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Reversed-phase liquid chromatographic determination of phytometallophores from Strategy II Fe-uptake species by 9-fluorenylmethyl chloroformate fluorescence

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Abstract

An HPLC method to quantitate phytometallophores (phytosiderophores) exuded from roots of barley (*Hordeum vulgare* L.) growing in nutrient solution culture was developed. 9-Fluorenylmethyl chloroformate (FMOC) derivatives of phytometallophores were separated on a C_{18} reverse-phase column using a sodium acetate (pH 7.2) and acetonitrile–methanol gradient over 20 min followed by fluorescence detection. Detection limits ranged from 15 to 370 pmol depending on the particular phytometallophore. The effectiveness of this method was demonstrated using the response of barley seedlings to Fe-sufficient and Fe-deficient nutrient solution conditions. Phytometallophores collected in root washings of Fe-deficient barley seedlings increased with plant age while phytometallophore release from Fe-adequate roots was negligible. Published by Elsevier Science B.V.

Keywords: Derivatization, LC; Amino acids, non-protein; Phytometallophores; 9-Fluorenylmethyl chloroformate; Mugineic acid

1. Introduction

Uptake of iron from alkaline soils is limited by the low availability of iron in the soil [1] and often results in iron deficiency, impaired growth and failure to produce seed for many crop species. To enable iron uptake from soils with limited available iron pools, graminaceous monocots (*Poaceae*) have evolved an iron-uptake mechanism in which ferric-chelating substances are released from the roots (Strategy II species; [2]). In addition to complexing ferric iron, these compounds can form stable hexadentate complexes with several other divalent transition metal ions, notably Zn^{2+}, hence our preference for the name phytometallophore [3]. Phytometallophores (Fig. 1) are non-proteinaceous amino acids that solubilize Fe(III) from insoluble soil pools via chelation. This results in the mobilization of certain insoluble transition metal ions from soil mineral pools followed by transport to root cell plasma membrane uptake sites. These metal complexes are then absorbed by root cells in toto allowing iron and

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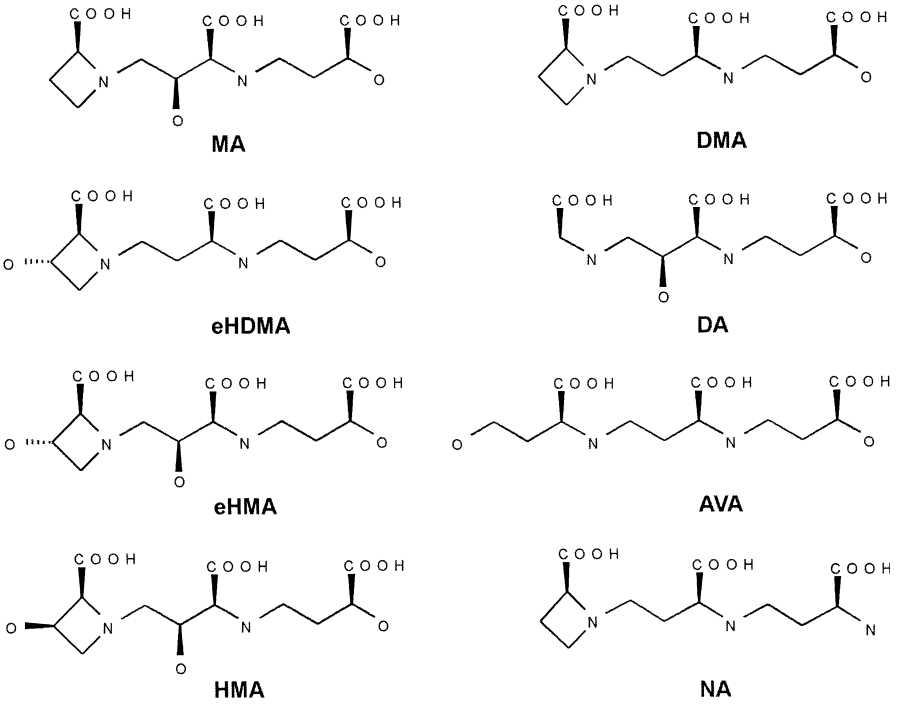

Fig. 1. The known phytometallophores in root exudates from Strategy II species: MA=mugineic acid, DMA=2′-deoxymugineic acid, eHDMA=epihydroxy-2′deoxymugineic acid, DA=distichonic acid, eHMA=epihydroxymugineic acid, AVA=avenic acid, HMA=hydroxymugineic acid), and the ubiquitous intracellular and intercellular Fe(II)-chelating phytometallophore — nicotianamine (NA).

other transition metals to enter root cells [4]. Phytometallophores released by cereal roots are synthesized from the precursor, nicotianamine, the only known low molecular mass Fe(II) complexing compound found in all higher plant species [5]. Nicotianamine is biosynthetically derived from methionine via *S*-adenosylmethionine [6]. Seven phytometallophores have been identified in root exudates from Strategy II species [7,8].

Published methods for phytometallophore analyses are based on HPLC methodologies or on the total metal complexing ability of root exudates using ion-exchange resins and/or metal chromophores. These resin-based methods only determine the total amount of transition metal ions that can be mobilized by root exudates [9–11] and do not distinguish between different phytometallophores and non-phytometallophore transition metal chelating substances in root exudates. They only determine the total amount of transition metal ions that can be mobilized by root exudates [9–11]. Current HPLC methods require extensive sample preparation including separate injections of oxidized and non-oxidized samples [12–14]. Other methods cannot readily quantify the different phytometallophores in a single sample (TLC, [15]). More complex methods using NMR [7,16], can determine molecular characteristics of root exudate compounds precisely, but are not easily adapted to rapid processing of multiple samples, and the instrumentation required is not available to most laboratories.

To determine phytometallophores rapidly and accurately, we developed an HPLC method utilizing fluorescence detection after pre-column derivatization. FMOC was used for derivatization as it has several advantages over other fluorescent derivatives (OPA [12], PITC [14]). FMOC can be observed with UV/Vis equipment or by fluorescence detection under conditions that selectively ignore non-amine compounds that may be present in exudates. Because

FMOC reacts with both secondary and primary amines, sample handling is reduced compared to OPA methods (i.e., OPA methods require sample pretreatment to oxidize secondary amines to primary amines before derivatization [13,17]). FMOC forms more stable derivatives than OPA [18], allowing FMOC-derivatized samples to be prepared ahead of time and sampled by automated equipment.

The purpose of this paper is to describe the development and implementation of an HPLC method for analyzing phytometallophores and to use the method to determine the effects of Fe-deficiency on phytometallophore release by barley (*Hordeum vulgare* L.) seedling roots.

2. Methods

2.1. Plant growth

Seeds of barley (cv. Herta) were germinated for 72 h in aerated 18 MΩ-cm H_2O until seminal roots were 1–2 mm long. Each germinated seed was placed in a 1.5-ml conical microcentrifuge tube with the base removed so that roots could grow unimpeded. The tubes were inserted into 9-mm diameter holes in the lids of 0.8-l black plastic pots containing 0.75 l of nutrient solution. Solution height was adjusted so that the roots of each plant were in contact with the solution. Black plastic beads were placed on top of the seed to reduce light exposure to the nutrient solution. The solution was aerated continuously with filtered compressed air supplied through black plastic tubing. Plants were grown in a controlled-temperature water bath at 20°C with a 14-h light/10-h dark period at a light intensity of 130 $\mu M\ m^{-2}\ s^{-1}$ supplied by ten 115 W Osram Gro-Lux fluorescent tubes (Sylvania Corp., USA[2]).

Nutrients were supplied to plants using a chelate-buffered nutrient solution [19] at the following concentrations: 0.5 m*M* KNO_3, 1.5 m*M* $Ca(NO_3)_2$, 0.02 m*M* $NH_4H_2PO_4$, 0.25 m*M* $MgSO_4$, 0.1 m*M* NH_4NO_3, 50 μ*M* KCl, 12.5 μ*M* H_3BO_3, 0.1 μ*M* H_2MoO_4, 10 μ*M* $ZnSO_4$, 1 μ*M* $CuSO_4$, 0.4 μ*M* $MnSO_4$, 0.1 μ*M* $NiSO_4$. To maintain constant micronutrient metal activity, HEDTA–KOH (*N*-(hydroxyethyl)ethylenediaminetriacetic acid buffered with KOH to pH 6.0) was added in equimolar amounts with a 20-μ*M* excess (31.5 μ*M* HEDTA total). Solution pH was buffered to 6.1 with 2 m*M* MES (2-morpholinoethane sulfonic acid). Solutions were replaced every 7 days.

Treatments were imposed on day 14 after severe iron deficiency symptoms had developed on the seedling leaves (10–14 days). Treatments consisted of adding either 5 μ*M* Fe(III)-HEDTA (pH 6.0) or 100 μ*M* Fe(III)-HEDTA to the nutrient solution.

2.2. Exudate collection and derivatization

Root exudates were collected from plants on days 16, 17 and 18 after germination. Prior to this collection, the root system of each plant was rinsed in 18 MΩ-cm H_2O, then immersed in 0.1 m*M* K_3HEDTA for 30 s to remove metal ions adhering to the root surface, then rinsed again in 18 MΩ-cm H_2O to remove the K_3HEDTA. Root exudates were collected by suspending the root system in 7.5 ml 18 MΩ-cm H_2O for 3 h, starting 2 h after the onset of the light period, previously determined to be the time of greatest phytometallophore release [20]. Immediately after collection, plants were harvested for dry matter and exudate samples were frozen for subsequent lyophilization. The lyophilized residues of root exudates were dissolved in 750 μl H_2O and stored at −20°C before use. No additional sample purification was performed.

To derivatize samples for HPLC analysis, 10 μl of solubilized root exudate was added to 50 μl of 1.2 *M* boric acid buffer, pH 10.2 (Hewlett-Packard, Waldbronn, FRG) in a 200-μl vial. The solutions were vortexed before adding 10 μl of 0.01 *M* FMOC-Cl in acetonitrile (Fluka, Buchs, Switzerland). The sample vials were sealed with a polyethylene cap and vortexed again to ensure complete mixing. The resultant FMOC derivatives were stable for at least 14 days at 4°C (data not shown) supporting previous results [18].

[2]Mention of trademark, proprietary product or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

2.3. HPLC conditions

The HPLC system consisted of a Waters (Milford, MA, USA) model 717 autosampler, a model 626 pump operated by a model 600+ controller and a model 474 fluorescence detector using the following settings: λ_{ex}=266 nm, λ_{em}=305 nm, gain=10, attenuation=64. The column was a Waters Symmetry® 250 mm×4.6 mm diameter, 5 μm particle-size RP-C_{18} amino acid analysis column maintained at 40°C. Solvent flow was maintained at 1 ml min^{-1}. The system was operated through a desktop computer running Waters Millennium[32] software.

The chromatographic procedure was adapted from the Hewlett-Packard ''AminoQuant'' amino acid detection method [21], because the original procedure gave poor resolution of phytometallophore standards. The following eluents were used: 0.02 *M* sodium acetate (Sigma, St. Louis, MO, USA)+180 μl l^{-1} triethylamine (Fluka)+3 ml l^{-1} tetrahydrofuran (Fluka) adjusted to pH 7.2 with 2% (v/v) acetic acid. To prevent precipitation of sodium acetate in the column during rinse stages, 18 MΩ-cm H_2O was used in place of the buffer. Two organic modifiers, HPLC-grade acetonitrile (Mallinkrodt, Paris, KY, USA) and HPLC-grade methanol (Mallinkrodt), were used and kept in separate reservoirs. All solvents were sparged with 10 ml He min^{-1}.

Elution times for phytometallophores were determined using purified samples (gifts from Dr S. Mori, University of Tokyo, Japan) of DMA (deoxymugineic acid), MA (mugineic acid), eHMA (epihydroxymugineic acid) and DA (distichonic acid A). Verification of standards and eluted compounds was performed by mass spectrometry. Electrospray (ESI) mass spectra were obtained by direct infusion of solutions into a Waters Micromass ZMD mass detector at a flow rate of 5 or 10 μl/min using a model 55-1199 syringe pump (Harvard Apparatus, Holliston, MA). Negative ion spectra were obtained in the negative ion mode using a cone voltage of 9 V, and a capillary voltage of 2.5 kV. Source block temperature was 100°C and desolvation gas (H_2) was 350°C.

HPLC calibration plots for phytometallophores were calculated using peak area. The method was checked by standard additions of 6.0 nmol MA+1.9 nmol DMA to concentrated root exudates.

3. Results and discussion

3.1. Gradient conditions

Separation of FMOC-derivatized phytometallophores was optimized for complete baseline separation of compounds using a linear gradient over the initial 20 min of the procedure. The organic modifiers in the gradient were increased by 20% and the starting and finishing proportions adjusted. The optimized procedure consisted of a sequence of linear and fixed gradients running for a total time of 45 min. The initial linear gradient was run for 20 min, from 84:0:8:8 (aqueous buffer: water: acetonitrile: methanol) to 64:0:18:18 (0–20 min). The next gradient immediately substituted water for the sodium acetate buffer to prevent precipitation of acetate in the column as the acetonitrile proportion increased (64:0:18:18–0:10:90:0 in 5 min, from 20 to 25 min). The rinse stage (fixed composition; 0:10:90:0) ran for 10 min from 25 to 35 min to elute the nonderivatized FMOC from the column. The eluent composition was returned to starting conditions (0:10:90:0–84:0:8:8 in 3 min from 35 to 38 min) and the column backpressure allowed to stabilize (fixed composition 84:0:8:8 for 7 min from 38 to 45 min).

A typical chromatogram for a mixture of the tested phytometallophore standards (MA, DMA, eHMA, DA) shows excellent peak resolution for each compound, with near complete baseline separation (Fig. 2). The peak area per mol of derivatized compound varied between compounds. Therefore, a separate calibration plot was used for each phytometallophore (Table 1). Calibration plots using peak area were

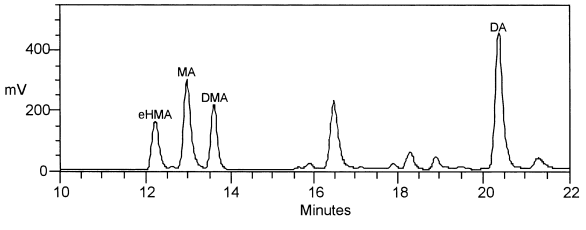

Fig. 2. Chromatogram of a mixture of phytometallophore standards. Peaks indicated contained the following amounts of phytometallophore standards: eHMA (100 nmol), MA (25 nmol), DMA (1 nmol), DA (2.5 nmol). Abbreviations are the same as in Fig. 1.

Table 1
Calibration plots and mass spectrometric data for phytometallophore standards with detection limits, linear range of detection for each phytometallophore determined

Determination	eHMA	MA	DMA	DA
Minimum detection (pmol)	100	370	70	15
Linear range (nmol)	0–50	0–20	0–5	0–1
R^2	0.99	0.99	0.99	0.99
m/z	335.1	318.9	303.3	292.7
Mol. mass	337	320	304	294

R^2 is the linear regression coefficient squared for peak area against mass injected into HPLC. Mass to charge data (m/z) was determined with phytometallophore standards dissolved in 3 m*M* borate buffer, pH 10.2, in negative ion mode. Abbreviations are the same as in Fig. 1.

linear ($R^2 \geq 0.99$) over concentration ranges of 0–1 nmol DA, 0–5 nmol (DMA), 0–20 nmol MA and 0–50 nmol (eHMA). Detection limits for phytometallophores ranged from 15 pmol for DA to 370 pmol for MA (Table 1).

Mass-spectrometric data indicated that the MA, and DMA standards used were pure, but the eHMA and DA standards contained small amounts of unknown impurities that did not interfere with chromatography (data not shown). Mass/charge data for the phytometallophores in negative ion mode indicated four compounds (m/z: 335.1, 318.9, 303.3, 292.7), that corresponded to the known masses of phytometallophores (Table 1).

When 6.0 nmol MA and 1.9 nmol DMA standards were added to concentrated root exudate samples from low-iron barley seedlings of differing ages (Table 2), average percentage recovery of the additional MA was 81%, while recovery of additional DMA was lower at 59%. The smaller linear detection range for DMA and tailing of the MA peak may have reduced the recovery of DMA. Recovery of MA and DMA from a blank sample yielded 94 and 74% respectively (Table 2).

3.2. Influence of iron supply on amount of phytometallophore exuded by roots

The effect of Fe supply on phytometallophore release by roots was obvious (Fig. 3, Table 3). Low-Fe plants exuded much greater amounts of phytometallophores than high-Fe plants, while root weight was greater in plants treated with the higher

Table 2
Percent recovery of MA and DMA standards added to a blank sample (9.75 nmol MA + 3.1 nmol DMA) and to root exudate samples (6.0 nmol MA + 1.9 nmol DMA)

	eHMA	MA	DMA	DA
Blank	0	0	0	0
Blank + MA and DMA	0	9.16	2.30	0
% Recovery	–	94	74	–
Sample 1	0.55	0	0	0.03
Sample 1 + MA and DMA	0.65	4.38	1.12	0.02
% Recovery	118	73	59	69
Sample 2	2.49	0	0.19	0
Sample 2 + MA and DMA	3.74	5.19	1.11	0.12
% Recovery	150	87	58	–
Sample 3	1.59	0.37	0	0.02
Sample 3 + MA and DMA	2.36	4.78	1.10	0.12
% Recovery	148	80	58	629
Sample 4	1.59	0	0.10	0.02
Sample 4 + MA and DMA	3.12	5.03	1.17	0.07
% Recovery	196	84	61	384

Data indicate nmol phytometallophore. Abbreviations are the same as in Fig. 1.

Fe supply. Phytometallophore release increased with plant age as soon as 2 days (day 16) after Fe treatments began (Table 3). In addition, low-Fe

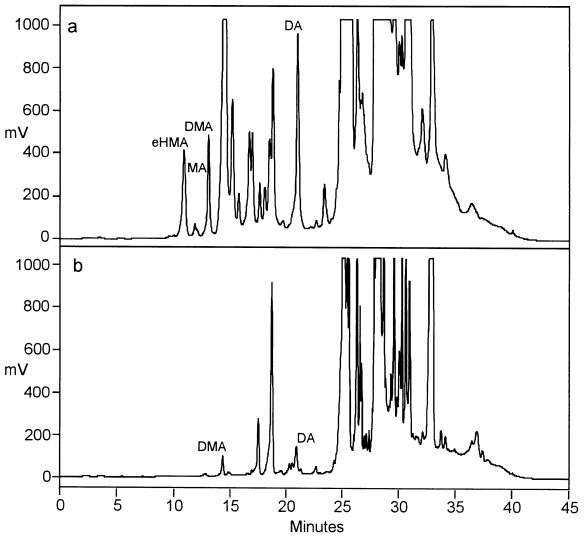

Fig. 3. Typical chromatograms of barley root exudates as influenced by Fe availability and measured by FMOC fluorescence HPLC: $a = 5$ μ*M* Fe(III)-HEDTA, $b = 100$ μ*M* Fe(III)-HEDTA. Abbreviations are the same as in Fig. 1.

Table 3
Phytometallophore production (μmol exuded g^{-1} dry mass root per 3 h, ±SD, $n=4$) by barley roots over 3 days when grown in low FeHEDTA (5 μmol l^{-1}) or adequate FeHEDTA (100 μmol l^{-1}) nutrient solutions

Day	16		17		18	
Fe	5	100	5	100	5	100
Root dry mass (mg $plant^{-1}$)	81±9	97+26	86±10	104±14	91±5	110±15
eHMA	1.54±0.83	–	7.59±4.42	4.20	11.45±7.20	2.00
MA	0.81±0.02	–	0.54±0.85	–	0.62±0.40	–
DMA	0.26±0.15	–	0.33±0.15	0.07	0.89±053	–
DA	0.06±0.01	0.02±0.01	0.34±0.30	0.04±0.04	1.15±0.74	0.03±0.01

Values with no SD indicate single replicate. Abbreviations are the same as in Fig. 1.

plants exuded all four identified phytometallophores simultaneously, while high-Fe treated plants produced mostly DMA and DA in small quantities.

Results for distichonic acid in root exudates are less accurate than for other compounds as peak heights exceeded the limit of the fluorescence detector. However, it was clear that DA was exuded by low-Fe plants in far larger quantities than high-Fe-treated plants of the same age (Table 3).

The amounts of phytometallophores exuded by Herta barley on a per plant basis (3.3–14.1 μmol $plant^{-1}$ 3 h^{-1}) are greater than the amounts reported elsewhere, e.g. (0.8–1.9 μmol $plant^{-1}$ 3 h^{-1} [22], 11.25 μmol $plant^{-1}$ 3 h^{-1} [10] and 0.75–1.5 μmol $plant^{-1}$ 3 h^{-1} [14]). Expressed on a per gram root basis however, Herta barley produced up to 155 μmol g^{-1} 3 h^{-1} while Europe barley achieved rates of 140 μmol g^{-1} 3 h^{-1} [22]. Although differing by up to an order of magnitude from the results given here, Cakmak et al. [22] found a 4- to 7-fold range in phytometallophore production by cv. Europe barley depending upon light conditions. The data in Table 3 were obtained from barley differing in age, cultivar, growth conditions (temperature, light intensity and composition of nutrient solution) to plants from other studies. These differences, along with the present method of phytometallophore detection may account for the apparent discrepancy in total yield of phytometallophores per plant.

4. Conclusions

The method described herein is a more rapid and effective method of determining phytometallophores in cereal root exudates than previously described methods [7,9–16], as it requires no purification of samples other than sample concentration. The method can be more easily adopted by conventional HPLC-equipped laboratories than other techniques [16], but yields results similar to other quantitative and qualitative methods. Derivatization of samples with FMOC is rapid and does not require extensive sampling handling for derivative preparation as with OPA and PITC methods [12–14]. Depending on the phytometallophore, detection limits as low as 15 (DA) to 370 pmol (MA) can be achieved. The results of a short-term experimental response confirm that Fe-deficient barley seedlings produced much greater amounts of phytometallophores than those supplied adequate levels of Fe.

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