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Short Communication: Isolation and biochemical characterization of *Rhizobium* strains from nodules of lentil and pea in Tarai agro-ecosystem, Pantnagar, India

SANDEEP PRAKASH UPADHAYAY[1,♥], NAVNEET PAREEK[1], GAURAV MISHRA[1,2,♥♥]

[1]Department of Soil Science, College of Agriculture, G. B. Pant University of Agriculture and Technology, Pantnagar, India-263 145. ♥email: sandeepupadhyay383@gmail.com

[2]Scientist, Rain Forest Research Institute, Jorhat, Assam, India-785001. ♥♥email: gaurav.mishra215@gmail.com

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Abstract. *Upadhayay SP, Pareek N, Mishra G. 2015. Isolation and biochemical characterization of Rhizobium strains from nodules of lentil and pea in Tarai agro-ecosystem, Pantnagar, India. Nusantara Bioscience 7: 73-76.* Root nodules were collected from young and healthy seedling of *Pisum sativum* L and *Lens culinaris* L from the field at different locations of Norman E. Borloug Crop Research Center, G.B.P.U.A. & T., Pantnagar, Uttarakhand state, India. Fifteen *Rhizobium* strains were isolated from the root nodule of *P. sativum* and *L. culinaris* and characterized by standard tests. All strains were gram-negative and did not absorb red color when cultured in YEMA containing congo red. In the ketolactose test yellowish zone of Cu_2O not found. Also, isolates showed either poor or no growth on the glucose peptone medium after one day which is indicating character of rhizobia Thirteen isolates were fast grower and only two were slow growers which is confirmed by bromothymol blue test. Results confirmed that isolated strains were *Rhizobium*.

Keywords: Lentil, pea, root nodules, *Rhizobium*, YEMA

INTRODUCTION

Pulses are unique, having god gifted ability to fix nitrogen in symbiosis with rhizobial soil bacteria. Role of this symbiosis is clearly defined; in nodules, bacteria reduce atmospheric nitrogen to ammonia for plant nitrogen nutrition. So, pulses are known to increase nitrogen status in soil and improving the soil fertility (Riah et al. 2014). There are about 750 genera of legumes (Young and Haukka 1996). Although most rhizobia are host specific, but it is also true that several different bacterial species are also isolated from a single legume species and it is only from limited hosts which have been examined as far as micro-symbionts are concerned (Arora et al. 2001). Lentil is known for its human nutrition ability and to maintain soil fertility (Rashid et al. 2014) while pea for its short term and highly beneficial nature (Wadhwa et al. 2011). There is huge amount of literature available, reporting rhizobia from different pulses (Riah et al. 2014; Wadhwa et al. 2011; Hou et al. 2009 and many others) but limited studies are there about the biochemical characterization of rhizobia inhabiting lentil and pea. Rhizobia are characterized on the basis of biochemical tests. So, this study was aimed to isolate and identify rhizobia on the basis of biochemical tests from root nodules of lentil and pea for better agriculture growth.

MATERIALS AND METHODS

Sample collection

Plants samples along with roots and nodules were collected at 50 days after sowing from various pea and lentil fields at Norman E. Borloug Crop Research Center, G.B.P.U.A. & T., Pantnagar, Uttarakhand, India. The roots along with mature nodules were thoroughly washed in running water until the removal of adhering soil particles. Big sized and pink colored nodule preferably on tap root were selected and transported to the laboratory for further investigation by following the method given by Vincent (1974).

Isolation of *Rhizobium* from root nodules

Selected root nodules were dipped in 0.1% mercuric chloride ($HgCl_2$) solution for 30 sec and later washed successively ten times with sterilized distilled water to remove the traces of toxic $HgCl_2$. Surface sterilized nodules were transferred in test tube containing 5 mL sterilized distilled water. These nodules were crushed with the help of sterilized glass rod to obtain a milky suspension of bacteroids. These were streaked on YEMA containing congo red. The plates were sealed by parafilm to avoid contamination and incubated at 28 $\pm 1^{o}$ C for 24-48 h. Brady *Rhizobium* or *Rhizobium* colonies were remained white, translucent, elevated and mucilaginous, after 24-72 h, whereas contaminations turned red. The colony was picked up and transferred to YEMA slant for further investigation.

Biochemical tests

Biochemical tests such as Congo red test (Vincent 1974), Ketolactose test (Bernaerts and De Ley 1963), Bromothymol blue (BTB) agar test (Somasegaran and Hoben 1994) and glucose-peptone agar test (Kleczkowska et al. 1968) were done to differentiate *Rhizobium* and *Agrobacterium.*

RESULTS AND DISCUSSIONS

A total of 15 bacterial strains were isolated from root nodules of Lentil (*Lens culinaris* L.) and Pea (*Pisum sativum* L.). Phenotypically isolated rhizobial colonies were cream colored with slime/mucoid transparent appearance on CRYEMA plates with marked distinction from red colored colonies of *Agrobacterium* (Figure 1). Further conformity of rhizobia was performed on ketolactose agar showed, all 15 isolates were negative for the production of 3 ketolactose from lactose no yellow zone of Cu_2O around the colonies were observed (Figure 3) which was indicative of *Agrobacterium* (Table 1). Production of 3-ketolactose from lactose is limited to species of *Agrobacterium*. Similarly, Gachande and Khansole (2011) isolated *Rhizobium japonicum* and Brady *Rhizobium japonicum* colonies, which were circular in shape with whitish pink color on CRYEMA medium. Absence of 3 ketolactose in rhizobial colonies on CRYEMA was also in agreement with earlier work carried out by Sadowsky et al. (1983) and Sharma et al. (2010).

While further confirming these all fifteen isolates showed either poor or no growth on the glucose peptone medium after one day indicating character of rhizobia as a conventional rule (Figure 5). *Rhizobium* strains growth is found poor on glucose peptone agar media while the other bacteria grow well on this media. Further purified isolates were classified as fast (turn medium yellow) and slow growing (turn medium blue) rhizobia on YEMA supplemented with BTB (Figure 4). The rhizobial isolates in the current study were further tested on YEMA plates containing BTB indicated that thirteen fast growing isolates (SMFP 1 II, SMFP 2 I, SMFP 2 II,

SMFP 3 I, ACFL 1, ACFL 2, ACFL 3, ACFL 4, ACFL 5, CL 1, CL 2, CL 5, GL 1) were found to produce yellow colonies due to acid production on the medium with high amount of mucus after 2 days of incubation (Table 1). Whereas, remaining two isolates along with reference strains SMFP 2 IV and GL 2 produced blue color colonies, which indicated the presence of alkali producers, considered as slow growing rhizobia. The use of YEMA-BTB medium for categorizing indigenous root nodulating fast and slow growing rhizobia based on acid/alkali production was also carried out by Saeki et al. (2005) in Vietnam and Sharma et al. (2010) in India. On BTB agar plates both fast and slow growing rhizobia formed circular, convex, colonies. The isolates were classified tentatively as fast (medium turn yellow) and slow growers (medium turn blue) based on their reaction on the yeast extract mannitol agar supplemented with bromothymol blue (Somasegaran and Hoben 1994). These isolates were similar in terms of reaction on the YEMA (BTB) when compared with reference strains which produced yellow and blue color in fast and slow growing strains, respectively according to Hungria et al. (2001).

Table 1. Results of different tests of *Rhizobial* isolates

Isolates	CRYEMA test (Growth After 2 days)	Ketolactose test (After 4 days growth)	Bromthymol Blue test (After 2-10 days, color/growth)	Glucose Peptone Agar test (Growth after 1 day)
Pea				
SMFP 1 II	Light pink/semi transparent	-ve	Yellow/fast	Poor growth
SMFP 2 I	Light pink/semi transparent	-ve	Yellow/fast	Poor growth
SMFP 2 II	Light pink/semi transparent	-ve	Yellow/fast	Poor growth
SMFP 2 IV	White/transparent	-ve	Blue/slow	Poor growth
SMFP 3 I	Light pink/semi transparent	-ve	Yellow/fast	Poor growth
Lentil				
ACFL 1	White/transparent	-ve	Yellow/fast	No growth
ACFL 2	Watery white/transparent	-ve	Yellow/fast	No growth
ACFL 3	White/transparent	-ve	Yellow/fast	No growth
ACFL 4	White/semi transparent	-ve	Yellow/fast	No growth
ACFL 5	White/semi transparent	-ve	Yellow/fast	No growth
CL 1	White/transparent	-ve	Yellow/fast	No growth
CL 2	White/transparent	-ve	Yellow/fast	No growth
CL 5	White/transparent	-ve	Yellow/fast	Poor growth
GL 1	White/semi transparent	-ve	Yellow/fast	Poor growth
GL 2	White/semi transparent	-ve	Blue/slow	No growth

Our findings congruence with Gachande and Khansole (2011)as reported for soybean rhizobia. Sadowsky et al. (1983) also reported that production of 3 ketolactose from lactose is limited to species of *Agrobacterium*, a genus that is closely related to *Rhizobium*. Mahana et al. (2000) also showed negative chemical reaction for 3 ketolactose productions by isolates of *Rhizobium* from *Vigna* mung. Similarly, Shetta et al. (2011) mentioned that *Rhizobium* strains failed to absorb congo red stain in the CRYEMA medium. *Rhizobium* is symbiotic bacteria which form nodule in leguminous plant. But *Agrobacterium* infects the root and forms false nodule (pseudo nodule) therefore, biochemical tests are essential to differentiate *Rhizobium* and *Agrobacterium.* All the biochemical test and cited literature suggested that isolated strains were *Rhizobium.*

In conclusion, all 15 *Rhizobium* strains did not absorb red color when cultured in YEMA containing congo red medium. Pseudo-nodule forming bacteria *Agrobacterium* utilized congo red but *Rhizobium* strains didn't utilize congo red. This test is essential to differentiate *Rhizobium* and *Agrobacterium*. Other biochemical tests confirmed that isolated strains were *Rhizobium.*

Figure 2. Different isolates of *Rhizobium* strains on YEMA

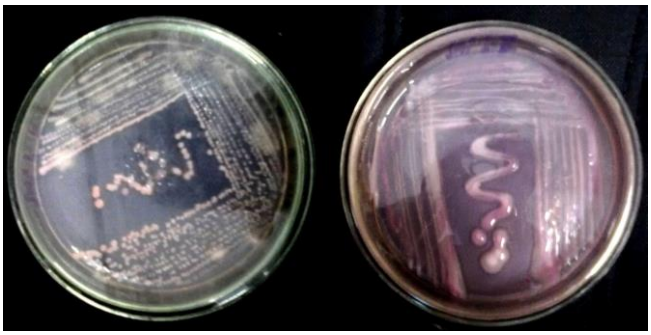

Figure 1. Growth of *Rhizobium* strains on CRYEMA media

Figure 3. Ketolactose test (No yellow ring of Cu2O around the growth of *Rhizobium*)

Figure 4. Bromthymol Blue test (Yellow and Blue color change of media after 2. 10 days).

Figure 5. Glucose peptone agar test (No growth of *Rhizobium* is observed in 1 day).

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