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# 重金屬過剩 水稻根에 있어서 Mugineic acid 및 그 類似 Phytosiderophore의 分泌特性

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# Secretion Characteristics of Mugineic Acid and Its Analogous Phytosiderophore from High Heavy Metal-Induced Rice Roots

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## Abstract

We established HPLC method for MAs (mugineic acid and its analogous phytosiderophore) determination. It was found that major phytosiderophores existed in high heavy metal-induced rice roots were MA (mugineic acid) and DMA (2'-deoxymugineic acid). The two MAs (MA and DMA) were simultaneously secreted at maximum rate at 12 : 00－14 : 00. On the other hand, the secretion of amino acids gradually decreased from after sunrise (7 : 00) to sunset (21 : 00). Fluctuaton in daily MAs secretion had rhythmic variation which occurred at intervals of about 4 days. The stimulation of MAs secretion from the roots was attributed not only to the light/dark cycle but to the increase in temperature. Temperature variation played a more important role than a photoperiod in MAs secretion from the roots. The initiation time of MAs secretion was positively correlated with rising time in temperature(20－30℃) during the dark period.

## Introdution

Plants require essential elements for their life support and proper growth. Among these elements, heavy metals such as Cu and Zn, are one of the essential metals used as a component of various enzyme systems. For all these trace elements, there exist a fairly narrow "concentration window" between the essential and toxic levels. An element which is indispensable for normal function may be highly toxic when present at higher concentration.

Heavy metal toxicity results in a reduction in root growth and leaf expension which is followed

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by chlorosis[1,2,3]. Heavy metals, in particular Cu and Zn, are also known to displace Fe from chelate complexes forming corresponding heavy metal chelates. This may be important in limiting Fe uptake and utilization, either by reducing Fe chelate translocation to roots or within the plant itself by the effect of the heavy metals on centers of physiological activity for Fe[4]. Heavy metals induce iron stress and cause iron chlorosis which turns the young leaves to yellow or white[5,6]. As a result, the plants cease to grow and eventually they wither up.

For the purpose of effective incorporation of iron in soil, gramineous plants have been found to excrete novel amino acids from their roots[7]. Mugineic acid, (2S, 2'S, 3'S, 3"S)-N-[3-carboxyl-3-{(3-carboxy-3-hydroxypropyl)amino}-2-hydroxypropyl]azetidine-2-carboxylic acid, an amino acid isolated from the roots of water-cultured barley (*Hordeum vulgare* L. var. Minorimugi), is the first phytosiderophore to be shown to play a role in the uptake and transport of iron in higher plants[8]. Several new amino acids posessing iron-chelating activity have since been isolated from other species of gramineous plants : 2'-deoxymugineic acid from wheat[9], 3-hydroxymugineic acid from rye[10], isomugineic acid from the growth depressed barley[11], avenic acid A from oat[12], distichonic acid A from beer barley[10].

On the other hand, the root of rice plants was found to secrete less iron chelating substances than other gramineous plants[13]. Therefore, the susceptibility of rice plants towards iron chlorosis may be attributed to the lack of this chelating substance[14]. The amount and kind of MAs (Mugineic acid and analogous phytosiderophores) secreted by rice plant are still not elucidated because of the difficulty of obtaining the root exudates in required quantities aseptically.

The objective of this work was to study, in more detail, not only the secretion characteristics but also the analytical method of phytosiderophore from high heavy metal-induced rice roots.

## Materials and Methods

### Plant culture

Rice plants (*Oryza sativa* L. var. Fujiminori) were cultured in a growth cabinet with a photoperiod of 14 hrs. light/10 hrs. dark ; temperature 30℃/20℃ ; relative humidity 75% ; light intensity 15000 lux. Young seedlings of rice plants were transplanted into holes of plastic plates covered 21 liter PVC pots. There were 42 rice plants per plate. Each pot contained 20 liters of nutrient solution (pH 5.1) with the composition shown in Table 1.

Table 1. Composition of nutrient solution

| Element | Concentration(mg/l) |
|---|---|
| $(NH_4)_2SO_4$ | 48.2 |
| $KH_2PO_4$ | 24.8 |
| $KNO_3$ | 18.5 |
| $K_2SO_4$ | 15.9 |
| $Ca(NO_3)_2$ | 59.9 |
| $MgSO_4$ | 65.9 |
| $H_3BO_3$ | 2.9 |
| $MnCl_2.4H_2O$ | 1.8 |
| $Na_2MoO_4.2H_2O$ | 0.13 |
| $ZnSO_4.7H_2O$ | 0.22 |
| $CuSO_4.5H_2O$ | 0.08 |
| $FeCl_3$ | 14.52 |
| $SiO_2$ | 10.0 |

The nutrient solution was aerated continuously during cultivation. When the rice plants had fully expanded their 2nd and 3rd leaves after a week or more, they were transferred to a high heavy metal level "preculture solution". The composition of preculture solution contained 230 mg/l $ZnSO_4$ $7H_2O$ and 4 mg/l $CuSO_4.5H_2O$. The pH of the solution was maintained at 5.5+0.3 by the addition of 0.5 N-NaOH or 0.5 N-HCl, 4 times a day.

### Collection of root washings

When chlorosis occured, after a week or more, root washings were collected. The rice plants from each pot, together with the plastic plate supporting them, were transferred to shallow quadrangular plastic dish (25×40×6 cm). In order to collect the compounds secreted from the roots, the rice roots were immersed in the distilled water. After root washing, the rice plants were returned to their original pots so that they might be used repeatedly for next collect of washings on other days.

### MAs isolation

According to the Takagi's procedure[14], the root washings obtained were immediately filtered through filter paper (Toyoroshi No. 5C), and the filtrates, after shaking with a small amount of thymol, were eluted through an Amberlite IR-120 ($H^+$) column (3×15 cm). The resin was then eluted with 2N-$NH_4OH$ to release MAs, the eluate was evaporated to dryness in vacuum evaporator, and the residue was dissolved in 1 ml of distilled water. This was eluted through a DE23 cellulose columm(1.2×20cm). The resin was then eluted with 5mM-HCOOH to release MAs, the eluate was collected by fraction collector. It was filtered through a microfilter (Fuji film microfilter FM-45, Pore size 0.45um), and then 20 ul of filtrate was injected to HPLC.

MAs in roots and shoots of rice plant were extracted by 80% ethanol. The extract was directly injected to HPLC by the microfilter syringe.

### MAs determination

We established HPLC method for MAs determination. The HPLC system used was the Shimadzu LC-6A which incorporates a liquid pump of CDQR system. The column was housed in the Shimadzu CTO-6A column oven. The flow of the mobile phase was changed by means of the Shimadzu SCL-6A step gradient elution unit. Samples were introduced by Shimadzu SIL-6A injector. The detector used was the Shmadzu RF-530 fluorometric detector. The conditions for MAs analysis is shown in Table 2, 3.

The sensitivity of MAs to orthophthalaldehyde (OPA) by using recrystallized N-acetylcystein as

Table 2. Conditions for MAs determination by HPLC method

| | |
|---|---|
| Column : | Shmadzu Li-ISC-07/S 1504(100×6.0mm I. D) |
| | Temperature ; 38℃ |
| Buffer : | A solution ; pH 2.60(42.3g Li-citrate dihydrate+210ml Methyl cellosolve×40ml $HClO_4$, total 3000ml) |
| | B solution ; pH 10.0(28.2g Li-citrate dihydrate×12.4g $H_3BO_3$+30ml 4N-LiOH, total 1000ml) |
| | C solution (4.2g LiOH, total 500ml) |
| | pH adjustment by 60% $HClO_4$ or 4N-LiOH, Flow rate ; 0.5ml/min. |
| Reagent : | A solution ; pH 10.0 (20.4g $Na_2CO_3$+9.4g $K_2SO_4$+6.8g $H_3BO_3$+0.2ml NaClO, total 500ml) |
| | B solution ; pH 10.0(0.8g OPA* +14ml ethanol+1g N-Acethyl-cystein+2ml 10% Brij-35# + 20.4g $Na_2CO_3$+9.4g $K_2SO_4$+6.8g $H_3BO_3$, total 500ml) |
| | PH adjustment by 60% $HClO_4$ or 45% KOH |
| | Temperature ; 50℃, Flow rate ; 0.4ml/min. |
| Wavelenght : | 345 nm for excitation and 455 nm for emission |

*Orthophthalaldehyde, #Polyoxyethylene lauryl alcohol ether

a coreactant was about 5 times higher than that by mercaptoethanol. As shown in Table 2, MAs were quantitated fluorometrically after reacting with OPA[15]. There was linear correlation between amounts of MAs and intensities of peak area. By this HPLC method, both MAs and amino acids could be simultaneously determined to nmol concentration (recovery 96%) by means of stepwise-pH gradient elution.

Standard MAs got from Prof. Takagi Sei-ichi, Iwate Univ., Japan.

## Results and Discussion

Chromatogram of 80% ethanol extract of high heavy metal-induced rice roots is shown in Fig. 1. It was found that major phytosiderophore existed in rice roots were mugineic acid(MA) and 2'-deoxymugineic acid(DMA). MA and DMA were equivalent to 21% and 79% of phytosiderophore existed in roots, respectively.

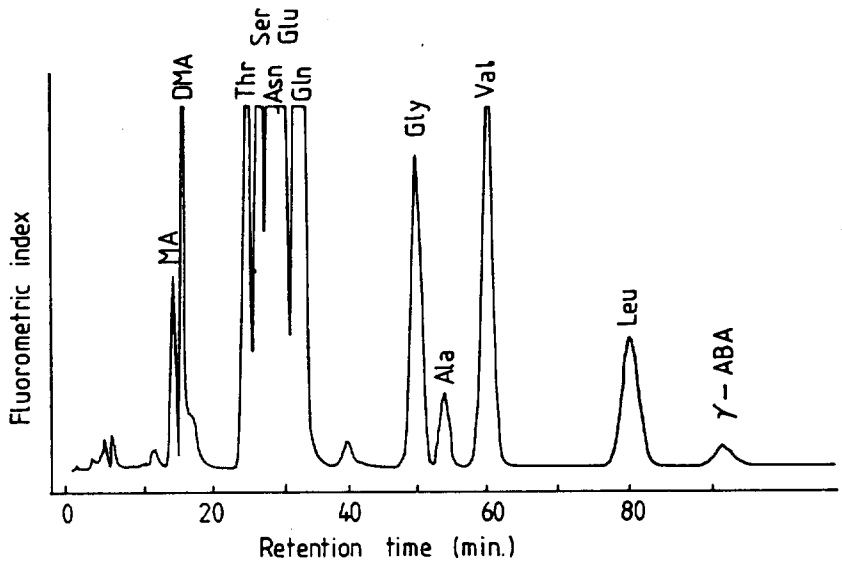


Fig 1. Chromatogram of 80% ethanol extract of high heavy metal-induced rice roots.

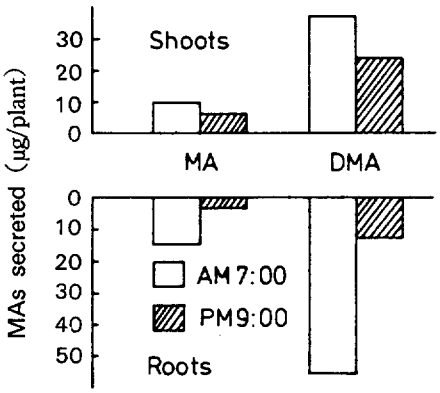


Fig 2. Change of MAs contents in high heavy melal-induced ria mots and shoots foom surise(7 : 00) to sumset(21 : 00)

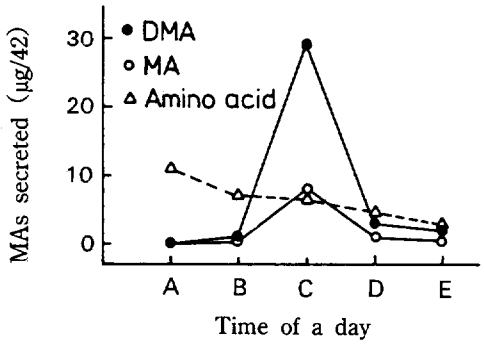


Fig 3. Diurnal variation of MAs secretion from high heavy metal-induced rice root. A ; 7 : 00−10 : 00 B ; 10 : 00−12 : 00 C ; 12 : 00−14 : 00 D ; 14 : 00−16 : 00 E ; 16 : 00−19 : 00

As shown in Fig. 2, MA and DMA remained in the shoots after sunset were 6.46 ug and 23.70 ug per plant. Whereas MA and DMA in roots after sunset decreased to 3.45 ug and 12.63 ug per plant, respectively.

As shown in Fig. 3, the secretion of MAs from high heavy metal-induced rice roots was observed at certain hours (3hrs. after the sunrise) in the morning every day. The two MAs were simultaneously secreted from high heavy metal induced-rice roots at maximum rate at 12 : 00−14 : 00. On the other hand, the secretion of amino acids was gradually decreasing from after sunrise (7 : 00) to sunset (21 : 00). Decrease of MAs in roots was due to secretion of MAs from the roots. This characteristic diurnal variation in MAs secretion, which may represent challenging problems on plant photophysiology, has been confirmed repeatedly in growth chamber experiments. We concluded, therefore, the quantity of daily MAs secretion can be determined rapidly with as described in "Materals and Methods".

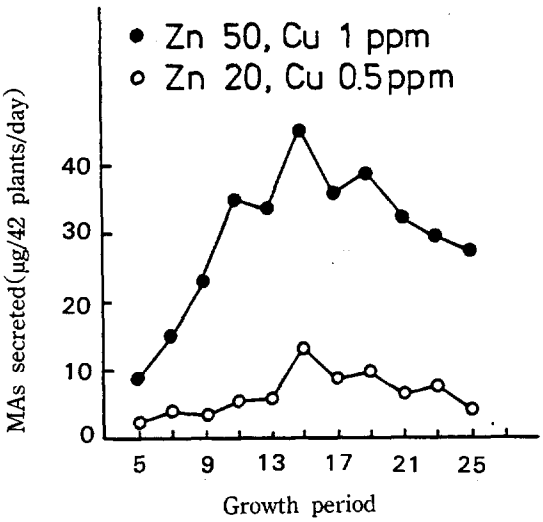


Fig 4. Fluctuation in daily MAs secretion from high heavy metal-induced rice roots.

Fig. 4 illustrates the fluctuation in daily MAs secretion by rice roots influenced by the growth period and heavy metal concentration of the nutrient solution. At higher heavy metal concentration, the amount of MAs secreted daily increased, with an increse in growth period, much more steeply than at lower heavy metal concentration. The amount of daily MAs secretion, however, was gradually decreasing after 15th day. The amount of MAs secreted per day, on the 15th day, reached as much as 45.3 ug/42 plants. Rhythmic variations in daily MAs secretion occurred with an interval of 4 days. These phenomena suggest that MAs secretion from rice roots is controlled by feedback mechanism which might be dependent on intracellular heavy metal level.

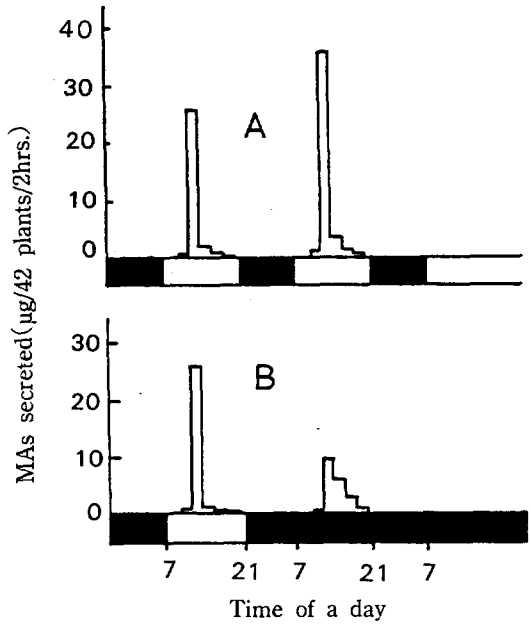


Fig 5. Secretion characteristics of MAs from high heavy metal-induced rice roots under 10h-dark/14h−light (A) and under 10h−dark/14h−light cycle followed by continuous darkness(B)

Fig. 5 illiustrates the effect of continuous darkness on the secretion pattern of MAs from high heavy metal-induced rice roots. The secretion of MAs under normal condition (A) reached maximum in 2−6hr. after sunrise. Under continuous darkness (B), the initiation time of MAs secretion was delayed and the duration of secretion was lengthened. From the 3rd time on the secretion was not observed.

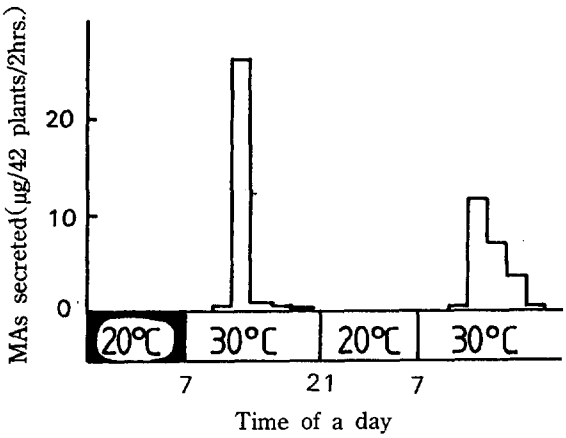


Fig 6. Secretion characteristics of MAs from high heavy metal-induced rice roots under continuous light condition.

Fig. 6 illustrates the effect of continuous light condition with temperature variation on the secretion pattern of MAs from high heavy metal-induced rice roots. Under continuous light condition with regular temperature alternations (30−20−30℃), the secretion pattern similar to that obtained under normal light/dark cycle was observed.

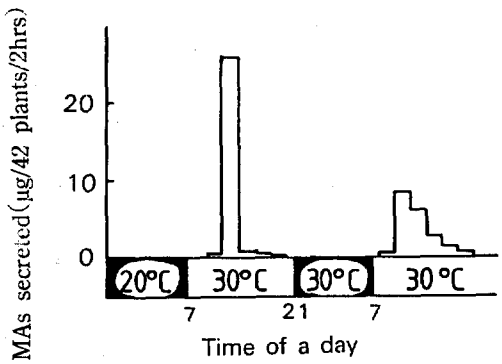


Fig 7. Secretion characteristics of MAs from high heavy metal-induced rice roots under uniform temperature.

Fig. 7 illustrates the effect of uniform temperature with normal photoperiod on the secretion pattern of MAs from high heavy metal-induced rice roots. Under normal photoperiod with uniform temperature, broad secretion peak appeared, indicationg that stimulation of MAs secretion was attributed not only to the light/dark cycle but to the increase in temperature.

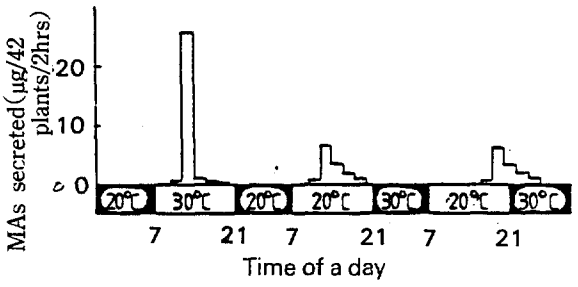


Fig 8. Secretion characteristics of MAs from high heavy metal-induced rice roots Under temperature of day and night reversed.

Fig. 8 illustrates the effect of the temperature of day and night reversed with normal photoperiod on the secretion pattern of MAs from high heavy metal-induced rice roots. Under temperatures of day and night reversed with normal photoperiod, broad secretion peak was also obtained late in the afternoon, indicating that drop in temperature by sunset might be another factor facilitating MAs secretion. These results suggest that the temperature variation may play more important role than the photoperiod in MAs secretion from high heavy metal-induced rice roots.

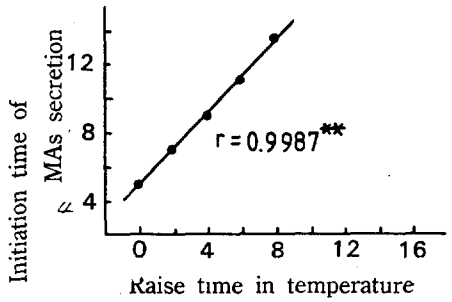


Fig 9. Relation ship between raising time in temperature and initiation time of MAs secretion from high heavy metal-induced rice roots.

Fig. 9 illustrates the effect of rising time in temperature on initiation time of MAs secretion from high heavy metal-induced rice roots. Under normal photoperiod, the initiation time of MAs secretion was positively correlated with advanced rise in temperature (10−17℃) during the dark period.

From the results above, the secretion of MAs from high heavy metal-induced rice roots seems to be controlled depending on time not only by endogenous rhythm in rice plant but external environment such as variations in temperature and photoperiod.

## 要 約

重金屬 過剩 狀態에서 栽培한 水稻 (*Oryza sativa* L. var. Fujiminori)根으로부터 分泌되는 Mugineic acid와 그 類似 Pytosiderophore들의 分泌特性을 調査하였다. 그리들의 迅速 正確한 定量을 위하여 기존의 HPLC method를 수정 보완하였다. 水稻根으로부터 分泌되는 Phytosiderophore는 Mugineic acid와 2'-deoxymugineic acid로 日出後 3時間以後 부터 여러 種類의 Amino acid와 同時에 分泌되었으며 그 分泌量은 日出後부터 日沒時까지 계속 減少하는 傾向이었다. 水稻根으로부터 分泌되는 Phytosiderophore의 量은 約 4日間의 周期로 變化하는 傾向이었으며 重金屬 過剩 培地에 移植한 後 15日째 最高에 달하였다.

重金屬 過剩狀態에서 栽培한 水稻根으로부터 分泌되는 Phytosiderophore들의 分泌 Pattern에는 明暗周期의 交替뿐만 아니라 이에 따른 溫度의 上昇이 주된 影響을 미치고 있으며 溫度條件의 變化가 光周期 條件의 變化보다 더 큰 影響을 미치고 있었다.

Phytosiderophore들의 分泌와 暗期中 氣溫의 上昇 사이에는 有意性 있는 正의 相關이 存在하였다.

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