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RESEARCH ARTICLE

ISOLATION AND PHENOTYPIC CHARACTERIZATION OF RHIZOBIUM SPECIES FROM ROOT NODULES OF HARICOT BEAN (*PHASEOLUS VULGARIS L.*) AT ARBA MINCH, SOUTHERN ETHIOPIA

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ABSTRACT

Haricot bean (*Phaseolus vulgaris L.*) is the most widely distributed and has the broadest range of genetic resources. In Ethiopia, the yield of haricot bean is extremely low mainly due to nitrogen deficiency and also little information is available regarding diversity of rhizobia nodulating haricot bean. Hence, this study was conducted to isolate and characterize of Rhizobium Species phenotypically and biochemically from Root Nodules of Haricot bean (*Phaseolus vulgaris L.*) at Arba Minch, Southern Ethiopia to know their diversity, efficiency of infectivity and effectiveness using haricot bean variety Omo-90. The results of the study revealed that isolates were able to form nodules on the roots of the haricot bean variety. The colony morphology of isolates was found to be viscous in consistency, round in shape and watery translucent pigmentation. The cells were also found to be gram negative and cocci in shape. The biochemical test revealed that the bacterial isolates were positive to catalase test. The result of this project activity is believed to provide information for isolating efficient nitrogen fixing strains of rhizobium species from Arba Minch and it also have some contribution for the effort being made in using leguminous plants to increase soil fertility as a substitutes for commercial fertilizers, which are costly and not environmentally friendly. However, to come with sound conclusion, the study should be repeated.

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INTRODUCTION

Haricot bean (*Phaseolus vulgaris L.*) is one of the most widely cultivated legumes in the world. According to Onwueme and Sinha (1991), it is probably native to tropical South America and might have been cultivated with maize. Shortly after the discovery of Americans it was introduced to Europe, Africa and Asia by the Spanish and Portuguese. It is now widely cultivated in many parts of the tropics and throughout temperate regions. It is the most important pulse crop throughout the tropical America and many parts of the tropical Africa including Ethiopia (Onwueme and Sinha, 1991; Westphal, 1974). Tenaw (1999) reported that it is also widely cultivated Southern Nations Nationalities and Peoples' Regional State (SNNPRs) including Gamo Gofa zone. It grows best at an altitude between 900-2100 meters above sea level (M.A.S.L) and demands free drainage soil with reasonably high nutrient content (Acland, 1971). Although haricot bean is the most widely distributed crop and has broadest range of genetic resource in Ethiopia, the yield is extremely low including the study area mainly due to nitrogen deficiency and limited knowledge on diversity of rhizobia nodulating haricot bean (Anteneh *et al*., 2007). Haricot beans (*Phaseolus vulgaris L.*) is nodulated by diverse rhizobial genotypes, all of which so far alpha proteobacteria (Amarger, 2001; Martinez-Romero, 2003). To date, five *rhizobium* species have been recognized as microsymbionts of *P. vulgaris* forming nodules immature: *R. etli* by *Phaseoli, R. leguminosarum* by *phaseoli, Rhizobium tropici, R. gallicum* by. *Phaseoli* and *gallicum, and R. giardinii* by *phaseoli and giardinii* (Amarger, 2001). *Rhizobium tropici* and *R. gallicum* have been found to be associated with beans in conditions of acid soils (Amarger, 2001). Zahran (1999) stated that the symbiosis between *Rhizobium* or *Bradyrhizobium* and legumes are a cheaper and visually more effective of agronomic practice for ensuring an adequate supply of N for legume based crop production than the application of fertilizer N. The symbiosis involving rhizobia and leguminous plants have major environmental and agricultural importance. They are responsible for most of the atmospheric nitrogen fixation on earth (Marta *et al*., 2004). Following the above fact, this research was initiated to isolate and characterize *rhizobium* species from root nodules of haricot bean.

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MATERIALS AND METHODS

Experimental site description

The study was conducted at Arba Minch. It is located at 505 km south of Addis Ababa and 5km from Arba Minch town, at coordinates between 50^0 55', and 60^0 15' latitude and 37^0 18' and 37^0 36' longitude and at an altitude of 1250 meter above sea level (m.a.s.l). The mean annual rain fall is 569 mm and its average temperature ranges between 17-29.5^{0}c.

Methods

Land preparation and seedling establishment: The land was prepared very well in order to support the plant in a good manner and the seed bed was prepared with a dimension of 1.2 meter width and 3m length. Haricot bean variety Omo-90 variety was planted at a spacing of 40 cm between rows and 10cm between plants. All important agronomic practices like fertilizing, watering, weeding and thinning were practiced uniformly for all treatments

Preparation of Rhizobium medium

Following the laboratory procedures outlined by Somasegaran and Hoben (1994) YEMA medium was prepared by mixing 2 g of Mannitol, 0.1 g of K_2HPO_4, 0.04 g of $MgSO_4.7H_2O$, 0.02 g of NaCl, 4.6 g of Yeast extract agar, 0.025 g of Congo red, and 200 ml of Distilled water. Then, the mixture was steamed to melt the agar by hot plate and sterilized in an autoclave for 30 minutes at 115^0. After these, the mixture was aseptically poured into sterile Petri-dishes in 15-20 ml and left undisturbed until agar sets. After the medium was solidified, the Petri-dishes were turned upside down to avoid excess water and finally the name of the medium and the date of preparation were labeled on the peripheral surfaces of the Petri-dishes.

Isolation and inoculation of Rhizobium media

Root nodules from haricot bean at 42 days of planting (about 50% flowering) were collected and washed thoroughly to remove the soils adhered to the roots of the bean. Root nodules having physically good appearance were taken by a test tube having silka jell in order to preserve them until they were crushed and isolated into *rhizobium* medium. Then, they were sterilized using 95% alcohol and rinsed with sterile distilled water. The sterilized root nodules were placed in an empty sterile watch glass and one drop of sterile water was dropped on it to facilitate crushing. The root nodules were crushed by sterilized glass rod till it became turbid and milky. After that, some solution from the crushed root nodules were taken with a sterile loop and streaked on the *rhizobium* medium (YEMA) plate. Finally, the plate was incubated in upside down position for 3 days at 30^0c.

Procedure to obtain pure culture

Four separate colonies were selected from culture to get pure cultures for further examination. The colonies were labeled as colony number 1, 2, 3, and 4. Sterile nutrient broth was prepared in five test tubes and labelled as test tube 1, 2, 3, 4, and 5. By using sterile loop, some culture were taken from colony number 1, 2, 3, and 4 and incubated aseptically into the test tubes 1, 2, 3, and 4, respectively. However, test tube number 5 was not incubated; rather it was taken as a control in refrigerator.

Fig 1. Uprooting root nodules from soil

Fig. 2. Washed root nodule of haricot bean to remove adhered soil

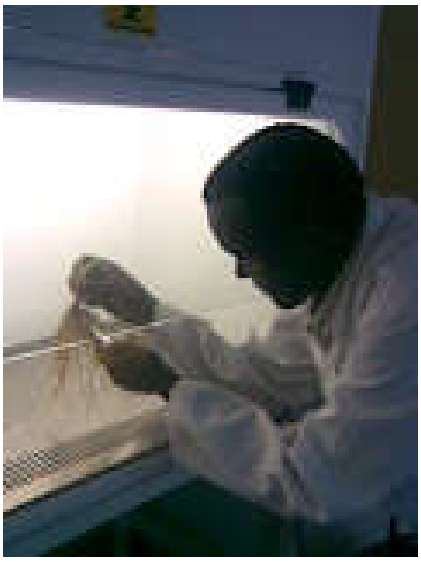

Fig. 3. Separation of nodule from root of haricot bean

The date of inoculation was labeled on the side surface of inoculated test tubes. They were then, incubated for 48 hours at 30^0c. After 48 hours of growth in test tube, each culture was streaked on *Rhizobium* medium plates with sterile loop. The plates were again further incubated for 72 hours at 30^0c to get more purified cultures and the above procedures were repeated for several times until pure culture of *Rhizobium* species was obtained.

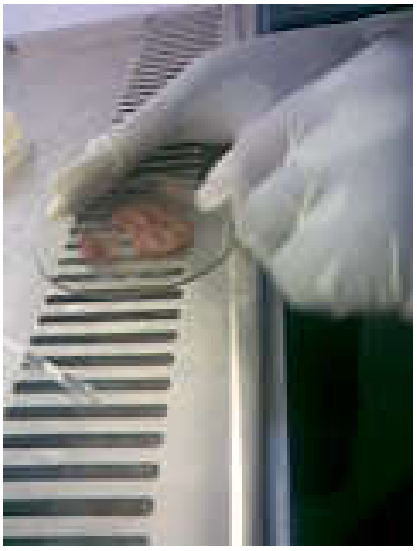

Fig. 4. Crushing of root nodules on watch

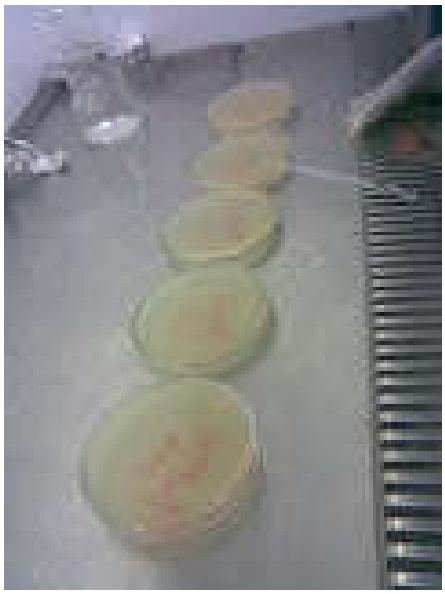

Fig. 5. Poured rhizobium medium (YEMA

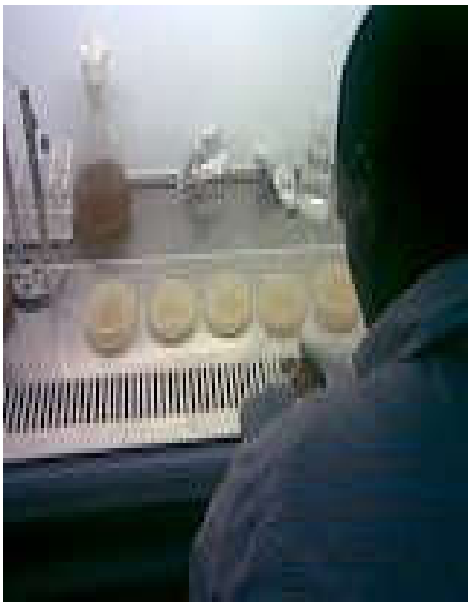

Fig. 6. Inoculation of the bacteriod on YEMA medium

Fig. 7. Incubating the bacteriod

After obtaining pure cultures, the colony morphology characteristics of bacterial isolates were studied.

Characterization of colony morphology

Size of the colony was measured by using ruler, shape, color, edge, and surface of colonies were observed with the help of magnifying glass. Consistency was detected by the help of loop.

Gram stain

A small drop of water was placed on a clean slide and some culture was taken from the pure culture by a sterile loop and then the drop of water on the slide was touched with the loop until it become turbid. Then, the remaining bacteria on the loop were burned and suspension was spread to form a thin film over the entire slide. Finally, this was allowed to completely air dry and blot on flame and the slide was passed through a Bunsen burner flame for 3 to 4 times. The heat fixed smear was placed on a staining rack and stained with crystal violet for 1 minute and then lugol's iodine was added and left for 3 minutes. After leaving for 3 minutes, it was decolorized with 95% alcohol for about 30 seconds. Finally, the entire film was covered with safranin for 3 minutes. Then, the slide was picked up by one end and hold at angle over the staining rack to wash off the excess safranin by tap water from the slide. The cells of the isolate were then examined under microscope as out lined by Brock *et al*. (1984).

Biochemical test

A small bacterial culture was transferred on a clean slide with the help of sterile loop. A drop of 3% hydrogen per oxide (H_2O_2) was added on the culture. Formation of air bubbles were considered as the presence of catalase enzyme which was produced by *rhizobium* species as a biochemical test procedure of Singleton and Sainsbury (1981) indicated.

RESULT AND DISCUSSION

Results: The selected physico-chemical properties of experimental soils, morphological characteristics of root

nodules, morphology of the isolates grow on *rhizobium* medium plate, the cell morphology of the isolates, the biochemical test of the isolates, and the exopolysacharide production ability of the isolates are presented in Table 1, Table 2, Table 3, Table 4, Table 5,and Table 6,respectively.
Table1. Selected soil physico-chemical properties of experimental site at Arba Minch, in 2010

Table 1. Selected soil physico-chemical properties of experimental site at Arba Minch, in 2010

Soil characteristics	Result
Soil P^H	7.82
Soil electric conductivity (EC)	0.68ds/m
Soil moisture content (MC %)	5.04
Soil textural class	clay loam

Table 2. Morphological characteristics haricot bean root nodules at Arba Minch, in 2010

Nodule characteristics	Result
Color	Pinkish
Number/plant	262-454
Arrangement	Lateral
Size (cm)	1-2
Shape	Oval to circular

Table 3. Colony morphology of isolates grow on YEMA medium incubated for 3 days at $3O^0$c at Arba Minch, in 2010

Colony characteristics	Result
Size	2-4mm
Shape	round
Pigmentation	watery transluycent
Edge	Smooth
Elevation	Raised
Surface	Smooth
Consistency	viscous

Table 4. Cell morphology of the isolates at Arba Minch, in 2010

Characteristics	Result
Gram stain	Negative
End	Smooth
Shape	Cocci

Table 5. Biochemical test of the isolates at Arba Minch, in 2010

Biochemical Characteristics	Result
Catalase test	Positive

Table 6. Exopolysaccharide production characteristics at Arba Minch, in 2010

Characteristics	Result
Copious Medium Low	positive

DISCUSSION

Selected physico-chemical properties of experimental soils

Laboratory analysis of physico-chemical properties of soil sample (Table 1) revealed that the textural class of the soil was clay loam. The P^H of the experimental soil was 7.82 which could be classified as slightly alkaline according to Herrera (2005) who classified soil P^H as strongly acidic (3-5.6), moderately acidic (5.6-6.2), slightly acidic (6.2-6.7), neutral (6.7-7.3), slightly alkaline (7.3-7.9), moderately alkaline (7.9-8.5) and strongly alkaline (>8.5). The range of P^H is favorable for activities of microorganisms (Brady and Weil, 2002). FAO (2000) reported that the P^H ranges in between 4 and 8 is generally optimum for most crops. Therefore, based on this classification P^H of the experimental soil was optimum for growth of haricot bean. The electrical conductivity of soil was 0.68 ds/m indicating that it was no salinity problem (Herrera, 2005). Similarly, Landon (1991) also reported that effects of EC value from 0-2 ds/m are mostly negligible. Also moisture content of the soil was 5.04%. Moisture greatly affects nitrogenase activity in legumes and in situations of extreme stress; nitrogenase activity ceases (Guerin *et al.,* 1990). Even a mild stress can significantly reduce nitrogenase activity in legumes (Rao & Venkateswarlu, 1987). A decrease in nitrogenase activity leads to low production of ammonia and to a decrease in ammonia-assimilating enzymes under water stress (Kaur *et al.,* 1985).

Characteristics of the isolates

Morphology and Cultural characteristics

Study of morphological characteristics of isolates (Table 3) has shown that most colonies were displayed medium sizes, viscous consistency and with watery translucent pigmentation. In addition, as indicated in the same table, most of colonies were with round shape, raised elevation and with smooth surface and edge. The reports of Marteinez Romero *et al. (*1991) and Silva *et al. (2003*) indicated that Smooth and gummy colonies of haricot bean rhizobia could be due to *Rhizobium leguminosarum or Rhizobium etli.* This result is in contrary to Alemayehu Workalemahu (2006) who reported that almost all haricot bean rhizobia of Southern Ethiopia isolates displayed smooth gummy colony except some isolates from Wolaita Soddo areas displayed rough colony morphology whereas isolate from Arba Minch areas which displayed creamy colony morphology on PY-medium. Colony morphology of *rhizobium* species isolated from haricot bean at Arba Minch area revealed as watery translucent appearance. The difference in result might be due to the difference in the growth media used.

Growth on YEMA-medium: According to the classification of *Rhizobiaceae* in Bergey's Manual (Jordan 1984), almost all isolates (Table 6) categorized in to fast growing rhizobia based on their generation time, acid production and large growth with production of copious exopolysaccharid and colony diameter greater than 2mm except few isolates (Table 3) at optimum temperature ($25\text{-}30^0$C) and pH of the medium (6-7). Fast growing bacteria nodulating haricot bean were also identified by several workers (Alemayehu Workhalemahu 2006; Amarger *et al.,* 1997; Aguilar *et al.,* 1998 and Desta Beyene *et al*., 2004). Isolates of *Bradyrhizobium*-like bacteria was also reported by Hungria *et al*. (1993) from Latin american soils that nodulate haricot bean.

Gram staining and catalase test of the isolates: Examination of cell morphology of isolates (Table 4) indicated that they were with smooth ends and with cocci shape. Besides, the cells were classified as gram negative according to Batzing, (2002) who characterized the cell morphology of *rhizobium* as gram negative if do not retain the crystal violet-iodine complex as a result of the presence of thin peptidoglycan layer in *rhizobium* species. Catalase tests of the isolates indicated that they were catalase positive based on the classification of Singleton and

Sainsbury (1981) who classified isolates as catalase positive, if they have air bubbles which is produced due to break down of hydrogen peroxide into water and oxygen and as catalase negative if they do not have air bubbles.

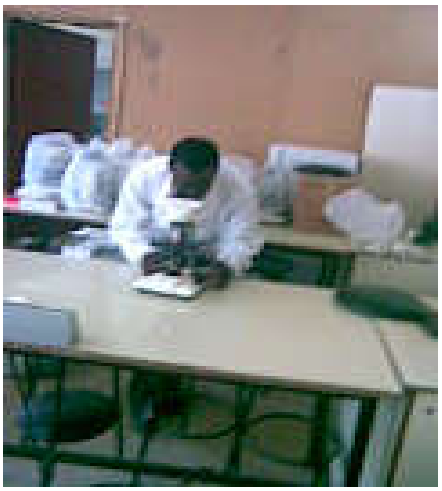

Fig 8. Morphological characterization rhizobial colony cell Isolates

Summary and Conclusion

Experiment was conducted at Arba Minch to isolate and characterize *rhizobium* species from the root nodules of haricot bean variety Omo-90. The preliminary phenotypic and its characteristics analysis performed with the strains indicated presence of rhizobia nodulating haricot bean at Arba Minch area. The isolates were able to show positive response to catalase test by showing oxygen bubbles tested on a clean microscope slide and with gram negative stain result. These abilities may favour the establishment of the rhizobia and may represent an advantage of these over other inoculate used for haricot bean. Nevertheless, rhizobial strains having the above characteristics in laboratory may not be effective in nitrogen fixation and survive the adverse condition under external environment. Thus, it needs to do intensive evaluations under field condition. The experiment has also showed that the isolates nodulating haricot bean isolated from the study area are effective and availability of effective strains of rhizobia. This shows that effective isolates could be used as an inoculum in other haricot bean growing area of the country. However, the experiment should be repeated and soils from other locations not covered in this study should also be investigated in order to get further information about the species of rhizobia that effectively nodulate haricot bean and developing a multi-inoculum for haricot bean.

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