BioMetals **15:** 167–174, 2002.

The extremely high Al resistance of *Penicillium janthineleum* F-13 is not caused by internal or external sequestration of Al

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Received 28 May 2001; accepted 23 August 2001

Key words: Al toxicity, Al tolerance, Al resistance, *Penicillium janthinellum* F-13

Abstract

Penicillium janthinellum F-13 has been isolated in previous work as a fungus tolerating the presence of high concentrations of Al (as high as 100 mM $AlCl_3$). Here its growth rate and yield in three acidic (pH 3.0) media of different composition with varying concentrations of Al are reported. The presence of Al did not affect these parameters, except that the growth yield was somewhat lower in GM (a glucose/peptone/yeast extract-containing medium) with the highest concentration tested (100 mM $AlCl_3$). The amount of Al found in the mycelium was so low that it cannot lead to a significant decrease in the medium for the higher Al concentrations applied. Although citric acid was excreted at growth on GM, and the presence of Al even promoted this, the concentration of this was far too low to diminish (by chelation) the high Al concentrations in the medium to a non-toxic level, i.e. the level (of approx. 1 mM) that is tolerated by low-resistance fungi. At growth on SLBM (a peptone/yeast extract/soil extract-containing medium), a rise in pH occurred. The same was found for SM (a glucose/mineral salts-containing medium), although in this case the picture was more complicated because the initial rise in pH was followed by a lowering due to the excretion of oxalic acid. Although both phenomena can diminish Al toxicity (by decreasing the external concentration of monomeric Al, regarded to be the toxic species), again the decrease is far too low to attain a non-toxic level when high Al concentrations are applied. Therefore, although in principal the metabolic phenomena observed for *P. janthinellum* F-13 at growth on different media can diminish Al toxicity, the tolerance of this organism for high external Al concentrations must be caused by another mechanism.

Introduction

Al is the most abundant metal in the earth crust. Most of it is present in the form of oxides and silicates that are nearly insoluble under neutral or slightly acid conditions. However under acid conditions these compounds become more soluble in water, leading to the formation of inorganic monomeric species (mAl) such as $Al(OH)_2^+$, $Al(OH)^{2+}$ and Al^{3+} (Haug 1984). Since the mAl species are phytotoxic already in the micromolar range, and such concentrations are easily attained at low pH, the wide occurrence of acid soils in the world forms a serious problem in agriculture (Myrold & Nason 1992). Some progress has been achieved in recent years since cultivars have been obtained that are somewhat more tolerant. The mechanisms responsible for the improvement include: (1) symplasmic tolerance, chelation of symplasmic Al with low molecular weight ligands or Al-binding proteins in the cytoplasm, or sequestration of Al within an internal compartment (e.g., the vacuole); (2) Al exclusion, the release of low molecular weight, Al chelating ligands into the rhizosphere, root-induced increases in rhizosphere pH, binding of Al within the cell wall, decreased permeability of the plasma membrane to Al influx, and binding of al within the mucigel associated with the root apex (Kochian 1995; Matsumoto 2000). However, since the increase in tolerance is limited (the upper limit is usually below 0.1 mM), further improvement is still needed.

Since soil is also populated by microbes, studies on these organisms could reveal additional insight into mechanisms underlying Al toxicity and resistance. Recently, a number of yeasts and fungi have been isolated from acid soil that tolerate the presence of Al salts in the growth media in concentrations as high as 100 mM (Kanazawa *et al.* 1996; Kawai *et al.* 2000). However, the mechanism responsible for this extremely high tolerance is unknown. Therefore, we attempted to study this by using one of the organisms isolated by us (Kawai *et al.* 2000), the fungus *P. janthinellum* F-13, as a model organism.

Since the ratio of mAl to tAl (the total concentration) depends on the presence of chelators and the pH value, it is understandable that the composition of the growth medium used strongly affects the Al-tolerance level of a microbe (Flis *et al.* 1993). Therefore, a synthetic medium and 2 different yeast extract/peptone-containing media, all having a pH of 3.0, were used to determine the effect of varying concentrations of $AlCl_3$. In principle, an increase in Al tolerance could originate from sequestering of the toxic mAl species (either internally or externally), or to an increase in the ratio of tAl to mAl induced by a rise in the pH value. Therefore, not only production of chelating organic acids but also the pH of the medium and accumulation of Al in the mycelium were monitored during growth. To obtain insight into the Al resistance levels of fungi, a number of culture collection strains having similarity to the isolates described in the previous paper were investigated.

Materials and methods

Microorganism and cultivation

All fungi used and their origin are listed in Table 1.

Glucose medium (GM) contains (per l): glucose, 10 g; NaCl, 10 g; peptone, 0.5 g; yeast extract, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g. Soil extract-containing medium (SLBM) contains (per l soil extract): NaCl, 10 g; peptone, 0.5 g; yeast extract, 0.2 g. The soil extract was prepared as follows: 100 g of neutral soil, collected from a field at our institute, was suspended in 1.0 l of distilled water and stirred for 40 min. The mixture was centrifuged at 8,000 rev min^{-1} for 20 min. The supernatant was filtered using a membrane filter of 0.45-μm-pore size. The tAl and PO_4 concentrations in this extract were 6.6 μM and 6.3 μM, respectively. The synthetic medium (SM) used here was a modification of that described by Sherman (1991). The medium contains (per l): glucose, 20 g; $NaNO_3$, 5 g; KH_2PO_4, 0.14 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; NaCl, 0.1 g; $CaCl_2$, 0.1 g; and trace element solution, 1 ml. The trace element solution contained (μg ml^{-1}): H_3BO_4, 500; $CuSO_4 \cdot 5H_2O$, 40; KI, 100; $FeCl_3 \cdot 6H_2O$, 200; $MnSO_4 \cdot 5H_2O$, 400; $Na_2MoO_4 \cdot 2H_2O$, 200; $ZnSO_4 \cdot 7H_2O$, 400. The pH of all media was adjusted to 3.0, except for that used for the citrate excretion experiment where the pH was 3.2.

Culture media (100 ml) were transferred into 500-ml shaking flasks, and these were autoclaved at 121 °C for 20 min. After that, an appropriate amount of Al stock solution was added aseptically. However, in experiments where the highest Al concentration (100 mM) was applied, it was added in the form of solid $AlCl_3$ $6H_2O$. The Al stock solution consisted of filter-sterilized 1 M $AlCl_3$.

For growth experiments, spores were collected from a GM agar plate on which the fungus was grown and suspended in 100 ml medium. The medium with spores was shaken for 12 h at 30 °C, giving the seed culture with which flasks for experiments with the same medium were inoculated. Growth experiments were carried out in triplicate. The flasks were incubated at 30 °C with shaking.

Analyses

The growth yield values were derived from dry weight determination of the mycelia. The mycelia were collected by filtering on a paper (Wipers S-200), washed 4 times with 10 mM citric acid and dried to constant weight at 105 °C. In those cases where precipitation of Al occurred (as was judged from the cloudy filtrate), the collected mycelium was suspended in citric acid-containing solution and the mixture stirred until the precipitate had dissolved.

Al concentrations in the mycelia collected as indicated above were determined as described by Guida *et al.* (1991). Total Al (tAl) was measured by an atomic absorption spectrophotometer (Hitachi Z-8200, Japan) and mAl was measured by the pyrocatechol violet method (Kerven *et al.* 1989).

The identity and quantity of the organic acids excreded in the medium were determined as described by Ma *et al.* (1997).

Table 1. Al resistance levels of some fungi when grown on GM.

Fungi	Source	Al tolerance*
Trichoderma asperellum F-15	Tea field	>200 mM
Penicillium sp. F-8b	Tea field	>100 mM
P. janthinellum F-13	Tea field	>200 mM
P. janthinellum IFO 4651	IFO[a] culture collection	>100 mM
P. janthinellum IFO 6581	IFO culture collection	>100 mM
P. janthinellum IFO 7905	IFO culture collection	>100 mM
P. janthinellum IFO 31133	IFO culture collection	>100 mM
P. janthinellum IFO 31969	IFO culture collection	>100 mM
P. lilacinum AKU 3414	AKU[b] culture collection	>100 mM
P. oxalicum AKU 3403	AKU culture collection	<0.6 mM
P. crysogenum IFO 4626	IFO culture collection	<0.6 mm
P. crysogenum AKU 3407	AKU culture collection	<0.6 mm
Aspergillus flavus Link F-6b	Tea field	>100 mM
A. flavus IFO 7540	IFO culture collection	>100 mM
A. niger IAM 3010	IAM[c] culture collection	<1 mM
A. oryzae IFO 4176	IFO culture collection	<1 mM
Cryptococcus humicola Y-6	Tea field	>100 mM
Cryptococcus humicola IFO 0076	IFO culture collection	>100 mM

*At the Al tolerance level indicated, at least 50% of the growth yield in the absence of Al was obtained (after 7 days). The fungi isolated from a tea field have been previously described (Kawai *et al.* 2000). [a]IFO, Institute of Fermentation, Osaka, Japan; [b]AKU, Faculty of Agriculture, Kyoto University, Kyoto, Japan; [c]IAM, Institute of Applied Microbiology, University of Tokyo, Japan.

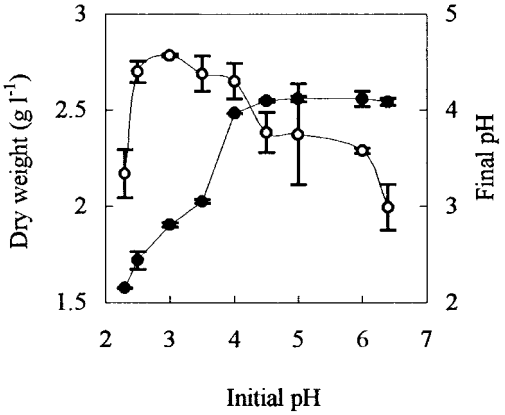


Figure 1. Growth yield (○) of *P. janthinellum* F-13 in GM and final pH (●) of the spent medium Mycelia were collected after 7-day growth. Error bars represent ± SD ($n = 3$).

Results

Effects of Al at growth on GM

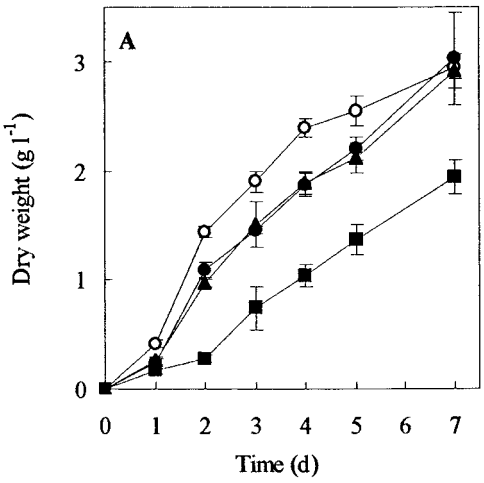
The growth yield of *P. janthinellum* F-13 was nearly constant in the pH range 2.5 to 4.0 (Figure 1), in agreement with the acid-resistant character of this organism (Kawai *et al.* 2000). The pH of the medium dropped only slightly during growth for media having an initial pH in this range, but substantially above this range (Figure 1). The drop in pH resulted from acid production (see next paragraph) but, since the GM was buffered by 10 mM citrate in these experiments, the drop can be clearly seen only when the initial pH of the medium was above the buffering range of citrate.

To determine the Al resistance of *P. janthinellum* F-13 on GM, this growth medium was supplemented with varying concentrations of $AlCl_3$. As appears from Figure 2A, growth rate and yield (after 7 days) were lowered only in the presence of 100 mM $AlCl_3$ and the drop in pH of the medium was slightly higher in the presence of Al (Figure 2B) (see below for the explanation). The data in Table 2 indicate that some uptake or binding of Al occurred in the mycelium, the amounts increasing with the concentration applied in the medium, but neither in a linear relationship nor in a growth phase-dependent way. The data also indicate that the binding or uptake scarcely contributed to diminishing the high outside concentrations of Al. HPLC analysis of the spent medium (data not shown) revealed that citric acid was excreted during growth and the yield and rate of citric acid production in-

Table 2. Incorporation of Al into mycelia of *P. janthinellum* F-13 grown on GM.

	$AlCl_3$ (mM) added				
	0	0.1	1	10	100
tAl incorporated (mg g^{-1} dry weight mycelia)	0	0.113	0.293	0.506	0.95
Decrease of the external Al concentration (μM)	0	8.5	24.4	28.9	22.9

The Al content of mycelia samoles taken at day2, 5 and 8 did not vary.



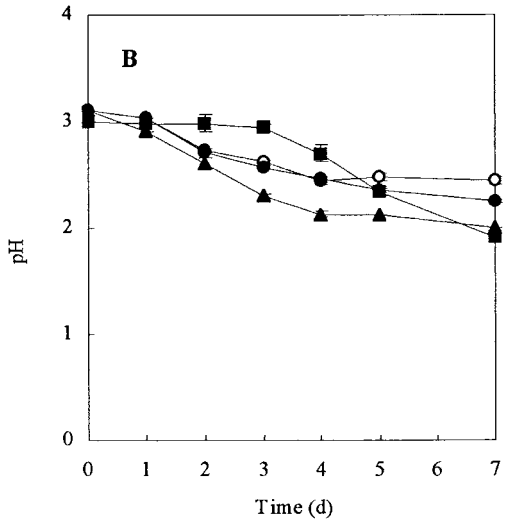


Figure 2. The effect of Al on *P. janthinellum* F-13 during growth in GM. A: growth curves; B: the pH of the medium during growth. Al added: ○, 0 mM Al; ●, 1 mM Al; ▲, 10 mM Al; ■, 100 mM Al. Error bars represent ± SD ($n = 3$).

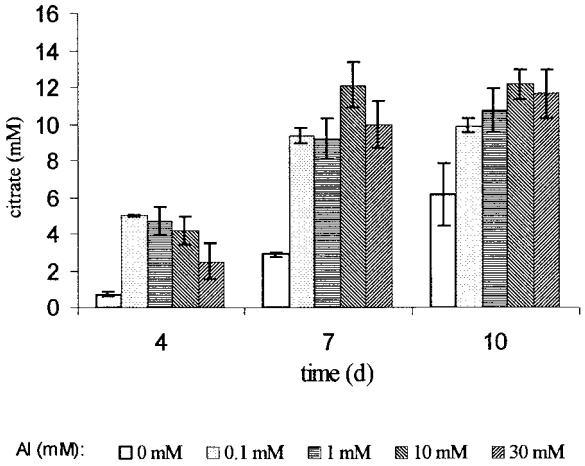


Figure 3. The effect of Al on citrate excretion by *P. janthinellum* F-13 during growth in GM in the presence of various concentrations of $AlCl_3$. Error bars represent ± SD ($n = 3$).

creased in the presence of Al (Figure 3), explaining the effect of Al on the pH of the spent medium (Figure 2B). A concentration of 0.1 mM $AlCl_3$ produced the maximum effect on the secretion of citric acid. Since the yield of citric acid (about 10 mM) was 100 times higher than the smallest Al concentration applied, the stimulation is not due to removal of citric acid as a complex with Al. complete complexation of mAl by citric acid was achieved when a concentration of 1 mM $AlCl_3$ was applied but not for higher concentration (Figure 4).

Fungi and yeast isolated from a tea field, and tolerating the presence of high concentrations of Al, were compared with respect to the Al resistance level at growth on GM with their relatives present in Culture Collections (Table 1). The results show that Culture Collection strains show either the extremely high tolerance of the organisms isolated from tea fields (of at least 100 mM) or a much lower one (of approx. 1 mM).

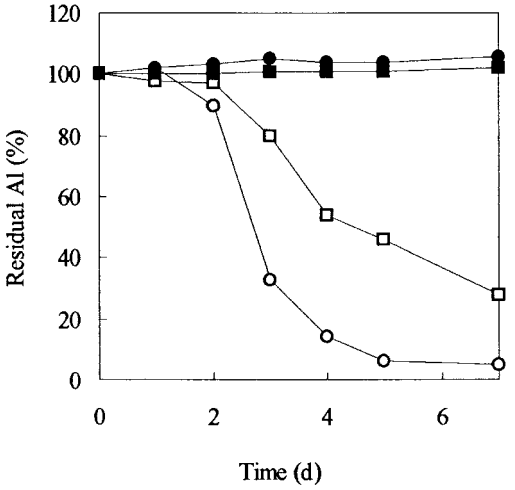


Figure 4. Changes in tAl (filled symbols) and mAl (open symbols) in the spent medium during growth of *P. janthinellum* F-13 in GM in the presence of 1 mM (○, ●), and 10 mM (□, ■) $AlCl_3$. Standard deviation ranged from 0.08–2.46%.

Effects of Al at growth on SLBM

As shown in Figure 5A, the growth yield of *P. janthinellum* F-13 on SLBM was somewhat increased in the presence of Al. Most probably this is due to a buffering effect preventing the pH of the medium from reaching a sub-optimal value for growth (Figure 5B), the buffering leading to precipitation of $Al(OH)_3$. However, this effect was relevant for lowering the mAl concentration only when 1 mM $AlCl_3$ was applied (Figures 6 & 7). The fact that 100 mM $AlCl_3$ did not decrease the growth yield, as it did in GM, can be explained by assuming that such a high concentration is harmful at pH 2 (Figure 2B) but not at pH 3 (Figure 5B). At growth on SLBM in the presence of 1 mM $AlCl_3$, no significant removal of external Al by binding to or uptake in mycellium occurred (Figure 7), although the amount (2.5 mg g^{-1} dry mycelium) was about 9 times higher than that found for growth on GM (0.293 mg g^{-1}).

Effects of Al at growth on SM

Growth yields on SM in the presence of Al were slightly higher than in its absence (Figure 8A). Just as in the case of SLBM, it seems that the buffering capacity of Al, preventing the rise in pH normally occurring (Figure 8B), is responsible for this. Removal of Al from the medium by precipitation occurred when 1 mM $AlCl_3$ was applied, but only a little when 10 mM was applied (Figure 9). However, the precipitate even-

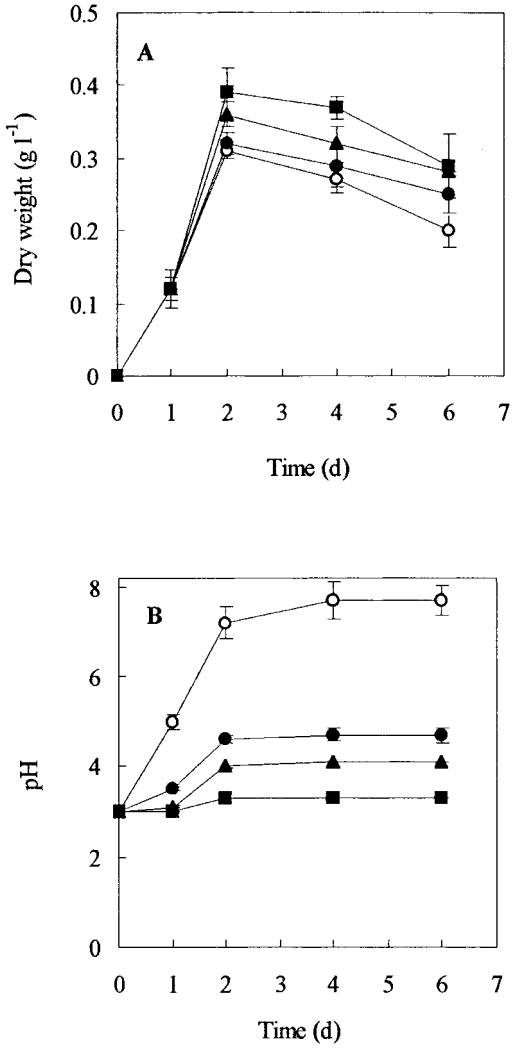


Figure 5. The effet of Al on *P. janthinellum* F-13 during growth in SLBM. A: growth curves; B: the pH of the medium during growth. Al added: ○, 0 mM Al; ●, 1 mM Al, ▲, 10 mM Al; ■, 100 mM Al. Error bars represent ±SD ($n = 3$).

tually disappeared by the production of oxalic acid (data not shown), leading to a rise in tAl but not in mAl concentration of the medium due to sequestration by the oxalic acid.

Combinations of Mg (0.1 μM–10 mM), Ca (0.1 μM–2 mM) and Fe (ferro, 0.1 μM–3 mM) at varying concentration with 10 mM Al showed no effect on growth (results not shown).

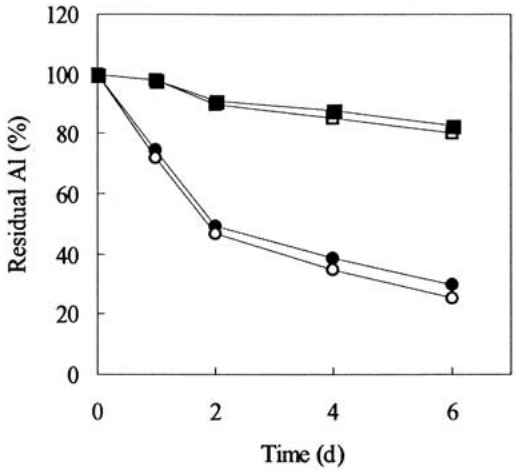


Figure 6. Changes in tAl (filled symbols) and mAl (open symbols) in the spent medium during growth of *P. janthinellum* F-13 in SLBM in the presence of 1 mM (○, ●), and 10 mM (□, ■) $AlCl_3$. Standard deviation ranged from 0.1–0.2%.

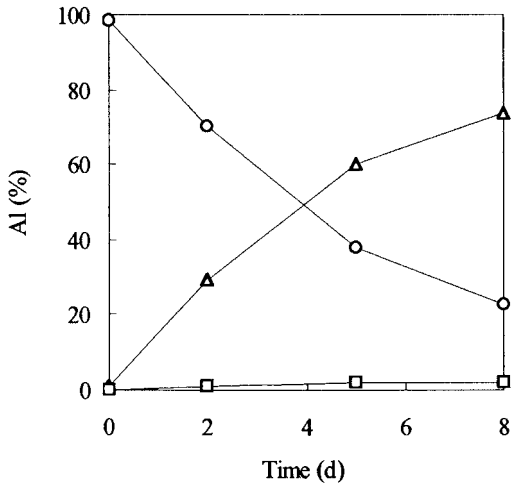
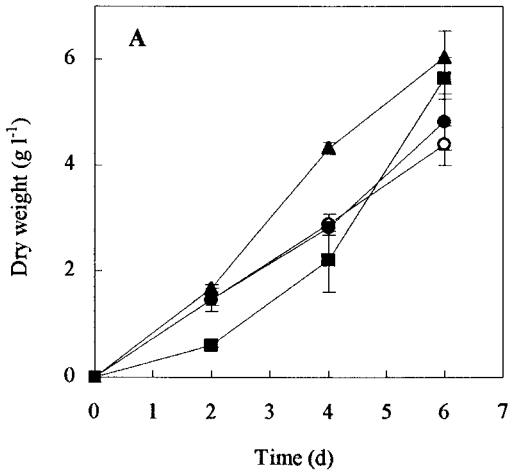


Figure 7. Distribution of tAl during growth of *P. janthinellum* in SLBM in the presence of 1 mM $AlCl_3$. tAl in the supernatant of the medium (○), precipitate (△) and mycelium (□). Standard deviation ranged from 0.05–2.8%.

Discussion

P. janthinellum F-13 grew well on GM having a starting pH between 2.5 and 4.5, confirming the previous finding that it concerns an acid-tolerant organism. Thus the pH range for optimal growth overlaps with that where the maximal toxicity of Al salts or soil is exerted. However, the results show that using a pH in this range (pH 3.0), the organism is resistant to Al on all the media tested, except on GM where the growth yield is somewhat lowered by the presence of 100 mM $AlCl_3$. Since also fungi exist which have a tolerance



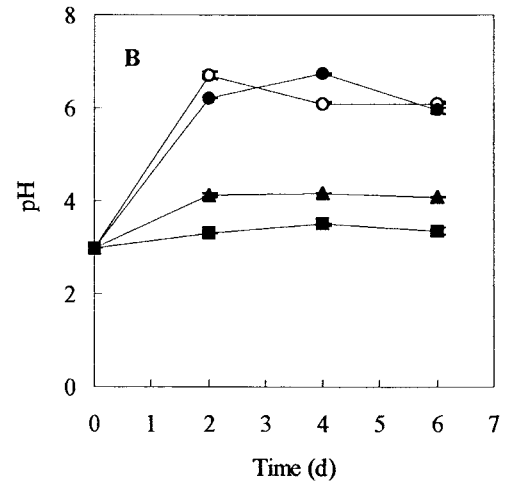


Figure 8. The effect of Al on *P. janthinellum* F-13 at growth in SM. A: growth curves; B: the pH of the medium during growth. Al added: ○, 0 mM Al; ●, 1 mM Al; ▲, 10 mM al; ■, 100 mM Al. Error bars represent ± SD ($n = 3$).

for Al on GM below 1 mM (Table 1), it is concluded that *P. janthinellum* F-13 has an extremely high Al resistance.

Citric acid was excreted at growth on GM, a property exhibited by many fungi when grown on a glucose-containing medium. Production of this led to sequestering of mAl in the medium, as shown in Figure 4. When it is assumed that only unsequestered mAl in the medium is toxic for fungi, this implicates that secretion of citric acid detoxifies Al. However, since the production levels were around 10 mM, in the case that 100 mM Al is applied only 10% of this is detoxified. It could be reasoned that production of chelating

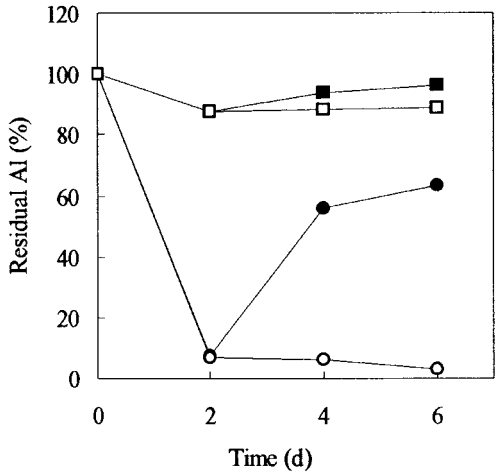


Figure 9. Changes of residual tAl (filled symbols) and mAl (open symbols) in the spent medium at growth of *P. janthinellum* F-13 in SM in the presence of 1 mM (○,●), and 10 mM (□, ■) $AlCl_3$. Standard deviation ranged from 0.15–3.12%.

organic acids is an important mechanism for protecting low-resistance fungi against Al. However, from the data obtained for *P. janthinellum* F-13, this can be the case only under certain growth conditions. As illustrated in Figure 3, excretion of citric acid occurs rather late in the growth cycle so that with batch wise growth, in the beginning this metabolic phenomenon provides no protection at all. Moereover, it should be noted that for non-acid tolerant fungi, excretion of these acids leads to a lowering of the medium pH, thereby counteracting the beneficial effect by shifting the mAl/tAl ratio to a higher value.

As can be expected from their composition, growth on SM and SLBM led to an increase of the culture medium pH. Since it lowers the mAl/tAl ration, this metabolic phenomenon will have a detoxifying effect on Al. However, the presence of Al counteracts the rise in pH due to conversion of Al^{3+} into $Al(OH)^{2+}$, $Al(OH)_2^+$ and $Al(OH)_3$, the latter precipitating at a certain concentration. The data shown here confirm this, the higher the Al concentration applied the less rise in pH occurred. To illustrate how this affects the detoxification caused by metabolic alkalizing of the medium, the growth experiments in SM and SLBM in the presence of 10 mM $AlCl_3$ are taken as example. As shown in Figure 5 and 8, the final pH of the growth medium was 4.0 so that it can be calculated (Pina & Cervantes 1996) that only 10% of the original mAl concentration has disappeared by the pH shift. It will be clear from this example that the detoxifying effect of this metabolic phenomenon is without significance when high Al concentrations are applied. Also here it should be realized that no effect can be expected at the beginning of batch wise growth so that alkalization of the medium will protect low-resistance fungi against low Al concentrations only under certain growth conditions.

Since no significant amounts of tAl were found in the mycelia, the toxicity of high external Al concentrations for *P. janthinellum* F-13 is not diminished by internal sequestering of it into a harmless form. Since also external sequestering can be excluded (see above), the conclusion is that the data presented here give no clue about the mechanism underlying the extremely high Al resistance of this organism. However, when it is assumed that the cellular machinery of high- and low-resistance fungi has similar sensitivity to Al, the high resistance of *P. janthinellum* F-13 may originate from a special system, preventing either import or providing in export of Al. Investigations on the acquirement and identity of such a system are in progress.

Acknowledgements

This research was supported by a grant from the Bio-oriented Technology Research Advancement Institution, Japan. We are grateful to Professor H. Matsumoto and his co-workers from our Institute for their help in measuring tAl and the organic acids.

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