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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