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Organic Phytotoxic Components in Digested Chicken Manure Effluent and Their Physiological Effects on Germination of Lettuce (*Lactuca sativa* L.)

鸡粪嫌气发酵液毒害植物的有机成分及其对莴苣种子萌发生理影响

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Abstract The results of the gas chromatograph analysis showed that the major organic phytotoxic compounds that were taken out by activated charcoal were isovaleric acid, butyric acid and isobutyric acid, sharing 45%, 35% and 20%, respectively. Bioassays were performed to identify the effects of the charcoal extracts of anaerobically digested chicken manure effluent (ADCME) and the three volatile fatty acids as well as their mixture on seed germination, radical growth and biochemical activity in the seedlings of lettuce (*Lactuca sativa* L.). All three fatty acids and ADCME extracts strongly inhibited seed germination, radical elongation and reduced the fresh weight of lettuce seedlings. Biochemical analysis showed that the fatty acids and ADCME extract retarded reusing the protein in lettuce seed, which resulted in the significant inhibitions in activities of Mg^{2+}-ATPase, Ca^{2+}-ATPase, polyphenol oxidase and peroxidase, and in inorganic Pi that might be released by ATPase activity. Among the three fatty acids, the strongest effect was found in isovaleric acid treatment, followed by butyric acid treatment, and synergistic effects were observed in the organic mixture treatment.

Key words lettuce, *Lactuca sativa* L., anaerobically digested chicken manure effluent (ADCME), germination, growth, metabolism, phytotoxicity, fatty acid

摘要 气相色谱分析的结果表明,由活性碳分离出来的鸡粪嫌气发酵液(ADCME)对植物产生毒害的主要有机化合物是异戊酸、丁酸和异丁酸,分别占45%、35%和20%。用生物分析来鉴定ADCME的活性碳提取物和这三种挥发性脂肪酸及其混合物对莴苣(*Lactuca sativa* L.)种子萌发、根芽生长和幼苗中的生化活动的效应。生化分析表明这些脂肪酸和ADCME提取物均抑制莴苣贮藏蛋白质的重新利用,导致其对 Mg^{2+}-ATP酶、Ca^{2+}-ATP酶、多酚氧化酶和过氧化物酶活性以及ATP酶的作用产物之一的无机 P_i 的释放都受到显著的抑制。这三种脂肪酸中,异戊酸处理的抑制作用最强,丁酸处理次之,并观察到有机混合物处理具有协和效应。

关键词 莴苣 鸡粪嫌气发酵液 萌发 生长 代谢 (植物)毒性 脂肪酸
中图法分类号 S 636.2; S 141.3; S 831.

Anaerobic digestion of livestock wastes is increasingly practiced by farmers to deodorize animal manures and generate methane gas, which can be used to generate heat and electricity. Because water has to be added to animal manures to facilitate digestion, farmers using anaerobic digesters have to dispose significant quantities of liquid effluent. The liquid effluent appears to be suitable as a fertilizer material, as it contains significant quantities of plant nutrients. However, besides inorganic toxic elements that could be regulated by suitable dilution, the effluent also contains potentially toxic levels of organic substances that are detrimental to plant growth, which can be partly taken out by activated charcoal treatment[1]. This paper reports the identified organic components that were taken out by activated charcoal from raw anaerobically digested chicken manure effluent (ADCME), and their effects on seed germination, seedling growth and metabolism of lettuce (*Lactuca sativa* L.).

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1 Materials and methods

1.1 Charcoal filtration of ADCME

The original ADCME, which was produced by the process described by Yang et al[2], was filtered with 6 sheets of cheese cloth. Then concentrated sulfic acid was added into ADCME while stirring until the pH reached to about 5.5. The effluent then was filtered by sand. And about 6 liters of the sand filtered ADCME was filtered with about 2 kg 0.5 mm to 0.85 mm activated charcoal[1]. The ADCME treated charcoal was washed by deionized water until the eluat became clear, and kept in plastic bottles and stored at 0℃.

1.2 Elution and identification of components

About 1 000 ml wet ADCME treated charcoal (365.5 g in dry weight) and 1 000 ml methanol were mixed in flask. The mixture was stirred and covered and kept overnight. The charcoal was washed 4 times with 500 ml, 100 ml, 100 ml and 200 ml methanol, respectively. All the methanol eluats were mixed and condensed to 330 ml in a rotary evaporator at 50℃. This product was defined as "methanol eluat". The methanol eluat 180 ml was extracted 3 times with 180 ml, 100 ml, and 50 ml methylene chloride respectively. The residue was defined as "aqueous residue". The methylene chloride extract were mixed and condensed to 90 ml in a rotary evaporator at 40℃, and further dehydrated with anhydrous sodium sulphate, and the final product was defined as "methylene chloride extract". Pure charcoal (468.2 g) treated methanol (1 000 ml) was used as the control following the same procedure as above. The methylene chloride extract was used for identification of volatile organic components on a computer-controlling gas chromatograph (GC).

1.3 Bioassay

For ADCME extract, certain amounts of different fractions were added into a 55 mm diameter Whatman No. 1 filter paper disk in a 55 Petri dish, and 1 ml deionized water was added as soon as the paper had been dried. Then 30 lettuce seeds (*Lactuca sativa* L., cv. Anuenue) were placed in each Petri dish, covered and sealed with Parafilm, and incubated in the dark at 25℃.

As the results of GC analysis, the major components in ADCME extracts are volatile fatty acids. So bioassays for the effects of organic acids on lettuce were also studied. So-called "organic acid mixture" in the context consisted of 45% isovaleric acid, 35% butyric acid and 20% isobutyric acid, in accord with their proportion in ADCME extract.

Time-dependent seed germination bioassay was conducted with 1 ml deionized water instead of chemicals.

1.4 Biochemical analysis

The three-day old lettuce radicals were used for biochemical analyses. Enzyme extraction was conducted with a mortar and pestle at 0℃ in an ice-bath. Samples was homogenized with 0.05 mol/L Na-phosphate buffer (pH 6.8). The homogenate was centrifuged at 10 000 r/min at 0℃ to 8℃ for 15 min, and the supernatant was used to determine the activities of polyphenol oxidase and peroxidase. The two enzymes were assayed at 25℃ in 3 ml reaction solutions. The activity of olyphenol oxidase was determined as described by Benjamin and Montgomery[3] in a reaction mixture containing 10 mmol/L catachol, 0.05 mol/L Na-phosphate (pH 6.8) and 0.02 ml enzyme extract. Changes of absorption were measured at 398 nm. The activity of peroxidase was determined at 470 nm[4] in a mixture including 0.5% guaiacol, 0.6% H_2O_2, 0.05 mol/L Na-phosphate (pH 6.8) and 0.1 ml enzyme extract.

Samples for ATPase activity assay was prepared the same as above except 0.05 mol/L Tris-HCl buffer (pH 7.5) containing 5 mmol/L DTT and 2 mmol/L EDTA was used. The supernatant was used as the source of Mg^{2+}-ATPase and Ca^{2+}-ATPase. Both enzymes were assayed as described by Li[5].

Protein content in the enzyme extract was determined by the method of Bradford[6].

2 Results

2.1 Identification of major components in ADCME extract

The results of GC analysis showed that the major components in ADCME extract were isovaleric acid, butyric acid and isobutyric acid, which made up 41% to 43%, 32% and 18% to 19% of the total components, respectively. The proportions of the three components were about 45%, 35% and 20% among

Table 1 Effects of ADCME extract on seed germination and radical growth of lettuce*

Charcoal extract of ADCME		Seed germination			Radical length at three days
		One day	Two days	Three days	
Methanol eluat	0.5 ml	96.63[a]	98.89[a]	98.89[a]	53.30[a]
	1.0 ml	85.23[a]	95.45[a]	95.45[a]	48.41[b]
	1.5 ml	48.27[b]	95.45[a]	95.45[a]	16.79[c]
	2.0 ml	7.06[c]	7.06[b]	7.06[b]	1.08[d]
Methylene chloride extract	0.5 ml	60.24[a]	95.45[a]	95.00[a]	17.15[a]
	1.0 ml	0[b]	0[b]	5.62[b]	2.87[b]
	1.5 ml	0[b]	0[b]	1.12[b]	0.10[b]
	2.0 ml	0[b]	0[b]	6.74[b]	1.13[b]
Aqueous residue	0.5 ml		95.56[a]	95.56[a]	73.64[a]
	1.0 ml		95.56[a]	95.56[a]	44.90[b]
	1.5 ml		98.13[a]	98.13[a]	6.66[c]
	2.0 ml		59.55[b]	59.55[b]	1.88[c]

* Data are percentage (%) of the controls. Data among each fraction in each column followed by the same letter are not significantly different at Duncan's 5% levels, and the same in the other tables.

Table 2 Effects of ADCME extracts and organic acids on seed germination and radical growth of lettuce*

Treatment	Germination(G)(%)	Fresh weight (FW)(mg/plant)	Radical length(cm)	Vigor idex(G× FW)
0.05% isobutyric acid	66.67^{c}	3.06^{e}	0.11^{c}	204^{d}
0.05% butyric acid	71.11^{c}	4.04^{e}	0.28^{c}	287^{e}
0.05% isovaleric acid	18.89^{d}	1.98^{f}	0.04^{c}	37^{e}
0.05% organic acid mixture	11.11^{e}	1.88^{f}	0.02^{c}	21^{e}
Water control	97.78^{a}	10.17^{b}	3.07^{b}	994^{a}
Methanol eluat of ADCME extract	87.78^{b}	4.56^{c}	0.36^{c}	400^{b}
Control for ADCME extract	98.98^{a}	10.60^{a}	3.42^{a}	1049^{a}

* Data for germination are from the investigations 1 day, others 3 days after treatment.

Table 3 Enzyme activities in lettuce seedlings sampled 3 days after treatment*

Treatment	Mg^{2+} -ATPase	Ca^{2+} -ATPase	Polyphenol Oxidase	Peroxidase
0.05% isobutyric acid	10.55^{e}	8.10^{e}	5.44^{d}	0^{e}
0.05% butyric acid	12.99^{d}	13.60^{d}	12.35^{b}	0.63^{d}
0.05% isovaleric acid	6.58^{f}	5.26^{f}	2.05^{e}	0^{e}
0.05% organic acid mixture	4.62^{g}	3.71^{g}	1.57^{e}	0^{e}
Water control	29.09^{a}	23.71^{b}	15.52^{a}	1.03^{b}
Methanol eluat of ADCME extract	15.56^{c}	17.62^{c}	10.59^{c}	0.78^{c}
Control for ADCME extract	24.96^{b}	25.06^{a}	13.12^{b}	1.13^{a}

* Data are specific activities of ATPase (μmol Pi/mg Pro. h), polyphenol oxidase (ΔA_{398}/mg Pro. min) and peroxidase (ΔA_{470}/mg Pro. min), respectively.

them. Total of other components such as propionic acid, valeric acid and hexanoic acid, only occupied 9% or less.

2.2 Effects of ADCME extract and organic acids on seed germination and radical growth

The three fractions of ADCME extract, i.e. methanol eluat, methylene chloride and aqueous residue significantly inhibited seed germination and radical elongation of lettuce (Table 1). Similarly, isobutyric acid, butyric acid and isovaleric acid also inhibited the seed germination and radical elongation, and isovaleric acid had the strongest effect among the three acids, and the acid mixture even showed a stronger effect (Table 2). As the rate of acid increased, the effects became more and more detrimental (Table 1). While without chemical treatment, the fresh weight and radical length of lettuce seedlings kept to increase until sixth days (data not shown).

2.3 Effects of ADCME extract and organic acids on enzyme activity

Normally, activities of Mg^{2+} -ATPase and Ca^{2+} -ATPase in lettuce seeds were very low, and those in the seedlings increased gradually within 6 days (data not shown). Activity of polyphenol oxidase was also very low in the seeds, and began to increase sharply from 1 to 3 days, and slowly since 3 days (data not shown). Activity of peroxidase was indetectable before two days, and increased slowly from 2 to 4 days, but no further increase was observed then (data not shown). The treatments with isobutyric acid, butyric acid, isovaleric acid, organic acid mixture and methanol eluat of ADCME extract inhibited the activities of Mg^{2+} -ATPase, Ca^{2+} -ATPase, polyphenol oxidase and peroxidase in lettuce seedlings. In general, isovaleric acid treatment showed the strongest effects as compared with other two individual acids, but the acid mixture showed even stronger effects (Table 3).

2.4 Effects of ADCME extract and organic acids on soluble protein and Pi

In lettuce seeds, soluble protein content is rather high, that is, about 320μg each seed. It decreased to about 260μg each plant in the first day, and continued to decrease gradually with the germination and radical growth until the fourth day when it went down to about 175μg each plant. In the present study, the treatments with organic acids or ADCME extract showed much higher soluble protein content in the radicals than the controls as measured 3 days after treatment (Table 4). In the first day of lettuce radical growth, the inorganic Pi content in the seedlings was as low as the seeds, which was below 0.2μg each seed

Table 4 Contents of soluble protein extracted with Tris-HCl buffer and Pi in lettuce seedlings sampled 3 days after treatment

Treatment	Soluble protein (μg/plant)	Pi (g/plant)
0.05% isobutyric acid	220.7^{c}	2.48^{e}
0.05% butyric acid	224.2^{c}	3.78^{d}
0.05% isovaleric acid	281.9^{a}	2.02^{e}
0.05% organic acid mixture	252.0^{b}	1.80^{e}
Water control	140.1^{e}	9.36^{a}
Methanol eluat of ADCME extract	195.0^{d}	5.26^{c}
Control for ADCME extract	150.8^{e}	7.97^{b}

or plant. It increased rapidly later, and it was about 4.5μg, 8.0μg, 10.8μg and 13.2μg each plant for the second, third, fourth and seventh day, respectively. In the present study, Pi content in the seedlings was much lower in the treatments with organic acids or ADCME extract than in the controls as measured 3 days after treatment (Table 4).

3 Discussion

Our previous investigation showed the charcoal treated ADCME could improve the growth of plant root system as compared with raw ADCME treatment[1]. In the present study, we further found that these phytotoxic substances taken out by activated charcoal are volatile organic acids, mainly isovaleric acid, butyric acid and isobutyric acid. All these compounds are water soluble, and they existed in every eluat fraction that showed strong toxicity to lettuce seed germination and radical growth. In the present study, among the three major components, isovaleric acid was the most detrimental, followed by butyric acid. Several researchers[7~11] reported that effects of individual phenol acids in mixtures may be synergistic, additive or antagonistic. In the present study, the effects of isovaleric acid, butyric acid and isobutyric acid were obviously synergistic.

In lettuce seeds, enzyme activities and inorganic Pi content are very low but soluble protein content is rather high. During germination and seedling growth, the soluble protein could be reused for synthesis of new protein such as enzymes and other nitrides for plant tissue structure and metabolism. The present study provides with the evidences that isovaleric acid, butyric acid and isobutyric acid as well as ADCME extracts severely retarded reusing the soluble protein, which resulted in strong inhibition of the increases in activities of Mg^{2+}-ATPase, Ca^{2+}-ATPase, polyphenol oxidase and peroxidase, and finally severe retardation of the release of inorganic Pi. In result, the retardation of seed germination and seedling growth would be inevitable. Previous studies also reported the inhibition of other allelochemicals on enzyme activity in plants such as phenolic compounds on ATPase that might have resulted in the inhibition of K^+ and Cl^- absorption in excised oat roots[12,13], flavones and flavone glycosides on mitochondrial ATPase[14], root secretions of *Lupinuus album* and *Zea mays* on catalase and peroxidase in two weed species, *Chenopodium album* and *Amaranthus retroflexus*, and tannins on polyphenol oxidase and other enzymes[15]. As the major organic phytotoxic substances in ADCME are volatile fatty acids, which may be degraded by continuing fermentation[16], the ADCME which is thoroughly fermented could be a good fertilizer in plant production.

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