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Quantification and enzyme targets of fatty acid amides from duckweed root exudates involved in the stimulation of denitrification

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ABSTRACT

Fatty acid amides from plant root exudates, such as oleamide and erucamide, have the ability to participate in strong plant-microbe interactions, stimulating nitrogen metabolism in rhizospheric bacteria. However, mechanisms of secretion of such fatty acid amides, and the nature of their stimulatory activities on microbial metabolism, have not been examined. In the present study, collection, pre-treatment, and determination methods of oleamide and erucamide in duckweed root exudates are compared. The detection limits of oleamide and erucamide by gas chromatography (GC) (10.3 ng mL^{-1} and 16.1 ng mL^{-1}, respectively) are shown to be much lower than those by liquid chromatography (LC) (1.7 and 5.0 μg mL^{-1}, respectively). Quantitative GC analysis yielded five times larger amounts of oleamide and erucamide in root exudates of *Spirodela polyrrhiza* when using a continuous collection method (50.20 ± 4.32 and 76.79 ± 13.92 μg kg^{-1} FW day^{-1}), compared to static collection (10.88 ± 0.66 and 15.27 ± 0.58 μg kg^{-1} FW day^{-1}). Furthermore, fatty acid amide secretion was significantly enhanced under elevated nitrogen conditions (>300 mg L^{-1}), and was negatively correlated with the relative growth rate of duckweed. Mechanistic assays were conducted to show that erucamide stimulates nitrogen removal by enhancing denitrification, targeting two key denitrifying enzymes, nitrate and nitrite reductases, in bacteria. Our findings significantly contribute to our understanding of the regulation of nitrogen dynamics by plant root exudates in natural ecosystems.

1. Introduction

Nitrogen losses from agricultural systems often lead to eutrophication in natural waterways. As one of the world's most widespread water quality problems, the over accumulation of nitrate (NO_3^-) and nitrite (NO_2^-) is well known to cause excessive algal growth, kill aquatic animals, and pose very significant direct health risks to humans (Camargo and Alonso, 2006; Cameron et al., 2013; Jensen et al., 2014). Therefore, it is urgent to control nitrogen pollution in ecological systems. Plant-microbe interactions play a significant role in controlling the rate of nitrogen transformation in natural soils and water bodies and, thus, can be used as an effective means to remove nitrogen from water bodies (Feng et al., 2012; Payne et al., 2014). One of the important plant materials receiving wide attention in the context of removal of nitrogen is duckweed, as, compared with other aquatic plants, it can generate a large biomass with high protein content, and tolerate a wide range of nutrient conditions (Caicedo et al., 2000; Lasfar et al., 2007; Xu and Shen, 2011; Mohedano et al., 2012). In addition to nitrogen assimilation during growth, plant roots can secrete compounds to stimulate denitrification, thus improving nitrogen removal (Henry et al., 2008). Much research suggests that root exudates can fuel denitrification as organic carbon sources (Salvato et al., 2012; Zhai et al., 2013), but root exudates also serve as signals that initiate and modulate dialogue between roots and microbes residing in the rhizosphere (Badri and Vivanco, 2009; Hartmann et al., 2009). Our previous research revealed that two fatty acid amides, oleamide and erucamide, from duckweed root exudates can stimulate nitrogen removal by the denitrifying bacterium *Pseudomona fluorescens* (Lu et al., 2014), but the specific enzymatic targets of the exudates remained unknown.

Fatty acid amides are a group of nitrogen-containing, lipid-soluble fatty acid derivatives (Kim et al., 2010) and have received

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much attention as critical endogenous signaling molecules in animals. Oleamide is a brain lipid that serves as a chemical modulator of sleep (Lerner et al., 1994; Cravatt et al., 1995; Ezzili et al., 2010). Erucamide was isolated from mammalian tissues and has been shown to regulate fluid volumes in various organs, inhibit intestinal diarrhea, and stimulate angiogenesis (Hamberger and Stenhagen, 2003). In recent years, the signaling role of fatty acid amides in plants has also received growing attention, with roles emerging in early seedling development and in plant-microbe interactions (Tripathy et al., 1999; Teaster et al., 2007; Kim et al., 2010). However, research concerning the influence of fatty acid amides on microbes is still in its infancy. In our previous study, erucamide exuded from duckweed root exudates was the most efficient biological compound stimulating bacterial nitrogen removal (Lu et al., 2014), and it was postulated that erucamide might stimulate bacterial nitrogen assimilation as well as denitrification. Nitrate and nitrite reduction are critical processes in the removal of nitrogen from soils and water bodies, mediated by the enzymes nitrate reductase (NAR) and nitrite reductase (NIR) (Toyofuku et al., 2008). Thus, the effect of erucamide on these two key denitrifying enzymes required examination.

Reliable collecting and determining methods are essential for quantitative root exudate assays. Static collection has frequently been used with hydroponic systems to acquire root exudates of various plants such as alfalfa and lupine (Muratova et al., 2015; Valentinuzzi et al., 2015); nutrient solution is then typically extracted with organic solvents. The use of static collection, however, is limited to research of low-concentration compounds and water-insoluble molecules. Because of these limitations, Tang and Young (1982) first developed a continuous root exudate trapping system to extract bioactive metabolites from the rhizosphere, and continuous collection has now been widely adopted to study root exudates. Several studies have discussed the advantages and pitfalls of various determination methods for oleamide and erucamide, including liquid chromatography (LC), gas chromatography (GC), GC hyphenated to mass spectrometry (GC–MS), and Fourier transform infrared spectroscopy (FTIR) (Farajzadeh et al., 2006; Garrido-López et al., 2007; ASTM, 2009). Among these, GC has emerged as more sensitive than LC, as long as the samples are derivatized prior to analysis (Gee et al., 1999; Hanus et al., 1999; Lv et al., 2009). The direct determination by GC of oleamide and erucamide without derivation has been favored for its simplicity and reliability (Farajzadeh et al., 2006; Garrido-López et al., 2007). However, direct assays by both GC and LC have not thus far been examined, and they are therefore compared here, in particular with respect to the analysis of perform nanogram quantities.

The secretion of root exudates is strongly affected by nutrient status (Bowsher et al., 2015). Organic acid exudation by plants has been observed to be stimulated under P-deficient conditions (Ohkama-Ohtsu and Wasaki, 2010). Likewise, nitrogen deficiency can decrease amino acids exudation (Carvalhais et al., 2011). Some metabolites contained in root exudates have exhibited higher abundance under high-nutrient conditions (Bowsher et al., 2015). As a small aquatic plant widely-distributed in eutrophic water bodies, duckweed is adapted to a wide range of nutrient conditions (Caicedo et al., 2000; Lasfar et al., 2007). In this study, we hypothesize that the secretion of specific nitrogen removal stimulators, oleamide and erucamide, from duckweed can greatly increase under adequate- and high-nitrogen conditions. The principal objectives of our study were to establish methods to determine fatty acid amides in duckweed root exudates, to examine whether secretion of oleamide and erucamide are regulated by nitrogen levels, and to explore the mechanisms underlying the stimulation of nitrogen removal specifically by erucamide.

2. Materials and methods

2.1. Plant and bacteria materials and their cultural conditions

Duckweed (*Spirodela polyrrhiza*) was collected from Huzhou city, Taihu Lake region, in October 2008 (Zhou et al., 2010), and was cultivated in a controlled-environment chamber with 23 °C temperature, 70% humidity, 16 h/8 h light/dark photoperiod, 100 μmol m^{-2} s^{-1} light intensity, and 400 ppm CO_2 concentration. The nutrient solution for duckweed was modified according to Steinberg medium (Zhou et al., 2010; Lu et al., 2014) as follows (mg L^{-1}): $Ca(NO_3)_2{\cdot}4H_2O$ 295, KNO_3 350, NH_4Cl 12.5, KH_2PO_4 90, $MgSO_4{\cdot}7H_2O$ 100, $MnCl_2{\cdot}4H_2O$ 0.18, H_3BO_3 0.12, $NaMoO_4{\cdot}2H_2O$ 0.044, $ZnSO_4{\cdot}7H_2O$ 0.18, $FeCl_3{\cdot}6H_2O$ 0.76, $Na_2EDTA{\cdot}2H_2O$ 1.5, MES 100, pH = 6.8.

The denitrifying bacterium *Pseudomonas fluorescens* (*P. fluorescens*) 01047 was obtained from the Agricultural Culture Collection of China (ACCC). Bacteria were activated in LBN medium (Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}, KNO_3 0.72 g L^{-1}, pH 7.0). The denitrifying medium (DM) was as follows (g L^{-1}): KNO_3 0.72, KH_2PO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.2, $C_4H_4Na_2O_4{\cdot}6H_2O$ 2.8, pH = 7.0. Bacteria were cultivated anaerobically at 30 °C, 120 rpm in the dark.

2.2. Static collection of duckweed root exudates

Duckweed fronds were rinsed with deionized water several times and soaked in Milli-Q water for use. Nutrient solution was prepared with Milli-Q water and 1.5 L solution was added into a 20 cm wide-mouth glass jar which was wrapped with silver paper to avoid light infiltration from the side. 6.0 g fresh duckweed was weighed and cultivated in this jar for 24 h with coverage of approximately 100%. The fresh weight of duckweed was recorded following collection. The solution was then filtrated with a 0.22-μm filter membrane and kept in the filter flask. A large volume sampling tube was used to connect solution to a solid phase extraction (SPE) device (CNW, Germany). A C18 SPE column (CNW, 1 g/6 mL) was used to collect target substances in the filtrate. The column was finally eluted with HPLC methanol into glass tubes and evaporated under nitrogen. The residue was redissolved in 200 μL dichloromethane or methanol, stored in −20 °C and was determined within a week.

2.3. Continuous collection of duckweed root exudates

A modified root exudate-trapping system was used to collect duckweed root exudates (Lu et al., 2014). 1.5 L of nutrient solution and 1.0 g fresh duckweed fronds were added into a 14-cm incubator, and the fronds had water coverage by approximately 1/3. Amberlite XAD-4 macroporous adsorptive resin (Sigma, USA) was used to collect root exudates, which was pre-treated with methanol and water before use. The resin column had a volume of 60 cm^3 and was wrapped with silver paper. The flow rate was 10 mL min^{-1} controlled by a peristaltic pump and water evaporation was compensated during collection. After 5 d, fresh duckweed was weighed and the resin column was detached and eluted with 600 mL Milli-Q water and 120 mL HPLC methanol. The methanol extraction was evaporated under vacuum on a rotary evaporator at 40 °C, and frozen to dryness in a freeze drier. The residue was redissolved in 5 mL methanol and filtered with a 0.22-μm filter membrane. The filtrate was evaporated to dryness under nitrogen and was redissolved in 200 μL dichloromethane or methanol for determination.

2.4. Comparison of GC and LC analysis

Authentic compound controls of oleamide and erucamide were dissolved in dichloromethane without derivation. The mixtures

were subjected to GC. The GC analysis was performed on an Agilent 6850 gas chromatograph equipped with a fused silica capillary column HP-5 (25 m × 0.2 mm × 0.33 μm) and a FID detector. Splitless injection was performed at 290 °C; the oven temperature was initially 150 °C for 2 min and was raised to 300 °C at a rate of 20 °C min^{-1} and was kept for 3 min. The carrier gas was helium at a flow rate of 1.0 mL min^{-1}, and sample size was 2 μL.

For LC analysis, authentic samples were dissolved in methanol and were subjected to a Shimadzu UFLC-20 liquid chromatograph equipped with a ZORBAX SB-C18 column (250 mm × 4.6 mm × 5 μm) and a UV detector. The mobile phase consisted of a mixture of acetonitrile: methanol (60:40, V/V), at a flow rate of 1.0 mL min^{-1}, and injection volume of sample was 20 μL. The separation was carried out at 30 °C for 15 min, and the wavelength of UV detector was 202 nm.

2.5. Determination of oleamide and erucamide and calculations

According to the comparison of the GC and LC features (above), the GC method was chosen to determine oleamide and erucamide in duckweed root exudates. The oleamide and erucamide unit secretion amounts were calculated as the total secretion amounts, using the following two equations:

$$A(1+k)^{d} = B. \quad (1)$$

$$Q = \int_{t=0}^{t=d} qAe^{kt} d_t \quad (2)$$

where A was the initial duckweed mass (g, FW), B was the final duckweed mass after collection (g, FW), d was collection time (days), k was growth rate per day and was assumed to remain constant during collection, q was unit secretion amounts (μg kg^{-1} FW day^{-1}), and Q was total secretion amount during collection (μg).

2.6. Oleamide and erucamide secretion as affected by nitrogen levels

Duckweed was cultivated in 87 mg L^{-1} nitrogen prior to starvation. Modified Steinberg medium with varying nitrogen concentrations (45 mg L^{-1}, 87 mg L^{-1}, 300 mg L^{-1} and 900 mg L^{-1}) was used prior to collection of duckweed root exudates, to reflect varying N status of duckweed (Lasfar et al., 2007). Nutrient conditions except for nitrogen were kept identical among treatments, and ammonium and nitrate ratio was consistent with that of the medium during mass cultivation. Following a one-day nitrogen starvation, the steps were the same with those used in the continuous collecting method. Relative growth rate (RGR, day^{-1}) was calculated according to the following equation:

$$RGR = (\ln B - \ln A)/d$$

where A was the initial duckweed mass (g, FW), B was the final duckweed mass after collection (g, FW), and d was collection time (days).

2.7. Mode of erucamide stimulating denitrifying bacteria of nitrogen removal

One milliliter of fresh *P. fluorescens* cells in DM were transferred into a 50-mL Erlenmeyer flask containing 19 mL DM, and then 20 μL erucamide dissolved in dichloromethane was added into the flask (erucamide concentration was 20 mg L^{-1} and initial NO_3^--N concentration was 100 mg L^{-1}). The mixture was introduced into an incubation shaker (120 rpm, 30 °C). After 48 h, 1 mL of culture mixture was centrifuged at 10,000 rpm for 10 min. Total nitrogen (mg L^{-1}) of both the supernatant and the culture mixture were determined using ultraviolet spectrophotometry following persulfate oxidation (APHA, 1998). Nitrogen absorbed by *P. fluorescens* (mg L^{-1}) and nitrogen removed by denitrification were calculated using the following equations.

(1) Nitrogen absorbed by bacteria (mg L^{-1}) = total nitrogen concentration of culture mixture after cultivation (mg L^{-1}) − total nitrogen concentration of supernatant after cultivation (mg L^{-1})
(2) Nitrogen removal by denitrification (mgL^{-1})=initial total nitrogen concentration (mgL^{-1})−total nitrogen concentration of supernatant after cultivation (mgL^{-1})−nitrogen absorbed by bacteria (mgL^{-1})

2.8. The effect of erucamide on denitrifying enzyme activities

P. fluorescens were transferred to 100 mL of LBN medium, and the air in the flask was replaced with 100% He under vacuum for anaerobic treatments, and replaced with gas mixture (79% He + 21% O_2) for aerobic treatments. Cells were maintained in incubation shakers at 30 °C, and 180 rpm in the dark, and were collected during the exponential growth phase (8-h incubation time, $OD_{600} \approx 1.0$). The extraction of cell fractions and the enzyme activity assays were according to Toyofuku et al. (2008). *P. fluorescens* collected were centrifuged at 5000 *g* at 4 °C, and washed twice with 100 mM potassium phosphate buffer (pH 7.5, containing 10% glycerol). Cells suspended in 20 mL of buffer were sonicated (300W for 10 min in an ice bath, for 3 s, in 5-s intervals). The sonicated cells were centrifuged for 10 min at 7000 *g* at 4 °C to remove unbroken cells (sediments). The supernatants were then centrifuged at 186,000 *g* at 4 °C for 90 min. The supernatants were soluble fractions of the cells for NIR activity assay, and the sediments were resuspended with the same buffer as membrane fractions for NAR activity assay.

NAR activity (μmol NO_2^- produced min^{-1} μg^{-1} protein) was assayed by determining the reduction product NO_2^-. Two hundred microliters of 100 mM KNO_3, 200 μL of 2 mM methylviologen, 400 μL of 250 μg mL^{-1} membrane fractions, and 200 μL of 100 mM $Na_2S_2O_4$ were added into 1 mL of 100 mM potassium phosphate buffer. The reaction volume was 2 mL containing 10 mM KNO_3, 0.2 mM methylviologen, 50 μg mL^{-1} enzymes, and 10 mM $Na_2S_2O_4$. According to our previous work (Lu et al., 2014), erucamide (40–60 mg L^{-1}) has the strongest stimulatory effect on nitrogen removal. As such, erucamide (40 mg L^{-1}) was dissolved in potassium phosphate buffer as treatment and dichloromethane as CK. The air in the reaction mixtures was replaced with nitrogen before adding $Na_2S_2O_4$. The reaction was carried out at 30 °C for 10 min in a water bath and was stopped by boiling water. The mixture was vortexed until its color became clear, indicating methylviologen oxidation, and NO_2^- production was measured.

NIR activity (μmol NO_2^- reduced min^{-1} μg^{-1} protein) was assayed by measuring NO_2^- consumption. Two hundred microliters of 2 mM KNO_2, 200 μL of 2 mM phenazine methosulfate, 200 μL of 250 μg mL^{-1} soluble fractions, 400 μL of 5 mM NADH were added into 1 mL potassium phosphate buffer containing 80 mg L^{-1} erucamide. The reaction volume was 2 mL containing 200 μM KNO_2, 200 μM phenazine methosulfate, 25 μg mL^{-1} soluble fractions, 1 mM NADH and 40 mg L^{-1} erucamide. The air in the reaction mixtures was replaced with nitrogen before adding NADH. The reaction was carried out at 30 °C for 10 min, and NO_2^- consumption was measured.

2.9. Data processing and statistical analyses

GC data was processed using Agilent ChemStation B.04.03, and LC data was analyzed using Shimadzu LC Solution. The experimental data were subjected to Excel 2007 for calculation and SPSS 18.0

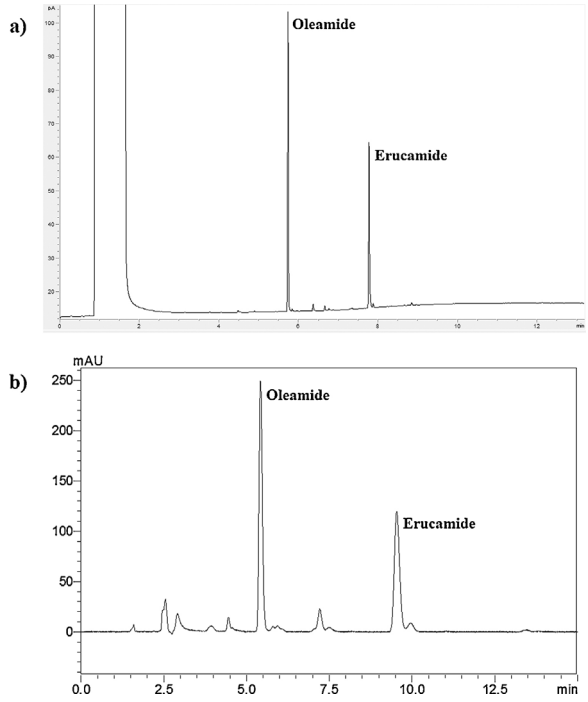

Fig. 1. a) GC chromatogram of oleamide (5.74 min) and erucamide (7.78 min) authentic compounds (5 μg mL^{-1}). b) LC chromatogram of oleamide (5.42 min) and erucamide (9.54 min) authentic compounds (500 μg mL^{-1}).

for analyses using Student's *t*-test, Duncan's multiple range test, and Pearson correlation analysis, as appropriate. Significance levels and other data formats were as illustrated in legends.

3. Results

3.1. GC and LC features for determination

Oleamide and erucamide both have a polar amino (-NH_2) group, and have poor solubility in non-polar solvents such as hexane. Thus, the moderately polar solvent dichloromethane was used to prepare stock solutions. Both authentic compounds produced sharp GC peaks, with oleamide showing a stronger response at an identical concentration (Fig. 1a). Retention times were 5.74 min and 7.78 min for oleamide and erucamide, respectively. Both compounds retained excellent linearity ($r^2 > 0.999$) in a concentration range of 0.05–50 μg mL^{-1} (Table 1), and the detection limits were 10.3 and 16.1 ng mL^{-1} for oleamide and erucamide, respectively. Relative standard deviations (RSDs) for both compounds were less than 5%, illustrating excellent reproducibility.

The American Society for Testing and Materials (ASTM) recommends the LC method for the determination of erucamide and other slip additives in PP products (ASTM, 2009). The response of erucamide, however, is poor compared to other additives. A modified LC method (Farajzadeh et al., 2006) was thus used, with methanol as the solvent and acetonitrile: methanol as the mobile phase. Both compounds showed a good response using LC, with oleamide responding better (Fig. 1b). Retention times were 5.42 min and 9.54 min and detection limit 1.7 and 5.0 μg mL^{-1}, respectively. Oleamide (5–125 μg mL^{-1}) and erucamide (10–250 μg mL^{-1}) retained excellent linearity ($r^2 > 0.999$) in the set concentration range (Table 1). RSDs for both compounds were less than 5%.

Based on the results of the methodological comparison, the direct GC method was recommended for further determination of fatty acid amides in duckweed root exudates.

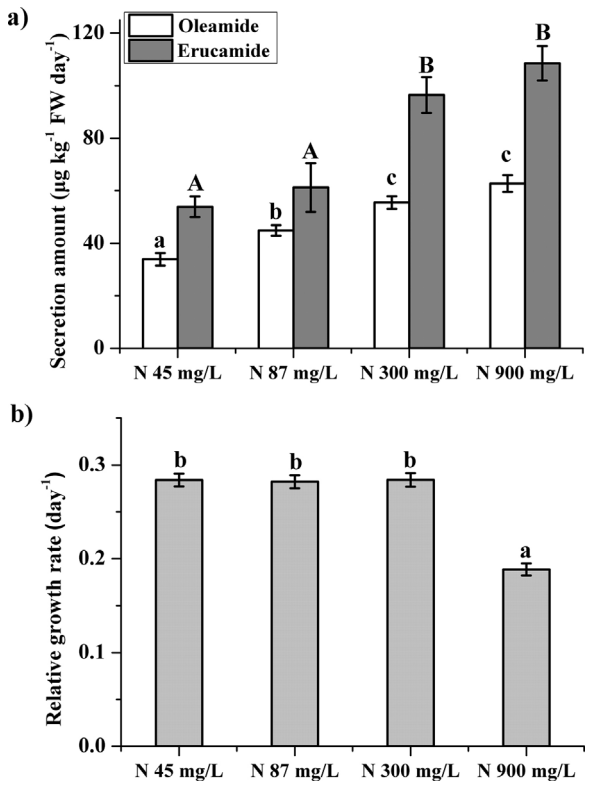

Fig. 2. a) Oleamide and erucamide amounts (μg kg^{-1} FW day^{-1}) in duckweed root exudates collected at different nitrogen concentrations (mg L^{-1}). b) Relative growth rate (day^{-1}) of duckweed at different nitrogen concentrations (mg L^{-1}) during collection. Data are presented as means and standard errors, n = 3. a) Lower-case and capital letters represent statistical differences (Duncan's test, at $P < 0.05$) of oleamide and erucamide amounts in root exudates, respectively; b) lower-case letters represent significant differences (Duncan at $P < 0.05$) of relative growth rate.

3.2. Determination of oleamide and erucamide in duckweed root exudates

Oleamide and erucamide in duckweed root exudates were determined by both the static and the continuous method. The concentration values of oleamide and erucamide in root exudate samples were calculated according to the equations listed above. The results of the calculations are shown in Table 2. Oleamide and erucamide secretion by static collection were 10.88 ± 0.66 μg kg^{-1} FW day^{-1} and 15.27 ± 0.58 μg kg^{-1} FW day^{-1}, respectively, and 50.20 ± 4.32 μg kg^{-1} FW day^{-1} and 76.79 ± 13.92 μg kg^{-1} FW day^{-1}, respectively, by continuous collection. The amounts of both compounds by continuous collection were significantly higher (5-fold) than the amounts by static collection. As a result, continuous collection was used for the determination of oleamide and erucamide in further research, due to the high efficiency of the method.

3.3. Oleamide and erucamide secretion as affected by nitrogen levels

Oleamide and erucamide secretion by duckweed was significantly higher in plants reared on 300 mg L^{-1} nitrogen concentration or above, compared with 45 and 87 mg L^{-1} (Fig. 2a). The exuded amounts were the highest from plants grown on 900 mg L^{-1} N, although the corresponding relative growth rate of duckweed was the smallest at this nitrogen level (Fig. 2b). Data were then subjected to Pearson correlation analysis (Table 3). Four factors were pairwise-correlated significantly. Nitrogen levels and fatty acid amide secretion had a positive correlation, whereas relative growth rate showed a negative correlation with the other three fac-

Table 1
Features of the GC and LC methods for oleamide and erucamide authentic compounds. The correlation coefficient was calculated with n = 10 by GC and n = 8 by LC. The detection limits were calculated by using a signal-to-noise ratio of 3.%RSD was determined at 1 μg mL^{-1} with GC and at 40 μg mL^{-1} with LC, n = 6.

Method	Compounds	Concentration range (μg mL^{-1})	Correlation coefficient (r^2)	Detection limit (ng mL^{-1})	% RSD
GC	Oleamide	0.05–50	0.9994	10.3	3.18
	Erucamide	0.05–50	0.9993	16.1	4.75
LC	Oleamide	5–125	0.9993	1.7×10^3	3.61
	Erucamide	10–250	0.9995	5.0×10^3	4.13

Table 2
Oleamide and erucamide exuded by the duckweed species *Spirodela polyrrhiza*. Data are presented as means ± SE, n = 4. Lower-case and capital letters represent respective differences of oleamide and erucamide secretions between different collecting methods (*t*-test at $P < 0.05$).

Root exudate samples	Oleamide (μg kg^{-1} FW day^{-1})	Erucamide (μg kg^{-1} FW day^{-1})
By static collection	10.88 ± 0.66 a	15.27 ± 0.58 A
By continuous collection	50.20 ± 4.32 b	76.79 ± 13.92 B

Table 3
Correlation values from Pearson analysis of nitrogen levels, oleamide, and erucamide amounts in root exudates, and relative growth rate of duckweed.

Item	Nitrogen levels	Oleamide	Erucamide	Relative growth rate
Nitrogen levels	1	0.826**	0.814**	−0.931**
Oleamide	0.826**	1	0.855**	−0.594*
Erucamide	0.814**	0.855**	1	−0.607*
Relative growth rate	−0.931*	−0.594*	−0.607*	1

** Denotes significance at the 0.01 level.

Table 4
The effect of erucamide (20 mg L^{-1}) on nitrogen removal pathways of *P. fluorescens*. Initial nitrogen concentration (NO_3^-) was 100 mg L^{-1}. Data are presented as means ± SE, n = 3.

Nitrogen removal pathways	CK	Erucamide
Nitrogen absorbed by bacteria (mg L^{-1})	13.15 ± 0.76	13.91 ± 0.32
Denitrification (mg L^{-1})	21.01 ± 1.34	27.46 ± 0.51*
Total nitrogen removal (mg L^{-1})	34.16 ± 1.02	41.37 ± 0.66*

* Denotes significant differences ($P < 0.05$) between CK and erucamide treatment, determined by *t*-test.

tors. These results indicate that oleamide and erucamide secretion responded positively to nitrogen levels.

3.4. Mode of erucamide stimulating denitrifying bacteria

The removal of nitrogen through denitrification was significantly increased when 20 mg L^{-1} erucamide was added (Table 4), while no significant difference via bacterial absorption was found between the erucamide treatment and the dichloromethane control. Thus, the bacterial nitrogen removal enhanced by erucamide was mainly through stimulation of denitrification activity.

3.5. The effect of erucamide on denitrifying enzyme activities

The denitrifying enzyme activities in an anaerobic environment were much higher than those in an aerobic environment (Table 5). Data were compared on a percentage basis, in order to examine the effect of erucamide addition on NAR and NIR activities (Fig. 3). The denitrifying enzyme activities of CK samples were set as 100%. With the addition of 40 mg L^{-1} erucamide, NAR activity increased by 44.5% and 25.9% under anaerobic and aerobic conditions, respectively, while NIR activity increased by 164.6% and 243.0%, respectively. Both NAR and NIR activities were significantly enhanced by erucamide.

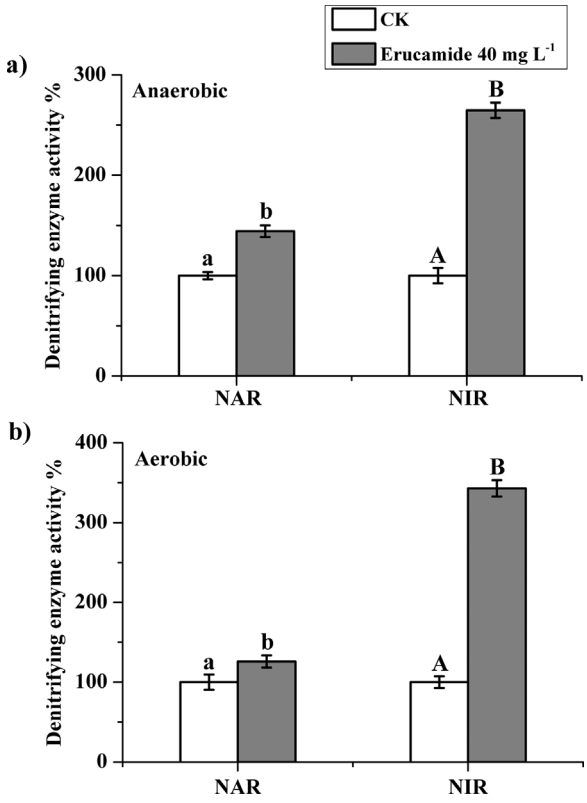

Fig. 3. The effect of erucamide on NAR and NIR denitrifying enzyme activities anaerobically (a) and aerobically (b). The denitrifying enzyme activity of CK was 100%. Lower-case and capital letters represent significant differences (*t*-test at $P < 0.05$) between CK and erucamide treatments of NAR and NIR activities, respectively, n = 3.

4. Discussion

Evidence is gathering that plant root exudates play important roles in influencing nitrogen transformations in the rhizosphere, and, in particular, stimulating nitrogen removal by denitrification (Jahangir et al., 2014; Lu et al., 2014). Some exudates act as simple carbon substrates for denitrifying bacteria (Salvato et al., 2012; Zhai et al., 2013; Jahangir et al., 2014), while others may act as signaling molecules enhancing microbial activity (Lu et al., 2014). A

Table 5
The effect of erucamide (40 mg L^{-1}) on NAR and NIR denitrifying enzyme activities anaerobically and aerobically. Data are presented as means ± SE, n = 3.

Denitrifying enzyme activities	Anaerobic		Aerobic	
	CK	Erucamide	CK	Erucamide
NAR (μmol NO_2^- produced min^{-1} μg^{-1} protein)	1.731 ± 0.063	2.501 ± 0.101*	0.586 ± 0.056	0.738 ± 0.045*
NIR (μmol NO_2^- reduced min^{-1} μg^{-1} protein)	0.065 ± 0.005	0.172 ± 0.006*	0.027 ± 0.002	0.093 ± 0.004*

* Denotes significant difference ($P < 0.05$) between CK and erucamide treatment, determined by *t*-test.

previous study by our group (Lu et al., 2014) demonstrated stimulation of bacterial denitrification by oleamide and erucamide from duckweed root exudates. However, the secretion amounts of the two fatty acid amides, and the specific targets of their actions on microbes, remained unresolved.

4.1. Reliable methods for exudate collection and chemical identification

The exudation of many compounds is significantly affected by growth conditions and sampling procedures (Oburger et al., 2013). The collection of oleamide and erucamide secretion by continuous collection was more efficient than static collection, likely due to the fact that fatty acid amides are lipid-soluble and are well retained by the XAD-4 resins used in continuous collection. Additionally, some compounds can readily decompose, on account of microbial degradation, in static collection protocols, whereas the chemical stability of these compounds increases once adsorbed by the resin particles employed in continuous collection (Tang and Young, 1982). Axenic cultural conditions were suggested in Tang and Young's method (1982); however, our previous work suggested oleamide and erucamide were present in duckweed root exudates under both natural and aseptic conditions (Lu et al., 2014). Therefore, a simple collection condition was employed here in natural conditions. The low amounts of fatty acid amides in secretions can present a limitation for determination, and we have found the most effective method to increase exudate concentration was to increase duckweed inputs rather than extending collection time, as nutrient solution stability over time is otherwise difficult to maintain.

The detection limits for oleamide and erucamide by the direct GC method (10.3 and 16.1 ng mL^{-1}) were lower by two orders of magnitude compared with similar methods reported by others (Farajzadeh et al., 2006; Garrido-López et al., 2007). The use of capillary columns with low polarity, such as HP-5, is suitable for the direct determination of oleamide and erucamide by GC. Moreover, pure HPLC solvents and clean glass tubes and cuvettes should be used to obtain a steady baseline. The detection limits by LC (1.7 and 5.0 μg mL^{-1}) were much higher than by GC, but could still be improved in our study compared with the reference method (20 and 30 μg mL^{-1}) by Farajzadeh et al. (2006). Although we do not recommend LC overall for the assay due to insensitivity, it can still be used to perform other microgram analyses.

4.2. Oleamide and erucamide secretion regulated by nitrogen levels

Oleamide and erucamide secretion increased with the nitrogen status of duckweed, with strong similarity in the degree of increase, indicating that both compounds respond simultaneously and are up-regulated at high nitrogen levels. Similarly, Bowsher et al. (2015) found that some metabolites contained in root exudates increase at high nutrient concentrations. There are also many studies that have focused on the response of root exudates to limited nitrogen supply (Vranova et al., 2013). Carvalhais et al. (2011) found that amino acid release decreases under nitrogen deficiency. It is, thus, reasonable to assume, and borne out by precedent, that the amounts of nitrogen-contained compounds released by roots be positively correlated with nitrogen levels in the environment. Our results, however, show that the growth of duckweed is inhibited at high nitrogen concentration (900 mg L^{-1}), with the secretion of fatty acid amides still increasing. The contrary responses in growth at high nitrogen, on the one hand, and fatty acid amid release on the other, indicate that the release of oleamide and erucamide is likely subject to active regulation rather than representing a passive response coincident with plant nitrogen status. The increase of secretion of compounds stimulating nitrogen removal may well be a strategy in aquatic plants to adapt to high nitrogen concentrations in eutrophic water bodies. Thus, these compounds may play an important role in the self purification of natural ecosystem.

4.3. Mechanisms underlying the stimulatory effect of denitrification by erucamide

Bacterial nitrogen removal can be achieved by the denitrification pathway as well as by microbial assimilation (Kim et al., 2008; Park and Craggs, 2011). In our present study, erucamide was chosen as the representative nitrogen-removal stimulant to elucidate the mechanism, as it displayed superior efficiency compared to oleamide (Lu et al., 2014). We found that erucamide could stimulate nitrogen removal mainly by enhancing denitrification rather than bacterial uptake (Table 4). This conclusion strengthens the notion that compounds such as erucamide act as signals rather than simple carbon skeletons in regulating nitrogen dynamics (Lu et al., 2014). In mammalian systems, chemicals such as erucamide have been shown to act in a well-defined receptor-mediated fashion (Hamberger and Stenhagen, 2003; Divito and Cascio, 2013). The lack of a significant difference in OD_{600} at final DM between control and erucamide treatments in our study also supports the observation that erucamide does not act as a simple carbon "nutrient" (data not presented), but has a more sophisticated signaling role. Similarly, Kim et al. (2008) found that an increase in the tissue C/N ratio could accelerate nitrate reduction rates by the aerobic denitrifying bacterium *Pseudomonas putida* AD-21, with no impact on bacterial nitrogen assimilation.

In order to further investigate the process of denitrification, we compared the influence of erucamide on the activities of two key denitrification enzymes under both aerobic and anaerobic conditions, those of bacterial nitrate and nitrate reductases (NAR and NIR). NAR and NIR activities were both enhanced by erucamide at 40 mg L^{-1} (Fig. 3). This observation is in agreement with Toyofuku et al. (2008), who studied *Pseudomonas aeruginosas* and found NIR activity to increase some 1.8-fold in cultures incubated with a quorum-sensing *Pseudomonas* quinolone signal. Since the synthesis of both NAR and NIR is greatly inhibited by oxygen (Korner and Zumft, 1989), the activities of the two enzymes in *Pseudomonas fluorescens* under anaerobic conditions were much higher than those under aerobic conditions (Table 5). Moreover, the stimulation of NIR activity by erucamide (1.6–2.4 fold) was much stronger than the stimulation of NAR (0.2–0.5 fold), under both conditions (Fig. 3), indicating a particularly significant role of erucamide in modulating NIR. Considering that nitrite reduction catalyzed by NIR is a key rate-limiting step in denitrification, erucamide from duckweed root exudates may limit nitrite accumulation to maintain the balance of nitrite and nitric oxide in denitrifying bacteria, which may

be of benefit as high nitric oxide levels (above 50 nM) can inhibit the expression of key genetic elements, such as *nirSTB* and *norCB* (Vollack and Zumft, 2001). Future work is needed to characterize the modulation of these genetic elements in more detail. As a key intermediate step in denitrification, nitrite accumulation can also have a toxic effect on denitrifying bacteria, and has been implicated centrally in the nitrogen toxicities to aquatic animals and humans (Camargo and Alonso, 2006). Thus, the elimination of excessive nitrite, and specifically the stimulation of NIR activity, by erucamide in our study suggests its potential to remediate nitrogen-polluted waterways in both agricultural and natural ecosystems.

5. Conclusions

This study recommends the continuous collection and direct GC methods for the determination and quantification of root exudates such as oleamide and erucamide in duckweed exudates, due to the method's superior efficiency and sensitivity. Our study shows that the increase of fatty acid amide excretion is an active response upon challenge by high nitrogen loads. Erucamide is shown to stimulate nitrogen removal through enhancing denitrification, by targeting the two key denitrifying bacterial reductases NAR and NIR. The understanding of the stimulatory mechanisms involved in nitrogen removal induced by fatty acid amides, such as the present compounds from duckweed, could be of great importance for the development of future wastewater clean-up methods via the use of beneficial root exudates.

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