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Effects of pH and Aluminium Ion Concentration on Spore Germination and Growth of Some Soil Fungi

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Abstract: Concentrations of three aluminium compounds significantly affected the mycelial extension of *Trichoderma viride* with low pH values growing on agar media, and the largest effect was recorded with aluminium chloride. Experiments in this work showed that there were significant relationships between Al concentration and pH in the Al inhibition of fungal activity on agar media. Different effects of aluminium concentration on the mycelial extension rate of eight fungal species inoculated on pH gradient plates were recorded. The spore germination of *Phoma exigua* and *Cladosporium herbarum* was reduced by increasing aluminium and acidity, and low pH increased the inhibitory effect of aluminium. Aluminium has an inhibitory effect itself at low pH and it may have an important effect on soil chemistry and microbial activity in the soils where air pollution is high.

Key Words: Aluminium, buffering capacity, fungi germination, inhibition, pH.

Bazı Toprak Mantarlarının Spor Çimlenmesi ve Gelişimi Üzerine pH ve Alüminyum İyon Konsantrasyonunun Etkisi

Özet: Üç alüminyum bileşiğinin artan konsantrasyonları düşük pH değerlerinde agar ortamında yetişen *T. Viride*'nin miselyum gelişimini önemli ölçüde etkiledi ve en büyük etki alüminyum klorür ile tesbit edildi. Bu çalışmadaki deneyler agar medyadaki fungal aktivitenin inhibisyonu üzerinde artan Al konsantrasyonu ile pH'nın istatistiksel olarak ilişkili olduğunu göstermiştir. pH dereceli ortam üzerine inoküle edilen 8 fungi türünün miselyum gelişimi üzerine alüminyumun farklı etkileri tespit edilmiştir. *Phoma exigua* ve *Cladosporium herbarum* sporlarının çimlenmesi artan asitlilik ve alüminyum konsantrasyonu ile azalmış ve düşük pH değerleri alüminyumun inhibitör etkisini artırtırmıştır. Alüminyum tek başına düşük pH'larda inhibitör etkisine sahip olup hava kirliliğinin fazla olduğu yerlerde toprak kimyası ve mikrobiyal aktivite üzerinde önemli etkiye sahiptir.

Anahtar Sözcükler: Alüminyum, tamponlama kapasitesi, fungi çimlenmesi, inhibisyon, pH.

Introduction

Acid deposition is hypothesised to cause changes in soil chemistry and to damage terrestrial ecosystems primarily through its effects on soil acidification (1,2). Wainwright (3) found that soil respiration was reduced by 50% over a one-year period by dry and wet acid deposition. The potential effects of acidic deposition on soil are perceived to be greater for forest ecosystems than for more intensively managed agricultural ecosystems, principally because many forest soils already have a low pH (4,5). Precipitation and other forms of atmospheric deposition are natural factors involved in the processes of soil weathering that lead to leaching of mobile elements and progressive acidification of soils. Anthropogenically generated mineral acidity may also contribute to mineral weathering, thereby affecting the chemical distribution and bioavailability of Al^{3+} and Mn^{2+} in forest soils (6). Acidic deposition, however, is only one of many concurrent stresses that have influenced forests in eastern North America and Western Europe in recent times (7).

Leaching of Al^{3+} from insoluble $Al(OH)_3$ increases following rain on the sites highly polluted by atmospheric SO_2 (8). In most podsolised soils, acidification of the soil solution and organometal complexing mediated by organic acids results in movement of Al and Fe ions into lower horizons (9), but the relative importance of various sources of H^+ in acid forest soils subject to air pollution is

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difficult to assess. Such factors can inhibit forest trees directly, e.g. 0.3 mM Al has been shown to reduce conifer growth by decreasing Ca and Mg uptake (10). Further, forest ecosystem function may be altered by effects of metals on microorganisms (11,12).

Research has not yet provided a lucid explanation of the role of atmospheric deposition in soil chemical processes. One of the many lacunae concerns the indirect effects of acid deposition; here we examine the interactive effect of Al^{3+} and pH on fungal growth and spore germination on agar media.

Materials and Methods

Extension rate of Trichoderma viride *on agar media with different combinations of pH and Al concentrations*

Malt extract agar (MEA; 2% malt extract and 1.5% agar in distilled water) media were prepared at eight pH values between 2 and 9 and with three aluminium compounds [$Al_2(SO_4)_3$, $Al(NO_3)_3$, and $AlCl_3$; Aldrich Chemical Co. Ltd., UK] at five different concentrations (0 to 200 µmol Al kg^{-1} medium), i.e. 120 media were tested. The chemicals (acid, alkaline and Al solutions) were sterilised separately at 121°C for 15 min. To adjust the pH of the media, predetermined amounts of the acid or alkaline solutions were added to the Petri dishes (13). One millilitre of sterilised Al solution was then added to each non-vented, 9 cm diam. Petri dish, and 20 g sterilised MEA at 60°C was poured into the Al solution. The Petri dishes were then rotated to mix the contents. Three replicates were prepared for each Al concentration and pH value, and after 24 h the Petri dishes were inoculated centrally with a single plug (4 mm diam.) of *Trichoderma viride* Pers. mycelium cut from the margin of a one-wk-old fungal colony on MEA using a No. 2 cork borer, and incubated at 15°C. Mycelial extension was measured along two diameters at right angles at 12 or 24 h intervals until the colony reached the edge of the Petri dish.

Combined effect of Al and pH on spore germination on two-dimensional plates

Preparation: Two-dimensional gradient plates were prepared using square Petri dishes (10 x 10 cm, Sterilin Ltd., UK) to examine the combined effects of Al and pH on the germination of fungal spores, using the method of Boddy *et al.* (14). Each plate consisted of four 22 ml layers of 2% MEA to which the following were added: layer one - 17.8% (v/v) 0.5 M H_2SO_4, 100 mM l^{-1} Tris [tris (hydroxy methyl) methylamine] and 50 mM citric acid; layer two - 28% (v/v) 1 M NaOH, 100 mM l^{-1} Tris, 50 mM l^{-1} K_2HPO_4 and 50 mM l^{-1} citric acid; layer three - 2 mM $AlCl_3$; layer four - only MEA. The chemicals were added after sterilisation of the MEA and solutions. All plates were prepared on a level surface. The first layer was poured into the plate with one edge raised on a 3 mm diam. glass-rod, which allowed the medium to solidify as a wedge. Once the first layer had set, the plate was placed flat and the second layer poured. After setting, the plate was rotated through 90° and the above process was repeated for layers three and four. The plates were left at room temperature for 24 h to allow the layers to equilibrate vertically.

Measurement of pH and Al concentrations in gradient plates: The pH gradient was measured at nine points, 1 cm apart, across the plate using a pH meter (Dulas Engineering, UK) with a surface electrode (Orion Research Incorporated, East Sussex, UK), on a 24-h-old uninoculated plate (Fig. 1). The Al-gradient was quantified spectrophotometrically at 535 nm with the reagent Erichrome Cyanine-R (15,16). Nine plugs (7 mm diam.) were cut from nine points 10 mm apart across the

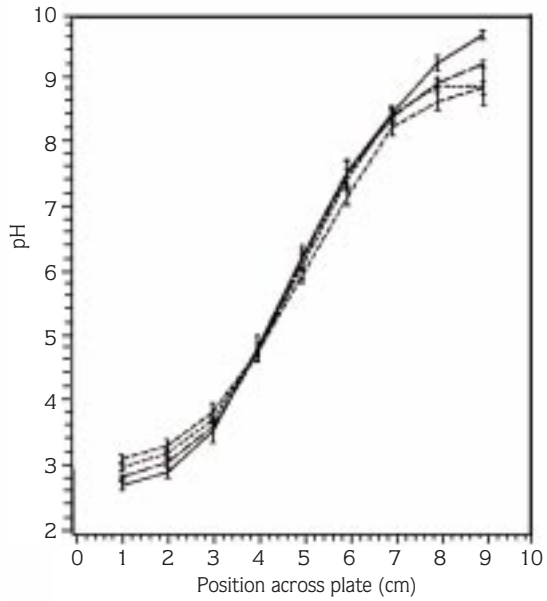


Figure 1. pH gradient across a two-dimensional gradient plate (—, day 1;— —,day 3; - - - - -, day 5; — . — . , day 7) (n=3, bars are standard error of mean).

plate with a No. 3 cork borer, and dissolved separately in 10 ml of boiling distilled water. After cooling, the volume of the solution was adjusted to 25 ml with distilled water or diluted further if necessary. Twenty-five millilitres of the sample solution containing not more than 15 µg of Al was taken and added to 2 ml of 1% ascorbic acid solution (in distilled water). The pH of the sample solution was adjusted to about 2.0, and after 5 min added to 5 ml of the Erichrome Cyanine-R solution (0.1% solution in distilled water, adjusted with HCl to about pH 2.5) and 5 ml of 50% ammonium acetate solution (in distilled water) with stirring. The solution was adjusted to pH 6.1-6.2 with dilute ammonia (in distilled water), the solution being added dropwise. The sample solution was diluted to 50 ml in a volumetric flask with distilled water, and the absorbance measured at 535 nm using a blank solution as reference with a Unicam PS 1800 ultra-violet spectrophotometer (Pye Unicam Ltd., UK). The concentration of Al was determined from a calibration curve, prepared using seven different concentrations of Al solution (between 0 and 40 µg l^{-1} Al), using the same procedure as described above with a blank solution as reference. The actual concentrations were calculated using plug weights (Fig. 2).

Inoculation: Spore suspensions of *Phoma exigua* Desm. and *Cladosporium herbarum* (Pers.) Link were prepared by adding 10 ml of sterile distilled water to 3-wk-old cultures, shaking for 5 min with sterile glass beads to separate spores from their conidiophores, and transferring 5 ml of the supernatant to a sterile McCartney bottle. The suspension was then filtered through sterile muslin (85 µm mesh size) into a sterile McCartney bottle.

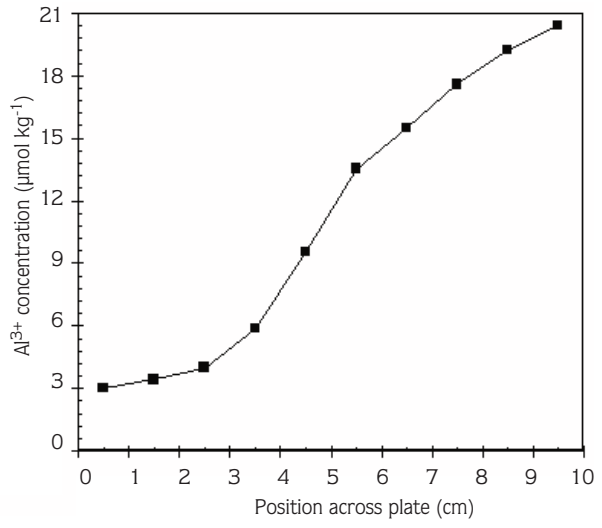


Figure 2. Aluminium concentration across a one-dimensional gradient plates.

The spore suspension (0.5 ml) was added to 4.5 ml half-strength, cool, molten MEA medium in a sterile McCartney bottle. This was shaken gently to distribute the spores, and then poured over the surface of a gradient plate prepared 24 h earlier. To prevent loss of the gradients by lateral diffusion, a polytetrafluoro-ethylene grid, divided into 81 1.1 x 1.1 cm by 1.0 cm deep squares, was inserted in each plate. The plates were incubated at 15°C in darkness, germination being assessed after 24 and 48 h.

One-dimensional pH gradient plates with constant Al concentrations

Preparation: One-dimensional pH gradient plates were prepared with agar media containing one of five different concentrations of $AlCl_3$ (between 0 and 2000 µmol Al kg^{-1} medium). The first and second layers were the same as those above for two-dimensional gradient plates. However, the plates were not sloped when preparing the third and fourth layers. For each one-dimensional gradient plate, the Al concentration was constant on the surface of the medium. The pH gradient of these plates was measured as described above.

Inoculation: Cladosporium herbarum, Colletotrichum dematium (Pers.) Grove, *Coniothyrium quercinum* Sacc. var. *glandicola* Grove., *Cylindrocarpon orthosporum* (Sacc.) Wollenw., *Fusarium avenaceum* (Fr.) Ces., *F. lateritium* Nees, *Mortierella ramanniana* (Möller) Linnem. and *Phoma exigua* were cultured on MEA at 15°C in darkness for 2 wk. The one-dimensional pH gradient plates were inoculated with plugs (4 mm diam.) of mycelia cut from the margin of these fungal colonies using a No. 2 cork borer. Each plate was inoculated with 9 plugs of mycelium placed 1 cm apart along a line parallel to the pH gradient, so that each plug was inoculated in medium of a different pH. Mycelial extension was measured every 24 h.

Statistical analysis

Mycelial extension rate was estimated by linear regression analysis of distance extended with time. Significance of differences in mean mycelial extension rate between fungi grown on different Al concentrations and

pH was assessed by ANOVA (17) and *a posteriori* comparisons made using an S-test (18). Also, interaction, if any, between the effect of pH and Al concentration on fungal extension was investigated by ANOVA.

Results

Effect of pH and three Al compounds on mycelial extension of T. viride

Trichoderma viride extended rapidly between pH 3.0 and 6.0 in the absence of Al and at low Al concentrations (Figs. 3-5). Aluminium chloride had no significant ($P>0.05$) effect at concentrations up to 75 µM, between pH 3.0 and 8.0. At 100 µM $AlCl_3$, low pH became a limiting factor for growth, extension rates being significantly ($P \leq 0.05$) lower at pH 3.0 than at pH 4.0-8.0. The lowest extension rate was at pH 2.0, being significantly ($P \leq 0.001$) slower than at other pH values. With $Al_2(SO_4)_3$ and $Al(NO_3)_3$, pH affected the mycelial extension rate differently. For example, with $Al(NO_3)_3$, there was no significant ($P > 0.05$) difference in the extension rate at pH values between 3.0 and 9.0 and concentrations of Al between 200 and 1000 µM, but the extension rate was significantly ($P \leq 0.001$) lower at pH 2.0. Similarly, with 2000 µM $Al_2(SO_4)_3$, only at pH 2.0 was the mycelial extension rate significantly ($P \leq 0.001$) lower than at other pH values.

Aluminium salts affected the mycelial extension rate of *T. viride* in the order $AlCl_3 > Al(NO_3)_3 > Al_2(SO_4)_3$, and mycelial extension was not significantly affected by the maximum concentration of $Al_2(SO_4)_3$ (2000 µM) at pH values between 6.0 and 9.0 (Fig. 5). Growth occurred at all Al concentrations tested with all three compounds. *T. viride* grew well up to 100 µM Al at all pH values, but mycelial extension rates began to be affected at 200 µM Al. Maximum inhibition by the three compounds occurred at pH 2.0 and inhibition increased with increasing concentration of Al (Figs. 3-5).

Effect of Al^{3+} and pH on mycelial extension

Phoma exigua grew at all Al^{3+} concentrations and pH values tested and was second after *T. viride* in tolerance to pH. Mycelial extension rates were significantly higher ($P \leq 0.001$) at pH 6.4-9.1 than at other pH values (Fig. 6) with all Al concentrations used. The effects of Al^{3+} concentrations on mycelial extension were only significant ($P \leq 0.05$) at pH 4.48.

Growth of *M. ramanniana* occurred at pH 2.5-6.4, with maximum mycelial extension occurring at pH 3.1 and 4.5 (Fig. 7). The pH had a significant ($P \leq 0.001$) effect on mycelial extension at all Al concentrations tested, but Al did not significantly ($P > 0.05$) inhibit the extension rate at any of the pH values between 2.5 and 6.4.

Cladosporium herbarum grew at pH values between 2.5 and 4.5, with the maximum extension rate at pH 3.1 (Fig. 8). The most significant effects were at the lower Al concentrations (0.125 and 0.25 µM; $P \leq 0.001$), the effect of pH decreasing with increasing Al concentration ($P \leq 0.05$). Aluminium had no significant ($P > 0.05$) effect

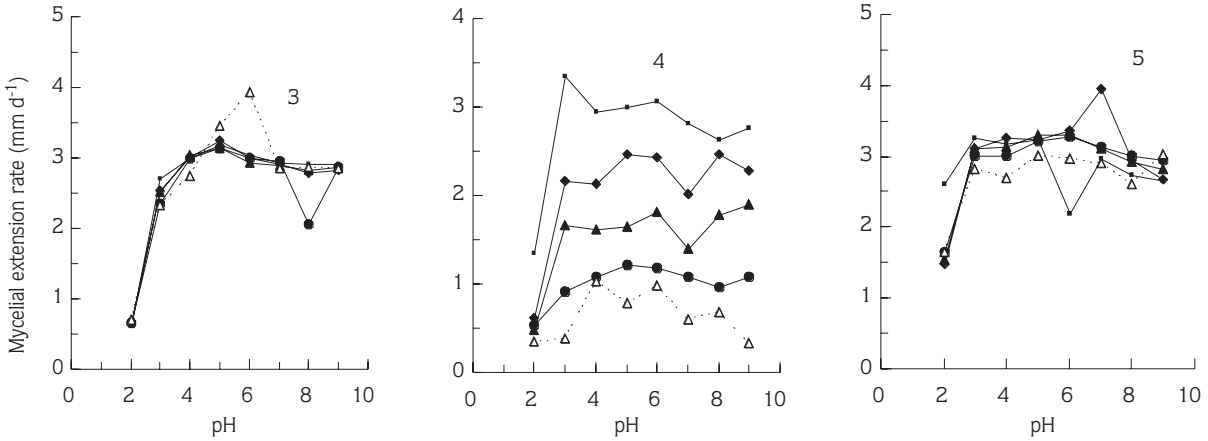
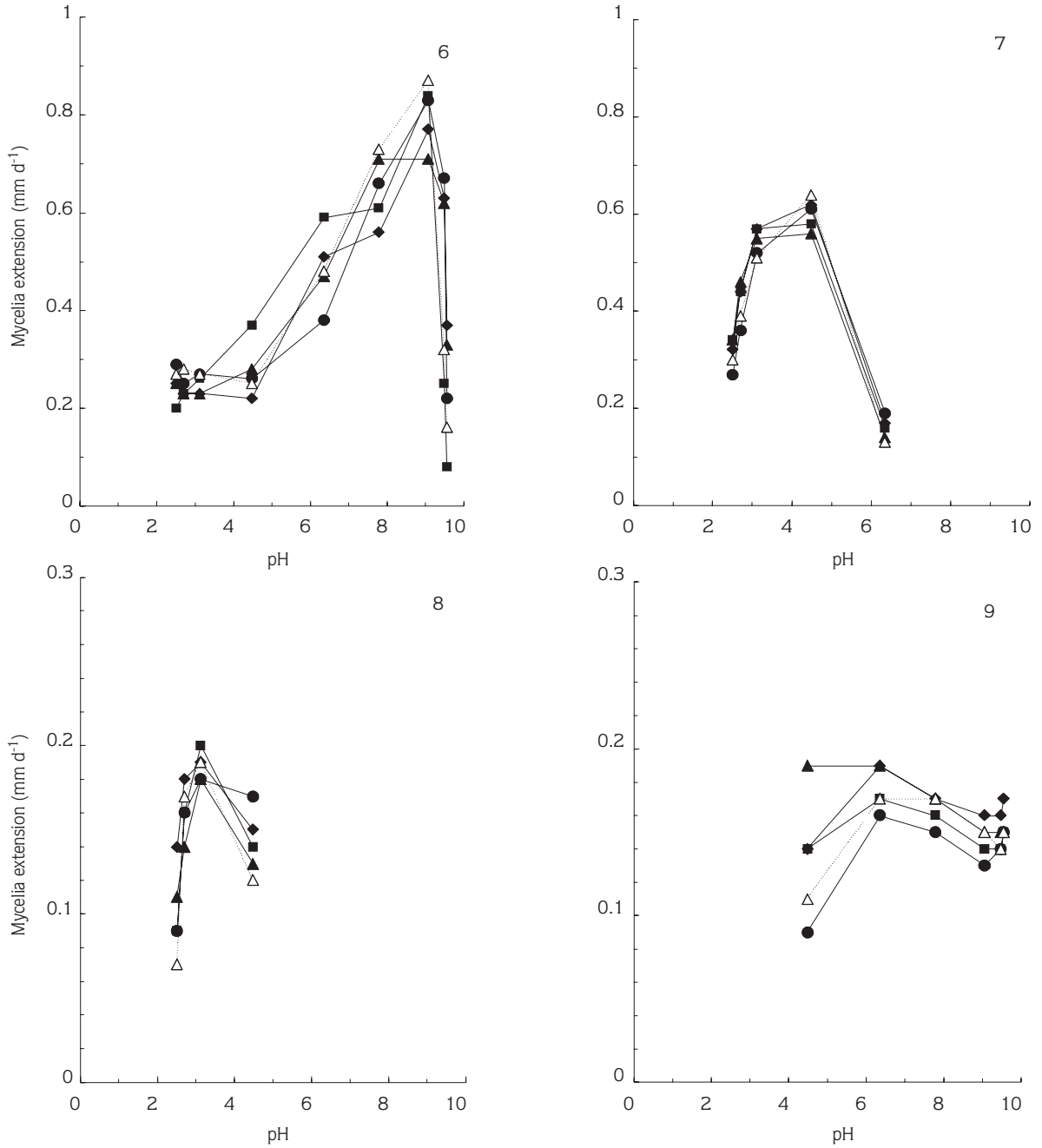


Figure 3. Effects of pH and Al^{3+} concentration on mycelial extension rate of *T. viride* (n=3; standard errors of the means were usually within 10% of the mean). **Fig. 3.** $AlCl_3$ (■, 5; ◆, 15; ▲, 50; ●; 75, Δ, 100 µmols Al Kg^{-1}); **Fig. 4.** $Al(NO_3)_3$, concentrations as in Fig. 1; and **Fig. 5.** $Al_2(SO_4)_3$ (■, 0; ◆, 200; ▲, 500; ●; 1000; Δ, 2000 µmols Al Kg^{-1}).

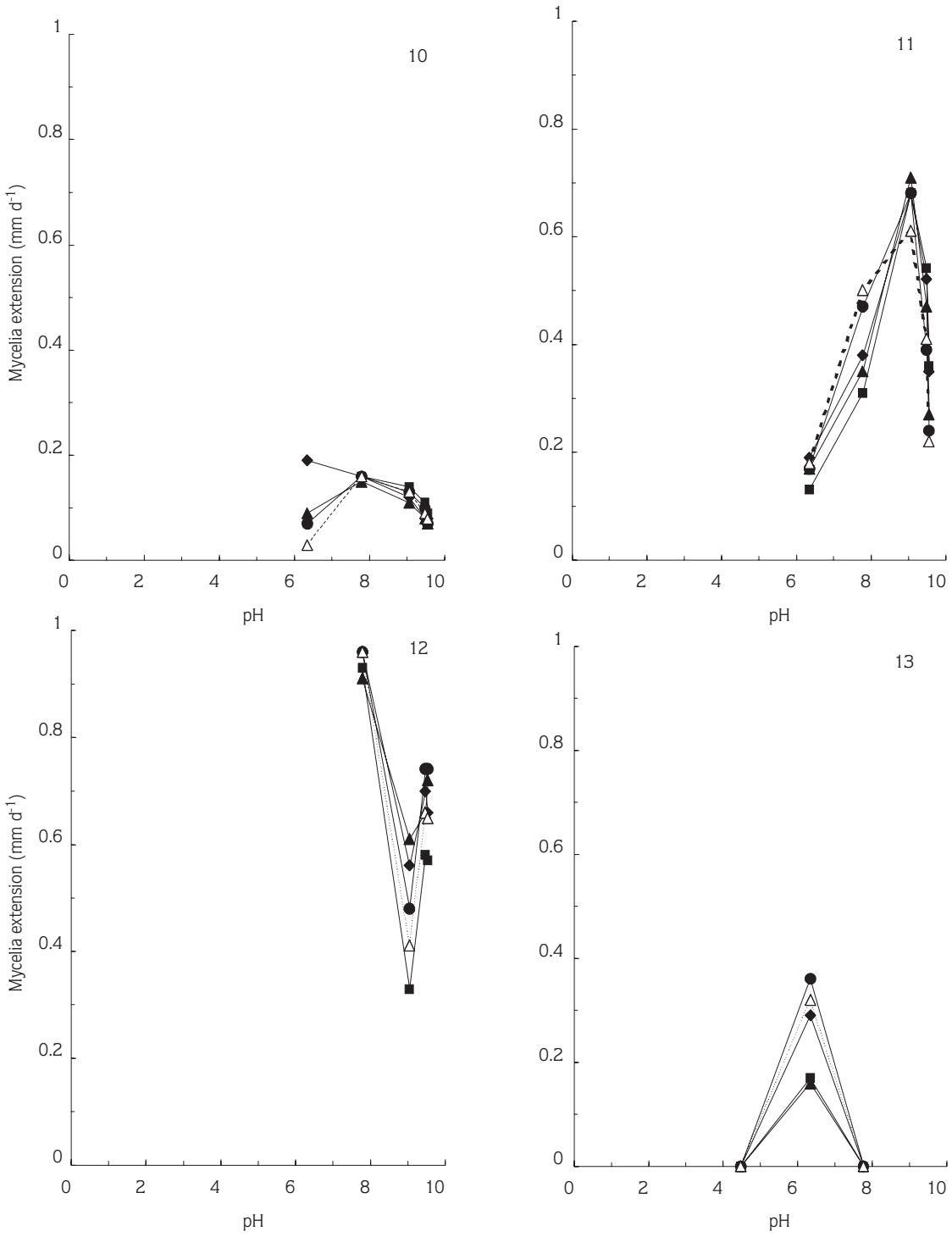


Figs 6-9. Effects of pH (2.5-9.55) and Al^{3+} concentration on mycelial extension rate on pH gradient plates (n=3; standard errors of the means were usually within 10% of the mean). **Fig. 6.** *P. exigua* (■, 0.031; ◆, 0.125; ▲, 0.313; ●, 0.625, Δ, 1.250 µmols Al Kg^{-1}); **Fig. 7.** *M. ramanniana* (■, 0.05; ◆, 0.20; ▲, 0.50; ●, 1.0, Δ, 2.0 µmols Al Kg^{-1}); **Fig. 8.** *C. herbarum* (■, 0.125; ◆, 0.250; ▲, 0.500; ●, 1.0, Δ, 2.0 µmols Al Kg^{-1}); **Fig. 9.** *C. orthosporum* (■, 0.031; ◆, 0.125; ▲, 0.313; ●, 0.625, Δ, 1.250 µmols Al Kg^{-1}).

on mycelial extension at any of the pH values between 2.5 and 4.5.

Cylindrocarpon orthosporum grew between pH 4.5 and 9.6 (Fig. 9). Mycelial extension rates were significantly ($P \leq 0.05$) lower at pH 4.5 than at pH 6.4, but no other significant ($P > 0.05$) differences were detected. Aluminium had a significant ($P < 0.05$) effect on the extension of this species only at pH 7.8.

Mycelial extension of *F. avenaceum* and *C. dematium* was recorded only at pH 6.4 or above (Figs. 10-11). The



Figs. 9-13. Effects of pH (2.5-9.55) and Al^{3+} concentration on mycelial extension rate on pH gradient plates (n=3; standard errors of the means were usually within 10% of the mean). **Fig. 10.** *F. avenaceum* (■, 0.0; ◆, 0.031; ▲, 0.063; ●; 0.125, Δ, 0.250 µmols Al Kg^{-1}); **Fig. 11.** *C. dematium* (■, 0.0; ◆, 0.031; ▲, 0.063; ●, 0.125, Δ, 0.250 µmols Al Kg^{-1}); **Fig. 12.** *F. lateritium* (■, 0.125; ◆, 0.250; ▲, 0.500; ●; 1.0, Δ, 2.0 µmols Al Kg^{-1}); **Fig. 13.** *C. quercinum* (■, 0.031; ◆, 0.63; ▲, 0.125; ●; 0.25, Δ, 1.25 µmols Al Kg^{-1}).

pH had a significant (P ≤0.05) effect on mycelial extension of *C. dematium* at all Al concentrations, but it only affected *F. avenaceum* in the absence of Al. There were no significant (P >0.05) inhibitory effects of Al concentration on mycelial extension of either species, except *F. avenaceum* growing at pH 6.35 (P ≤0.001). *Fusarium lateritium* grew between pH 7.8 and 9.6 with the maximum mycelial extension rate at 7.8 (Fig. 12). The pH significantly (P ≤0.001) affected mycelial extension at all Al concentrations. Al concentration significantly (P ≤0.05) affected mycelial extension at pH 9.1 and 9.5 but not at 7.8 or 9.6.

Coniothyrium quercinum grew only at pH 6.4 at all Al concentrations tested, being the most sensitive to pH of the species tested (Fig. 13). There was a significant (P <0.05) effect of Al on mycelial extension.

There was a highly significant interaction (P <0.001) between pH and Al concentrations on inhibition of mycelial extension of *T. viride, F. avenaceum, F. lateritium*, and *P. exigua* but not (P >0.05) of *C. dematium, C. herbarum, C. orthosporum*, or *M. ramanniana.*

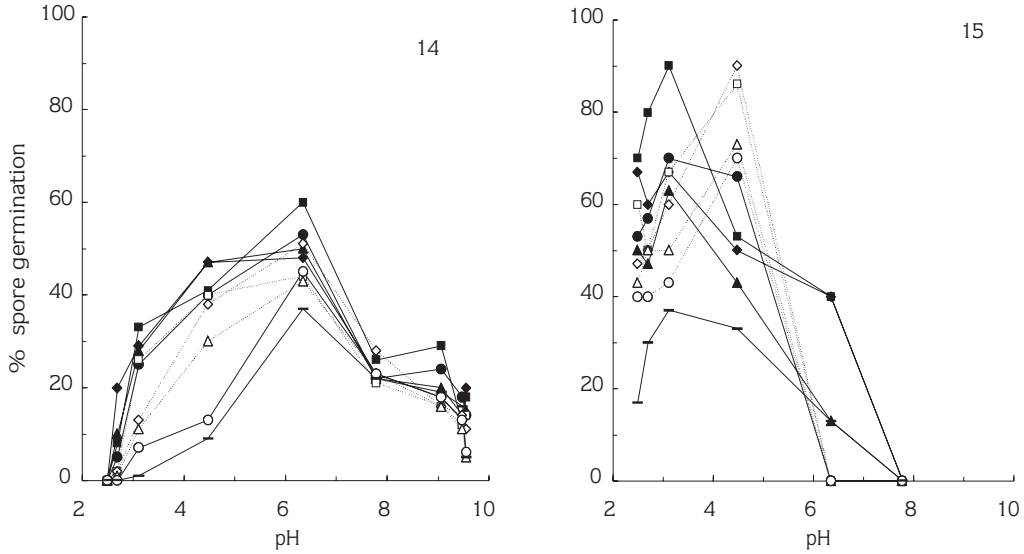
Effects of pH and Al^{3+} concentration on spore germination on two-dimensional gradient plates

The pH had a significant (P ≤0.001) effect on germination of *P. exigua* spores at all Al concentrations tested, germination occurring between pH 3.1 and 9.7 after 2 d, the optimum being between pH 4.5 and 6.4 (Fig. 14). Aluminium significantly (P <0.001) inhibited the germination of spores of *P. exigua* except at pH 7.8 and 9.5 (P >0.05).

Germination of *C. herbarum* spores was more sensitive to pH than that of *P. exigua*, occurring only above pH 6.4 at Al concentrations between 3.27 and 19.83 µM after 48 h (Fig. 15). There were significant (P ≤0.001) effects of Al and pH separately except that of Al at pH 3.1. Also, there were significant (P ≤0.001) interactions between pH and Al in the effects on germination of spores of both *C. herbarum* and *P. exigua.*

Discussion

The results of this study show that pH is an important factor affecting spore germination and growth of decomposer fungi on agar media. The toxicity of Al concentration on mycelial extension was significant at pH values 6 and lower, when fungi were grown on pH gradient plates. It is known that the pH of the natural milieu influences the microbial community of a habitat, and that the pH of soil and decomposing materials is lowered by dry and wet acid deposition (19-21). Bååth *et al.* (22) showed that soil biological activity was significantly reduced by treatment with simulated acid



Figs. 14-15. Effects of pH and Al^{3+} concentration on germination of spores of *P. exigua* **(Fig. 14)** and *C. herbarum* **(Fig. 15)** inoculated onto two-dimensional gradient plates. (■, 3.27; ◆, 3.76; ▲, 4.99; ●; 7.79, ❑, 11.55; ◊, 14.01; Δ, 16.54; ❍; 18.27, ▬, 19.83 µmols Al Kg^{-1}) (n=3; standard errors of the means were usually within 10% of the mean).

rain, and acid treatments have been shown to impair the decomposition of both deciduous and coniferous leaf litters (23,24). Most fungi do not grow at very low pH values (2.0), and mycelial growth and spore germination rates of the fungi tested are very low at pH values below 3.5 (25).

Fungal communities, both species composition and biomass, were strongly affected along a heavy-metal gradient described by Nordgren *et al.* (26). Thornton and Davey (27) found that the growth of *Rhizobium trifolii* strains in culture media was affected by 15-40 µM Al at pH 6.3 and toxicity was higher at pH 4.2 with the same Al concentration. About 500 µM Al was required to inhibit *Aspergillus flavus* growth in the studies of Firestone *et al.* (28). Again, Thompson and Medve (12) found inhibition by three Al compounds (SO_4^{2-}, NO_3^- and Cl^-) of several mycorrhizal fungi, $AlCl_3$ having the greatest effect. Most of the fungi showed significant reductions in growth at 50 ppm, and several cultures ceased growth at 150 ppm Al. In the present investigation, $AlCl_3$ was also the most toxic of the three Al compounds to the growth of *T. viride.* At low pH, the effect of Al concentration was greater than at high pH (i.e. alkaline), and the minimum mycelial extension was found at pH 2.0. The pH was clearly an important factor for mycelial extension of this fungus. The Al concentrations used were higher than in natural conditions, but some fungal growth was observed at all concentrations and *T. viride* appeared to be tolerant of high concentrations of Al.

In contrast to the results with *T. viride* growing on MEA with Al additions, experiments with Al in gradient plates to test the toxicity effects on mycelial extension of some other species did not reveal significant inhibition of mycelial growth. Indeed, growth of some species increased with higher concentrations of Al. This may be evidence that agar media and other chemicals used to prepare pH gradients decreased the inhibitory effect of Al on the growth of the fungi tested. Boddy *et al.* (14) used two-dimensional pH/KCl gradients to investigate the effects of both factors on spore germination. However, a disadvantage to use this two-dimensional gradient technique to determine effect of Al inhibition on fungi is that, it is necessary to use buffers during the preparation of the pH gradient. Firestone *et al.* (28) also suggested that the use of a small volume of test solution in agar does not provide the most sensitive bioassay for Al toxicity, but it does provide a simple method for determining the relative sensitivities of different microorganisms to Al. More realistic effects of low Al concentration could be obtained using soil or decomposing plant litter, because the availability of Al in agar media depends on changing pH values and other components of the media.

The effects of acidification on both terrestrial and aquatic systems are complex and the mechanisms are still poorly understood. The application of fully controlled experiments in terrestrial and aquatic systems is very difficult, and Al ionisation is also complex with a large pH gradient. In recent experiments, non-toxic chemicals were used to control media pH. Some of the species used in this work could be decomposed by the buffer chemicals and this may affect the pH values of media. These fungi were not as strongly inhibited as others. On the other hand, different Al inhibition was found between the fungi used in this work, because uninhibited species may prefer high pH and the toxic effect of Al was not seen with low Al concentrations.

Low concentrations of Al have been shown to inhibit the spore germination of *Alternaria tenuis* and *Botrytis fabae* at pH 5.0 and below (29). Also, germination of *Neurospora tetrasperma* spores was inhibited when exposed to >24 µ mol l^{-1} total Al in either latisol soil extracts (soil:water, 1:1) or artificial media containing various sources of Al (11). In the present study, Al concentration significantly affected the spore germination of both *P. exigua* and *C. herbarum* in a short-term experiment. The toxicity of Al on the spore germination of *P. exigua* and *C. herbarum* appeared to cease above pH 7 and 5 respectively. At the pH values 4-6, solutions of Al salts contain mostly compounds bound to OH^- ions. The significant effect of pH on inhibition by Al was due presumably to the chemistry of Al complexes, the highest toxicity being found at the lowest pH (2.5). However, it is not easy to explain the inhibitory effect at high pH values, where Al may not be in a bioavailable form, with most of the Al probably being bound with organic compounds in the media.

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