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ORIGINAL ARTICLE

Assessment of rice rhizosphere-isolated bacteria for their ability to stimulate plant growth and their antagonistic effects against *Xanthomonas arboricola* pv. *juglandis*

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Abstract
This study looked at the possibility of using bacteria that were separated from the rhizosphere of rice plants to promote plant development and offer biological control against pests that affect agriculture. A total of 119 bacteria were isolated from rice rhizospheres collected from six different locations. Of these, 15.47% showed phosphate solubilization, 47.05% showed IAA, 89.07% showed siderophore, and 10.08% showed ACC deaminase activity. Generally, high siderophore production was observed in strains showing ACC deaminase activity. The antagonistic behavior of all strains against the walnut pest *Xanthomonas arbiricola* was also studied, and eight (6.7%) isolates suppressed the growth of this pathogen (7–43 ± 2 mm zone diameter). It was also noted that these eight isolates showed almost exclusively siderophore activity. In contrast to IAA and siderophore synthesis, the study demonstrated reduced activity levels for phosphate solubilization and ACC deaminase. The 16S rRNA sequence results of some of the bacteria selected in this study and AFLP analysis based on some restriction enzymes showed that the diversity was quite high. According to the 16S rRNA analysis, the high antagonistic effect of strain 71, which is one of the members of the *Enterobacter* genus, shows that it can be used as a biocontrol agent. In this study, it was revealed in detail that bacteria can be preferred as alternative biological agents for plant growth instead of synthetic fertilizers. This is the first study on this subject in this region, which is one of the important points of the country in terms of rice production.

Keywords Biocontrol · PGPB · ACC deaminase · AFLP · IAA · Phosphate-soluble

Introduction

Global population projections indicate that by 2055, there will be a projected 7.7 billion people on Earth, and this number will likely rise to 10 billion. The Earth and its finite resources are under stress due to the world's growing population. Water, air, and soil pollution are thought to be the primary causes of 40% of global mortality, and environmental deterioration and population expansion are the main drivers of the sharp rise in human illness rates. Arable, fertile land is more important as the population rises to feed everyone. Much more land will not be harmed, though, if substitutes that boost productivity in already-existing arable regions are successful (Glick 2012; Ferreira et al. 2019b). The Farm to Fork (F2F) plan, unveiled by the European Union in May 2020, attempts to lessen reliance on pesticides, antibiotics, and overuse of fertilizers. New agro-ecological strategies for managing agricultural biodiversity have been the subject of current study in this area (Timofeeva et al. 2022).

It is now crucial to look for better, more affordable, and ecologically friendly alternative procedures to boost output on arable land or address issues related to agriculture. In this situation, naturally, non-green or unsustainable methods like the heavy use of agrochemicals in chemical fertilization (field fertilization) or pesticide use (fighting phytopathogens) should be replaced by the search for alternatives. To increase productivity in agricultural areas, high concentrations of fertilizers, including nitrogen (N), phosphorus (P), or potassium (K) are applied. The excessive use of these artificial fertilizers has seriously harmed the environment's quality and the characteristics of the soil. Nevertheless, there are not many studies examining the connection between fertilizer

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use and the harm it does to the ecosystem. In order to preserve the sustainability of the agroecosystem and guarantee competitive crop yields and environmental health, it is now crucial to investigate and find alternative approaches (Shen et al. 2016). Accordingly, it is not surprising that, especially in recent years, there has been an increasing interest and focus on plant growth promotion bacteria (PGPB) as an alternative proposal for a more useful and environmentally friendly development of agriculture (Ferreira et al. 2019b). PGPB are proposed to be used as biofertilizers as well as to replace existing methods such as pesticides, herbicides, and fungicides. PGPB enhance plant productivity through various plant growth-promoting activities such as nitrogen fixation, efficient nutrients utilized by the plant, hormonal modulation and inhibition of the growth of harmful microbes (Ji et al. 2019). To make this clearer, PBGBs have been reported to affect plant growth through both direct and indirect pathways. Nitrogen fixation, phosphate solubilization, iron absorption, phytohormones known as auxins including IAA, gibberellins and cytokinin production are shown as direct mechanisms, while ACC deaminase, cell wall inhibitors, antimicrobial compounds or siderophores produced by bacteria to inhibit the growth of phytopathogens are considered indirect mechanisms (Ratnaningsih et al. 2023). Because it is involved in several metabolic processes, such as lipid metabolism and the formation of cell membranes and nucleic acids, phosphorus (P) is a vital nutrient for plants. It is also one of the most scarce nutrients in agricultural environments across the world. Since there is virtually no P mobilization in the soil, P absorption by the plant in the rhizosphere is essential. In order to allow plants to use and absorb soil phosphorus, phosphorus-solubilizing bacteria (PSB) produce phosphatase enzymes that hydrolyze physiologically inaccessible organic phosphorus (about 95–99%) into bio-available inorganic phosphorus (Chen et al. 2022; Yu et al. 2022). By keeping phosphorus from seeping from the soil and generating eutrophication, these bacteria also play a significant role in converting it into a form that is accessible to plants (Yu et al. 2022). Soil generally has little accessible phosphorus (0.4–1.2 g kg–1). The plant is unable to absorb around 80% of the phosphorus supplied by artificial fertilizers. This is because, according to Janati et al. (2022), the majority of applied phosphorus leaves the plant-soil system. PSB quantity and presence in the soil might vary based on a number of circumstances. Human activity, in particular, may be detrimental to PSB populations. Variations in PSB populations in soil can be attributed to many variables. These variables include the pH of the soil (Raymond et al. 2021), the misuse of fertilizers high in phosphorus, chemical and pesticide residues, and land deterioration. The use of biofertilizers containing beneficial bacteria with PSBs that boost plant accessibility to phosphorus is one of the most significant answers to these issues. Furthermore, Bargaz et al. (2021) noted in their assessment of the literature that several studies have demonstrated that co-applying PSB and phosphate fertilizer together can significantly lower P adsorption in the soil, increasing plant accessibility.

The use and commercialization of PGPB as a biological fertilizer in agriculture began worldwide in the 1950s. The current biofertilizer market is reported to cover about 5% of the total chemical fertilizer market (Ji et al. 2019; Chu et al. 2019). There are some studies in which such bacteria have been used in rice production and have been found to be effective, including the following: In order to assess the impact of ACC-producing strains on the germination and growth of inoculated rice seeds under conditions of salt stress and ethylene production, Bal et al. (2013) isolated ACC deaminase-producing PGPB strains from the rhizosphere of rice plants growing in coastal saline soils. In comparison to the uninoculated control, the results showed that inoculation with specific PGPB isolates significantly improved a number of rice growth parameters, such as germination percentage, shoot and root growth, and chlorophyll content. Additionally, inoculation with strains that produce ACC deaminase decreased the production of ethylene under salinity stress. According to Mwashasha et al. (2016), who looked at how plant growth-promoting microorganisms affected rice production, using isolates of Aspergillus, Bacillus, and Enterobacter as inoculant biofertilizers was helpful for rice production. In order to track the changes, Bao et al. (2013) infected rice seeds with *Azospirillum* sp. B510 and intended to sow the seeds in the paddy field. According to their findings, *Azospirillum* sp. B510 changed the proportion of a few specific bacteria linked to rice in addition to promoting rice development.

It has been reported that the root microbiome consists of commensal, pathogenic and plant-beneficial microbes, that these microbiomes have a microbe-associated molecular structure similar to that of plant pathogens, and that their recognition results in activation of host immunity and suppression of plant growth due to deforming traumas (Yu et al. 2019). According to Yu et al. (2019), immune suppression promoted the colonization of beneficial microorganisms on the roots, beneficial *Pseudomonas* suppressed root immunity by reducing ambient pH, and 42% of the root microbiota they studied was able to overcome local root immune responses. Vandana et al. (2018) highlighted that *Bacillus* spp. may be a viable alternative in organic tea plant farming due to its spore-forming capacity and sensitivity to various climatic conditions, and *Pseudomonas* spp. due to its specific colonization behavior and antagonistic activity against potential fungal pathogens. Their study evaluated plant growth-promoting bacteria from the rhizosphere of tea plants.

Four goals were set for this study: (i) separate culturable bacteria that have the potential to promote plant growth

from different rice-producing areas; (ii) evaluate these isolates' PGP activities, such as phosphate solubilization, IAA production, ACC deaminase, and siderophore activity; (iii) identify these isolates using molecular and classical microbiological techniques; and (iv) investigate their antagonistic interactions against the walnut plant pathogen, *Xanthomonas arboricola* pv. *juglandis*.

Methods

Collection of samples and bacterial isolation

Rhizospheric soil samples were collected from rice cultivation areas in Sinop center and Boyabat district. The necessary permits for the isolation of bacteria and the collection of soil samples were obtained from the Ministry of Agriculture and Forestry, the General Directorate of Nature Conservation and National Parks. Paddy fields were identified as three in Sinop Center and three in Boyabat district and samples were collected from these locations between July and August 2021. Supporting Table S1 shows the locations (Fig. 1) and conditions where the samples were collected. Plants were taken from the paddy fields together with their roots, and care was taken to take 8–11 roots from different points by zigzagging from each sampled area. The roots were placed in sterile snap nylon bags and kept in a cold chain bag. The samples were brought to the laboratory within 3 h after collection and analysis were started.

Soil samples were taken briefly as follows; rice plant roots were removed from the soil and then the plant roots were shaken by hand for 5–10 min to remove the soil pile. The remaining adherent soil was considered as rhizospheric soil, for which the roots were collected in a sterile plastic container by hand shaking for 5–10 min (Suarez-Moreno et al. 2019). To isolate bacteria, 1 g of the rhizosphere soil sample was mixed with 9 mL of phosphate buffer (20 mM, pH 7.0) and incubated at 30 ℃ for 30 min at 150 rpm on a shaker. Serial dilutions of 10^{-7} of the processed soil samples were prepared. They were mixed by hand with gentle hand shaking and incubated on Nutrient Agar (NA), Tryptic Soy Agar (TSA), Kings B Agar and Burks N-free Agar in triplicate at 37 ℃ for 3–5 days. After the incubation phase, isolates were selected based on their morphology, purified and stored at – 80 ℃ in 17% glycerol stock until further use (Vandana et al. 2018).

Fig. 1 Map showing the stations where the samples were taken (generated from google maps). S1, S2, and S3 represent samples from Sinop Center, while B1, B2, and B3 represent samples from Boyabat District

Indole acetic acid production

The ability of bacteria cultured fresh overnight to produce indole acetic acid (IAA) was determined based on the colorimetric method. Isolates were inoculated into LB medium containing 5 mM tryptophan and incubated for 7 days at 28 ℃ at 200 rpm on a continuous shaker. After incubation, bacterial cultures were centrifuged at 6000 rpm for 10 min and 1 mL of supernatant was mixed with 4 mL of Salkowski's reagent (0.5 M $FeCl_3$ + 70% perchloric acid). The absorbance of the mixture was allowed to stand in the dark for 20 min at room temperature before being measured at 535 nm using a spectrophotometer. The IAA concentration of each sample was determined from a standard curve of pure indole-3-acetic acid at 0–100 µg mL^{-1}. The study for IAA was performed in two replicates (Gupta and Pandey 2019).

ACC deaminase production

1-Aminocyclopropane-1-carboxylate (ACC) deaminase activity was detected by monitoring the amount of α-ketobutyric acid produced from the cleavage of ACC. ACC deaminase activity is induced after growing bacterial cells to the logarithmic phase in a minimal medium containing ACC as the sole nitrogen source. Isolates cultured overnight in TSB medium were treated with 15 mL DF salt (per liter: 4 g KH_2PO_4, 6 g Na_2HP0_4, 0.2 g $MgSO_4$. $7H_2O$, 2 g glucose, 2 g gluconic acid and 2 g citric acid with trace elements: 1 mg $FeSO_4.7H_2O$, 10 mg H_3BO_3, 11.19 mg $MnSO_4$. H_2O, 124.6 mg $ZnSO_4.7H_2O$, 78.22 mg $CuSO_4.5H_2O$ and 10 mg MoO_3, pH 7.2). The cultures were incubated overnight at room temperature at 200 rpm^{-1} in a shaking water bath. The whole sample was then centrifuged at 6000×g for 10 min at 4 ℃ to obtain a dense pellet. The supernatant was removed, and the pellet was washed with 5 mL of DF minimal medium. Again, the sample was centrifuged at 6000×g for 10 min at 4 ℃ and suspended in 7.5 mL of DF salt minimal medium. For the next step, ACC (sterilized with a 0.2 µm membrane filter and stored at − 20 ℃ until use) was added to the tubes to a final concentration of 3 mmol L^{-1}. The tubes were then incubated overnight in a shaking water bath at 200 rpm^{-1} at room temperature. The sample was centrifuged at 6000×*g* for 10 min at 4°C. After removing the supernatant, cells were suspended in 5 mL 0.1 mol L^{-1} Tris–HCl pH 7 and transferred to a sterile 1.5 mL eppendorf tube. The sample was centrifuged at 12,000×*g* for 5 min and the supernatant was removed. The pellet was suspended in 600 µL of 0.1 mol L^{-1} Tris–HCl pH 8.5. 30 µL toluene was added to the sample and vortexed at high setting and 100 µL toluninized sample was stored at 4 ℃ for protein determination. ACC deaminase activity was continued with the remaining amount. 200 µL of the toluinized sample was taken, 20 µL of 0.5 mol L^{-1} ACC was added and it was incubated at 30 ℃ for 15 min with gentle vortexing. Then, 1 mL of 0.56 mol L^{-1} HCl was added, vortexed and centrifuged at 12,000×g for 5 min at room temperature. 800 µL of 0.56 mol L^{-1} HCl was vortexed with 1 mL of the supernatant and 300 µL of 2,4-dinitrophenyl hydrazine reagent was added to the sample in a glass tube, vortexed and incubated at 30 ℃ for 30 min. Following this step, it was mixed with 2 mL 2 N NaOH and measured at 540 nm. The α-ketobutyrate produced by the reaction was determined by comparing the absorbance at 540 nm of the sample with a standard α-ketobutyrate curve ranging from 0.1 to 1.0 µmol. ACC deaminase activity was expressed as the amount of α-ketobutyrate produced per mg protein per hour. The study was performed in triplicate for each sample (Saravanakumar and Samiyappan 2007).

Phosphate solubilization activity

Bacteria were inoculated in the National Botanical Research Institute's Phosphate (NBRIP) medium and incubated at 30 ℃ for 7 days. The formation of a clear zone around the colony after the incubation period indicated phosphate solubilizing activity. As a result of this test, the isolates were classified as follows: (–) no zone or activity; (+) low activity (up to 2 mm); (+ + +) moderate activity (2–4 mm); (+ + + +) high activity (zone larger than 4 mm) (Carpentieri-Pipolo et al. 2019).

Detection of siderophore production

The Chrome Azurol S (CAS) method was used to estimate bacterial siderophore production (Alexander and Zuberer 1991). To prevent iron contamination before starting the tests, all glassware was soaked in 10% nitric acid overnight and then washed twice with deionized water before use. Bacteria were incubated in 10 mL of minimal medium broth (MM) at 29 ℃ for 24 h. MM content per liter was as follows: 10 g glucose, 1.47 g glutamic acid, 3.0 g potassium hydrogenophosphate (K_2HP0_4), 1.0 g potassium dihydrogenophosphate (KH_2PO_4), 0.5 g ammonium chloride (NH_4Cl), 0.1 g ammonium nitrate (NH_4NO_3), 0. 1 g sodium sulfate (Na_2SO_4), 10 mg magnesium sulfate heptahydrate ($MgSO_4$-$7H_2O$), 1 mg magnesium sulfate tetrahydrate ($MnSO_4$-$4H_2O$), 0.5 mg calcium chloride ($CaCl_2$). Overnight, fresh cultures of bacteria were centrifuged at 2500×g for 10 min to obtain a pellet. The supernatant was carefully removed, filtered with a 0.45 µm pore diameter filter and stored at − 20 ℃ until siderophore detection.

The CAS solution was prepared as follows: 21.9 mg of hexadecyltrimethylammonium bromide (HDTMA) was dissolved in 25 ml of water with continuous stirring at a low temperature. In a separate container, 1.5 mL of 1 mM

$FeCl_3.6H_20$ (in 10 mM HCl) was mixed with 7.5 mL of 2 mM CAS. This solution was slowly added to the HDTMA solution with stirring and the mixture was transferred to a 100 mL flask. To prevent precipitation of the dye, 9.76 g MES (2-[N-morpholino] ethanesulfonic acid) was dissolved in 50 ml of water, the pH was adjusted to 5.6 with 50% KOH and then the buffer solution was added to the metered flask containing the dye solution. dH_2O was added to bring the volume to 100 ml. For the so-called "Shuttle" solution, 87.3 mg of 5-sulfosalicylic acid was added to the above solution just before use to speed up the reaction (Alexander and Zuberer 1991).

The siderophore concentration of the previously filtered supernatant was measured by mixing 100 µL of CAS solution with 100 µL of supernatant in a 96-well microplate plate. The solutions were allowed to reach chemical equilibrium at room temperature in the dark for 4 and 24 h. Absorbance was then measured at 630 nm with a microplate reader (Thermo Scientific Multiskan Go Version 1.01.10). A zero-absorbance solution was also prepared by mixing 100 µL of CAS solution with 100 µmol L^{-1} desferrioxamine mesylate salt (desferal). Siderophore production was determined by comparison with a standard curve of desferal absorbance ranging from 100 to 10 µmol L^{-1}, expressed as µmol L^{-1} desferal equivalent (Ferreira et al. 2019a).

Determination of protein content

Protein content was determined based on the Bradford method (Bradford 1976). Bovine serum albumin was used as a standard and protein content was determined based on the curve generated by considering the absorbance at 595 nm.

Antagonistic effects of the tested isolates against the walnut bacterial blight agent (*Xanthomonas arboricola* pv. *Juglandis*—Xaj)

Overnight fresh cultures of 119 bacteria and *X. arboricola* tested in the study were prepared in NB medium. Mc.Farland scale of fresh bacterial cultures was prepared and antagonistic effect analysis was started. Wells with a diameter of 6 mm were made in Mueller Hinton Agar (MHA) and *X. arboricola* culture was spread with a swab. The fresh culture of each PGP bacterial sample was then centrifuged at 12,000 rpm for 7 min and 70 µL of the supernatant was placed in the wells and 3 µL of each bacterial sample was poured into the center of the petri dish with a micropipette. The plates were incubated at 30–37 ℃ for 24–48 h and the results were recorded by measuring the zone diameters around the well with a millimeter ruler. Tests were performed in triplicate (Iqbal et al. 2018).

Identification of PGP bacteria

After the bacteria isolated from the plant rhizosphere were tested for their plant growth-inducing activities, morphological, physiological and some biochemical tests (Gram staining, catalase and oxidase) were performed on the isolates with the highest activity. Genomic DNA isolation from pure cultures was performed according to the method of Sambrook et al. (1989). Sequence analysis of the 16S rRNA gene region based on 27F-1492R primers of the isolates with possible genus/species definitions was performed through service procurement. Sequence chromatograms were analyzed using the Chromas Lite program. Sequences were compared with the database (GenBank) using the NCBI Blast program. Editing and alignment of sequences were performed using the Clustal W program. Phylogenetic trees were drawn with the molecular evolution genetic analysis (MEGA11) program. GenBank access numbers for 18 isolates were determined in the range PP504606 to PP504623.

For Amplified Fragment-Length Polymorphism (AFLP) analysis, the 16S rRNA PCR products were pooled and then run on a 1.8% agarose gel prior to purification. Gel slices containing the fragments were excised and DNA was recovered using the Quick Gel Extraction Kit (Invitrogen) as specified by the manufacturer. Purified PCR products were quantified on a 1.5% agarose gel. The purified PCR products of all isolates were digested by HinfI (NEB), BsgI (NEB) and MspI (NEB) restriction enzymes. Enzyme digestion was performed in a 31 µl mixture containing 10 µl of the PCR product, 1 µl (10U) of enzyme, 2 µl buffer and 18 µl of nuclease-free water at 37 ℃ (55 ℃ for BsgI) for 4 h. After digestion with enzymes, the presence of the PCR product was analyzed by a 3% agarose gel and visualized under UV light.

Results

Preparation of samples for isolation of bacteria

Plant samples were first thoroughly beaten by hand to remove coarse soil and/or sludge supporting Fig. S1A, then soil samples around the plant roots were weighed at 1 g and homogenized in 9 mL of physiological saline (FTS) and then serial dilutions up to 10^{-7} were prepared. Immediately after the dilutions were prepared, the samples were sown on Nutrient Agar (NA), Tryptic Soy Agar (TSA), KingB Agar and N-Burks Agar media for bacterial isolation by broadcast inoculation method and incubated at 37 ± 2 ℃ for 24–48 h for NA, TSA and KingB and at 25 ℃ for N-Burks Agar for up to 7 days. After the incubation phase, distinct colonies were selected (supporting Fig. S1B-C), inoculated into Nutrient Broth (NB) and incubated at 37 ± 2 ℃ for 24 h. The

following procedures were continued from liquid to solid and from solid to liquid until pure colonies were obtained. After the purification step, 119 bacteria were obtained as indicated in supporting Table S2. Colony morphology, oxidase (supporting Fig. S1D), catalase (supporting Fig. S1D) and Gram-staining of the bacteria were performed and given in supporting Table S2.

Evaluation of the properties of bacteria that contribute to plant growth

In this study, 119 bacteria isolated from the rhizosphere of rice plants from 6 randomly selected locations were studied for some of their properties that contribute to plant growth. Their antagonistic effects against a selected plant pathogen were also examined. It is clear from analyzing the data in the context of the whole framework that not all bacteria have significant PGP characteristics. However, as demonstrated by the experiments listed in Table 1, certain bacteria are significantly successful at exhibiting particular properties.

For phosphate solubilization activity, 13 isolates (15.47%) out of 119 isolates showed activity (Table 1). In addition, according to the test results, only one isolate (no. 50) showed high activity (Fig. 2D), five isolates (48, 87, 99, 104 and 109) showed moderate activity and seven isolates (4, 66, 84, 84, 85, 86, 105 and 112) showed low activity (Table 1).

According to Indole Acetic Acid test results, 56 (47.05%) isolates showed activity. Values between 0 and 47.02 ± 0.09 µg mL^{-1} were observed for IAA (Table 1). Only 12 (10.08%) isolates tested showed ACC deaminase activity between 0 and 46.8 ± 0.5 nmol ketobutyrate mg^{-1} (Table 1). For siderophore, the majority of the isolates (106–89.07%) showed activity between zero and 167.1 ± 0.7 µmol L^{-1}. In addition, the protein content of 84 (70.58%) isolates was tested based on the Bradford method, and protein content data were obtained and given in Table 1. When evaluated in terms of phosphate solubilization, IAA, ACC and siderophore activity, no bacteria producing all of them could be detected. It was observed that bacteria with phosphate solubilization activity (except those coded 48 and 50) showed IAA and siderophore activity. Isolates showing ACC deaminase activity (except for isolates coded 113 and 81) were generally positive for IAA and siderophore. In addition, isolates with ACC deaminase activity (except 113) were found to produce relatively high levels of siderophores. Isolate 88, which gave the highest siderophore activity of 167.1 ± 0.7 µmol L^{-1}, was found to have no activity for the other three factors. Apart from these, 11 (9.24%) isolates did not show any activity. In addition, standard curves were prepared for each of the four different properties (r^2 values were all above 0.99) and the axis values of each curve are shown in supporting Fig. S2.

In addition to these characteristics, antagonistic activity of the isolates against *X. arboricola* pv. *juglandis* was studied and 8 (6.72%) isolates showed activity against this pathogen (Table 1 and Fig. 2A, B). Especially isolate 71 showed very high activity with a zone diameter of 43 ± 2 mm (Fig. 2B). In addition, isolate 23 showed a zone diameter of 21 mm and the others showed activity ranging between 7 and 15 mm. Crucially, the production of siderophores was nearly always linked to every isolate that exhibited antagonistic activity against *X. arboricola*.

In addition, identification of some selected strains (18) based on 16S rRNA sequence analysis was aimed and the phylogenetic tree drawn in MEGA is shown in Fig. 2. Among these strains, it is noteworthy that strain 50, which was found to have high phosphorus solubilizing activity, is close to the *Acinetobacter* species selected as a reference. In addition, strains 2, 81 and 84 are close to the reference strain *Pseudomonas* and strains 66, 92 and 99 are close to the reference strain *Bacillus*. Based on 16S rRNA sequence analysis within the Enterobacteriaceae family, strain 71, which showed the strongest antagonistic activity, was closely connected to the Enterobacter genus. Furthermore, the isolates discovered by 16S rRNA analysis showed strong activity in the synthesis of siderophores, IAA, ACC deaminase, and phosphate solubilization, indicating a considerable taxonomic variety.

The results of AFLP analysis to determine genetic diversity among the tested strains are shown in Fig. 3A–C. Accordingly, all strains were tested with *Msp*I and *Bsg*I restriction enzymes, but results were obtained for 104 and 100 strains, respectively. For *Hinf*I, 27 strains that gave the best results for PGP were tested and a band profile was obtained from all of them. When the results are evaluated according to Fig. 3A–C, it was determined that the strains showed a diversity of 50% or more for *Msp*I and slightly more than 50% or more for the other two enzymes. When the affinity relationships in the phylogenetic tree generated for 18 strains in Fig. 2 are compared with the AFLP results, we can state that AFLP is effective in determining the genetic distance between species. In addition, we can say that cutting with different enzymes has an important role in determining genetic diversity.

Discussion

It would be accurate to compare these two key points by taking Sinop, Center, and Boyabat districts into consideration before generating a judgment based on the six places where bacteria were found. In light of this, Table 1 analysis suggests that the bacteria isolated from Sinop Center samples are comparatively more effective in promoting the growth of rice plants. However, only four bacteria from Sinop Center

Table 1 Results for traits that contribute to plant growth

Isolates	Phosphate colony size (mm)/zone diameter (mm)/result	Indole acetic acid (µg mL^{-1})	ACC deaminase per nmol ketobutyrate mg^{-1}	Siderophore (µmol L^{-1})	Bradford protein content	Antagonistic relationship against *X. arboricola* (Zone diameter mm)
1	–	1.33 ± 0.84	–	125.4 ± 5.0	–	–
2	–	1.84 ± 0.34	26.3 ± 0.19	96.3 ± 10.4	339.8 ± 26.1	–
3	–	–	–	–	–	–
4	6 / 2 ± 0.15 / +	0.21 ± 0.02	–	87.4 ± 2.2	321.2 ± 10.7	–
5	–	6.55 ± 0.36	46.8 ± 0.5	107.9 ± 6.2	1117.8 ± 16.1	–
6	–	–	–	101.5 ± 10.1	539.2 ± 17.0	–
7	–	–	–	–	–	–
8	–	1.00 ± 0.34	–	82.9 ± 8.9	50.53 ± 4.1	–
9	–	1.69 ± 0.16	–	131.8 ± 7.0	670.5 ± 12.4	–
10	–	–	35.9 ± 12.16	67.9 ± 0.4	500.5 ± 24.1	–
11	–	–	–	153.2 ± 23.6	354.5 ± 8.9	–
12	–	0.87 ± 0.16	–	138.5 ± 23.0	668.5 ± 47.8	–
13	–	5.35 ± 0.25	–	107.9 ± 6.9	883.8 ± 7.36	–
14	–	–	–	–	–	–
15	–	–	–	–	–	–
16	–	5.66 ± 0.50	27.9 ± 0.33	113.8 ± 1.7	1055.8 ± 22.1	–
17	–	–	–	119.9 ± 17.5	555.8 ± 10.8	–
18	–	–	–	144.6 ± 34.0	259.2 ± 8.6	–
19	–	–	–	65.1 ± 17.0	–	–
20	–	–	–	–	–	–
21	–	–	–	129.9 ± 26.7	593.2 ± 17.9	–
22	–	–	–	–	–	–
23	–	6.00 ± 0.45	–	114.9 ± 20.7	–	21 ± 0.57
24	–	0.25 ± 0.04	–	109.6 ± 40.4	–	–
25	–	–	–	99.9 ± 13.76	558.5 ± 35.9	–
26	–	1.94 ± 0.05	29.6 ± 0.19	113.5 ± 13.1	1141.8 ± 6.7	–
27	–	–	–	28.8 ± 26.3	–	–
28	–	0.49 ± 0.02	–	87.4 ± 14.2	351.2 ± 1.6	–
29	–	–	–	–	–	–
30	–	0.51 ± 0.04	–	84.3 ± 40.9	259.2 ± 40.4	–
31	–	2.32 ± 0.02	–	144.0 ± 31.6	345.2 ± 8.16	–
32	–	–	–	126.8 ± 11.3	–	–
33	–	–	–	64.6 ± 8.0	13.86 ± 18.2	–
34	–	–	–	47.9 ± 16.8	87.2 ± 4.32	–
35	–	–	–	126.0 ± 6.3	–	–
36	–	–	–	142.4 ± 11.0	–	–
37	–	4.41 ± 0.07	–	112.6 ± 14.1	688.5 ± 19.3	–
38	–	4.60 ± 0.05	25.3 ± 0.19	116.5 ± 12.5	1008.5 ± 12.2	–
39	–	1.62 ± 0.22	28.4 ± 0.19	100.4 ± 8.1	1156.5 ± 7.3	–
40	–	–	–	106.8 ± 8.0	89.86 ± 8.05	–
41	–	–	–	106.8 ± 14.5	–	–
42	–	–	31.7 ± 0.19	147.1 ± 12.9	1100.5 ± 6.5	–
43	–	–	–	136.5 ± 9.0	79.86 ± 1.88	–
44	–	–	–	88.8 ± 19.5	255.2 ± 2.82	–
45	–	0.07 ± 0.04	–	118.2 ± 6.8	365.8 ± 6.79	–
46	–	–	–	–	–	–
47	–	–	–	90.7 ± 5.1	742.5 ± 7.36	–

Table 1 (continued)

Isolates	Phosphate colony size (mm)/zone diameter (mm)/result	Indole acetic acid (µg mL^{-1})	ACC deaminase per nmol ketobutyrate mg^{-1}	Siderophore (µmol L^{-1})	Bradford protein content	Antagonistic relationship against *X. arboricola* (Zone diameter mm)
48	7/3 ± 0.1/+ +	–	–	55.4 ± 8.5	354.5 ± 4.98	–
49	–	–	–	97.9 ± 8.3	395.8 ± 2.49	–
50	5/5 ± 0.26/+ + +	–	–	58.8 ± 7.9	260.5 ± 10.4	–
51	–	–	–	146.5 ± 13.6	222.5 ± 7.71	–
52	–	–	–	129.9 ± 2.4	91.2 ± 2.82	–
53	–	–	–	139.9 ± 3.7	109.2 ± 2.82	–
54	–	–	–	114.3 ± 18.7	–	–
55	–	–	–	130.7 ± 10.6	–	–
56	–	–	–	113.5 ± 8.8	277.8 ± 16.7	–
57	–	–	–	–	–	–
58	–	–	–	96.3 ± 3.3	–	–
59	–	–	–	142.6 ± 9.8	183.8 ± 86.7	–
60	–	–	–	89.6 ± 19.0	435.8 ± 71.0	–
61	–	–	–	139.3 ± 16.3	–	–
62	–	–	–	119.0 ± 6.1	337.8 ± 1.8	12 ± 0.76
63	–	–	–	90.1 ± 8.2	239.8 ± 6.5	15 ± 1.5
64	–	–	–	101.8 ± 6.1	–	11 ± 0.23
65	–	–	–	99.0 ± 3.1	–	12 ± 0.11
66	7/1 ± 0.1/+	47.2 ± 0.09	–	71.5 ± 7.6	406.5 ± 17.9	–
67	–	21.7 ± 0.20	–	49.9 ± 7.6	407.8 ± 9.9	–
68	–	–	–	108.8 ± 11.5	267.2 ± 12.3	–
69	–	–	–	124.6 ± 14.3	338.5 ± 26.3	–
70	–	0.35 ± 0.04	–	142.4 ± 4.1	482.5 ± 35.1	7
71	–	–	–	136.03 ± 4.6	737.2 ± 7.48	43 ± 2
72	–	–	–	131.5 ± 7.1	–	–
73	–	–	–	136.8 ± 5.7	743.8 ± 16.1	–
74	–	2.55 ± 0.05	–	118.8 ± 17.3	103.2 ± 16.5	–
75	–	–	–	151.5 ± 12.3	387.8 ± 11.1	–
76	–	–	–	126.5 ± 1.8	–	–
77	–	1.51 ± 0.13	–	131.0 ± 7.8	144.5 ± 7.71	–
78	–	–	–	110.4 ± 6.3	569.2 ± 20.8	–
79	–	7.57 ± 0.45	–	70.7 ± 15.8	583.2 ± 19.8	–
80	–	4.95 ± 4.16	35.5 ± 1.5	131.8 ± 18.5	983.8 ± 26.5	12 ± 0.2
81	–	–	25.8 ± 0.3	77.9 ± 11.8	811.2 ± 33.1	–
82	–	–	–	134.9 ± 20.6	883.2 ± 50.6	–
83	–	–	–	–	–	–
84	6/1 ± 0.24/+	12.4 ± 0.50	–	104.0 ± 6.0	725.2 ± 10.1	–
85	5/1 ± 0.25/+	2.43 ± 0.00	–	109.0 ± 13.2	802.5 ± 63.1	–
86	11/1 ± 0.1/+	4.30 ± 0.13	–	88.5 ± 5.1	757.8 ± 11.4	–
87	5/2 ± 0.05/+ +	4.03 ± 0.07	–	105.1 ± 2.8	667.2 ± 28.6	–
88	–	–	–	167.1 ± 0.7	–	–
89	–	8.01 ± 8.17	–	123.2 ± 10.2	732.5 ± 4.1	–
90	–	2.60 ± 0.05	–	131.0 ± 25.7	520.5 ± 8.05	–
91	–	–	–	116.5 ± 8.6	571.2 ± 7.1	–
92	–	0.28 ± 0.04	25.3 ± 0.5	152.6 ± 13.8	–	–
93	–	0.37 ± 0.02	–	141.3 ± 17.2	486.5 ± 9.8	–
94	–	10.2 ± 0.14	–	134.9 ± 2.3	487.2 ± 24.7	–

Table 1 (continued)

Isolates	Phosphate colony size (mm)/zone diameter (mm)/result	Indole acetic acid (μg mL^{-1})	ACC deaminase per nmol ketobutyrate mg^{-1}	Siderophore (μmol L^{-1})	Bradford protein content	Antagonistic relationship against *X. arboricola* (Zone diameter mm)
95	–	0.68 ± 0.07	–	101.3 ± 6.9	660.5 ± 28.6	–
96	–	3.13 ± 0.09	–	128.2 ± 14.3	–	–
97	–	–	–	160.1 ± 18.6	–	–
98	–	4.01 ± 0.39	–	86.8 ± 2.7	1105.8 ± 8.3	–
99	8/3 ± 0.07/ + +	17.5 ± 0.02	–	108.8 ± 0.7	96.53 ± 13.1	–
100	–	–	–	–	–	–
101	–	43.3 ± 0.02	–	158.5 ± 2.7	969.2 ± 86.3	–
102	–	1.30 ± 0.31	–	162.9 ± 11.7	–	–
103	–	3.46 ± 0.48	–	91.8 ± 3.2	837.8 ± 4.1	–
104	6/3 ± 0.25/ + +	3.63 ± 0.25	–	66.3 ± 5.2	1137.2 ± 44.4	–
105	10/1 ± 0.1/ +	1.66 ± 0.45	–	35.4 ± 1.7	669.8 ± 41.3	–
106	–	1.43 ± 0.23	–	162.4 ± 14.7	439.8 ± 36.7	–
107	–	1.98 ± 0.43	–	95.4 ± 17.3	935.8 ± 16.4	–
108	–	–	–	–	580.5 ± 18.5	–
109	11/2 ± 0.14/ + +	18.31 ± 0.02	–	84.3 ± 4.9	–	–
110	–	–	–	127.6 ± 5.2	33.8 ± 26.5	–
111	–	1.86 ± 0.13	–	99.08 ± 5.3	115.8 ± 4.9	–
112	11/1 ± 0.1/ +	14.2 ± 0.48	–	85.7 ± 4.2	327.8 ± 21.3	–
113	–	0.43 ± 0.22	26.8 ± 0.5	–	963.2 ± 16.9	–
114	–	0.56 ± 2.65	–	103.5 ± 7.2	63.2 ± 6.5	–
115	–	4.08 ± 0.14	–	99.0 ± 5.8	–	–
116	–	2.74 ± 0.11	–	98.2 ± 3.1	79.8 ± 2.4	–
117	–	0.59 ± 0.05	–	91.8 ± 11.2	111.8 ± 3.3	–
118	–	4.25 ± 0.05	–	109.9 ± 10.9	322.5 ± 4.1	–
119	–	7.28 ± 0.1	–	102.9 ± 14.3	1391.2 ± 3.2	–

exhibited ACC deaminase activity, compared to eight bacteria isolated from the Boyabat region. The strong phosphate solubilizing activity of isolates 48 and 50 recovered from B3 (Boyabat 3rd point) is noteworthy when the six isolated areas are assessed. With the exception of isolate 45, all 21 of the bacteria that were isolated from the same location were shown to lack ACC and IAA activities. The most noteworthy circumstance pertaining to the bacteria isolated from Sinop Center initial point (S1) is that this area produced 5 of the 8 isolates that showed activity against *X. arbiricola*. In terms of phosphate solubilization, IAA, and siderophore activities, it was discovered that points S2 and S3 contained productive bacteria.

From a broad perspective, the highest phosphate solubilization, IAA, ACC deaminase, and siderophore activities were observed at points B3, S1, B1 and S2, respectively. In this context, when regional significance and good characteristics are compared, we can say that bacteria are distributed. However, we can say that the bacteria isolated from the rice rhizosphere of the Sinop region did not contribute much in terms of phosphate solubilization or ACC deaminase in general, but they were especially effective in terms of siderophore. In addition, it is known how important the antagonistic relationship between bacteria is. In this direction, the high activity of the isolates tested against the walnut bacterial blight disease agent *X. arbiricola*, especially the bacteria isolated from the S1 point, shows that this area should also be focused on. The concentration of a biological drug agent(s) in bacteria living in rhizosphere samples collected from one point (S1) and the purification of bacteria with high activity (43 ± 2 mm) (bacterium 71) is a great chance for this study. The fact that bacterium 71 was not considered very significant at first, especially until the antagonistic relationship test, because it did not show phosphate solubilization, IAA or ACC deaminase activity, showed once again how important bacteria are.

Xanthomonas arboricola is a bacterial species that causes diseases in various plants and includes corylina, juglandis, fragariae, populi, pruni and celebensis pathovars. Among these pathovars, Xaj (*X. arboricola* pv. *juglandis*) manifests itself with walnut blight disease in most parts of the world where walnuts are produced. Within Europe, it has

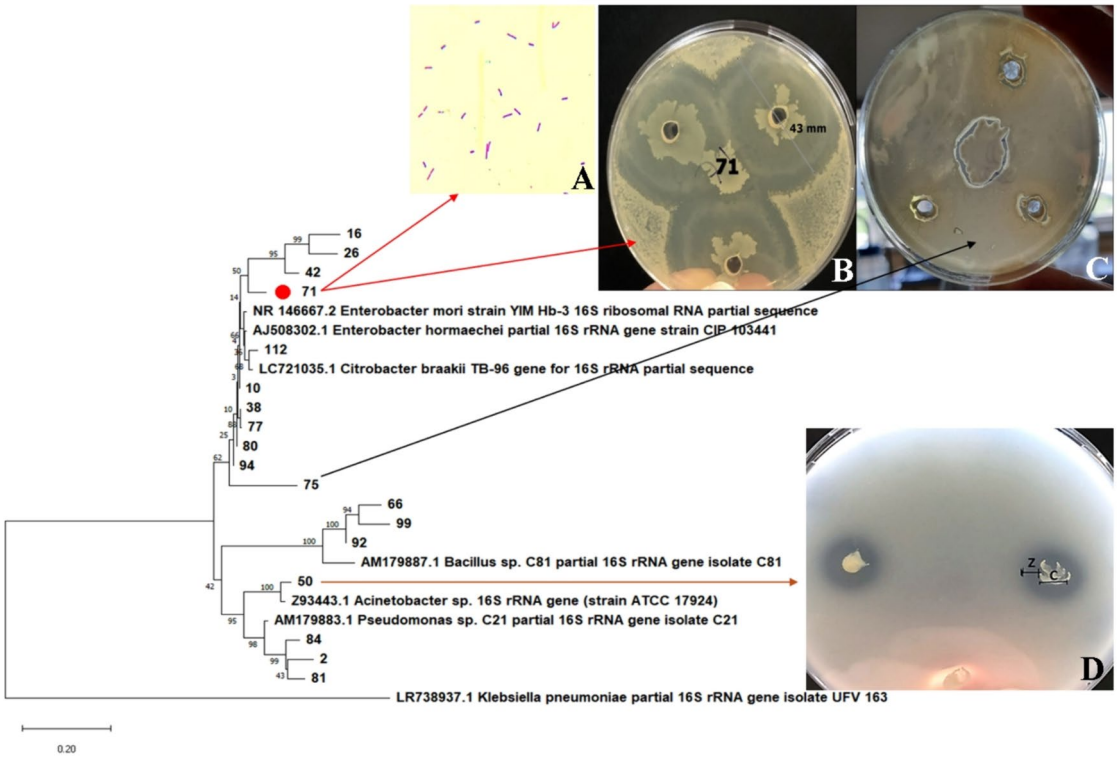

Fig. 2 Phylogenetic tree constructed based on 16S rRNA sequence analysis, numbers refer to the strains in this study, while bacteria with NCBI code at the beginning refer to those selected as outgroups; **A** Gram-stain image of strain 71 showing the highest antagonistic effect; **B**, **C** Antagonistic activity of the tested bacteria against *X. arbiricola* (left petri isolate 71 and right petri isolate 75); **D** Petri image of the phosphate solubilization activity test of isolate 50. Zone (Z), colony (C). The Neighbor-Joining approach was used to infer the evolutionary history (Saitou and Nei 1987). The ideal tree is displayed. Next to the branches are the percentage of duplicate trees where the related taxa grouped together in the bootstrap test (1000 replicates) (Felsenstein 1985). With branch lengths expressed in the same units as the evolutionary distances used to estimate the phylogenetic tree, the tree is depicted to scale. The evolutionary distances are shown in base substitutions per site and were calculated using the Maximum Composite Likelihood approach (Tamura et al. 2004). There were 25 nucleotide sequences in this investigation. For every sequence pair, all unclear places were eliminated (pairwise deletion option). The resulting dataset had 1137 locations in total. In MEGA11 (Tamura et al. 2021), evolutionary analyses were carried out

been reported to cause excessive losses in Turkey, France, Spain, Italy and Greece (Kaluzna et al. 2014). Based on these important considerations, the antagonistic behavior of a bacterium isolated from the rice rhizosphere against another pest, walnut, shows how many alternatives are available for biological control.

The contribution of bacteria to plant development has been demonstrated in many studies. When the studies on siderophore production among the properties provided by bacteria are examined, it is seen that this factor provides important advantages, especially resistance to plant pathogens. Some of the studies are as follows: it was reported that the *Bacillus aryabhattai* strain, which produced high levels of siderophores under iron deficiency and high salt concentration stress, increased rice productivity by 60% and 43% in salt-free and 200 mM concentration salt environments, respectively (Sultana et al. 2021). In another study, they found that a mutant *Pseudomonas fluorescens* WCS374r strain producing pyoverdin-type siderophore developed resistance against *Magnaporthe oryzae*, which causes infection in rice. In the same study, they inoculated siderophore-producing and non-producing strains of the same species into the soil and found that the infectious agent caused damage to rice leaves within a few days in the mutant strains that did not produce siderophore (De Vleesschauwer et al. 2008). In another study, they reported that the strain they isolated from the tobacco rhizosphere and identified as *Pseudomonas koreensis* produced phosphate-soluble, siderophore, ammonium, protease, amylase and cellulase, but not IAA, and also played a role in resistance to plant fungal pathogens (Gu et al. 2020). In the study conducted by Sheng et al. (2020), it was determined that the *Brevibacillus brevis* GZDF3 strain isolated from the *Pinellin* rhizosphere produced an effective antagonistic activity against *Candida albicans* causing fungal diseases due to its high siderophore production capacity. Based on these studies,

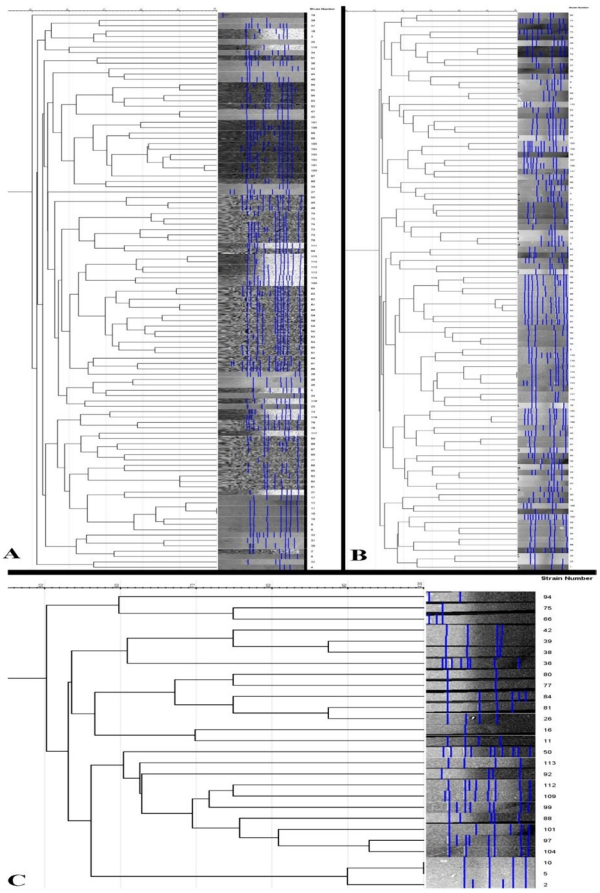

Fig. 3 UPGMA dendrogram analysis of band profiles obtained with *Msp*I (**A**), *Bsg*I (**B**) and *Hinf*I (**C**) restriction enzymes. The dendrograms were drawn in GelJ (Heras et al. 2015)

it can be concluded that there is a significant relationship between the antagonistic effect of strain 71 tested in our study against *X. arbiricola* (43 ± 2 mm) and the high siderophore produced (136.03 ± 4.6 µmol L^{-1}). In addition, Zhou et al. (2023) demonstrated that natural soil microbes support plant growth and fight against disease agents in the soil and that inoculating these microbes into the soil restores the natural capacity of the soil to fight against diseases. The results of these comprehensive studies reveal the importance of the strain 71 obtained from our study and show that it can be used in the suppression of the relevant disease agent.

It is now known as an undeniable fact that phosphorus is important for plant development. To ensure this, producers support agricultural areas with phosphorus-containing fertilizers. However, considering the increase in costs and the damage caused by fertilizers in the soil and its environment in the long term, there is a need to explore methods that will provide phosphorus uptake from the soil to the plant biologically. The bacteria tested in the present study did not contribute much to plants in terms of phosphorus solubilizing activity. This suggests that in the region where the study was conducted, plants were not supported by bacteria in terms of phosphorus uptake. However, considering the fact that *Arbuscular mycorrhizal* fungi (AMF) play a major key role in the uptake of phosphorus from the soil by plants by providing almost 90% of it, as stated by Edlinger et al. (2022), biological control can be carried out with alternative microbial groups by identifying soils with bacterial problems in phosphorus solubilization.

This study shows that some bacteria should be particularly emphasized, whether their role in plant growth or their activity against plant pathogens is evaluated. As a result, these bacteria provide a valuable biological substitute for synthetic fertilizers. 119 bacteria strains isolated from a significant rice-producing agricultural area had exceptional activity in the formation of siderophores, IAA, ACC deaminases, and phosphate-solubilizing activity. The presence of strain 71, a closely related member of the Enterobacter genus, among the studied bacteria was another noteworthy aspect. It is planned to study the effect of the most active bacteria to be selected for each feature on plant growth in the laboratory environment in future studies. It should be inevitable to support natural production through biological means, especially in this period when we are faced with a problem that has reached the point of damaging soil and water due to the excessive use of synthetic fertilizers. In terms of the tests conducted in this study, would the most active bacteria for each trait have the ability to colonize together? Can they survive against the resistance of the existing microorganism flora of the soil when they are inoculated into the soil again? Will these bacteria show the same activity when a mixed culture is prepared? Will isolates showing antagonistic relationships against walnut plant pathogens be effective against rice pathogens? The results of this study raised these questions and paved the way for further studies.

Accession numbers

GenBank access numbers for 18 isolates were determined in the range PP504606 to PP504623.

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Data availability The author has approved using the relevant sequence data uploaded to the Genbank database by this journal.

Declarations

Conflict of interest The author declares no conflict of interest.

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