Nitrogen fixing bacteria isolated from *Pisum sativum* L.

Yin Yin Waing[1], Khin Thuzar Myint[2], Sandar[3] & Cho Cho Nyunt[4]

Abstract

The plant of *Pisum sativum* L. is one of the most important vegetable crops. It belongs to the subfamily Faboideae of the pea family Fabaceae. It is a cool weather crop and therefore the plant could not survive during the rainy season. To get the root nodules, the seeds were tested with the concentrations 250 mg/1 kg of calcium chloride solution. The germination type of *Pisum sativum* L. is a hypogeal germination. The experiments were made during the winter and rainy seasons. The root nodules were obtained during the sowing period (45 DAS). Thus, the nitrogen fixing bacteria were isolated from the root nodules of *Pisum sativum* L. and examined for the macroscopical and microscopical characters of nitrogen fixing bacteria. The creamy white color and rod shape of the isolated nitrogen fixing bacteria were observed from the study. Then, the isolated nitrogen fixing bacteria were used and its effect on germination of *Hibiscus cannabinus* L. during the rainy season was examined. Good results were obtained by using nitrogen fixing bacteria solution. The germination type of *Hibiscus cannabinus* L. was described as epigeal germination.

Key words: Root nodules, nitrogen fixing bacteria, hypogeal and epigeal germination

INTRODUCTION

The plant, *Pisum sativum* L. (sadaw-pe) is one of the most important vegetable crops for the local consumption which belongs to the subfamily Faboideae of the family Fabaceae. The garden peas are cool weather plants but can be grown either in the winter months or at higher elevations in tropical regions (Mortensen and Bullard, 1970).

The resumption of active growth on the part of the embryo resulting in the rupture of the seed coats and emergence of the young plant is known as germination (Meyer and Anderson, 1952).

In many areas of the world, intensive farming practices that accomplish high yields need chemical fertilizers, which can not only cost a lot but also create environmental problems. The extensive use of chemical fertilizers in agriculture is currently under debate due to environmental concern and fear for consumer's health. Consequently, there has recently been a growing level of interest in environment friendly sustainable agricultural practices and organic farming systems which include the use of bio-fertilizers as a substitute of chemical fertilizers. Thus, in the development and implementation of sustainable agriculture techniques, bio-fertilization is a major importance of decreasing environmental pollution and the conservation of nature (Poonia, 2011).

Legume plays an important role in sustainable management of dry arid regions. On the other hand, leguminous crops are important cash crops, and demand for domestic consumption and export is increasing in Myanmar. About one-third of human dietary protein is derived from grain legumes. In addition to their being a rich source of protein, legumes are important because they have the unique ability to produce substantial amounts of organic

[1] Associate Professor, Department of Botany, Hinthada University
[2] Associate Professor, Department of Botany, Hinthada University
[3] Associate Professor, Department of Botany, Hinthada University
[4] Associate Professor, Department of Botany, Hinthada University

nitrogen through symbiotic biological nitrogen fixation. Therefore legume-rhizobia symbiosis can provide an easy, inexpensive way to maintain soil fertility and improve crop production (Aung Zaw Htwe *et al*., 2019).

Nitrogen is important for maintaining and improving crop growth and yield. However, the long-term excessive use of chemical fertilizers in agriculture has unanticipated environmental impacts, including soil fertility degradation, soil organic matter deterioration and decreases water nutrient holding capacities and nutrient use efficiency. An alternative to nitrogen fertilizer is effective, efficient rhizobial N-fixing bacteria alone or together, *Rhizobium* inoculants are relatively inexpensive for leguminous crop production (Aung Zaw Htwe *et al*., 2019).

Nitrogen is an essential nutrient for growth and development. It is one of the essential elements required for the synthesis of amino acids which, in turn, are used by the plant to form protein. Leguminous plants are also able to utilize nitrogen derived from the symbiotic relationship they form with root nodule bacteria (Poonia, 2011). The high protein content of *Pisum sativum* L. is related to the presence of root nodules containing nitrogen fixing bacteria, *Rhizobium* (Pandey, 1981). Therefore, it is suggested that the plants of *Pisum sativum* L. can support the essential nutrient nitrogen for the soil fertility.

Calcium is important for neutralizing the acid in the soil. Moreover, the absence of calcium causes rapid changes in the root tips of *Pisum sativum* L. Calcium is a constituent of the protoplast essential for normal mitotic division in the meristematic region (Day, 1929). Severe calcium deficiency restricts the amount of nitrogen fixed by legumes through restriction of host plant growth. On the other hand, when calcium deficiency is less severe it may have a specific effect on the symbiotic system (Banath *et al*., 1965).

Therefore, the experiments were also studied and isolated the nitrogen fixing bacteria from the root nodules of *Pisum sativum* L. The aim and objectives of the study are to know the calcium chloride solution effect on growth of *Pisum sativum* L., to isolate the nitrogen fixing bacteria from the root nodules of *Pisum sativum* L., to know the nitrogen fixing bacteria solution effect on germination of *Hibiscus cannabinus* L. during the rainy season and to support the students majoring in Botany and other researchers in future.

MATERIALS AND METHODS

Soil analysis of the natural field soil

The soil samples were diagonally collected from the field according to the method of soil analysis, Department of Agriculture (Land use), Soil Interpretation of Results. Firstly, the collected soil samples were thoroughly mixed and analysed in the soil laboratory, Department of Geography, Kalay University. A portion of the soil samples was analysed for soil physical-chemical parameters like soil texture, pH, available nitrogen, phosphorus and potassium.

Preparation of basal soil and Maintaining of the sowing seeds

In this study, the basal medium (natural field soil) was prepared for the opened polyethylene pots (25 cm x 36 cm) containing the concentrations 250 mg/1 kg of calcium chloride solution before sowing the seeds. The basal media were saturated with water before sowing. The seeds were buried in the basal medium and the sowing depth of soil in containers was about 1.0 cm. In 20 DAS, the germinated plants were treated with the calcium chloride solution respectively. The experiment included three replications and fifteen seeds in one replicate. Therefore, the total seeds of the treatment included 45 seeds. All the sown seeds were kept at temperature 16±27°C and relative humidity 39-68% during the winter

season. The temperature and relative humidity of the rainy season were 27 ± 42°C and relative humidity 60-88%. Watering was done daily as necessary.

Isolation of nitrogen fixing bacteria from the root nodules of *Pisum sativum* L.

The healthy and mature root nodules of *Pisum sativum* L. treated with the concentration of calcium chloride solution were collected during winter season. According to the method of Dubey and Maheshwari (2002), the nitrogen fixing bacteria were isolated. The root nodules were washed in running tap water for 5 minutes. They were separated and cut into small pieces 0.2 cm in length. They were successively washed with ethanol for 15 seconds and then rinsed with sterile water. After rinsing with sterile water, they were dried on sterile tissue paper and cultured on agar plate (including YEMA medium). They were incubated from two days to one week.

According to Dubey and Maheshwari (2002), the Yeast Extract Mannitol agar (YEMA) media were prepared for the isolation of nitrogen fixing bacteria. The compositions of YEMA medium included Mannitol 10.0 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, Yeast extract 0.5 g, Agar 15.0 g and Distilled water 1000 ml. The pH adjusted as 6.8-7.0. After autoclaving, Nystatin 0.8 g was added to the medium to inhibit fungi.

After the isolation of nitrogen fixing bacteria from the root nodules of *Pisum sativum* L., the isolated bacteria were cultured in the liquid Yeast extract Mannitol agar medium (YEMA) and incubated for one week. Then the cultured medium (about 2 ml) was mixed in the beef solution to make up the final volume 1 litre and again incubated for four days. After the incubation period 4 days, the solution was used as fertilizer including nitrogen fixing bacteria.

Maintaining the seeds by applying nitrogen fixing bacteria solution

After 4 days incubation of the nitrogen fixing bacteria, the seeds of *Hibiscus cannabinus* L. were treated with nitrogen fixing bacteria solution and sown on tissue paper in petridish. The experiments were compared with control (water). Each treatment contains ten seeds and the total seeds were twenty seeds. They were kept at room temperature. Watering was done daily as necessary.

Then, the seeds of *Hibiscus cannabinus* L. were sown in polyethylene bags including natural field soil and the seeds treated with nitrogen fixing bacteria solution. The experiments were compared with the control of water. Each treatment contains five replications and each replicate with five seeds. The total number of seeds was fifty seeds. They were kept under shaded building and, the temperature 27±42°C and relative humidity 60-88%. Watering was done daily as necessary. The germination percentage was collected during 7 DAS. The germination percentage (GP %) was recorded in this study. The germination percentage calculated by using the formula. According to Mostafavi *et al*. (2011), the germination percentage formula was used as follows:

$$\text{Germination Percentage (GP, \%)} = \frac{\text{Number of germinated}}{\text{Total number of sowing}} \times 100$$

RESULTS

Soil analysis

The results of the natural field soil analysis indicated a neutral soil pH (6.5 – 7.0), low concentrations of nitrogen, phosphorus and potassium. The soil texture was sandy loam or loam.

Examination of nitrogen fixing bacteria isolated from *Pisum sativum* L.

After 45 DAS, the root nodules of *Pisum sativum* L. were observed in the treatment including $CaCl_2$ 250 mg/1 kg. The nitrogen fixing bacteria were isolated from the root nodules and cultured on Yeast Extract Mannitol agar (YEMA) media. After one-week incubation period, the isolated bacteria were examined under light microscope. The examination of macroscopical characters of the isolated nitrogen fixing bacteria were creamy white in color and the microscopical character was rod shaped in appearance under light microscope.

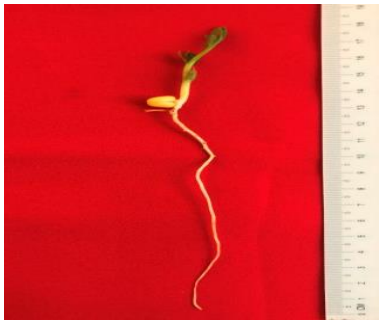
The results of the experiment showed that the germination percentage of T_0 (control) were 84% in 2 DAS and 4 DAS, and 88 % in 6 DAS. The germination percentage of T_1 (nitrogen fixing bacteria solution) were 92% in 2 DAS and it was observed 96% until 7 DAS. Therefore, the treatment T_1 (nitrogen fixing bacteria solution) was more percentage than that of the treatment T_0 (control).

Treatment	Germination Percentage (%)		
	2 DAS	4 DAS	6 DAS
T_0 (Control)	84%	84%	88%
T_1 (nitrogen fixing bacteria solution)	**92%**	**96%**	**96%**
F test	ns	ns	ns
5% LSD	28.2660	13.5785	13.5785
CV%	18.3	8.6	8.4

Table 1. Different germination percentage of *Hibiscus cannabinus* L.

ns – Non-significant

A



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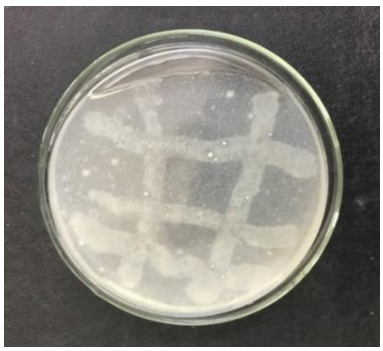

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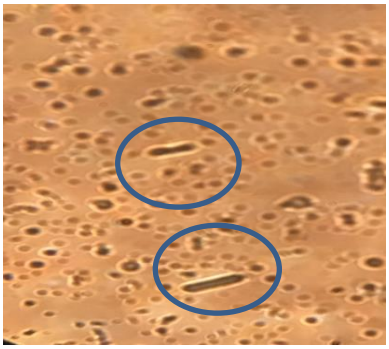
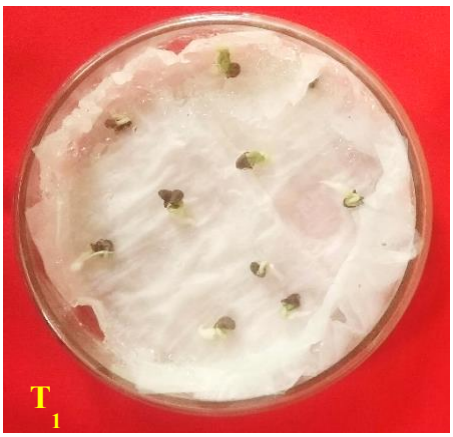


F

Figure 1. Isolation of nitrogen fixing bacteria from *Pisum sativum* L.

A. Seeds of *Pisum sativum* L.
B. Hypogeal germination of *Pisum sativum* L. in 8 DAS
C. Potted plants of *Pisum sativum* L. in 45 DAS
D. Formation of root nodules after 45 DAS
E. Macroscopical character of nitrogen fixing bacteria
F. Rod shaped nitrogen fixing bacteria

Effect of nitrogen fixing bacteria solution on germination test of *Hibiscus cannabinus* L.



A

B

Figure 2. Seedling growth of *Hibiscus cannabinus* L. in 2 DAS

A. Seedling growth of T_1 (nitrogen fixing bacteria solution)
B. Seedling growth of T_0 (control)

Figure 3. Comparison seedling growth of *Hibiscus cannabinus* L. in 7 DAS

DISCUSSION AND CONCLUSION

The present study was undertaken with the isolated nitrogen fixing bacteria taken from the root nodules of *Pisum sativum* L. treated with calcium chloride solution. The fertilizer was prepared by using the isolated nitrogen fixing bacteria as biofertilizer. Then the effect of nitrogen fixing bacteria solution treatments (T_1) and water control (T_0) on the germination and seedling growth of *Hibiscus cannabinus* L. were studied. The experiment was done in the rainy season. The germination percentage was recorded in this experiment. The germination percentage was calculated by using the formula and analysed by using IRRI software. The statistical analysis was done and led to the Fisher's test (F-test) and Coefficient of Variation (CV%).

It was indicated that the seedling emergence of *Pisum sativum* L. was important and, the soil including the concentration 250 mg/1 kg of calcium chloride solution was used as basal soil medium. The root nodules of *Pisum sativum* L. were obtained from 45 DAS.

In this study, the nitrogen fixing bacteria were isolated from the root nodules of *Pisum sativum* L. and cultured on Yeast Mannitol agar nutrient (YEMA) medium. It was consistent with Dubey and Maheshwari (2002), and Hamza and Alebejo (2017). The results of the macroscopical and micorscopical characters of nitrogen fixing bacteria were examined. It was observed that the macroscopical characters of isolated bacteria were observed as creamy white color. It agreed with Hamza and Alebejo (2017). The microscopical characters of nitrogen fixing bacteria were rod shaped in appearance. It coincided with Bhattacharya *et al.* (2013).

In germination experiment, the seeds of *Hibiscus cannabinus* L. were treated with T_0 (Control) and T_1 (nitrogen fixing bacteria solution). The sandy loam soil was used as basal nutrient media. It was suggested that the sandy loam soil would allow aeration in the soil media for good gas exchange between the germinating embryo and the soil. If the soil was not well aerated due to overwatering or compaction, the carbon dioxide would not dissipate. And the seeds could suffocate, and then oxygen was also found in soil pore spaces. The above suggestions were agreed with Raven *et al*. (2005). It indicated that the germination

type of *Hibiscus cannabinus* L. was epigeal germination. It was consistent with Meyer *et al*. (1960).

According to the statistical analysis results, the variability in samples from population of *Hibiscus cannabinus* L. were compared with the different means, Fisher's test (F test) and the coefficient of variation (CV%) in this experiment. According to the statistical analysis results, the F test of the germination percentage of the treatments was non-significant. The CV% of the germination of the treatments was 18.3% in 2 DAS, 8.6% in 4 DAS and 8.4% in 6 DAS respectively. The level of dispersion of the data was mostly <10% or 10-35% which was low or medium level of dispersion data. It agreed with Bowman (2001) and Banerjee (2004). Therefore, the mean separation of the treatments was representative for the data sets. Moreover, the germination experiment of *Hibiscus cannabinus* L. was regarded as the scientific research based on the statistical analysis.

Therefore, it could be concluded that the germination type of *Pisum sativum* L. was hypogeal germination. The seedling growth of *Pisum sativum* L. was preferable in light textured soils or sandy loam soil and pH 6.5-7.0 for germination. The root nodules were observed from the concentration of calcium chloride 250 mg/1 kg in soil and therefore it was indicated that the concentration could induce root to be nodulated.

Moreover, it could be concluded that nitrogen is an essential nutrient for plant growth and development. Therefore, *Pisum sativum* L. plays an important role in sustainable management of agriculture. Most legumes could provide enough nitrogen for their physiological needs. The plants *Pisum sativum* L. could be nodulated by nitrogen fixing bacteria. They were fast growing bacteria which were rich sources of nitrogen to the crop. Increasing usage of nitrogen fixing bacteria fertilizer would decrease the need for chemical fertilizers and reduce adverse environmental effects.

Finally, it could be concluded that the shape of nitrogen fixing bacteria isolated from the root nodules of *Pisum sativum* L. was rod shaped. The seedling growth of *Hibiscus cannabinus* L. was preferable in light textured soils (sandy loam soil) for germination. The good results of germination percentage of *Hibiscus cannabinus* L. were obtained from the treatment T_1 (nitrogen fixing bacteria solution). The germination type of *Hibiscus cannabinus* L. was epigeal germination. The statistical analysis of mean separation (5% LSD), Fisher's test (F-test) and the Coefficient of Variation (CV%) were essential levels of scientific experiments. The scientific experiments should be determined by using statistical analysis.

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