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ORIGINAL ARTICLE

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Effect of low molecular weight lignin fragments including oxalic acid in alkaline-oxygen stage waste liquor on Al toxicity

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Abstract Our previous study indicated the existence of some low molecular weight contaminants in separated fractions (F1 to F4) of alkaline-oxygen stage waste liquor by spectroscopic analysis. In the present study, the quantities of these compounds were determined by capillary electropheresis (CE). Substantial amounts of oxalate and acetate were found in F3. The complexes between Al and oxalate as well as F3 were characterized by ^{27}Al-nuclear magnetic resonance (NMR) spectroscopy, and the spectra of Al-oxalate complexes demonstrated the ability of oxalate to chelate Al and to produce different forms of complexes at varied molar ratios. Plant growth experiments in the presence of Al-oxalate complexes suggested that at a proper range, oxalate has a favorable effect on the detoxification of Al toxicity. It can be assumed that oxalate in F3 also plays an important role in the efficiency of removing Al toxicity. ^{27}Al-NMR was proved to be a useful method for the study of complexes between Al and organic compounds without disturbing their equilibrium conditions.

Key words Alkaline-oxygen delignification stage waste liquor · Al toxicity · Soil-conditioning agent · Oxalic acid · ^{27}Al-NMR

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Introduction

Acid soil characteristically contains a large amount of soluble Al which exists mainly in the trivalent form at low pH.[1] It is important to note that poor root development and drought susceptibility observed in acid soil seem to be primarily due to the toxicity of Al. Because of Al toxicity, rooting depth and the degree of root branching of plants are known to be limited.[2,3] The seriousness of soil acidity and Al toxicity has attracted much attention in recent years, because acid soils are prevalently distributed in tropical regions where people in many developing countries heavily rely on seasonal crops for survival.[4]

In our previous article, four different lignin fractions with different molecular weights were prepared by gel filtration chromatography (GFC). The fractions were found to be capable of alleviating Al toxicity and improving the performance of radish root growth.[5] Therefore, it seemed to be promising to use those lignin fragments as soil-conditioning agents from the standpoint of better utilization of waste liquor from pulp and paper production. However, separated fractions showed different performance in removing Al toxicity. In particular, in the case of fraction 3(F3), the alleviating efficiency decreased with higher dosages. The peculiar behavior of F3 may indicate the existence of some contaminants that have negative effects on plant growth.

In this study, capillary electrophoresis (CE) was applied to determine the contaminants in each separated fraction. CE is a versatile and powerful tool with high separation efficiency and selectivity, and is particularly suitable for the analysis of charged species like inorganic and organic cations and anions.[6] In addition, concerning the detoxifying mechanism of the fractionated waste liquors, Al complexes were characterized by ^{27}Al nuclear magnetic resonance (NMR), which can provide a rapid, direct, and nondestructive analysis of Al.[7–10]

The objectives of this research were to further discuss the different performances of the separated fractions, with special attention to the behavior of F3, and to characterize

the nature of Al complexes in terms of their effects on plant root growth.

Experimental

Preparation of lignin fractions of alkaline-oxygen stage waste liquor

Alkaline-oxygen stage waste liquor was subjected to GFC of Sephadex G-25. Four fractions (F1 to F4) of lignin fragments with different molecular weights, as well as properties, were obtained: a relatively high molecular weight fraction (F1) and three relatively low molecular weight fractions (F2, F3, F4).

Determination of low molecular weight contaminants in F1 to F4

Contaminants in each of the fractions (F1 to F4) were determined by the use of a capillary electrophoretic instrument (CE, Hewlett Packard HP), a relatively new and highly efficient analytical technique,[11] with an 80.5-cm-long untreated fused silica capillary column (i.d. 75 μm) and ultraviolet (UV) detector. Separation was performed at 20°C and 25 kV using a buffer solution of 2,6-pyridinedicarboxylic acid and cetyltrimethylammonium bromide, which can provide a high background absorbance, because most small organic compounds do not absorb in the UV region. The samples were injected with a pressure of 100 mbar. Each sample was analyzed five times.

The calibration curve for organic anions, such as oxalate, formate, and acetate, were linear over the range of 5 ppm to 50 ppm with correlation coefficients higher than 0.9900. The reproducibility for migration time and peak areas as shown by the relative standard deviation (RSD) were found to be less than 1% and 5%, respectively.

Effect of oxalate and Al-oxalate complexes on plant growth

Oxalate and Al-oxalate complexes with different molar ratios of Al to oxalate were examined in terms of the effect on root elongation of radish after 3 days of cultivation. Oxalate solutions with concentrations of 30 μM, 50 μM, 100 μM, and 200 μM were prepared from 250 mM stock solution, and were adjusted to pH 4.8. Solutions of Al-oxalate complexes were prepared by adding 0.5 ml of 25 mM $AlCl_3$ and different amounts of sodium oxalate solution (pH 4.8) to the culture solution at molar ratios of 1:0.5, 1:1, 1:2, 1:3, 1:4, 1:6, and 1:10. The solutions were adjusted to pH 4.8 with dilute HCl or KOH solution before the plant growth experiments, which were conducted as described in the previous report.[5]

Pretreatment of F3 with $CaCl_2$

$CaCl_2$ was added dropwise to the F3 solution, and the solution was allowed to stand for 2 h. The calcium oxalate precipitate was removed by filtration. The clear filtrate thus obtained was examined by CE to confirm that oxalate was removed, and was then used in the plant growth experiment after adjusting the pH to 4.8.

^{13}C-NMR spectroscopic measurement

^{13}C-NMR spectra were recorded on a Jeol JNM-A500 FT-NMR system in 5-mm (o.d.) NMR tubes and with 1,4-dioxane as an internal standard. The measurement parameters were as follows: observed frequency 125.65 MHz, acquisition time 0.57 s, pulse width 4.70 μs.

^{27}Al-NMR spectroscopic measurement

^{27}Al-NMR, which can directly measure different forms of Al without disturbing the chemical equilibrium of the system, was applied to examine the Al species, as well as Al concentration in the sample solutions. The ^{27}Al-NMR spectra were recorded on a Jeol JNM-A500 FT-NMR system. The measurement parameters were as follows: observed frequency 130.20 MHz, acquisition time 0.039 s, pulse width 11.05 μs. According to the method described by Komatsu and Kubota,[12] potassium hexathiocyanatoaluminate (III) ($K_3[Al(SCN)_6]$) was prepared and used as its acetone-d_6 solution in a 3-mm (o.d.) capillary as the reference (−33.4 ppm) and for quantification of signals of interest.

All spectra were obtained under identical conditions, and ^{27}Al-NMR measurements were replicated three times for each sample.

Preparation of Al-complex solutions for Al-NMR measurements

Stock solutions of Al and oxalate (pH 4.8) of 100 mM were prepared. To prepare a calibration curve, 1 mM, 2.5 mM, 5 mM, and 12.5 mM Al solutions were prepared from 100 mM Al stock solution and the pH was adjusted to 3 and 4 separately for each solution. The different forms of Al in Al-oxalate complexes were quantified based on the calibration curve. Two sets of complex solutions were prepared according to the following procedure.

For the first set of the Al-complex solutions, to a solution containing 250 μmol Al, appropriate volumes of the oxalate stock solution were added to give different molar ratios (Al/oxalate) of 1:1, 1:2, 1:3, and 1:4. After allowing the complex solutions to stand for 2 h, the pH was adjusted to 3 and 4 separately with stirring. The resulting solutions were kept overnight at room temperature to ensure that the solution had reached equilibrium. Then pH of the solution was checked again and the Al concentration was measured by ^{27}Al-NMR. A similar procedure was applied for another set

Table 1. Content of low molecular weight compounds in each fraction

Fraction	Oxalate	Acetate	Formate	Glycolate	NaCl	Na_2SO_4
F1					2.80	1.25
F2	0.49		0.08		1.83	3.64
F3	15.78	29.66	0.12	2.70	0.63	9.20
F4	1.22		0.68		94.69	0.23

Content expressed as mmol/10 g dry matter

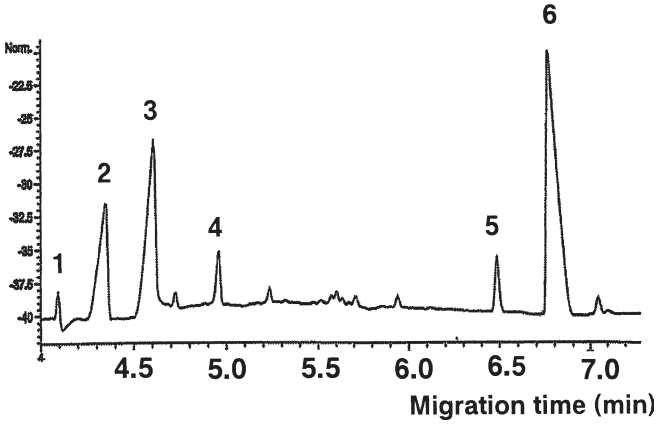

Fig. 1. Electrophoregram of F3. *1*, Cl^-; *2*, SO_4^{2-}; *3*, oxalate; *4*, formate; *5*, glycolate; *6*, acetate

Table 2. Acidic groups content in each fraction

Sample	Strongly acidic group (A)[a]	Weakly acidic group (I)[b]	Weakly acidic group (II)[c]	Total
F1		0.39	0.29	0.68
F2	0.502	1.024	0.288	1.81
F3	0.650	1.64	0.230	2.52
F4	0.253	2.5		2.78

Content expressed as mol/200 g organic matter
[a] pK_a: 2.21 to 2.36
[b] pK_a: 4.20 to 5.02
[c] pK_a: 6.18 to 9.61

of complex solutions containing 25 μmol Al with Al to oxalate molar ratios of 1:0.5, 1:0.6, 1:0.7, 1:0.8, 1:0.9, and 1:1.

Two sets of sample solutions containing different amounts of F3 or low molecular weight organic compounds were also prepared; the first set of sample solutions contained 25 μmol Al and 13.70 mg, 27.39 mg, or 41.09 mg of F3, and the second set of sample solutions contained 25 μmol Al and acetate, formate, at molar ratios of 1:1, 1:2, and 1:3 and the equimolar mixture of acetate, formate, and oxalate. All sample solutions were adjusted to pH 4.

Results and discussion

Determination of low molecular weight contaminants by CE in separated fractions

In the previous study, ^{13}C-NMR, ^{1}H-NMR, and FT-IR spectra of each separated fraction of alkaline-oxygen stage waste liquor indicated the existence of some low molecular weight organic compounds, namely oxalate.[5] The existence of low molecular weight organic compounds was further examined and four kinds of anions, oxalate, formate, acetate, and glycolate, were determined by CE in this study. At the same time, the inorganic anions Cl^-, SO_4^{2-} were also determined. Figure 1 shows the electrophoregram of F3, which shows separation based on the electrophoretic mobilities of anions. Anions with highest charge/mass ratio migrate first (such as oxalate), followed by the anions with reduced ratios (such as formate, glycolate, acetate).[13] These anions were confirmed by adding authentic compounds to the sample solution, and were quantified by the use of calibration curves and are summarized in Table 1.

As shown in Table 1, F3 contained substantial quantities of oxalate and acetate, which may be the result of degradation of lignin during alkaline-oxygen treatment. A certain amount of oxalate was also found in F2 and F4. Formate was found in F2, F3, and F4, of which F4 showed the highest content. However, these low molecular weight organic compounds were not found in F1.

In addition, Cl^- and SO_4^{2-}, which probably originated from kraft pulping or bleaching chemicals, were found in all fractions. Sodium sulfate was mainly found in F3, much less was found in F1 and F2, whereas sodium chloride was found mostly in F4, and some amounts in F1, F2, and F3. It is important to note that the effect of these inorganic salts on the plant growth experiments at the confirmed levels of concentration was found to be negligible.

Because of the presence of a considerable amount of inorganic salts, the levels of acidic groups obtained by potentiometric and conductrometric titrations in our previous work[5] were recalculated based on the organic matter and are shown in Table 2. It is obvious that the lower molecular weight fraction contains a higher amount of acidic groups, indicating a higher level of oxidation. These results are in good agreement with the contents of low molecular weight compounds found in F1 to F4 by CE. These low molecular weight organic compounds could be responsible to the performance of each fraction in alleviating Al toxicity, because they were reported to form complexes with Al. Oxalate has been identified as a common and strong Al chelator,[14] whereas formate and acetate are weak.[15–17] Chelating behaviors of low molecular weight contaminants are discussed in the following section.

Table 3. Concentration of different forms of Al in Al–oxalate complex (mM)

Chemical shift (ppm)	Molar ratio of Al to oxalate				
	1:0.5	1:1	1:2	1:3	1:4
−0.032[a]	7.86	1.89			
6.213[b]	4.57	8.59	1.12		
11.481[b]		2.13	8.07	1.95	1.00
16.165[b]		0	3.08	10.56	11.39
Total	12.43	12.62	12.27	12.51	12.39

Concentration of original Al: 12.5 mM, pH of complex solution is 3
[a] Al^{3+}
[b] Al in Al–oxalate complexes

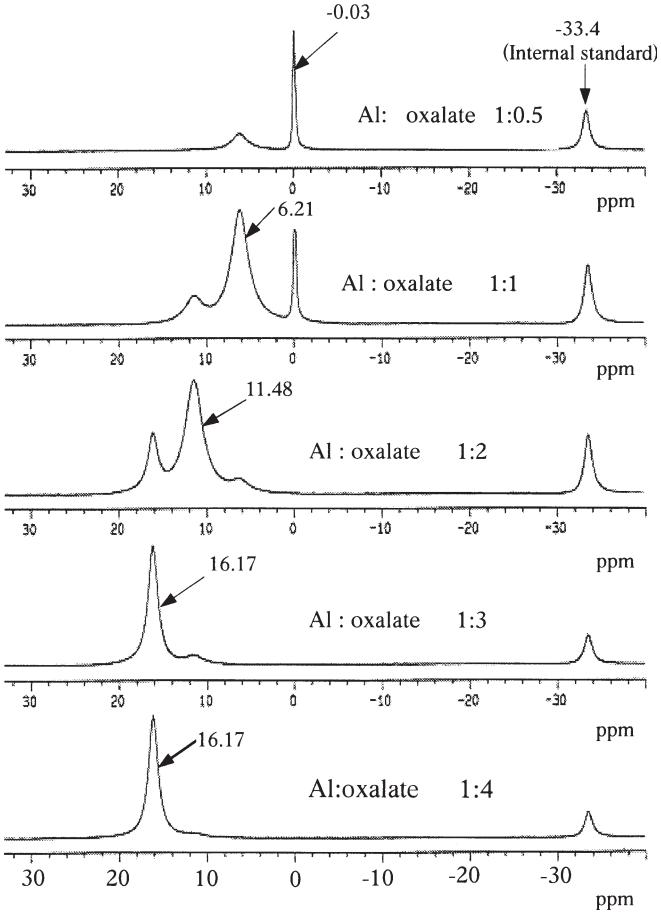

Fig. 2. ^{27}Al nuclear magnetic resonance (NMR) spectra of Al and oxalate at various molar ratios

^{27}Al-NMR spectra of Al complexes

^{27}Al-NMR spectra of Al-oxalate complexes and the concentrations of different forms of Al in complexes at varied molar ratios are shown in Fig. 2 and Table 3, respectively. Several forms of Al can be distinguished in Fig. 2. A signal at a chemical shift of −0.032 ppm is attributed to the monomer cation of Al, which is Al^{3+}. A signal at 6.213 ppm could be assigned to a complex of $Al(OX)^{+2}$, where OX designates a ligand molecule. When the Al–oxalate molar ratio increased to 1:1, the intensity of the Al^{3+} signal decreased, and the signal at 6.213 ppm increased. If Al–oxalate molar ratio was further increased to 1:2, the monomer signal of Al disappeared and another two signals appeared at 11.481 ppm and 16.165 ppm, and a signal at 11.481 ppm, which may be assigned to $Al(OX)_2^{+1}$, showed higher intensity. Further increase of Al–oxalate molar ratio to 1:3 resulted in higher intensity of a signal at 16.165 ppm, which should correspond to another type of complex. The ^{27}Al-NMR spectra of Al-oxalate complex at different Al–oxalate molar ratios showed that Al may exhibit different forms. The formation of complexes of acetate and formate with Al at pH 4 was also observed by ^{27}Al-NMR. Although the complex of Al with formate at a molar ratio of 1:3 could be observed at 4.066 ppm, 80% of Al remained uncomplexed. A similar situation was also found for acetate. As shown in Table 4, 70.4% of Al remained uncomplexed at an Al–acetate molar ratio of 1:2. Complexes of Al with formate and acetate at a molar ratio of 1:1 were also confirmed by ^{27}Al-NMR, but the signals were extremely weak. Furthermore, the ^{27}Al-NMR spectrum of Al with an equimolar mixture of oxalate, acetate, and formate was almost the same as the spectrum of Al with oxalate at the same molar ratio. This indicates that oxalate was much more reactive toward binding Al than acetate or formate. This should be the reason why the signals of complexed Al with acetate and formate are not observed on the ^{27}Al-NMR spectra of Al with F3.

^{27}Al-NMR spectra of Al in the presence of F3 showed signals at exactly the same chemical shifts as those of the complex of Al with oxalate indicating the presence of oxalate or other similar ligand in F3.

Table 4. Concentration of different forms of Al in Al–acetate and Al–formate complexes (mM)

Chemical shift (ppm)	Molar ratio of Al to acid	
	1:2 (acetate)	1:3 (formate)
−0.032	8.86	9.96
3.676	3.72	
4.066		2.51
Total	12.58	12.47

Concentration of original Al: 12.5 mM, pH of complex solution is 4

Quantification of oxalate in F3 by ^{27}Al-NMR

Quantitative estimation of Al presenting as different forms in the Al–oxalate system was conducted by integrating each signal in the ^{27}Al-NMR spectrum (Table 3). Based on the calibration curve, the complex of Al and the ligand in F3 was assumed to be 1:0.82 when the concentration of Al was 25 mM and 13.70 mg of F3 in 5 ml was used, if the ligand in F3 is predominantly oxalate. The amount of oxalate estimated by this method was consistent with the data determined by CE. Therefore, ^{27}Al-NMR measurement should be a useful method for quantifying free or complexed Al in a sample solution without disturbing its equilibrium system.

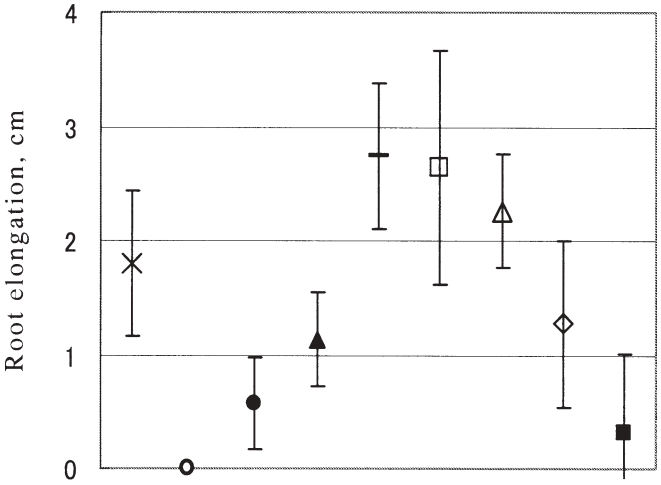

Fig. 3. Effect of Al-oxalate molar ratio on root elongation. *Cross*, control (no Al, no oxalate); *open circle*, Al (no oxalate); *filled circle*, Al:oxalate 1:0.5; *filled triangle*, Al:oxalate 1:1; *minus sign*, Al:oxalate 1:2; *open square*, Al:oxalate 1:3; *open triangle*, Al:oxalate 1:4; *diamond*, Al:oxalate 1:6; *filled square*, Al:oxalate 1:15

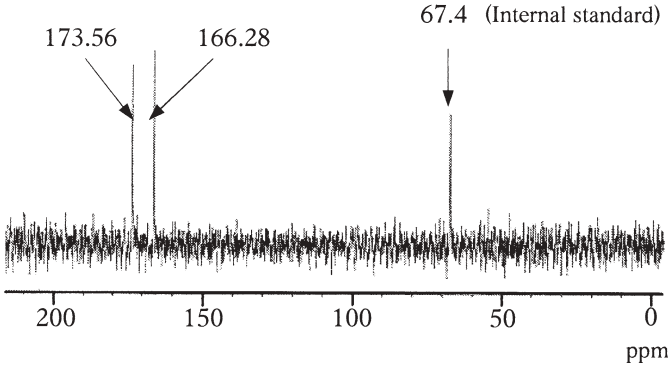

Fig. 4. ^{13}C-NMR spectrum of Al–oxalate complex at molar ratio of 1:6. 173.56 ppm: free oxalate; 166.28 ppm: Al–oxalate complex

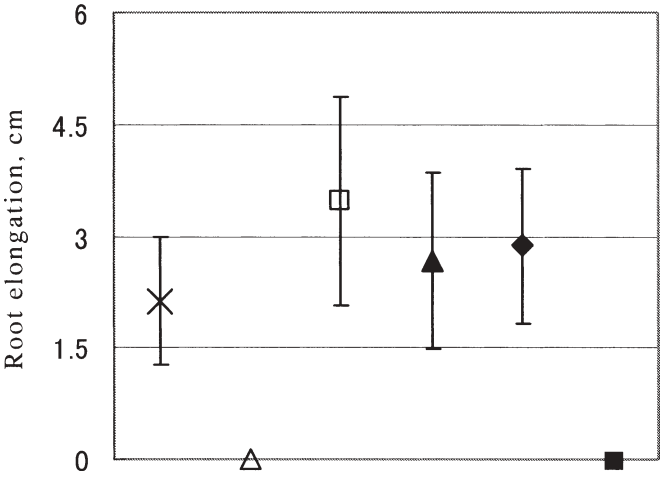

Fig. 5. Effect of F3 on radish root elongation. *Cross*, control (no Al, nolignin); *open triangle*, 0.5 ppm Al; *open square*, 0.5 ppm Al and 12.5 ppm F3; *filled triangle*, 0.5 ppm Al and 25 ppm F3; *diamond*, 0.5 ppm Al and 50 ppm F3; *filled square*, 0.5 ppm Al and 100 ppm F3

Effect of oxalate and Al–oxalate complexes on plant growth

No positive effect of oxalate on the growth of radish was observed in the absence of Al. Oxalate alone at 100 μM showed a negative effect on root elongation by 60%, and almost no root elongation was observed in the case of 200 μM oxalate. When Al was present, however, the effect of oxalate on plant growth was dependent on the Al–oxalate molar ratio (Fig. 3). At an Al concentration of 0.5 ppm, root growth was practically inhibited as reported in the previous article.[5] When oxalate was presented at the Al–oxalate molar ratio of 1:0.5, root elongation was slightly improved, but not much different from the case of no oxalate. This can be explained by the fact that about 63% of Al is still present as free Al^{3+} under this condition (Table 3). When the ratio of Al to oxalate was changed to 1:1, only about 15% of Al was found to be free Al^{3+} and the Al-induced inhibition of root elongation was partially reduced.

Significant amelioration effects were observed at Al–oxalate molar ratios of 1:2 and 1:3. However, when the molar ratio was changed to 1:4, the reverse effect started to appear, which was caused by an inhibitory effect of oxalate itself on root elongation, because free oxalate in 1:4 and 1:6 Al–oxalate mixtures was confirmed at 173.56 ppm by ^{13}C-NMR (Fig. 4).

Explanation for the behavior of F3

Considering the effects of each fraction in the plant growth experiments, F3 was quite different from the others. Although F3 showed increased efficiency in removing Al toxicity at a certain level of its dosage, the efficiency decreased remarkably at higher dosages. This peculiar performance of F3 could be related to its high oxalate content because the complex formed between Al and oxalate in F3 was verified by ^{27}Al-NMR spectroscopy.

Based on the CE experiment, the Al–oxalate molar ratios in F3 were calculated. The Al–oxalate molar ratios in solutions containing 0.5 ppm Al and 12.5 ppm, 25 ppm, and 50 ppm of F3 in plant growth experiments (Fig. 5) were 1:1.07, 1:2.03, and 1:4.26, respectively, under which F3 showed an obvious positive effect in protecting radish root from Al toxicity. However, the positive effect of F3 was significantly decreased when the dosage was increased to 100 ppm, which corresponds to an Al–oxalate molar ratio of 1:8.52 in F3. This phenomenon could be interpreted by the facts that at a proper range, oxalate could have a positive effect on the amelioration of Al toxicity. These behaviors of oxalate in F3 against Al were in good accordance with early reports on the Al detoxification mechanism of some kinds of plants.[18–20]

Plant growth experiment with F3 pretreated with $CaCl_2$

The above information provides impetus for further investigation of the effect of oxalate in F3 on Al toxicity by means of removing oxalate. Oxalate was removed selectively by Ca^{2+}, because calcium oxalate is not soluble in water. The precipitate formed after adding Ca^{2+} to F3 solution was verified as calcium oxalate by comparing its FT-IR spectrum with authentic compound. The plant growth experiment suggested that the Al alleviation effect of original F3 could not be reproduced when oxalate was removed by $CaCl_2$. In other words, oxalate in F3 plays an important role in the efficiency of removing Al toxicity.

Conclusions

The conclusions of this study are summarized as:

1. Alkaline-oxygen stage waste liquor exhibited the potential to function as a soil-conditioning agent.
2. Some low molecular weight contaminants, especially oxalate, in the separated fractions of alkaline-oxygen stage waste liquor could play important roles in the Al alleviation efficiency.
3. Oxalate showed a favorable detoxifying effect on Al toxicity over a particular oxalate concentration range, while excess oxalate showed the reverse effect. Therefore, for the utilization of alkaline-oxygen stage waste liquor to remediate acid soil, the content of low molecular weight organic compounds, especially oxalate, should be controlled to the required concentration.
4. Quantitative ^{27}Al-NMR measurement is very useful for the characterization of complexes between Al and organic compounds.

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