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Stigmasterol root exudation arising from *Pseudomonas* inoculation of the duckweed rhizosphere enhances nitrogen removal from polluted waters☆

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ABSTRACT

Rhizospheric microorganisms such as denitrifying bacteria are able to affect 'rhizobioaugmention' in aquatic plants and can help boost wastewater purification by benefiting plant growth, but little is known about their effects on the production of plant root exudates, and how such exudates may affect microorganismal nitrogen removal. Here, we assess the effects of the rhizospheric *Pseudomonas* inoculant strain RWX31 on the root exudate profile of the duckweed *Spirodela polyrrhiza,* using gas chromatography/mass spectrometry. Compared to untreated plants, inoculation with RWX31 specifically induced the exudation of two sterols, stigmasterol and β-sitosterol. An authentic standard assay revealed that stigmasterol significantly promoted nitrogen removal and biofilm formation by the denitrifying bacterial strain RWX31, whereas β-sitosterol had no effect. Assays for denitrifying enzyme activity were conducted to show that stigmasterol stimulated nitrogen removal by targeting nitrite reductase in bacteria. Enhanced N removal from water by stigmasterol, and a synergistic stimulatory effect with RWX31, was observed in open duckweed cultivation systems. We suggest that this is linked to a modulation of community composition of *nirS- and nirK*-type denitrifying bacteria in the rhizosphere, with a higher abundance of *Bosea, Rhizobium, and Brucella*, and a lower abundance of *Rubrivivax*. Our findings provide important new insights into the interaction of duckweed with the rhizospheric bacterial strain RWX31 and their involvement in the aquatic N cycle and offer a new path toward more effective bio-formulations for the purification of N-polluted waters.

1. Introduction

Nitrogen (N), as the mineral macronutrient required in largest abundance for plant growth and crop yield, is the principal component of chemical fertilisers and has been heavily applied in agriculture since the onset of the first Green Revolution. However, N loss from agricultural non-point sources can lead to eutrophication in aquatic ecosystems. Furthermore, excess nitrate (NO_3^-) and nitrite (NO_2^-) in ground and surface waters are toxic to many aquatic organisms and induce adverse effects on human health and the economy (Lundberg et al., 2004; Camargo and Alonso, 2006; Poulsen et al., 2018; Min et al., 2021). Plant-microbe interactions fundamentally shape N transformation processes in soil and water (Coskun et al., 2017a/b; Lu et al., 2014; Azreen et al., 2017), and optimising such interactions is thus considered an important strategy to reduce both the cost of fertiliser application and minimize environmentally detrimental N loss from agroecosystems and N accumulation in natural waterbodies (Feng et al., 2012; Coskun et al., 2017b).

Over the past few decades, aquatic plants have been applied worldwide for the purification of agricultural and industrial wastewater. The duckweed family, a family of small floating macrophytes (family *Lemnaceae*), is particularly desirable for this purpose, due to its widespread availability, high fuel-conversion rate, ease of harvesting, and excellent adaptation to a wide range of nutritional conditions (Zimmo et al., 2004; Mohedano et al., 2012; Zhao et al., 2014; Sun et al., 2019; Gusain and Suthar, 2020). In recent decades, a number of microbial inoculants have been isolated from the duckweed rhizosphere, some of which could contribute to enhanced N removal (Zhou et al., 2013), degradation of organic pollutants, phytoremediation of heavy metals (Toyama et al., 2009; Yamaga et al., 2010; Kristanti et al., 2014), and

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promotion of biomass production in aquatic systems (Toyama et al., 2017). However, the mechanisms underlying the bioaugmentation by rhizospheric microorganisms to remove pollutants have not been extensively investigated.

Several studies have shown that microbes strengthen rhizosphere biological function by directly supporting root development, plant growth, nutrient and water acquisition, and induce systemic resistance against biotic and abiotic stresses (Bulgarelli et al., 2013; Pii et al., 2015; Debiec-Andrzejewska et al., 2020). In return, plant roots can secrete organic compounds that can function as carbon sources and chemical signals for microbial growth (Bais et al., 2006). Several microbial traits and mechanisms involved in these interactions have been identified, including the production of enzymes and excretion of metabolites into the rhizosphere (Dodd et al., 2010). However, the processes by which rhizosphere-resident microorganisms alter plant biochemistry and whether such biochemical/metabolic changes affect pollutant removal is not well understood yet.

Root exudates, which act as rhizosphere chemical signals, are centrally important to plant-bacterial interactions and to improving nutrient transformation (Badri and Vivanco, 2009; Venturi and Keel, 2016; Sun et al., 2016a). As a symbiotic partner, rhizospheric microorganisms significantly modulate the biosynthesis and secretion of root exudates (Etalo et al., 2018). The impact on root exudation in the model species *Arabidopsis thaliana* and in major crops (rice, wheat, soybean, tomato, cotton) following the colonization by *Bacillus* sp. (Liu et al., 2017; Rekha et al., 2018), *Pseudomonas* sp. (Ankati et al., 2019; Kandaswamy et al., 2019), *Chryseobacterium balustinum* (Dardanelli et al., 2010), *Kosakonia radicincitans* (Witzel et al., 2017), arbuscular-mycorrhizal fungi (Zhang et al., 2012), and *Nitrosomonas* (Zhang et al., 2019) has been described. However, little is known about the effects on metabolism in aquatic plants. Moreover, the composition and concentration of specific root exudates, such as flavonoids (quercetin or naringenin), fatty acids and their derivatives (myristic acid, stearic acid, palmitic acid, and 1,9-decanediol), organic acids (malic acid) and amino acids (tryptophan), are affected by bacterial colonization, suggesting that these compounds are involved in below-ground interactions, even though their biological functions and mechanisms have remained largely unclear (Liu et al., 2017; Dardanelli et al., 2010; Ren et al., 2015; Ankati et al., 2019; Zhang et al., 2019).

Pseudomonas sp., which are among the most common denitrifying microbes, are widely distributed and in occur in high densities in many soils (Gamble et al., 1977; Tiedje et al., 1982). In our previous study, we revealed that fatty acid amides and fatty acid methyl esters from duckweed root exudates can stimulate nitrogen removal by the denitrifying bacterium *Pseudomonas fluorescens* (Lu et al., 2014). We also isolated an aerobic denitrifying strain, *Pseudomonas* sp. RWX31, from the rhizosphere of *Lemna minor*. It was shown that inoculation with this strain stimulates nitrate removal rates in a duckweed-based water--purification system (Zhou et al., 2013). As far as we know, no work exists concerning the effects of denitrifying bacteria on the root exudate profile of duckweed. Therefore, we hypothesized that the rhizospheric bacterium RWX31 can alter the specific pattern of duckweed root exudates to strengthen the nitrogen removal capacity and efficiency of a coupled duckweed-denitrifying bacteria system. The main objectives of our study were to: (1) investigate if the stimulation of nitrogen removal by rhizospheric bacteria in duckweed is linked to root exudates; (2) verify whether specific metabolites can stimulate nitrogen removal capacity in pure bacterial cultures and in open duckweed-based systems; (3) reveal the microbial mechanism underlying denitrification stimulation by specific root exudates.

2. Methods and materials

2.1. Plant and bacteria materials

Duckweed (*Spirodela polyrrhiza*) colonies were collected from a paddy field drainage in Huzhou City on the south bank of the Taihu Lake region (30°53′N, 120°24′E), and species identification was performed based on morphology (Zhou et al., 2010); flattened fronds were of wide obovate shape, 5–8 mm long and 4–6 mm wide, with purple undersides, and 8–10 roots. Whole duckweed plants were gently washed with sterile water for 5 min to remove impurities on the plant surface and were then cultured in 250-mL flasks containing modified Steinberg medium (Lu et al., 2014), with the following composition: $Ca(NO_3)_2{\cdot}4H_2O$ 295 mg L^{-1}, KNO_3 350 mg L^{-1}, NH_4Cl 12.5 mg L^{-1}, KH_2PO_4 90 mg L^{-1}, $MgSO_4{\cdot}7H_2O$ 100 mg L^{-1}, $MnCl_2{\cdot}4H_2O$ 0.18 mg L^{-1}, H_3BO_3 0.12 mg L^{-1}, $NaMoO_4{\cdot}2H_2O$ 0.044 mg L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.18 mg L^{-1}, $FeCl_3{\cdot}6H_2O$ 0.76 mg L^{-1}, $Na_2EDTA{\cdot}2H_2O$ 1.5 mg L^{-1}; pH = 6.8, in a growth chamber (JYC-412, Yuejin, China) at 23 ± 1 °C, with a light intensity of 100 μmol m^{-2} s^{-1}, and a photoperiod of 16 h/8 h (light/-dark) cycle.

Pseudomonas sp. RWX31, isolated from the duckweed rhizosphere, was used for the study. The strain RWX31 was previously characterized in terms of high denitrification capacity and stimulation of nitrogen removal by a duckweed system (Zhou et al., 2013). *Pseudomonas* sp. RHZ1 was also isolated from duckweed roots and was identified as *Pseudomonas rhodesiae*, which was not the same strain as RWX31. The non-rhizospheric *Pseudomonas stutzeri* strain ATCC 17588 was obtained from the American Type Culture Collection (Manassas,VA, USA). These strains were cultivated in Luria–Bertani medium and shaken at 180 rpm at 30 °C until the cultures reached the late exponential phase.

2.2. Nitrogen removal of duckweed inoculated with denitrifying bacteria

The bacterial cells were harvested by centrifugation (8000 *g*, 4 °C, 10 min), washed twice with sterilized modified Steinberg medium, and then re-suspended in the same medium. The density of the bacteria was measured and adjusted to an OD_{600} value of 1.0. For the duckweed-only (non-inoculated) treatment, ten duckweed plants were transferred into a 150-mL flask with 50 mL of modified Steinberg medium supplemented with 50 mg L^{-1} KNO_3 and were further cultivated under the photoperiod at 23 °C. Then, 0.5 mL of bacterial suspensions of the three denitrifying bacterial strains, RWX31, RHZ1, and *Pseudomonas stutzeri* were added to the duckweed cultivation systems, respectively. Culture medium (0.25 mL) was collected on day 5 and subjected to total N (TN) content analysis using an ultraviolet spectrophotometric method following persulfate oxidation (American Public Health Association (APHA), 1998). Nitrogen removal efficiency (NRE) of duckweed systems was then calculated following Eq. (1):

$$NRE(\%) = (TN_0 - TN_t) / TN_0 \times 100\% \quad (1)$$

where TN_0 is the initial total N concentration (mg L^{-1}), and TN_t is the total N concentration (mg L^{-1}) after 5 days.

2.3. Identification of duckweed root exudates

The static collection of root exudates was performed as described earlier with some modifications (Sun et al., 2016b). In short, 50-mL exudate solutions, collected after 5-days cultivation, were passed through a 0.22-μm filter membrane to remove cellular debris and external microorganisms. The filtered solutions were then subjected to a solid-phase extraction (SPE) with a C18 cartridge. The columns were finally eluted with HPLC methanol into glass tubes and evaporated under nitrogen. The residues were subjected to N, O-bis(trimethylsilyl) trifluoroacetamide (Sigma-Aldrich) derivatization at 60 °C for 1 h. Duckweed root exudate profiling was performed by gas chromatography/mass spectrometry (GC/MS) based on previously published protocols (Lu et al., 2014), with minor changes.

The GC-MS analysis was carried out using an Agilent 6890 gas chromatograph equipped with a DB-5 (30 m × 0.25 μm × 0.25 mm) capillary column and coupled to an Agilent 5975 mass spectrometer.

Derivatized extracts (2 μL) were injected in splitless mode at 280 °C, using helium as the carrier gas (1.0 mL min^{-1}). A GC oven temperature program was applied, starting from 80 °C for 1 min up to 300 °C at 15 °C min^{-1}, holding for 10 min. The mass-selective detector was operated at an ionization energy of 70 eV and in a range of 20–650 amu. Compounds were identified based on the comparison of their retention time and mass spectra of GC-MS peaks with the authentic standards.

2.4. Effect of sterols on bacterial N removal

Authentic stigmasterol and β-sitosterol were obtained from Sigma-Aldrich (St. Louis, MO, USA). They were dissolved in dimethyl sulfoxide (DMSO). Bacterial cells grown in LB from the late exponential phase were recovered by centrifugation at 6000 g for 10 min, at 4 °C, and resuspended in sterile denitrifying medium (DM, 0.72 g L^{-1} KNO_3, 1.0 g L^{-1} KH_2PO_4, 0.20 g L^{-1} $MgSO_4{\cdot}7H_2O$, 2.8 g L^{-1} $C_4H_5NaO_4{\cdot}6H_2O$, pH 7.0). An aliquot (0.5 mL) of bacterial cells, and 20 μL of the two identified sterols (20, 40, and 100 μM) were added to a 50-mL flask with 19.5-mL DM, stirred, and incubated on a shaker at 120 rpm and 30 °C. Control experiments were performed by substituting DMSO for sterols. Each treatment was run in triplicate, and the experiments were performed twice. After a 24-h incubation period, 1 mL of the reaction mixture was centrifuged (at 10,000 *g* for 10 min) for TN determination. The stimulation of the nitrogen removal efficiency (NRE) by sterols was calculated using Eq. (2) and Eq. (3) according to Lu et al. (2014).

$$\mathrm{NRE}(\%) = [(\mathrm{TN}_0 - \mathrm{TN}_t) / \mathrm{TN}_0] \times 100\% \quad (2)$$

where TN_0 is the initial total N concentration (mg L^{-1}), and TN_t is the total N concentration (mg L^{-1}) after 24 h.

$$\text{Stimulation of NRE } (\%) = [(\mathrm{NREsterol} - \mathrm{NREcontrol}) / \mathrm{NREcontrol}] \times 100\% \quad (3)$$

where NREsterol is the NRE of the sterol, and NREcontrol is the NRE of the DMSO control after 24 h.

2.5. Effect of stigmasterol on bacterial biofilm formation

A biofilm formation assay was performed as described previously (Yamaga et al., 2010), with some modifications. An overnight culture in LB was diluted to OD_{600} 0.3 and inoculated (1%) into 500 μL of DM in a 1.5-mL microcentrifuge tube. Stigmasterol was added to obtain a final concentration of 20, 40, and 100 μM. Three replicates were included for each treatment. After cultivation at 30 °C without shaking for 24 h, liquid was discarded, and the tubes were washed. Biofilms adhering to the surface were subjected to staining. Biofilm cells were stained with 400 μL of 0.1% crystal violet for 30 min. Subsequently, the tubes were washed twice with distilled water. The crystal violet attached to the biofilm was dissolved in 500 μL of 95% ethanol and the optical density (OD) was measured at 590 nm.

2.6. Effect of stigmasterol on activities of denitrifying enzymes

The influence of stigmasterol on denitrification was also determined by assaying for activities of two key denitrifying enzymes, nitrate reductase (NAR) and nitrite reductase (NIR), based on our previously described methods (Sun et al., 2016b). Briefly, the bacteria were collected after an 8-h incubation period during the exponential growth phase (OD_{600} 1.0), washed with phosphate-buffered saline (PBS) solution, and resuspended at 4 °C. The crude cell extracts were obtained by sonication for 10 min (300 W in an ice bath, for 3 s, in 5-s intervals) and centrifuged at 6000 *g* for 10 min. The supernatants were then centrifuged at 186,000 *g* at 4 °C for 90 min, with the supernatants as soluble fractions for NIR activity assay, and the sediments as membrane fractions for the NAR activity assay. Protein concentrations of the cell extracts were measured using a Protein Assay Kit (Bio-Rad). The reaction volume of NAR was 2 mL containing 10 mM KNO_3, 0.2 mM methyl-viologen, 10 mM $Na_2S_2O_4$, and 100 μM stigmasterol. The reaction volume of NIR contains 200 μM KNO_2, 200 μM phenazine methosulfate, 1 mM NADH, and 100 μM stigmasterol. The cell extract (100 μL) was injected to initiate the reaction, and was incubated for 30 min at 30 °C. The activities of NAR and NIR were determined by measuring the production or consumption of nitrite (NO_2^- min^{-1} g^{-1} protein), respectively. The relative activities of NAR or NIR were expressed relative to the untreated control.

2.7. Nitrogen removal of duckweed as affected by stigmasterol and RWX31

A flask-scale batch cultivation experiment was conducted to evaluate the stimulatory effect of stigmasterol on the nitrogen removal efficiency of duckweed systems and the potential synergism with RWX31. For the duckweed-only (non-inoculated) treatment, ten duckweed plants were transferred into a 150-mL flask with 50 mL of modified Steinberg medium supplemented with 50 mg L^{-1} KNO_3. Then, 100 μM stigmasterol and 0.5 mL of RWX31-bacterial suspensions, alone and in combination, were added to the different duckweed cultivation systems. Culture medium (0.25 mL) was collected at different times (0.5, 1, 2, 4, 5, and 7 d) and subjected to TN analysis. Duckweed growth was monitored by counting the number of fronds after 7 d of cultivation. The growth-promoting effects of three treatments are expressed as % increase of the frond numbers in comparison with the corresponding duckweed control. All treatments were replicated 3 times, and the experiment was repeated twice to ensure reproducibility.

2.8. DNA extraction of duckweed rhizosphere bacteria

The duckweed samples (about 0.75 g fresh weight) obtained from the flask-scale batch cultivation experiment (section 2.7) after 7 d were immediately used for rhizosphere bacteria collection, according to Xie et al. (2015) and Chen et al. (2020). 0.5 g of whole duckweed plants (FW) were rinsed twice with sterile deionized water and transferred into a 150 mL-flask containing 50 mL 100 mM phosphate buffered saline solution supplemented with 0.02% Tween 20, and shaken at 200 rpm at 20 °C in a shaking incubator for 2 h. The washing solution was centrifuged at 8000 *g* for 30 min at 4 °C. The supernatant was discarded, and the pellet was used for DNA extraction using the MoBio PowerSoil DNA-isolation kits (MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. The quantity and quality of extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. Three biological replicates were used for each treatment. All DNA samples were stored at −20 °C.

2.9. PCR amplicon and sequence analysis

PCR amplification of the *nirS,* and *nirK* genes was performed using the primer pairs *nirS*-cd3aF(5′-GTSAACGTSAAGGA RACSGG-3′) and *nirS*-R3cd(5′-GASTTCGGRTGSGTCTTGA-3′), *nirK*-F1aCu(5′-ATCATGGTSCTGCCGCG-3′) *and nirK*-R3Cu(5′-GCCTCGAT-CAGRTTGTGGTT-3′) (Throback et al., 2004), respectively. The reaction solution volume was 25 μL, comprised of 5 μL of Q5 reaction buffer (5 ×), 5 μL of Q5 High-Fidelity GC buffer (5 ×), 0.25 μL of Q5 High-Fidelity DNA Polymerase (5 U per μL), 2 μL (2.5 mM) of dNTPs, 1 μL (10 μM) of each forward and reverse primer, 2 μL of DNA template, and 8.75 μL of ddH_2O. Thermal cycling consisted of initial denaturation at 95 °C for 2 min, followed by 35 cycles consisting of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension of 5 min at 72 °C. PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual

quantification step, amplicons were pooled in equal amounts, and paired-end 2 × 300 bp sequencing was performed using the Illlumina MiSeq platform at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

The sequencing data has been deposited in the NCBI Sequence Read Archive (SRA) database under accession number PRJNA657643. Sequencing reads were processed with QIIME version 1.8.0 and R version 3.2.0. Low-quality sequences that had a quality score <20,

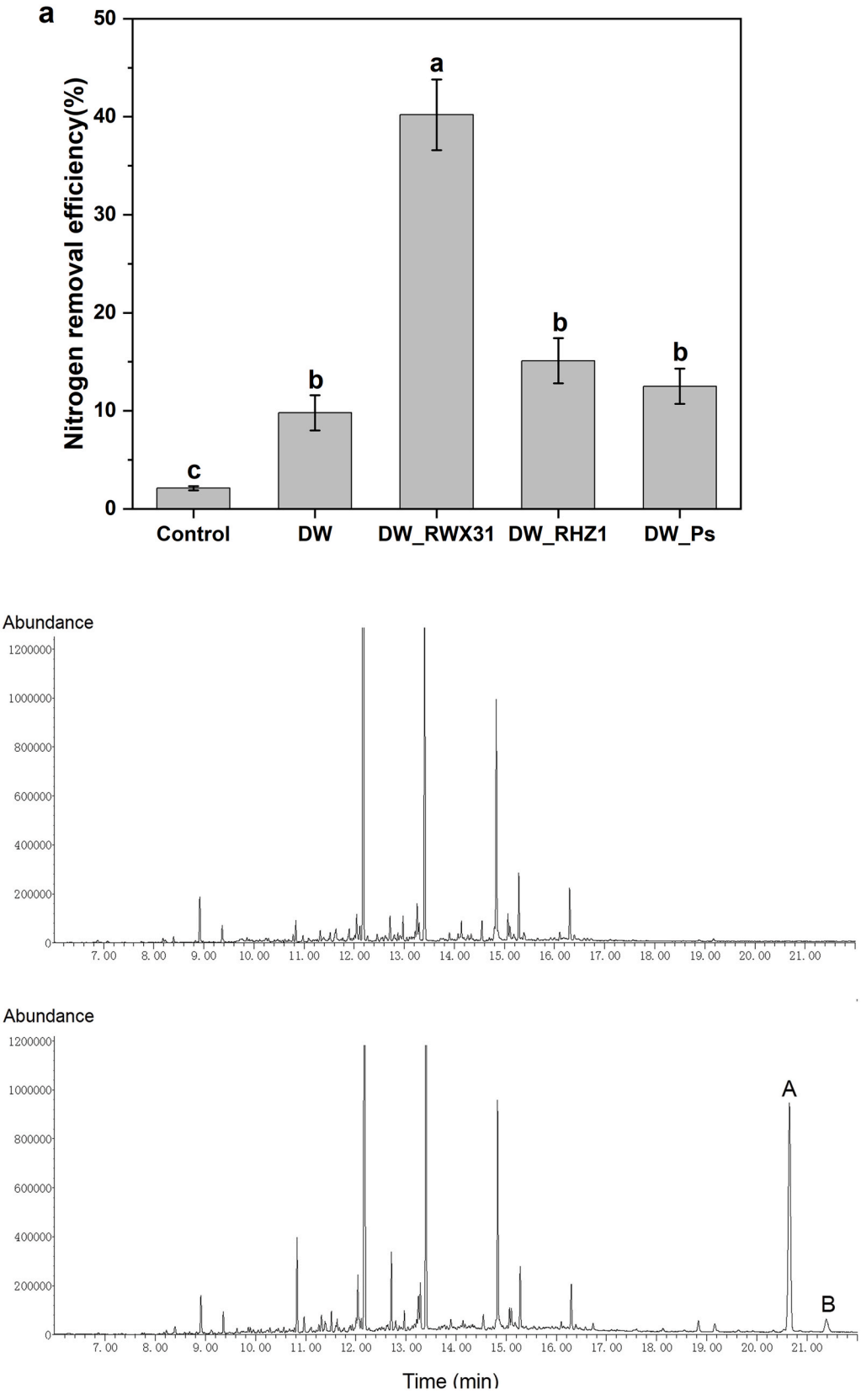

Fig. 1. Effect of different denitrifying bacteria on the nitrogen removal by a duckweed system after 5 days (a). Different letters indicate significant differences between treatments at $P < 0.05$ based on an LSD test. Control was set for the experiment without duckweed and denitrifying bacteria. DW: duckweed, DW_Ps: duckweed with *Pseudomonas stutzeri*; Ion chromatogram of root exudates of duckweed (b), and duckweed inoculated with RWX31 (c). A. stigmasterol (20.65 min), B. β-sitosterol (21.40 min).

contained ambiguous nucleotides, or did not match the primer and barcode were removed. Paired-end reads were assembled using FLASH version 1.2.7 (Magoc and Salzberg, 2011). After quality control, the validated sequence reads were classified into different operational taxonomic units (OTUs) based on a 97% identity level. A representative sequence in an OTU was selected based on the most abundant sequence. Representative sequences in the OTUs were taxonomically classified by the BLAST algorithm-based search within GenBank, using the following settings: E-value cutoff 1e-5, number of alignments 1, output format 0, number of descriptions 1, and percent identity threshold 97%.

2.10. Statistical analysis

Statistical analyses were carried out using the SPSS 18.0 software package (SPSS, Chicago, IL, USA) for Windows. Normality of data distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test) assumptions were satisfied. The relationships between the relative abundance of members of the nitrite-reducing bacterial community and TN removal were assessed using Pearson correlation analysis. Statistically significant differences among treatments were determined by one-way ANOVA and least significant difference (LSD) calculations at a 5% confidence level.

3. Results

3.1. Effect of Pseudomonas sp. inoculation on N removal by duckweed

In comparison with the non-inoculated duckweed control, at 5 d, root inoculation of duckweed resulted in significantly higher nitrogen removal efficiency with the rhizosphere denitrifying bacteria RWX31 (about 38%) (Fig. 1a). By contrast, inoculation with another duckweed rhizosphere denitrifying bacterium, RHZ1, and a non-rhizosphere denitrifying bacterium, *Pseudomonas stutzeri*, had no significant effect on the nitrogen removal efficiency of duckweed system.

3.2. Identification of duckweed root exudates by GC-MS

To examine whether the response to different denitrifying bacterial strains is related to root exudate composition, the profile of duckweed exudates with or without denitrifying bacterium inoculation was

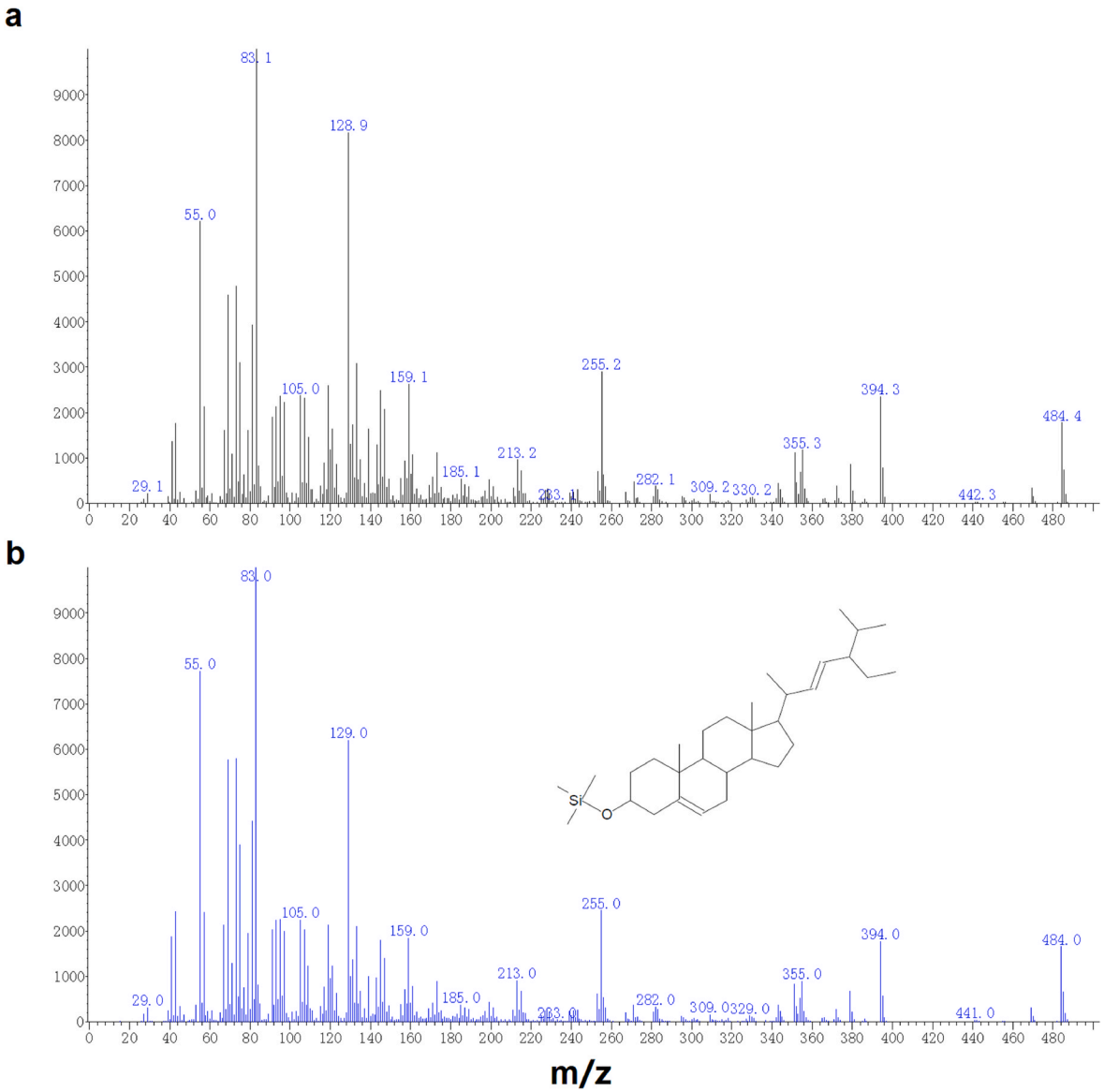

Fig. 2. Mass spectrum fragment patterns of stigmasterol in root exudates (a) and the corresponding mass spectra of its authentic standard (b). The mass spectral similarity of stigmasterol was 99%.

determined by GC/MS analysis. Overall, more than 30 metabolites were found in the duckweed root exudate. The chemical composition of duckweed root exudates was greatly influenced by RWX31 (Fig. 1b). Of particular interest were two peaks, at 20.65 min and 21.40 min, detected in RWX31-inoculated root exudates (Fig. 1c). Comparing against the NIST mass-spectral library revealed that the two compounds belonged to the sterol class of chemicals, and that they were stigmasterol (20.65 min, Fig. 2a) and β-sitosterol (21.40 min, Fig. S1), specifically, and the similarity of their mass spectra was 99% and 93%. This chemical identity was then ascertained by comparing their retention times with authentic standards.

The chemical structures of the two peaks are shown in Fig. 2b and Fig. S1b. However, these two sterols were not detected in the chemical exudates under inoculation with the rhizospheric strain RHZ1 and the non-rhizospheric *Pseudomonas stutzeri* (Fig. S2a, 2b), suggesting a specific induction by RWX31. Moreover, stigmasterol and β-sitosterol were not found in natural duckweed root exudates (Fig. 1b) or in RWX31 bacterial exudates (Fig. S2c).

3.3. Effect of stigmasterol on N-removal efficiency

To test whether the two sterol exudates are responsible for the stimulation of N-removal efficiency, pure bacterial cultures were supplemented with authentic stigmasterol and β-sitosterol. After 24 h, both sterols had no significant influence on the growth of RWX31(OD_{600}) (Table S1). Notably, the addition of 40 μM and 100 μM stigmasterol significantly stimulated N-removal efficiency ($P < 0.05$) of RWX31, while the presence of β-sitosterol had barely any effect among treatments (Fig. 3). Dose-response relationships showed that 100 μM stigmasterol resulted in the maximum stimulation of N-removal efficiency, by 42%, compared to the DMSO control. These results clearly demonstrate that stigmasterol, but not β-sitosterol, can act as an N-removal stimulant.

3.4. Effect of stigmasterol on biofilm formation and denitrifying enzymes

As denitrification has been shown to play an important role in rhizosphere colonization (Redondo-Nieto et al., 2013), and biofilm formation is an indicator for efficient root colonization (Ramey et al., 2004), we then examined whether biofilm development of RWX31 could be affected by stigmasterol in the bacterial culture assay. Compared with the control, the addition of stigmasterol at 40 μM and 100 μM significantly enhanced biofilm formation with RWX31 by 24.5% and 36.0% ($P < 0.05$), respectively, while 20 μM stigmasterol showed no significant effect (Fig. 4a). These results show a similar trend to N-removal efficiency of RWX31 affected by stigmasterol.

To clarify whether stigmasterol directly targets denitrifying enzymes, the activities of the two key denitrification enzymes, nitrate reductase (NAR) and nitrite reductase (NIR), in RWX31 were examined in in-vitro experiments. As shown in Fig. 4b, the activity of NIR increased to 208% of levels observed in the control after exposure to 100 μM stigmasterol ($P < 0.05$). However, no significant effect was found on NAR activity. Therefore, stigmasterol led to enhanced NIR activity, and ultimately affected nitrate reduction.

3.5. N removal by plant-microbe–root exudate partnerships in water

For acceleration of N removal by plant-microbe-root exudate partnerships in water, strain RWX31, stigmasterol, and the RWX31-stigmasterol association were introduced into a sterile modified Steinberg medium containing 50 mg L^{-1} nitrate, with duckweed plants introduced as the control. After 7 days, the individual introduction of duckweed plants removed a small amount of TN (about 10%). Co-introduction of duckweed and RWX31 removed about 40% of the TN

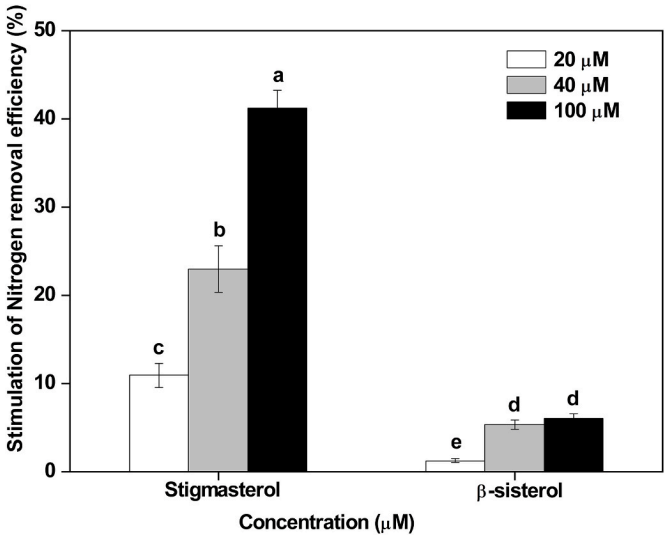

Fig. 3. Stimulation of the nitrogen-removal efficiency of RWX31 at different concentrations of the two identified sterols. The nitrogen-removal efficiency of the DMSO control was 44.1 ± 1.4%. Mean values and standard errors are shown (n = 3). Different letters indicate significant differences between treatments at $P < 0.05$ based on an LSD test.

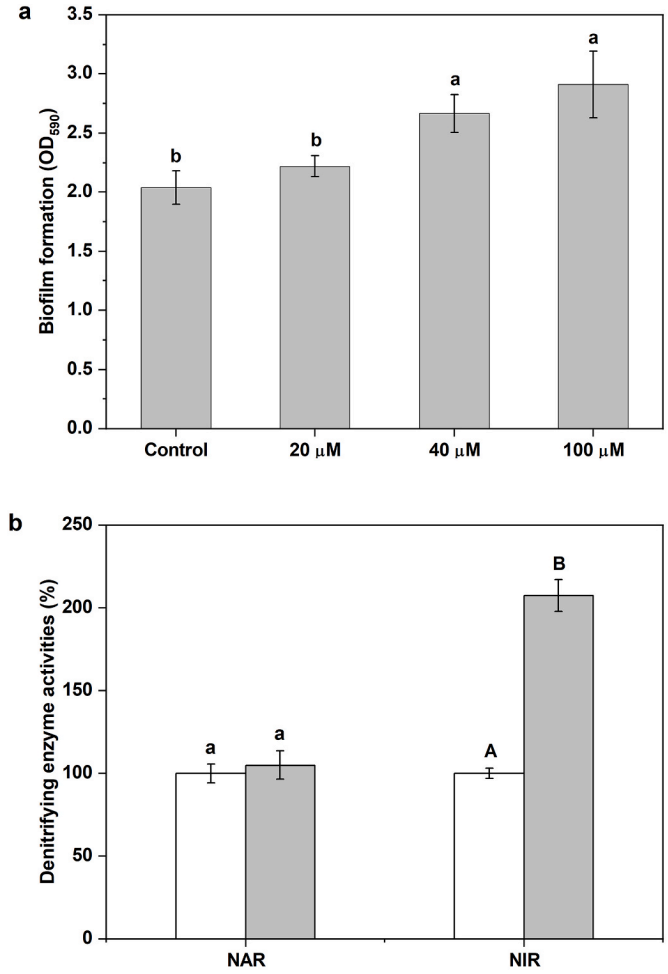

Fig. 4. The effect of stigmasterol on biofilm formation abilities(a), and NAR and NIR denitrifying enzyme activities (b) in RWX31. The denitrifying enzyme activity of the control was 100%. Lower-case and capital letters represent significant differences between control and stigmasterol treatments of NAR and NIR activities, respectively, at $P < 0.05$ based on an LSD test (n = 3).

content in water, similar to experiments involving treatments with 100 μM of exudates containing stigmasterol (Fig. 5). A synergistic stimulatory effect was found in the combination of stigmasterol and RWX31, which led to the highest N-removal efficiency (about 64%) in water. Moreover, we counted duckweed frond number after 7 d of cultivation, and found that stigmasterol and the RWX31 strain stimulated duckweed growth by less than 20% (Table S2). These results underscore the application potential of stigmasterol alone and in partnership with RWX31, to accelerate N removal in an aquatic plant-based water-treatment system.

3.6. Composition of the nitrite-reducing bacterial community and relationship with N removal

Since nitrite reduction is the key rate-limiting step in the denitrification process, the community composition of nitrite-reducing bacteria, based on *nirS* and *nirK* genes, in the duckweed rhizosphere at the genus level was analyzed, as shown in Fig. 6. Within the *nirS* microbial community, *Rubrivivax* and *Ideonella* were the dominant communities in the duckweed control, accounting for 45.01% and 16.87% of the relative abundance, respectively. Stigmasterol altered the community composition by decreasing the relative abundance of *Rubrivivax* and increasing that of *Ideonella* and *Polymorphum* (Table S3, $P < 0.05$). Inoculation with the RWX31 strain significantly increased the proportion of *Pseudomonas* and decreased the abundance of *Rubrivivax* (Table S3, $P < 0.05$). In the *nirK* community, the most dominant genera were *Pseudomonas* (22.80%), *Bosea* (21.33%), and *Mesorhizobium* (17.17%) in the control. The higher relative abundance of *Bosea, Rhizobium,* and *Brucella*, together with the lower abundance of unidentified Burkholderiales were found under all the treatments (Table S4, $P < 0.05$).

The correlation analysis between nitrogen removal and the proportion of the *nirS* and nir*K* community at the genus level was further carried out (Table S5; S6). It was found that *Polymorphum* in the *nirS* community, and *Bosea, Rhizobium,* and *Brucella* in the *nirK* community, were positively correlated with TN removal by duckweed systems. By comparison, *Rubrivivax* in the *nirS* community and unidentified Burkholderiales in the *nirK* community showed a significantly negative relationship.

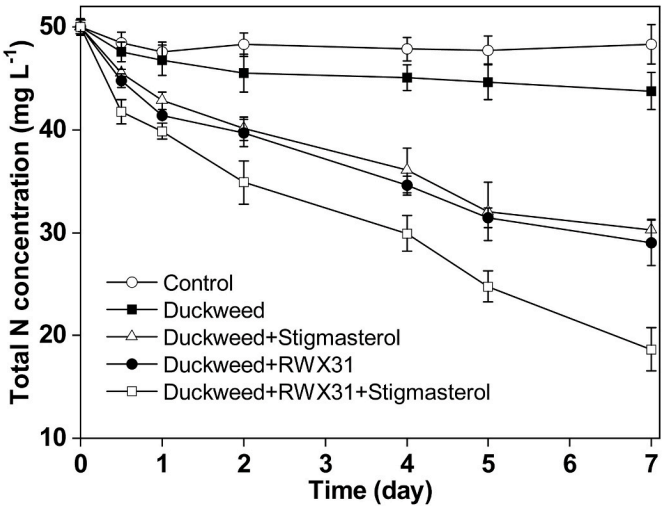

Fig. 5. Long-term performance test of nitrogen removal by different duckweed and RWX31/stigmasterol combination systems. Control was set for the experiment without duckweed, RWX31, and stigmasterol. Each data point is the average of triplicate experiments.

4. Discussion

4.1. Plant sterol exudation induced by rhizospheric denitrifying bacteria

Over the past decade, the application of microbial biostimulants isolated from plant rhizospheres has been increasing in aquatic wastewater treatment systems, driven by the interest of farmers, purification technology companies, and researchers in the quest to boost treatment efficiency while reducing cost, in a sustainable manner, with especially much progress in aquatic plants such as duckweed (Yamaga et al., 2010; Kristanti et al., 2014; Yamakawa et al., 2018). A bio-stimulatory effect in duckweed species was observed both in the present study involving *Spirodela polyrrhiza* (Fig. 1a), and in our previous study involving *Lemna minor* (Zhou et al., 2013), demonstrating that inoculation with the rhizospheric *Pseudomonas* sp. RWX31 strain significantly improves N-removal efficiency in duckweed compared with untreated plants. Some proposed mechanisms for the bio-stimulatory activity of rhizospheric bacteria in duckweed are the modulation of the root system (in particular root length) and frond number (Yamaga et al., 2010). Another important component, however, could be enhanced production and/or improved functioning of phytometabolites involved in the chemical dialog between plant and microbes. Such effects may be linked to the secretion of root exudates that act on rhizosphere bacteria and, in turn, enhance duckweed performance.

The present results show the secretion of two specific phytosterols from duckweed roots, which are the result of plant-metabolic processes triggered by *Pseudomonas* sp. RWX31 inoculation of the rhizosphere. During the interaction of duckweed plants with RWX31, stigmasterol and β-sitosterol were exuded from roots (Fig. 2). Other as yet unidentified compounds may also be involved in this signaling, as relative amounts of several compounds showed an increased or decreased response to RWX31 compared to control. However, sterols were the most responsive and most abundantly secreted chemical class, appearing only under inoculation condition with denitrifying bacteria RWX31. Sterol lipids in the root exudates were modulated in the context of microbial interaction, in agreement with previous literature on beneficial fungi (Lucini et al., 2019). Accumulation of the phytosterol stigmasterol in Arabidopsis leaves has also been observed following exposure to the pathogenic *Pseudomonas syringae* and fungal infection with *Botrytis cinerea* (Griebel and Zeier, 2010). Thus, it is likely that sterols are exuded in a range of mechanistically different plant-microbe interactions.

When collecting exudates with the coexistence of bacteria, one must also consider that rhizospheric denitrifers could be the origin of sterols. To exclude this possibility, exudates of RWX31 were collected and identified. GC/MS analysis of the RWX31 exudates revealed the absence of stigmasterol and β-sitosterol in cultures (Fig.S2c), which does, however, not rule out the possibility that bacteria may produce sterols only when interacting with plant roots. In view of the absence of the two phytosterols in natural duckweed root exudates (Fig. 1a), and exudates when inoculated with RHZ1 and *Pseudomonas stutzeri* (Fig. S2a, 2b), our data indicate that stigmasterol and β-sitosterol are exuded from duckweed only as a result of the interactions with specific denitrifiers. Couto et al. (2011) also found that metabolites such as linalyl acetate, menthyl acetate, and α-farnesene were only identified in *Glycine* max inoculated with *Bradyrhizobium japonicum*. It is likely that, once the plant receives signals from denitrifying bacteria RWX31, an array of metabolic processes is induced in the plant, with one specific target being stigmasterol synthesis, followed by release into the rhizosphere. However, the chemical classes of other signals exuded under the influence of denitrifying bacteria, as well as chemicals released by the bacteria themselves when in symbiosis, will need to be delineated in future studies, to gain a more comprehensive picture.

4.2. Mechanism of the stimulation of N removal by stigmasterol

As the major structural components of plant membranes, phytosterols in the rhizosphere are reported to have diverse biological roles,

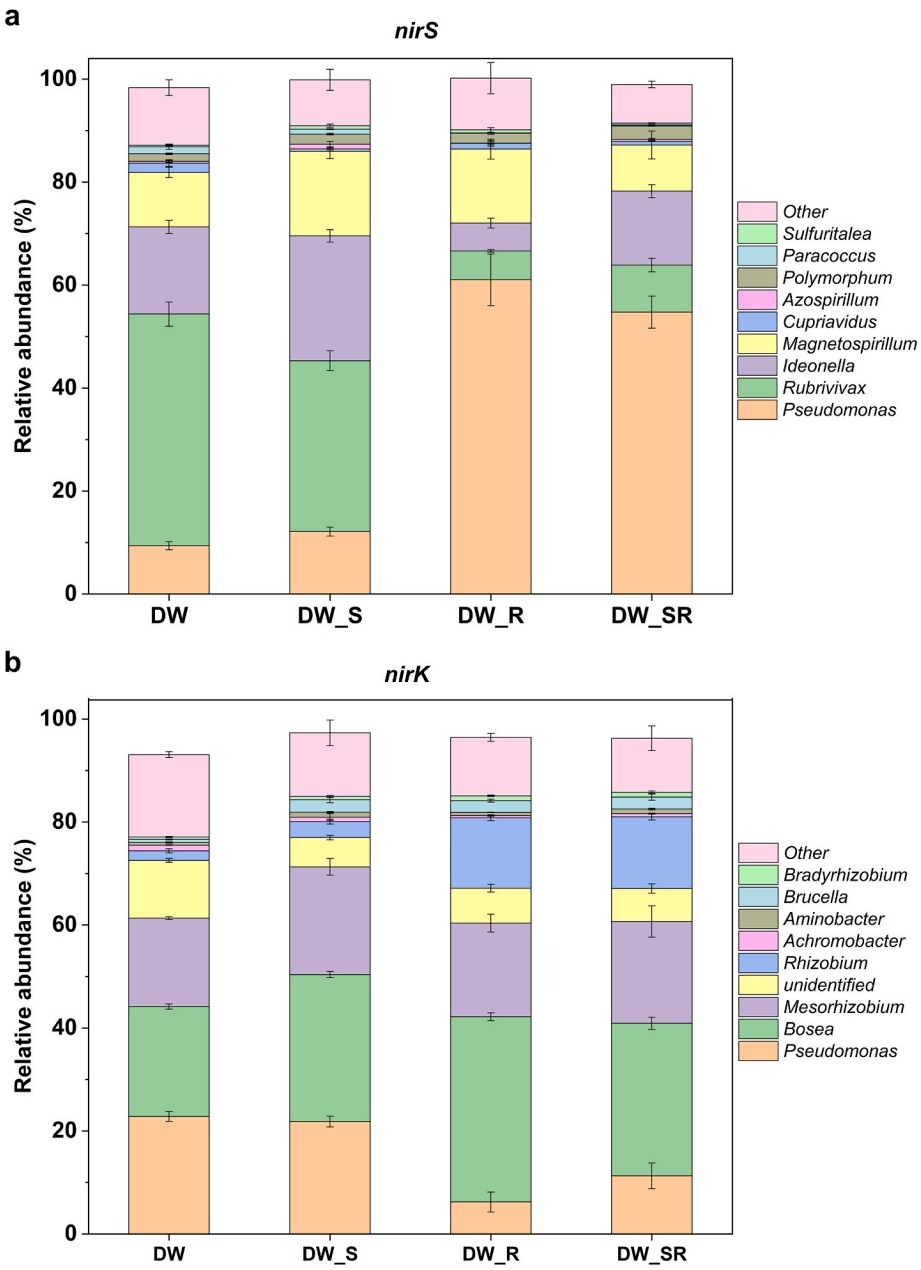

Fig. 6. Composition (relative abundances > 0.5%) of *nirS* (a) and *nirK* (b) communities at the genus level from the rhizosphere of duckweed under different treatments on day 7. DW: duckweed, DW_S: duckweed with stigmasterol, DW_R: duckweed with RWX31, DW_SR: duckweed with stigmasterol and RWX31. Data are means ± standard errors (n = 3).

acting as antioxidants, modulators of enzymes, and cell receptors (Yoshida and Niki, 2003; Schaller, 2003), as well as being involved in plant growth and biotic stress resistance (Clouse, 2002; Griebel and Zeier, 2010; Wang et al., 2012). As far as we know, this is the first report to show the function of stigmasterol in the promotion of denitrification both in pure bacterial cultures and in symbiotic systems of aquatic duckweed associated with rhizospheric bacteria. Moreover, our study shows that the biofilm formation ability of RWX31 was enhanced by stigmasterol, which could establish conditions for improved root colonization and, consequently, denitrification in the duckweed rhizosphere. Denitrification ability is strongly positively correlated with rhizosphere colonization and plant growth promotion (Ghiglione et al., 2002; Ghirardi et al., 2012), and we therefore propose that stigmasterol may be central to the competitive strength and fitness of both duckweed and several specific denitrifers in aquatic ecosystems.

Despite sharing similar structures, the sterol stigmasterol and β-sitosterol elicit rather distinct physiological responses to *Pseudomonas* sp. (Fig. 3). This may be due to the additional double bond at C22 in the sterol side chain of stigmasterol. This renders the alkyl chain more hydrophilic, and therefore influences the solubility and membrane fluidity, and the ability to interact with denitrifying reductases. Such an effect of structural features of the sterol molecule on the activity of membrane-bound enzymes has been previously observed. For instance, H^+-ATPase activity in maize roots was stimulated by stigmasterol, whereas the C22-saturated counterpart β-sitosterol did not stimulate (GrandmouginFerjani et al., 1997).

We further show a particularly significant role of stigmasterol in modulating bacterial nitrite reductases (NIR) rather than nitrate reductases (NAR). This observation is in agreement with a previous study that showed that the stimulation of NIR activity by the exudate chemical

erucamide was much stronger than that of NAR (Sun et al., 2016b). Similarly, Toyofuku et al. (2008) reported that NIR activity increased 1.8-fold in cultures incubated with a quorum-sensing *Pseudomonas quinolone* signal. Further corroboration comes from a community analysis of denitrifying microbes in the duckweed rhizosphere, which shows stigmasterol affects the composition of *nirS*- and *nirK*-denitrifying microbial communities (Fig. 6), underscoring the role of stigmasterol in regulating rhizosphere-bacterial nitrite reduction. Since high nitrite and nitric oxide levels can be toxic to denitrifying bacteria, aquatic animals, and even humans (Vollack and Zumft, 2001), the elimination of excess nitrite by stigmasterol's shaping of the nitrite-reducing bacterial community and of bacterial NIR activity highlights its potential to remediate N-polluted wastewaters, and in particular its potential to reduce the problem of nitrite accumulation in the environment.

Rhizosphere microbial composition is closely associated with the improvement of N removal (Zhao et al., 2015; Li et al., 2020; Zuo et al., 2020). We observed that stigmasterol and RWX31 both increased the relative abundance of *Bosea, Rhizobium,* and *Brucella* (member of Rhizobiales) in the *nirK* community, which were positively correlated with TN removal in duckweed systems. These genera are well-known beneficial partners in plant-microbe interactions and known to contribute to plant growth, to be important to symbiotic N fixation, and to be effective in biocontrol contexts (Lugtenberg and Kamilova, 2009). Interestingly, *Rubrivivax* in the *nirS* community and unidentified Burkholderiales in the *nirK* community (member of Burkholderiales), which are negatively correlated with TN removal, exhibited a decreasing trend following treatment with stigmasterol and RWX31. As several species of the Burkholderiales can act as opportunistic pathogens in humans (Chiarini et al., 2006), our results indicate stigmasterol might also be helpful in optimising the rhizospheric community profile from perspectives other than plant growth stimulation and N-removal stimulation. We, thus, suggest that the stimulation by stigmasterol and RWX31 of duckweed N removal may be attributed to the positive regulation of *Bosea, Rhizobium, and Brucella* and the negative regulation of *Rubrivivax*. Considering that the core duckweed microbiome is highly conserved, with strong resilience, inoculation with the single PGPB strain did not have a long-lasting impact (Trabelsi and Mhamdi, 2013; Acosta et al., 2020), and biochemical regulation by stigmasterol in shaping the rhizosphere microflora may enhance the stability and persistence of inoculated microbial strains. Such chemical-microbial interplay in duckweed-associated bacterial communities warrants further characterization in future studies, given the practical importance of such relationships.

Despite the regulation on duckweed-associated denitrifying microbiota, the observation that stigmasterol increased the frond number by 10.3% (Table S2) indicates its potential for promoting the growth of duckweed plants, which could further enhance duckweed N uptake. However, it has often been emphasized that the nitrification-denitrification process is the most important pathway for N removal rather than direct plant N uptake in duckweed wastewater treatment systems (Zimmo et al., 2004). In this context, duckweed growth promotion would only be expected to have a small contribution to N removal stimulation, against the background of the 3- to 5- fold increase in TN removal observed (Fig. 5). Moreover, it should be noted that the root exudate stigmasterol acts on key denitrifying enzymes rather than affecting the growth of RWX31 (Table S1), and we therefore suggest that the addition of low-dose stigmasterol might not have a significant impact on microbial growth in the duckweed rhizosphere. It would, however, be desirable to elucidate bacterial growth contributions to N removal in future examinations.

4.3. Future potential application of stigmasterol in N-polluted environments

Selection of optimized root microbiomes is a novel tool for pollutant removal from contaminated water and soil (Stout and Nusslein, 2010; Segura and Ramos, 2013). As previous studies mainly focused on screening highly efficient microbial and aquatic plant genotypes (Yamaga et al., 2010; Li et al., 2015), our study focused on the bioactive chemical signals secreted from roots, a central connection point between plants and microorganisms, the understanding and optimization of which could lead to a significant strengthening of rhizosphere interactions and the capacity for, and efficiency of, pollutant removal. Here, we identify principal biochemical players in the bioaugmentation of the rhizosphere by the rhizobacterium RWX31 in the context of N removal from duckweed-based wastewater treatment systems, with a specific focus on the significant role of the root exudate stigmasterol in strengthening this biostimulation process. The contribution of components of duckweed root exudates has also been observed in organic pollutant degradation by symbiotic plant-bacterial complexes, showing increased remediation of phenol, pyrene, and benzo[a]pyrene from water by plasmid introduction improving plant-bacterial partnerships (Toyama et al., 2011; Jin et al., 2019). Thus, the synergism of stigmasterol with RWX31 in stimulating N removal makes it possible to develop superior bio-formulations that will enhance the N-bioremediation capacity of aquatic plants.

Different from root exudates acting as simple carbon sources for bacterial growth, stigmasterol, given its low effective dose, appears to act as a chemical signal directly affecting enzyme activity in rhizospheric bacteria RWX31, similar to the non-nutritional role of another duckweed exudate, erucamide, we identified previously (Lu et al., 2014). Given its low dose, it is unlikely for stigmasterol to affect the reproduction or survival/necrosis of denitrifying microorganisms during the biological N-removal process. We believe the application of stigmasterol to be highly promising as a novel low-dose denitrification bio-stimulant amenable to permeable reactive barrier (PRB) technology in groundwater remediation, avoiding the clogging problems often encountered in PRB (Gibert et al., 2008).

5. Conclusions

Our results demonstrate that inoculation with the rhizospheric denitrifying bacterial strain RWX31 can distinctively modulate the chemical composition of root exudates in aquatic duckweed, and specifically induce the secretion of stigmasterol. Furthermore, our data show that low-dose stigmasterol (100 μM) can stimulate the N-removal efficiency of duckweed wastewater treatment systems by about 3-fold, and we suggest that this occurs by a combination of effects on bacterial enzyme activity, and composition of the microbial community responsible for nitrite reduction. We provide novel insight into the role of specific chemical signals exuded from roots in strengthening plant-bacterial interactions involved in N removal from aquatic ecosystems. From a practical point of view, the identification of the specific exudate stigmasterol and its synergism with the rhizospheric denitrifying bacterial strain RWX31 may be of interest in the context of developing novel strategies and technologies aimed at improving the N-removal efficiency of nitrate-contaminated wastewater with or without aquatic plants.

Author statement

Yufang Lu: Investigation, Funding acquisition, Writing – original draft, **Herbert J. Kronzucker**: Reviewing and Editing, **Weiming Shi**: Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117587.

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