Infection, Colonization, and Disease of *Amaranthus hybridus* Leaves by the *Alternaria tenuissima* Group

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ABSTRACT

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With the increased use of *Amaranthus hybridus* as a leafy-vegetable crop in Africa and the recent identification of Alternaria leaf spot on this host in southern Africa, the role of this potentially damaging pathogen was investigated. The goals of this study were to test the pathogenicity of the *Alternaria tenuissima* group, determine how these fungi infect *Amaranthus hybridus* leaves, and examine the colonization pattern within host tissues. Asymptomatic leaves of *Amaranthus hybridus* were collected from two field sites in South Africa. Eight *A. tenuissima* group isolates collected from these leaves were used in inoculation experiments conducted in both greenhouse and growth chamber studies. Scanning electron microscopy revealed *A. tenuissima*–like conidia germinating on leaf surfaces and mycelia entering leaves only through stomata of both field-collected and artificially inoculated leaves. Unwounded, inoculated leaves had no symptoms, and light-microscopy observations of both asymptomatic field-collected and unwounded and inoculated leaves revealed hyphae in mesophyll tissue growing intercellularly with no host cell penetration or host-cell response. Seven of the eight isolates produced brown to black, circular to oval, necrotic lesions only at the wound site of injured and inoculated leaves. These results confirm that isolates of the *A. tenuissima* group can infect and colonize *Amaranthus hybridus* leaves in a manner consistent with other endophytic fungi, and suggest that these fungi can act as latent leaf pathogens when the host is altered by wounding.

Additional keywords: amaranth, asymptomatic persistence, environmental conditions, latent pathogen, smooth amaranthus, wound response

Amaranthus species are dicotyledonous pseudocereals identified as alternative crops with potential for expanded commercial application (15,22). The leaves and seeds of these species are edible and are high in vitamins A and C, calcium, iron, protein, carbohydrates, and lipids (19,22). *Amaranthus hybridus* (common names: smooth amaranthus or amaranth) can grow up to 1.5 m tall, produce a large amount of biomass in a short time, and complete up to six generations per year. This species is a nutritious leafy vegetable well suited for cultivation in semiarid regions throughout the world.

It has been postulated that all living plants contain fungi that live within their tissues without causing any visible symptoms on their hosts. These fungi are called endophytes, and they display varying degrees of association and nutritional interdependence with their host plants, including positive, neutral, or even negative relationships (20,28,35,36). Possible benefits of endophytes to plants can include antagonism to fungal pathogens and a decrease in palatability of plant tissues for herbivorous insects and large animal herbivores. This suggests mutualistic relationships between various plants and their associated endophytic fungi. Some grass endophytes have been shown to provide protection from insects and pathogenic fungi (12,16,28). On the other hand, some endophytic fungi can act as pathogens when induced by certain environmental or nutritional conditions, or by host maturity (35,36). However, if the stressful environmental or host condition never occurs, these fungi complete their life cycle after the host dies, thus never causing disease.

Alternaria tenuissima–like species are both endophytes and latent pathogens of *Amaranthus hybridus*. The *A. tenuissima* group consists of the most common fungi isolated from asymptomatic, surface-disinfested leaves of *Amaranthus hybridus* collected from the field in South Africa (8). This group was represented by more than 85% of the isolates collected from leaves, but was seldom recovered from the leaves of greenhouse-grown plants (less than 0.2%). Brown to black, circular to oval, necrotic lesions were observed on leaves of cultivated, 6-month-old *Amaranthus hybridus* plants in Potchefstroom, South Africa, in 1998; and members of the *A. tenuissima* group were isolated frequently from the lesions (7). In artificial inoculation tests, the *A. tenuissima* group caused leaf-spot symptoms comparable to those observed in the field.

Although small leaf spots might be of minor importance on many vegetable crops, *Amaranthus hybridus* is a leafy-vegetable crop. Even a few small spots can cause complete economic losses. This complete crop loss has only been observed at two sites in South Africa, where nearly every leaf on every plant in two fields had minor to extensive leaf-spot symptoms.

The increased use of *Amaranthus hybridus* as a leafy vegetable necessitates the understanding of the fungi associated with this host and their potential roles as both endophytes and pathogens. The objectives of this study were to: (i) test if isolates of the *A. tenuissima* group can act as latent pathogens, (ii) examine if water stress or wounding stimulates infection/colonization, and (iii) determine how endophytic fungi infect *Amaranthus* leaves and the subsequent colonization pattern within leaf tissues. Field and greenhouse observations and microscopic examinations using both asymptomatic field-collected leaves and artificially inoculated leaves were used to examine the *A. tenuissima–Amaranthus hybridus* relationship.

MATERIALS AND METHODS

Greenhouse pathogenicity experiments. Smooth amaranthus plants were established in 500-ml pots in a soil mix (vol/vol) of 50% sand-loam (10 to 15% clay) and 50% peat moss. Soils were steam-sterilized twice for 1 h at 80°C before planting seeds 10 mm deep. Plants were watered to field capacity daily and were fertilized by adding 50 ml per pot of a 3 g/liter hydroponic nutrient solution (6.5:2.7:13 N:P:K with micronutrients) once a week as a soil drench. Six-week-old plants were transplanted into 2-liter pots containing the same soil mix as the 500-ml pots.

Two weeks after transplanting, plants were supplemented with artificial light to provide a 16-h photoperiod. Photon flux density of the supplemented light averaged 18 $\mu E \cdot s^{-1} \cdot m^{-2}$, with a maximum recorded ambient greenhouse photon flux density of 1,233 $\mu E \cdot s^{-1} \cdot m^{-2}$. The average greenhouse temperature was 25°C during the day and 17°C at night, and the average relative humidity (RH) was 78% during the day and 94% at night. The floors and walls of the greenhouse were sprayed with water

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twice a day, 7 days a week, to maintain the high RH. Plants were placed on a bench flooded with water to allow continuous water contact with the bottom of the pots.

For the first greenhouse experiment, seven replications per combined treatment were used. Combined treatments consist of all combinations of five inoculation treatments (including a mock inoculation), two water treatments, and two high humidity treatments (time in plastic bags). All combined treatments were assigned randomly, and the experiments were repeated in two independent trials 7 weeks apart. Therefore, a completely randomized experimental design was used in the analyses.

Four weeks after transplanting (mid-April and early June for the repetition, 1998), half the plants (70 plants per trial) were placed on stands suspending them above the water (water stressed), and the remaining plants were allowed continuous water contact with the bottom of the pots (non-water-stressed). A pressure bomb (26) was used periodically to measure the mean predawn leaf water potentials (ψ_{PD}) of five randomly selected plants of each water treatment. When the mean ψ_{PD} of the water-stressed plants fell below –2.0 MPa, all plants were watered to field capacity. A ψ_{PD} of –2.0 MPa was reached twice in both trials.

Cultures of four single-spore *A. tenuissima* group isolates (Table 1), collected previously from asymptomatic *Amaranthus hybridus* leaves (8), were grown on V8 juice agar in petri plates for 3 weeks at ambient room temperature (approximately 24°C) in the dark. These isolates were randomly selected. Culture plates then were washed several times with cold, sterile distilled water from flasks on ice. A glass rod was used to agitate cultures with water between each wash. The conidial suspensions, decanted from plates, were filtered through two layers of cheesecloth, collected in cold sterile flasks, and diluted to 10^5 conidia per ml. Noncolonized plates containing V8 juice agar were washed in a similar manner, and the extract was used for controls. Conidial suspensions were maintained in iced flasks for approximately 1 h before inoculation.

After 1 week under the different watering regimes (i.e., water-stressed or non-stressed), conidial suspensions of each of the four isolates and the V8 juice agar extract (control) were applied to five leaves of each of 14 stressed and 14 non-stressed plants. The tops and bottoms of inoculated leaves were saturated with approximately 1.3 ml of conidial suspension (or control) using an atomizer, while covering the remainder of the plant with plastic to prevent cross-contamination. The conidial suspensions also were applied to 1.5% water agar (WA; Oxoid, Basingstoke, England) plates to test conidial germination, which ranged from 96 to 100%. Leaves were allowed to dry between subsequent leaf inoculations on a plant. Plants were then sealed in plastic bags. Metal loops were placed in the pots to suspend the plastic bags above and around the plants. Half the bags (70 plants per trial) from each water treatment were removed after 16 h and the other half after 3 days.

In a second greenhouse experiment, seven replications per combined treatment were used. Combined treatments consist of all combinations of one inoculation or mock inoculation treatment, the two water treatments, and a wounded or nonwounded leaf treatment. All combined treatments were assigned randomly, and the experiments were repeated in two independent trials 7 weeks apart. Therefore, a completely randomized experimental design was used in the analyses.

The second greenhouse experiment was conducted at the same time and in the same greenhouse as the first experiment. The same methods were used with three exceptions: in this experiment, one single-spored isolate (no. 279; Table 1) and the control were applied to four individual leaves (two inoculated and two control leaves) of each of seven water-stressed and seven non-stressed plants per trial; all plastic bags were removed after 3 days; and half of the

Table 1. Origin of the single-spore *Alternaria tenuissima* group isolates used to inoculate *Amaranthus hybridus* leaves[a]

Isolate[b]	Date collected	Geographic origin	Experiment[c]
2	11 April	Bloemfontein	ND
46	11 April	Bloemfontein	D
130	11 April	Bloemfontein	NMD
157	11 April	Bloemfontein	D
224	25 April	Potchefstroom	D
279	25 April	Potchefstroom	NWMD
297	25 April	Potchefstroom	ND
321	25 April	Potchefstroom	D

[a] All isolates were collected in South Africa from individual, asymptomatic *Amaranthus hybridus* leaves in 1997.
[b] Culture collection numbers.
[c] N = isolate was used in the nonwounded greenhouse inoculation experiment. W = isolate was used in the wounded greenhouse inoculation experiment. M = leaves inoculated with the isolate were examined with both the scanning electron and light microscopes. D = isolate was used in the detached-leaf assay experiment.

Table 2. Percentages of inoculated *Amaranthus hybridus* leaves with symptoms and percentages of leaves from which the *Alternaria tenuissima* group was recovered[a]

Experiment Treatment	Symptoms (%)[b]	*A. tenuissima* group recovery (%)[c]
Nonwounded greenhouse experiment		
Inoculated[d], 16 h[e]	0	0
Inoculated[d], 3 days[e]	0	72
Control, 16 h[e]	0	0
Control, 3 days[e]	0	2
Wounded greenhouse experiment		
Wounded[f] and inoculated[d]	82	71
Nonwounded and inoculated[d]	0	73
Wounded[f] controls	0	2
Nonwounded controls	0	0
Detached-leaf assay		
Wounded[f] and inoculated[g]	56	95
Nonwounded and inoculated[g]	0	60
Wounded[f] controls	0	0
Nonwounded controls	0	0

[a] All single-spore isolates were collected in South Africa from individual, asymptomatic *Amaranthus hybridus* leaves in 1997. For the greenhouse experiments, results are pooled across trials and across water treatments since those main effects were not significant. For the detached-leaf assay, results are pooled across trials. Although there were differences between the two trials in lesion size, the trial-inoculation treatment interaction was not significant, indicating a similar relative response among isolates between trials.
[b] Symptoms included dark brown to black, circular to oval, necrotic lesions. Larger lesions had tan centers, and tissues beside the leaf spots remained green.
[c] The presence of *A. tenuissima* group isolates in leaves was confirmed from two surface-disinfested 5-mm-diameter leaf disks.
[d] Inoculated with conidial suspensions at 10^5 conidia per ml.
[e] After inoculation, plants were placed in plastic bags for 16 h or 3 days.
[f] Leaves were wounded with a needle (0.5 mm diameter) at leaf centers (midvein; equal distance from leaf tip and leaf base).
[g] A 5-mm-diameter, colonized potato dextrose agar plug cut from the margin of an actively growing culture was placed mycelium-side-down on the center of the leaf (midvein; equal distance from leaf tip and leaf base).

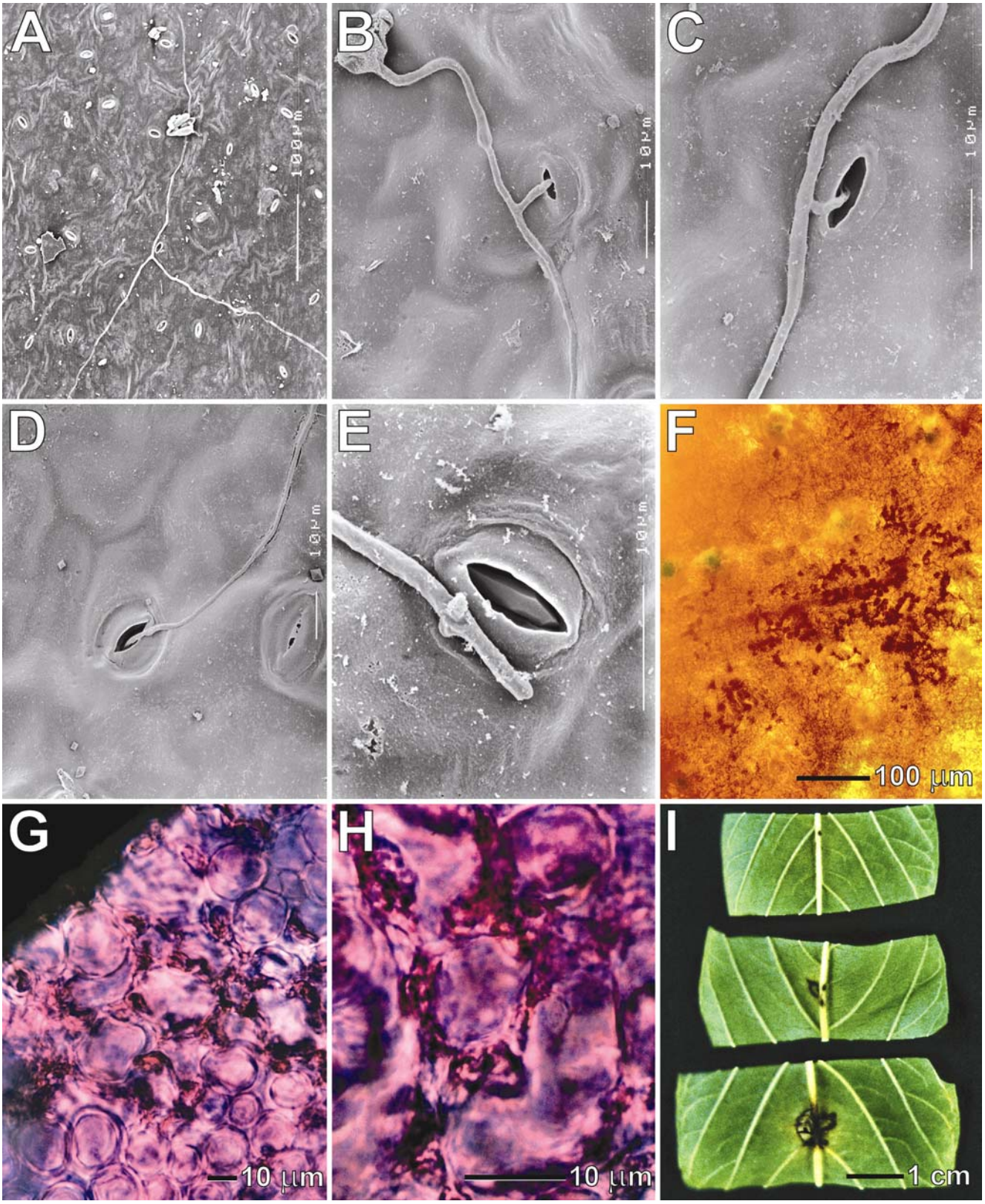

Fig. 1. Scanning electron micrographs of *Alternaria tenuissima*–like conidia germinating on the epidermal surface of *Amaranthus hybridus* leaves collected from **A,** the field, and **B,** an inoculated greenhouse experiment. Scanning electron micrographs of **C,** the 90 degree hyphal branching associated with the *A. tenuissima* group stomatal entry, **D,** direct growth into a stomata, and **E,** the 90 degree hyphal branching with no stomatal entry on *Amaranthus hybridus* leaves collected from an inoculated greenhouse experiment. Light micrographs of the *A. tenuissima* group internal hyphal net around *Amaranthus hybridus* leaf mesophyll tissues collected from the field and **F,** unstained at ×100, **G,** stained with acid fuchsin-malachite green at ×400, and **H,** collected from an inoculated greenhouse experiment and stained with acid fuchsin-malachite green at ×1,000. A camera photo of **I,** the range of symptoms observed on wounded and inoculated greenhouse leaves.

inoculated and control leaves were wounded with a needle (0.5 mm diameter) at leaf centers (midvein; equal distance from leaf tip and leaf base) after the conidial suspension or control was applied. Leaves were allowed to dry between subsequent leaf inoculations on a plant, and before wounding. The isolate was randomly selected.

For both greenhouse experiments, leaf lesions were counted and the diameters were measured (if present) 4 weeks after inoculation. All leaves then were surface-disinfested in a series of 1 min in 96% ethanol, 5 min in a 3.5% NaOCl solution (wt/vol), and 30 s in 96% ethanol. Two leaf disks (5 mm diameter) were then cut from each leaf using a cork-borer. One leaf disk was taken from the leaf center, and the other disk was centered 12 mm lateral to the leaf center. The disks were transferred to WA and incubated at ambient laboratory temperature (approximately 24°C) and light. The presence of the *A. tenuissima* group was confirmed after 21 days from resulting conidia. Leaves inoculated with two of the single-spore isolates (Table 1) and control leaves were saved from the greenhouse experiments for later microscopic observations.

Lesion diameters were analyzed by four-factor analysis of variance with all interactions. Factors used as main effects were: (i) inoculation treatment (isolate and control treatments); (ii) watering regime (water-stressed and non-water-stressed treatments); (iii) trial (trial 1 and 2); and (iv) either the high humidity treatment (16 h or 3 days in plastic bags) for the first greenhouse experiment, or the wounded/nonwounded treatment for the second greenhouse experiment. Lesion diameters were analyzed both untransformed and after natural-log(x + 1) and sqrt(x + 1) transformations were applied. The P values and resulting conclusions were similar for all forms of analysis. Therefore, results are reported only for the untransformed data. ψ_{PD} from the two experiments were analyzed by one-way analysis of variance with watering regime as the factor. Chi-square analysis was used to analyze recovery of the *A. tenuissima* group in relation to watering regime. Analysis of variance (using general linear model procedure) and chi-square analysis were performed using Minitab for Windows, release 10.2 (Minitab Inc., State College, PA).

Microscopic observations. Fields were sampled from two sites (20 km apart) in Bloemfontein, South Africa, in 1997. One noninoculated, asymptomatic *Amaranthus hybridus* leaf was collected from each of 12, 6-month-old plants from each site for later microscopic observations. Our previous study (8) confirmed the presence of the *A. tenuissima* group in noninoculated, surface-disinfested, asymptomatic *Amaranthus hybridus* leaves. The *A. tenuissima* group was isolated from 88% of 1,000 asymptomatic leaf tissues sampled at one of the sites in 1997 (8), and from 86% of 100 asymptomatic leaf tissues sampled at the other site the same year using the same isolation methods (J. T. Blodgett and W. J. Swart, *unpublished*).

For surface observations (infection patterns), leaf pieces (5 × 5 mm) from both the field and the greenhouse experiments were examined using scanning electron microscopy (SEM). Twelve leaf pieces were examined from each of the two fields, and eight leaf pieces were examined from each of the greenhouse experiments for both an inoculated and control treatment. Immediately after collecting, the tissues were fixed in cold (4°C) 3% glutaraldehyde in a 0.1 M sodium-phosphate buffer (pH 7.0). Tissues were fixed for approximately 2 months in the dark at 4°C. The leaf pieces then were dehydrated in a graded ethanol series to 100% ethanol (50, 70, 80, 95, and 2 times at 100%); critical-point dried in liquid CO_2 from 100% ethanol (Polaron Critical Point Dryer Circulatot C400; Polaron Equipment Limited, Watford, England); attached to aluminum stubs with two-way tape; and sputter-coated with gold (SEM sputter coater unit E5000; Polaron Equipment Limited). Leaf pieces were then examined with a scanning electron microscope (JEOL WINSEM JSM-6400 Scanning Microscope; JEOL Limited, Tokyo, Japan) at 5 kV.

For confirmation of fungal mycelium within host tissues (colonization patterns) and also surface observations, leaf pieces (10 × 10 mm) from both the field and the greenhouse experiments were examined using light microscopy. Twelve leaf pieces were examined from each of the two fields, and eight leaf pieces were examined from each of the greenhouse experiments for both an inoculated and control treatment. Immediately after collecting, the tissues were fixed in cold (4°C) ethanol: dichlormethane (3:1 vol/vol) with 0.15% trichloroacetate. Tissues were fixed for approximately 2 months in the dark at 4°C. The leaf pieces then were cleared in nearly saturated chloral hydrate (5:2 wt/vol) and stored in 50% glycerol with trace lactophenol at 4°C in the dark. Leaf pieces were stained for 24 h with acid fuchsin–malachite green (1), which stains fungal mycelia bluish-purple. The stained pieces were placed on glass slides, covered with coverslips, and viewed at ×100, ×400, and ×1,000 using a blue filter (NCB10) mounted before the light source. Fixed, cleared, but unstained leaf pieces also were examined.

Detached-leaf assay. Smooth amaranthus plants were established in 500-ml pots according to procedures described for the greenhouse experiments. Plants were watered to field capacity daily and were fertilized according to procedures described for the greenhouse experiments. Leaves from 6-week-old *A. tenuissima*–free plants were then collected from the greenhouse.

Seven replications per combined treatment were used. Combined treatments consist of all combinations of 10 inoculation treatments (including a mock inoculation and a nontreated control leaf), and either a wounded or nonwounded leaf treatment. All combined treatments were assigned randomly, and the experiments were repeated in two independent trials 8 weeks apart. Therefore, a completely randomized experimental design was used in the analyses.

Eight single-spore *A. tenuissima* group isolates (Table 1) were randomly selected for pathogenicity tests from a previous field experiment (8). Ten leaves were placed in each of 14 sterile, clear plastic, moist chambers (24.5 × 24.5 × 2.5 cm; A/N Nunc, Roskilde, Denmark). For each of the eight isolates, one 5-mm-diameter, colonized potato dextrose agar plug (PDA; Oxoid) cut from the margin of an actively growing culture was placed mycelium-side-down on the center of a leaf (midvein; equal distance from leaf tip and leaf base). A sterile PDA plug was used as a control, as was an untreated leaf (no plug). Half the leaves from each inoculation treatment were wounded with a needle (0.5 mm diameter) at leaf centers before placement of the PDA plugs. The moist chambers were placed in plastic bags, and the bags were tied closed. Sealed chambers were then placed in a growth chamber set at 25°C during the day and 20°C at night, and provided artificial light (photon flux density 13 $\mu E \cdot s^{-1} \cdot m^{-2}$) for 16 h/day.

Leaf lesions were counted and the diameters were measured (if present) 4 weeks after inoculation. Isolations were then made from all leaves, and the presence of the *A. tenuissima* group was confirmed as described in the greenhouse experiments.

Lesion diameters were analyzed by three-factor analysis of variance with interactions. Factors used as main effects were: (i) inoculation treatment (isolate and control treatments), (ii) wounded/nonwounded treatment, and (iii) trial (trial 1 and 2). Lesion diameters were analyzed both untransformed and after natural-log(x + 1) and sqrt(x + 1) transformations were applied. The P values and resulting conclusions were similar for all forms of analysis. Therefore, results are reported only for the untransformed data. Analyses of variance (using general linear model procedure) were performed using Minitab for Windows, release 10.2 (Minitab Inc., State College, PA).

RESULTS

Greenhouse pathogenicity experiments. All unwounded, artificially inoculated leaves remained green and asymptomatic in both greenhouse experiments. All wounded and unwounded controls also remained green and asymptomatic in both greenhouse experiments. However, symp-

toms were observed on 82% of the wounded and inoculated leaves (Table 2). Symptoms were only observed at the wound site of wounded and inoculated leaves, and were first observed after 18 and 17 days (trials 1 and 2, respectively). The isolate produced dark brown to black, circular to oval, necrotic lesions with diameters ranging from <1 mm up to 7 mm, with an average diameter of 3.1 mm (0.7 standard error). Larger lesions had tan centers, and tissues adjacent to the leaf spots remained green. Symptoms were the same as those previously observed in the field in which species of the *A. tenuissima* group were identified as the causal agent of disease in this host (7). The inoculation treatment effect (control versus isolate) for lesion size was significant ($P < 0.001$), but not the trial effect ($P = 0.159$) or the trial–inoculation treatment interaction effects ($P = 0.872$).

Significant differences in water potentials ($P < 0.001$ for all comparisons) between plants of the two watering regimes were measured in both trials of both greenhouse experiments. The lowest water potential readings (before watering) of stressed plants averaged –2.1 MPa in both trials of both experiments, and the nonstressed plants averaged –0.1 MPa for all readings. However, the effect of water treatment was not significant on lesion size ($P = 0.631$) for wounded, inoculated leaves. The effect of water treatment also was not significant on the recovery of fungi ($P = 1.000$, 0.285, and 0.704; nonwounded experiment with plastic bags for 16 h or 3 days, and wounded experiment, respectively).

In the first greenhouse experiment, the *A. tenuissima* group was recovered from leaves of the unwounded and inoculated plants that were sealed in plastic bags for 3 days. The *A. tenuissima* group was recovered from 68% of the leaves sampled at the leaf center and from 55% of the leaves sampled 12 mm from the leaf center, with a total recovery of 72% (Table 2). However, the *A. tenuissima* group was never recovered from unwounded, inoculated leaves that were kept at high RH for only 16 h. For wounded and inoculated leaves, the *A. tenuissima* group isolate was recovered from 69% of the leaves sampled at the wound site and from 20% of the leaves sampled 12 mm from the leaf center (beyond the symptomatic areas), with a total recovery of 71%. For nonwounded inoculated leaves in the second greenhouse experiment, the *A. tenuissima* group was recovered from a total of 73% of the leaves. The *A. tenuissima* group was seldom recovered from control plants in either greenhouse experiment.

Microscopic observations. Surface examinations, using both SEM and light microscopy, revealed *A. tenuissima*–like conidia germinating and resultant mycelial growth on leaf surfaces of the field-collected leaves (both sites) and on the artificially inoculated greenhouse leaves (Fig. 1A and B). Hyphae were observed entering leaves only through stomata, and this was only observed on the field-collected leaves (both field sites) and on leaves from the greenhouse experiments when plants were maintained at high RH for 3 days. Hyphae were never observed penetrating epidermal tissues, and appressoria were never observed. Stomatal entry was observed as often as six times on a single 5 × 5 mm field-collected leaf section and as often as 12 times on a single 5 × 5 mm inoculated greenhouse leaf section. A 90 degree hyphal branching pattern was associated with approximately 80% of the observed stomatal entries (Fig. 1B and C); however, direct growth into stomata was also observed (Fig. 1D). For artificially inoculated greenhouse leaves, when plants were maintained at high RH for only 16 h, *A. tenuissima*–like conidia were observed germinating on leaf surfaces with mycelial growth; however, stomatal entry was never observed. Still, the 90 degree hyphal branching was observed at the stomata (Fig. 1E). Fungal hyphae were never observed on surfaces of control leaves.

Light microscopy (internal leaf observations) revealed a net of hyphae surrounding mesophyll tissues in field-collected leaves from both sites (Fig. 1F and G). A similar net of hyphae surrounding mesophyll tissues (Fig. 1H) was observed in leaves from the artificially inoculated, unwounded leaves in the greenhouse experiment, but only when the plants were maintained at high RH for 3 days. Hyphae were growing only intercellularly among the mesophyll tissues, with no host cell penetration and no observed host cell response. Tissue colonization was never observed in the artificially inoculated, unwounded greenhouse plants maintained at high RH for only 16 h, and fungal hyphae were never observed in control leaves.

For the inoculated and wounded leaves in the greenhouse experiment, tissues around the wounds were collapsed and colonized (Fig. 1I). Internal examinations at the wound sites were not possible using our techniques because tissues remained dark brown and opaque. Extensive mycelial growth was observed on leaf surfaces around the wounds, and *A. tenuissima*–like conidia were observed germinating on the leaf surfaces.

Detached-leaf assay. Unwounded, inoculated leaves and both wounded and unwounded controls remained green and asymptomatic. However, symptoms were observed on 56% (Table 2) of the wounded and inoculated leaves (excluding one isolate that did not produce symptoms). Symptoms were only observed at the wound site of wounded and inoculated leaves, and were first observed after 14 and 12 days (trials 1 and 2, respectively). Seven of the eight isolates produced dark brown to black, circular to oval, necrotic lesions with diameters ranging from <1 mm up to 7 mm (average diameter 2.4 mm [0.5 standard error] in trial 1; 3.0 mm [0.7 standard error] in trial 2). Leaf-spot symptoms were comparable to those observed in the greenhouse wound-inoculation experiment and to those previously observed in the field (7). One isolate (no. 321; Table 1) never produced symptoms in either trial.

Differences in lesion diameters were significant ($P = 0.002$) among inoculation treatments; however, when only the seven isolates that produced symptoms were included in the analyses, inoculation treatment was not significant ($P = 0.682$). Although there were differences between the two trials in lesion size ($P = 0.013$), the trial–inoculation treatment interaction was not significant ($P = 0.234$), indicating a similar relative response among isolates between trials.

For unwounded and inoculated leaves, the *A. tenuissima* group was recovered from 60% of the leaves sampled at the leaf center (inoculation site) and from 13% of the leaves sampled 12 mm from the leaf center, with a total recovery of 60% (Table 2). For wounded, inoculated leaves, the *A. tenuissima* group was recovered from 93% of the leaves sampled at the wound site and from 32% of the leaves sampled 12 mm from the leaf center (beyond the symptomatic areas), with a total recovery of 95%. For the isolate that never produced symptoms, recovery from wounded, inoculated leaves averaged 92% from the wound site and 46% 12 mm from the leaf center, with a total recovery of 93%. The *A. tenuissima* group was never recovered from unwounded or wounded controls.

DISCUSSION

Endophytic fungi have been reported to infect hosts either through stomata or by direct epidermal penetration (23). Epidermal penetration may include the formation of appressoria on leaf surfaces and infection pegs through the epidermis. Many pathogenic *Alternaria* species can penetrate the cuticle and epidermis directly, but others only enter through the stomata or seem to require wounds (24). Consistent with the greenhouse experiments, we found higher *Alternaria* recovery at leaf centers compared with leaf margins from field-collected *Amaranthus hybridus* leaves (J. T. Blodgett and W. J. Swart, *unpublished*). These recovery differences might be attributed to the higher frequency of stomata at leaf centers compared with leaf margins on this host. Our current study indicates that infection of *Amaranthus hybridus* by the *A. tenuissima* group is restricted to entry through stomata of unwounded leaves, but may also occur through wounds.

Duration of high humidity and/or wetting of leaf surfaces are important factors in determining infection success or failure.

Although these factors have been extensively studied for various foliar pathogenic fungi, little research has been conducted with endophytic fungi. For endophytic fungi, the more humid microclimate within a tree crown compared with the edge of the crown was suggested to account for higher recovery of endophytes within the crown (13). The minimum wetting periods reported for infection by different pathogenic *Alternaria* species range from 3 to 73 h (24). Our results suggest that high RH for more than 16 h, but possibly less than 72 h, is required for both germinating and subsequent infection of *Amaranthus hybridus* leaves by the *A. tenuissima* group.

The surface-disinfestation methods used in this study are consistent with those used in other studies of endophytic fungi. In the greenhouse experiment, when leaves were kept at high RH for 16 h, conidia and hyphae were on leaf surfaces; yet there was no infection or colonization of leaves and no *Alternaria* recovery. Recovery of other fungal species is also extremely rare from noninoculated, surface-disinfested *Amaranthus hybridus* leaves of plants grown in a greenhouse (8). Our results clearly show that the surface-disinfestation methods used in this study are sufficient to kill fungi growing on *Amaranthus hybridus* leaf surfaces.

Infection and colonization patterns of fungi can be diagnostic in determining the organisms present in a host. Both intercellular and intracellular colonization of host tissues by endophytes have been described for other hosts (23). Cabral et al. (10) describe several forms of host colonization in field-collected *Juncus* and correlate the various forms to the frequencies of fungal recovery. In the current study, observations of both artificially inoculated leaves and field-collected leaves using both SEM and light microscopy revealed the same results. There was only one common pattern of host infection and colonization in both the field and artificially inoculated leaves. The net of hyphae surrounding mesophyll tissues and the 90 degree hyphal branching pattern at the stomata are unique, yet were frequently observed in or on leaves. This is also consistent with the single, dominant *A. tenuissima* group recovered from leaves of this host at two field sites in South Africa (8). Therefore, isolates belonging to the *A. tenuissima* group are clearly the dominant fungal species in asymptomatic field leaves. The minor species previously reported in leaves of this host (8) might be restricted to substomatal chambers, as was observed for some fungal species in annual *Juncus* (10).

Although *A. tenuissima* is found on a wide range of plants and is extremely common, it has never been previously identified in high frequencies from asymptomatic host tissues. It was previously shown that *A. alternata* occurs in low frequencies in *Juncus* spp. and *Phaseolus* spp. with little host colonization (10,18). In contrast, isolation frequencies of *A. tenuissima* isolates from *Amaranthus hybridus* leaves collected from the field were as high as 88% (8), and extensive colonization occurs in this host. The unique colonization pattern of the *A. tenuissima* group isolates in unwounded leaf tissues of *Amaranthus hybridus* and the high recovery of this fungus suggests a strong specificity of this fungus with this host. This internal net of hyphae in asymptomatic leaves has never been previously reported for *A. tenuissima* on any host. These results are consistent with the conclusions of Petrini (20), who suggested that a limited number of endophytic fungal taxa dominate within a single plant species.

Environmental conditions including water stress are known to influence many plant diseases. Drought stress is associated with enhancement of disease development on several species by many pathogens (2–4,6,21,25). However, water stress also may have a neutral or negative influence on the development of certain plant diseases (5,14,33,34). *Alternaria* diseases can be enhanced by high or low soil moisture extremes (24). A field survey of tree endophytes (11) found lower frequencies of endophytic fungi from leaves on dry sites than on sites of moderate moisture. We found that the abundance of endophytic fungi in *Amaranthus hybridus* is influenced by changes in the soil environment, and potentially by soil moisture (J. T. Blodgett and W. J. Swart, *unpublished*). Stomatal closure reduces water loss and might result in reduced host infection. Since infection of unwounded leaves occurs only through the stomata in this host–fungus system, infection can occur if water potentials are not too extreme and leaf surfaces are exposed to very high RH, such as in the early phases of our greenhouse experiments.

Wounding can trigger disease expression and might result in increased infection. However, it is clear from both the field-collected leaves and from the unwounded, inoculated experimental leaves that infection and colonization do not require wounding. Without wounding, though, no symptoms develop. Therefore, wounding causes quick changes in the host–fungal system, resulting in a leaf-spot disease. It might simply be the momentary nutritional imbalance in the system that is quickly overcome, resulting in the distinct leaf spot. The recovery of the *A. tenuissima* group in asymptomatic tissues beyond the leaf spot in both the greenhouse experiments and the detached-leaf assay show that colonization does occur beyond the symptomatic area.

This leaf-spot disease was previously reported on this host from a field in South Africa, and the *A. tenuissima* group was identified as the causal agent with the same range of symptoms as in this present study (7). Wounding resulting from wind and sand damage have been attributed to increased disease of potato, tomato, and onion leaves by various *Alternaria* spp. (9,17). Skiles (32) found that *Alternaria porri* can infect unwounded onion leaves, but causes more disease when the tissues are damaged. Injury was suggested as a requirement for infection by *A. alternata* and *A. tenuissima*, two weak pathogens of onion (32). Feeding damage by the bean leaf beetle (*Cerotoma trifurcata*) has also been associated with disease of soybean pods caused by *A. tenuissima* (27).

Alternaria is prevalent in unwounded *Amaranthus hybridus*, yet leaves appear completely healthy. Our results confirm that the *A. tenuissima* group can infect and colonize *Amaranthus hybridus* leaves in a manner consistent with other endophytic fungi or latent pathogens. However, in this host–pathogen relationship, infection and colonization does occur in nonwounded-inoculated leaves, yet no symptoms develop. This asymptomatic persistence of pathogenic fungi may be more common than is generally believed in other host–pathogen systems.

The same range of symptoms was observed on wounded leaves in both the detached-leaf assay for seven of eight isolates, and for one isolate in the second greenhouse experiment (wounded leaves). Five distinct morphological forms of *Alternaria* were previously identified from this host (8), the most common belonging to the *A. tenuissima* group. This species grouping is further divided into temporary subgroupings (29,30,31). Differences observed in pathogenicity might be helpful in further examinations of the *A. tenuissima* group. The detached-leaf assay offers a relatively easy method to screen isolates of the *A. tenuissima* group quickly, and potentially to screen susceptible/resistant species and varieties of *Amaranthus*.

The definition of endophytic fungi used in this paper does not exclude endophytic fungi acting as latent pathogens. The *A. tenuissima* group in *Amaranthus hybridus* seems to fit both the endophytic and the latent pathogen lifestyle (depending on host condition). Extensive asymptomatic colonization (growing intercellularly) with no host cell penetration and no symptoms is not typical of most pathogens. The disease is not typical of most endophytic fungi. Therefore, at least some isolates of the *A. tenuissima* group do persist asymptomatically in *Amaranthus hybridus*, yet when triggered by wounding can act as pathogens.

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