Identification of Fungi and Fungal Pathogens Associated with *Hypolixus haerens* and Decayed and Cankered Stems of *Amaranthus hybridus*

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ABSTRACT

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Discoloration, cankers, and decay in branches, stems, and root collars of *Amaranthus hybridus* were observed in Bloemfontein, South Africa. Examination of symptomatic stems revealed larval galleries of the pigweed weevil (*Hypolixus haerens*). The objectives of this study were to: identify the most common fungal species associated with this damage, determine if the adult pigweed weevil might be a vector for the fungi, and test if the associated fungi can cause the stem canker disease observed in the field. The most common fungal species isolated were *Fusarium subglutinans* from discolored tissues adjacent to insect galleries (42%), *F. subglutinans* from weevil larvae (29%), the *Alternaria tenuissima* group from adult weevils (31%), and the *A. tenuissima* group from cankered stems (40%). Three of the seven most common fungal species produced cankers following wounding and inoculation, with *F. sambucinum* and *F. oxysporum* being the most aggressive. Although fungal species compositions differed ($P < 0.01$) among the four tissue/insect stage combinations tested, all four had the same major fungal species, suggesting the pigweed weevil as a vector for the *Fusarium* pathogens. There is significant potential for yield loss affiliated with this insect-fungal association. The identification of this insect-fungal relationship and the pathogens involved in disease set the stage for further research on the etiology and disease management of this important insect-fungal relationship.

Additional keywords: amaranth, *Fusarium equiseti*, *F. solani*, insect vectoring, smooth amaranthus

Amaranthus hybridus (common names: smooth amaranthus or amaranth) is a fast-growing vegetable crop with edible leaves that are highly nutritious (30,33). *Amaranthus* species are used as alternative crops throughout the world, growing well in semiarid regions (21,22,33). In southern Africa, cultivation of this crop as a leafy vegetable is increasing. *A. hybridus* has considerable potential for increased commercial application worldwide. However, a sustainable integrated pest/disease management approach for the cultivation of *A. hybridus* requires an understanding of abiotic and biotic factors that can affect this important food crop.

The pigweed weevil, *Hypolixus haerens* (Coleoptera: Curculionidae), is the main insect pest of *A. hybridus* in South Africa (26). The adult weevil primarily lays its

eggs in branch crotches, and the larvae bore through stems to the root collars, causing a hollowing of stems. This larval feeding results in stems that are more susceptible to wind breakage, increasing crop losses. The weevil overwinters in the dying and deteriorating stems as mature larvae and/or pupae. In the spring, weevils eclose and immediately feed on leaves of *A. hybridus* and other plants.

Insect-fungal interactions have long been known to result in severe crop losses of many host species (1,3,11,17). In autumn 1997, extensive tissue discoloration and decay were observed in most branches, stems, and root collars of mature *A. hybridus* in two plots in Bloemfontein, Free State Province, South Africa (9). Symptoms included discolored phloem, xylem, and pith; black cankers on stems and root collars; and weakened stems prone to wind breakage. Examination of these tissues revealed larval galleries of the pigweed weevil in all symptomatic stems. The adult weevil causes wounds to plant tissues during feeding and egg laying, and the larvae cause wounds to plant tissues during feeding (26). Insect involvement in the transmission of fungal pathogens is well documented (1). However, the possibility that the pigweed weevil might be a vector of the observed canker disease has never been described.

The potential role of the weevil-associated fungi in causing disease of *Amaranthus* stems colonized by *H. haerens* was investigated in this study. The objectives of this study were to identify common fungal species in the following settings: (i) associated with tissue decay and discoloration in larval galleries of the pigweed weevil, (ii) carried on the larvae, (iii) carried by the adult insects as they fly to new hosts, and (iv) on the cankered stems and root collars; (v) to test if the more common fungi identified might be involved in the branch, stem, and root collar diseases observed in the field; and (vi) to examine if water stress can stimulate colonization of stems. Water stress was investigated since *A. hybridus* is often grown in semiarid regions (21,22,33), and soil moisture extremes have been associated with many diseases, including those caused by *Fusarium* species (5,27,37). Studies were conducted in cultivated monoculture fields at two sites in South Africa, and in a greenhouse using plants grown from seed.

MATERIALS AND METHODS

Field plot establishment. *A. hybridus* fields were established at two sites (20 km apart; sites A and B) in Bloemfontein, South Africa. The fields were mechanically cultivated using tine implements at both sites. Smooth amaranthus seeds were sown in seed trays in a sand-loam (10 to 15% clay) and peat moss (Les Tourbes Niron, Rivière du Loup, Québec, Canada) mixture (1:1 vol/vol). Soils were steam-pasteurized 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 seed tray of a 3 g/liter hydroponic nutrient solution (6.5:2.7:13 N:P:K plus micronutrients) once a week as a soil drench for 30 days. Thirty-day-old plants were transplanted to the field in mid-November at both sites. After placing plants in planting holes, the holes were filled with water and then with soil. At both sites, fields were 40.8 m long and 20 rows wide, with an intra-row spacing of 0.3 m and 1.5-m spacing between rows, resulting in approximately 2,720 plants per field. A drip-line irrigation system delivered approximately 40 mm of water for 2 h every other day at each site.

Field isolations and identification. Ten cankered, 6-month-old *A. hybridus* stems

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were collected from each of the two sites in June 1997. Stems/root collars were split and eight small samples of approximately 64 mm³ (approximately 4 × 4 × 4 mm) of discolored pith, xylem, or phloem tissues adjacent to larval galleries were aseptically transferred from each stem to cornmeal agar (CMA; Oxoid, Basingstoke, England) containing 0.3 ml of streptomycin sulfate (at 1 g a.i. per 3 ml) per liter and incubated at 24°C. A total of 160 isolation attempts were made. CMA is a general purpose medium for isolating fungi. The associated larvae were extracted from the same stems/root collars (48 larvae from site A; 40 larvae from site B), decapitated with a tweezer, and plated directly on CMA in petri plates that were incubated at 24°C. After 4 to 7 days, fungi growing from the tissues or larvae were transferred from colony margins onto separate 1.5% water agar (WA; Oxoid) plates. Isolates were later transferred to half-plates of carnation leaf agar/potato dextrose agar (Oxoid) for identification (28). Carnation leaf agar and potato dextrose agar work well for identification of many fungal species. Therefore, the same methods were used to identify all fungal species. Plates were incubated in a growth chamber with 16 h of light per day and temperatures of 25°C day and 20°C night. Percent recovery can exceed 100% due to multiple isolates from individual tissue samples or larvae. Reporting percentages above 100% clearly show when multiple species co-occur in tissues and also clearly show the percentage of times each species is recovered.

Five additional asymptomatic stems not harboring insect larvae were collected from each site at the same time. The stems/root collars were split, and eight small samples of approximately 64 mm³ (approximately 4 × 4 × 4 mm) of healthy-appearing pith, xylem, or phloem tissues were aseptically transferred from each stem to CMA and incubated at 24°C. A total of 80 isolation attempts were made. After 4 to 7 days, any fungal colonies growing from the tissues were transferred from colony margins, incubated, and identified as previously described.

Three cankered stem segments (approximately 5 cm long) from each of 10 stems were collected from each of the two sites 2 weeks later. Segments were placed in moist chambers for 3 weeks. Fungal hyphae growing from the stem surfaces were collected with a scalpel, transferred to CMA, and incubated at 24°C. A total of 60 such isolation attempts were made. After 5 to 7 days, mycelia from the colony margins were transferred, incubated, and identified as previously described.

In October 1997, stems/root collars collected from the same two sites were split, and pre-emergent adult weevils and recently emerged adult weevils were aseptically transferred, after decapitation with a tweezer, to CMA and incubated at 24°C.

At the time of collection, the adult weevil population was exiting stems. Forty adults from site A and 33 adults from site B were collected. After 4 to 7 days, fungal colonies growing from weevils were transferred from colony margins, incubated, and identified as previously described.

Inoculation experiment: planting and greenhouse conditions. 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-pasteurized 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 plus 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 $\mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ with a maximum recorded ambient greenhouse photon flux density of 1,233 $\mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. 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 twice a day, 7 days a week, to maintain the high RH. High RH was used to reduce water loss in plants, thus extending the period of water stress. Plants were placed on a bench flooded with water to allow continuous water contact with the bottom of the pots.

Inoculation experiment: water stress. Four weeks after transplanting (mid-April and early June for the repetition, 1998), half the plants (45 plants; 90 total plants in each of two trials) 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). Host water stress was included in this study since *A. hybridus* is often grown in semiarid regions, such as South Africa, and soil moisture extremes have been associated with various diseases, including those caused by *Fusarium* species (5,27,37). A pressure bomb (36) 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.

Inoculation experiment: wounding and inoculation. After 1 week under the different watering regimes, stems were inoculated with seven of the more common fungal species isolated from the stems and insects. One single-spore isolate of each of

the following species was used: *Fusarium subglutinans*, *F. oxysporum*, *F. sambucinum*, *F. solani*, *Alternaria tenuissima* group, a *Phomopsis* sp., and a *Phoma* sp. (five replications per watering regime/isolate treatment combination). Inoculations involved wounding a stem by removing approximately 6 × 6 mm of the epidermis 5 cm above the soil with a scalpel. Wound inoculations were used in this study because both the adult weevil and the larvae cause physical damage to *A. hybridus* and other plant species. Five-mm-diameter WA plugs cut from margins of actively growing cultures were placed mycelium-side-down on the wounds. Parafilm (American National Can Co., Chicago, IL) was wrapped around the plug and stem. Wounded and nonwounded (untreated) controls were also included. Noncolonized WA plugs were applied to wounded controls but not to nonwounded controls. Five plants per watering regime/isolate treatment combination, and five wounded and nonwounded control plants for each watering regime, were used in each of two completely randomized independent trials, separated by 2 months. A total of 180 plants were used. Since all treatments were assigned randomly, a completely randomized experimental design was used in the analyses.

Inoculation experiment: observations and measurements. Canker lengths and percentage of the stem circumference cankered were measured (if present) 4 weeks after inoculation. Fungal recovery also was attempted 4 weeks after inoculation from all stems, including stems of the two control treatments. Stem segments 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. Tissues (approximately 5 × 5 mm) from the wound sites or from a canker (if present) were then cut from the stems using a scalpel. The tissues were aseptically placed in individual WA plates and incubated for 21 days at ambient laboratory temperature (approximately 24°C) and light. Presence of the inoculated fungi in the incubated stem tissues was confirmed by examining resulting mycelia and spores.

Statistical analyses. Chi-square analyses were used to examine the composition of fungal species isolated from stems/insects in relation to geographic location (site A and B) and source of isolation (stems/insects). For the inoculation experiment, chi-square analyses were also used to examine incidence of disease and recovery after inoculation in relation to watering regime (two levels), inoculation treatment (nine levels), and trial (two levels). Canker lengths and circumferences were analyzed by three-factor analyses of variance with all interactions. Factors used as the main effects were watering regime (two levels), inoculation treatment (nine levels), and trial (two levels). Five replica-

tions were used per watering regime–inoculation treatment–trial combination, with a total of 180 plants used in the inoculation experiment. The canker lengths and circumferences were analyzed both untransformed and after $\log_e(x + 1)$ and $\sqrt{x + 1}$ transformations were applied. Because the *P* values and resulting conclusions were similar for all forms of analysis, results are reported only for the untransformed data. ψ_{PD} from the inoculation experiment were analyzed by one-way analyses of variance with watering regime (two levels) as the factor. Chi-square analyses and analyses of variance using general linear model procedure were performed using Minitab for Windows, release 10.2 (Minitab Inc., State College, PA).

RESULTS

Field isolations. Fungal isolation from symptomatic *A. hybridus* and from *H. haerens* larvae and adults was successful. However, few isolates were recovered from asymptomatic stem tissues (5% recovery from site A and 0% from site B). Recovery from: larval galleries yielded isolates 100% of the time from site A and 108% from site B; larvae yielded isolates 108% of the time from site A and 104% from site B; adult weevils yielded isolates 193% of the time from site A and 176% from site B; and cankered stems yielded isolates 117% of the time from site A and 93% from site B.

Several fungal species were isolated from symptomatic *A. hybridus* tissues and from *H. haerens* larvae and adults (Table 1). The most common species isolated from discolored tissues adjacent to insect galleries was *F. subglutinans* (42%); from weevil larvae was *F. subglutinans* (29%); from adult weevils was the *Alternaria tenuissima* group (31%); and from cankered stems was the *A. tenuissima* group (40%). Other common species isolated

from the stems and weevils included *F. oxysporum*, *F. equiseti*, *F. solani*, *F. sambucinum*, *Epicoccum* spp., a *Phomopsis* sp., and *Phoma* spp. Only two isolates were recovered from asymptomatic stem tissues, a *Chaetomium* sp., and a *Penicillium* sp.

There were significant differences between the two sites in fungal species compositions for larval gallery tissues ($P < 0.01$), adult weevils ($P = 0.02$), and cankered surface stem tissues ($P < 0.01$), but not for larvae ($P = 0.10$). However, several of the same species were isolated from both sites (Table 1). Significant differences ($P < 0.01$) in fungal species composition also occurred among *A. hybridus* tissues, larvae, and adults. Although species compositions differed among the four tissue/insect stage combinations examined, all four had the same major species.

Inoculation experiment. Significant differences in mean ψ_{PD} ($P < 0.001$) between plants of the two watering regimes were measured in both trials of the greenhouse experiment. Before watering, the lowest water potential readings of stressed plants averaged -2.1 MPa in both trials, while the nonstressed plants averaged -0.1 MPa for all readings. However, the effect of water treatment was not significant on canker length ($P = 0.94$), canker width ($P = 0.99$), incidence of symptoms ($P = 0.87$), or recovery of fungi ($P = 0.76$). The trial effect also was not significant on canker length ($P = 0.11$), canker width ($P = 0.65$), incidence of symptoms ($P = 0.64$), or recovery of fungi ($P = 0.37$). Therefore, results for canker length, canker width, incidence of symptoms, and recovery of fungi are combined for plants inoculated with the same fungal isolate, regardless of water treatment and trial.

The inoculation treatment was significant ($P < 0.001$) for incidence of symp-

toms, canker length, canker width, and recovery of fungi (Table 2). Only three of the seven species tested, *F. sambucinum*, *F. oxysporum*, and *F. subglutinans*, produced cankers. Lesions were never observed on stems inoculated with the other isolates, and lesions were never observed on either wounded or nonwounded control stems. Discoloration and canker symptoms were the same as symptoms observed in the field, and symptoms were first observed 5 days after inoculation in both trials. The isolates were recovered from stems inoculated with all species (Table 2), with slightly higher recoveries from some of the species that did not produce symptoms. None of the *Fusarium* spp. were recovered from either of the two control treatments ($n = 20$ for each species tested).

DISCUSSION

The present investigation has demonstrated that numerous species of fungi are associated with *H. haerens*, a phytophagous weevil on *A. hybridus*. Three of the common fungal species isolated from four tissue/insect stages, *F. sambucinum*, *F. oxysporum*, and *F. subglutinans*, were shown in artificial inoculations to be independently pathogenic to the host plant. These *Fusarium* species are likely the cause of the discoloration, decay, and cankers observed in branches, stems, and root collars of mature *A. hybridus*. However, *H. haerens* larvae were observed in all symptomatic stems in the field.

Although the *Alternaria tenuissima* group was the most common isolated from cankered stems, this species group did not cause stem cankers in our study. *Fusarium* spp. also were isolated from cankered stems, and from the weevil larval galleries and larvae. The low recovery of fungi from asymptomatic, epidermal stem tissues collected from the same sites 1 month

Table 1. Species composition (percentages) of fungi isolated from *Hypolixus haerens* larval galleries, larvae, and adults, and cankered stems of *Amaranthus hybridus*

Fungal species/genera	Larval galleries		Larvae		Adults		Cankered stems	
	Site A	Site B	Site A	Site B	Site A	Site B	Site A	Site B
<i>Fusarium subglutinans</i>	32	51	26	31	6	7	11	9
<i>F. oxysporum</i>	11	15	22	19	3	8	11	6
<i>F. equiseti</i>	1	... ^w	9	10	5	14	18	3
<i>F. solani</i>	2	8	6	8	2	4	8	15
<i>F. sambucinum</i>	7	...	5	3	14	4	13	4
<i>F. proliferatum</i>	3	1	2	3	...	3	...	4
<i>F. culmorum</i>	...	...	...	...	...	...	2	...
<i>Alternaria tenuissima</i> group	6	14	3	9	38	24	33	47
<i>Epicoccum</i> spp.	...	...	...	4	16	24	...	...
<i>Phomopsis</i> sp.	21	...	8	...	...	...	...	...
<i>Phoma</i> spp.	6	4	2	1	8	4	...	...
<i>Microdochium</i> spp.	5	2	6	3	1	...	...	...
Other identified isolates ^x	5	1	4	2	4	3	1	4
Unidentified isolates ^y	1	4	7	7	3	5	3	8
N ^z =	80	86	52	42	77	58	35	28

^w Fungi were not isolated.

^x Less common species including: *Rhizopus* spp., *Alternaria* spp. other than the *A. tenuissima* group, basidiomycete species, *Phytophthora* spp., *Chaetomium* spp., *Acremonium* spp., *Penicillium* spp., oomycete species, and *Trichoderma* spp.

^y Includes nonsporulating mycelial fungi.

^z Total number of isolates.

prior to this study (10) suggests that the *A. tenuissima* group and other non-*Fusarium* species infect during or soon after the *Fusarium* species.

Plant diseases caused by *Fusarium* species have been associated with several insects (14,23,24). Weevils, in particular, have been associated with various fungal pathogens (13,15,29), including *Fusarium* spp. (18,20,25). In the insect-fungal-plant relationship we investigated on amaranth, adult pigweed weevils might act as a vector for *Fusarium* species, and/or might produce the infection site by host wounding when eggs are laid. Healthy, insect free pith, xylem, and phloem stem tissues yielded few fungal isolates, and never yielded the *Fusarium* species. Fungi are also infrequent or absent in asymptomatic, epidermal stem tissues collected from the field (10). *Fusarium* was the most common genus associated with the discolored stem tissue around larval galleries, and the most common genus associated with *H. haerens* larvae. The recovery of many of the same *Fusarium* species from cankered stems and from the adult insects, although in lower percentages than the *Alternaria tenuissima* group, supports the hypothesis of introduction of these pathogens by the weevil. Isolation attempts from the spotted maize beetle (*Astylus atramaculatus*) and the tarnished plant bug (*Lygus lineolaris*), other common insects on *A. hybridus*, did not result in the recovery of *Fusarium* species (J. T. Blodgett, W. J. Swart, and S. vdM. Louw, unpublished). This indicates that *H. haerens* might be an important vector of the *Fusarium* pathogens of *A. hybridus*.

Water stress is known to influence many plant diseases. Water stress is associated with enhancement of disease development on several host species by many pathogens

(2,4,6,8,32,34,35). However, water stress also may have a neutral or negative influence on the development of certain plant diseases (7,19,38,39). *Fusarium* diseases can be enhanced by high or by low soil moisture extremes (5,27,37). In our study, results indicate that even extreme water stress of *A. hybridus* plants does not significantly affect the development of stem cankers by any of the seven fungal species tested.

In a previous study (10), some of the same fungal species associated with *A. hybridus* were described as being endophytic or quiescent in this host. Endophytic or latent *Fusarium* spp. have also been reported from other plant hosts (12,16,31). The potential ability of endophytic *Fusarium* isolates in *A. hybridus* to act as latent pathogens when induced by wounding such as weevil damage therefore deserves further investigation.

It is not known if these fungal species cause significant disease on this host independent of the weevil. However, the fact that these insect-fungal associations were firmly established is important information when developing an integrated pest management program for the cultivation of *A. hybridus* in South Africa. Further studies are therefore needed to determine the role of these pathogenic fungi in weakening *Amaranthus* stems colonized by the pigweed weevil.

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Table 2. Incidence of symptoms^a, canker lengths, and canker widths on inoculated^b *Amaranthus hybridus* stems, and percentages of stems from which the inoculated fungi were recovered^c 4 weeks after inoculation

Inoculation treatment ^a	Incidence of symptoms (%)	Canker length (mm)	Canker circumference (%)	Recovery (%)
<i>Fusarium sambucinum</i>	100 ^d	30	75	65
<i>F. oxysporum</i>	100	26	43	50
<i>F. subglutinans</i>	65	10	17	60
<i>F. solani</i>	0	0	0	70
<i>Alternaria tenuissima</i> group	0	0	0	75
<i>Phomopsis</i> sp.	0	0	0	95
<i>Phoma</i> sp.	0	0	0	55
Significance ^e	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001

^a Symptoms included black cankers and were the same as symptoms observed in the field.

^b Inoculations involved wounding a stem by removing approximately 6 × 6 mm of the epidermis 5 cm above the soil with a scalpel and inoculating with a 5-mm-diameter water agar plug cut from margin of actively growing cultures.

^c Presence of inoculated fungi were confirmed from surface-disinfested tissues.

^d All single-spore isolates. Results are pooled across trials and across water treatments since those effects were not significant.

^e *N* = 20 for each species tested with a total of 180 plants used in the study, including wounded and nonwounded (untreated) controls.

^f Chi-square analyses were used to examine incidence of symptoms and recovery after inoculation. Canker lengths and circumferences were analyzed by analyses of variance.

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