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ACID AND ALUMINUM TOLERANCE OF MICROORGANISMS IN ACID SOIL ENUMERATED BY A DILUTION PLATE METHOD

Kazuhira YOKOYAMA, Hiroaki TANAKA, Akira YOSHIDA,
Norihiro AMIMOTO, Satoshi AWATA and Yutaka KAWADA

The numbers of microorganisms in acid soils were enumerated by plate method with both neutral (pH 7.0) and acid (pH 3.5) agar media. A decline of media pH decreased the numbers of CFUs. Strains picked up from colonies on agar plates at neutral pH were tested for its ability to grow in acid liquid media. While fungi were often able to glow in acid media, more than a half of actinomycetic and bacterial strains failed to grow under acid conditions. Under acid conditions, bacterial isolates which grew in full-strength of nutrient broth did not grow in the 100-fold diluted medium.

All of fungal isolates were tolerant to Al as well as acidity. Whereas an addition of Al to acid liquid media inhibited even the growth of acid tolerant bacteria. Aluminum-sensitivity of bacteria was partly correlated to Al^{3+} activity, however, treatments by monomeric Al compounds was the most principal for the growth inhibition of most soil bacteria.

Key words : Acid soil, Aluminum species, Media pH, Microorganisms, Plate method

Introduction

Application of excess fertilizers lowers soil pH. In Japan, tea soil often becomes very acidic, for example pH (H_2O) falls below 4.0. In addition to low pH, dissolution of Al is one of the most important problems in acid soils because Al precipitates phosphate and is toxic to organisms. WATANABE *et al.*[1,2] determined the number of soil microorganisms (as colony forming units, CFUs) in acid tea soil by means of conventional agar media at neutral pH. It is interesting that the numbers of microorganisms were almost at a same level as other soils at rather neutral pH nevertheless acid and Al stresses are expected to repress microbial activity.

Agar media at neutral or nearly neutral pH have been used to determine the CFUs of soil microorganisms. Some common compositions of media ensured the comparison of data obtained by different authors. However, it has been demonstrated that modification of the composition of agar media is necessary to allow colony formation of unfamiliar microorganisms in extreme environments[3]. At first, we examined the effect of pH at which acid soil was subjected on the number of microorganisms, and, then, tolerance of microbial isolates to acid conditions either in the presence or absence of Al was examined.

Materials and Method

Acid soil was collected from Mannou Branch Station of Kagawa Agricultural Experiment Station (Kagawa) and Kagoshima Tea Experiment Station (Kagoshima) in Japan (Table 1).

Table 1. Properties of soils used

	Kagawa	Kagoshima
Texture	Light clay	Loam
pH (H_2O)[1]	3.40	3.35
pH (KCl)[1]	3.03	3.01
Total C (g Kg^{-1})[2]	7.2	128
Total N (g Kg^{-1})[3]	0.6	13.3
CEC (cmol (+) kg^{-1})[4]	8.1	6.6
Exchangeable cations (cmol (+) kg^{-1})[5]		
Calcium	—	0.5
Potassium	0.2	1.1
Magnesium	0.2	0.3
Sodium	0.1	0.7
Exchangeable aluminum (cmol Kg^{-1})[6]	0.6	0.4
Soluble aluminum (cmol Kg^{-1})[7]	6.5	10.3

1) soil : solution = 1 : 2.5, 2) dicromate oxidation and titlation, 3) Kjeldahl digestion and distillation, 4) successively equilibrated in 1 mol L^{-1} then 0.05 mol L^{-1} ammonium acetate (pH 4.0), 5) 1 mol L^{-1} ammonium acetate (pH 4.0) extractable, 6) 1 mol L^{-1} KCl extractable, 7) 1 mol L^{-1} sodium acetate (pH 4.0) extractable

Kagawa soil was tentatively classified as Ultisol (Hupludult) and Kagoshima soil as Inceptisol (Dystrandept)[4]. Both were collected from the top 5 cm layer of mineral soil under tea-bushes. Kagawa soil was subjected to the determination of microbial numbers after passing through a 2 mm mesh sieve at a field moisture content while Kagoshima soil was pre-incubated for 4 weeks at 28℃ in the dark at 60% of maximum water holding capacity after storage of sieved soil at 5℃.

The number of microorganisms was determined by the plate count method on a rose-bengal agar medium for fungi[5], an egg-albumin agar medium for actinomycetes[6] and NB and DNB (at 10^{-2} strength of NB) agar media for bacteria[7]. At neutral conditions, soil suspension was diluted by using sterilized deionized water after reciprocal shaking for 10 min and 1 mL of an aliquates of a suitable dilution (almost pH 6) was poured with neutral media (pH 7.0). At the same time, sub-samples of the soils were suspended and diluted by pH 3.5 H_2SO_4 solution and inoculated in agar media with a sterilized dilute H_2SO_4 solution in order to adjust pH at 3.5. Colony froming units were counted after 4 days (fungi and NB bacteria), 7 days (actinomycetes) and 4 weeks (bacteria on DNB agar plates) at 28℃ for neutral media while much longer incubation was necessary for acid media. Colonies on DNB agar medium were counted under a stereo microscope at a magnification of 10.

Bacterial and actinomycetic colonies on neutral counting plates were isolated onto neutral agar slants which had the same composition as counting agar media while fungi were maintained on a CZAPEK agar medium at pH 6.0. Bacteria isolated from NB agar plates were

defined as NB bacteria. Isolates from DNB agar plates were tested for their ability to grow in the neutral NB liquid medium up to 2 weeks to differentiate NB and DNB bacteria[8].

The growth experiment of each isolate was carried out in acid liquid media at pH 3.5 adjusted by 0.05 mol L^{-1} H_2SO_4. Inocula were transferred from slants to 5 mL of acid liquid media whose composition was identical with agar media for slants but did not contain agar. Cultures were incubated at 28℃ up to 3 weeks. The increase in turbidity as proliferation of microorganisms was examined at suitable time intervals by naked eyes. The culture was prepared in duplicate. Isolates which grew in acid liquid media were regarded as acid-tolerant species.

For the examination of a response to Al, the acid-tolerant isolates were pre-cultured in the acid media (pH 3.5) prepared as above. At a late logarithmic phase, cells were harvested by centrifugation at 4000 × g for 15 min at 25℃ and washed by 2 mL of an autoclaved H_2SO_4 solution (pH 3.5) three times. Pellets were suspended aseptically in 5 mL of a 5×10^{-5} mol L^{-1} Al solution prepared from $Al_2(SO_4)_3 \cdot 17H_2O$ (an average composition) at pH 3.5 adjusted by H_2SO_4. At the same time, cell washing and an Al treatment were carried out in a similar manner with the solutions osmotically balanced by either 0.3 mol L^{-1} mannitol or 0.15 mol L^{-1} $MgSO_4$. After 48 h cells were harvested and washed three times as above and finally suspended in 2 mL respective sterilized Al free solutions. A small portion (0.1 mL) of the vortexed suspension was inoculated to new acid liquid media (Al-free) and the development of a turbidity was observed up to 4 weeks. A microscopic observation showed that cells were not destroyed during the treatments.

Except for pH adjustment of neutral medium, a dilute H_2SO_4 solution was used to adjust pH throughout this study, as sulfate ion has been very frequently added to soil as a component of fertilizers.

Results and Discussion

Table 2 shows the number of microorganisms. On acid media, the number of CFUs declined to less than 30 % of that on corresponding neutral media and the reduction was more prominent for bacteria and actinomycetes than fungi. It is obvious that some of the isolates from neutral agar media could not grow in acid liquid media (Table 3). We calculated the number of CFUs of acid-tolerant microorganisms by multiplying the factors of frequency of acid tolerant isolates in total isolates of fungi, actinomycetes and NB or DNB bacteria (Table 3) and CFUs of Kagawa soil determined by neutral agar media (Table 2). Comparing with the number of CFUs counted on acid agar plates, the calculated values often still high (Table 2), suggesting that some of microorganisms once exposed to neutral conditions became more tolerant to acid condition. Another possibility is that some of acid tolerant soil microorganisms in a resting form did not begin to form a colony unless once experienced neutral conditions.

Under both neutral and acid conditions, the number of bacterial CFUs determined on DNB agar plates did not exceed those on NB plates (Table 2). Especially in Kagoshima soil, CFU based on neutral DNB agar was one-fourth of that on neutral NB plates. In contrast, the number of NB bacteria fell closely to that counted on DNB agar plate when the experiment was carried out under acid conditions. Bacteria which could not grow on NB agar medium (ie.

DNB bacteria) usually formed colonies on DNB agar medium and thus higher counts were obtained on a DNB than NB agar[8]. More than a half of isolates of NB bacteria from neutral NB agar plates were able to grow in an acid NB liquid medium whereas those from neutral DNB agar plates generally could not grow in an acid DNB liquid medium (Table 3). This was confirmed that 9 of 14 NB bacterial isolates which grew in an acid NB liquid medium failed to grow in an acid DNB liquid medium (Table 3). Thus, as well as pH of media on which the isolates were maintained, the concentration of nutrients affected microbial growth under acidic conditions.

Table 2 Effect of pH on the number of CFU determined

		CFU g^{-1} dry soil		
		neutral	acid	calculated[1]
Kagawa	Fungi ($\times 10^6$)	3.9 (0.5)[2]	0.65 (0.30)	3.5
	Actinomycetes ($\times 10^7$)	57 (14)	0.71 (0.25)	7.4
	Bacteria (on NB agar) ($\times 10^7$)	6.8 (1.5)	1.8 (1.7)	4.1
	Bacteria (on DNB agar) ($\times 10^7$)	6.8 (1.4)	1.3 (0.7)	0.6
Kagoshima	Fungi ($\times 10^6$)	2.4 (0.7)	0.54 (0.36)	—
	Actinomycetes ($\times 10^7$)	5.4 (0.7)	0.27 (0.14)	—
	Bacteria (on NB agar) ($\times 10^7$)	13 (2.0)	0.23 (0.18)	—
	Bacteria (on DNB agar) ($\times 10^7$)	2.7 (0.5)	0.20 (0.07)	—

1) Multiplication of CFUs on neutral media and frequency of acid tolerant isolates
2) Values in parenthes show a standard deviation.

Table 3. Frequency of acid or acid plus Al tolerant strains isolated from Kagawa soil

	Liquid medium (pH 3.5)	No tested	No. tolerant			
			Acid	Al[2]		
				50 Al[3]	50 Al + $MgSO_4$[4]	500 Al[5]
Fungi	CZAPEK	10	9	9	9	9
Actinomycetes	Egg alubmin	8	1	1	1	—
NB bacteria (from NB agar)	NB	23	14	5	7	—
NB bacteria (from DNB agar)	DNB	8	1	1	1	—
DNB bacteria	DNB	3	0			
NB bacteria (from NB agar)[1]	DNB	14	5			

1) NB bacteria which grew in a NB liquid medium (pH 3.5) were tested.
2) All isolates examined were acid tolerant
3) The solution contained 5×10^{-5} mol L^{-1} Al at pH 3.5. The addition of mannitole at 0.3 mol L^{-1} did not show an additional effect.
4) The solution contained 5×10^{-5} mol L^{-1} Al and 0.15 mol L^{-1} $MgSO_4$ at pH 3.5.
5) The solution contained 5×10^{-4} mol L^{-1} Al at pH 3.5. Only fungi were tested.

The ability of Al tolerance did not fully associate to that of acid tolerance (Table 3). Only 5 of 14 acid tolerant NB bacteria isolated from neutral NB plates of Kagawa soil grew after the treatment at 5×10^{-5} mol L^{-1} Al. While the addition of mannitol did not show any additional efficiency on microbial growth as far as examined, the addition of $MgSO_4$ to the Al solution reduced inhibitory effect of 5×10^{-5} mol L^{-1} Al on 2 NB bacteria. In contrast, all of acid tolerant fungi examined grew after the treatment at 5×10^{-5} mol L^{-1} Al regardless of $MgSO_4$ addition and even after the treatment by the solution containing 5×10^{-4} mol L^{-1} Al. These notable characteristics of fungi compared with bacteria may be one of the reason for the fact that fungi and their activity essential in acid soil[9].

The inhibitory effect of Al determined here is direct to microbial cells rather than precipitation of nutrients in media. It has been established that the activity of Al species in a solution correlates more directly to Al toxicity to organisms than a total Al concentration[10]. Following LINDSAY's equations[11], we proximated the composition of Al species by using his data for equilibria of Al and Al hydroxides and Al-sulfate complexes except for the equiriblium constant for $Al(SO_4)_2^-$ of KINRAIDE and PARKER[12] (Table 4). If once microbial cells absorbed soluble ions or excreted charged substances to a bulk solution, the composition of Al species would fluctuate. Moreover, the value of ion strength was not critically calculated when the solution contained salt at such a high concentration that was employed here[11]. Thus, the values shown in Table 4 is only a rough proximation of composition of Al species just before cells addition. According to these values, expected species of Al in the solutions were Al^{3+}, $Al(OH)^{2+}$, $Al(SO_4)^+$ and $Al(SO_4)_2^-$. The addition of $MgSO_4$ lowered the activity of the former two species and increased those of Al-sulfate complexes. The activity of Al^{3+} in the testing solution at a concentration of 5×10^{-5} mol L^{-1} Al without $MgSO_4$ was within a range sufficient to inhibit the root elongation of barley[13] and wheat[14]. The activity of Al-sulfate complexes was comparable or higher than those employed for the study of KINRAIDE and PARKER[12] to ensure the non-phytotoxicity of Al-sulfate complexes.

The results in Table 3 and 4 suggest that fungi were surprisingly tolerant to Al^{3+} and Al hydroxides and Al-sulfate complexes at a given activity. Some of NB bacteria were also tolerant to these Al species as far as examined with the solution containing 5×10^{-5} mol L^{-1} Al. However, Al^{3+} and monomeric Al hydroxides considered to be toxic and Al-sulfate complexes non-toxic to NB bacteria which could grow after the treatment of Al in the presence of $MgSO_4$. Thus, the ameliorative effect of $MgSO_4$ was attributed more likely to the reduction of the activity of these toxic Al-species rather than the competition of Mg^{2+} with positively charged Al complexes on negatively charged sites of cell surface. In contrast, isolates not growing after any treatment appeared to be sensitive

Table 4. Activity (10^{-6} mol L^{-1}) of Al species estimated

	50 Al [1)]	50 Al + $MgSO_4$ [1)]	500 Al [1)]
Al^{3+}	28.8	0.2	167.9
$AlOH^{2+}$	0.9	—	5.1
$Al(OH)_2^+$	0.1	—	0.8
$Al(OH)_3^0$	—	—	—
$Al(OH)_4^-$	—	—	—
$Al(SO_4)^+$	9.3	9.6	182.4
$Al(SO_4)_2^-$	0.2	23.1	10.2
$Al_2(SO_4)_3^0$	—	—	—

1) Abbrebiations are identical with those used in Table 3

even to Al-sulfate complexes under starvation or extremely sensitive to Al^{3+} and monomeric Al hydroxides compared with other NB bacteria. We could not compare our result directly with others for Al toxicity to *Rhizobium* sp.[15,16,17,18], because, in these studies, cells were grown in media containing Al, which made the activity of Al species unclear.

BRADLEY and PARKER[19] suggested the specific other than ionic binding of Al to bacterial cells. This could be occurred in this study as the density of negatively charged sites on microbial cell surface would be very low at pH 3.5 and washing of cells by the pH 3.5 solution after the Al treatment replaced exchangeable Al cations with H^+. Once Al enters in a microbial cell, DNA double strands may be one of the most important target of Al[20]. Apart from DNA, organic and inorganic substances forming stable complexes and precipitates with Al would be also attacked by Al complexes.

We defined acid and Al tolerance as microorganisms could proliferate after each treatment. This definition may not be appropriate to evaluate all living microorganisms in acid soil. Some of microorganisms might maintain their viability in acid soil but could not grow under conditions given here (Table 3). Moreover, there might be isolates living but unable to result in turbidity in liquid media after the Al treatment because an initial cell density of bacteria in Al solution affected the production of turbidity[18] and Al prolonged doubling time and inhibit complete doubling of microbial cells[16]. Thus, we might examine the most actively proliferating fraction of microorganisms in acid soil.

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希釈平板法による酸性土壌の微生物計数値と得られた微生物単離株の耐酸・耐アルミニウム性の検討

横山和平・田中浩晶・吉田　明・網本典浩・粟田　智・川田　裕

酸性土壌中の微生物数の測定値に対する寒天培地のpHの影響を検討した。酸性培地（pH3.5）で得られる微生物数は中性培地（pH7.0）での値に比べ小さかった。

中性培地上のコロニーから得た単離株のうち，ほとんどの糸状菌株は酸性液体培地中で生育したが，放線菌・細菌単離株の半数以上は生育しなかった。酸性下では培地の養分濃度が細菌単離株の生育に強い影響を与えた。

耐酸性の糸状菌単離株は，すべてアルミニウム耐性であったが，耐酸性細菌の多くはアルミニウム感受性であった。Al^{3+}の活動度は一部の細菌に対し影響を与えたが，多くの場合，Al^{3+}以外の単量体アルミニウム化合物も細菌の生育を阻害した。