EFFECTS OF DIFFERENT SUBSTRATE ADDITIONS ON GROWTH STATUS OF SUBMERGED PLANTS AND RHIZOSPHERE MICROORGANISMS IN SEDIMENTS

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Abstract. Sediment is an important factor affecting the growth of aquatic plants. The effects of sediment substrate application on aquatic plants and rhizosphere microorganisms are poorly understood. This study examined the effects of four substrates (maifanite, vermiculite, ceramsite, and volcanic rock gravels) on the growth and rhizosphere microorganisms of *Vallisneria natans* (Lour.) Hara using Illumina high-throughput sequencing technology. The results showed that the root activity of *V. natans* in the four treatment groups was higher than that in control group (CK) during the whole experimental period. Catalase activity in leaves in the same treatment groups, except the maifanite group, was significantly different during different periods ($P < 0.05$). All four treatment groups exhibited faster reduction in malondialdehyde content in leaves than the CK group, indicating that substrate addition could rapidly enhance plant stress adaptation. Redundancy analysis showed that root vitality positively correlated with biomass, plant height, and root length, and that different substrates had different effects on the growth of *V. natans*. Relative abundance of *Desulfobacterota* and *Nitrospirota* was higher in the treatment groups than in the CK group. Our results indicated that substrate addition, especially maifanite, into lake sediments could promote the growth of submerged plants and improve the rhizosphere microbial community structure.
Keywords: *submerged macrophyte, sediment modifying, growth and ecophysiological, synergistic effect, microenvironment*

Introduction

Lake eutrophication is a process driven by the accumulation of excessive nutrients in lakes, resulting from both natural factors and human activities (Zou et al., 2020). Lake eutrophication leads to the massive growth and reproduction of algae in water, consequently reducing the stability of water ecosystems, and posing a global environmental problem (Le Moal et al., 2019; David et al., 2020). At present, the prevention and control strategies against lake eutrophication mainly include physical,

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chemical, and biological ones. Specific technologies mainly include aeration, oxygenation, mechanical removal of phytoplankton, dredging, and sediment removal, addition of flocculants and algaecides, phytoremediation (Schindler et al., 2008; Qin et al., 2013; Niemistö et al., 2020; Zhang et al., 2020). However, physical and chemical methods are not effective in solving lake eutrophication, in contrast, biological and ecological methods can continuously reduce pollutants in lakes, while maintaining the stability of the ecological environment.

Submerged macrophytes are the main primary producers in aquatic ecosystems and play an important role in maintaining biodiversity and water ecosystem stability (Malecki-Brown et al., 2010; Phillips et al., 2016). These plants can provide habitat for organisms, absorb nutrients from the water body and sediments, and slow sediment suspension (Horppila and Nurminen, 2003; Veraart et al., 2011; Xian et al., 2022). Le Bagousse-Pinguet et al. (2012) found that interactions between submerged macrophytes can promote plant growth and reproduction in harsh environments, and also facilitate the growth of target macrophytes threatened by severe eutrophication. Submerged macrophytes are also very important for improving the eutrophication state of lakes (Gao et al., 2017).

The ability of submerged macrophytes to establish good roots in sediment is a key element for their growth. The physical and chemical properties of sediments play a very important role in the root growth of submerged macrophytes (Lin et al., 2020), and they affect the process of rooting, germinating, and stable growth of plants (Rattray et al., 1991; Xie et al., 2005). Submerged macrophytes can also absorb nutrients directly from the sediment to meet their own nutritional needs (Carr and Chambers, 1998). However, high contents of organic matter, nitrogen, phosphorus, and other nutrients in eutrophic sediments accelerate eutrophication and water bloom (Qiu et al., 2016), thus limiting the growth and distribution of submerged plants (Soana et al., 2012; Zhu et al., 2016). Therefore, it is necessary to improve the sediment for the stable growth of submerged macrophytes.

Nutrients in sediments maybe easily enter the water under certain conditions (Peng et al., 2021). Addition of the substrates onto the sediment surface can inactivate sediments and slow down the release of pollutants and nutrients into water (Wang et al., 2019). The common sediment-modifying substrates have been widely used for ecological restoration due to their environmentally friendly nature (Larsen et al., 2004; Liu et al., 2021). Moreover, substrate not only affects the removal efficiency of pollutants from the water body by aquatic plants, but also affects the growth rate of plants (Zotina et al., 2014). Some studies have found that the substrate is the key factor to promote the growth of submerged macrophytes (Jones et al., 2012; Xu et al., 2016). However, there is still a lack of understanding of the links between the substrate and the growth state of submerged macrophytes or the sediment microenvironment.

Considering it, this study added four different kinds of commonly used substrates to the sediment and planted *Vallisneria natans* (Lour.) Hara. The aim of this study was to: (1) investigate the effects of different substrates on the growth, physiological, and biochemical properties of *V. natans*, and (2) compare the effects of different substrates on microorganisms in rhizosphere sediments.

Materials and methods

Sampling sites and sediment collection

In June 2022, 15-cm-thickness surface sediment samples were collected using a Peterson grab sampler from the center location of Shangjinhu Lake (114°11′31″ N,

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30°38′22″E), one sub-lake of Jinyinhu Lake, located in Wuhan, China. Then, the collected samples were immediately transported to the laboratory for preservation. A portion of the sediment samples were used for the determination of physicochemical properties. The remaining sediment samples were filtered with a sieve to remove large dinas and residues of animals and plants for subsequent planting experiments. The contents of organic matter (OM), total nitrogen (TN), and total phosphorus (TP) in the sediments in Shangjinhu Lake were 77.50 g kg^{-1}, 3.13 kg^{-1}, and 0.82 g kg^{-1}, respectively.

Experimental plants and substrates

V. natans is a common submerged macrophyte species, which is widely distributed in the middle reaches of the Yangtze River. It is widely used for the restoration of lake ecosystems due to its rapid growth and developed root system, and it can absorb excess nutrients from the water body (Yan et al., 2013). The *V. natans* was incubated in a constant temperature room (25°C) at a light intensity of 3500 lx with the light-dark duration ratio of 1:1 for a week. After incubation, the healthy and equal-sized submerged plants were selected, pruned to a uniform plant height of 10 cm, and transplanted into the polyethylene column (15 cm in diameter, and 50 cm in height).

The experiments were carried out in polyethylene column, each containing five *V. natans* plants (10 cm in height) and a sediment layer of 20 cm thickness. The 4 treatment groups consisted of maifanite (ME), vermiculite (VE), ceramsite (CE), and volcanic rock gravels (VR) substrates (3-5 mm in particle size). The group without additional substrate was control group (CK). All substrates were produced in Henan province, China. The sediment surface was covered with 1 cm of substrate, and then distilled water was added to make the column water depth reach 24 cm. The experiment was performed in triplicates. *V. natans* planting experiment started on June 25, 2022, and lasted for 40 days with plant sampling every 10 days.

Determination of plant growth indicators

One *V. natans* plant was randomly selected from each column at each sampling time, and after being cleaned, their growth indicators were determined including height, plant biomass, leaf number, and root length. The chlorophyll content and malondialdehyde (MDA) content of leaves were determined by the Lichtenthaler–Arnon method and thiobarbituric acid method, respectively. The superoxide dismutase (SOD) and catalase (CAT) activity were determined by guaiacol method, and root activity was determined by the triphenyl tetrazolium chloride (TTC) method (Yang et al., 2012).

Determination of microorganisms

The 0.4 g sediment samples were taken at the final sampling (2022.8.4) and stored at −80°C for DNA extraction. Sediment DNA was extracted using a Fast DNA Spin Kit for Soil (Qbiogene Inc., Carlsbad, CA, USA) according to the manufacturer's instruction. The extracted DNA was used as template for PCR amplification. The 20 μL PCR reaction system contained 4 μL of 5×FastPfu Buffer, 2 μL of dNTPs (2.5 mmol L^{-1}), 0.4 μL of FastPfu Polymerase, 0.8 μL of each primer (5 μmol L^{-1}), 0.2 μL of BSA, 1 μL of template DNA, and 10.8 μL of ddH_2O. In order to investigate the dynamic variations of the microbial community structure, DNA samples were PCR amplified with the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-

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GACTACHVGGGTATCTAATCC-3′) (Herlemann et al., 2011) and pyrosequenced using a HiSeq sequencer of Illumina by the standard PCR methods and protocols. PCR was performed as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 45 s. The amplified products were detected by 2% agar gel electrophoresis. The purified amplicons were mixed in equimolar amounts and sequenced on an Illumina Hiseq2500 PE250 platform.

Statistical analysis

The differences in the physiological and biochemical properties of the *V. natans* samples among different groups were determined by one-way ANOVA and Tukey's test using SPSS 20.0 software ($P < 0.05$). Detrended correspondence analysis (DCA) (Canoco 4.5) was used to examine the correlation between different substrate and physiological characteristics of the plant. Since the length of the gradient value obtained by DCA in this study was less than three, the redundancy analysis (RDA) was further conducted.

Results

Effect of different substrates on plant growth

Except for plant height, no significant difference in the growth status of *V. natans* was observed within the same substrate group at different times ($P > 0.05$) (*Fig. 1a*). The mean plant height in the VE group (26.33 ± 1 cm) was higher than that in other groups (CK: 18.56 ± 0.48 cm, CE: 20.44 ± 4.06 cm, ME: 18.96 ± 2.71 cm, VR: 21.90 ± 1.90 cm) during the whole experimental period. After a 40-day culture, the maximum plant height was VE group (30.5 ± 0.51 cm), and the maximum number of leaves was CE group (13.5 ± 1.53). In addition, the mean number of leaves in all 4 treatment groups (CE: 12.38 ± 1.87, ME: 12.63 ± 1.38, VE: 11.50 ± 1.25, VR:11.88 ± 2.38) was higher than that in the CK group (7.63 ± 0.63) during the whole experimental period (*Fig. 1b*). During the growth of *V. natans* under the four substrate treatments, the change trend of root length was consistent with that of leaf number ($P > 0.05$) (*Fig. 1c*). The mean root length in all 4 treatment groups (CE: 10.93 ± 1.68 cm, ME: 10.38 ± 1.38 cm, VE: 13.75 ± 1.00 cm, VR: 13.94 ± 0.77 cm) was higher than that in the CK group (8.45 ± 0.29 cm) during the whole experimental period (*Fig. 1c*). During the culture period, no significant difference in the biomass of *V. natans* was observed among all the groups at different times ($P > 0.05$) (*Fig. 1d*). The biomass of VE and VR groups was higher than that of the CK group at different times, and the mean biomass in all 4 treatment groups (CE: 2.84 ± 1.49 g^{-1}, ME: 2.72 ± 0.81 g^{-1}, VE: 4.74 ± 0.73 g^{-1}, VR: 3.45 ± 0.75 g^{-1}) was higher than that of the CK group (2.70 ± 0.98 g^{-1}) during the whole experimental period.

Effect of different substrates on physiological properties of plants

In ME group, the contents of Chl-a and Chl-b showed the same change trend, which increased first and then decreased (*Fig. 2a, b*). On the 20th day post culture, the contents of Chl-a and Chl-b in the ME group reached the maximum, which were 2.01 ± 1.22 mg g^{-1} and 1.37 ± 0.78 mg g^{-1}, respectively. On the 40th day post culture, the total chlorophyll content in all the groups except group VE (which was slightly

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decreased) was increased, compared with the initial value (on day 10), and the highest total chlorophyll content was observed in ME group (2.4 ± 1.21 mg g^{-1}) (*Fig. 2c*). During the whole experiment, the average total chlorophyll content in *V. natans* leaves in CE, ME, VE, VR, and CK groups was 0.99 ± 0.20 mg g^{-1}, 2.40 ± 1.28 mg g^{-1}, 1.10 ± 0.09 mg g^{-1}, 1.03 ± 0.09 mg g^{-1}, and 0.99 ± 0.05 mg g^{-1}, respectively.

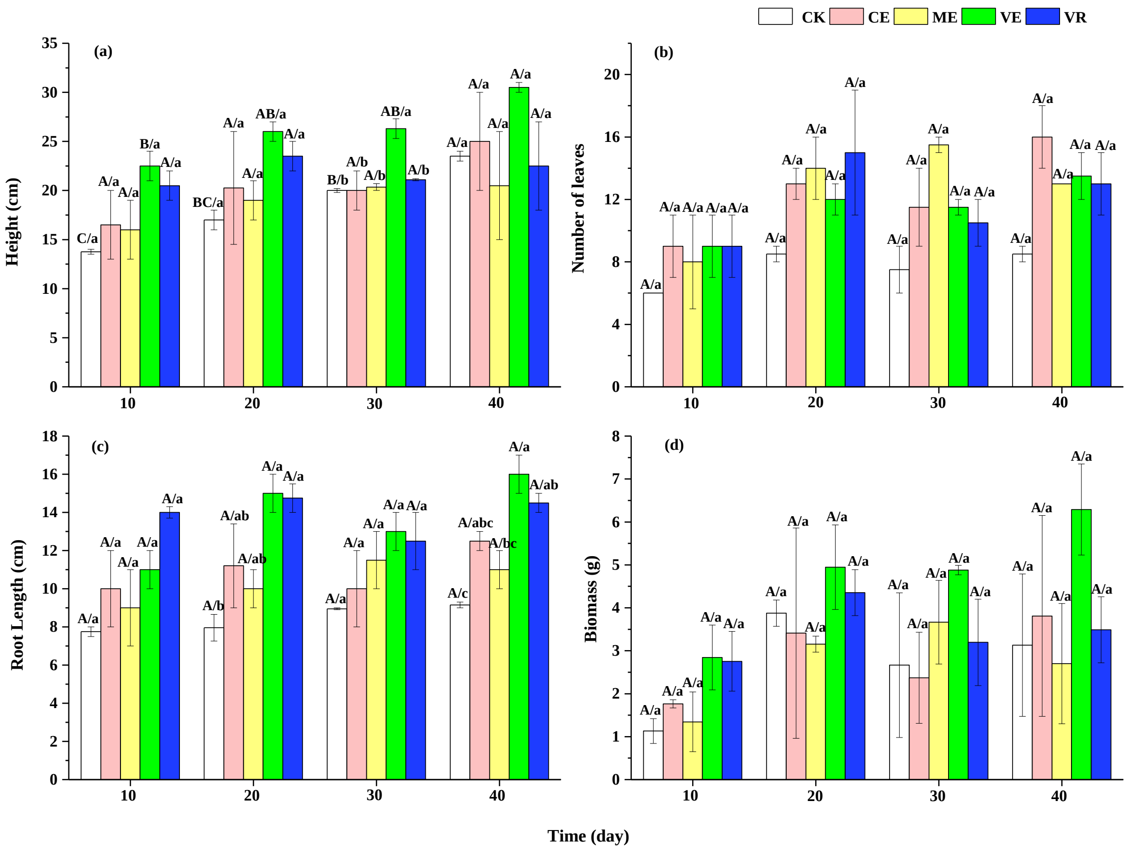

Figure 1. *Effects of different substrates on V. natans growth. (a) Height. (b) Number of leaves. (c) Root length. (d) Biomass. The bar chart uses different capital letters to indicate significant differences at the P < 0.05 level within the same treatment group at different times and different lowercase letters to indicate significant differences at the P < 0.05 level among different treatment groups at the same times. Error bars indicate standard deviation*

From the early to middle stage of culture (day 10-20), CAT activity was increased in all groups, and the highest CAT activity was observed in VR group (489.83 ± 46.13 U g^{-1} min^{-1}) (*Fig. 3a*). However, on the 40th day post culture, the CAT activity in all four treatment groups (CE: 200.0 ± 30.2 U g^{-1} min^{-1}, ME: 196.95 ± 42.5 U g^{-1} min^{-1}, VE: 92.49 ± 11.82 U g^{-1} min^{-1}, VR: 161.70 ± 15.04 U g^{-1} min^{-1}) was lower than that of the CK group (224.27 ± 11.12 U g^{-1} min^{-1}). No significant difference in the SOD activity in *V. natans* leaves was observed among CE, ME, or VR groups during the whole culture period ($P > 0.05$) (*Fig. 3b*), and on day 30, the SOD activity in CE, ME, and VR groups reached the maximum, which was 331.18 ± 108.72 U g^{-1} min^{-1}, 458.50 ± 58.53 U g^{-1} min^{-1}, and 215.72 ± 82.99 U g^{-1} min^{-1}, respectively. On day 40, SOD activity in *V. natans* leaves in all the treatment groups reached the lowest value, which was similar to the change trend of CAT activity.

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The MDA content in *V. natans* leaves was significantly different among all five groups during the different culture period (P < 0.05) (*Fig. 3c*), and the MDA content in CE and ME groups was higher than that in VE and VR groups during all the periods. Compared with the CK group, the 4 treatment groups reached the maximum MDA content earlier. On day 10, there were significant differences in MDA content among all groups (P < 0.05), and the MDA content in *V. natans* leaves in CE, ME, VE, and VR groups reached the maximum, which was 53.73 ± 73 nmol g^{-1}, 61.38 ± 6.88 nmol g^{-1}, 48.37 ± 1.07 nmol g^{-1}, and 41.08 ± 4.94 nmol g^{-1}, respectively.

The root activity of *V. natans* in all five groups increased first and then decreased (*Fig. 3d*), and it reached the highest from 20 to 30 days post culture. The root activity of *V. natans* in the four treatment groups was higher than that in the CK group throughout the entire period, but without statistical significance (P > 0.05) during the different culture period. During the whole experiment period, the mean root activity of *V. natans* in CE, ME, VE, VR, and CK groups was 67.14 ± 49.42 U g^{-1}, 34.78 ± 12.53 U g^{-1}, 74.58 ± 58.01 U g^{-1}, 85.28 ± 37.68 U g^{-1}, and 28.48 ± 13.91 U g^{-1}, respectively.

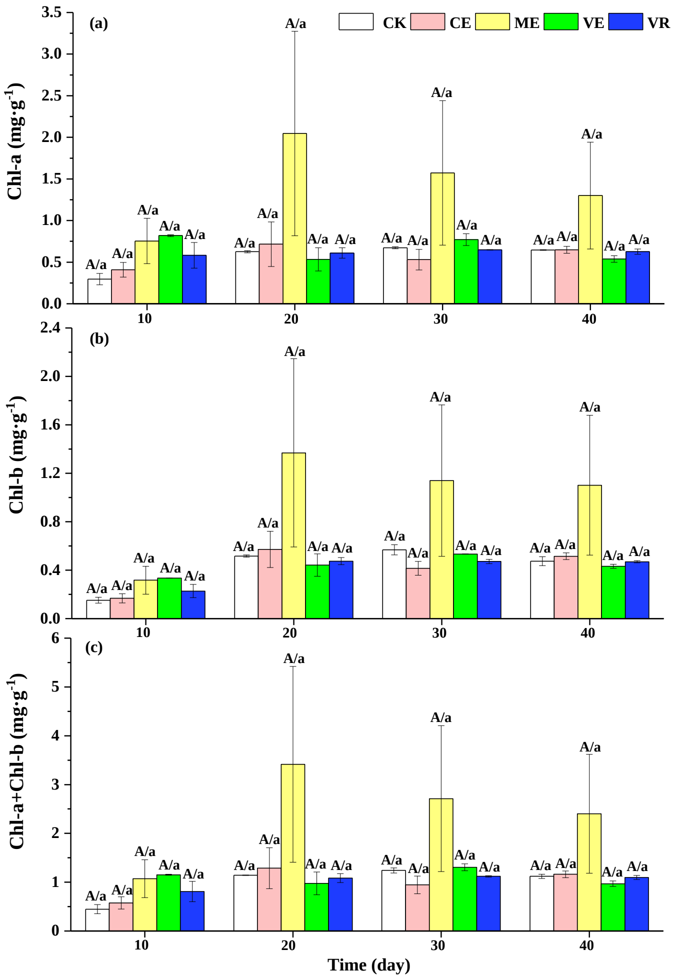

Figure 2. *Effects of different substrates on chlorophyll content of V. natans. (a) Chl-a. (b) Chl-b. (c) Chl-a + Chl-b. The bar chart uses different capital letters to indicate significant differences at the P < 0.05 level within the same treatment group at different times and different lowercase letters to indicate significant differences at the P < 0.05 level among different treatment groups at the same times. Error bars indicate standard deviation*

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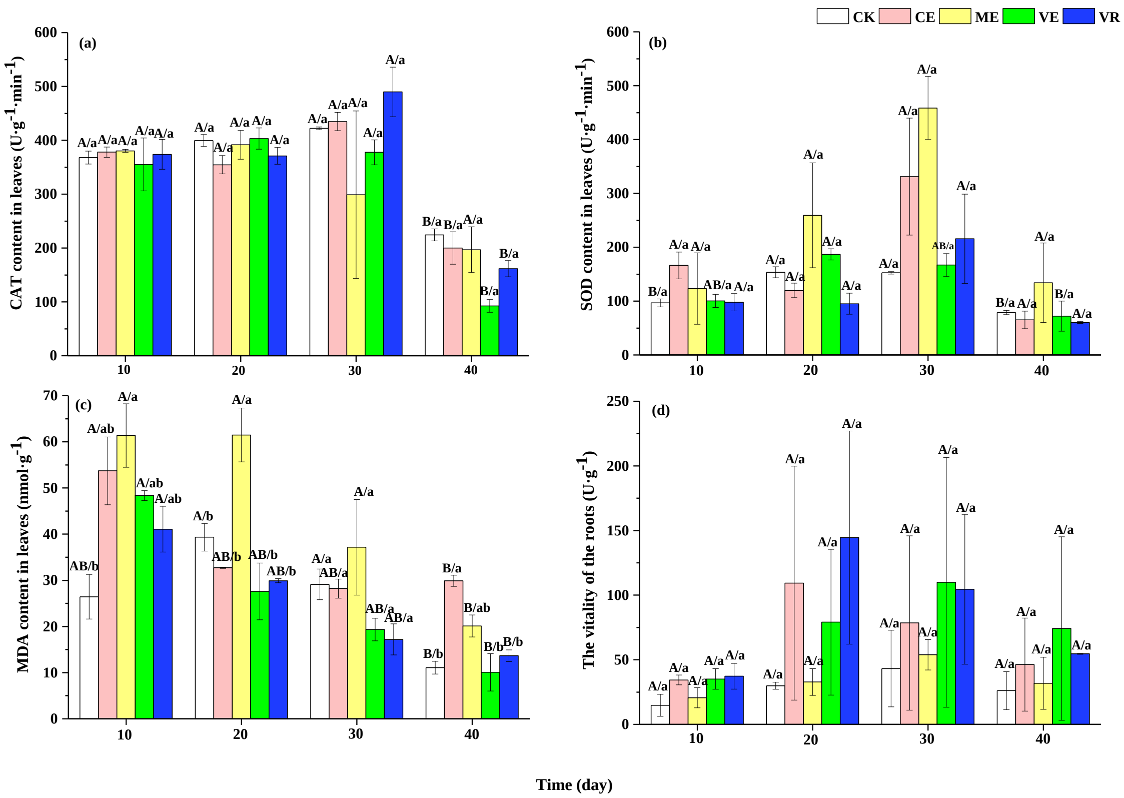

Figure 3. *Effects of substrates on enzymes and root vitality of V. natans. (a) CAT activity. (b) SOD activity. (c) MDA content. (d) Root vitality. The bar chart uses different capital letters to indicate significant differences at the P < 0.05 level within the same treatment group at different times and different lowercase letters to indicate significant differences at the P < 0.05 level among different treatment groups at the same times. Error bars indicate standard deviation*

Redundancy analysis

The RDA plot showed that the first two axes jointly accounted for 93.9% of the variance (*Fig. 4*). The results showed that CAT activity of submerged plants in each group was negatively correlated with other physiological indexes. Root vitality was positively correlated with biomass, plant height, and root length. SOD activity was positively correlated with Chl-a, Chl-b, Chl-a + Chl-b, and MDA contents. The great distance among the substrate groups indicated that different substrates had different effects on the plants.

Effects of different substrates on rhizosphere microbial communities

The dominant bacterial species of each group were similar (*Fig. 5*). At the phylum level, *Proteobacteria* and *Chloroflexi* were the top two dominant bacteria in all samples, with *Proteobacteria* accounted for 16.94%-21.55%, and *Chloroflexi* accounted for 14.82%-22.48%. However, the influences of different substrates on the proportion and composition of dominant bacteria were different. No difference in the relative abundance of *Chloroflexi* among ME, VE, or VR groups, but it was lower in these three treatment groups than in CK group, whereas the relative abundance of *Chloroflexi* in the CE group was higher than that in CK group. Additionally, the

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relative abundance of *Desulfobacterota* and *Nitrospirota* in four treatment groups was higher than that in CK, while the relative abundance of *Acidobacteriota* was the opposite.

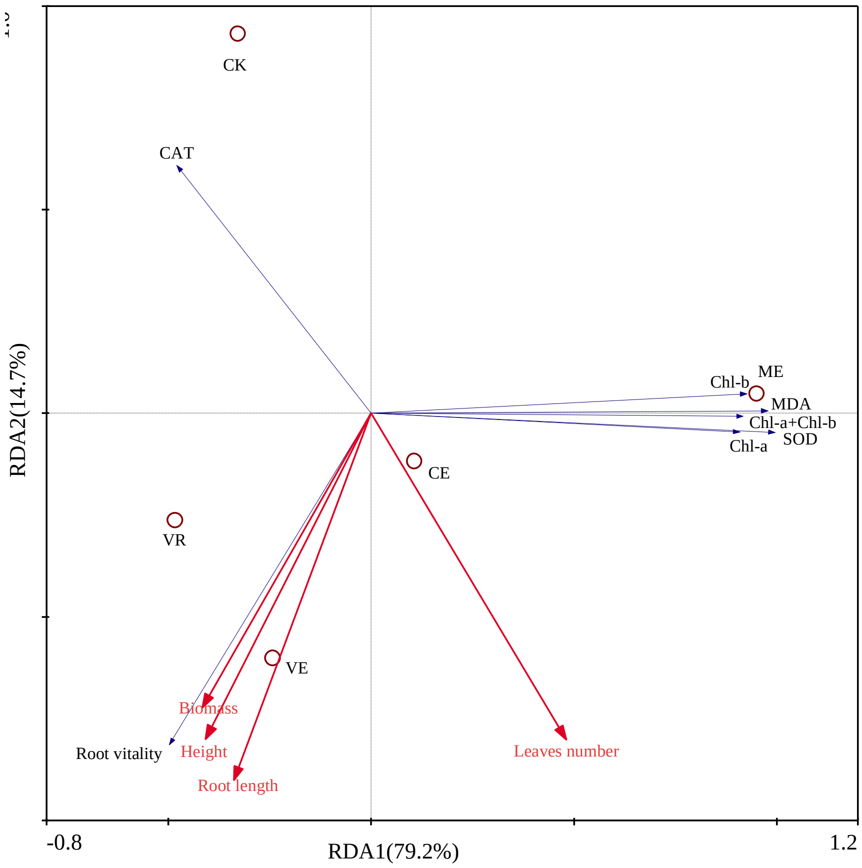

Figure 4. *RDA ordination plot of relationship between different substrates and physiological properties of V. natans*

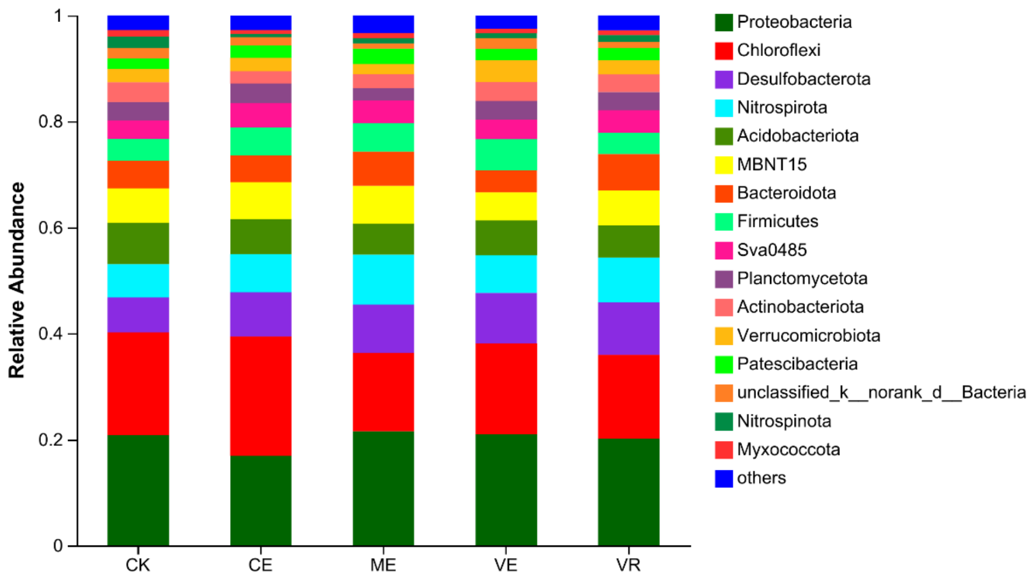

Figure 5. *Distribution of microbial community for each group at phylum level*

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On day 40, Ace, Chao, Sobs, and Shannon indexes in all four treatment groups were higher than CK (*Table 1*), among which group VE was the highest, followed by group VR. The ME, VE, and VR groups exhibited a lower Simpson index than CK group, while CE group displayed a higher one than CK. These results indicated that the substrate addition could increase diversity and abundance of rhizosphere microorganisms.

Table 1. *Sediment microbial community diversity based on 97% OTU similarity*

Sample	ACE	Chao	Shannon	Simpson	Sobs
CK	1492.10	1452.99	5.76	0.0092	1029
CE	1650.74	1635.96	5.80	0.011	1163
ME	1604.22	1582.20	5.83	0.0083	1088
VE	1739.42	1695.61	5.87	0.0081	1333
VR	1657.47	1667.64	5.86	0.0081	1146

Discussion

The growth indexes of plants can reflect their adaptability to these substrate materials to some extent (Chi and Liu, 2016). It has been reported that the addition of biochar to sediments significantly promotes the growth of *V. natans* and *Ceratophyllum demersum* (Li et al., 2020), and the addition of maifanite to sediments increased root weight by more than 93% in *Vallisneria spiralis* and *Hydrilla verticillate* (Liu et al., 2020b). In this study, the addition of all four substrates resulted in an increase in the biomass of *V. natans*. Furthermore, lake sediments with high viscous strength can reduce the rooting ability of submerged plants (Handley and Davy, 2002). However, the sediments in this study were mainly clay with high viscosity, and the addition of substrate has been reported to reduce the sediment viscosity (Bai et al., 2022), which may explain the enhanced root length of *V. natans* in the treatment groups compared to the CK group. Additionally, the added substrates are rich in trace elements such as Na^{+}, Fe^{2+}, Mg^{2+}, and Ca^{2+} (Liu et al., 2018), which can provide nutrients for the growth of submerged plants (Li et al., 2020; Wegrzyn et al., 2022). This could be another contributing factor to the improved plant growth observed in the treatment groups.

Our results showed that the contents of chlorophyll Chl-a and Chl-b reached the maximum value at 20-30 days post culture. At the early stage (day 10), there was no significant difference in chlorophyll content among different treatment groups (*Fig. 2*). At the late stage (day 40), chlorophyll content in maifanite treatment group was significantly higher than that in other groups. Chlorophyll reflects the photosynthetic capacity of plants, and the chlorophyll content in maifanite group was higher than that of other groups at the late stage of plant growth, which indicated that the maifanite as substrate could improve the photosynthesis of plants, thus promoting the growth of aquatic plants.

When plants are exposed to external environmental stresses, reactive oxygen species will accumulate in the plant organelles, thus disturbing the original oxidation reduction system of plant cells, eventually damaging the structure and functions of the cell membrane system (Ahmad et al., 2016). Therefore, plants will increase one or more antioxidant enzymes such as SOD and CAT to remove reactive oxygen species so as to reduce this damage (Panda and Choudhury, 2005; Asaeda et al., 2022). Some studies

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have found that under the extended environmental stresses, the accumulation of lipid peroxidation product MDA increased in plant cell membrane, and its content increased with the increasing environmental stresses (Gao et al., 2012; Chen et al., 2018). The accumulation of MDA even leads to the inactivation of the enzymes related to photosynthesis, respiration, and some metabolic processes in plant cells (Song et al., 2015). In this study, MDA content was significantly different at the same group at different times ($P < 0.05$). On day 10, MDA content in each treatment group reached the maximum, then gradually decreased, and finally became stable, and such a change trend occurred earlier than CK group (*Fig. 3c*). This might be due to the beginning of the experiment, the transplantation caused damage to *V. natans*, which produced a large amount of MDA. However, the addition of substrates enabled plants to rapidly generate antioxidant enzymes, thus inhibiting the MDA production and promoting the adaptation of *V. natans* to the new environment after transplantation, eventually promoting the rapid growth of plants. Our results were consistent with a previous report that environmental stress can cause plant tissues to initiate stress response mechanisms (Hao et al., 2020). Our study also showed that MDA content in vermiculite and maifanite groups on day 40 reduced by 38.2 nmol g^{-1} and 40.1 nmol g^{-1}, respectively, compared with that on day 10, indicating that these two substrates could promote the adaptation of plants to adverse environment stress.

When MDA content is elevated in plants, plants will activate an antioxidant system to avoid damage caused by oxidative stress (Singh et al., 2020). The antioxidant enzyme SOD can convert O_2^- into O_2 and H_2O_2 in plants, and CAT further decomposes H_2O_2 into non-toxic H_2O_2 and O_2 in plants (Yin et al., 2008; Jiang et al., 2011). In the middle stage of culture (day 30), *V. natans* in all treatment groups produced a large amount of SOD to decompose O_2^-, thus avoiding the possible harm by O_2^-. The maifanite group exhibited the highest and the most obvious reduction of O_2^- in plants, and SOD content in maifanite group was always higher than that in the control group during the whole experiment period, indicating that addition of maifanite could better improve plants' adaptation to the environment.

Root vitality can reflect the ability of plants to absorb water and nutrients, and plays a key role in determining plant growth status (Rewald and Meinen, 2013). In this study, the root activity was positively correlated with the plant growth indexes, and the root activity in each group reached the maximum at the middle stage of culture (day 20-30). This might be because trace elements in the substrate promoted the plant's root activity and enzyme defense, thus weakening the damage to plants under adverse conditions and promoting the growth of plants in changing environments (Liu et al., 2020a).

In this study, the diversity of rhizosphere microorganisms and the relative abundance of *Desulfobacterota* and *Nitrospirota* were higher than those of CK group after substrate addition onto the surface of the sediment. Han et al. (2019) also found similar results, which might be mainly due to the low oxidation reduction potential in the surface layer of sediments, thus leading to the accumulation of sulfide. Mg^{2+}, Fe^{2+}, and other trace elements in the form of inorganic salts in the substrate can directly participate in electron transport, oxidative stress, nitrogen fixation, and hormone synthesis (Liu et al., 2018), which promotes the growth of *Desulfobacterota* and *Nitrospirota* and possibly facilitates the conversion of certain nitrogen-containing sulfides.

In this study, the dominant bacterial phyla in all the groups were basically the same, among which *Proteobacteria* was the largest dominant bacterial phylum in all the

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groups, followed by *Chloroflexi*, which was consistent with the report by Guan et al. (2015). *Proteobacteria* was considered to be the main dominant phylum in many ecosystems such as constructed wetlands and biofilter-treated surface water (Feng et al., 2013; Ansola et al., 2014). This may be because *Proteobacteria* includes a variety of bacteria contributing to the carbon and nitrogen cycle, and these bacteria are involved in the biodegradation or biotransformation of organic compounds (Liu et al., 2014; Santos et al., 2019).

Conclusion

In this study, four different substrates were added to the surface of the sediments to investigate their effects on the growth of submerged plant *V. natans* and rhizosphere microorganisms. The results showed that the addition of substrates to the sediment increased leave number, root growth, and root activity, thus promoting the growth of plants. The addition of maifanite had the most significant promotion effect on photosynthesis and plant growth. Under adverse transplantation conditions, the maifanite addition group exhibited the highest mean SOD activity level, and drastically decreased MDA content, and thus this group had a strong adaptability to the environmental changes. The increased relative abundance of *Desulfobacterota* and *Nitrospirota* in rhizosphere sediments of substrate addition groups indicated that the addition of substrates had promoted the conversion of nitrogen sulfide in the sediments. Overall, our results indicate that the substrate addition, especially maifanite addition, could promote the growth of submerged plants and improve the rhizosphere microbial community structure on the sediment surface. Our findings provide valuable reference for screening sediment substrate improver.

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Conflict of interest. The authors have no relevant financial or non-financial interests to disclose.

Data availability. The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

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