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# Isolation and Authentication of Local Rhizobia Nodulating Common Bean (*Phaseolus vulgaris* L.) in Different Agro-Ecological Zones of Côte d'Ivoire

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## Abstract

Legumes such as common bean (*Phaseolus vulgaris* L.) have been introduced into cropping systems for sustainable soil management. Consequently, the loss of fertility of the latter remains a major constraint to bean production because this legume is rarely fertilized, yet it is considered to be a poor nitrogen fixer in the absence of inoculation. To overcome this, this study was undertaken with the objective of seeking efficient local rhizobia in order to propose a bean inoculum formulation. To do this, soil samples taken from twelve localities in the Centre, North and West areas of Côte d'Ivoire were used to trap bean nodulating rhizobia. The ROBA1 bean accession used was sown in pots containing the sampled soils. Seedlings were uprooted at the start of flowering and nodulation was assessed. The isolates obtained were purified and then characterized phenotypically. The infectivity and symbiotic efficacy of these isolates were determined *in vitro* by the authentication test in which the purified isolates were reinoculated to their original host plant. A total of 24 rhizobium isolates were obtained from the soils of six localities. During morphological characterization, the isolates showed typical characteristics of Rhizobium. With the exception of RPC501, RPC505 and RPC522, all isolates were authenticated and able to nodulate the host plant in controlled culture. Isolates RPC502, RPC507, and RPC508 were effective and significantly increased ($P < 0.05$) nodule number and weight, height, and plant biomass. This study has, therefore, revealed the presence of effective local rhizobia in Ivorian soils and capable of nodulating common beans. A genetic characterization of efficient rhizobia identified after experimentation in different environmental

conditions should be considered before being recommended as bean rhizobia inoculant.

## Keywords

Trapping, Native Rhizobia, Nodulation, Common Bean, Côte d'Ivoire

## 1. Introduction

Agriculture occupies a central place in the development and economy of Côte d'Ivoire. It represents 40% of the country's export earnings and generates nearly two-thirds of jobs. It is mainly based on export crops [1] [2]. Today, accelerated urbanization and the explosion of Ivorian demography have led to strong pressure on arable land [3]. To increase agricultural production, methods of natural soil fertilization by fallowing have been abandoned in favor of those based on the use of synthetic fertilizers and pesticides. These chemical inputs applied to the soil are costly and often inappropriate with harmful consequences on the environment and human health [4]. Thus, to sustainably restore soils while fighting food insecurity, the introduction of grain legumes such as dry beans (*Phaseolus vulgaris* L.) into cropping systems has been recommended [5]. Dry beans are a popular legume for their high protein (22.9%) and carbohydrate (60.6%) content. It is an inexpensive protein source in the diet of populations in developing countries [6]. In Côte d'Ivoire, bean production is estimated at 40,322 tons and is a source of income for rural households during lean periods [7]. Dry bean also has the ability to establish symbiotic associations with atmospheric nitrogen-fixing bacteria [8]. This symbiotic association, therefore, represents an economical and ecological way to improve agricultural productivity [9]. In Côte d'Ivoire, dry beans are used empirically by farmers as a crop precedent to fertilize the soil. However, studies have indicated that this legume has the least symbiosis with local strains of rhizobium [10]. In addition, the chemical inputs used in conventional agriculture would constitute a potential threat to the survival and efficiency of bacterial strains [11].

Thus, bean inoculation is necessary in the absence of compatible and efficient rhizobia for nitrogen fixation. Although many studies have been carried out in various countries on the performance of local rhizobia strains on bean productivity, there is little information on the use of local rhizobia strains in Côte d'Ivoire on beans [12] [13]. The present study, therefore, aims to select compatible and competitive local rhizobia for the cultivation of dry beans in Côte d'Ivoire.

## 2. Material and Methods

### 2.1. Experimentation Site

The study was conducted at the Food Crops Research Station of the National Center for Agronomic Research (SRCV/CNRA) in Bouaké located in the center

of Côte d'Ivoire at 7˚46' North latitude, 5˚06' west longitude and 375 m altitude [14].

## 2.2. Plant Material

The plant material consists of a bean accession HARI35/GHA19. It comes from the collection of genetic resources of the Vegetable and Protein Crops Program of the CNRA.

## 2.3. Methods

### 2.3.1. Soil Sampling and Rhizobia Trapping

Trapping rhizobia makes it possible to verify the absence or presence of the latter in a given soil. To do this, soil samples were taken from 12 localities in Côte d'Ivoire (Figure 1). They are: Béoumi, Botro, Bouaké, Bouaflé, Daloa, Djébonoua, Ferkéssédougou, Gagnoa, Korhogo, Sakassou, Sinématiali, and Yamoussoukro. None of the plots sampled had beans as their previous crop.

At each site, five soil samples were taken in a zig-zag auger at a depth of 0 to 20 cm depth to form a composite sample.

The composite soil samples were homogenized and distributed in buckets of 4 L capacity, then three seeds were sown therein. The buckets were then arranged in a block arrangement under a shelter.

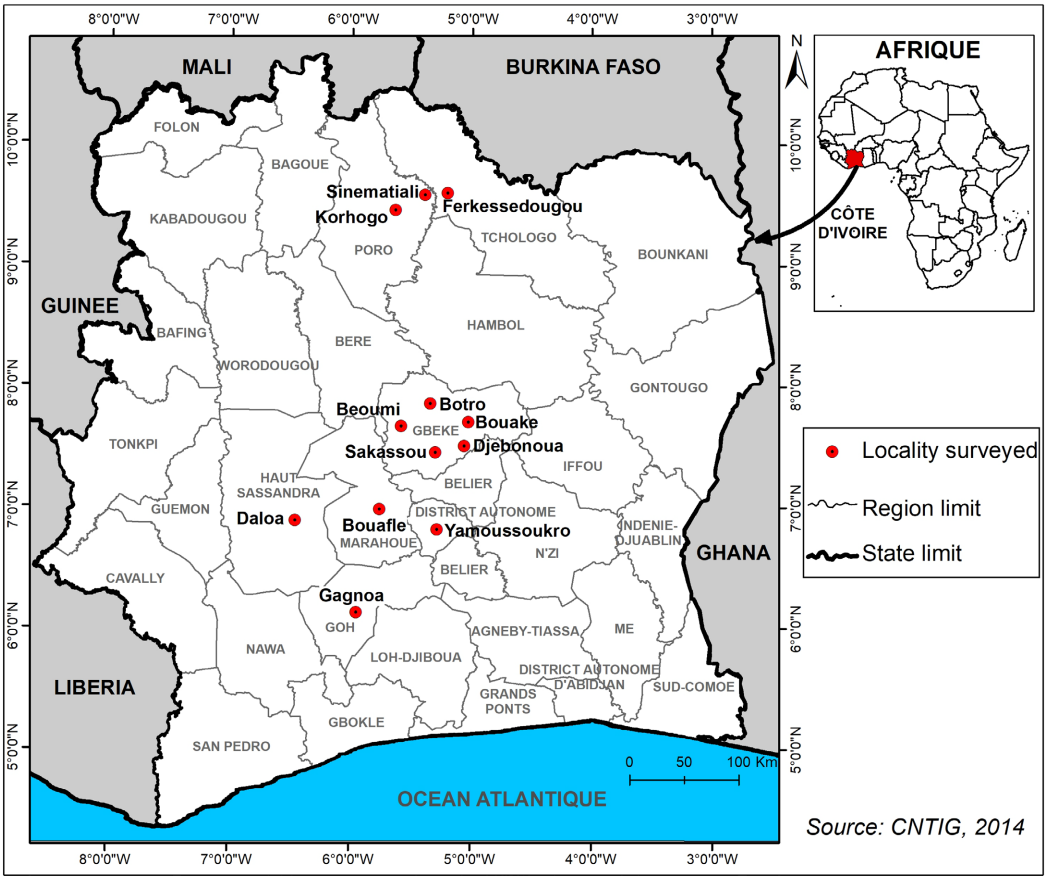


**Figure 1.** Map of agro-ecological sampling zones.

### 2.3.2. Isolation and Purification of Isolates

Rhizobia were isolated from sterile nodules and purified with the protocol using Yeast Extract Mannitol (YEM) agar supplied with 0.02% Congo red [15]. At flowering, the plants were de-potted and then their roots were examined to detect the presence of nodules. These were then detached from the roots to be sterilized on the surface with a 0.1% mercury chloride ($HgCl_2$) solution for 3 minutes. This disinfection was followed by rinsing in 10 successive baths of sterile distilled water. The nodule thus treated was crushed in 1 ml of sterile distilled water using a previously sterilized glass rod. A volume of 0.1 ml or 100 µL of the supernatant from the homogenate was withdrawn aseptically with a sterile pipette and spread using a spreader on the pre-poured and solidified YEM agar. It is composed of Mannitol (10 g/L), Yeast extract (1 g/L), $K_2HPO_4{\cdot}3H_2O$ (0.46 g/L), $KH_2PO_4$ (0.12 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L), NaCl (0.1 g/L) and Agar (14 g/L). The Petri dish was then incubated in an oven at 28˚C.

After three to eight days of incubation, bacterial colonies with a gummy and translucent appearance, representative of rhizobia, were selected to be purified. Thus, the selected colonies were inoculated by transverse streaks on the YEM medium and incubated at 28˚C for 2 to 6 days. The operation was repeated until pure isolate was obtained.

### 2.3.3. Authentication of Isolates

The bacterial colonies obtained after isolation and purification were inoculated into their original host plant in a sterile medium (substrate). This step is necessary to demonstrate that the isolates obtained are capable of inducing nodulation in their host plant. The identification of the isolates was carried out with 3 repetitions per isolate and an uninoculated control to ensure the aseptic conditions of the culture of the plant. The substrate used for this purpose was washed sea sand, sterilized and then transferred to greenhouse pots. Bean seeds were sterilized and sown as previously described.

The bacterial suspension which served as inoculum was obtained by subculturing the bacterial isolates in the YEM medium and incubated at 28˚C for 48 hours. The seeds were inoculated one week after sowing with 1 ml of the broth solution of the inoculum. After sowing, the pots were watered preferably twice with distilled water and once with nutrient solution every week [16].

Three to four weeks after sowing, the plants were unpotted. The formation of root nodules indicates that the isolates are indeed bean nodulating rhizobia. Nodulation parameters and biomass were evaluated.

The pure bacterial colonies were inoculated to their original host plant in a sterile medium (substrate). This step is necessary to demonstrate that the isolates obtained are capable of inducing nodulation in their host plant. The identification of the isolates was carried out with 3 repetitions per isolate and an uninoculated control to ensure the aseptic conditions of the culture of the plant. The substrate used for this purpose was washed and sterilized sea sand then transferred to greenhouse pots. Bean seeds were sterilized and sown as previously de-

scribed. After sowing, the pots were watered preferably twice with distilled water and once with nutrient solution every week [16]. Upon seed emergence, the seedlings were inoculated with a bacterial suspension at the rate of 1 ml of the inoculum broth solution per seedling. This suspension was obtained by subculturing the bacterial isolates in YEM medium and incubated at 28˚C for 48 hours.

Three to four weeks after sowing, the plants were unpotted. The formation of root nodules indicates that the isolates are indeed bean nodulating rhizobia. Nodulation parameters (number and weight of nodules) and biomass (fresh and dry biomass) as well as seedling size were evaluated.

### 2.4. Statistical Analyses of Data

All data were entered with Excel 2007 spreadsheet and analyzed using Statistica 7.1 software. The comparison of the means was made by an Analysis of Variance (ANOVA) at the probability threshold of 5%. When a significant difference was found between the treatments for a given parameter, Fisher's Least Significant Difference (LSD) test was performed at the 5% level to separate the means. All these analyses have been carried out.

## 3. Results and Discussion

### 3.1. Results

#### 3.1.1. Assessment of the Rhizobial Potential of the Soils of the Côte d'Ivoire

The presence of symbiotic bacteria in the sampled soils, evidenced by the presence of nodules (**Figure 2**) on the roots of the bean, was observed in the plants tested. Indeed, plants grown on trapping soil formed functional nodules of red color (**Figure 2**) with local rhizobia.


**Figure 2.** Nodules induced on roots of common bean by local rhizobia. (1) Roots of bean plants showing bean nodules; (2) Longitudinal section of nodules: (a) Non-functional green nodule, (b) Nodule functional red nodule.

The analysis of variance revealed a significant difference ($P < 0.05$) between the nodulation and the origin of the soils (Table 1). The accession tested produced nodules in the soils of the localities of: Botro, Djebonoua, Ferkéssédougou, Gagnoa, Sakassou and Yamoussoukro. The number of nodules obtained with soils from Sakasou (31.17 nodules formed) was greater than those of other towns. However, no nodules were observed on the roots of plants from the soils of Béoumi, Bouaflé, Bouaké, Daloa, Korhogo and Sinématiali.

### 3.1.2. Isolation and Morphological Characterization of Bean Rhizobia

24 bacterial isolates were obtained from the nodules from the soil samples. These isolates presented various morphological aspects representative of bacteria belonging to the genus Rhizobiaceae (Figure 3). Indeed, the latter were composed of colonies of circular, convex or flat shape with a regular outline with a smooth transparent, gummy, translucent or opaque surface. They were whitish in color with sizes varying from 2 to 7 mm in diameter. In addition, all bacteria were found to be Gram negative.

### 3.1.3. Authentication of Bean Bacterial Isolates

The bacterial isolates obtained were authenticated on the basis of infectivity, *i.e.* the presence or absence of nodules on the roots of their host plant. The isolates were coded RPC (Rhizobia isolated from *Phaseolus vulgaris* in Côte d'Ivoire) followed by a number.

Of 24 bean bacterial isolates obtained from different soils in Côte d'Ivoire, 21 (representing a rate of 87.5%) induced nodulation of the roots of their host plant (Table 2). RPC501, RPC505, RPC522 and the non-inoculated control did not give nodules on the roots of the host plant. The isolates significantly influenced

**Table 1.** Average number of nodules per plant on the HARI35/GHA19 accession tested.

| Sampling site | Average number of nodules per plant |
|---|---|
| Béoumi | 0 |
| Botro | 14.50 ± 8.2 |
| Bouaflé | 0 |
| Bouaké | 0 |
| Daloa | 0 |
| Djébonoua | 27.50 ± 6.6 |
| Ferkéssédougou | 9.67 ± 12.6 |
| Gagnoa | 11.17 ± 19.3 |
| Korhogo | 0 |
| Sakassou | 31.17 ± 10.2 |
| Sinématiali | 0 |
| Yamoussoukro | 13.33 ± 9.3 |
| Mean | 8.95 |

**Figure 3.** Bacterial colonies obtained after purification of the isolates.

**Table 2.** Authentication of isolates and their effect on the number and weight of nodules in the HARI35/GHA19 accession.

| Isolates | Presence of nodules | Number of nodules | Nodule weight (g) |
|---|---|---|---|
| Witness | − | 0 | 0 |
| RPC501 | − | 0 | 0 |
| RPC502 | + | 9 ± 2.6defghi | 0.26 ± 0.1abc |
| RPC503 | + | 7 ± 2.6fghij | 0.05 ± 0.01bc |
| RPC504 | + | 17 ± 2.6abc | 0.28 ± 0.2abc |
| RPC505 | − | 0 | 0 |
| RPC506 | + | 2.7 ± 1.2ijk | 0.26 ± 0.1abc |
| RPC507 | + | 18 ± 2ab | 0.36 ± 0.1ab |
| RPC508 | + | 13.3 ± 2.3bcdef | 0.28 ± 0.1abc |
| RPC509 | + | 18 ± 1.7ab | 0.41 ± 0.2a |
| RPC510 | + | 7.7 ± 1.5fghi | 0.27 ± 0.1abc |
| RPC511 | + | 0.7 ± 0.6jk | 0.26 ± 0.1abc |
| RPC512 | + | 8.7 ± 0.6efghi | 0.27 ± 0.1abc |
| RPC513 | + | 15 ± 3.6bcde | 0.5 ± 0.2a |
| RPC514 | + | 9 ± 3.5defghi | 0.26 ± 0.1abc |
| RPC515 | + | 4 ± 1.7hijk | 0.26 ± 0.1abc |
| RPC516 | + | 6.7 ± 1.5ghij | 0.26 ± 0.1abc |
| RPC517 | + | 15.3 ± 1.5bcd | 0.28 ± 0.01abc |
| RPC518 | + | 10.7 ± 2.1cdefg | 0.26 ± 0.1abc |
| RPC519 | + | 10.7 ± 1.5cdefg | 0.27 ± 0.01abc |
| RPC520 | + | 10 ± 3.0defgh | 0.26 ± 0.1abc |
| RPC521 | + | 23 ± 3.6a | 0.54 ± 0.1a |
| RPC522 | − | 0 | 0 |
| RPC523 | + | 5.7 ± 1.5ghijk | 0.25 ± 0.01abc |
| RPC524 | + | 7 ± 2ghij | 0.26 ± 0.1abc |
| Mean | | 8.76 | 0.25 |
| Probability | | <0.05 | <0.05 |

Note: +: Presence of nodule; −: Absence of nodule. In each column, the means followed by the same letter are not significantly different at the 5% level according to Fisher's LSD test; T0: Control not inoculated.

($P < 0.05$) the number and weight of nodules (Table 2). In fact, the RPC521 isolate induced the formation of the greatest number of nodules (23 nodules). With regard to the weight of the nodules, the largest were observed in the RPC509, RPC513 and RPC521 isolates with values ranging from from 0.41 to 0.54 g.

### 3.1.4. Evaluation of the Efficacy of Indigenous Bean Isolates

The 21 authenticated rhizobium isolates were selected for their ability to induce high biomass.

Bean inoculation with various rhizobium isolates significantly influenced fresh/dry biomass as well as seedling height compared to the uninoculated control (Table 3). Fresh biomass values varied from 1 g with isolate RPC502 to 5.16 g with isolate RPC508. As for the dry biomass, the largest material (0.74 g) was obtained with isolate RPC507. Regarding the height of bean seedlings, it varied between 9.7 cm with RPC514 and 37.6 cm with RPC507.

**Table 3.** Impact of local rhizobia on fresh/dry biomass and bean plant height.

| Isolates | Fresh biomass (g) | Dry biomass (g) | Plant height (cm) |
|---|---|---|---|
| Witness | 2.06 ± 0.7ab | 0.33 ± 0.2ab | 14.7 ± 1.3hijk |
| RPC502 | 1 ± 0.1b | 0.32 ± 0.1ab | 19.0 ± 4.9efghi |
| RPC503 | 1.56 ± 1ab | 0.40 ± 0.3ab | 19.9 ± 0.9efghi |
| RPC504 | 2.55 ± 0.7ab | 0.50 ± 0.2ab | 20.3 ± 1.7defghi |
| RPC506 | 1.58 ± 0.2ab | 0.50 ± 0.1ab | 14.5 ± 1.3ijk |
| RPC507 | 4.42 ± 2.3ab | 0.53 ± 0.1ab | 37.6 ± 3.7a |
| RPC508 | 5.16 ± 0.6a | 0.74 ± 0.3a | 27.3 ± 2.5bc |
| RPC509 | 4.44 ± 2.2ab | 0.50 ± 0.2ab | 25 ± 1.2bcde |
| RPC510 | 2.16 ± 1.6ab | 0.23 ± 0.1b | 19.7 ± 1.6efghi |
| RPC511 | 2.17 ± 1.7ab | 0.43 ± 0.3ab | 14.7 ± 0.6hijk |
| RPC512 | 1.58 ± 1.4ab | 0.40 ± 0.2ab | 25.5 ± 0.5bcde |
| RPC513 | 3.49 ± 1ab | 0.50 ± 0.2ab | 26.9 ± 1.9bcd |
| RPC514 | 2.55 ± 1.2ab | 0.60 ± 0.1ab | 9.7 ± 1.6k |
| RPC515 | 2.5 ± 0.9ab | 0.23 ± 0.1b | 17.8 ± 2.7fghij |
| RPC516 | 3.31 ± 0.4ab | 0.51 ± 0.2ab | 20.4 ± 0.9defghi |
| RPC517 | 2.58 ± 0.1ab | 0.41 ± 0.1ab | 19.5 ± 2.1efghi |
| RPC518 | 2.41 ± 0.7ab | 0.40 ± 0.1ab | 23 ± 1.4bcdef |
| RPC519 | 3.31 ± 1ab | 0.50 ± 0.2ab | 22.6 ± 3.6bcdefg |
| RPC520 | 3.25 ± 0.9ab | 0.20 ± 0.1b | 23.3 ± 2bcdef |
| RPC521 | 4.03 ± 1.8ab | 0.40 ± 0.1ab | 29 ± 1.7b |
| RPC523 | 2.28 ± 1ab | 0.40 ± 0.1ab | 21.7 ± 1.6cdefg |
| RPC524 | 2.36 ± 0.3ab | 0.50 ± 0.1ab | 21.4 ± 3.3cdefgh |
| Mean | 2.65 | 0.43 | 20.82 |
| Probability | <0.05 | 0.01 | <0.05 |

Note: In each column, the means followed by the same letter are not significantly different at the 5% level according to Fisher's LSD test; T0: Control not inoculated.

### 3.2. Discussion

In this study, the results show the presence of local rhizobia that nodulate common bean in Ivorian soils without a history of common bean cultivation or inoculation. Kawaka *et al.* (2014) [12] in Kenya also demonstrated the presence of local rhizobia with no history of inoculation that can nodulate and fix nitrogen with the bean. These results show that the soils of Côte d'Ivoire in particular and of Africa in general naturally possess bean rhizobia. However, the seedlings from the soils of six sampled localities (Béoumi, Bouaflé, Bouaké, Daloa, Korhogo and Sinématiali) were free of nodules. These observations indicate that the Ivorian soils are poor in rhizobia capable of nodulating beans. Similar results were obtained by Elbana *et al.* (2009) [17] in Egypt. According to these authors, Egyptian soils do not have a sufficient number of Rhizobium spp compatible with beans. Indeed, the bean is not a plant native to Africa but rather to Central and South America [18]. This reason could explain this weak nodulation outside its original areas. Furthermore, it could also be due to biotic and abiotic factors such as high nitrogen availability, low phosphorus availability, soil salinity or micronutrient deficiency as well as competition from rhizobium strains and/or predation of rhizobia by protozoa or phages. The nodules collected were red in color indicating the presence of leghemoglobin, a protein essential for the nitrogen fixation process [19]. These nodules are known to contain and actively express the *nifH genes* which code for the synthesis of nitrogenase enzymes responsible for the reduction of N to NH3 [20].

The morphological characteristics of the isolates based on Gram stain results and growth on YEMA medium are typical of rhizobia and confirmed the standard morphocultural characteristics of Rhizobium species as described by Vincent, 1970 [15]. The morphological characteristics of the isolates in the present study are similar to those reported by Kawaka *et al.* (2014) [13]. Previous studies have shown that the bean nodulates mainly with fast-growing rhizobia grouped under *R. gallicum* and *R. giardini* [21], *R. leguminosarum biovar phaseoli* [22], *R. tropici* [23] and *R. etli* [24]. The bacterial colonies obtained were gummy in appearance, a sign of production of Exopolysaccharides (EPS) which could be an adaptive characteristic providing bacterium with protection against environmental factors such as: temperature, salinity and pH fluctuations in the soil [25]. Rhizobia able to withstand such environmental stresses might be suitable for inoculant development.

However, one of the important characteristics for identifying nodular isolates as rhizobia is to prove their ability to reinfect and form nodules on their host plant under controlled bacteriological conditions [26]. Thus, bacterial isolates isolated from bean nodules were authenticated on the basis of their infectivity. Following this test, 87.5% of the isolates obtained induced nodulation of the roots of their host plant. Isolates capable of reinfecting beans under controlled conditions are then considered bean Rhizobium isolates. These observations corroborate with those of Yadav *et al.* (2018) [13] in India. These authors have authenti-

cated 35 bacterial isolates of green bean capable of reinfecting their original host plant. For isolates RPC501, RPC505 and RPC522 that did not form nodules on the bean roots, several explanations can be given. It may have a deficiency in their invasive competence and requires the presence of helper microorganisms. In addition to the specificity between the two partners (bacteria-plant), several parameters can condition this phenomenon; the concentration of the inoculum, the presence of certain mineral elements, the loss of infectivity of the isolates and the uncontrolled experimental conditions [27]. Furthermore, several investigators have reported that the failure of nodulation and nitrogen fixation of the isolates could be attributed to the possibility of loss of rhizobial plasmids carrying the *nod* and *nif* genes [28] [29].

The absence of nodulation in the non-inoculated control demonstrates that the aseptic conditions were met. However, authentic isolates showed great diversity in their ability to infect the host plant and improve plant growth. They significantly influenced ($P < 0.05$) the number and weight of nodules. Indeed, the average nodulation ranged from 0.7 nodules to 23 nodules, showing the different ability of rhizobium isolates to infect the host plant. Almost all of the inoculated bean seedlings had fresh/dry biomass and higher height compared to the control, indicating that inoculation with native isolates improved plant growth and is, therefore, effective in the fixation of nitrogen. Houssain *et al.* (2012) [30] attributed this improvement in nodulation and biomass of inoculated plants to high nitrogen fixation incorporated in nitrogen biosynthesis. However, these parameters were not directly related to nodule count or nodule dry weight. This is in agreement with previous work, which showed that the number and dry weight of nodules are not appropriate to determine the effectiveness of a rhizobium-legume association [31]. The significant differences observed in fresh/dry biomass and bean seedling height show variability in the nitrogen-fixing capacity of the isolates and are among the essential characteristics to determine their symbiotic efficacy [32].

## 4. Conclusion

Soil samples taken from different localities in the northern, central and western regions of Côte d'Ivoire made it possible to isolate 24 bean bacterial isolates. 87% of these were able to re-infect the host plant. Of these isolates, RPC502, RPC507, and RPC508 were more efficient and effective. The genetic identity of these native rhizobia with promising symbiotic and agronomic traits should be established. Therefore, these should be subjected to further field trials to verify their stability under different environmental conditions before being recommended as a bean rhizobium inoculant.

## Conflicts of Interest

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

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