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

Preangka Saha Briste¹* 💿 and Chowdhury Nafisa Tabassum²

Address

¹Department of Plant Pathology, Faculty of Agriculture, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh

²Department of Agriculture, Faculty of Science, Noakhali Science and Technology University, Noakhali, Bangladesh

* Corresponding author: Preangka Saha Briste, Email: briste@bsmrau.edu.bd

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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 EnAgM₃ 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 EnAgM₂ 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 $H_2PO_4^-$ or HPO_4^2 . These anions get fixed in the soil if the soil contains cations like Ca^{2+} , Mg^{2+} , Fe^{3+} and Al^{3+} 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 phyllosphere, 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 phyllosphere, 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 growth and 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 10⁻¹, 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.

SL No	Isolate Name	Associated weed	
01	EnAgK ₂	Cyperus rotundus	
02	EnAgC₅	Cyperus rotundus	
03	EnAgM₂	Cyperus rotundus	
04	EnAgC₃	Amaranthus spinosus	
05	EnAgC₅	Scirpus mucronatus	

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

In vitro efficacy of the bacterial isolates on phosphate solubilization attributes:

Pikovskaya mediums 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).

Phosphate Solubilizing Efficiency (%) =
$$\frac{2}{C} \times 100$$

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 μ L 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 (%):

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Percentage of Germination = <u>Number of germinated seeds</u> ×100
Number of total seeds
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The formula which was used to calculate the vigour index (VI) is given underneath:

VI= (RE+ SE) × 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.

Sl. No.	Name of the Isolate	Colony morphology (color)	Shape	Odor	Catalase test	Gram reaction
1.	EnAgK ₂ (P*)	Orange	Rods	Odorless	Positive	Negative
2.	EnAgC₅ (P*)	White	Rods	Odorless	Positive	Negative
3.	EnAgM ₂ (P*)	Orange	Rods	Odorless	Positive	Negative
4.	EnAgC ₃ (P*)	White	Rods	Odorless	Positive	Negative
5.	EnAgC₅ (P*)	Yellow	Rods	Odorless	Positive	Negative

Table 2. Biochemical test and identification of the bacterial isolates

*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).

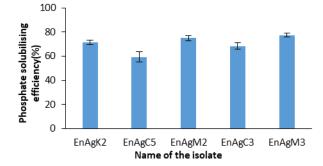


Fig 1. Phosphate solubilizing efficiency of the isolates of Pseudomonas sp.

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 then control. According to our study, a significantly higher germination percentage was observed in $EnAgM_3$ (90.00%) treated seed of wheat over control (63.33%) (Fig. 2). Other treatments showed no significant differences over control.

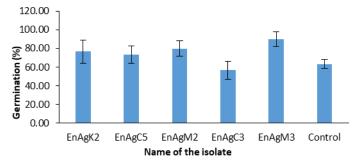
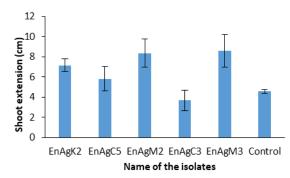


Fig 2. Impact of the isolates of Pseudomonas sp. on wheat seed germination (%)

Impact on the shoot and root extension of wheat:

Significantly higher shoot length was found in the seedlings in which seeds were treated with $EnAgM_3$ (8.59 cm) isolate & lower shoot length was found in $EnAgC_3$ (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 $EnAgM_3$ (9.17cm) treated seedlings & lower root length was observed in the seedlings whose seeds were treated with $EnAgC_3$ (4.33cm) bacterial isolate.



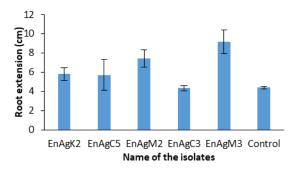


Fig 3. Impact of the isolates of Pseudomonas sp. on the shoot and root extension of wheat

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.

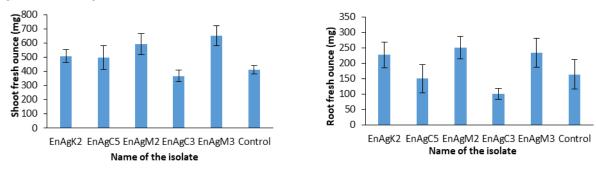


Fig 4. Impact of the isolates of Pseudomonas sp. on the shoot and root fresh ounce of wheat

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).

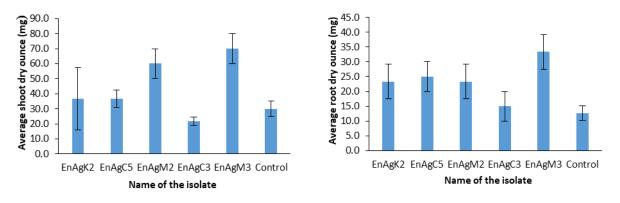


Fig 5. Impact of the isolates of Pseudomonas sp. on the shoot and root dry ounce of wheat

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) isolates & lower vigour index was observed in EnAgC₃ (444.0) treated seeds over control (567.60) (Fig 6).

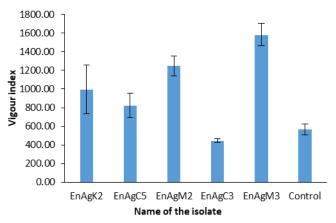


Fig 5. Effect of the isolates of Pseudomonas sp. on the vigour index

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

¹*برينجكا ساحة بريست ²شوذر نفيسة تبسم ¹قسم أمراض النبات ، بانغاباندو جامعة شيخ مجبور الرحمن الزراعية ، غازيبور 1706- ، بنغلاديش ²قسم الزراعة ، جامعة نواخالى للعلوم والتكنولوجيا ، نواخالى ، بنغلاديش ***البريد الإلكتروني للمؤلف المراسل:**briste@bsmrau.edu.bd

العربى الملخص

بحثت الدراسة الحالية في إمكانية إذابة الفوسفات وقدرة تعزيز نمو النبات في المختبر لخمسة عزلات بكتيرية من Amaranthus وCyperus rotundus وعن المنطقة الداخلية لثلاثة أعشاب مختلفة هي Cyperus rotundus و Cyperus البكتيرية على و Scirpus mucronatus يتم جمعها من المنطقة الساحلية لبنغلاديش .تم تحديد الأنواع البكتيرية على أنها .Pseudomonas sp بناءً على خصائصها المورفولوجية .لمزيد من التأكيد سيتم إجراء التوصيف الجزيئي .عزلات أنها .Pseudomonas sp أظهرت درجة متفاوتة من إمكانية إذابة الفوسفات على وسائط بيكوفسكايا .أظهرت النتائج أن عزل EnAgM3 كان له القدرة القصوى على إذابة الفوسفات بكفاءة50.0 ± 77.43 / (بينما احتلت العزلة 2 المرتبة الثانية بكفاءتها 20.5 ± 74.92 // (ثم استخدمت العزلات لتقييم قدرتها على تعزيز النمو في المختبر للقمح . المرتبة الثانية بكفاءتها 20.5 ± 74.92 // (ثم استخدمت العزلات لتقييم قدرتها على تعزيز النمو في المختبر للقمح . المرتبة الثانية بكفاءتها 20.5 ± 74.92 // (ثم استخدمت العزلات لتقييم قدرتها على تعزيز النمو في المختبر للقمح . المرتبة الثانية بكفاءتها 20.5 ± 74.92 // (ثم استخدمت العزلات لتقييم قدرتها على تعزيز النمو في المختبر للقمح . باستثناء الأوقية الطازجة للجذر بينما عزز EnAgM3 أيضًا بشكل كبير جميع المعلمات التي تمت دراستها باستثناء الأوقية الطازجة للجذر بينما عزز EnAgM3 أيضا بشكل كبير جميع المعلمات التي تمت دراستها باستثناء الأوقية الطازجة للجذر بينما عزز EnAgM3 أيضًا بشكل كبير جميع المعلمات التي تمت دراستها باستثناء الأوقية الطازجة للجذر بينما عزز EnAgM3 أيضًا بشكل كبير جميع المعلمات التي تمت دراستها باستثناء الأوقية الطازجة للجذر بينما عزز EnAgM3 أيضًا بشكل كبير جميع المعلمات التي تمت دراستها باستثناء الأوقية الطازجة للجذر بينما عزز EnAgM3 أيضًا بشكل كبير جميع المعلمات باستثناء نسبة الإنبات مقارنة والسليم في مالمالي قائبة أعراب . وتقيقة محتملة لها ذوبان الفوسفات وإمكانية تعزيز نمو النبات .وبقدر ما نعلم ، فهي أول دراسة شاملة أجريت لعزل دقيقة محتملة لها ذوبان الفوسفات وأمكانية مناز مو النبات .وبقدر ما نعلم ، فهي أول دراسة شاملة أجريت لعزل لإذابة الولي المات وكذلك تعزيز نمو نبات القمح. لإذابة الفوسفات وكذلك تعزيز نمو نبات القمح.

الكلمات المفتاحية : بكتيريا Pseudomonas sp. ، Endophytic، قمح ، وسط بيكوفسكايا، قوة تذويب الفوسفات