Endophytic *Pseudomonas* species from coastal weeds affecting *in vitro* phosphate solubilization and growth of wheat (*Triticum aestivum*) in Bangladesh

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**ABSTRACT**

The present study investigated the phosphate solubilizing potential and *in vitro* plant growth-enhancing capability of five bacterial isolates of *Pseudomonas* sp. isolated from the endophytic region of three different weeds namely *Cyperus rotundus*, *Amaranthus spinosus*, *Scirpus mucronatus* which were collected from the coastal region of Bangladesh. The bacterial species were identified as *Pseudomonas* sp. based on their morphological characteristics. For further confirmation, molecular characterization will be done. The isolates of *Pseudomonas* sp. showed varying degrees of phosphate solubilizing potential on Pikovskaya’s media. The results showed that isolate EnAgM1 had the utmost capability of phosphate solubilization with the competency of (77.43±0.65 %) and the second place was occupied by the isolate EnAgM2 with their competency of (74.92±0.95 %). Then the isolates were used for the assessment of their *in vitro* growth-promoting capability of wheat (*Triticum aestivum*). Isolate EnAgM3 showed the best growth promotion in respect of all the parameters studied except root fresh ounce whereas EnAgM1 also significantly enhanced all the parameters except germination percentage in comparison to control. Conclusively, this study revealed that the unexplored coastal region of Bangladesh hold potential microorganisms having phosphate solubilizing and the potential of plant growth enhancement. As far as we know, it is the first comprehensive study conducted for isolating *Pseudomonas* sp. as the endophytic bacteria from the coastal weeds of Bangladesh and to identify their *in vitro* potential of phosphate solubilization as well as plant growth enhancement of wheat.

**Keywords:** Endophytic bacteria, *Pseudomonas* sp., Wheat, Pikovskaya’s media, Phosphate-solubilizing bacteria

**INTRODUCTION**

The world population is increasing day by day while the cultivable land is decreasing at the same rate. Ensuring food for this increasing world population is one of the main challenges faced by the world. To cope up with this increasing population and to feed them, it has become necessary to boost up the crop yield. The use of chemical fertilizer for the improvement of soil fertility and crop yield in a short possible way has become a suitable alternative chosen by most farmers. Wheat (*T. aestivum*) is one of the major cereal crops that are up-taken by a big portion of the world population. The bread made from wheat is regarded as the staple food by people from different countries. In cultivation of wheat, the most crucial fact faced by the farmers is a deficiency of phosphorus in the soil as they easily get fixed in the soil and become unavailable for plants. Especially, where there is calcareous soil, the problem is more prominent (Ashrafuzzaman et al., 2009).

Phosphorus is up-taken by plants as orthophosphate ion: either H2PO4- or HPO42-. These anions get fixed in the soil if the soil contains cations like Ca2+, Mg2+, Fe3+ and Al3+ and become unavailable for the plants. For this reason, a very small amount of phosphorus mostly below the belt of optimum level remains available for the plants (Khan et al., 2010). To correct this deficiency, farmers apply a huge amount of phosphatic fertilizers to soil. In return, knowingly or unknowingly they are hampering soil health; ultimately the environment. Therefore, it has become hay time to generate new alternative ways to correct the situation in an inexpensive, eco-friendly as well as sustainable manner (Ashrafuzzaman et al., 2009). Introduction of microbial inoculants may prove to be a suitable replacement for chemical fertilizer in cultivation of wheat.

Researchers have been working for decades to un-riddle the situation and they already have explored the phylosphere, rhizosphere and endosphere regions to find out the startling microbial agent having not only the capability of plant growth promotion but also having the potential of disease suppression (Abbamondi et al., 2016). Luckily, we do have such types of microbial agents occurring naturally in the environment. They have a tremendous capability of phosphate-solubilization, nitrogen fixation, siderophore production ultimately growth promotion. Among all the microbes occurring in phylosphere, rhizosphere and endosphere; the microbes prevailing in the endosphere region are the most sophisticated ones, as they remain inside the plants. They may be found in roots, stems or leaves (Ray et al., 2018). The endosphere of plants is the playground of a wide range of microbial agents and most of the plants occurring in nature act as the Boniface of numerous endophytic microbes (Strobel et al., 1993). The natural openings or injuries may pave the way for establishment of them inside a plant or they may enter directly (Hallmann et al., 1997). The involvement of endophytic bacteria in some noble acts like plant growth promotion and making insoluble phosphorus in their soluble form has drawn the attention of
researchers towards them. Till today, a wide range of economically important crops have been studied for the isolation of endophytic bacteria with noble traits (Bacon and Hinton, 2006).

Therefore, the application of endophytic bacteria having phosphate solubilizing efficiency may replace phosphatic fertilizers in the production of wheat (Ashrafuzzaman et al., 2009; Sial et al., 2015). Among the bacterial genera, the names which dominate in the section of phosphate solubilization as well as plant growth promotion are Pseudomonas, Bacillus, Rhizobium, Burkholderia, Agrobacterium, Flavobacterium, Erwinia, Achromobacter, Micrococcus etc. They have the potential to solubilize the insoluble forms of phosphate such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, etc. and bring back them in their soluble form which can be uptaken by the plants. For these reasons, they may be used as biofertilizer in the field. Application of them as biofertilizer in crop fields may enhance the yield of economically important crops such as cereal crops, legumes, oil crops, a wide range of vegetables etc. (Khalimi et al., 2012; Viruel et al., 2011). There is a huge amount of them existing in the plant rhizosphere or in the plant endosphere which should be explored extensively and formulate them as biofertilizer. Already there are ongoing researches on this topic around the world. But information regarding, isolation of native root-associated endophytic bacteria from the weeds of the coastal region and their application as bio-inoculant in Bangladesh is scanty. That’s why the study was focused on the isolation and screening of some indigenous endophytic bacteria from weeds collected from the coastal region of Bangladesh and assessment of phosphate solubilizing capabilities as well as in vitro plant growth enhancement attributes of those isolates on a local variety of wheat.

MATERIALS AND METHODS

Sampling and isolation of endophytic root-associated bacteria from the coastal weeds:
The weed samples used in this experiment were collected from the coastal area of Bangladesh (Noakhali). The weeds specimens were randomly picked, put in plastic zip carrier, brought to the University laboratory & preserved at refrigerator maintaining temperature of 4° C. About ten samples of weed were collected. Among them, three weeds (Cyperus rotundus, Amaranthus spinosus, Scirpus mucronatus) were used for the experiment (Table 1).

To isolate endophytic bacteria, the samples were washed thoroughly in tap water then the sterilization procedure was done. For this purpose, the samples were sequentially dipped in 70 % ethanol for 1min then they were washed with sterilized distilled water, again they were immersed in 1 % sodium hypochlorite solution for 40 seconds and were rinsed with sterilized distilled water for removing residual sodium hypochlorite from the samples. Then they were air-dried. After that 1 mg root sample was taken from each type of weeds and ground to make a paste using sterilized distilled water in a mortar pastel. Then a mixture was put in an Eppendorf tube containing 1.5 ml distilled water and was undergone to vortex for one minute and it was considered as stock solution. Then series of dilutions 10^0, 10^-1, 10^-2, 10^-3, 10^-4 were made. From the diluted suspension, 0.1 ml of suspension from 10^-4 dilution was taken for plating by following spread plate technique in nutrient agar medium. The plates were looked on after 2 days of incubation for the enumeration of bacterial colonies. Well-separated, single colonies were taken for re-streaking onto the nutrient agar media. This procedure was repeated until the pure culture was obtained.

Table 1: List of the weeds and their associated bacterial isolates

| SL No | Isolate Name | Associated weed               |
|-------|--------------|--------------------------------|
| 01    | EnAgK1       | Cyperus rotundus               |
| 02    | EnAgC2       | Cyperus rotundus               |
| 03    | EnAgM2       | Cyperus rotundus               |
| 04    | EnAgC3       | Amaranthus spinosus            |
| 05    | EnAgC4       | Scirpus mucronatus             |

In vitro efficacy of the bacterial isolates on phosphate solubilization attributes:
Pikovskaya medium were used for the growth of bacteria on them to find out their phosphate solubilizing attribute (Pikovskaya, 1948). After spotting them on the medium, they were observed for halo formation around the colony of the isolates. It took about 5 days of incubation for the formation of a halo which was then measured in cm. This noble attribute of phosphate solubilization of the bacterial isolates were calculated by using specific formula (Premono et al., 1996).

\[
\text{Phosphate Solubilizing Efficiency (Z)} = \frac{Z - C}{C} \times 100
\]

Here,

- \( Z \) = zone of phosphate solubilization (the halo)
- \( C \) = radius of the colony

Morphological and biochemical study (gram reaction and catalase test) of the isolates:
Morphological characters of the isolates were identified by the colony color and odor. Gram staining of the isolates was studied following the standard procedure (Archana et al., 2013). After gram reaction catalase tests were performed by following a standard protocol (MacFaddin, 1980).

Identification of the bacterial isolates based on morphological and biochemical characteristics:
Based on the morphological and biochemical characteristics the bacterial isolates were identified by comparing the isolates with the standard species described in Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994). But for further confirmation and characterization, the isolates will be identified based on molecular characterization by using 16S-rRNA gene sequencing. For the evaluation of the genetic diversity among the isolates, Randomly Amplified Polymorphic DNA fingerprinting and similarity index will be measured.
**In vitro effect of the bacterial isolates on the growth parameters of wheat:**
To assess the influence of the isolates on the growth parameters of wheat, the seeds of wheat were treated with each of them separately. For this purpose, five falcon tubes were taken where 1500 µL sterilized distilled water was added & one loopful of the bacterial isolate was suspended in the respective tubes. After that, they were put in the vortex mixer for about 5 seconds to evenly distribute the bacterial cells in the water. About thirty treated wheat seeds were taken from each of the falcon tubes and kept overnight inside the laminar airflow on dried filter paper. On the next day, the treated seeds were placed onto dampened filter paper on Petri plates.

Wheat seeds, normally washed with sterile distilled water were maintained as control. Then the parameters audited were as follows a) germination percentage, b) shoot & root extent (cm), c) root-shoot fresh ounce & dry ounce (mg), d) vigour index. The following formula was used for the calculation of germination (%):

\[
\text{Percentage of Germination} = \frac{\text{Number of germinated seeds}}{\text{Number of total seeds}} \times 100
\]

The formula which was used to calculate the vigour index (VI) is given underneath:

\[\text{VI}= (\text{RE+ SE}) \times \text{GP}\]

Here,
- RE= Root Extent (cm)
- SE= Shoot Extent (cm)
- GP= Germination Percentage

**Statistical analysis and design of experiment:**
Data recorded for germination percentage and growth parameters were compiled & analyzed statistically at 5% probability level by using one way ANOVA test as well as LSD test in Statistix 10 software & Microsoft Excel 2016. Completely Randomized Design (CRD) was followed for arranging the experiment with 3 replications.

**RESULTS**

**Morphological and biochemical study of the isolates:**

Around 16 bacterial isolates were found. Among them, 5 isolates were used for bio-chemical test and morphological characterization (Table 2) as well as for further study. Based on the morphological and biochemical test and after comparing with the standard species described in Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994), the bacterial isolates were identified preliminary as *Pseudomonas* sp. However, for further confirmation, molecular characterization was needed. And the isolates are already in the process of molecular characterization. For partial confirmation, 16S-rRNA gene sequencing will be used.

**Table 2. Biochemical test and identification of the bacterial isolates**

| Sl. No. | Name of the Isolate | Colony morphology (color) | Shape | Odor | Catalase test | Gram reaction |
|--------|---------------------|---------------------------|-------|------|---------------|---------------|
| 1.     | EnAgK2 (P*)         | Orange                    | Rods  | Odorless | Positive     | Negative      |
| 2.     | EnAgC5 (P*)         | White                     | Rods  | Odorless | Positive     | Negative      |
| 3.     | EnAgM2 (P*)         | Orange                    | Rods  | Odorless | Positive     | Negative      |
| 4.     | EnAgC3 (P*)         | White                     | Rods  | Odorless | Positive     | Negative      |
| 5.     | EnAgM3 (P*)         | Yellow                    | Rods  | Odorless | Positive     | Negative      |

*Bacterial dilutions 10−4, *P= Pseudomonas sp.

**Phosphate solubilizing efficiency of the isolates of *Pseudomonas* sp.**

The noble trait of phosphate solubilization of the isolates was determined by observing the clear halo around the colony. EnAgM3 exhibited the highest phosphate solubilizing efficiency of 77.43% on PVK medium among the isolates in in vitro conditions. Isolates EnAgM2 & EnAgK2 showed phosphate solubilizing efficiency of 74.92% & 71.43% respectively (Fig. 1).
In vitro effect of the bacterial isolates on the growth parameters of wheat:
Effect on wheat seed germination percentage (%) of the isolates of *Pseudomonas* sp.

Results revealed that germination percentage of wheat increased, while the seeds were treated with the bacterial isolates than control. According to our study, a significantly higher germination percentage was observed in EnAgM3 (90.00%) treated seed of wheat over control (63.33%) (Fig. 2). Other treatments showed no significant differences over control.

![Germination Graph](image)

**Fig 2.** Impact of the isolates of *Pseudomonas* sp. on wheat seed germination (%)

Impact on the shoot and root extension of wheat:
Significantly higher shoot length was found in the seedlings in which seeds were treated with EnAgM3 (8.59 cm) isolate & lower shoot length was found in EnAgC3 (3.67 cm) treated seedlings (Fig. 3). Like the significant extension of shoot, root extension also took place at a significant rate after treating the seeds with the bacterial inoculum. Significantly higher root length was found in EnAgM3 (9.17 cm) treated seedlings & lower root length was observed in the seedlings whose seeds were treated with EnAgC3 (4.33 cm) bacterial isolate.

![Shoot and Root Extension Graph](image)

**Fig 3.** Impact of the isolates of *Pseudomonas* sp. on the shoot and root extension of wheat

Impact on the shoot and root fresh ounce of wheat (mg) :
Seed treatment with the isolated bacteria significantly (P<0.05) enhanced the shoot fresh ounce of wheat. The seedlings treated with the EnAgM3 isolate produced significantly higher shoot fresh ounce (653 mg), which was superior to other treatments & the lower shoot fresh ounce was found in EnAgC3 (367 mg) treated seedlings (Fig. 4). Significantly higher root fresh ounce was found in EnAgM2 (250.00 mg) treated seedlings & the lowest shoot fresh ounce was found in EnAgC3 (100 mg) treated seedlings.

![Shoot and Root Fresh Ounce Graph](image)

**Fig 4.** Impact of the isolates of *Pseudomonas* sp. on the shoot and root fresh ounce of wheat
Impact on the shoot and root dry ounce (mg) of wheat:
Significantly higher shoot and root dry ounce was observed in the seedlings which were treated with EnAgM3 isolate over control, where shoot dry weight was obtained 70.0mg and root dry weight were obtained 33.3mg (Fig 5).

Impact on the vigour index of wheat:
In our study, it was found that the endophytic bacterial isolates have an impressive effect on the vigour index of the wheat seedlings, while they were tested *in vitro*. Significantly higher vigour index was found from the seedlings whose seeds were treated with EnAgM3 (1584.0) isolates & lower vigour index was observed in EnAgC3 (444.0) treated seeds over control (567.60) (Fig 6).

**DISCUSSION**

Microbes that reside inside the plant parts (root, shoot, leaf etc.) without causing any damage to the respective plant are known as endophytic microbes. The interaction of novel endophytic microbes especially bacterial species and plants can promote plant health directly through N2 fixation which promote biomass production; production of phytohormones, they help in elongation of root and shoot length; through phosphorus, potassium, and zinc solubilization which help in seed germination, flowering ultimately increase the yield of crops (Hallmann et al., 1997; Rosenblueth and Martínez-Romero, 2006; Verma et al., 2013). Sustainable production of crops has become an indispensable need of today’s century. It has been indicated several times that the world population may get doubled in the upcoming decade. To feed this additional population, we have no other option but to increase the yield of the major crops up to 50 % (Godfray et al., 2010). In Bangladesh, the amount of wheat-based product up-taken by the people, position just after rice. Phosphorus deficiency in soil may obstruct wheat production or may lessen yield as they are needed for the proper root & shoots elongation of wheat (Kochian, 2012). From the applied phosphatic fertilizers, after getting fixed in the soil, an only one-fifth portion of phosphorus is reprieved by the wheat plants (Rodriguez and Fraga, 1999). This scenario is louder in the coastal areas due to the presence of Ca2+ and Na+ ions. In this regard, phosphate-solubilizing bacteria may play a great role in solubilizing inorganic phosphate in the best eco-friendly manner. It has been one of the mooted topics of research by researchers around the world to explore various plants in search of endophytic bacterial isolates with phosphate solubilizing capability. A wide range of bacterial isolates of different species has been found while exploring the endophytic region of plants and they’ve been found to enhance plant growth as well as have the capability to bring back the phosphorus to its soluble form (Rodriguez and Fraga, 1999). This revolutionary characteristic of the bacterial isolates has a significant impact on crop yields (Ponmurugan and Gopi, 2006).
In our study, we isolated endophytic bacteria (Pseudomonas sp.) from coastal weed and about sixteen isolates were obtained from which five bacterial isolates were identified as Pseudomonas sp. based on their morphological and biochemical characteristics. It was found as plant growth-promoter. It has been proved in several studies that the species of Pseudomonas is a potential phosphate solubilizer. They solubilize phosphate by producing organic acids in the culture media which in return lower the pH of the media (Whitelaw et al., 1999). There are some Pseudomonas species which has gain recognition of efficient phosphate solubilizer as well as a plant growth promoter. Pseudomonas putida (Kumar et al., 2001), Pseudomonas fluorescens (Choi et al., 2008) and Pseudomonas stutzeri (Vazquez et al., 2000) are the short-listed ones.

In several studies it was found that the endophytic bacteria having phosphorus solubilizing potential were able to promote root and shoot growth, biomass production, ultimately increase the yield of different agriculturally important crops (Kumar et al., 2001; Capper, 1986). They may enhance growth by producing plant growth hormones (Patten and Glick, 2002; Bottini et al., 2004). It was also found by several authors that the bacteria Pseudomonas sp. have the potential to enhance the growth of wheat (Rosas et al., 2009). In our study, we found similar results. The isolates were not only able in solubilizing phosphorus but also promoted the growth of wheat. Among the five isolates, EnAgM3 was found the best in enhancing germination (%) (90.00 %), shoot and root extent (8.59 cm and 3.67 cm), shoot and root fresh ounce, shoot and root dry ounce and vigour index. In our study, we identified the isolates based on their morphological characteristics. However, their molecular characterization is needed. After their molecular-based characterization, we may use the bacterial isolates as potential biofertilizers at the field level.

CONCLUSION

Five bacterial isolates of Pseudomonas sp. having the potential of phosphate solubilisation were isolated from different weeds, collected from coastal regions of Bangladesh and were assessed for their in vitro growth enhancement of wheat. The outcome of the study indicated that the EnAgM3 isolate of Pseudomonas sp. was an efficient phosphate solubilizer, and was also able to promote the growth parameters of wheat. Further molecular studies and field level trials of the isolates may help in the introduction of new bio-inoculants having both the characters of phosphate solubilisation and growth promotion of wheat; which may lessen the use of excessive fertilizers in the field. And this will lessen the cost of production and will sustain the production of wheat.

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Endophytic Pseudomonas sp., a promising source of phosphate-solubilizing and growth-promoting bacteria in Bangladesh coastal areas

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 انواع من بكتيريا السيدوموناس النباتية الداخلية المعزولة من بعض الحشائش الساحلية وتأثيرها في المختبر على إذابة الفوسفات وتعزيز نمو القمح في بنغلاديش

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الملخص

بحثت الدراسة الحالية الفعالية إذابة الفوسفات وعقارية نمو النبات في المختبر لخمسة عزلات بكتيرية من Pseudomonas sp. معزولة من المنطقة الداخلية لثلاثة أعشاب مختلفة هي: Amaranthus، Cyperus rotundus، Scirpus mucronatus، Spinosus amaranthus، Cyperus. تم تحديد الأنواع البكتيرية على بناء على خصائصها المورفولوجية. لمزيد من التأكد، سيتم إجراء التوصيف الجزيئي. عجزت عزلات Pseudomonas sp. أنها أظهرت درجة متفاوتة من إمكانية إذابة الفوسفات على وسائط بيوكوفسكايا. أظهرت النتائج أن عزلة 3 EnAgM 2 كان له القدرة القصوى على إذابة الفوسفات بكفاءة 63 ± 4.75% (بينما احتلت العزلة EnAgM 3 المرتبة الثانية بكفاءتها 95 ± 7.42%). تم استخدام العزلات لتقسيم قدرتها على تعزيز نمو القمح في المختبر للقمح Triticum aestivum. أفضل عزلة تعزيز للنمو فيما يتعلق بجميع المعلمات التي تمت دراستها أظهر عزلة EnAgM 3. أيضاً، أظهرت كفاءة عزلة EnAgM 2 في تأثير سلبي على نمو الوراثة. كشفت هذه الدراسة أن المنطقة الساحلية غناء المكتشفي في بنغلاديش تحتوي على كائنات دقيقة محتملة لها دوران الفوسفات وعقارية نمو النبات. وتعزيز ما نعلم، فهي أول دراسة شاملة أجريت لعزل Briste Tabassum

كلمات المفتاحية: بكتيريا Pseudomonas sp.، Endophytic، القمح، نمو نبات الفوسفات.