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PRODUCTION OF BIOFERTILIZERS USING *RHIZOBIUM* ISOLATED FROM *PHASEOLUS VULGARIS* ROOT NODULES

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ABSTRACT: *Rhizobia are special bacteria that can live in the soil or nodules formed on the roots of legumes. Phaseolus vulgaris is an important legume vegetable belonging to the family Fabaceae. Rhizobia are Gram-negative bacteria, aerobic, and non-sporulating which are associated symbiotically with the roots of leguminous plants. Overuse of chemical fertilizer and biodiversity loss are serious problems challenging the sustainable development of modern agriculture. As organic fertilizers are increasingly used in agriculture today, there is an imperative need to preserve the health of humans and the environment. This study was aimed at isolating and characterizing Rhizobium bacteria from the nodules of Phaseolus vulgaris to produce nitrogen biofertilizer that can be used to substitute chemical fertilizer. Specifically, nodules were collected from Phaseolus vulgaris roots at different stages of development in the school garden of the University of Bamenda, sterilized and used to purify and characterise morphologically and microscopically Rhizobium bacteria. These were then used to produce nitrogen biofertilizer using charcoal powder. As a result, morphological characterization of the bacteria isolated from the bean nodules revealed fast-growing bacteria in 72 h in Yeast Extract Mannitol Agar with Congo red medium. They do not absorb Congo Red, are translucent, mucoid, bulging in the Petri Dish and gram-negative which corresponds to Rhizobium bacteria. Only Yeast Extract Mannitol broth showed good multiplication of bacteria for inoculant production. The pH of charcoal powder was 7.9 which is good for rhizobia growth and three concentrations of 225g, 165g and 145g of Rhizobium biofertilizers were produced with a shelf life of six months.*

KEYWORDS: *Phaseolus vulgaris*, Bean, *Rhizobium* bacteria, Biofertilizer.

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INTRODUCTION

Rhizobia are special bacteria that can live in the soil or nodules formed on the roots of legumes (Emane *et al.*, 2019). They are Gram-negative bacteria, aerobic, and non-sporulating. Phylogenetically, they belong to the alpha subdivision of *Proteobacteria* (Helene *et al.*, 2022) that exist in a symbiotic relationship with several grain legumes as a host plant. In this association, the host plant provides the bacteria symbiont with sugars and a protected environment, while the bacteria fix nitrogen from the air and make it available to the plant in the form of ammonia (Ngakou *et al*., 2010). The genus *Rhizobium* was the first described group of these bacteria, and that is why this name has been frequently used for the nitrogen-fixing bacteria of legumes. This group of bacteria are scattered in 18 genera in the Rhizobiaceae (Lindström and Mousavi, 2020). The genus *Rhizobium* accommodates 112 species and represents the largest genus of rhizobia (Ledermann *et al*., 2021; Lajudie *et al*., 2019). Plants usually depend upon combined, or fixed forms of nitrogen, such as ammonia and nitrate because it is unavailable in its most prevalent form as atmospheric nitrogen (Al-Mujahidy *et al*., 2013). Rhizobia invade their host plants via root hair or crack entry infection to enter the root nodule, which the host is constructing for the potentially beneficial microsymbionts (Lindström *et al*., 2015).

In agriculture, 80% of the biologically fixed nitrogen comes from symbiosis involving leguminous plants and bacteria of the family Rhizobiaceae (Datta *et al*., 2015). The family Rhizobiaceae currently involves six genera: *Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium and Bradyrhizobium*, which are collectively referred to as Rhizobia (Gonzalez *et al*., 2015; Bonaldi *et al.*, 2010). *Rhizobium* species have been successfully used worldwide as a biofertilizer facilitating the effective establishment of N_2-fixing symbiotic association with leguminous plants (wang *et al.*, 2018; Remigi *et al.*, 2016). In nature, there is a close relationship between plants and microorganisms in general. Among these relationships, there is a symbiotic between legumes and bacteria (Pradesh *et al*., 2022). In this relationship, bacteria assist the plant by fixing atmospheric nitrogen necessary for their growth and development (Chen *et al*., 2021). The imprudent use of chemical fertilizers, pesticides and fungicides has resulted in the deterioration of soil health (Afkir *et al.*, 2020) and also harmful effects on soil biota (Singh *et al*., 2011). In chemical fertilizers, nitrogen is one of the essential compounds. If nitrogen-fixing bacteria makes nitrogen available to plants why do farmers continue to fertilize their soils with inorganic nitrogen? Is it possible that the quantity of organic nitrogen is sufficient for plant development? What could be the characteristics of these rhizobia bacteria species? Can *Rhizobium* bacteria represent an alternative solution to nitrogen chemical fertilizers in agriculture? It would therefore be necessary to find out if a maximum input of nitrogen-fixing bacteria could remedy the use of organic nitrogen. This study aims to isolate and characterize *Rhizobium* bacteria from the root nodules of *Phaseolus vulgaris* to produce nitrogen biofertilizers that can be used to substitute chemical fertilizers.

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STUDY SITE

This research was carried out in the research farms of the Department of Biology, Higher Teachers Training College (HTTC), The University of Bamenda at the Bambili campus. Bambili is located in the Tubah sub-division, Mezam division of the Northwest region of Cameroon. The town has a total surface area of about 250.69 km^2. It is located between latitudes 5° 60' 0'' and 6° 05' 0'' north of the equator and between longitudes 10° 12' 0'' and 10° 22' 0'' east of Greenwich Meridian. It has a humid tropical climate with an average annual rainfall of about 2200 mm. The temperature is about 20.7°C Bambili has an undulating topography with altitudes varying between about 900 and 2270 m above sea level. The climate is characterized by two distinct seasons: a long-wet season (March to October) with high winds followed by a short dry season (November to March) with high light intensity (Melle *et al*., 2016).

MATERIALS AND METHODS

Sampling period

The samples of beans cultivated without any manure were collected from the research farms.

Figure 1: Beans plant cultivated in the school garden at the University of Bamenda

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COLLECTION AND DESCRIPTION OF NODULES IN DIFFERENT STAGES OF DEVELOPMENT OF ROOT NODULES

After germination, which lasted about 12 days, the growth phase followed, during which the trifoliate leaves developed. The root system was observed with the appearance of very small whitish nodules of spherical shape (branching stage). During the ripening stage when pods and seeds are formed, there is also an increase in the volume and weight of the nodules which become brown. To collect the nodules, the soil was dug about 15 cm around the plant and 20 cm into the soil to remove the plant and its root system; then the entire plant was collected and put in a plastic bag. In the laboratory, the aerial part of the plant was removed and the root part was carefully washed under running water. Nodules were cut at 1-2 mm from the site of attachment and then dried with absorbent paper.

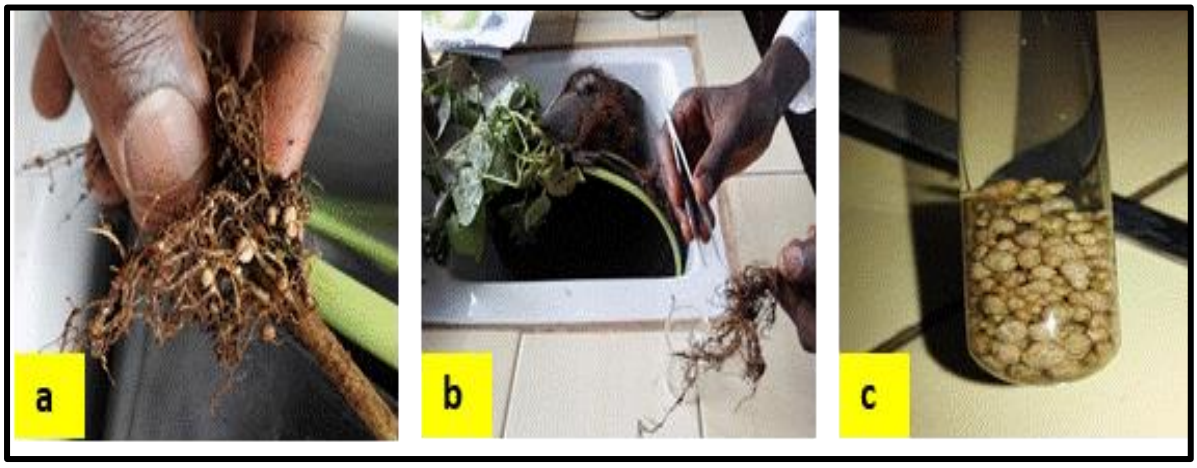
Nodules were collected at different stages of bean development: during the branching stage, flowering stage and fruiting stage. Parameters such as colour, shape, and weight of a defined quantity of nodules were measured each time accompanied by images. These colourations reflect the colour of leghemoglobin which is an indication within a root nodule that Nitrogen-fixation is taking place (Linderman, 2008; Ott, 2005). The nodules on which the bacteria were isolated were collected during the flowering phase of the beans to have a large quantity of mature nodules.

Preparation of Yeast Extract Mannitol Agar (YEMA) medium

After preparation of the YEMA medium, pH was adjusted at 7 using NaoH (4%). The medium was heated with agitation over a Bunsen flame until it completely dissolved before sterilising by autoclaving at 121°C for 15 minutes. After that, the culture medium was distributed in a petri dish (20 ml per Petri Dish) in sterilized conditions.

Preparation of the sample

Root nodules were sterilized in 95% ethanol for 10 seconds and then washed 7 times with sterile distilled water. The nodules located on the roots were spherical (2-4 mm) and pink in colour (fig.2).



a: Nodules; **b**: selection of nodules; **c**: sterilized nodules

Figure 2: Selection and sterilization of nodules roots

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Individual nodules were ground in the laboratory mortar with a few drops of distilled water. Then, the concentration juice was streaked on Yeast Extract Mannitol Agar containing 7ml of Congo red. After incubation for 2-3 days at 30°C, single colonies were selected and retreated on YEM agar for characterization. After isolation and purification, isolates were multiplied in a YEM medium and stored at −20°C until they were used (Paudyal *et al*., 2021; Datta *et al.*, 2015).

Morphological characterization of the different isolated species

The colony morphology of isolates was studied taking into consideration the colour, the size of colonies, the shape, the texture, the opacity, the pigmentation, the elevation and their margins in YEMA + Congo Red medium.

MULTIPLICATION OF *RHIZOBIUM* BACTERIA ISOLATED

Solid medium

To amplify *Rhizobium* bacteria on solid media, YEMA media was prepared, then sterilised by autoclaving at 121°C for 15 minutes. The media was cooled to approximately 42°C, and then almost 20 ml under aseptic conditions was poured into each sterile Petri dish and allowed to gel. A metal loop was used to take a pellet of the stored sample and spread it on the surface of the gelled agar over a small area at the periphery; then the inoculum was finely distributed over the plate by spreading it with a loop in a series of parallel lines in different segments of the plate. The plates were covered and incubated in an inverted position at 38°C for 18-24 hours.

Liquid medium

Two media were used to multiply isolated bacteria: sugarcane concentrated juice and Yeast Extract mannitol broth (YEM).

Yeast Extract mannitol broth (YEM)

After growth in solid YEMA medium, liquid medium (YEM) was prepared and sterilized in an autoclave at 120 °C for 20 minutes. Bacteria were collected and inoculated in a liquid medium in dishes containing 200 ml and incubated at 30°C for 72 hours. A few drops of the medium were taken and spread on the slides. After that, gram staining was done.

Preparation of sugarcane juice

The concentrated juice of sugarcane obtained was filtered through the filter paper to obtain 1000 ml of fresh cane juice, with a pH of 6.38. The content was introduced into an Erlenmeyer flask and 24 h later, the supernatant was poured and the remaining concentrated juice was distributed in 4 glass bottles (200 ml) and sterilized in an autoclave at 120 °C for 15 min. After cooling the *Rhizobium* bacteria were collected and inoculated into the medium and incubated at 30 °C for 24 hours.

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RHIZOBIUM BIOFERTILIZER DEVELOPMENT

Preparation of starter culture

After the multiplication of the bacteria in a liquid medium (YEM and concentrated sugarcane juice), gram staining and optical density measurement were performed to ensure the effectiveness of bacterial growth. Subsequently, only strains multiplied in YEM medium for formulation were used.

PREPARATION OF CARRY MATERIAL

Charcoal purchased at the local market was brought to the biology laboratory and dried in the sun for three days. It was put in a clean bag, crushed and sieved. The resulting powder was also dried for two days in the sun. The charcoal powder (320 g) was then introduced into an Erlenmeyer (1000 ml) flask and autoclaved at 121°C for 25 min, together with the polythene bags prescribed by Hermann *et al*. (2013). The sterile charcoal powder (10 g) was mixed with distilled (20 ml) water and the pH (4.6) was measured using the pH meter. To neutralise the medium, 3 g of calcium carbonate ($CaCO_3$) powder was added to the mixture recommended by Dound and Rathod (2021). The YEM medium (80 ml) containing pure Rhizobium bacteria was mixed with 320g of sterile charcoal powder in the 1000 ml Erlenmeyer flask around the heat and shaken until a homogeneous semi-solid mixture was obtained and incubated at 29 °C for 24h.

PACKAGING

The mixture was immediately introduced into the sterilized polythene bags and the package was sealed immediately to prevent contamination, weighed and stored at a temperature of 4 to 15 °C for a maximum of 6 months. The procedure for field application was developed to be glued to the packaging.

RESULTS

Morphological description of nodules at different stages of development of root nodules

The size of the nodules was very large (0.33g) during the maturation phase (fruiting stage) while it was small during the growth phase when the nitrogen demand of the plant was also low (0.14g in the flowering stage and 0.06 in the branching stage). Table 2 shows the different characteristics of the root nodules collected.

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Table 1: Morphology description of nodules collected in bean plant

	Fruiting stage	Flowering stage	Branching stage
Beans plant			
Nature of nodules			
Colour	Brown	Pinkish	Whitish
Shape	Oval	Spherical	Spherical
Weight (SD)	0.33 ± 0.2 g	0.14 ± 0.3g	0.06 ± 0.1 g

Isolation and morphological identification of bacteria-isolated strains

Morphology Characterization

The strains were found to be fast-growing. All strains were motile and rod-shaped. Colonies on YEM agar were rod-shaped, non-spreading, translucent, convex, smooth, and mucoid in texture (Table 2) after 48 hours of incubation at 29±1°C. These isolates were characterised by non-absorption of Congo red dye up to 24 hours of growth (fig.3)

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Table 2: Morphology characteristic of rhizobia bacteria onYEMA+CR

Isolate name	Colony characteristics on YEMA+CR						
	Shape	Size	texture	opacity	Pigmentation	Margin	Elevation
Red beans	Extended	Medium	mucoid	Translucent	White	undulate	Smooth
White beans	Extended	large	mucoid	Translucent	White	undulate	Raised

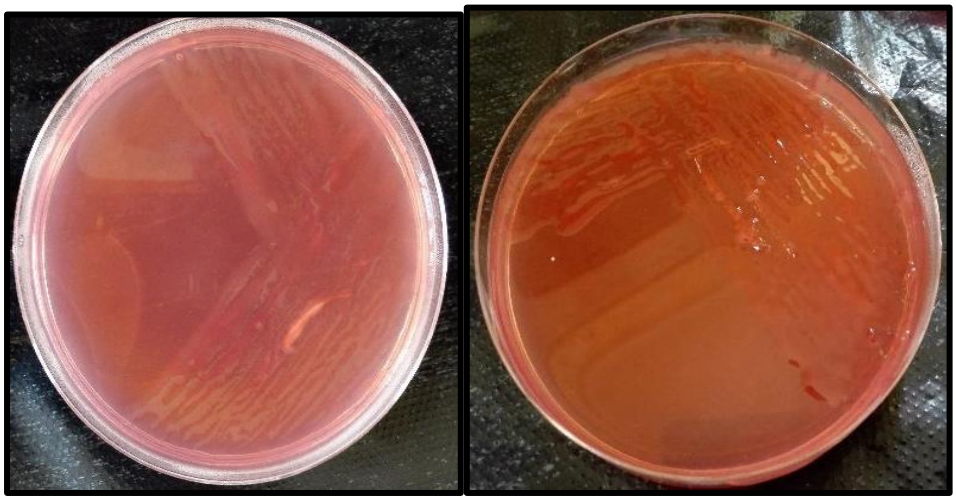

Figure 3: Isolated rhizobacteria on YEMA+CR medium

Multiplication of isolated strains and gram staining test.

After 24-48 hours, the colonies of RB (red beans) and WB (white beans) isolates on the YMA medium had a very large and rapid growth and a domed and smooth surface. They were viscous and shiny, with a homogeneous texture. Colonies were white or beige, circular, convex, semi-translucent, raised and mucilaginous (table 3). Gram staining (fig.4) performed on two different media (liquid and solid) showed that *Rhizobium* bacteria were coloured pink (isolated bacteria are gram-negative).

Table 3: Morphology characteristic of rhizobia bacteria on YEMA medium

Isolate name	Colony characteristics on YEMA						
	Size	Shape	Texture	Opacity	Pigmentation	Margin	Elevation
Red beans	Medium	Medium	Bright	Translucent	White	undulate	Smooth
White beans	Large and circular	Extended	Viscose homogenous	Translucent	White	undulate	Curved and smooth

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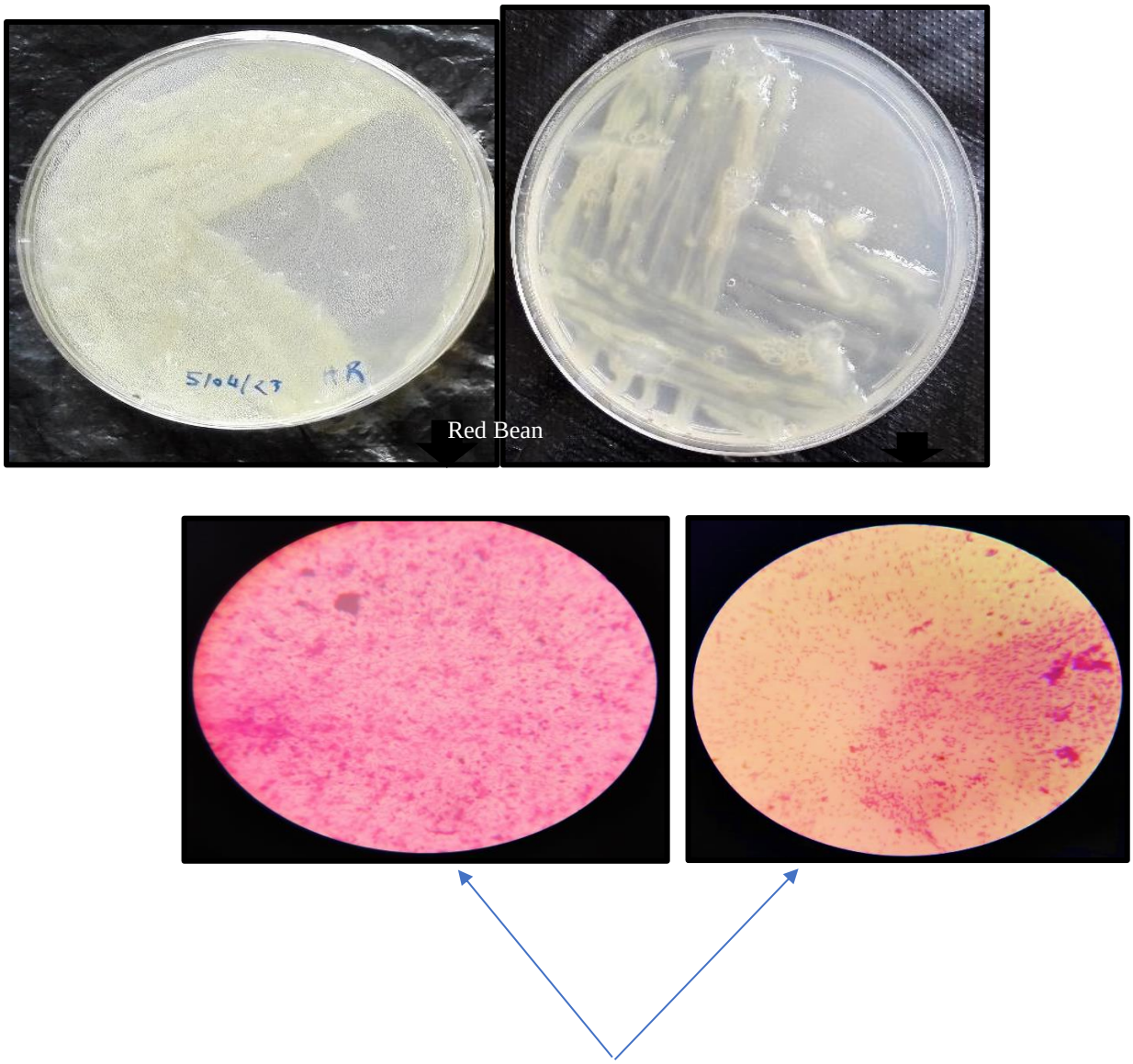

Figure 4: Microscopic observation of isolated bacteria in YEM broth (G x100)

Rhizobium biofertilizer development

Bacterial growth in liquid medium

The optical density was 0.122 and 0.184 respectively for strains isolated from red bean (RB) and white bean (WB) roots in YEM. The measured pH of the inoculum was 7.9.

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Packaging

Three bags of 225g, 165g and 145g of *Rhizobium* biofertilizers were produced. The mixture was stored at a temperature of 4-15 °C for a maximum of 6 months. The instructions for use were given on the leaflet glued to the sealable polythene bags (fig.5a and fig.5b)

Figure 5a: Rhizobium biofertilizers in polythene bags

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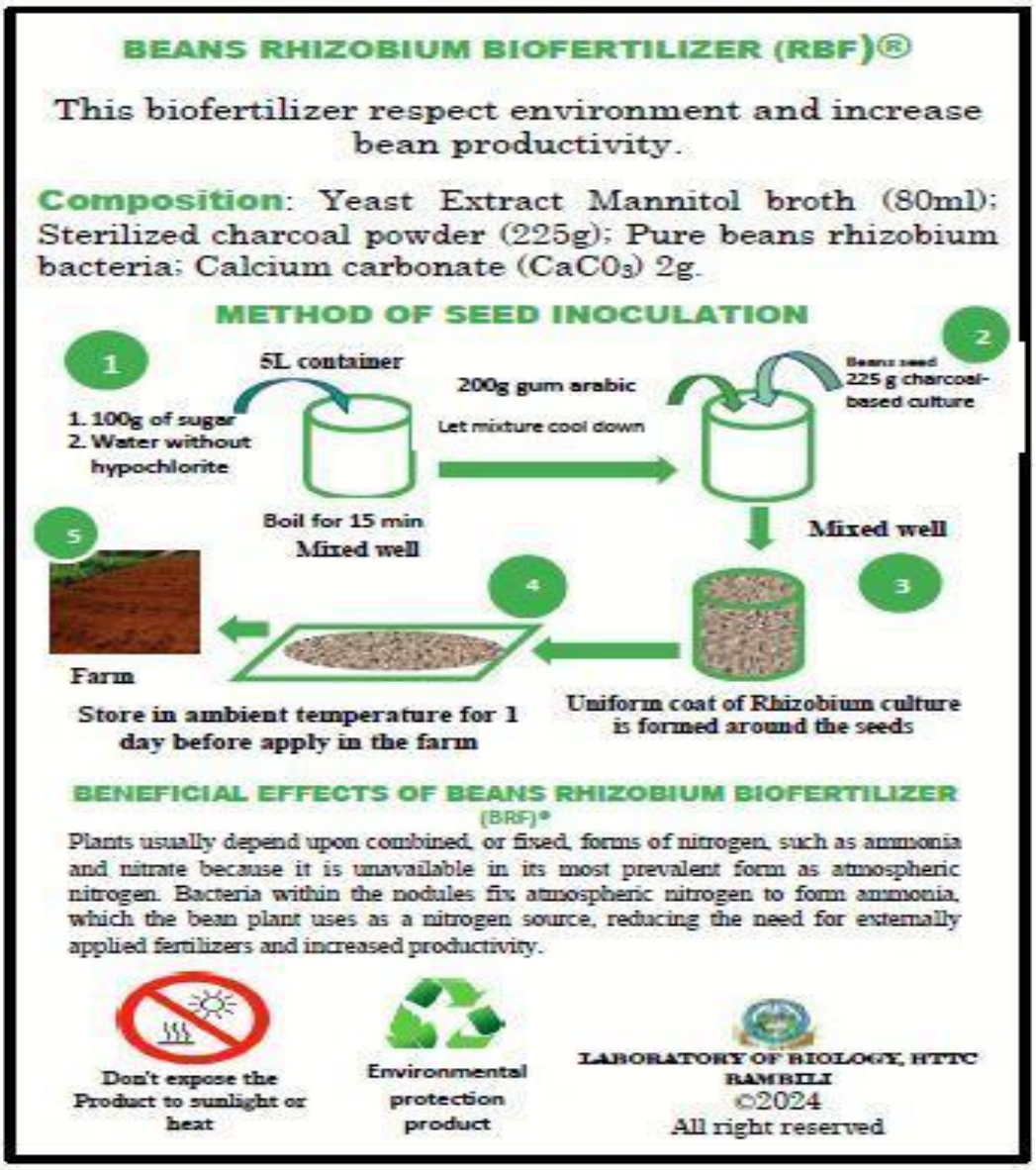

Figure 5 b: instructions for use *of Rhizobium* biofertilizer

DISCUSSION

The results of this study showed that the formation of nodules on the roots of bean plants is essential for their survival. Nodule development is another key component of *Rhizobium (*Herrmann and Lesueur, 2013 *).* Our findings revealed three main periods of bean plant growth after germination, namely the branching stage, flowering stage, and fruiting stage. According to our results, nodule formation starts during the branching stage, characterized by the appearance of leaves on the plant, infection of the roots and the development of small white oval nodules. This is followed by the flowering stage, which is characterized by a differentiation of the different parts of the plant and an increase in the volume of the nodules, which become pinkish and spherical. This could result in an increase in the plant's demand for

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nitrogen. These results are in agreement with those of Daniel *et al*., (2022) who reported that, to accommodate the symbiotic bacteria *Rhizobium*, most bean plants generate root lateral organs de novo, known as "root nodules" (Mazouz, 2018). Symbiotic bacterial infection of the legume plant stimulates the creation of new organs (Yoon *et al*., 2014), such as nodules, by altering the fate of differentiated cortical cells (Wang *et al*., 2018). According to Chen *et al*., (2021), the rate of nitrogen fixation by nodules changes with soybean plant growth. Nodules begin to fix nitrogen at the expression of the leghemoglobin (Lb) gene (branching stage) (Linderman, 2008; Ott *et al.,* 2005); the rate of nitrogen fixation then gradually increases relatively high at the flowering and fruiting stages, and then gradually weakens from the pod stage; and at the harvest stage. The nodules are senescent and almost unable to fix nitrogen (Chen *et al.,* 2021; Walker *et al*., 2015). Biological nitrogen fixation is a critical component of rhizobia activities. Only prokaryotes, which can be symbiotic or free-living in nature, are able to produce the nitrogenase enzyme to fix nitrogen biologically (Berrabah *et al*., 2019).

Morphological characterization of the bacteria isolated from the bean nodules revealed fast-growing bacteria in 72 h in the YEMA+CR medium. They do not absorb Congo Red. This result is in conformity with Tahkoubit (2018) where bacteria belonging to the *Rhizobiaceae* family do not absorb Congo Red. They have the particularity of producing acids in the presence of mannitol as a single source of carbon in the medium. Results showed that bacteria are translucent, mucoid, bulging in the petri dish and gram-negative. According to Bhattacharya *et al*. (2021); Datta *et al.* (2015); and Teresa *et al*. (2021), who did a similar study, this description is consistent with the bacteria of the genus *Rhizobium*. These characteristics were even more visible in the YEM growth medium and fast growth. Results obtained are similar to Küçük *et al*., (2006) which reported that bacteria able to modulate and establish an effective symbiosis with beans are fast-growing.

Results also showed that the process of nitrogen biofertilizer development involves several steps, like isolation and characterization of *Rhizobium* in root nodules of leguminous plants, followed by preparation of starter culture which is also known as mass culture and quality control. This step is followed by the selection and sterilization of appropriate carrier material. The last step of the formulation is the packaging. These results are in line with that of Herrmann *et al*., (2013) who reported that the formulation of an effective inoculant is a multi-step process which results in one or more strains of microorganisms contained in a particular carrier together with sticking agents or other additives which help in the protection of the cells during storage and transport. The values of optical density increased after two days in YEM broth. This means that bacteria were very concentrated in the medium. This result is in conformity with a study which stipulated that the optical density of the solution increased linearly with the number of bacteria (Charles *et al*., 2021). Charcoal powder was used as carry material to produce our nitrogen biofertilizer because of its pH= 7.9 and other particular properties. Analysis of the physico-chemical properties of charcoal has shown that it would be a good substrate for biofertilizers because it contains few quantities of Nitrogen (N) Phosphorous (P), Potassium (K) and good moisture (Lakshmi, 2016). Charcoal also maintains good viability of bacteria for a long period and is locally available at low cost (Paudyal *et al*., 2021; Pradesh *et al*., 2022). However, the choice of the carrier defines the physical form of the inoculant and it is obvious that there cannot be a perfect and universal carrier for all microorganisms (Daniel *et al*., 2022; Mishra *et al.,* 2020). Later, quality control of *Rhizobium* biofertilizer would permit us to know how it can be enhanced in different manners. Finally, biofertilizers provide a number of advantages, including being a low-cost source of nutrients, excellent suppliers of micro-

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compounds and micronutrients, organic matter suppliers, growth hormone producers, and a means of counteracting the negative effects of chemical fertilizers.

CONCLUSION

This study shows that the morphology of the root nodule of a bean begins to develop in the branching stage, followed by the flowering and fruiting stage during which the colour, shape and weight change. Flowering stage is the appropriate period to collect nodules for bacteria isolation. The bacterial strains isolated from the root nodules of beans on YEM agar do not absorb Congo Red and it is gram-negative bacteria. According to these, the bacteria isolated are genus *Rhizobium.* Only *Rhizobium trifolii, R. etli, and R. phaseoli* can infect root nodules of beans. Preparation of the bio-inoculants of Rhizobia and inoculant carriers for the development of biofertilizers is essential for substitutes for synthetic chemical fertilizers. The symbiotic nitrogen fixation from the leguminous can be enhanced by the artificial amendment of rhizobia with the inoculation of effective rhizobial strains. Inoculant carriers (with charcoal) can also be prepared for commercial purposes as nitrogen biofertilizers. The fact that legumes uniquely associate with the symbiotic bacterium *Rhizobia,* plays a vital role in the fixation of the atmospheric free nitrogen in forms that can be directly used by plants.

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