempts have been made to conduct research on soybean growth and production. However, the studies have focused solely on evaluating the effective compatibility of soybean varieties with the *Bradyrhizobium japonicum* (IRAT FA3) provided by France for two decades for inoculums production (N'Gbesso *et al.,* 2010). Recent results from these studies have shown differences in efficacy between *B. japonicum* strain IRAT FA3 and introduced soybean genotypes in the country (N'Zi *et al.,* 2015; N'Gbesso *et al.,* 2017). In addition, there is little information available on native symbionts of soybeans. Thus, to obtain highly effective symbiotic soybean bacteria, it is necessary to search for native *Bradyrhizobium* in the laboratory and in greenhouse conditions. Hence, the objective of our investigation is to isolate and evaluate the symbiotic efficacy of soybean *Bradyrhizobium* sp from Côte d'Ivoire.

Materials and Methods

The study was conducted on greenhouse in Jean Lorougnon Guédé University (Daloa, Côte d'Ivoire) experimental site (6°54'27.37''N; 6°26'11.26''W) in 2017-2018. The soybean [*Glycine max* (L.) Merrill] cultivar Piramama was used for the isolates trapping and authentication tests.

Soil sampling and Rhizobia trapping

Soil samples were collected from six localities in Daloa area in the fields which soybean and over leguminous were previously growing in the three latest year (Table 1). Sampling was done following the procedure outlined by Barker and Pilbean (2007). Soil samples were randomly collected from several places at 0-20 cm depth from each plot and they were placed in pots (4 Kg/pot) previously disinfected by sodium hypochlorite. Pots were arranged in a completed randomly block design in greenhouse with five repetitions.

Isolation and morphological characterization of soybean Rhizobia

The Rhizobia were isolated from sterile nodules and purified with the protocol using Yeast Extract Mannitol (YEM) agar supplied with 0.02 % Congo red (Vincent, 1970). At the harvest, soybean root nodules were washed first by water and then immersed in 0.1 % acidified $HgCl_2$ for 5 minutes. The nodules were transferred in a beaker containing 10 ml of 95 % ethanol for 2-3 minutes. The nodules were rinsed in 6 changes of sterile water and each nodule was transferred in hemolysis tube containing 1 ml sterilized distilled water. Each nodule was crushed with sterile glass rod. The aliquot of the suspension was transferred on YEM agar plate. The plates were incubated at 28 ºC for 4-7 days. Some characteristics colony, morphology and gram staining properties were observed.

Confirmation of isolated Rhizobia through nodulation test

The nodulation capacity of the bacteria isolated from soybean was confirmed by inoculation tests on sterile sand. Each isolate was grown on YEM liquid medium to exponential phase. Seeds of soybean cultivar Piramama were surface-sterilized as before and sown on plastic pots containing sterile sand. Seven days after sowing, each plant of a pot except the controls was inoculated by 1 ml of broth culture of each isolate. Control pots were included for an unfertilized and an uninoculated negative control (TO) and uninoculated but nitrogen fertilized (0.05 % KNO_3) positive control (TN). Plants were supplied with distilled water every two days, and they were saturated once a week with a nitrogen-free nutrient solution. Furthermore, TN control received weekly 0.05 % (w/v) KNO_3 as nitrogen source.

Plants were harvested 45 day after sowing (DAS), evaluating nodule number, nodule weight, plant height and biomass weight. The experiment was statistically laid out with three replications using randomized black design (Somasegaran and Hoben, 1994).

Physiological characteristic: Salt tolerance

Salt tolerance was determined on YEM agar plates containing from 0 to 10 % (w/v) NaCl concentrations. This test was carried out on YEM agar plates. Petri dishes containing defined medium were subdivided into squares and each square was inoculated with 10 µl of 48 h bacterial YEM broth Konate *et al.,* 2015). After 7 days of incubation at 28 °C, bacterial growth was compared to the controls.

Statistical analysis

The data of measured parameters recorded were pooled together and subjected to statistical analysis using the STATISTICA program (7.1). The strains tolerance was subjected of Chi^2 of Pearson test. Plant growth and nodulation parameters were subjected by analysis of variance. The difference between the treatments means were evaluated at 5 % level of significance using Fisher's LSD test.

Results and Discussion

Soil sampling and Rhizobia trapping

Analysis of the variance revealed a significant difference ($P < 5\%$) between nodulation and soils samples provenance. Indeed, soybean [*Glycine max* (L.) Merrill] cultivar Piramama was able to induce nodule formation only on soils which were previous soybean cultural. The best nodulation was obtained on Gonate SODEFOR soil with an average of 38 nodules per plant (Table 2). However, soils with a history of cowpea, green gram, groundnuts and bean cultural have been unable to promote nodules formation. These observations indicate that soybean can form nitrogen fixing nodules only in symbiosis with a very narrow range of symbionts. These results corroborate those of Karaboneye (2013), Kumar and Reddy (2018). According to those authors, there are very few native rhizobia strains that are compatible with soybean lines in tropical soils. Thus, nodules formation on soybean roots by native rhizobia is closely related to the cultural history of soils, the cultivated variety and compatibility between a variety and the bacterial strain present in the soil (N'Gbesso *et al.*, 2017).

Isolation and morphological characterization of soybean Rhizobia

Thirty (30) isolates bacteria were obtained from the root nodules of soybean grown on soils from various sites of center-west of Côte d'Ivoire (Table 3). Isolates obtained in this study were coded RSC (Rhizobia isolated from Soybean in Côte d'Ivoire) and were generally circular, mucilaginous, pink or white color on YEM agar medium. Microscopic examination revealed that all isolates were rod shaped and gram negative. Similar characteristics were observed by over authors on isolates of soybean root nodules in several parts of the world (Sharma *et al.*, 2010; Jadhav, 2013; Kapembwa *et al.*, 2016). These characteristics have been described by Jordan (1982) as distinctive features of Rhizobia. In addition, colonies diameter and generations times had varied from 1 to 7 mm and 2 to 7 days respectively. Based on these latest, isolates could belong to the genus *Bradyrhizobium* (slow growth) and *Sinorhizobium* (intermediary growth) (Sadowasky *et al.*, 1983; Hossain *et al.*, 2012).

Confirmation and preliminary screening of soybean Rhizobia effectiveness through nodulation test

All isolates bacteria, except RSC 327 and RSC 330 were authenticated as soybean symbiont (Table 3). Therefore, authentic isolates showed a large diversity in their capacity to infect the host plant and to improve plant growth.

Isolates influenced significantly (P <5%) number and weight of nodules. Isolates RSC 325 and RSC 309 produced the highest number of nodules (39 and 42.33 plant^{-1} respectively) (Figure 1). Isolates RSC 323 and RSC 309 induced the highest weight of nodules 423.33 mg and 446.67 mg respectively).

Table.1 Soils sampling origin and cultural history

Sampling area	Geographical Coordinates		Cultural history
Daloa	6°54'31.76''N ; 6°26'14.99''W		greengram
Bribouo	6°52'22.12''N ; 6°30'11.96''W		cowpea
Gbetitapea	6°47'31.35''N ; 6°27'7.84''W		groundnuts
Gonate SODEFOR	6°53'59.14''N ; 6°10'54.89''W		Soybean
Gonate North	6°55'36.59''N; 6°15'31.76''W		Soybean
Tapeguhe	6°57'25.48''N; 6°28'22.69''W		Common bean

Table.2 Nodules means number according to soils origin

Soil sampling sites	Bribouo	Daloa	Gbetitapea	Gonate N	Gonate S	Tapeguhe
Nodules number	0	0	0	07±2	38±4	0

N = North; S = South

Table.3 Effect of native soybean root nodule isolates on nodule number, nodule weight, plant height and biomass of soybean on sand pot

Treatment	Nodule number	nodule weight (mg plant^{-1})	plant height (cm)	Fresh biomass (g plant^{-1})	Dry biomass (g plant^{-1})
RSC 301	10.67f	83.33fg	31.33ab	5.52cde	1.07de
RSC 302	3.33g	53.33g	16.33d	5.15de	1.05e
RSC 303	4g	61g	17.8d	5.43de	1.36cde
RSC 304	5.33g	68.33g	26.67c	6.13de	1.22de
RSC 305	3.33g	46.67g	26c	4.50de	0.86e
RSC 307	3.67g	51.66g	30bc	6.70bc	1.22de
RSC 308	4g	40g	27.67c	7.06bc	1.25de
RSC 309	42.33a	446.67a	37.23a	7.82ab	2.11a
RSC 310	38b	403.33b	32.26ab	7.07bc	1.88b
RSC 312	27d	380c	27.80c	6.32cd	1.48cd
RSC 313	10f	86.67fg	28bc	5.12de	1.05e
RSC 314	7.33fg	81.67fg	29bc	6.52c	1.33cde
RSC 315	6.67fg	66.67g	29.67bc	6.77bc	1.33cde
RSC 316	3.33g	53.33g	27c	5.47de	1.21de
RSC 317	3.67g	58.33g	27.5c	5.77cd	1.35cde
RSC 318	20.33e	261.67d	32.33ab	8.2a	2.97a
RSC 319	7.33fg	96.67fg	24.33c	5.55cd	1.22de
RSC 320	6g	70g	26.67c	4.9de	0.92e
RSC 321	4.33g	51.66g	28.73bc	5.24de	1.09de
RSC 322	7.66fg	93.33fg	31ab	6.06cd	1.24de
RSC 323	38b	423.33ab	34.57ab	7.51ab	1.96b
RSC 324	31.67c	390bc	30.67ab	7.50ab	2.01b
RSC 325	39.00b	408.33b	33.67ab	7. 79ab	1.94b
RSC 326	11f	140e	31.1ab	6.13cd	1.77bc
RSC 327	-	-	27.90c	6.03cd	1.35cde
RSC 328	7.33fg	83.33fg	27.80c	5.58cd	1.26de
RSC 329	5.67g	76.67g	28.67bc	6.62c	1.33cde
RSC 330	-	-	27.90c	5.92cd	1.19de
RSC 331	10.66f	113.33ef	29bc	5.72cd	1.07de
TN	-	-	30.50bc	7.07bc	1.73bc
TNN	-	-	25.33c	4.62e	0.86e
LSD (5 %)	**4.33**	**34.33**	**6.58**	**0.8**	**0.42**

In the column, means followed by the same letter did not differ significantly at 5 % level by the LSD test.
TN: uninoculated and fertilized control; T0: uninoculated and unfertilized control.

Table.4 Tolerance of soybean Rhizobia to different concentrations of NaCl

	Salt tolerance									
Isolates	**0 %**	**0.5 %**	**1 %**	**1.5 %**	**2%**	**3%**	**4%**	**5%**	**8%**	**10%**
RSC 301	3+	2+	2+	1+	-	-	-	-	-	-
RSC 302	3+	2+	2+	2+	1+	1+	-	-	-	-
RSC 303	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 304	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 305	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 307	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 308	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 309	3+	2+	2+	2+	1+	1+	1+	-	-	-
RSC 310	3+	2+	2+	2+	1+	1+	1+	1+	1+	1+
RSC 312	3+	2+	2+	2+	1+	1+	1+	1+	-	-
RSC 313	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 314	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 315	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 316	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 317	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 318	3+	2+	2+	2+	1+	1+	-	-	-	-
RSC 319	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 320	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 321	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 322	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 323	3+	2+	2+	2+	1+	1+	1+	1+	-	-
RSC 324	3+	2+	2+	2+	1+	1+	1+	-	-	-
RSC 325	3+	2+	2+	2+	1+	1+	1+	-	-	-
RSC 326	3+	2+	2+	2+	1+	1+	-	-	-	-
RSC 327	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 328	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 329	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 330	3+	2+	2+	2+	1+	-	-	-	-	-
RSC 331	3+	2+	2+	2+	1+	-	-	-	-	-

3+: Very good growth; 2+: Good growth; 1+: moderate growth; -: no growth

Fig.1 Soybean cultivar Piramama plants inoculated by the isolates RSC 309 (A) and RSC 325 (B) and the uninoculated plants (C)

A **B** **C**

Considering the growth of plants, the results revealed that all isolates produced significantly higher biomass and plant height compared to negative control (T0). The higher biomasses were recorded by the isolates RSC 318 (2.97 g) and RSC 309 (2.11 g). The isolates RSC 310, RSC 323, RSC 324 and RSC 326 were found statistically similar and stimulated plant growth than synthetic nitrogen (KNO_3) applied on positive control (TN). The lowest was found in negative control and isolates RSC 305 and RSC 320. These observations are in agreement with previous reports by N'Gbesso *et al.,* (2017) on inoculation of over soybean cultivars with native selected *Bradyrhizobium* strains, which showed increased survival percentage in seedlings and greater biomass production in all inoculated plant. Some researchers attributed the higher nodulation and biomass yields of inoculated plants to high nitrogen fixation incorporated into nitrogen biosynthesis (Sharma *et al.,* 2000; Hossain *et al.,* 2012).

Physiological characteristic: salt tolerance

Isolates exhibited a wide tolerance to salt stress (Table 4). All isolates grew on the YEM agar medium containing up 2 % of NaCl excepted isolate BSC 301. Beyond this concentration, the percentage of tolerant strains decreased rapidly. Only 26 % of strains supported 3 % of salt concentrations. Of these, isolates RSC 309, RSC 324 and RSC 325 tolerated 4 % NaCl while RSC 312 and RSC 323 showed tolerant to 5% NaCl. Isolate RSC 310 exhibited the highest tolerance of salt (10 %). Thus, the salt inhibitory concentrations varied among strains.

This finding corroborated previous reports which stipulated that Rhizobia could grow up to 3 % of salt concentrations (Chen *et al.*, 2000; Raza *et al.*, 2001; El Hilali, 2006). However, over work showed that Rhizobia tolerate low salt concentrations (Zerhari *et al.*, 2000; Maâtallah *et al.*, 2002).

In conclusion, this study clearly reveals that the soils of Côte d'Ivoire are very poor in rhizobia, which nodules of the soybean [Glycine max (L.) Merrill], a legume introduced in 1970. However, the soils that have previously received the cultivation of this legume could generate compatible rhizobia. In fact, thirty rhizobia were isolated from the nodules of the soybean Piramama cultivar in the soils of two localities in Côte d'Ivoire (center-west). More than 93 % of the isolates were authenticated, capable of nodulating the host plant and very close to *Bradyrhizobium* and *Sinorhizobium*. Symbiotic and physiological data showed that isolates RSC309, SRC310, RSC312, RSC323, RSC324 and RSC325 were particularly infectious, efficient and salt-tolerant at 4-5% of NaCl. These six isolates have promising symbiotic and agronomic traits and could be recommended as native inoculants for the biological inoculation of soybeans under soil and climatic conditions in Côte d'Ivoire.

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