Differential Sensitivity of *Phytophthora parasitica* var. *nicotianae* and *Thielaviopsis basicola* to Monomeric Aluminum Species

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ABSTRACT

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Aluminum (Al) is toxic to many plant pathogens, including *Thielaviopsis basicola* and *Phytophthora parasitica* var. *nicotianae*. Because fungitoxicity of Al has been described in soils over a wide pH range, multiple species of Al may be responsible for pathogen suppression. The goals of this work were to determine the sensitivity of *T. basicola* and *P. parasitica* var. *nicotianae* to Al over a range of pH values, quantify the toxicity of monomeric Al species to production of sporangia of *P. parasitica* var. *nicotianae* and chlamydospores of *T. basicola*, and detect the accumulation of Al in pathogen structures. A complete factorial treatment design was used with Al levels ranging from 0 to 100 μM and pH levels ranging from 4 to 6 in a minimal salts medium. The chemistry of test solutions was modeled using GEOCHEM-PC. Colonies were grown in

5% carrot broth, and after 1 or 2 days, the nutrient solution was removed, colonies were rinsed with water, and Al test solutions were added to each of four replicate plates. After 2 days, propagules were counted and colonies were stained with the Al-specific, fluorescent stain lumogallion. The oomycete *P. parasitica* var. *nicotianae* was sensitive to multiple monomeric Al species, whereas sensitivity of *T. basicola* to Al was pH-dependent, suggesting that only Al^{3+} is responsible for suppression of this fungal pathogen. Chlamydospore production by *T. basicola* was inhibited at pH values <5.0 and Al levels $>20 \mu\text{M}$, whereas sporangia production by *P. parasitica* was inhibited at Al levels as low as 2 μM across all pH values tested. The lumogallion stain was an effective technique for detection of Al in fungal tissues. Aluminum accumulated in sporangia and zoospores of *P. parasitica* var. *nicotianae* and in nonmelanized chlamydospores of *T. basicola*, but not in cell walls of either organism. The differential sensitivity of the two organisms may indicate that true fungi respond differently to Al than members of the oomycota, which are more closely related to plants.

Acid soil chemistry and the associated aluminum (Al) toxicity to both micro- and macroflora have been the subject of many interdisciplinary studies emphasizing the dynamic interactions between the soil chemical environment and biological communities in natural and agroecosystems (12,24). Approximately 40% of the earth's arable lands are located on acid soils, and the phytotoxicity caused by high levels of Al is one of the major factors limiting crop productivity in acid agricultural soils (28). In soils, dissolved Al levels range between 10 and 350 μM Al, with solubility mainly dependant on pH (41). Aluminum is also one of the major factors limiting microbial activity in acid soils. For example, an Al-mediated reduction of soil microbial biomass and nitrogen mineralization has been observed in acid forest soils (19).

In some agricultural soils, abiotic suppression of plant pathogens has been related to the toxicity of Al to soilborne fungi and bacteria. Aluminum-mediated suppression of plant diseases has been reported in a number of pathosystems, including potato scab caused by the actinomycete *Streptomyces scabies* (35), Verticillium wilt of eggplant (17,21,39), Rhizoctonia damping-off (27), root and bulb rots caused by *Fusarium* sp. (15,47), and potato late blight caused by *Phytophthora infestans* (2). Acid soils in North Carolina confer Al-mediated suppression of the tobacco diseases, black shank, caused by *P. parasitica* var. *nicotianae* (syn. *P. nico-*

tianae var. *nicotianae*) (7,43), and black root rot, caused by *Thielaviopsis basicola* (syn. *Chalara elegans*) (31–34). In both pathosystems, suppressive soils had a characteristic low pH and high levels of exchangeable Al, conditions promoting high Al^{3+} activity. Soils suppressive to *T. basicola* have pH values <5.0 , low base saturation ($<70\%$), low calcium levels, and high exchangeable Al levels (1 meq 100 g^{-1} of soil or 1 $\text{cmol}_c \text{ kg}^{-1}$) (32). In vitro growth of *T. basicola* is suppressed with 1.1 $\text{cmol}_c \text{ kg}^{-1}$ of Al, and chlamydospore germination is inhibited at Al levels of 0.55 $\text{cmol}_c \text{ kg}^{-1}$ and higher (33); however, the species of Al responsible for suppression of *T. basicola* have not been determined. Suppression of *P. parasitica* also has been observed in Al-amended, horticultural potting mixes at pH 5.2 and low levels of exchangeable Al (4,5). Further investigation of Al sensitivity of *P. parasitica* in an Al-amended peat system demonstrated suppression of pathogen populations at pH >5.2 and 1 μM Al g^{-1} of peat (14). In the peat system, the predicted dominant monomeric Al species was $\text{Al}(\text{OH})_2^+$, suggesting that Al-hydrolysis species may play a role in toxicity of Al to *P. parasitica*. An additional study on *P. parasitica* incorporated both abiotic and biological pathogen suppression in a horticultural potting medium by amendment with both $\text{Al}_2(\text{SO}_4)_3$ and composted swine waste, respectively (13). In this system, Al-mediated suppression was only observed at $>2 \mu\text{M}$ Al g^{-1} of medium, and abiotic suppression was lost as K-exchangeable Al levels decreased over time. In agar medium, sporangia production of *P. parasitica* var. *nicotianae* was inhibited by 100 μM Al, and radial growth of the pathogen was inhibited by 300 μM Al (46).

Interpretation of published accounts of Al-mediated pathogen suppression across pathosystems is nearly impossible due to differences in soil type and environmental conditions associated with

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open, dynamic systems. In soils, the solubility of Al is controlled by precipitation and dissolution reactions, adsorption onto inorganic minerals, reactions with organic matter, and the formation of soluble complexes (12,41). The processes governing solubility of Al vary between soil systems, thus necessitating the use of closed systems with predicted chemical speciation to elucidate the role of individual Al species in pathogen toxicity. Furthermore, interpretation of the literature on Al toxicity is thwarted by the inappropriate use of units based on equivalents of charge, such as milliequivalents (meq) and centimoles (cmol). Such units should be avoided due to the pH dependence of Al hydrolysis species and charge distribution. Conversely, the use of solutions free of undefined organic substrates (e.g., potato dextrose agar, etc.) that contain defined molar ion concentrations allows for prediction of ionic strength and the activities of all soluble complexes. Soil scientists have recently introduced the use of a constructed acid subsoil solution with predicted chemical species activities to correlate inhibition of lateral root growth of soybean to the sum of monomeric Al species $[Al^{3+}, Al(OH)^{2+}, Al(OH)_2^+, Al(OH)_3^0, \text{ and } Al(OH)_4^-]$ activities (42). The use of a closed system with predictive chemical speciation properties has not been applied to the study of Al toxicity to plant pathogens.

The physiological mechanisms of Al toxicity to soilborne plant pathogens and the relative toxicity of monomeric Al species to these pathogens is unknown. The activities of monomeric Al species are pH-dependant; therefore, the baseline sensitivity of pathogens to Al must be documented over a wide pH range, generally bracketing the conditions where Al-mediated suppression is observed in the field. *T. basicola* and *P. parasitica* var. *nicotianae* were selected for basic toxicological studies because suppression of these pathogens has been observed under diverse soil chemical conditions. Furthermore, although both are pathogens of tobacco, the two organisms are taxonomically unrelated and may exhibit different mechanisms of Al tolerance or sensitivity.

Physiological studies addressing the mechanisms of Al toxicity to plant cells have documented the localized accumulation of Al in root apices using lumogallion (3-[2,4 dihydroxyphenylazo]-2-hydroxy-5-chlorobenzene sulfonic acid), an Al-specific fluorescent stain (18,22,23,44,45,48). Lumogallion also has been utilized to correlate Al accumulation with growth inhibition of soybean roots (23), and to illustrate localized Al accumulation at plant cell nuclei (44). Lumogallion also has been applied to the study of mechanisms of Al tolerance in soybean (45). Application of a technique for fluorimetric detection of Al in pathogen tissue may facilitate an understanding of Al toxicity to microorganisms and the role of Al in disease-suppressive soils.

The overall goal of this project was to investigate the toxicity of Al to two soilborne plant pathogens in a closed system with predicted chemical parameters. Specific objectives included: (i) the determination of baseline sensitivity of *T. basicola* and *P. parasitica* var. *nicotianae* to Al over a range of pH, (ii) the investigation of differential toxicity of monomeric Al species to *P. parasitica* and *T. basicola*, and (iii) the identification of regions of localized Al accumulation in *T. basicola* and *P. parasitica* var. *nicotianae* tissues using a new fluorimetric technique.

MATERIALS AND METHODS

Sensitivity of *T. basicola* to aluminum level and pH. An isolate of *T. basicola* was obtained from diseased roots of pansy (*Viola × wittrokiana*) and used in all experiments. Cultures of the fungus were grown for 4 days on 5% carrot agar, and a spore suspension was collected by rinsing endoconidia from the agar surface with sterile deionized water. A 7-ml aliquot of 5% carrot broth and a 1-ml aliquot of the spore suspension were pipetted into each of 125 petri plates (60 × 15 mm). Nylon mesh, 100 µm pore diameter (Sefar America Inc., Depew, NY), was cut into

4 cm² segments, autoclaved, and one piece of sterile mesh was submerged in the broth medium in each plate. The plates were incubated for 48 h at room temperature to allow for mycelial colonization of the mesh segment. After 48 h, the carrot broth was aspirated off each plate under a laminar flow hood, thus separating the colonized mesh from the nutrient solution, and the colonies were rinsed twice with sterile distilled water. After the water was aspirated off each plate, 8 ml of an Al test solution was added to each plate. Using a complete factorial treatment design, colonies were treated with Al test solutions at five pH levels (4.0, 4.5, 5.0, 5.5, and 6.0) and five Al levels (0, 10, 20, 50, and 100 µM Al), totaling 25 treatments with five replicate plates of each treatment. Colonies were incubated in Al test solutions for 48 h at room temperature, and chlamydospore production was assessed. The number of chlamydospores observed in four randomly selected 10× fields of view (each 1.4 mm²) of an inverted light microscope was counted utilizing the nylon mesh as a grid. Test solutions from replicate plates of each treatment were then combined and the final pH was measured in the bulked solutions. A subset of the mycelial samples was saved for staining with lumogallion. The experiment was run twice.

Aluminum test solutions were prepared with half-strength minimal salts (MS) medium (0.5 g of K₂HPO₄·3H₂O, 0.25 g of KCl, 0.25 g of MgSO₄, 0.005 g of FeSO₄·7H₂O, 0.5 g of (NH₄)₂SO₄, 0.22 g of CaCl₂, and 0.0005 g of thiamine hydrochloride per liter). Because one goal of the project was to document the ability of the pathogen to convert stored resources into resting structures, the MS medium was prepared without a carbon source. The five Al levels were established in the MS background solution by the addition of Al₂(SO₄)₃. Within each of the five Al levels, the five pH levels were established with the addition of 1 N KOH. Aluminum test solutions were filter sterilized through a 500-ml bottle-top filter with 0.2 µm pore diameter.

The program GEOCHEM-PC (40) was used to determine the chemical speciation of Al test solutions. The individual molar concentrations of cations and anions were calculated and recorded as the –log of the ion concentration. For the 0 µM Al control treatments, the –log[Al] was assigned the arbitrarily low value of 12 to avoid undefined functions, a routine practice to stabilize the program for the calculation of ionic strength and speciation of other ions in the system. Thiamine hydrochloride was excluded from the speciation analysis because it is not in the GEOCHEM-PC database and the quantity was negligible with respect to the total amount of Al in the system. Speciation of test solutions was determined for each treatment with pH entered as the initial pH established in test solutions. The initial pH was chosen over final pH for GEOCHEM-PC analysis because the goal was to assess Al sensitivity of the pathogen upon the removal of nutrients from the system (i.e., an introduced stress). In the GEOCHEM-PC analysis of test solutions, simple solid phases were allowed but not imposed, the pH was fixed, the convergence criterion was 1.00E-04, the maximum number of iterations was 50, and the input of estimated ionic strength was 3.600E-02. The activities of Al^{3+} , $Al(OH)^{2+}$, $Al(OH)_2^+$, $Al(OH)_3^0$, and $Al(OH)_4^-$ were recorded for each treatment and the sum of these activities was used to create the variable ΣAl_{mono} . Activity is a unitless value representing the effective concentration of an ion in solution, with its highest value equal to the molar concentration at infinite dilution. Activity is empirically related to the ionic strength (I) of a solution where $I = 1/2 \Sigma c_i z_i^2$ and c_i and z_i represent the molar concentration and valence of each ion, respectively. For input into SAS (SAS Institute, Cary, NC), activities of Al-hydrolysis species in the 0 µM Al treatments were given an arbitrarily low value of 7.04×10^{-14} , then all species activities were multiplied by 10^8 for simplification. A stepwise regression was used to correlate spore production with the activities of monomeric species within each pH level. Only monomeric Al species at the 0.15 significance level or higher were entered into the regression model. This statistical

technique was not employed to distinguish differences across pH levels because of the intrinsic correlation of Al species activities with pH. An analysis of variance, however, was used to view main effects and interactions of pH and Al level on chlamydospore production.

Sensitivity of *P. parasitica* var. *nicotianae* to aluminum level and pH. A race 0 isolate of *P. parasitica* var. *nicotianae* was used in all tests. This isolate was obtained from infested soil from a tobacco field in Duplin County, NC, and was provided by M. Sullivan, N.C. State University, Raleigh. After growth for 1 week on oatmeal agar (Difco Laboratories, Detroit), the mat of aerial hyphae was scraped from the agar surface with a surface-sterilized, double-edge razor blade. The hyphal mats from four petri plates were placed in a sterile blender with 500 ml of sterile deionized water and the mixture was blended for 10 s. A 1-ml aliquot of the blended hyphal suspension was pipetted into each of 100 petri plates (60 × 15 mm), along with 7 ml of 5% carrot broth and the addition of a 4 cm² nylon mesh segment. Colonies of *P. parasitica* var. *nicotianae* remained in the carrot broth for 24 h at room temperature and were then rinsed twice with sterile deionized water. The water was aspirated from each plate and 8 ml of an Al test solution was added. Using a complete factorial treatment design, colonies were exposed to Al test solutions at four pH values (4.0, 4.5, 5.0, and 5.5) and five Al levels (0, 2, 5, 10, and 33 μM Al), with five replicate plates prepared for each treatment. Aluminum test solutions were prepared in the same manner as above except the (NH₄)₂SO₄ was excluded from test solutions. Colonies were incubated in Al test solutions for 48 h at room temperature, and then sporangia production was assessed. The number of sporangia observed in four randomly selected 10× fields of view (each 1.4 mm²) was recorded for each replicate plate. The Al test solutions from replicate plates were then combined within each treatment and the final pH was measured in the bulked solutions. A subset of mycelium was saved for staining with lumogallion. The experiment was run twice.

GEOCHEM-PC was used to model Al test solutions used in the sporangia production experiment. An analysis of variance was used to determine main effects and interactions of Al level and pH, and treatment differences were determined with a Waller-Duncan *K*-ratio test (*K* = 100).

Dose-response curve for *P. parasitica* var. *nicotianae*. Because of the high sensitivity of *P. parasitica* to Al, a dose-response study addressing the effect of Al level on sporangia production was conducted at pH 4.5. A 1-ml aliquot of a hyphal suspension and

7 ml of 5% carrot broth were pipetted into each of 20 petri plates (60 × 15 mm). After incubating for 24 h at room temperature, the cultures were rinsed twice with sterile distilled water and then bathed for 48 h in Al test solutions. Aluminum test solutions were prepared in half-strength MS medium excluding glucose and (NH₄)₂SO₄, and adjusted to 0, 0.2, 0.5, and 1.0 μM Al with the addition of Al₂(SO₄)₃. The pH of test solutions was adjusted to 4.5 with the addition of 1 N KOH before filter sterilization. After colonies were bathed 48 h in test solutions, the number of sporangia observed in four randomly selected 10× fields of view under an inverted light microscope in five replicate plates of each treatment was recorded. The experiment was run twice and an analysis of variance was used to determine main effect of Al level, and treatment means were separated using a Waller-Duncan *K*-ratio test (*K* = 100).

Duration in carrot broth and sensitivity of *P. parasitica* var. *nicotianae* to aluminum. A study was designed to determine if the duration in carrot broth affected sensitivity of *P. parasitica* var. *nicotianae* to Al. In this experiment, a 1-ml aliquot of a hyphal suspension was placed in each of 180 petri plates (60 × 15 mm) with 7 ml of 5% carrot broth. The carrot broth was aspirated off plates after 24, 48, or 72 h. Upon removal of carrot broth, colonies were rinsed twice with sterile distilled water, and then the water was aspirated off plates and 8 ml of an Al test solution was added to each plate. Test solutions were prepared as in the aforementioned *P. parasitica* var. *nicotianae* studies; however, in this experiment, test solutions were prepared at four pH values (4.0, 4.5, 5.0, and 5.5) and three Al levels (0, 2.0, and 33 μM Al). Five replicate plates were prepared for each treatment and the experiment was run twice. Sporangia production was determined as described above, and the main effects of pH and Al level were assessed for each incubation time using an analysis of variance. Treatment means were separated using a Waller-Duncan *K*-ratio test.

Fluorimetric aluminum detection in pathogen tissue. The accumulation of Al in vegetative and reproductive structures of *T. basicola* and *P. parasitica* var. *nicotianae* was observed using lumogallion, an Al-specific, fluorescent stain. After chlamydospores or sporangia were counted, colonies were separated from Al test solutions and stained with lumogallion in petri plates (60 × 15 mm). Colonies from control treatments were used to differentiate any background fluorescence from the Al-related fluorescence in colonies that had been exposed to the metal. The lumogallion staining technique was modified from a procedure used to

Fig. 1. Chlamydospore production of *Thielaviopsis basicola* colonies grown 48 h in 5% carrot broth, rinsed with distilled water, and incubated 48 h in Al test solutions. **A** and **B** represent runs 1 and 2 of the experiment. Error bars represent standard error.

observe Al accumulation in soybean roots (44). A 10 mM citrate solution was adjusted to pH 4.5 and 8 ml of the solution was added to each plate to remove apoplastic Al. Colonies were bathed in citrate for 30 min at 25°C on a rotary shaker at 75 rpm in the dark. The citrate solution was then aspirated off plates and 8 ml of deionized water was added to each plate. Colonies were bathed in the water for 15 min, and then the water was aspirated off each plate. An 8-ml aliquot of a 0.2 M sodium acetate buffer (pH 5.2) was added to each plate, and colonies were bathed in this solution for 15 min at 25°C. Buffer was then aspirated off the plates and a 4-ml aliquot of fresh buffer was added to each plate along with 4 ml of lumogallion (Molecular Probes, Eugene, OR) stock solution (10 mM lumogallion in 0.2 M sodium acetate buffer at pH 5.2) and allowed to incubate for 60 min at 50°C on a rotary shaker at 75 rpm in the dark. Hyphae were then mounted in glycerol on glass slides and stored in the dark. Metal tools were rinsed with 20% (vol/vol) HNO₃ prior to use to avoid trace contamination with Al, and lumogallion stock solution was stored in the dark. Slides were viewed within 2 h of preparation under an

Axiophot microscope (Carl Zeiss, Thornwood, NY) that was equipped for epifluorescence microscopy with an HBO 100-W/2 mercury burner and G365 nm exciter; LP420 nm barrier fluorescence filters.

RESULTS

Sensitivity of *T. basicola* to aluminum level and pH. There was a significant interaction of pH and Al level ($P \leq 0.01$) on chlamydospore production by *T. basicola* over both runs of the experiment (Fig. 1). Aluminum sensitivity was consistently observed at pH 4.0 and 4.5 in treatments containing $\geq 20 \mu\text{M}$ Al. In the first run of the experiment (Fig. 1A), chlamydospore production was similar across all treatments in the higher pH range (pH 5.0 to 6.0); whereas in the second run (Fig. 1B), chlamydospore production was higher in the control treatment than in all Al treatments at pH 5.0 and 5.5.

The pH of Al test solutions increased after 48 h incubation of *T. basicola* colonies (Table 1). For example, Al test solutions with an initial pH of 4.0 and 4.5 equilibrated at pH values between 5.3 and 5.9 after incubation of the pathogen. Al test solutions with an initial pH of 6.0, however, did not exhibit a significant pH change after pathogen incubation.

Thermodynamic modeling indicated that Al test solutions at pH 4.0 and 4.5 exhibited a sequential increase in predicted monomeric Al species activities (Fig. 2) with an increase in Al level. Aluminum test solutions at pH 5.0 (Fig. 2) reached the threshold of Al solubility by 50 μM Al ($-\log(\text{Al}^{3+}) = 6.9$). Aluminum test solutions at pH 5.5 and 6.0 were saturated with Al at the 10 μM Al level ($-\log(\text{Al}^{3+}) = 8.40$); therefore, higher concentrations of Al did not result in a change in monomeric Al species activities. Similarly, the sum of monomeric Al species activities ($\Sigma\text{Al}_{\text{mono}}$) increased sequentially with Al level (0 to 100 μM Al) at pH 4.0 and 4.5 (Fig 3). Across all Al test solutions, the highest predicted $-\log(\Sigma\text{Al}_{\text{mono}})$ was 5.67 and this was in the pH 4.0 test solution

TABLE 1. Final pH^y of Al test solutions^z after 48 h incubation of *Thielaviopsis basicola*

Al level	Initial pH				
	4.0	4.5	5.0	5.5	6.0
0 μM	5.45	5.90	5.65	5.65	6.15
10 μM	5.85	5.70	5.75	5.80	6.15
20 μM	5.55	5.90	5.90	5.85	6.10
50 μM	5.35	5.90	5.25	5.80	5.65
100 μM	5.55	5.45	5.65	5.70	5.90

^y Final pH values were averaged over two experimental runs because differences within treatments were never greater than 0.1 pH units.

^z Aluminum test solutions were prepared in a minimal salts medium amended with Al₂(SO₄)₃. Colonies were incubated in test solutions at 25°C in the dark.

Fig. 2. Distribution of predicted monomeric Al species activities in Al test solutions used for study of chlamydospore production of *Thielaviopsis basicola*. Al species activities were predicted using GEOCHEM-PC (Parker). A, B, C, and D represent Al test solutions at pH 4.0, 4.5, 5.0, and 5.5, respectively. (Al³⁺ = Al³⁺, Al²⁺ = Al(OH)₂⁺, Al¹⁺ = Al(OH)₂⁰, Al⁰ = Al(OH)₃⁰, Al⁴⁻ = Al(OH)₄⁻).

containing 100 μM Al (Fig. 2). The ionic strengths of Al test solutions were calculated by GEOCHEM-PC and values ranged from 2.55×10^{-2} to 2.60×10^{-2} . No correlation between calculated ionic strength and chlamydospore production was observed.

The stepwise regression analysis was used to assess the relationship of individual monomeric Al species activities with reduced chlamydospore production of *T. basicola* within each pH level. The only species of Al entered into the regression model over both experimental runs was Al^{3+} , and this was only significant at pH 4.0 and pH 4.5 ($R^2 \geq 0.28$).

Sensitivity of *P. parasitica* var. *nicotianae* to aluminum level and pH. Sporangia production of *P. parasitica* var. *nicotianae* was reduced by at least 80% after exposure to $\geq 2.0 \mu\text{M}$ Al (Fig 4). Because there was no significant interaction of treatments and experimental runs, data were combined over the two runs to illustrate both a significant Al effect ($P \leq 0.0001$) and a pH effect ($P \leq 0.05$). The pathogen's response to Al was similar across pH levels, but sporangia production in colonies treated with 0 and 2.0 μM Al was approximately doubled at pH 4.5 compared with similar treatments at pH 4.0 (Fig. 4).

The pH of Al test solutions increased slightly after the 48 h incubation of *P. parasitica* var. *nicotianae* colonies (Table 2). Aluminum test solutions with an initial pH of 4.0 equilibrated at pH values between 4.11 and 4.84. Test solutions with an initial pH

Fig. 3. Sum of monomeric Al activities ($\Sigma\text{Al}_{\text{mono}}$) in Al test solutions used for study of chlamydospore production of *Thielaviopsis basicola*. Monomeric Al species activities were predicted using GEOCHEM-PC, then summed to form the $\Sigma\text{Al}_{\text{mono}}$ variable.

Fig. 4. Sporangia production of *Phytophthora parasitica* var. *nicotianae* grown 24 h in 5% carrot broth, rinsed twice with sterile distilled water, and incubated 48 h in Al test solutions. Data were combined over two runs of the experiment, and error bars represent standard error.

of 5.5 increased to values between 5.71 and 6.14. No definitive pH trends across Al levels were observed.

Aluminum test solutions used to study sporangia production of *P. parasitica* var. *nicotianae* remained unsaturated with monomeric Al species, except in the pH 5.5 test solutions. The activities of monomeric Al species were the same in pH 5.5 test solutions at 5, 10, and 33 μM Al levels, with the $-\log(\Sigma\text{Al}_{\text{mono}}) = 7.29$. Because sporangia production was affected by Al across a wide range of pH values, sporangia production data could not be examined using a stepwise regression model. Furthermore, the predicted ionic strength of test solutions used to study sporangia production in *P. parasitica* var. *nicotianae* was essentially invariant (1.7×10^{-2}).

Dose-response curve for *P. parasitica* var. *nicotianae*. Because a greater difference in sporangia production was observed between Al-treated and the no-Al control treatment at pH 4.5 (Fig. 4), a dose-response curve was generated at pH 4.5 to illustrate the effect of Al level on sporangia production (Fig. 5). In the first run of the dose-response experiment (Fig. 5A), the threshold for detection of reduced sporangia production ($P \leq 0.05$) was observed at 0.5 μM Al (75% reduction). In the second run of the dose-response experiment (Fig. 5B), the threshold for detection of reduced sporangia production was observed at 0.2 μM Al (56% reduction).

Duration in carrot broth and sensitivity of *P. parasitica* var. *nicotianae* to aluminum. Colonies of *P. parasitica* var. *nicotianae* grown for 24, 48, or 72 h on carrot broth all responded similarly to Al test solutions (Table 3). Sporangia production was generally reduced in Al test solutions of 2 μM Al and higher. In this experiment, pH did not have a significant effect on sporangia production; therefore the average number of sporangia produced was calculated and presented across pH values (Table 3). Although sporangia production was not statistically evaluated across durations in carrot broth, it is worth noting that more sporangia were produced in colonies grown for 24 h in carrot broth than those grown for either 48 or 72 h in carrot broth.

Fluorimetric aluminum detection in pathogen tissue. Lumogallion stain successfully detected Al accumulation in both *T. basicola* and *P. parasitica* var. *nicotianae* after colonies were bathed in Al test solutions. Aluminum accumulation was observed in colonies of *T. basicola* grown in a pH 4.0 test solution at 20 μM Al (Fig. 6A and B). Comparison of photographs under light microscopy with the same field of view under fluorescence illustrated localized fluorescence in basal cells of chlamydospores and in more hyaline cells of chlamydospores. The stain also detected Al in *P. parasitica* var. *nicotianae* exposed to 20 μM Al at pH 4.0 (Fig. 6C and D) and 2 μM Al at pH 5.5 (Fig. 6E and F). Hyphal tips of *P. parasitica* var. *nicotianae* (Fig. 6D) exhibited greater

TABLE 2. Final pH of Al test solutions^a after 48 h incubation of *Phytophthora parasitica* var. *nicotianae*

Al level	Initial pH			
	4.0	4.5	5.0	5.5
Run 1				
0 μM	4.31	5.13	5.40	5.84
2 μM	4.17	4.97	5.37	5.71
5 μM	4.19	5.24	5.35	5.89
10 μM	4.28	4.98	5.44	5.78
33 μM	4.25	4.98	5.62	5.73
Run 2				
0 μM	4.27	5.71	5.98	6.08
2 μM	4.11	5.61	5.88	6.06
5 μM	4.84	5.53	5.95	6.08
10 μM	4.75	5.59	5.79	6.14
33 μM	4.54	5.47	5.77	5.90

^a Aluminum test solutions were prepared in minimal salts medium amended with $\text{Al}_2(\text{SO}_4)_3$. Colonies were incubated in test solutions at 25°C in the dark.

fluorescence than older hyphae. The cell wall of *P. parasitica* var. *nicotianae* did not fluoresce, as is evidenced by the black outline of a sporangium containing zoospores (Fig. 6E).

DISCUSSION

These basic toxicological studies addressed the differential toxicity of monomeric Al species to *T. basicola* and *P. parasitica* and documented the baseline sensitivity of these organisms to the metal. Chlamydospore production of *T. basicola* and sporangia production of *P. parasitica* var. *nicotianae* were both inhibited in the presence of Al, but the conditions mediating toxicity were different for these respective pathogens. *T. basicola* was suppressed at Al levels ≥ 20 μM Al and suppression was pH-dependant, occurring only at pH 4.0 and 4.5. Suppression of *P. parasitica* var. *nicotianae* was pH-independent and occurred at Al levels < 1.0 μM .

The pH-dependent nature of Al-mediated suppression of *T. basicola* supports field studies conducted in North Carolina, Wisconsin, and Connecticut that documented suppression of black root rot of tobacco in acid soils (6,20,32). In general, severity of black root rot is enhanced at soil pH > 5.6 and suppressed in soils with pH < 5.2 (1,3,9,10,34,36). Pathogen-infested soils have been rendered suppressive to black root rot disease after acidification with nitric or sulfuric acid (9), but amendment with phosphoric acid, which reduces Al availability in soils, had no effect on disease severity. Due to the pH-dependant nature of pathogen suppression, the use of acidifying fertilizers has been recommended in fields with a high soil pH and history of the disease (36,37). Soils suppressive to *T. basicola* in North Carolina have a characteristic low pH < 5.0 , low base saturation ($< 70\%$), low calcium levels, and high exchangeable Al levels (32). Raising the pH of these suppressive soils to 6.5 nullified suppression (31), presumably due to the reduced activity of Al at elevated pH levels. In vitro suppression of growth of *T. basicola* has been observed in a dilute nutrient agar medium containing 333 μM Al, and chlamydospore germination was inhibited at 183 μM Al and higher (33). The activity of Al in the test media, however, is unknown and unpredictable because of the presence of unidentified organic acids in the nutrient broth and the potential for complexation of Al with organic acids and agar components.

Suppression of *Phytophthora* sp. has been reported over a range of pH values; however, individual studies have demonstrated a pH-dependent nature of disease suppression. For example, the 1935 study by Lambert and Crandall (29) reported that soil acidifi-

cation with $\text{Al}_2(\text{SO}_4)_3$, reducing pH from > 6.2 to 4.6, reduced wilt disease of black-locust seedlings caused by an unidentified *Phytophthora* sp. Similarly, disease of green pepper caused by *P. capsici* was increased after an acid soil was amended with lime (38). In these studies, a suppressive soil was rendered conducive to disease development upon an increase in pH, suggesting that Al may play a role in fungistasis and disease suppression. The pH-independent nature of suppression in the present study suggests that Al may also have a fungitoxic effect on *P. parasitica* var. *nicotianae*, inhibiting basic physiological processes. Further studies are necessary to determine the role of Al in soil fungistasis, inoculum efficiency, and the mechanisms of Al toxicity to fungi.

Suppression of *P. parasitica* by Al has been documented under diverse soil chemical conditions. For example, disease incidence

TABLE 3. Mean sporangia production of *Phytophthora parasitica* var. *nicotianae* in aluminum test solutions^x after incubation in 5% carrot broth for either 24, 48, or 72 h^y

Al level	No. of sporangia ^z	
	Run 1	Run 2
Duration in carrot broth: 24 h		
0 μM Al	24.9 a	14.2 a
2 μM Al	1.65 b	1.46 b
33 μM Al	0.88 b	1.56 b
Duration in carrot broth: 48 h		
0 μM Al	0.33 a	1.71 a
2 μM Al	0.09 b	1.19 ab
33 μM Al	0.08 b	0.68 b
Duration in carrot broth: 72 h		
0 μM Al	10.78 a	1.72 a
2 μM Al	0.94 b	0.55 b
33 μM Al	0.55 b	0.65 ab

^x Aluminum test solutions were prepared with a complete factorial treatment design composed of three Al levels (0, 2, 33 μM Al) and four pH levels (4.0, 4.5, 5.0, and 5.5).

^y Colonies were grown in 5% carrot broth for either 24, 48, or 72 h before being rinsed and bathed in Al test solutions.

^z The average number of sporangia produced per 1.4 mm^2 of nylon mesh was determined. Each value represents the average number of sporangia produced in four randomly selected 10 $\times$ fields of view under the light microscope over five replicate plates of each treatment. Spore production was averaged across pH for each Al level. Letters represent significant differences ($P \leq 0.05$) in sporangia production across Al levels within each experimental run and duration in carrot broth.

Fig. 5. Dose-response curve relating percent reduction in sporangia production of *Phytophthora parasitica* var. *nicotianae* with Al concentration in Al test solutions at pH 4.5. Colonies were grown for 24 h in 5% carrot broth, rinsed with deionized water, and incubated for 48 h in Al test solutions. A and B represent two runs of the experiment, and error bars represent standard error.

Fig. 6. Light and fluorescent photographs of *Thielaviopsis basicola* and *Phytophthora parasitica* var. *nicotianae* after 48 h incubation in Al test solutions and subsequent staining with lumogallion. **A and B,** Colonies of *T. basicola* were grown for 48 h in 5% carrot broth, rinsed with deionized water, and incubated in an Al test solution at pH 4.0 with 20 μ M Al. **A and B,** Images are of the same field of view, with **A** taken under light and **B** taken under fluorescence. **B,** Note the fluorescence in the hyaline chlamydospore cell (c) and the hyaline basal cell (b). **C and D,** Images were taken of a *P. parasitica* var. *nicotianae* colony grown 24 h in 5% carrot broth before incubation in an Al test solution at pH 4.0 with 20 μ M Al. **C and D,** Images are of the same field of view, with **D** captured under fluorescence. Note the fluorescence of the hyphal tip (ht) in **D**, suggesting a localized accumulation of Al. **E and F,** Images represent a *P. parasitica* var. *nicotianae* colony that was incubated in an Al test solution at pH 5.5 with 2 μ M Al. **E and F,** Images are of the same field of view under light and fluorescence microscopy, respectively. Note the lack of fluorescence in the sporangial cell wall (sw) and the fluorescence of individual zoospores within the sporangium (z) in **F**.

and severity of black shank, caused by *P. parasitica* var. *nicotianae*, has been related to soil pH and soil series (11,25,26, 30,43). Exchangeable Al levels were consistently higher in soils suppressive to black shank of tobacco than in conducive soils in North Carolina (7). Converse to Al-mediated suppression of *Phytophthora* sp. in mineral soils, suppression of *P. parasitica* was observed in horticultural potting mixes at pH values >5.2 and exchangeable Al levels as low as 1 μM Al g^{-1} of peat (14). Although earlier research on Al-mediated suppression of *P. parasitica* in horticultural potting media related the observed toxicity to the Al^{3+} ion (4,5), the predicted prevalence of $\text{Al}(\text{OH})_2^+$ in the Al-amended peat system investigated by Fichtner et al. (14) suggested that multiple species of Al may be responsible for suppression of pathogen populations. In the present study, Al-mediated inhibition of sporangia production was pH-independent, confirming the probability that multiple monomeric Al species are responsible for suppression.

The present study suggests that the relative toxicity of monomeric Al species to *T. basicola* and *P. parasitica* var. *nicotianae* is different for each pathogen. Suppression of *T. basicola* was correlated to Al^{3+} activity in Al test solutions with thermodynamically predicted $-\log(\text{Al}^{3+})$ of at least 6.82. Conversely, sensitivity of *P. parasitica* var. *nicotianae* to Al could not be correlated with the activity of any one monomeric Al species, suggesting that multiple species of monomeric Al are toxic to the pathogen. Similar to *P. parasitica* var. *nicotianae*, Al-mediated inhibition of lateral root length of soybean was closely correlated to the $\Sigma\text{Al}_{\text{mono}}$ (42). Inhibition of tap root length of soybean was correlated with a balance of Ca^{2+} activity and multiple monomeric Al species activities (42). In general, phytotoxicity of Al is mainly related to inorganic monomeric Al in the soil solution (28).

The similarity of Al species toxicity to plants and *Phytophthora* sp. may be due to similar mechanisms of toxicity in these two kingdoms. *Phytophthora* spp. are members of the kingdom Straminipila (8) and are closely related to plants. *Phytophthora* spp. differ from true fungi in part due to their diploid nature, morphology of mitochondrial cristae, cell wall biochemistry, and the flagellate nature of zoospores (16). *T. basicola* is a member of the kingdom Fungi and is not closely related to plants or straminipilous organisms.

The sensitivity of *T. basicola* to Al^{3+} , coupled with the pathogen's ability to increase the pH of test solutions, suggests that the pH change may offer a possible mechanism for amelioration of Al toxicity by the fungus. *P. parasitica* var. *nicotianae* did not alter the pH of test solutions; thus, similar levels of monomeric Al activities were maintained for the duration of incubation. The inability of the pathogen to alter the pH may contribute to its heightened sensitivity to Al. Other acidophilic organisms have exhibited multiple mechanisms for Al insensitivity. For example, *Penicillium janthinellum* excludes Al under conditions with high levels of labile carbon (24). Under these conditions, a net reduction of pH was observed with a concurrent reduction of inorganic Al, suggesting that Al was removed from solution by complexation with secreted organic acids. Under conditions with low levels of labile carbon, however, the pathogen tended to accumulate Al, resulting in a net increase of pH and a concurrent decrease of total Al and total inorganic Al in the system (24). The study of Kawai et al. (24) therefore illustrates that expressed mechanisms of Al insensitivity in a single organism may be influenced by background soil chemical conditions and related to both positive and negative changes in pH.

The lumogallion staining technique documented Al accumulation in tissues of *T. basicola* and *P. parasitica* var. *nicotianae*. Accumulation of Al was observed in *T. basicola* colonies exposed to a pH 4 test solution containing 20 μM Al. *T. basicola* cell walls remained black under fluorescence microscopy, illustrating a lack of accumulation of Al in cell wall tissue. Hyaline cells of chlamydospores exhibited more fluorescence than melanized cells,

suggesting heightened Al accumulation in the hyaline cells. It is uncertain whether the melanin simply masks fluorescence of lumogallion-Al complexes, thus, resulting in an underestimation of the accumulation of Al in these cells. Quantitative determination of Al accumulation in root tips has been achieved by inductively coupled plasma atomic emission spectrometry (44), and lumogallion fluorescence intensity has been positively correlated with quantitatively determined Al accumulation in plant tissue (22).

Aluminum accumulation also was observed in hyphae and sporangia of *P. parasitica* var. *nicotianae*. Because the hyphal tip is the active growing point of the pathogen, the intense fluorescence at hyphal tips suggests the initiation of a potential defense response. No Al accumulation was observed in the cellulose-composed cell walls of *Phytophthora* sp., as evidenced by the lack of a visible sporangial cell wall under fluorescence microscopy. Individual zoospores within a sporangium exhibit high fluorescence intensity. The influence of Al accumulation in zoospores on the viability and inoculum potential of these propagules is unknown.

Accumulation of Al in *P. parasitica* var. *nicotianae* was observed under both low pH (4.0) and high pH (5.5) conditions with Al levels as low as 2 μM Al. Al accumulation in *T. basicola* was only studied at pH 4.0 due to the pH-dependent nature of suppression. Future studies are necessary for detection of Al accumulation under multiple chemical conditions, and for determination of selective localization of Al at the subcellular level. For example, soybean roots have been stained with both 4,6-diamidino-2-phenylindole (DAPI) and lumogallion to identify localized accumulation of Al at plant cell nuclei (44), and Al accumulation in *P. parasitica* was observed in an Al-amended peat system containing complexes of Al with organic matter (12).

Conclusions. Both *T. basicola* and *P. parasitica* var. *nicotianae* exhibit sensitivity to Al; however, the chemical nature of Al sensitivity is different in the two pathogens. *T. basicola* exhibited a pH-dependent sensitivity to Al, with chlamydospore production inhibited at low pH and $\geq 20 \mu\text{M}$ Al. Reduction of chlamydospore production was correlated with Al^{3+} activity in solution. Conversely, sporangia production of *P. parasitica* var. *nicotianae* was inhibited by $\leq 2 \mu\text{M}$ Al over a wide pH range, suggesting that multiple species of monomeric Al are responsible for suppression. The differential toxicity of monomeric Al species to these pathogens suggests that multiple mechanisms of Al toxicity may be responsible for suppression of plant pathogens. The ability of *T. basicola* to increase the pH of its surroundings may in part aid in the amelioration of Al toxicity to the pathogen. The lumogallion staining technique identifies the accumulation of Al in pathogen tissue and may have future application for subcellular investigation of the mechanisms of Al toxicity to microorganisms.

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