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Article

Isolation and Molecular Diagnosis of Enterobacter cloacae and Kineococcus radiotolerans from Red Clover nodules and evaluation of the prepared inoculum from them as a stimulator for plant growth

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ABSTRACT

To obtain biological inoculum that promotes plant growth and contributes to a clean environment and sustainable agriculture, twentyone samples were collected from the root nodes of the red clover plant (Trifolium paratens) grown in gypsiferous soils at the Research Station of the Department of Soil Sciences and Water Resources - Faculty of Agriculture - University of Tikrit, samples were cultured in the medium of YEMA. Two isolates belonging to *Enterobacter cloacae* and *Kineococcus radiotolerans* were diagnosed phenotypically and molecularly. The diagnosis was confirmed by analyzing the nitrogen bases sequence of the DNA of the 16S rRNA gene. It was recorded in the National Center Biotechnology Information(NCBI) with the numbers MN310027.1 and KT216573.1 respectively, these isolates were activated and tested their efficiency as a bio-stimulant by testing their ability to dissolve insoluble phosphate compounds and produce indole acetic acid and chelating compounds, the results showed the ability of all isolates to produce indole acetic acid (IAA), chelating compounds, and solubility of phosphates, *E. cloacae* isolate outperformed *K. radiotolerans* isolates in these parameters, as it gave *E. coloaca* solubilization of phosphate and IAA production of 40.3 mg p L-1 and 11.2 µg ml-1 respectively, and it showed a high output of the chelating compounds compared to the treatment inoculated with *K. radiotolerans*, which gave 27.20 mg p L-1 and 7.21 µg ml-1, and medium production of iron chelating compounds, the results also showed the superiority of the inoculated treatments over the uninoculated treatments in the percentage of germination, the speed of germination, the length of tomato seedlings, the dry weight of the Shoot and root parts and the number of leaves, and the results showed the superiority of the inoculated treatment with *E. cloacae* significantly on K. radiotolerans isolate.

Keywords: *Enterobacter cloacae*; *Kineococcus radiotolerans*; Molecular Diagnosis; Bacterial inoculation; Eggplant seedlings.

INTRODUCTION

Producing healthy and robust seedlings of vegetable crops is the first pillar for obtaining early and high production, especially for crops that reproduce by seedling methods, such as eggplants, tomatoes and peppers, as this method provides an excellent opportunity for early production and economy in the number of seeds,

obtaining high production, and excluding diseased and weak seedlings [1]. The problem in the production of vegetable crops is their dependence on chemicals (fertilizers, pesticides and hormones) to encourage their growth, increase production, and resist various pathogens. Therefore, the trend was to use natural, non-chemical, organic or biological environmentally friendly products that stimulate seed germination, encourage plant growth, increase resistance to diseases, reduce the use of chemical products, and contribute to a clean environment and sustainable agriculture; the result is healthy and robust seedlings that are the basis for high production and early vegetable crops [2, 3]. Many studies demonstrated the ability of plant growth-promoting rhizobacteria PGPR species to stimulate the growth of different crops and increase their yield and resistance to diseases through various mechanisms such as nitrogen fixation, solubilization of insoluble phosphorous and potassium compounds and the production of growth regulators such as IAA and chelating compounds through that stimulate and encourage plant growth [4, 5].

Enterobacter cloacae is a bacterial genus that inhabits the Rhizosphere zone, in the crust and inside the root. The effectiveness of this bacteria can be utilized in the field of biofertilization and sustainable agriculture. It has been developed and counted among the best-studied genera of bacteria that encourage plant growth PGPR [6]. This bacterium encourages plant growth through various mechanisms, including nitrogen stabilization and the production of growth regulators such as Indol Acetic Acid (IAA), Cytokines, Gibberellins, and other amines. Thus, it helps to develop roots and to increase the absorption of water and nutrients by plants [7]. *K. radiotolerans* is a gram-positive, coccoid-shaped, motile organism isolated from a radioactive work area. It is an orange-pigmented bacterium that is catalase-positive, oxidase, and urease-negative; it can bear high levels of resistance to gamma-radiation[8]; the capabilities to produce secondary metabolites and enzymes for biotechnological and agricultural applications make it one of the most essential microbes among prokaryotes[9]. To produce healthy and active eggplant seedlings, this study evaluated the inoculum prepared from two local bacterial isolates in stimulating seed germination and encouraging the growth of eggplant seedlings.

MATERIALS AND METHODS

Isolation of Bacteria from root nodules

Twenty-one samples were collected from the root nodules of the red clover plant grown in gypsiferous soils at the Research Station of the Department of Soil Sciences and Water Resources - Faculty of Agriculture - University of Tikrit; the roots were thoroughly washed with water to get rid of the stuck dust, then the nodules were washed with water several times and then with 95% ethyl alcohol for 5-10 seconds, the nodules were immersed in 0.01% HgCl2 for 2 minutes [10], the nodules were transferred to sterile dishes and then pulverized with a small volume of sterile distilled water using glass rod to obtain a bacterial suspension, then 1 ml of bacterial suspension was planted on the medium of YEMA and then the dishes were incubated at a temperature of 28^0C for 3 – 7 days [11, 12].

Identification of Bacteria

Biochemical and Phenotypic Diagnosis

Phenotypical and microscopic tests were carried out that included colony shape, colony color, bacterial cell shape, and Gram stain, as well as some biochemical tests that involved the catalase, oxidase, and urea hydrolysis test [13].

Genomic DNA Extraction

To extract genomic DNA, a swab of 100 mg was used from a newly grown pure colony (24 hours old). DNA was extracted using Kit type ZR Fungal / Bacteria / Yeast DNA MiniprepTM (Zymo Research, USA, block number 17045), and then the 16s RNA gene was doubled using the PCR polymerase chain reaction. With the use of 5'- AGAGTTTGATCCTGGCTCAG-3 and 5'- GGTTACCTTGTTACGACTT-3 primers, the primers were prepared by Integrated DNA Technologies Company (Canada), the polymerase chain reaction was carried out with a volume of 25 µl as follows: 1.5 µl DNA, 5 µl Taq PCR PreMix (Intron, Korea), 1 µl from each primer (10 picols), and then distilled water was added to complete the volume of 25µl. As for the reaction program, it included 40 cycles, and each cycle included the following: a) Initial Denaturation at a temperature

of 95°C for 5 min, b) Denaturation -2 at a temperature of 95°C for 45 sec, c) Annealing at 58^{0}C for 45 sec. d) Extension-1 at 72^{0}C for 45sec, e) Extension -2 at 72°C for 7 min.
A thermal polymerization device (PCR system, Gene Amp Applied Biosystem, 9700) was used in the replication process. The PCR product was separated using electrophoresis on a 1.5% agarose gel. Then, the bands were revealed using ultraviolet light (302nm) after staining with a red stain (Intron Korea)[14].

Nucleotide Sequence and Congruence
The nucleotide sequencing process was carried out at the National Instrumentation Center for Environmental Management (http://nicem.snu.ac.kr/main/?en_skin=index.html) using a DNA sequencer (Applied Biosystem, DNA sequencer3730XL). To study the congruence of the resulting sequence of the genetic tree tag, the BLAST program available on the NCBI website (http://www.ncbi.nlm.nih.gov) and the Mega X program were used. The comparison was made with the nucleotide sequences recorded in the genome bank. The isolates were recorded in the genome bank with the numbers *E. cloacae*-1103 and *K. radiotolerans*-1100, as a global number was recorded for each isolate.

Efficiency of bacterial isolates in dissolving phosphate and producing indole acetic acid and iron chelating compounds:
The efficiency of the isolates in dissolving phosphate according to the method shown by [15], producing indole acetic acid described by [16] and the production of iron chelating compounds[17].

Steps for preparing the bacterial inoculum
Pure isolates of young bacterial culture 24 hours old *E. Cloacae* and K. *radiotolerans* were grown in 500 ml flasks containing 300 ml of nutrient broth liquid media. The cultures were incubated in a shaking incubator at 28° C at a speed of 100 cycles minute^{-1} for 3-5 days; the numbers of bacterial cells were counted by dilution and plate counting to ensure that the bacterial number reached the threshold number of 108 CFU.ml^{-1} [18]. Sterilize the seedling medium and adjust the pH by adding 5% calcium carbonate. Seedling dishes were filled with sterilized peat moss, and the eggplant seeds were sterilized by washing them well with water several times to remove the fumigated substance sticking to their surfaces and removing it entirely. Then, it was soaked in a solution of 0.01% HgCl2 for 30 seconds to eliminate the organisms on their surfaces; then, it was washed well with water 3-4 times to remove the remaining traces of the sterile material. Eggplant seed variety Kamer was sown in seedling dishes by placing a seed in each hole; then, the seedling dishes were inoculated according to the treatments by injecting 3 ml of the inoculum into each hole around the seeds; the experiment was carried out in the greenhouse within the design of the randomized complete design (CRD) with three replicate to study the effect of inoculation with two local bacterial isolates in promoting the growth of eggplant seedlings (Solanum melongena).

Studied traits
The following traits were evaluated: germination percentage, germination speed, according to Kotowski (1969), dry root and vegetative weight (gm seedling-1), number of plant leaves (leaf. plant-1), and seedlings height.

RESULT

Biochemical and Phenotypic Diagnosis

Two isolates belonging to the genera *E. cloacae* and K. *radiotolerans* were diagnosed from the root nodules of red clover. Table (1) shows the phenotypic characteristics and some biochemical tests adopted in the diagnosis. The results showed that *E. cloacae* were negative for Gram stain and oxidase and Urea hydrolysis tests. At the same time, they were positive for the Catalase test, and their colonies appeared in a circular white color 13. As for *K. radiotolerans*, it was positive for the Gram stain and Catalase test and negative for both the Oxidase and Urea hydrolysis test. As for their colonies, their shapes vary according to the age of the

cell. When they are young, the colonies appear in a smooth and wet circular shape, but with time, they turn into rough and dry colonies that appear in an irregular mass [8].

Characters	***Enterobacter cloacae***	***Kineococcus radiotolerans***
Colony morphology	Rounded colony- entire edge	Young colonies are moist, smooth and round but transition into a rough, dry, raised colony mass of irregular shape over prolonged incubation.
Pigmentation	White(yellowish)	Orange
Gram staining	Negative	Positive
Cells shape	Rod-shaped	Coccoid
Catalase	+	+
Oxidase	-	-
Urea hydrolysis	-	-

Table 1. Some morphological properties and biochemical tests of Enterobacter cloacae and Kineococcus radiotolerans.

Nucleotide Sequence and Congruence

Figure (1) shows the Phylogenetic tree that demonstrates the affinity of *E. cloacae* with global isolates recorded in the National Center for Biotechnology Information (NCBI). When comparing the genetic similarity with the nearest isolate, *E. cloacae* strain Xuyi_379_1, which carries the global number MN310027 from China, it shows that the similarity ratio reached 99.39, which indicates the accuracy of diagnosing bacteria recorded in this study with a genetic difference of 0.61%.

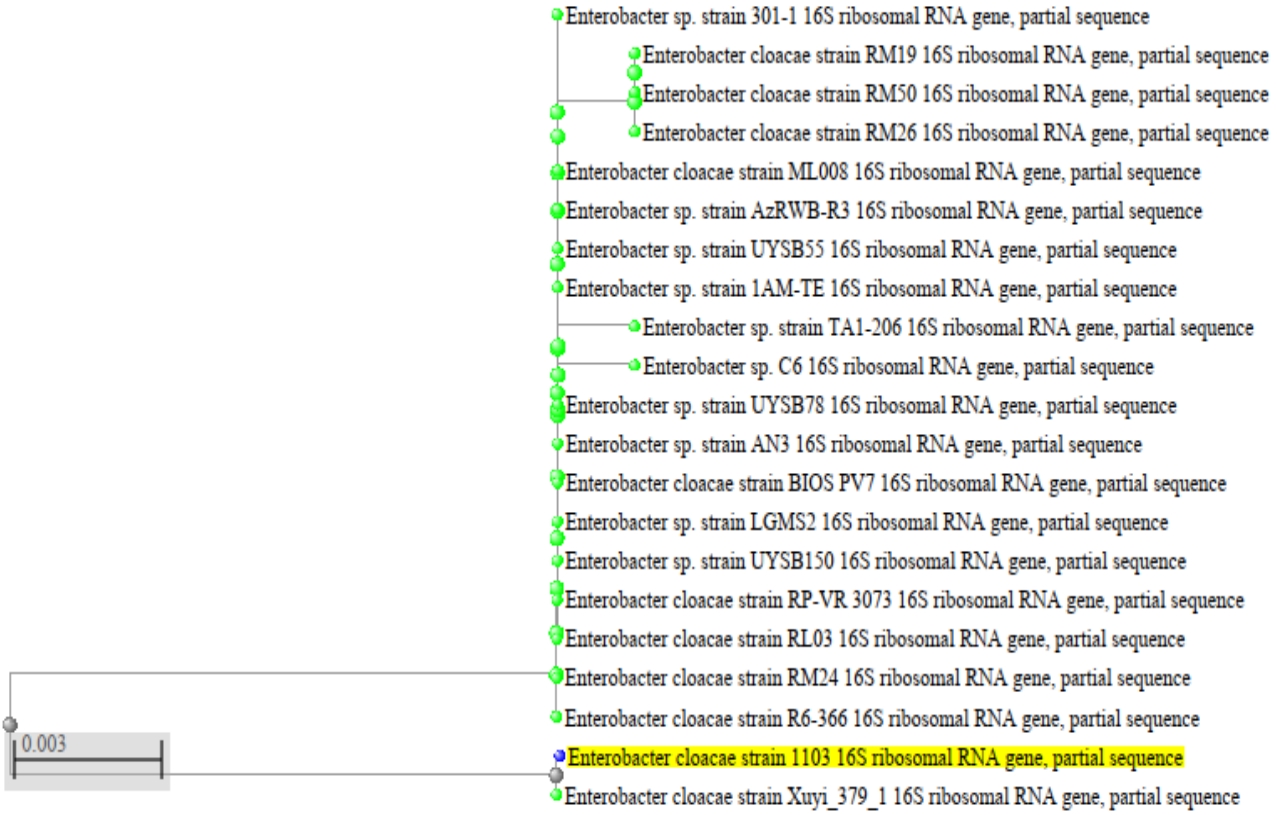

Figure 1. Genetic tree of *Enterobacter cloacae* isolate compared with world strain.

Figure (2) shows the genetic tree that demonstrates the affinity of *K. radiotolerans* bacteria with global isolates recorded in the National Center Biotechnology Information(NCBI) when comparing the genetic similarity with the nearest isolate, *K. radiotolerans* strain LAP2-29, which carries the global number KT216573.1 from Pakistan, it was found that the similarity ratio was 99.74, which indicates the accuracy of the diagnosis of the bacteria recorded in this study with a genetic difference of 0.26%.

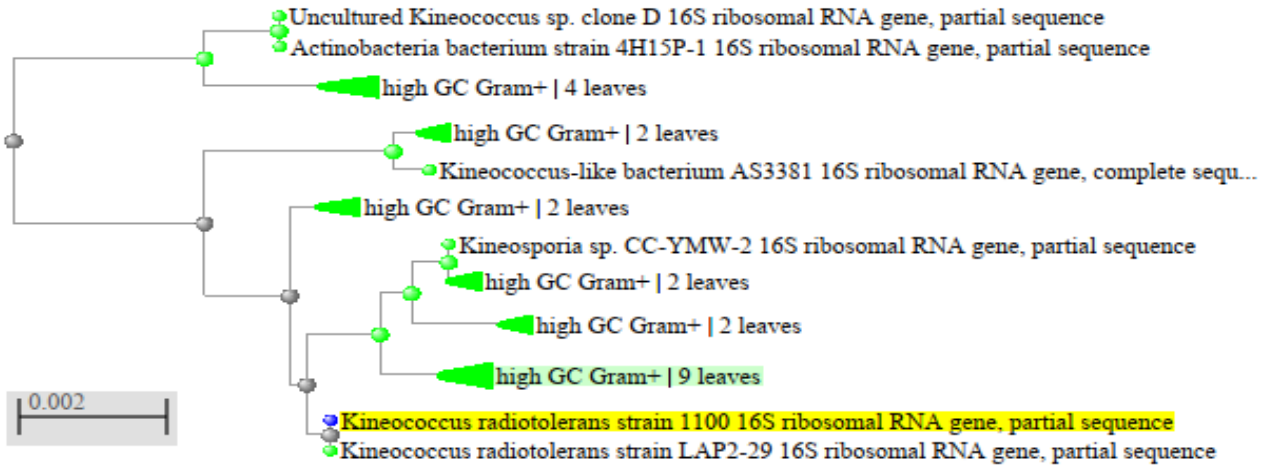

Figure 2. Genetic tree of *Kineococcus radiotolerans* isolates compared with world strain.

It is evident from Table (2) that all isolates could dissolve insoluble phosphate compounds. It appears from the Table that *E. cloacae* bacteria gave the highest solubility of phosphate at 40.3 mg p L^{-1} and outperformed the rest of the isolates *K. radiotolerans*, which gave 27.20 mg p L^{-1}, as it appears from the results that the gram-negative treatments are superior to the gram-positive treatments in phosphorylation, it appears from the Table the ability of two isolates to produce indole acetic acid and chelating compounds. *E. cloacae* is superior to *K. radiotolerans*, as it gave an indole production rate of 11.2 µg ml^{-1} compared to the value of 7.2 µg ml^{-1} for *K. radiotolerans*.

Isolates	Indole Acetic Acid (IAA)µg.ml	Phosphate solubilizing (mg p L^{-1})	Iron chelating
Enterobacter cloacae	11.2	40.3	++
Kineococcus radiotolerans	7.21	27.20	++

Table 2. Efficiency of bacterial isolates in solubilizing phosphate, Indole Acetic Acid and iron chelating compound production.

Percentage and speed of germination of eggplant seeds

Table (3) shows the effect of inoculation with bacterial inoculum on the speed and rate of germination of eggplant seedlings. It is clear that inoculation of seedling growth medium with the prepared inoculum had a significant effect on the rate and the speed of germination, the superiority of the inoculated treatments over the uninoculated treatment was significant in the rate of germination and the speed of germination, the Table shows the superiority of the treatment inoculated with bacteria *E. cloacae* over the rest of the treatments in the rate of germination as it gave 68.75%. In comparison, the treatments inoculated with *K. radiotolerans* and Control were 63.30% and 62.20% respectively.

Treatments	Germination speed(day)	Germination percentage
Control	14.20	62.20 %
E. cloacae	13.81	68.75 %
K. radiotolerans	14.00	63.30%x
LSD 0.05	0.37	0.47

Table 3. Effect of inoculation with Bacterial inoculum in percentage and speed of germination of eggplant seeds (seedling/ day).

Growth Traits

Table (4) shows that the inoculated treatments were significantly superior to the un-inoculated treatment in fresh and dry weight of the shoot and root system, it appears from the Table the superiority of the treatment inoculated with *E. cloacae* bacteria over the rest of the treatments in fresh and dry weight of the shoot and root system, as the treatment inoculated with *E. cloacae* bacteria gave the traits mentioned above 0.49 gm seedling-

$^{-1}$, 0.07 gm seedling^{-1}, 0.11 gm seedling^{-1}, 0.020 gm seedling^{-1}, respectively, compared with the uninoculated treatment which gave 0.28gm seedling^{-1}, 0.01 gm seedling^{-1}, 0.07gm seedling^{-1}, 0.001gm seedling^{-1} respectively.

Treatments	Root dry weight	Root fresh weight	Vegetative dry weight	Vegetative fresh weight
Control	0.001	0.07	0.01	0.28
E. cloacae	0.020	0.11	0.07	0.49
K. radiotolerans	0.010	0.08	0.02	0.30
LSD 0.05	**0.001**	**0.02**	**0.01**	**0.02**

Table 4. Effect of inoculation with Bacterial inoculum in fresh and dry weight of vegetative and root parts of tomato seedlings (gm seedling^{-1}).

Table (5) shows that the inoculated treatments were significantly superior to the uninoculated treatment in plant height and number of leaves, it appears from the Table, the superiority of the treatment inoculated with *E. cloacae* bacteria over the rest of the treatments in plant height, number of leaves, as the treatment inoculated with *E. cloacae* bacteria gave the traits mentioned above 6.22 cm plant^{-1}, 4.25 leaf plant^{-1} respectively, compared with the uninoculated treatment which gave 4.62 cm plant^{-1}, 3.25 leaf plant^{-1}respectively.

Treatments	plant height(cm seedling^{-1})	number of leaves(leaf plant^{-1})
Control	4.62	3.25
E. cloacae	6.22	4.25
K. radiotolerans	5.77	4
L.S.D 0.05	**0.19**	**0.24**

Table 5. Effect of inoculation of Bacterial inoculum in seedling height (cm seedling^{-1}) and number of leaves (leaf plant^{-1}).

DISCUSSION

Phosphorus is the second necessary element after Nitrogen in terms of importance and quantity needed by the plant; this element is found in the form of insoluble organic and mineral compounds that are not available for plants. The ability of bacterial isolates to dissolve insoluble phosphate compounds may be due to the ability of these isolates to produce organic acids [20, 21, 22], as it appears from the results that the treatments inoculated with gram-negative bacteria were superior to the gram-positive treatments in phosphate solubilizing, this confirmed by [23, 24], The hormone indole acetic acid is one of the essential plant hormones. It directly affects growth, cell division, and root formation. It increases the length and density of the root system, Which leads to an increase in the surface area of the root absorption zone, which enables the root to reach the nutrients in the medium in which it grows[25]. The difference in the amount of indole produced by different bacterial isolates can be attributed to the genetic variation of these isolates, as they belong to different species, which is reflected

in their biological properties, including their secretions in the growth medium. It was also evident from the results of Table 2 that all bacterial isolates are capable of producing chelating compounds because they were able to grow on an iron chelating medium and that the two isolates, *E. cloacae* and *K. radiotolerans*, showed medium susceptibility to iron chelation and gave medium growth on the medium, The difference in isolates in their production of chelating compounds is due to the variation in their genetic composition, which caused their difference in growth on the iron chelating medium, which is due to the difference in the amount of iron chelated from the medium[26]. The reason for the superiority of treatments inoculated with bacterial isolates over non-inoculated ones in the percentage of eggplant seed germination and the speed of germination can be attributed to the encouraging mechanisms for seed germination possessed by the bacterial isolates, especially the production of the hormone indole acetic acid IAA (Table 1), and the role of indole acetic acid in stimulating germination of seeds, cell division and elongation, and an increase in the rate and speed of germination[27], as for the superiority of the treatment of inoculation with *E. cloacae* bacteria over the rest of the treatments, it is due to its high production of indole acetic acid, while the decrease in the rate and speed of germination when inoculated with *K. radiotolerans* bacteria is due to its low production of IAA (Table 1).

The superiority of treatments inoculated with bacterial isolates over non-inoculated treatments in seedling height, number of leaves, and the fresh and dry weight of the vegetative and root part of tomato seedlings could be due to all bacterial isolates possessing different mechanisms to encourage plant growth, such as dissolving insoluble phosphate compounds and producing indole acetic acid IAA And iron chelating compounds (Table 1), phosphorous plays a crucial role in many vital processes such as photosynthesis, respiration, energy transfer, and enters the formation of DNA and RNA.

The reason for the superiority of the inoculated treatments over the uninoculated in the studied growth traits can be attributed to the possession of these isolates to various stimulating mechanisms such as the response of phosphate compounds, the production of indole acetic acid IAA and chelating compounds as shown in Table (1). These results are consistent with what was found by [28, 29]. The increase in seedling height, number of leaves, and fresh and dry weight of eggplant seedlings as a result of inoculation with the prepared inoculum from bacterial isolates may be due to the direct and indirect mechanisms possessed by these isolates to encourage plant growth (Table 1), which include the solubilizing of phosphate compounds, liberation of plant-ready phosphorous, and secretion of Stimulants to the medium such as IAA enhance the growth of running hairs and build a dense root system, which reflects positively on the process of absorbing nutrients from the growth medium, which is reflected in improving plant growth and increasing root and vegetative weight, both soft and dry[30], The increase in the studied growth characteristics of tomato seedlings can be attributed to the release of chelating compounds siderophores that contribute to the protection and protection of nutrients, especially iron, and making them available in the traction area and accessible for the plant [25].

CONCLUSIONS

We concluded from this study that the efficiency of *E. cloacae* and *K. radiotolerans* prepared inoculum encouraged the growth and production of eggplant seedlings. It also concludes the superiority of the inoculum prepared from *E. cloacae* bacteria over the *K. radiotolerans*. Thus, we recommend the adoption of this inoculum to produce robust, active, and healthy seedlings by relying on the biological inoculum, which will reduce high-cost chemical products and cause significant damage to the environment and human health.

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