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Plant growth-promoting rhizobacteria isolation from rhizosphere of submerged macrophytes and their growth-promoting effect on *Vallisneria natans* under high sediment organic matter load

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Summary

Sediment organic matter is a key stressor for submerged macrophyte growth, which negatively impacts the ecological restoration of lakes. Plant growth-promoting rhizobacteria (PGPR) were screened from the rhizosphere of submerged macrophytes and used due to their promoting effect on *Vallisneria natans* under a high sediment organic matter load. Root exudates were used as the sole carbon source to obtain the root affinity strains. Eight isolates were selected from the 61 isolated strains, based on the P solubilization, IAA production, cytokinins production and ACC deaminase activity. The analysis of the 16S rDNA indicated that one strain was *Staphylococcus* sp., while the other seven bacterial strains were *Bacillus* sp. They were all listed in low-risk groups for safety use in agricultural practices. The plant height significantly increased after inoculation with PGPR strains, with the highest rate of increase reaching 96%. This study provides an innovative technique for recovering submerged macrophytes under sediment organic matter stress.

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Introduction

The deterioration of water quality and degradation of the ecological structure are problems for water ecosystems worldwide (Ho *et al*., 2019). The restoration and reconstruction of water ecosystems depend on the dominance and community stabilization of submerged macrophytes (Sayer *et al*., 2010). The natural recovery of submerged macrophytes usually takes decades after the reduction in the water nutrition level (Sand-Jensen *et al*., 2008). Therefore, artificial-assisted recovery technologies have been used to accelerate the succession process. The germination and sprout growth of submerged macrophytes are commonly limited by sediment anoxia during restoration (Wu *et al*., 2009), and organic enrichment is a common issue in aquatic environments. Anoxic degradation pathways lead to oxygen exhaustion and the accumulation of potentially phytotoxic compounds, causing benthic vegetation decline (Soana *et al*., 2015). Decreases in the aquatic plant biomass of fertile sediments are related to high organic matter contents (Ni, 2001). Additionally, when the organic matter content is high enough to convert sediment into reductive sapropel, it threatens the survival and germination of aquatic plants (Phillips *et al*., 1978).

Plant growth-promoting rhizobacteria (PGPR) are symbiotic and free-living bacteria that live within the plant root and can directly or indirectly promote plant growth (Arruda *et al*., 2013; Vimal *et al*., 2017). Owing to their performance in plant growth promotion and biological control, the screening of region-specific and local PGPR strains has been explored for commercial use (Tabassum *et al*., 2017). PGPR can promote plant growth by dissolving potassium and phosphorus, secreting plant hormones, such as cytokinins and indole-3-acetic acid (IAA), and producing siderophore and 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Saleem *et al*., 2007), which enhance plant resistance to environmental

stresses (Pérez-Montaño *et al*., 2014). IAA is a common natural auxin in plants and can loosen their cell walls, increasing the production of root exudates to provide PGPR with some additional nutrients (Etesami and Maheshwari, 2018). The ACC deaminase produced by PGPR can help plants to decompose the synthesis precursor ACC of ethylene by metabolizing it into α-butanone acid and ammonia, which could reduce stress caused by ethylene accumulation (Ullah *et al*., 2015). Numerous PGPR strains have been isolated from rice, wheat and other crops (Bhattacharyya and Jha, 2012), as well as medicinal plants (Feng *et al*., 2016) and economic trees (Wang and Han, 2018).

Several studies have demonstrated that beneficial rhizospheric microorganisms can enhance the abiotic stress tolerance of plants, such as drouth, saline-alkali and heavy metal stresses, and nutrient deficits. The application of specific PGPR strains can alleviate salt stress in crops and improve their yields (Ramadoss *et al*., 2013; Qin *et al*., 2016; Ilangumaran and Smith, 2017). Inoculation with *Brevundiminas diminuta* NBRI012 increased the content of chlorophyll and absorption of phosphorus in the seedlings under arsenic pollution and significantly reduced the accumulation of arsenic in the aboveground rice (Singh *et al*., 2016). Additionally, *Streptomyces thermocarboxydus* can improve the drouth resistance ability of rice by increasing the dissolved phosphorus, secreting proline, phytohormone and ferritin contents (Lasudee *et al*., 2018). Therefore, based on terrestrial plant research of plant–microbe associations, the utilization of PGPR could be beneficial for the rehabilitation of submerged macrophytes under high sediment organic matter loads. However, little research has been conducted on this topic.

Owing to root exudation, the rhizosphere is a special niche where beneficial bacteria compete with other microbiota for organic carbon compounds and interact with plants and soils through root colonization (Haichar *et al*., 2014). These compounds can be used as chemical attractants for microorganisms, and the bacterial populations in the rhizosphere are 10–100 times larger than those in the bulk soil (Hassan *et al*., 2019). Amino acids and sugars secreted by roots are common chemical attractants for colonization of PGPR, which can use the specific compounds secreted by roots to synthesize plant growth hormones and promote plant growth (Asari *et al*., 2016). Root exudates provide important energetic materials for rhizosphere microorganisms that compete for the limited nutrition in this habitat. Some PGPR that seize some of the favourable sites of root surface exudates could effectively utilize the rhizosphere nutrients and root exudates (Haichar *et al*., 2014). Therefore, preliminary screening of PGPR based on root exudates as a selective medium could identify strains with a high competitive ability for rhizosphere nutrition.

Owing to the unique underwater life history, competition for abiotic resources, such as light availability, is more intense and the environmental conditions of freshwater ecosystems are typically beyond artificial control. The rehabilitation of macrophyte species and communities is more difficult than that of terrestrial plants. Owing to their adaptability to the aquatic environment and rapid reproduction rate, aquatic plants have good potential for habitat restoration and the removal of special pollutants, such as pesticide and toxic metals (Vidal Ribeiro *et al*., 2019; Saleh *et al*., 2019). This study aimed to explore the possibility of using the symbiotic relationship between PGPR and submerged macrophytes to improve their resistance to environmental stress, particularly propagule germination and seedling growth under a high sediment organic matter load. Rooted submerged macrophyte, *Vallisneria natans* (*V. natans*), was studied as it is widespread in tropical and subtropical areas and is commonly used in lake and river rehabilitation (Zhang *et al*., 2016).

This study attempts to identify PGPR strains that can promote the recovery of submerged plants in sediment with high organic matter content. However, we could not find naturally growing submerged plants in the high organic matter sediment of nearby lakes or rivers. In a microcosm system, submerged plants can grow in the sediments with both high and low organic matter contents; however, under a high organic matter content, the growth is inhibited (C. Wang, unpublished). Therefore, we collected rhizosphere samples from both field lake sediment and microcosm sediment to obtain strains with better growth-promoting effects. PGPR were screened from the rhizosphere of *V. natans* cultured under low and high sediment organic matter loads, where the root exudates of the host *V. natans* were the sole carbon source. Additional PGPR were screened from the rhizosphere of submerged macrophytes that naturally grew in West Lake, a shallow urban lake in China. The growth promotion effect of isolated PGPR strains was tested to provide an innovative approach for the artificially aided restoration of submerged macrophytes.

Results

PGPR isolation

After the primary screening of PGPR, strains with faster growth rates and larger colonies were selected from the medium of groups H (sediment with high organic matter levels), L (sediment with low organic matter levels) and S (sampling from field lake) respectively. A total of 61 strains, including 20 from group H, 27 from group L and 14 from group S, were obtained. The specific plant

species from which the PGPR strains were separated are shown in Table 1.

Determination of phosphorus solubilization

Quantitative analysis indicated that 50 strains could dissolve inorganic phosphorus, 20 of which were in group H, 19 in group L and 11 in group S. The specific results are shown in Fig. 1A. There was no significant difference between groups H and L; however, significant differences were found between groups S and H ($P = 0.012$) and groups S and L ($P = 0.002$). The amount of phosphorus dissolved in group S was significantly higher than that in groups H and L, with average amounts of 24.37, 14.03 and 11.55 mg/l respectively. MS4 had the strongest ability to dissolve phosphorus, with the dissolved amount reaching 59.35 mg/l.

IAA production ability

Indole-3-acetic acid (50 mg/l) and pure water were added to the colourimetric solution as positive and negative controls. In the colour reaction of the 61 tested strains, 50 could produce IAA, 19 of which were in group H, 18 in group L and 13 in group S, as shown in Fig. 1B. There was no significant difference in the IAA production ability between groups L and S. However, significant differences were found between groups H and L ($P = 0.036$) and groups H and S ($P = 0.007$). As shown in Fig. 1C, the strains separated from sediment with a high organic matter load had poor IAA production ability. The IAA production ability of group H was lower than that of the other two groups. Strain PC7 had the strongest ability to produce IAA, with the production amount reaching 46.80 mg/l.

ACC deaminase activity

Sixty-one strains were inoculated on the SMA solid medium. After five continuous passes, 26 strains could grow on the medium, with ACC as the sole nitrogen source. Six of these strains were in group H, 10 were in group L and 10 were in group S. There was no significant difference between the three groups. H19 had the highest ACC deaminase activity, reaching 0.047 U/mg. The specific activity of each strain is shown in Fig. 1E. Morphologically, the growth of *V. natans* was inhibited by the high organic load sediment after 70 days of acclimatization (Fig. 1D). The strains from group H may have had the highest activity of ACC deaminase to inhibit the synthesis of ethylene precursors and withstand sediment stress.

Table 1. Isolated PGPR strain numbers and their host plant species.

Group	Strain source	Strain number	Code name
H	*Vallisneria natans*	20	H1-H20
L	*Vallisneria natans*	27	L1-L27
S	*Hydrilla verticillata*	3	HV2 HV3 HV4
	Myriophyllum spicatum	3	MS1 MS3 MS4
	Vallisneria natans	1	VN3
	Potamogeton maackianus	2	PM1 PM2
	Potamogeton crispus	5	PC2 PC4 PC5 PC6 PC7

Cytokinin production ability

Following the protocol of the CTK ELISA quantitative detection kit (96T), 61 strains were tested, and all of them could produce cytokinins. The production ranged from 2.00 to 21.10 μg/l. H6 produced the most cytokinin (Fig. 1F), and there was no significant difference in the variance analysis of the three groups. The average amounts of cytokinin production were 7.14, 9.90 and 9.07 μg/l for H, L and S respectively.

Strain identification

The growth-promoting abilities of 61 strains were compared to select the optimal strains with the strongest ability of each function and strains with multiple functions. Finally, eight strains were selected and their growth-promoting effects on seed germination and the early growth of *V. natans* were tested. A phylogenetic tree was constructed based on the 16S rDNA sequences using MEGA7.0 (Fig. 2). The homology between the eight strains and the most similar model species in the database exceeded 99%. The isolates of H6 and H19 in group H *were Bacillus subtilis*, and L3 and L18 in group L were *Bacillus cereus*. The isolates in group S included *Bacillus stratosphericus*, *Bacillus thuringiensis* and *Staphylococcus xylosus*. The 16S rDNA sequences were uploaded on NCBI GenBank with submission ID SUB8104505 and accession numbers were obtained (Table 2).

Growth-promoting effects on seed germination and early growth of V. natans

The analysis of the growth-promoting effects of the eight selected strains indicated that the plants treated with PGPR were significantly taller than those of the non-inoculated control (Fig. 3A). Under the treatment with isolate PC2, the plant height was 96% higher than that of the control (Fig. 3B). The germination rate was either higher or lower than that of the control and did not show

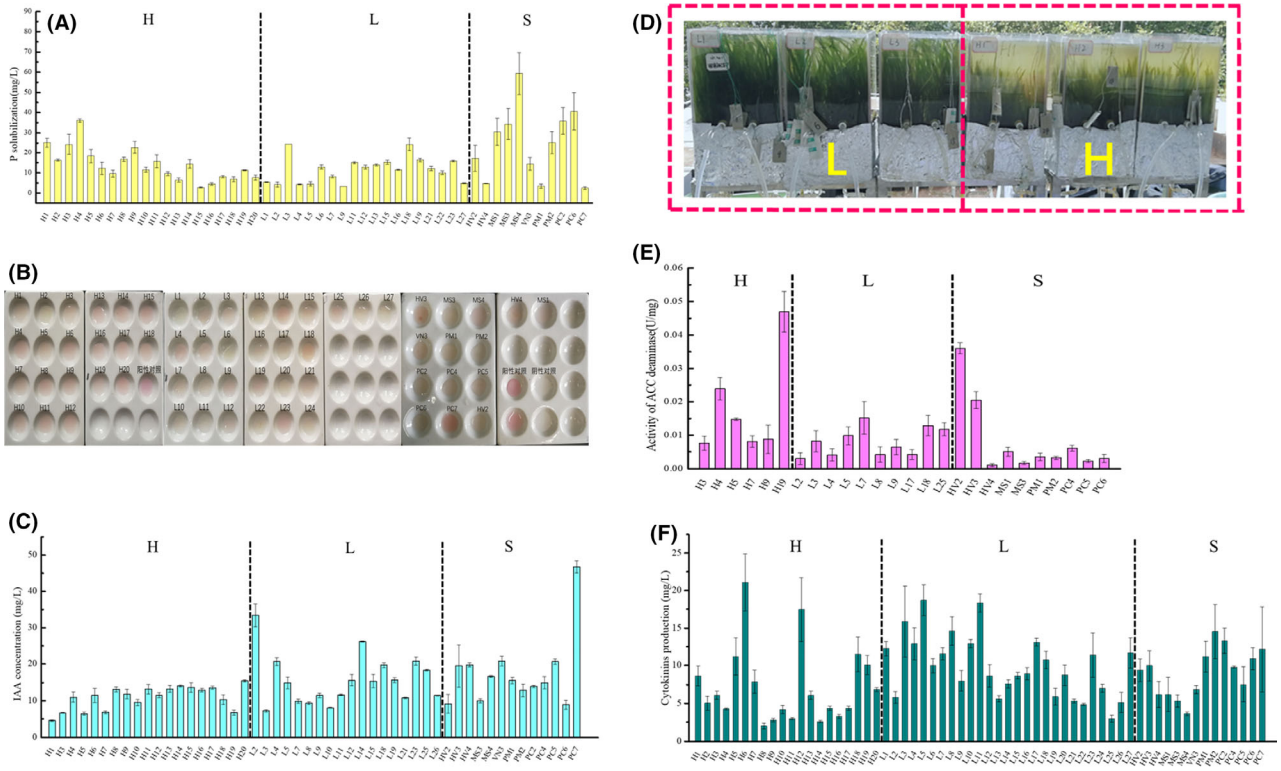

Fig. 1. Determination of the growth-promoting properties of the isolated strains. (A) Phosphorus solubility; (B) colour reaction of produced IAA; (C) IAA production ability of each strain; (D) growth performance of *V. natans* under low and high sediment organic matter load; (E) ACC deaminase activity; (F) ability to produce cytokinins. H: sediment with high organic matter; L: sediment with low organic matter; S: sampling from field lake.

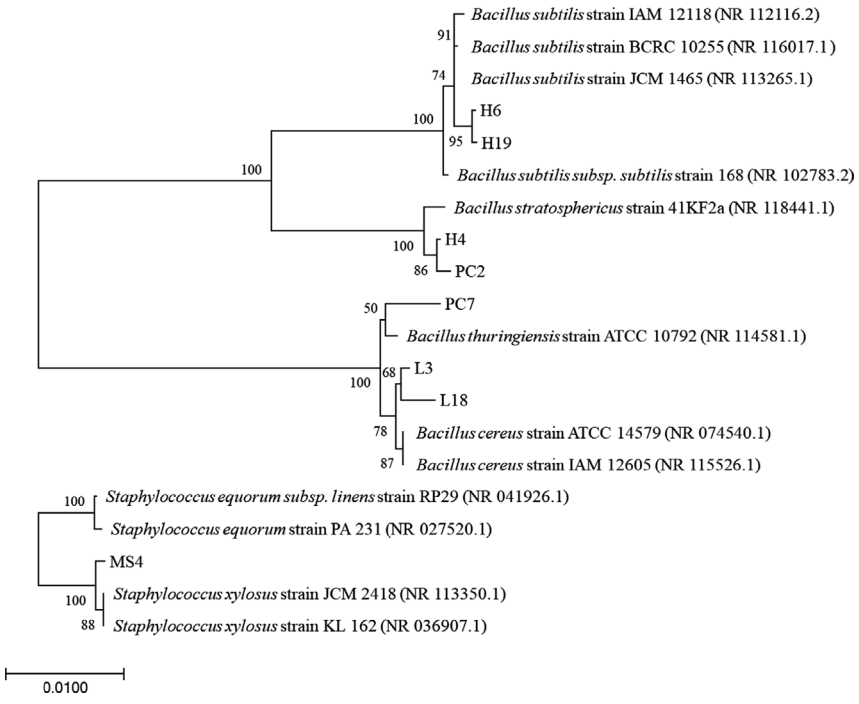

Fig. 2. Adjacency phylogenetic tree of the 16S rDNA sequence of the eight selected PGPR strains.

Table 2. Phylogenetic affiliation and the plant growth-promoting characteristics of each selected PGPR strain.

Group	Selected strains	Strain species	GenBank Accession number	P solubilization (mg/l)	IAA (mg/l)	ACC (U/mg)	Cytokinins (μg/l)	P.S.
High SOM	H4	*Bacillus stratosphericus*	MT974188	36.10 ± 0.87^{b}	10.99 ± 1.40^{ab}	0.024 ± 0.003^{a}	4.28 ± 0.13^{a}	Multiple function
	H6	*Bacillus subtilis*	MT974189	12.25 ± 3.00^{a}	11.48 ± 1.95^{d}	ND	21.10 ± 3.80^{a}	Optimum in cytokinins production
	H19	*Bacillus subtilis*	MT974190	11.38 ± 0.20^{a}	6.72 ± 0.68^{a}	0.047 ± 0.006^{b}	10.08 ± 1.29^{a}	Optimum in ACC deaminase activity
Low SOM	L3	*Bacillus cereus*	MT974191	24.45 ± 0.00^{ab}	7.25 ± 0.33^{a}	0.008 ± 0.003^{a}	15.87 ± 4.70^{a}	Multiple function
	L18	*Bacillus cereus*	MT974192	24.26 ± 3.13^{ab}	19.67 ± 0.67^{d}	0.013 ± 0.003^{a}	10.74 ± 1.16^{a}	Multiple function
Sampling from field lake	MS4	*Staphylococcus xylosus*	MT974193	59.35 ± 10.36^{b}	16.67 ± 0.20^{cd}	ND	3.62 ± 0.22^{a}	Optimum in P-solubilization
	PC2	*Bacillus stratosphericus*	MT974194	35.88 ± 6.56^{ab}	13.94 ± 0.35^{bc}	ND	13.31 ± 1.67^{a}	Multiple function
	PC7	*Bacillus thuringiensis*	MT974195	2.48 ± 0.74^{a}	46.80 ± 0.35^{e}	ND	12.17 ± 5.64^{a}	Optimum in IAA production

Letters a-e represent the significant difference among the selected strains for each indicator. ND, not detected; SOM, sediment organic matter.

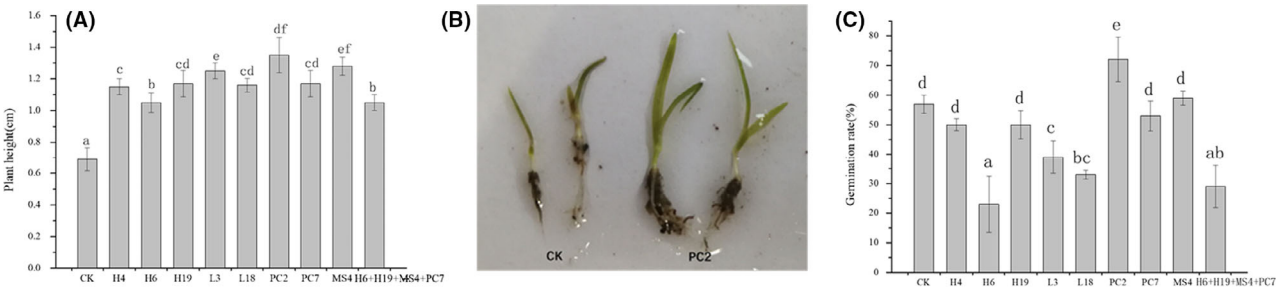

Fig. 3. Growth-promoting effects of *V. natans* in each inoculated treatment. CK in the x-axis represents the control treatment added by bacteria-free pure water, and the other abbreviations are tested strains. (A) Effects of PGPR inoculation on plant height; (B) morphological observation of the early formed sprout in the control and PC2 inoculated treatment; (C) effects of PGPR inoculation on germination rate.

an accordant-promoting effect with the plant height index among the tested strains (Fig. 3C). The plant growth-promoting characteristics of each inoculated treatment are shown in Table 2.

Discussion

Different PGPR bacterial genera have been isolated from food crops, cash crops and other plants (such as medicinal plants, industrial raw material crops and wild plants), and *Bacillus*, *Pseudomonas*, *Enterobacter* and *Burkholderia* are common (Karakurt *et al*., 2011; Liu *et al*., 2016). In this study, candidate PGPR were isolated by microcosm and field sampling. After testing their IAA production, P solubilization, ACC deaminase activity and CK production properties, eight PGPR strains with potential for growth promotion were selected from the 61 candidate strains (Fig. 4), one of which was *Staphylococcus* sp., and the other seven were *Bacillus* sp. *Staphylococcus* strain was screened from the *M. spicatum* rhizosphere in field sampling. *Bacillus* spp. were screened from the *V. natans* rhizosphere in the groups grown in sediment with high and low sediment organic matter contents and the *P. crispus* rhizosphere in the field sampling. According to the American Biological Safety Association (https://my.absa.org/Riskgroups) and the latest microbial safety review on PGPR (Ferreira *et al*., 2019), the strains tested for *V. natans* growth promotion efficiency were classified as biologically safe groups that are unlikely to cause human disease and have no risk of spreading to the human community.

Plant growth-promoting rhizobacteria could increase plant growth and resistance to abiotic stresses through various mechanisms, such as P solubilization, IAA production, cytokinin production and ACC deaminase activity (Etesami and Maheshwari, 2018). Additionally, the pollutant degradation, hormones and antibiotics or lytic enzyme production, heavy metal detoxification activities, salinity tolerance and biological phytopathogens and insects control properties of PGPR also contribute to the benefits they provide to plant growth (Gouda *et al*., 2018). Phosphorus is usually bound to Fe^{3+}, Ca^{2+} and

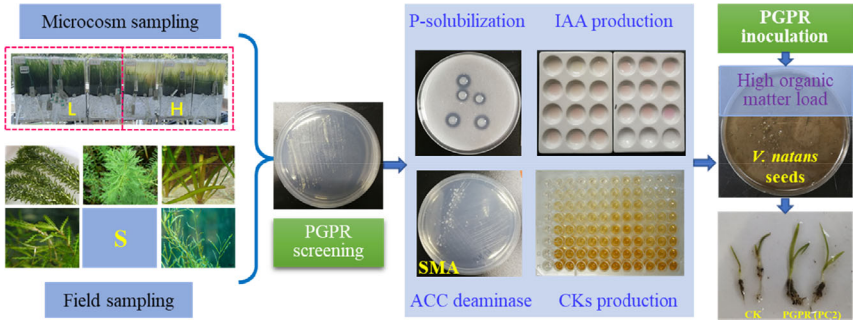

Fig. 4. The procedure of plant growth-promoting rhizobacteria (PGPR) screening, selection and inoculation. PGPR were screened from the rhizosphere of submerged macrophytes and selected by their plant growth promoting indicators. The excellent strains were inoculated and significantly promoted the growth of *Vallisneria natans* seedings under a high sediment organic matter load.

Al^{3+} in soil, and these compounds are water-insoluble and difficult to uptake by plants. Phosphorus-dissolving bacteria convert the insoluble form of phosphorus to a soluble form through two pathways: (i) reducing soil pH by secreting organic acids to dissolve insoluble phosphate or by secreting protons; and (ii) secretion of extracellular phosphatase to improve the utilization of phosphorus (Hu *et al*., 2004). MS4 from group S had the strongest ability to dissolve phosphorus, with the amount dissolved reaching 59.35 mg/l. After inoculation with MS4, the plant became 86% taller than the control. The promotion effect was greater than that of the crop plant inoculated with P-solubilizing bacteria, with the plant height increasing by 11.2–20.2% (Wang *et al*., 2017). Soil organic matter is crucial in regulating the effectiveness of phosphorus, because phosphorus could bind to soil organic matter via ternary complexes (Audette *et al*., 2020). Considering the availability of phosphorus may become a limiting factor for plant growth, we used the phosphorus selective medium for the first step of strains screening and thus obtained the strains with higher amount of dissolved phosphorus in group S. The IAA production abilities of groups L and S were significantly higher than that of group H, indicating that the IAA production ability of PGPR in high organic matter sediment was lower than that in low organic matter sediment. After the inoculation of the PC7 strain, the plant height increased by 70% compared with the control. IAA can be synthesized through numerous approaches, the most important of which is the tryptophan pathway (Xie *et al*., 2017). Tryptophan accumulates in plant leaves and roots under salinity and drouth stress (Llanes *et al*., 2016; Khan *et al*., 2019), and PGPR use tryptophan to synthesize IAA, which can significantly promote the growth of sugar beet, mustard, wheat and other plants (Asari *et al*., 2016). When ACC deaminase-producing bacteria were inoculated in wheat, the negative effects of drouth stress on wheat growth were significantly reduced (Bangash *et al*., 2013). In this study, plant growth in group H was inhibited at the later stage. This may be because the ethylene content of plants increased under high organic matter stress. Group H achieved the highest ACC deaminase activity, which might inhibit the synthesis of ethylene precursors and withstand this stress. ACC deaminase activity is relatively common in plant microbiomes, particularly in stressful environments, emphasizing its importance in the interactions between plants and PGPR (Orozco-Mosqueda *et al*., 2020). Cytokinins are also a plant hormone that can accelerate cell division and growth and promote plant growth (Ma *et al*., 2016). There was no significant difference between the H, L and S groups. Cytokinin-producing PGPR can enhance a plant's tolerance to environmental stress and growth inhibition due to aluminium could be alleviated (Zerrouk *et al*., 2020). The root length of maize seedlings increased by 40% when inoculated with *B. toyonensis*, which can produce cytokinin (Zerrouk *et al*., 2020).

We have done the correlation analysis between the growth-promoting efficiency with the data of IAA production, P solubilization, ACC deaminase activity and cytokinins production, respectively, however none significant correlations were found. PGPR act as a plant growth enhancer not only attribute to a single function but the combination of the direct mechanisms such as nutrient supply and the indirect mechanisms such as abiotic or biotic stress resistance. In this study, the tested strains that showing the best plant growth effect are multiple function strains in the four property indices rather than the ones optimum in the single index, which is in line with the opinion that the synthesis effects of PGPR properties makes them plant growth enhancers. After PGPR inoculation, the germination rate was either higher or lower than that of the control and was not as significantly promoted as the plant height index in this study. Carrozzi *et al.* (2012) reported that PGPR (*Azospirillum brasilense*) inoculation decreased the fraction of abnormal seedlings. This may be because PGPR inoculation intensified the survival of the fittest in the early development

of plant seeds. That is, only the well-developed seeds could germinate under these conditions. Therefore, the well-developed seeds germinated and the plant height was greatly promoted.

The mutually beneficial symbiosis between PGPR and plants agriculture, forestry and grassland industries has been studied for a long time. To resolve the issues of nutrition acquisition, resistance to environmental stress, pathogen control and transplanting, PGPR has been utilized in agricultural sustainability and vegetation protection (Singh and Singh, 2013; Franco and Castro, 2015; Castanheira *et al*., 2017). The mutually beneficial symbiosis between PGPR and plants may provide an innovative approach to the restoration of aquatic ecosystems and particularly resolve the recovery of submerged macrophytes under multiple abiotic stress factors. For example, PGPR that can produce IAA with ACC deaminase activity can enhance the ability of plants to withstand stress and promote the proliferation of plant cells and root elongation by reducing the production of ethylene. The inoculation of PGPR with frigostabile potential enhanced plant growth and landscape effects in winter (Chanway *et al*., 2000).

This study was conducted to explore effective PGPR strains for growth promotion under a high sediment organic matter load, thereby accelerating the recovery of submerged macrophytes in polluted water bodies. The plant height promotion rates for seed inoculation ranged from 52.17 to 95.65%. Three isolates that separately showed the highest growth promotion rate in group H, L and S were tested under non-sterilized condition for adult *V. natans* plants. The promoting effect was even more significant, with the highest increasing rate of the aboveground fresh weight reached 378.8% (C. Wang. unpublished). The experiment conducted with the selected PGPR strains on *V. natans* demonstrates that it is a feasible method for increasing plant biomass. This study provides an innovative approach to the restoration of submerged macrophytes, expanding the application of theoretical results of plant–microbe interactions in freshwater ecological restoration. Photosynthesis of submerged macrophytes is largely depended on water transparency and the recovery of submerged plants in saline-alkali waterbody is also challenging. Therefore, the idea could be further applied to isolate PGPR strains coping with low light, salinity and other specific stress conditions.

Conclusions

Plant growth-promoting rhizobacteria strains were screened from the rhizosphere of submerged macrophytes, and their IAA production, cytokinin production, P-solubilizing ability and ACC deaminase activity were detected. Eight strains of PGPR with better performance were isolated for *V. natans* seed inoculation. One strain was identified as *Staphylococcus* sp., and the other seven bacterial strains were *Bacillus* sp., which were all listed as biologically safe agents. The selected PGPR strains significantly promoted the growth of *V. natans* under a high sediment organic matter load, and the highest rate of increase in seeding *V. natans* after inoculation reached 96%. This study expands the application of PGPR in freshwater ecosystems and provides suggestions for the artificially assisted restoration of submerged macrophytes.

Experimental procedures

Rhizosphere soil collection

i. Microcosm sampling: According to the Guidelines for the Protection and Management of Aquatic Sediment Quality, the severe effect level at which the sediment concentration of a compound detrimental to the majority of benthic species for aquatic sediment is 17.24% (Persaud *et al*., 1993). Therefore, sediments with low (L) and high (H) organic matter levels (4.94% and 17.35% as loss on ignition, LOI) were obtained from Maojiabu and Xilihu, the two different areas of West Lake, Hangzhou, China. Microcosms of two groups (L and H) were established in 20 × 12 × 30 cm cubic structures composed of polymethyl methacrylate. Each group was tested in triplicate, and *V. natans* was planted in each microcosm. After 70 d, approximately 10 g of the *V. natans* roots and the surrounding sediment were collected for strain screening.
ii. Field sampling: The sediment organic load of the area inhabited by submerged plants in West Lake is approximately 10% (as loss on ignition, LOI). Therefore, we collected rhizosphere samples for PGPR isolation from submerged plants inhabiting sediment with low and medium contents of organic matter. Individuals of five species, including *Vallisneria natans*, *Potamogeton crispus*, *Myriophyllum spicatum*, *Hydrilla verticillata* and *Potamogeton maackianus*, were selected for rhizosphere soil (group S). Approximately 10 g of plant roots and the surrounding sediments were collected for strain screening.

Culture medium

The root exudate medium was used for the preliminary screening of the candidate strains, and the other media used in this work included LB medium, Monkina inorganic phosphorus medium and SMA medium.

Root exudate medium: The intact plants of *V. natans* in the H and L groups were washed and then soaked in

ultrapure water. The leaves were placed along the glassware wall for regular water spraying. After 12 h of hydroponic collection, the ultrapure water was lyophilized for root exudate collection. Approximately 0.51 mg of dry root exudate matter was obtained per gram of fresh *V. natans*. The medium was prepared with a final root exudate concentration of 20 mg/l. The dry matter of the root exudates was re-dissolved in ultrapure water, sterilized through a 0.22 μm filter and placed in a 60 °C water bath for preheating. The agar medium (40 g/l) was autoclaved and cooled to 60 °C in a water bath. The two solutions were then mixed with a volume ratio of 1:1 to obtain a preliminary screening medium.

LB medium (1 l): 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH = 7.0.

Monkina inorganic phosphorus medium (1 l): 10.0 g glucose, 0.5 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4{\cdot}7H_2O$, 0.03 g $MnSO_4{\cdot}4H_2O$, 0.3 g KCl, 0.03 g $FeSO_4{\cdot}7H_2O$, 0.3 g NaCl, 10.0 g $Ca_3(PO_4)_2$, 18.0 g agar, pH = 7.0–7.5; the liquid medium was prepared without agar.

SM medium (1 l): 1.0 g glucose, 1.0 g sucrose, 1.0 g sodium citrate, 1.0 g malic acid, 1.0 g mannitol, 1.0 g CH_3COONa, 0.4 g KH_2PO_4, 2.0 g K_2HPO_4, 0.2 g $MgSO_4$, 0.1 g $CaCl_2$, 1 mg $CuSO_4$, 1 mg $NiSO_4$, 5 mg $ZnSO_4$, 5 mg $FeSO_4$, 3 mg $MnSO_4$, 1 mg $CoSO_4$, 1 mg Na_2MoO_4, 2 mg H_3BO_3, pH 6.4; the liquid medium was prepared without agar.

The sterilized ACC was added to the SM medium to obtain the SMA medium with a concentration of 0.5 g/l.

Screening of candidate PGPR

Groups H and L: 10 g of *V. natans* roots from groups H and L and their surrounding sediments were weighed in a conical bottle, with 90 ml of sterile water and sterile glass beads. The mixture was incubated in a shaking incubator at 28 °C and 170 r/min for 30 min. Gradient dilutions of the bacterial suspensions (10^{-4}, 10^{-5} and 10^{-6}) were plated and applied to the root exudate medium for the initial screening of the PGPR. Each gradient was replicated three times. The culture dishes were then incubated at 28 °C for 5 days. The dominant strains were selected for separation and purification and stored in glycerinum (20%) at −80 °C before use.

Group S: Rhizosphere samples for each plant species were obtained following the screening method for groups H and L and applied to the Monkina inorganic phosphorus medium for the initial screening of the PGPR.

P solubilization ability

Qualitative determination: Each strain to be tested was activated in the LB medium, separately spotted onto the Monkina inorganic phosphorus solid medium following the dropping method, and cultured at 28 °C for 10 days. Whether the strain could dissolve inorganic phosphorus was determined by calculating the ratio of the diameter of the phosphate ring to that of the colony (D/d).

Quantitative determination: The strain to be tested was inoculated into the Monkina inorganic phosphorus liquid medium at a dose of 1% and incubated at 28 °C and 180 rpm for 7 days. Two millilitres of the bacterial suspension were then centrifuged to obtain the supernatant. The dissolved phosphorus content was then quantitatively determined following the antimony molybdenum anti-colourimetric method (Wu *et al*., 2012).

IAA production ability

Qualitative determination: Each strain to be tested was inoculated into the LB liquid medium containing L-tryptophan (200 mg/l) and incubated in a shaking incubator at 28 °C and 180 rpm for 4 d. The suspension (50 μl) was dropped on a white ceramic plate along with an equal volume of the Salkowski colourimetric solution and stored at 25 °C for 30 min. IAA production was indicated by whether the colour turned red.

Quantitative determination: The standard curve was prepared using analytically pure IAA. The absorbance (OD_{530}) was determined by Salkowski colourimetry using IAA as the standard solution at concentrations of 0, 3.5, 7, 14, 21, 28 and 35 mg/l. The standard curve was drawn with the OD value as the abscissa and IAA concentration as the ordinate.

Two millilitres of the bacterial suspension cultured for 4 days were collected in a centrifuge tube and centrifuged for 5 min at 9391 ($\times g$). The supernatant was transferred into an equal volume of the Salkowski colourimetric solution. After storage under darkness at 25 °C for 30 min, the OD_{530} values were detected, and the IAA content was calculated from the standard curve (Gordon and Weber, 1951).

ACC deaminase activity assay

Qualitative determination: Each strain to be tested was inoculated into SMA solid medium, and the strain that was grown on the medium with ACC as the sole nitrogen source five times was selected as the ACC deaminase-positive strain.

Quantitative determination: The strains were cultured in LB liquid medium for 24 h following the methods of Honma (1978) and Penrose and Glick (2003) and then centrifuged at 4 °C for 5 min at 8228 ($\times g$) to collect the cell pellet, which was washed and centrifuged twice with SM medium, resuspended in SMA medium, and then incubated in a shaking incubator at 28 °C and 180 rpm for 24 h to induce the production of ACC deaminase.

The bacteria were collected by centrifugation at 4 °C and 8228 × *g* for 5 min and washed twice with a 0.1-mol/l Tris-HCL buffer (pH 7.6) to remove the SMA medium. The bacteria were then suspended in 1 ml of the 0.1-mol/l Tris-HCL buffer (pH 7.6), transferred to a 2-ml centrifuge tube and centrifuged at 13 523 × *g* for 5 min to collect the cells, which were then resuspended in 600 μl of the 0.1-mol/l Tris-HCL buffer (pH 8.5). Approximately, 30 μl of toluene was added and the cells were disrupted by vortexing for 30 s. To determine the protein concentration, 200 μl of the crude enzyme solution was mixed with 20 μl of a 0.5-mol/l ACC solution and bathed at 30 °C for 15 min. After adding 1 ml of 0.56-mol/l HCL, the solution was mixed thoroughly and centrifuged for 5 min at 13 523 × *g*. One millilitre of the supernatant was transferred and added to 800 μl of 0.56-mol/l HCL. After thorough shaking, 300 μl of 2,4-dinitrophenylhydrazine (2 mol/l HCL dissolved, mass concentration of 2 g/l) was added at 30 °C for 30 min. Finally, the OD_{540} value was determined by adding 2 ml of 2 mol/l NaOH. Pure water was treated instead of the bacterial suspension as a control.

Standard α-butanone acid solutions with concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1 mmol/l were prepared with the Tris-HCL (pH 8.5) solution as a control. The standard curve was then drawn with the concentration of α-butyric acid solution as the ordinate and the OD_{540} value as the abscissa.

The amount (μmol) of the substance that catalyzed the ACC to produce α-butyric acid by ACC deaminase per minute was calculated based on the standard curve as one unit enzyme activity (U). The protein content was determined following the Coomassie Brilliant Blue G-250 method (Bradford, 1976), and the standard curve was drawn using a gradient solution of bovine serum protein. The ratio of the unit enzyme activity to total protein content was defined as the ACC deaminase activity in U/mg.

Cytokinins production ability

Quantitative detection of the microbial cytokinins was conducted following the protocol of the CTK ELISA quantitative detection kit (96T), and its detection range was from 0.3 to 14 μg/l.

Strain identification

16S rDNA sequencing was performed for the selected excellent strains, and the sequence was determined by Sangon Biotech (Shanghai) Co., Ltd. The 16S rDNA partial gene was amplified by PCR using the 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') universal primers. The relevant strain sequences were obtained from GenBank of the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov).

Promotion effect of selected strains on the V. natans seeds

Sediment with a high organic load (OM = 17.35%) was selected and sterilized by autoclaving for the germination experiments. The sterilized sediment was then laid in Petri dishes (diameter of 15 cm) at a height of 1.5 cm and covered with 0.5 cm of pure water. A total of 100 sterilized *V. natans* seeds were evenly placed on the sediment, and pure water was then added. Each strain was tested in triplicate. One millilitre of a bacterial solution (OD = 1) prepared from the pure water re-suspension that had been washed and centrifuged three times was added to each Petri dish every 5 days. Additionally, bacteria-free pure water (1 ml) was added every 5 days as a control. Pure water was added every 3 days to maintain the sediment moisture content. The Petri dish was placed in a 2000-lux light incubator for 12 h under light followed by 12 h under darkness. The experiment lasted for 10 days, and the germination rate and plant height were measured at the end of the experiment.

Statistical analysis

The PASW Statistics 18.0 software package was used to conduct a one-way ANOVA test in order to analyse the significant differences in P solubilization, IAA production, ACC deaminase and cytokinin production between groups H, L and S, and for the eight selected strains. The effects of PGPR inoculation on seed germination and plant growth were also tested by one-way ANOVA. The Student–Newman–Keuls (SNK) method was employed to conduct stepwise multiple comparisons. Column plots were created using OriginPro 9.0 (OriginLab Corporation, Northampton, MA, USA), and the phylogenetic tree was constructed using the neighbour-joining algorithm in MEGA 7.0 (Kumar *et al.*, 2016).

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Conflict of interest

The authors declare that they have no conflict of interest.

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