IDENTIFICATION OF ISOLATED INDIGENOUS BACTERIA FROM NODULES OF *PISUM SATIVUM* L.

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Abstract

The present study is to focus describe and identify the specific bacteria from the nodules of *Pisum sativum* L. The plant samples were selected from the cultivated field of Kachin State and it carried out to the laboratory of Agricultural Microbiology Section, Agricultural Research Center, Yezin, Nay-pyi-daw. This research was conducted from November 2018 to December 2019. The media YEMA, CRYEMA and BTBYEMA were used as selective media for isolate bacteria. After culturing, colony characters and cell morphological characters of isolate bacteria were studied. Colonies of isolates were sticky appearance, circular, convex (raised), smooth, entire margin and white (translucent). Cell morphological characters were medium size, rod shaped, motility and gram-negative. Moreover, the characterization was done by biochemical tests. As results, the isolated indigenous bacteria were faster grower. The positive chemical reaction were showed Indole, Methyl red, Catalase, Urease hydrolysis, starch hydrolysis, Glucose peptone agar, Glucose, Sucrose and Mannitol tests. The negative chemical reactions were showed in lactose fermentation, Citrate utilization, Gelatin hydrolysis, Methyl blue and Mannitol salt agar experiments. According to the character results, isolated indigenous bacteria from nodules of *Pisum sativum* L. was confirmed as genus *Rhizobium.*

Keywords: Root nodules, *Pisum sativum*, Biochemical, *Rhizobium*

Introduction

Pulses, fresh or dry, had a high nutritional values due to their high contents of carbohydrates, proteins, vitamins and minerals (Smart, 1990). The garden pea (*Pisum sativum* L.) is a cool season legume. A major resource of food in Myanmar and vital component of our daily dishes. Food legumes serve as a feed crop in farming systems and fetch higher prices compared to cereals. These crops are being grown more to supplement farmers' incomes. Pulses play an important and different role in food systems and in the diet of the poor, they are the best crop to reduce poverty and hunger, improving human health, nutrition and enhancing ecosystem resilience Akibode and Maredia(2011).

The crops from legumes are an important crop for consumption in developing countries. These are considered a vital crop for achieving food and nutritional security for both poor producers and consumers in many parts of the tropics countries, particularly where meat is scarce. Because they are higher in protein than any other food plant and are close to animal meat in quality. In fact, they are often called poor man's meat being an inexpensive source of high quality protein and also play an important nutritional role in supplying those essential amino acids.

Legume-*Rhizobium* symbiosis is the most promising plant bacterium association for immediate increase in grain yield through biological nitrogen fixation Gresshoff *et al.*, (2014) Desta *et al*., (2015) stated that by the inoculation of adaptable effective legume-*Rhizobium* effect on pulses production can be increase from lower point.

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Rhizobium plays a very important role in agriculture by inducing nitrogen fixing nodules on the roots of legumes such as peas, beans and clover. In 19th century, the scientific demonstration of this symbiosis was started and established that bacteria are present in legume root nodules and which are responsible for fixing atmospheric nitrogen Zsbrau, (1999). The *Rhizobium* species live inside the root nodules of host legumes so they are beneficial for the growth of the plants Oblisami, (1995). They easily colonize in the plant root and promote solubilizing activity, nitrogen fixation and biocontrol activity Deshwal *et al*., (2011).

Rhizobium inoculation increased the root nodulation through better root development and more nutrient availability, resulting in vigorous plant growth and dry matter production which resulted in better flowering, fruiting and pod formation and ultimately there was beneficial effect on seed yield Sardana *et al*., (2006) Rhizobia have been classified and characterized on the basis of biochemical tests Gachande and Khansole, (2011). The interest in biological nitrogen fixation and rhizobia-legumes symbioses, particularly those involving economically important legume crops in terms of food and forage is essentially for sustainable agricultural practices Laranjo *et al.*, (2014). The excessive use of nitrogen fertilizers increased the total costs of crop production, created pollution and increased deterioration of soil fertility Marschner, (1995). Use of these fertilizers has led to worldwide ecological problems as well as affects the human health Vitousek, (1997). Biological nitrogen fixation (BNF) micro-organisms is the cheapest and most environment friendly organisms that can be fixed aerobic nitrogen in the soil and interacting with leguminous plants. This symbiotic relationship reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops Gauri *et al*., (2011). The rhizobia are a group of Gram-negative bacteria that form species-specific symbioses with legume plant Bhatt *et al*., (2013).

The present study was investigated identification of isolated indigenous bacteria from nodules of *Pium sativum* L. (Garden pea). The aim and objectives of the present research is to study nature isolated indigenous bacteria and to evaluate their characteristics performed by various biochemical tests.

Materials and Methods

The fresh and healthy root nodules of *Pisum sativum* L. (garden pea) plants were collected from different places of Mogoung, Nanmati and Sahmaw in Kachin state. Plants possessing healthy nodule with pink colour were selected and the nodule samples were collected in plastic capped tube containing desiccant material, such as silica gel cover with a cotton plug. And then, carried out to the laboratory of Microbiology Section, Department of Agricultural Research Center, Yezin, Nay-Pyi-Taw during November 2018 to December 2019.

Table 1 Location of the samples collected sites

Collected Sites	Area	Location	Collected date
MOGAUNG	Kachin	25˚18′ 16.3″N 96˚56′11.2″ E	29. 11.2018
NANMATI	Kachin	25˚ 22′ 49 .2″N 97˚ 00′ 38 .3″ E	29. 11.2018
SAHMAW	Kachin	25˚ 13′ 37.2″N 96˚47′ 33 .4″ E	30. 11.2018

Figure 1 Root with nodules

Figure 2 Nodules in tube filled with dehydrated silica gel for transportation

Surface sterilization of the nodules

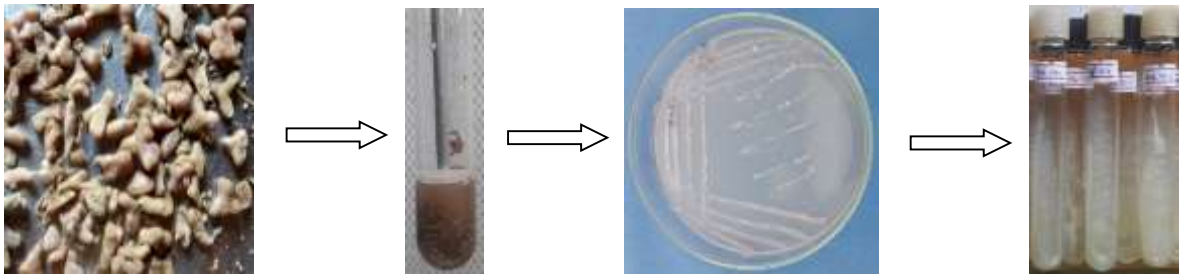
The samples of root nodules from *Pisum sativum* L. collected from different localities of Kachin state. Pea plants were uprooted carefully and as to get intact are obtained. Healthy pea nodules were detached from the root and further isolation of root nodulating rhizobia was carried out. The detached root nodules were washed in tap water to remove the adhering soil particles from nodule surface and surface treatment of nodules done with 95% alcohol for 30 sec. 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 of sterilized distilled water.

Extraction of indigenous root nodules bacteria

In this experiment, the selected plants have a healthy root nodules with pink color and are used as a material for isolation and further study the morphological characters of indigenous bacteria strain. These nodules were crushed with the help of sterilized glass rod to achieve a pink color suspension contain bacteriods. These were direct streaked on the media YEMA, CRYEMA and BTBYEMA. The plates were sealed by parafilm to avoid contamination and incubated at 28˚C. Typical isolated colonies were re-streaked on fleshy prepared YEMA slants, and use in order to obtain pure cultures for experiment. Finally, the pure culture slants were stored at 4˚C in refrigerator for further experiment works.

Colony morphological characterization of isolated indigenous bacteria

Colony morphology was studied by observing of various features such as size, shape, color, margin, elevation, surface and consistency. Microscopic examination was done by using Gram staining as described by (Arora,2003)



(A)Healthy fleshy root (B)Crushed root nodule (C)Culture on YEMA (D) Pure culture nodule maintain in slant

Figure 3 Extraction of indigenous bacteria from root nodules of *Pisum sativum* L.

Biochemical tests of isolated indigenous bacteria

All the collected samples were used in process of different biochemical tests such as Indole production test, Methyl red test Elsheikh E.A and wood, M (1989)., Methyl blue Wei *al.*, (2003), Citrate utilization Lupwayi and Hague (1994), Urea hydrolysis Lindstrom and Lehtomaki (1988), starch hydrolysis De O Liveria *et al*., (2007),Gelatin hydrolysis(Hunter *et al.*, 2007)., Glucose peptone agar(Kucuk *et al.*, 2006), Mannitol salt agar, Catalase test MacFaddin (2000), Glucose fermentation,Sucrose fermentation,Lactose fermentation Somasegaran & Hoben (1985) and Mannitol fermentation.

Results

Morphological characters of *Pisum sativum* L.

Scientific name - *Pisum sativum* L.
Family - Fabaceae
Sub-family - Papilionoidae
Common name - Sa-daw-pe
English name - Garden pea, Pea

(A)Cultivated field (B)Garden pea plant (C) Pod with seeds

Figure 4 Habit of ***Pisum sativum*** L**.**

Morphological characters of *Pisum sativum* L.

Pisum sativum L. is an herbaceous annual, with a climbing hollow stem. Leaves are alternate, pinnately compound, and consist of large leaf-like stipules. Flowers have five green fused sepals and five white to reddish-purple petals of different sizes. Fruit grows into a pod. that often has a rough inner membrane. The pod is a seed container which composed by two sealed valves and spilled along the seam which connects the two valves. Seeds are round, smooth, and green color.

Characteristics of nodules sample

Nodules are found on lateral roots of *Pisum sativum* L. as well as found a few Mostly on taproots. The nodules are about 2-5 mm in diameter. Active nitrogen fixing nodules contain a protein which is called leghemoglobin. When the nodules crushed the turned pink color because of the presence of leghemoglobin.

(A)Nodule

(B) L.s section

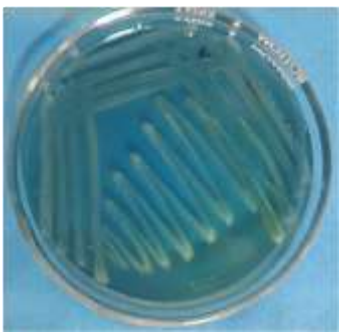
(C)Nodules

(D) L.s section

Figure 5 Transverse section of nodules of garden pea

(A)YEMA

(B)CRYEMA



(C)BTBYEMA

Figure 6 Isolation of bacteria culture on different medium

(A)Single colony(400X)

(B)Motility test

(C)Gram staining

Figure 7 Morphological characters of isolated indigenous bacteria

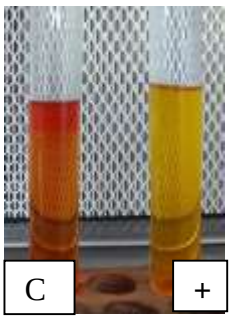
Table 2 Morphological colony characterization of isolated indigenous bacteria

Sr. No.	Colony character	MG	NM	SH
1.	Size (mm)	2-3	2-4	2-4
2.	Shape	Circular	Circular	Circular
3.	Color	white and translucent	white and translucent	white and translucent
4.	Margin	entire	entire	entire
5.	Elevation	convex (raised)	convex (raised)	convex (raised)
6.	Surface	Smooth	Smooth	Smooth
7.	Opacity	Opaque	Opaque	Opaque
8.	Consistency	Sticky	Sticky	Sticky
9.	Motility	Motile	Motile	Motile
10.	Gram nature	(- ve)	(- ve)	(- ve)

Table 3 Biochemical characterization of isolated indigenous bacteria

Sr. No	Biochemical Tests	MG	NM	SH
1.	Indole production test	+	+	+
2.	Methyl red test	+	+	+
3.	Methyl blue test	-	-	-
4.	Citrate utilization test	-	-	-
5.	Urease Hydrolysis test	+	+	+
6.	Starch Hydrolysis test	+	+	+
7.	Gelatin Hydrolysis test	-	-	-
8.	Glucose Peptone Agar test (GPA)	+	+	+
9.	Mannitol Salt Agar test	-	-	-
10.	Catalase test	+	+	+
11.	Glucose fermentation	+	+	+
12.	Sucrose fermentation	+	+	+
13.	Lactose fermentation	-	-	-
14.	Mannitol fermentation	+	+	+

(+) =Positive reaction (-) = Negative reaction



(A) Glucose fermentation

(B) Lactose fermentation

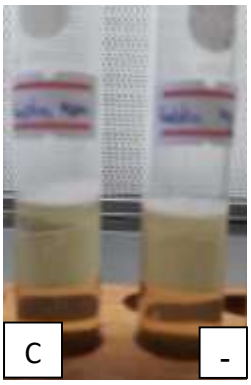
(C) Glucose Peptone Agar

(D) Citrate utilization

(E) Indole test

(F) Methyl red

(G) Methyl blue

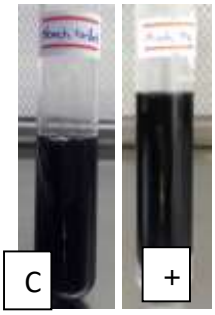


(H) Gelatin hydrolysis

(I) Urea hydrolysis

(J) Catalase test

(K) Mannitol salt agar



(L) Starch hydrolysis

Figure 8 Biochemical characterization of isolated bacteria

Discussion and Conclusion

The present study deals with identification of isolated indigenous bacteria from nodules of *Pisum sativum* L. The colonies of isolated *Rhizobium* bacteria were obtained by culture on the media YEMA, CRYEMA and BTBYEMA incubated after 3 days at 28˚C. The colonies were large (2-4 mm in diameter) mucilaginous, circular, convex with smooth edges **and** glistening translucent or white. This findings are in close agreement with Vincent, (1970) and Holt *et al.,* (1994). The isolated colonies were white and translucent, mucilaginous, circular, convex with smooth edges and sticky characters. However, the isolated bacteria were failed to absorb congo-red color in the CRYEMA and blue color in the BTBYEMA that is similar to the statements by researchers Shetta *et al.* (2011).

According to the results, colonies of isolated bacteria were appeared after 3 days and which are found like the fast grower species. Therefore, these findings are consistent with the findings of researchers Bala *et al* (2011). Microscopic examinations of the isolates were observed rod-shaped, pink colour gram- negative in nature and non-spore forming. These findings are similar to the nature of the findings of researchers Singh *et al*. (2008).According to biochemical tests results, the starch hydrolysis test was showed a positive response and the results are in line with the statement of researchers De Oliveria *et al.,* (2007).According to the results of Kucuk et al. (2006) Rhizobial cells have grown in GPA media. It has been suggested that Rhizobium uses glucose as a carbon source. The current result is consistent with the results of Kucuk *et al.*

However, pure *Rhizobium* isolates are unable to grow on lactose that can grow best on glucose, mannitol and sucrose. Therefore, the current findings are similar to the findings of the Somasegaran & Hoben (1985). In this experiment, It was observed that the rhizobial cells did not produce gelatinase enzymes as a medium containing gelatin.So, the result is agreement by findings of Hunter *et al.,* (2007) that negative gelatinase activity is a sign of Rhizobium. Besides, Current research has shown negative reactions to the growth of microorganisms in the methylene blue test and the gentian test.

These results consistent with the findings of Wei *et al.* (2003). In the catalase test, the formation of bubbles was clearly demonstrated. This finding is supported by the statement of MacFaddin (2000). Also in urea hydrolysis test, the isolated bacteria had a positive reaction and similar to the finding of Lindstrom and Lehtomaki (1988). In citrate utilization test the isolated bacteria reacted negatively reaction and in the line with the statement of Lupwayi and Hague. (1994). In Indole and Methyl red tests, the isolated bacteria were showed positive reaction and agreement with the findings of Elsheikh and Wood (1989). Mannitol salt agar test showed a negative reaction.

According to the results of the research, the isolated indigenous bacteria can be identified as *Rhizobium* species based on morphological and biochemical characteristics. These findings allow us a new scope for extensive research in Agricultural Biotechnology. The present study provides the valuable knowledge for young scientists to apply microbiology that can be applied and make available information for preparation of biofertilizer.

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