Inhibition of Germination and Growth of *Thielaviopsis basicola* by Aluminum

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ABSTRACT

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Soils suppressive to *Thielaviopsis basicola* typically have low pH values and high levels of exchangeable aluminum (Al). A series of experiments was conducted to determine the sensitivity of *T. basicola* to Al. All experiments were conducted in Al-amended carrot agar medium buffered at pH 5.0, except for experiments to determine effects of pH. Isolates of *T. basicola* from suppressive and conducive soils responded similarly to Al in most tests, but where differences were observed, the isolate from a suppressive soil was less sensitive to Al than was the conducive-soil isolate. Germination of endoconidia and chlamydospores of *T. basicola* decreased significantly when exposed to 0.55 or 1.1 meq of Al (1 meq = 9 ppm of Al) compared with Al-free controls. Effects of Al on germination were greatest at pH values <5.6 and at low nutrient levels. Radial growth of *T. basicola* was suppressed on agar amended with 1.1 meq of Al but not on that with 0.55 or 0.27 meq of Al. Development of colonies from chlamydospores was inhibited in agar medium amended with 0.55 or 1.1 meq of Al but not in that with 0.27 meq; colonies also took longer to develop in the presence of Al. $Al(NO_3)_3$ was slightly more inhibitory to germination of endoconidia than were other sources of Al. Radial growth was similar on medium amended with different sources of Al.

Additional keywords: *Chalara elegans, Nicotiana tabacum*.

Thielaviopsis basicola (Berk. & Broome) Ferraris is a soil inhabitant that attacks many important agricultural and horticultural hosts (21), including tobacco (*Nicotiana tabacum* L.). Black lesions on main and lateral roots are diagnostic for the black root rot disease on tobacco and may result in severe root pruning (13). The pathogen overwinters primarily as segmented, pigmented chlamydospores (26) but produces endoconidia in abundance soon after germination of chlamydospores (22) and root infection (5). The role of endoconidia in the epidemiology of the disease on tobacco is unknown.

Black root rot increases in severity on tobacco and other host species as soil pH increases (2,3,6,8,16–18). Soils with pH values >5.6 generally are considered conducive to black root rot, whereas soils with pH values 5.2 or lower are often suppressive (2,6,18). Suppression of black root rot in acid soil does not appear to be caused by pH alone (17,18), since *T. basicola* grows in culture at pH values of 3.3–8 (12) and causes disease in acid soil under conditions of high base saturation (17). In a previous study, soils conducive to black root rot became suppressive to the disease when amended with aluminum (Al) but not when pH was lowered without a subsequent increase in Al (18). The level of exchangeable Al in naturally suppressive field soil and in suppressive Al-amended soils was approximately 1 meq of Al per 100 g of soil or higher; Al concentrations in naturally conducive field soils were typically <0.3 meq (18). These observations led to the hypothesis that Al toxicity suppresses development of black root rot in certain field soils.

The role of Al in the ecology of soil microorganisms has not been studied extensively, and research has been focused mainly on the *Bradyrhizobium*-legume interaction in acid soils (24). Several root diseases, including those caused by *Phytophthora* spp., *Rhizoctonia* spp., and *Verticillium albo-atrum,* are known to be inhibited by Al, although the mechanism for this effect is not yet known (10,19,20). *Sclerotinia sclerotiorum,* a pathogen of sunflowers, was tolerant of high concentrations of Al (20). Al also is a primary factor in soil fungistasis in some acidic Hawaiian soils (9). It is not known whether *T. basicola* is sensitive to Al. This paper reports a series of experiments to determine the effects of Al on spore germination and hyphal growth of *T. basicola.*

MATERIALS AND METHODS

Spore germination. *Endoconidia.* Two isolates of *T. basicola* were used, one from a suppressive soil and one from a conducive soil (18). Suppressive soils are those in which *T. basicola* is present but causes little or no black root rot on susceptible cultivars of burley tobacco (17,18). In conducive soils, black root rot is severe, even at low initial inoculum densities (17).

Working cultures of *T. basicola* were maintained on 5% carrot agar (50 ml of canned Hollywood Carrot Juice [Pet, Inc., St. Louis, MO] and 18 g of agar per liter of deionized water) and routinely restarted from stock cultures to prevent cultural changes in the pathogen. Stock cultures consisted of chlamydospores stored in sterile soil. Agar plugs containing *T. basicola* were transferred to fresh plates of 5% carrot agar and incubated in the dark at 22–25 C for inoculum production. A suspension of endoconidia of each isolate was prepared by gently washing endoconidia from the surface of 1-wk-old colonies into a beaker with sterile deionized water. This procedure did not dislodge chlamydospores or hyphal fragments from the culture.

Three levels of nutrients and four levels of Al were prepared in a factorial treatment design with 0.25, 0.5, or 1% carrot agar amended with aluminum chloride ($AlCl_3$) to a final concentration of 0, 0.27, 0.55, or 1.1 meq of Al (1 meq = 9 ppm of Al). These concentrations of Al approximate those previously observed in titrations of conducive, moderately conducive, and suppressive soils, respectively (18). MES buffer (2-[*N*-Morpholino]ethanesulfonic acid, Sigma Chemical Co., St. Louis, MO) at a concentration of 50 mM and Al from a freshly prepared stock solution of $AlCl_3$ were added to flasks of media after the media had been autoclaved at 121 C for 20 min and allowed to cool to approximately 45 C. Media were poured into petri dishes (9 cm in diameter) after the pH was adjusted to 5.0 with 1 N NaOH or 1 N HCl. Although the suggested buffer range for MES is 5.5–6.7,

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MES was chosen because it does not affect germination, growth, or reproduction of *T. basicola* (H. D. Shew, *unpublished data*). In addition, pH measurements of media taken with a surface reading electrode (Fisher Scientific Co., Pittsburgh, PA) at the beginning and end of each test indicated that MES was an effective buffer at pH 5.0 for the duration of the studies conducted.

A 100-μl aliquot of a spore suspension containing approximately 2×10^4 endoconidia was spread on each of five replicate petri dishes for each isolate × nutrient × Al combination. After a 5- to 6-h incubation period in the dark at 22–25 C, percent germination was determined for the first 50 spores observed on a transect of each petri dish. A spore was considered germinated if the germ tube was equal to or greater than the width of the endoconidium. The test was run twice.

To test the effect of pH and the interactions between pH and Al on endoconidial germination, 16 combinations of Al and pH were prepared in a factorial treatment design by amending 0.5% carrot agar with $AlCl_3$ to a final concentration of 0, 0.55, 1.1, or 2.2 meq of Al per liter. MES buffer, 50 mM, was added, and the pH was adjusted to 4.8, 5.2, 5.6, or 6.0 after autoclaving. These pH values were representative of suppressive (<5.2) and conducive (>5.6) soils (18). Once again, pH measurements with a surface electrode indicated that the pH values of the media were constant over the duration of the tests. A 100-μl aliquot of a spore suspension was spread over three replicate dishes for each pH × Al combination. After incubation in the dark at 22–25 C, spore germination was determined as described above. The test was conducted twice.

The effect of Al source also was determined. Media were prepared by amending 0.5% carrot agar with 50 mM MES buffer and either aluminum nitrate ($Al[NO_3]_3$, Sigma), aluminum chloride ($AlCl_3$, Sigma), or an atomic absorption standard solution of Al in 1% HCl (Sigma) to establish a concentration of 0 or 1.1 meq of Al per liter, as described above. Aluminum sulfate ($Al_2[SO_4]_3$, Sigma) and aluminum potassium sulfate ($AlK[SO_4]_2$, Sigma) also were used in preliminary tests. The pH was adjusted to 5.0 as previously described. MES was an effective buffer at pH 5.0 for all Al sources. A suspension of endoconidia was prepared, and an aliquot of the spore suspension was spread over five replicate petri dishes per treatment combination. Incubation and determination of percent germination were conducted as described above. The experiment was run twice.

Tests also were conducted to determine whether the effect of Al on germination of endoconidia was fungistatic or fungitoxic. Suspensions of endoconidia were collected and washed five times on a millipore filter with sterile water. Spores were washed from filters into sterile test tubes with Al solutions at concentrations of 0, 0.27, 0.55, or 1.1 meq of Al per liter. There were three replicate tubes per treatment. After a 24- or 48-h incubation period in the Al solutions in the dark, aliquots of the spore suspension from each tube were placed on each of two petri dishes of 1% carrot agar (pH 5.0) at 25 C in the dark. Percent germination of endoconidia was determined at 24, 48, and 72 h after placement on the carrot agar by counting 500 spores from each petri dish (a total of 3,000 spores per treatment). The experiment was run three times.

Chlamydospores. A chlamydospore suspension was prepared by gently scraping the surface of 4-wk-old colonies of the conducive-soil isolate of *T. basicola* into a beaker of distilled water. The resulting suspension was washed through nested sieves of 105- and 27-μm pore sizes. Most hyphae were collected on the top sieve, endoconidia washed through both sieves, and chlamydospores were harvested from the bottom sieve. The chlamydospores and hyphal fragments from the 27-μm sieve were suspended in sterile deionized water, sonicated for 60 s on ice to lyse hyphal fragments and separate the chlamydospore chains from residual hyphae, and resieved. Chlamydospores retained on the 27-μm sieve were resuspended in sterile deionized water for no more than 1 h before use.

Agar media containing 12 combinations of nutrients (0.25, 0.5, and 1% carrot agar, pH 5.0) and Al (0, 0.55, 1.1, and 2.2 meq) were prepared. An aliquot of each spore suspension containing approximately 2×10^4 chlamydospores (each chlamydospore chain was considered a single propagule) was spread over each of five replicate dishes for each nutrient × Al combination. After incubation for 24 h in the dark at 22–25 C, percent germination was determined by counting 50 spores per replicate plate. A chlamydospore chain was considered to be germinated if one or more of the segments had germinated. The experiment was run three times.

Vegetative growth. Three replicate 250-ml Erlenmeyer flasks containing 120 ml of 5% carrot juice agar were autoclaved and then amended with $Al(NO_3)_3$ to give a final concentration of 0, 0.27, 0.41, 0.55, or 1.1 meq of Al per liter and adjusted to pH 5.0. Control flasks, also at pH 5.0, were prepared with 0, 39.5, 59, 79.2, or 157.5 mg of calcium nitrate per liter to give the same final concentration of nitrate as in the aluminum nitrate treatments.

A suspension of chlamydospores was prepared from each suppressive- and conducive-soil isolate of *T. basicola*. An aliquot of spore suspension containing about 100 chlamydospores was added to each flask and mixed by gentle swirling. The medium in each flask was dispensed among four petri dishes (approximately 30 ml of medium per dish). After a 2-wk incubation period at 25 C in the dark, the number of colonies of *T. basicola* that grew through the medium to the surfaces of the dishes was determined and summed for the four petri dishes. Treatment means and standard error of the means were calculated from three replicate flasks per test. The test was run three times with each isolate.

The effect of Al source on vegetative growth was determined by amending 0.5% carrot agar with $Al(NO_3)_3$, $AlCl_3$, or an atomic absorption standard solution of Al in 1% HCl to a final concentration of 0, 0.27, 0.55, or 1.1 meq of Al per liter, as described above. The pH was adjusted to 5.0 after adding 50 mM MES buffer. Plugs (1 mm in diameter) were cut from the edges of 1-wk-old colonies of *T. basicola* and transferred to Al-amended media. Five petri dishes were prepared for each Al source × Al concentration combination and arranged in a factorial treatment design. Final colony size was measured after a 7-day incubation period in the dark at 22–25 C. The experiment was conducted twice.

Statistical analysis. All tests were established in a randomized complete block design. Data were analyzed by analysis of variance, GLM, or regression procedures (SAS Institute, Cary, NC), and significant differences were determined by Waller-Duncan k ratio tests ($k = 100$) or by single degree of freedom contrast statements. When possible, results were combined across tests for analysis and presentation.

RESULTS

Spore germination. *Endoconidia.* Germination of endoconidia of both isolates of *T. basicola* was significantly affected by the concentrations of Al ($P < 0.001$) and carrot juice ($P < 0.001$) in the medium, but the effect of Al was dependent on nutrient concentration and isolate (Al × nutrient × isolate interaction, $P < 0.03$; Fig. 1). Inhibition of the suppressive-soil isolate was less than that of the conducive-soil isolate at all Al × nutrient combinations; however, the magnitude of the difference varied, which led to a significant interaction. The greatest inhibition of both isolates occurred at low nutrient levels (0.25 and 0.5% carrot agar) and at Al concentrations of 0.55 and 1.1 meq (Fig. 1). For example, at 1.1 meq of Al and 1.0, 0.5, and 0.25% carrot agar, percent inhibition was 26, 71, and 98% for the conducive-soil isolate and 16, 60, and 90% for the suppressive-soil isolate, respectively.

The pH of the medium ($P < 0.001$) and the concentration of Al ($P < 0.001$) affected germination of endoconidia (Fig. 2), but the level of inhibition by Al was dependent on the pH of the medium (pH × Al interaction, $P < 0.001$). Mean germination across all treatments for the two isolates was similar, 71 and 69% for the conducive- and suppressive-soil isolates, respectively, so data for the two isolates were combined for analysis and presentation (Fig. 2). Germination in the Al-free controls was 69% at pH 4.8 and near 100% at pH 5.6 and 6.0. Inhibition

of germination in the presence of added Al was greatest at pH values of 4.8 and 5.2. For example, germination was significantly inhibited at all concentrations of Al at pH 4.8, at 1.1 and 2.2 meq of Al at pH 5.2, and only at 2.2 meq of Al at pH 5.6 (Fig. 2). Germination was not inhibited at any concentration of Al tested at pH 6.0.

All sources of Al inhibited germination of endoconidia compared with Al-free controls ($P < 0.001$, Fig. 3). Overall, $Al(NO_3)_3$ was slightly more inhibitory than other sources of Al; mean inhibition of germination was 73% for $Al(NO_3)_3$ and 65% for the other sources of Al. The suppressive-soil isolate was slightly less sensitive to Al than was the conducive-soil isolate in these tests, but the effect was not significant, so results were combined for presentation. There were several small but significant interactions of isolate × Al source detected in one run of the experiment; germination of the suppressive-soil isolate was most sensitive to aluminum chloride, whereas the conducive-soil isolate was most sensitive to aluminum nitrate.

Incubation of endoconidia in Al solutions for 24 or 48 h before plating on carrot agar decreased germination from >90% observed in Al-free controls to <1.0% in Al treatments. All concentrations of Al resulted in similar levels of inhibition after 24 h on carrot agar, but small differences were observed after 72 h of incubation. For example, after 72 h on carrot agar, mean germination had increased to 3.2% for spores exposed to Al for 24 h, but germination was still below 1% for spores exposed to Al for 48 h. No germination was observed when spores were exposed to Al solutions for longer than 48 h.

Chlamydospores. Germination of chlamydospores also was inhibited by Al concentration ($P < 0.001$), but as observed with endoconidia, the effect was dependent on the level of nutrient present in the agar medium (Fig. 4). Germination in the Al-free controls also was proportional to the nutrient content of the medium ($P < 0.001$) and ranged from 61% in 0.5% carrot agar to 94% in 2% carrot agar. All concentrations of Al inhibited spore germination in 0.5% carrot agar, but only the treatment with 2.2 meq of Al inhibited germination in 1 and 2% carrot agar (Fig. 4). The suppressive-soil isolate germinated as well or better than the conducive-soil isolate in all Al × nutrient treatment combinations, but the difference was usually small (Fig. 4).

Vegetative growth. The number of colonies that developed from chlamydospores placed in the agar medium was inversely related to Al concentration (Fig. 5). Colonies also took longer to appear on the surface of the agar amended with Al than on unamended agar. Colony development of the conducive-soil isolate was inhibited at 0.41, 0.55, and 1.1 meq of Al but not at 0.27 meq ($P < 0.05$). Colony development of the suppressive-soil isolate was inhibited only at the treatment with 1.1 meq of Al. Calcium nitrate had no effect on vegetative growth of either isolate of *T. basicola.*

Radial growth of *T. basicola* on Al-amended medium was suppressed at Al concentrations of 1.1 meq ($P < 0.01$) but not at lower concentrations. Effects of Al sources on radial growth were similar, and isolates responded similarly to the presence of Al.

DISCUSSION

Spore germination and vegetative growth of *T. basicola* were inhibited in the presence of Al in carrot agar medium. These observations support previous reports that indicate that Al is

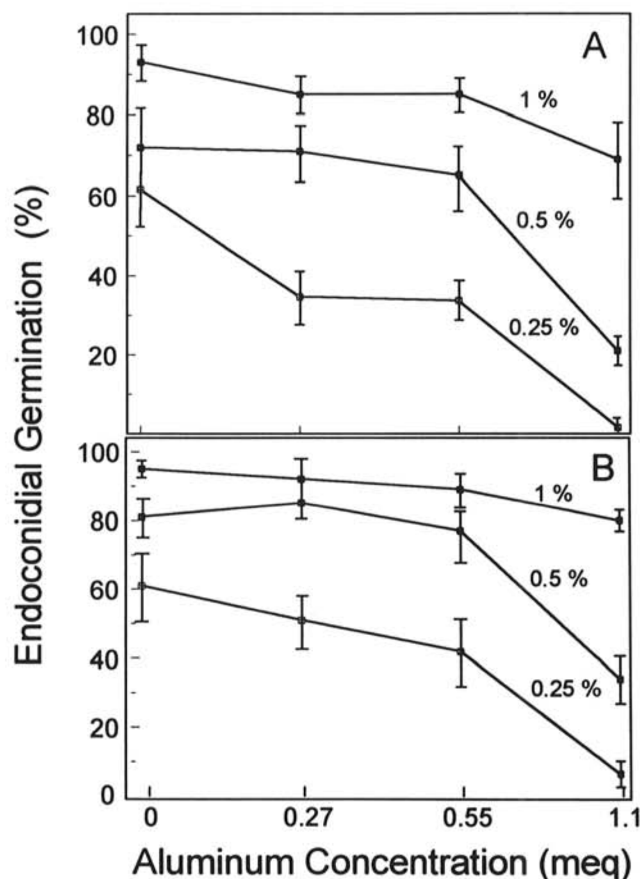

Fig. 1. Effect of aluminum concentration on germination of endoconidia of *Thielaviopsis basicola* on agar amended with 0.25, 0.5, or 1% carrot juice and buffered to pH 5.0. **A,** Isolate from a conducive soil, and **B,** isolate from a suppressive soil. Standard error of the mean is indicated for each concentration.

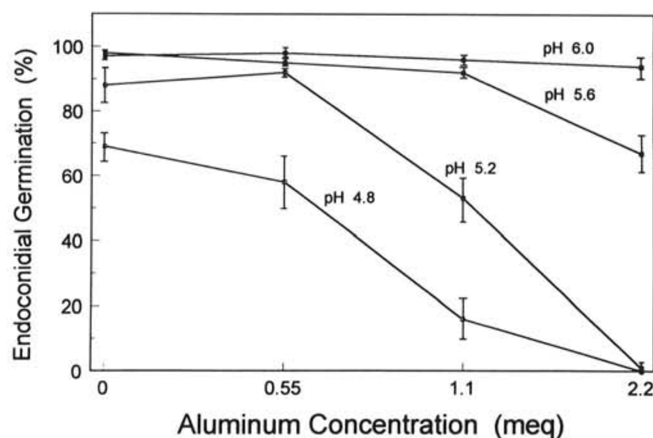

Fig. 2. Effect of aluminum concentration and medium pH on germination of endoconidia of *Thielaviopsis basicola.* Data from the suppressive- and conducive-soil isolates were similar and were combined for presentation. Standard error of the mean is indicated for each concentration.

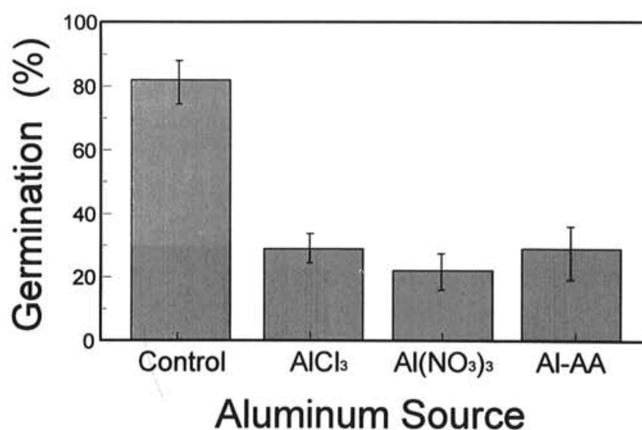

Fig. 3. Effect of aluminum from different sources (all at 1.1 meq) on germination of endoconidia of *Thielaviopsis basicola* on agar amended with 0.25% carrot juice and buffered to pH 5.0. Standard error of the mean is indicated for each source.

toxic to fungal plant pathogens (4,10,19,20). Different sources of Al had similar effects on germination and growth, indicating that Al, and not the associated anions, was responsible for the suppressive effects observed in these experiments. The baseline concentration of Al in the carrot juice used was not determined, but since nutrient level had a slightly positive effect on germination in the Al-free controls, it is unlikely that the carrot juice contained appreciable amounts of Al before amendment.

Propagules of *T. basicola* germinate in response to added nutrients and are sensitive to soil fungistasis; this sensitivity has been exploited for potential biological control of the organism (1,11,25). In these tests, germination of endoconidia and chlamydospores in the presence of Al was dependent on the concentration of nutrients in their environment. Chlamydospores were less sensitive than endoconidia to low levels of nutrients, perhaps because of the higher level of stored nutrients in the larger spores. In all tests with both spore types, germination was most inhibited by Al at low levels of nutrients. The role of added nutrients in the reduction of Al toxicity to *T. basicola* is unknown. The most likely explanation is that other ions or compounds affect the solubility of Al. Many organic compounds and ions in the environment precipitate or chelate Al (23). Some components of carrot juice (e.g., citrates) are very strong chelators of Al. Thus, as carrot juice concentration increased, the concentration of Al^{3+}, the form of Al most commonly recognized as toxic to organisms (23), probably decreased in the agar medium. A second possible nutrient

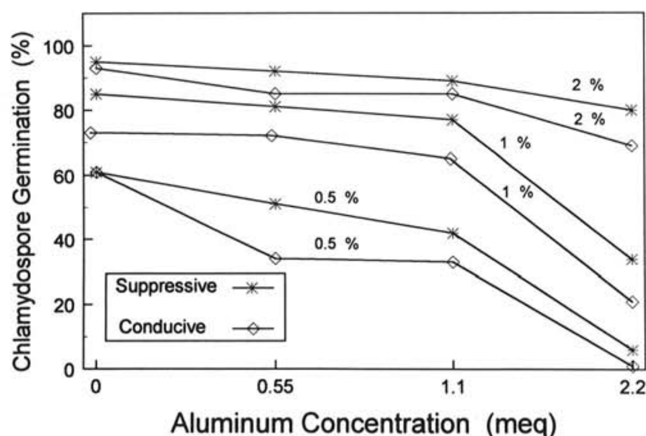

Fig. 4. Effect of aluminum concentration on germination of chlamydospores of *Thielaviopsis basicola* on agar amended with 0.5, 1.0, or 2% carrot juice and buffered to pH 5.0.

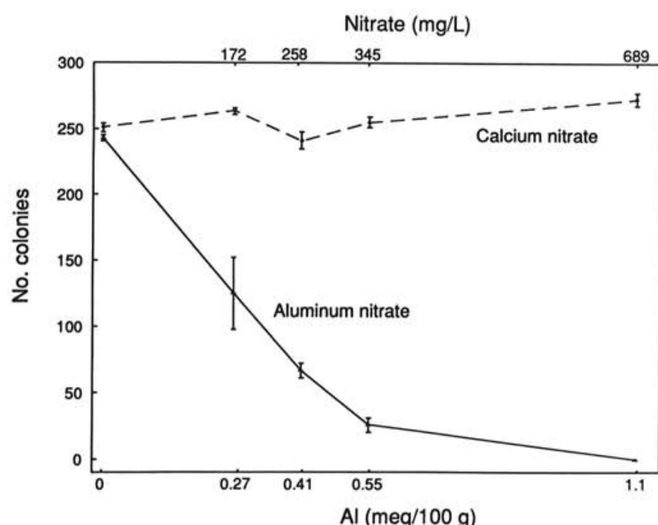

Fig. 5. Colony development of a conducive-soil isolate of *Thielaviopsis basicola* from chlamydospores placed in agar amended with aluminum or calcium nitrate and buffered to pH 5.0. Standard error of the mean is indicated for each concentration.

effect would be a direct physiological effect on germination or growth. Experiments are needed to determine the nature of the observed nutrient effect on sensitivity to Al. In the rhizosphere environment, nutrients such as organic acids, which very effectively precipitate Al, may exert a major influence on the concentrations of Al present in an infection court. This may partially explain the occasional occurrence of lesions on plants grown in suppressive soils (17,18).

An enhanced toxicity of Al in the absence of nutrients is supported by results from tests in which endoconidia were incubated in Al solutions. Endoconidia were much more sensitive to Al when incubated in Al solutions free of nutrients than when placed on Al-amended media with varying concentrations of carrot juice. Even the lowest concentration of Al tested (0.27 meq) almost completely suppressed germination of endoconidia upon removal after a 24-h incubation period in an Al solution free of nutrients. Not all spores were killed after this short incubation; 1–3% germination occurred after 48–72 h on carrot agar. With incubation periods longer than 48 h, no spores germinated on carrot agar following removal from the Al solutions. It appears that Al is fungistatic following short exposures but fungitoxic to *T. basicola* endoconidia following long exposures. High levels of Al in soil solution would thus suppress soil populations of *T. basicola*. A similar effect was recently reported for *Phytophthora parasitica* (4). Cultures obtained from spores exposed to Al were not less sensitive to Al than parental cultures (H. D. Shew, *unpublished data*).

In the absence of Al, a direct effect of pH on germination of endoconidia was observed. These results agree with earlier studies in which germination of endoconidia of *T. basicola* was reduced at low pH values (7,14). Endoconidia germination of nearly 100% at pH 5.6 and 6.0 may partially explain the highly conducive nature of soils with these pH values (13). Mathre and Ravenscroft (14) also observed the highest germination rates of endoconidia and chlamydospores on carrot extract agar at pH >5. However, the direct effect of pH observed in these tests does not appear to be great enough to explain disease suppression observed in acid soils (17,18). Toxicity of Al was much greater at pH 4.8 and 5.2 than at 5.6 and 6.0. As pH decreases, a higher percentage of the Al is in the highly toxic Al^{3+} form (23). As pH increases above 5.0, Al^{3+} makes up a very small proportion of soluble Al; the predominant Al occurs as Al hydroxides. The toxicity of these forms of Al to fungi have not been determined.

Isolates from conducive and suppressive soils responded similarly in most, but not all, tests conducted in this study. In general, where differences were observed, the suppressive-soil isolate was less sensitive to Al than was the conducive-soil isolate. In earlier studies, isolates from suppressive, conducive, and moderately conducive soils did not differ in survival or virulence in a range of soils (18). Although *T. basicola* is present in suppressive soil, the data presented here suggest that these isolates, while somewhat less sensitive to Al, do not have a high level of resistance to Al (18). Investigations into differences among populations of isolates of *T. basicola* from conducive and suppressive soils is warranted.

Results from this study indicate the possible significance of Al in the ecology of *T. basicola* in certain soils. Inhibition of hyphal growth and spore germination by soluble Al in soil could significantly affect the epidemiology of black root rot. *T. basicola* may grow through the soil or rhizosphere to reach the root and typically grows along the root surface (5). Soon after root infection, abundant chlamydospores and endoconidia are formed on the surfaces of infected roots (5,13,15). Results of this and previous studies suggest that the germination of primary and secondary inoculum is inhibited by Al (18).

These results also support the hypothesis that Al can act as a primary fungistatic and even fungitoxic factor in soil. The inhibition of germination and growth of *T. basicola* in the presence of Al may be great enough to partially explain the suppressiveness of acid soils to black root rot. Germination of both spore types and vegetative growth of *T. basicola* were inhibited by Al at concentrations >0.5 meq when pH was <5.2, conditions that

commonly characterize suppressive soils (18). Further studies are planned to explore biochemical mechanisms of Al effects on *T. basicola.*

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