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Low Molecular Weight Organic Acids in *Brassica pekinensis* Rupr. and Growing soil Influenced by Simulated Nitrate Deposition

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ABSTRACT: We investigated whether carboxylate exudation of *Brassica pekinensis Rupr*. was affected by nitrate deposition from simulated acid rain. A gas chromatographic (GC) analysis was employed for the determination of low molecular weight organic acids (LOA) in rhizosphere soils, bulk soil, roots and leaves of *Brassica pekinensis Rupr*.. Rhizosphere soils were collected after 8 weeks of plant growth by first removing the bulk soil from the root system and then by mechanical move off the rhizosphere soil that adhered to the root surface with soft brush. Soil and plant materials were simultaneously extracted with the mixture of methanol and sulfuric acid (100:7, v/v). Seven organic acids, oxalic, malonic, fumaric, succinic, maleic, L-malic and citric acid were identified and quantified by GC equipped with FID. Oxalic, L-malic, and citric acids were found in both the bulk and rhizosphere soils, while most LOAs were not detected in the control treatment. On the contrary, except maleic acid, all other organic acids were detected in the leaves and roots of cabbages treated with nitrate deposition.

Key Words: *Brassica pekinensis Rupr*., rhizosphere, nitrate deposition root exudates, GC, low-molecular weight organic acid

INTRODUCTION

In soils, organic acids may be derived from vegetal, fungal or microbial sources.

Aliphatic mono-carboxylic acids (formic, acetic, propionic, etc.) and the di- and tri-carboxylic acids, including oxalic, citric, malonic, malic, succinic and tartaric, are commonly found in soil and plant tissue (Fig. 1).[1)] The exudation of low molecular weight organic acids (LOAs) by plant roots can be affected by soil chemical properties, including mineral nutrient deficiency.

LOAs are usually present in root exudates and are part of nutrient mobilization strategy. LOAs play an important role in the solubilization of soil mineral nutrients, e.g. P, Mn, Fe, Cu and Zn. LOAs are recognized to be of major importance in a number of soil and plant processes.[2,3)] In recent years many works show that organic acids have been implicated in a number of soil processes excepting plant nutrition acquisition, including mineral weathering/dissolution, podzolization metal leaching, phytoavailability of metals, phosphate desorption and dissolution, metal adsorption and solubilization reactions, organic pollutants desorption, solubilization and phytoavailability.[4,5)] Now most previous

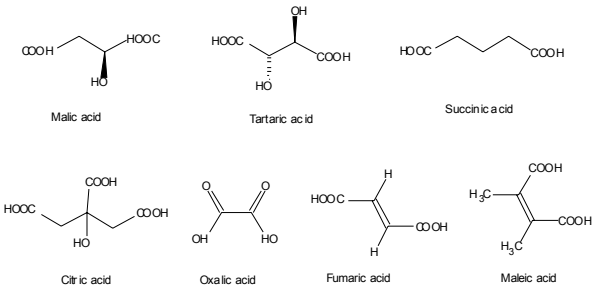

Fig. 1. Chemical structures of low molecular organic acids

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studies have focused on the role of nutrient deficiencies in triggering organic acid release.[6-8)]

There is little information on the effects of acid precipitation on the LOAs, but the importances of LOAs have long been recognized in the plant and soil environment. The direct consequences of potentially acidifying pollutants, especially N deposition, on plants, or soil mediated acidification effects, might cause significant changes is the functioning of exudation of LOAs of roots.

Thus, the purpose of this work was to evaluate the effect of simulated acid rain on the kinds and level of the organic acids in leaf, root, rhizosphere and bulk soil of *Brassica pekinensis Rupr*.. A methodology based on GC-FID of LOAs was applied to determine the proportion of different acid exuded by the roots of cabbage treated with simulated acid rain in pot experiments.

MATERIAL AND METHODS

Pot experiment design

Cabbages were grown in the greenhouse in the experimental farm of Chungnam National University (Daejeon, Korea). The seeds were put into a 50 mL sterile beaker and kept for 30 min in the 30℃ warm water. Afterwards, the seeds were thoroughly rinsed with autoclaved deionised water and kept in the last rinsing water for 1 day. Then the seeds were aseptically transferred to Petri dishes and covered by wet filtrate paper, which were stored in the dark at room temperature. To suppress bacterial infection carbamicillin (60 $\mu l \cdot mL^{-1}$) was added. A sufficient number of germinating seeds occurred within approximately 3 days for cabbage.

The soil was sifted (mesh size 2 mm) before use and loosely packed in freely drained PVC pots (600 mm × 160 mm × 110 mm, L × W × H). Ten even-sized seedlings were selected and transplanted to the pots and grown for 8 weeks under the greenhouse conditions before sampling.

The stock acid solution was prepared by nitric acid. At 14 days after transplanting, different amounts of NO_3^- were gradually supplied as nitric acid solution to the pots at 0, 3 and 24 g NO_3-N m^{-2} yr^{-1} on the basis of local seasonal rainfall, these treatments being designed as T_1, T_2 and T_3, respectively. The working solutions of simulated rain water with pH of 2.5, 3.5 and 5.0 were prepared in the volumetric flasks by diluting the stock solution with deionized water, whereas the treatment T_0 without plant was designed as a control.

Sample preparation

Cabbages were removed from the pots and separated into shoot and root part portion. Plant leaves and stems were carefully cut and collected. Then the roots were removed from the pots, and any

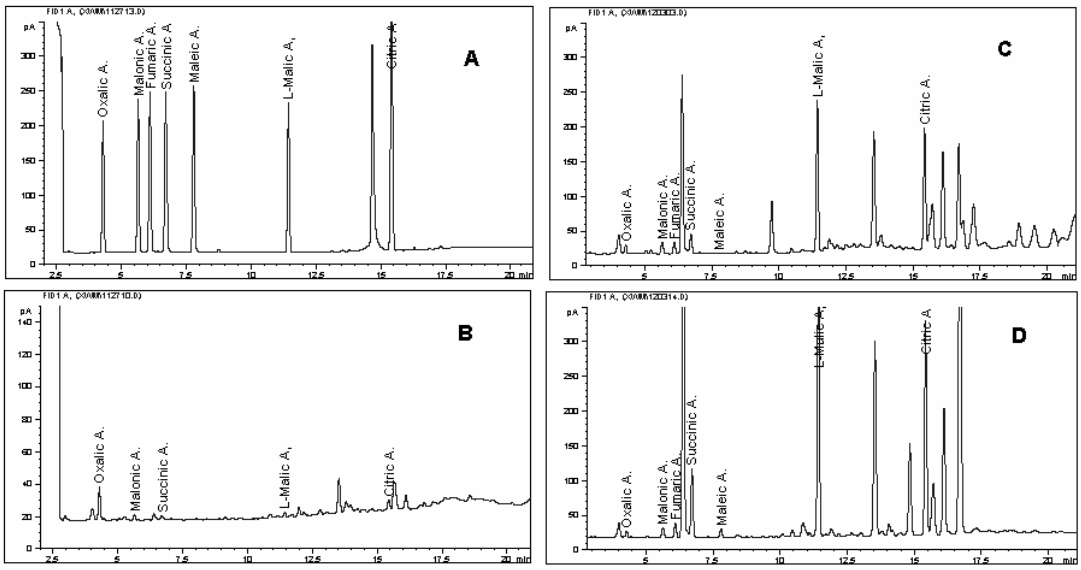

Fig. 2. Gas chromatograms of methylated standard mixtures of organic acids in standard, soil, root and leaf samples. A: Organic acid standards; B: Organic acids in soil; C: Organic acids in root; D: Organic acids in leaf

loosely adhered on the roots was gently shaken off back into the pot. The soil in the pot was mixed, and the sample was taken which represented as the bulk soil. The soil adhered on the roots was carefully removed with a soft brush, and this soil represented as the rhizosphere soil.[9] Samples of the respective plant fractions and rhizosphere and bulk soil were dried (50℃, 48 h), milled, passed through a sieve (<0.5 mm) and then, stored in a deep freezer at -24℃ prior to chemical analysis. The shoot and root portions were dried at 65℃ for five days and used sample for analysis of biomass and some other items.

Frozen samples were thawed. And then, 1g of bulk or rhizosphere soil was extracted. The extract methylated with 9 mL of the mixture of methanol and sulfuric acid (10:7, v:v) for overnight heated at 60℃. After cooling, it was filtered through a Buchner funnel, the filter cake was washed with 5 mL methanol. The filtrate was transferred to a 60 mL separatory funnel. Then, 1 mL of saturated sodium chloride solution and 20 mL of DI water were added. The container was washed with 2 mL of methanol, and the solution was poured into the separatory funnel. Then, 5 mL of chloroform was added.

The mixture was shaken vigorously for 60 seconds and left to stand until the layers separated. The chloroform layer was transferred to a flask. The water layer remained was extracted with another 3 mL of chloroform and combined the chloroform fraction. Then, the chloroform was evaporated with gentle nitrogen gas to neared dry at 40℃, The residue were dissolved in 1 mL of chloroform and injected onto GC. The same pre-treatment method was applied to the plant samples.

Recovery of the extraction and derivatization of LOAs procedure was evaluated in fresh roots, leaves, and rhizosphere soils of cabbage samples and bulk soils. Soil sample (1 g) and plant sample (0.5 g) were analyzed to determine the initial organic acid concentration.

Quantitative determination of LOAs was performed by an Agilent 6890N Series GC equipped with an Alltech AT-WAX capillary column (10 m × 0.53 mm i.d., film thickness 1.2 μm). The injector, column and flame ionization detector temperatures were 250, 80 and 250℃, respectively. Nitrogen gas was used as a carrier gas at a flow rate of 7.1 ML min^{-1} and the constant column and makeup combined flow at 45 ML min^{-1}. Sample was injected with an auto-injector in a splitless mode.

RESULTS AND DISCUSSION

Identification of organic acid

The chromatograms demonstrate that good separations of organic acids can be achieved in a short analysis time by GC-FID method. The seven low molecular weight dicarboxylic acids investigated in this study were identified within 16 min. The target organic acids peaks were identified by comparison with their retention time, which are listed in table 1.

Recovery of acids from the extraction, methylation and purification procedures were examined by spiking the soil, root and leaves with 100μg of target acids. A good recovery and reproducibility were obtained for the fortified LOAs (Table 2). All recoveries of LOAs were above 80% except for L-malic and citric acids. The results of methodology evaluation demonstrate

Table 1. Retention times and linear formulation of organic acid standards measured by gas chromatography

Organic acids	Average RT[1] min	Linear formulation	Correlation
Oxalic acid	4.287	y=3.65x+28.40	0.9997
Malonic acid	5.649	y=0.70x-13.99	0.9999
Fumaric acid	6.096	y=0.36x+5.24	0.9999
Succinic acid	6.713	y=9.66x-23.71	0.9997
Maleic acid	7.783	y=8.94x-11.99	0.9999
L-Malic acid	11.433	y=21.34x-64.17	0.9998
Citric acid	15.417	y=9.77x-254.14	0.9932

[1] Average of 6 replications

that the method developed in this study is accurate and precise. Chen reported that samples analyzed after 1 month of storage in the freezer (-24℃) showed no signs of decomposition.[10)]

Effects of nitrogen deposition and plant on soil pH

The pH of soil samples was measured in distilled water. The pH in rhizosphere soils of cabbage planted was lower than that in bulk soils except T_1 without nitrate deposition (Fig. 3). However, the pH of rhizosphere soils was decreased with the increase of nitrate deposition. On the contrary, the pH of bulk soil was no obvious difference for all three treatments. The pH of T_0 was a little lower than that of other treatments with cabbage.

Effects of nitrogen deposition and cabbage cultivation on LOAs concentration in soil and plant tissue

The effect of nitrate deposition on exudation of organic acids in rhizosphere soil showed that oxalic acid, L-malic acid and citric acid were detected in bulk and rhizosphere soils with cabbage cultivated (Table 3). Oxalic, L-malic and citric acid were also detected. But for T_1 irrigated with nitrate deposition with water, only oxalic and citric acid were found in rhizosphere and bulk soil. Exudation of oxalic and citric acids in rhizosphere were higher than that in bulk soil in T_1 and T_2. On the contrary, all the organic acids in bulk soil of T_3 were higher than that in the rhizoshphere soil.

An overview of low molecular weight organic acids identified in the root and leave tissue of cabbage in the experiment is presented in Fig. 4.

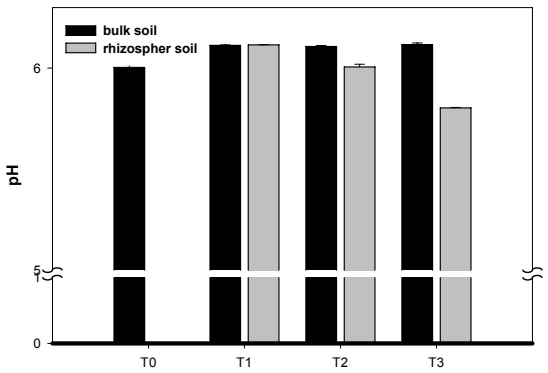

Fig. 3. The pH of bulk and rhizosphere soil (different litters indicate significant difference in pH value within a species, P≤0.05)

Table 2. Recovery of spiked of LOAs of soils, roots and leaves by methanol/ sulfuric acid extraction and methylation as determined by GC-FID

LOAs	Amount in the unspiked samples (μg)		Spiked amount (μg)	Amount in the spiked samples (μg)		Recovery %	
	Repeat 1	Repeat 2		Repeat 1	Repeat 2		
Oxalic	30.04	28.49	391.22	352.36	328.99	82.39	76.81
Malonic	68.87	65.31	2549.72	2239.02	2248.8	85.11	85.64
Fumaric	ND	ND	5373.84	5127.59	4606.9	95.42	85.73
Succinic	6.09	5.771	203.13	204.49	195.8	97.67	93.55
Maleic	ND	ND	218.04	202.07	179.68	92.68	82.41
L-malic	5.33	5.05	84.01	69.31	69.24	76.16	76.41
Citric	42.45	40.25	414.31	364.71	328.62	77.78	69.60

Table 3. Concentration of LOAs in rhizosphere and bulk soil (mg kg^{-1})

LOAs	T_0	T_1		T_2		T_3	
		Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk
Oxalic acid	ND	11.83	3.74	12.21	ND	11.16	34.02
Malonic acid	ND	ND	ND	ND	ND	ND	ND
Fumaric acid	ND	ND	ND	ND	ND	ND	ND
Succinic acid	ND	ND	ND	ND	ND	ND	ND
Maleic acid	ND	ND	ND	ND	ND	ND	ND
L-malic acid	ND	ND	ND	13.41	ND	11.476	14.83
Citric acid	ND	70.23	59.92	95.29	88.99	80.278	96.39

All the seven LOAs were detected in both root and leaf tissues of cabbage (Fig. 4). Fumaric acid was the major organic acid in roots and leaves. Maleic, oxalic and malonic acid were lowest levels in roots and leaves.

In root tissue, except oxalic acid T_1 have the higher concentration for the other kind of organic acids than that in roots of T_2 and T_3. On the contrary, fumaric, L-malic and citric acids in the leaves of T_1 of higher than that of T_2 and T_3 leaves. There were no obvious difference among T_1, T_2 and T_3 in the concentration of oxalic, malonic, succinic and maleic acids in leaves. Compared with the root the concentration of LOAs in the leaves were extremely high. Nitrate deposition probably would not contribute to the amount of LOAs in leave because no obvious increase in all of tested organic acids. But the nitrate deposition have great effect on the LOAs concentration in the root tissues. The total level of LOAs in roots were 12.1, 4.1 and 4.1 g kg^{-1} for T_1, T_2 and T_3, repectively. The total level of LOAs in leave were 56.6, 36.5 and 31.0 g kg^{-1} for T_1, T_2 and T_3, repectively.

The organic acid content of plants is governed primarily by their type of C fixation (e.g., CAM, C_3 or C_4), their nutritional status and age. Typically the total concentration of organic acids in roots is around 1 to 4% of total dry weight. One of the primary factors determining organic acid levels in roots is their degree of cation-anion imbalance. In situations where roots take up an excess of cations (particularly K^+), the negative charge required to balance this is often provided by organic acids, such as malate, malonate, citrate and aconitate.[11,12] Also roots grown on NO_3^- have higher organic acid concentrations than those grown on NH_4^+.[13]

Finally in Table 4 the change of staple characteristics of experimental soil was shown. The reason of the decrease on EC, available P_2O_5 and T-N might be a leaching process by irrigation during experimental periods. And the increase of NO_3^--N in T_0 should be resulted by microbial activity. It is natural that the cabbage cultivation reduced EC, available P_2O_5, T-N as shown in Table 4. Also, the NO_3^--N concentration in cabbage cultivation plots increased by proportional to NO_3^- deposition amount.

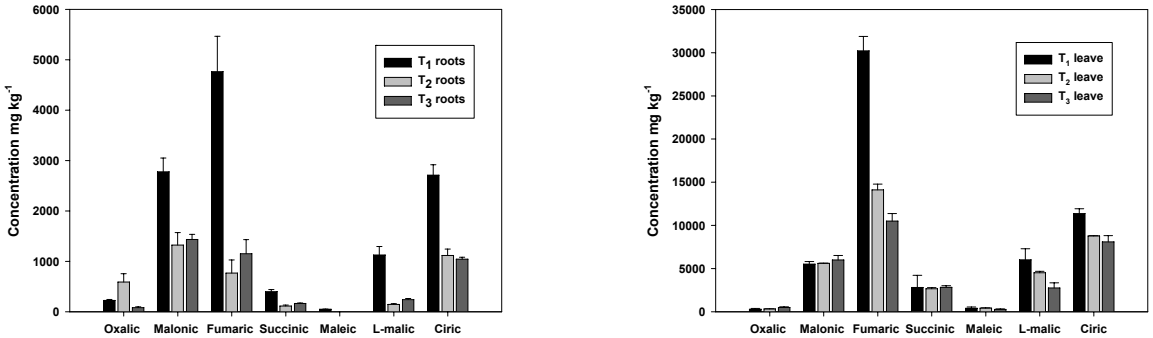

Fig. 4. Total exudation of LOAs from roots and leaves of Chinese cabbage, calculated as mg kg^{-1} dry weight and the percent of each acid exuded by plant. Mean±S.E. (n=3); n.d.=not detectable

Table 4. The change of staple characteristics of soil before and after experiment

Soil	EC	Organic matter	Available P_2O_5	Total-N	NO_3^--N
	mSm^{-1}	gKg^{-1}	$mgKg^{-1}$	gKg^{-1}	$mgKg^{-1}$
Before experiment	7297.5	15.5	742.6	1.27	190.5
After experiment					
T_0	70.0	15.8	536.1	0.70	238.4
T_1	18.5	15.4	464.1	0.56	162.6
T_2	24.0	15.4	441.0	0.45	195.8
T_3	28.5	15.2	402.6	0.67	226.8

Nitrate deposition may be attributed either to a change in the total amount of LOAs in the cabbage tissue of roots and leaves or to change the distribution of different kinds of LOAs.

ACKNOWLEDGEMENTS

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