# Etiology of Atypical Symptoms of Charcoal Rot in Sunflower Plants Parasitized by Larvae of *Cylindrocopturus adspersus*

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## ABSTRACT

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Sunflower plants (*Helianthus annuus* L.) parasitized by larvae of the sunflower stem weevil, *Cylindrocopturus adspersus*, developed black-to-brown discoloration of the stalks. *Alternaria alternata, Fusarium roseum, F. solani, Macrophomina phaseolina, Phoma macdonaldii,* and *Rhizopus arrhizus* were isolated from the discolored tissues of sunflower stalks parasitized by the larvae of *C. adspersus* as well as from the larval and adult weevils. Greenhouse-grown sunflower plants inoculated with one of five fungi, *A. alternata, F. roseum, F. solani, P. macdonaldii,* and *R. arrhizus* alone or in combination with *M. phaseolina*, developed black-to-brown discoloration. Sunflower plants, however, inoculated with *M. phaseolina* alone developed typical charcoal rot symptoms, a gray discoloration with many black sclerotia on the stalks. The results indicate that the five fungi are capable of contributing to the development of black-to-brown discoloration on sunflower stalks infected by *M. phaseolina* and parasitized by larvae of *C. adspersus*.

Cultivated sunflower (*Helianthus annuus* L.) plants, both infected by *Macrophomina phaseolina* (Tassi) Goid. and parasitized by larvae of the stem weevil, *Cylindrocopturus adspersus* (LeConte), developed black-to-brown discoloration with or without gray areas on the stalk (12). Yang and Owen (12) suggested that feeding in the stalk by tunneling larvae enhances the growth of saprophytic fungi which might contribute to the development of the black-to-brown discoloration.

The purpose of the experiments reported here was to isolate and identify fungi from stem weevil adults and larvae and from the discolored stalk tissues and to determine the role of these fungi in the development of the black-to-brown symptoms on sunflower stalks. Results of the preliminary study have been reported (11).

## MATERIALS AND METHODS

**Isolation of fungi from adults and larvae of *C. adspersus* and oviposition sites.** One hundred thirty-three adults of *C. adspersus* were collected from three sunflower fields at Dimmit and Halfway, TX in June 1981, and 45 surface-sterilized tissue samples from the oviposition sites (surface sterilized in 1% NaOCl solution for 5 min) were taken from 38 sunflower plants at Halfway, TX. The samples were plated (one plate per adult for overnight, and one plate for tissue samples from the same plant) on potato-dextrose agar (BBL, Microbiology Systems, Becton Dickinson & Co., Cockeysville, MD 21030) amended with additional agar (8 g/L), streptomycin (100 mg/L), and penicillin G potassium (30 mg/L) (PDA-SP, both antibiotics from Calbiochem-Behring, San Diego, CA 92112). This method has already been reported (13) for detecting *M. phaseolina* on adult *C. adspersus*.

Larvae were collected from the basal stalk and upper tap roots of 50 field-grown sunflower plants (Texas Triumph 894A [TT894A]). One to five larvae per plant were collected from 25 plants on 3 September, and again on 14 October 1981. Larvae were placed on separate PDA-SP plates overnight. The plates were then incubated at room temperature (24 ± 1 C) for 1–2 wk.

**Isolation of fungi from discolored sunflower stalks.** Pieces of epidermal tissues were taken from three basal internodes of the 50 plants after the larvae had been removed. Four surface-sterilized and four nonsurface-sterilized pieces from each stalk were incubated on PDA-SP.

**Identification of fungi from adults and larvae of *C. adspersus* and from the larvae-parasitized plants.** Fungi isolated from adults, larvae, oviposition sites, and larvae-parasitized sunflower plants were transferred from PDA-SP to PDA plates or slants. The fungi (except *Penicillium*) were identified to species by growing on appropriate media (1,3,4,7,10) under conditions specified by Toussoun and Nelson (10).

**Parasitization of sunflower by larvae of *C. adspersus*.** Sixty-two sunflower plants (TT894A) (two per 30-cm-diameter pot) at R2 budding stage (8) were artificially parasitized with nonsurface-sterilized larvae of *C. adspersus* (second to fourth instar) that were collected directly from the parasitized sunflower plants. Larvae that were free of fungi and alive were not available. The second internode from the base was rubbed with 70% ethanol before holes were made with sterile forceps. One or two larvae collected from field-grown sunflowers were placed in each hole (0.5 cm deep) and the hole was covered with plastic adhesive tape. Plants showing no discoloration near the site of infestation within 2 wk were reinfested with larvae near the first site. Sunflower plants with holes only served as controls. Discoloration of stalks was recorded 8 wk after the successful infestation of the plants with larvae. Attempts were made to isolate fungi from tissues of the discolored internodes. In addition, 20 sunflower plants, which were parasitized with larvae, were also inoculated with *M. phaseolina*.

**Inoculation of sunflower with fungi.** Sunflower plants (TT894A, two plants per isolate) at different stages of growth (V4 to R3 [8]) were inoculated with one to four of the isolates of each fungal species from the adults, larvae, and infested plants. Plants were grown in the greenhouse under the following conditions: 25–35 C, 50–85% RH, 14 hr of light (Sylvania cool white 215W, 2 ft above plant) (natural and fluorescent) daily, and irrigation when needed. Inoculum of *M. phaseolina* consisted of sclerotia from 2- to 8-wk-old cultures, and of the other fungal isolates in small agar blocks

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with mycelium cut from 2- to 3-day-old cultures. The inoculum was inserted into a hole 0.3–0.5 cm deep made with a sterile metal needle (0.2 cm in diameter) in the second basal internode, and the hole was covered with plastic adhesive tape for 3 days. The internodes had been rubbed with 70% ethanol before the holes were made. Plants inoculated with PDA blocks only at each inoculation date served as controls. Discoloration was recorded 8 wk after inoculation, and the fungi were reisolated on PDA-SP medium from the discolored tissues. Some inoculations were made by inoculating both with *M. phaseolina* and with one additional isolate of *A. alternata, F. solani, P. macdonaldii,* or *R. arrhizus*. Forty sunflower plants were doubly inoculated.

## RESULTS

**Isolation of fungi from adults and larvae of *C. adspersus* and from oviposition sites.** *Alternaria alternata* (Fries) Keissler, *Fusarium solani* (Mart.) Sacc., *M. phaseolina*, and *Rhizopus arrhizus* Fischer were most frequently isolated from the nonsurface-sterilized adult and larval weevils (Table 1).

*A. alternata, F. solani, M. phaseolina*, and *P. macdonaldii* were isolated, respectively, from 10, 15, 3, and 1% of the sample tissues from 45 oviposition sites, whereas other fungi, such as *Aspergillus niger* Van Tieghem and *Penicillium*, were recovered not more than once.

**Isolation of fungi from discolored sunflower stalks.** *A. alternata, F. solani, M. phaseolina, P. macdonaldii*, and *R. arrhizus* were the fungi most frequently isolated from discolored stalk tissue of sunflower hybrid TT894A (Table 2). The saprophytic fungi *C. cladosporioides* (Fresen) deVries, *C. sphaerospermum* Penz., and *R. arrhizus* were frequently isolated from the nonsurface-sterilized tissue pieces (Table 2).

**Parasitization of sunflower by larvae of *C. adspersus*.** Sunflower plants artificially parasitized with nonsurface-sterilized larvae in the greenhouse developed black-to-brown discolored areas (5–27 cm in length) on 43 stalks (Fig. 1b). The larvae caused different degrees of injuries in the stalks. Stalks of seven other plants and 20 control plants showed discoloration only near the site of the wounds (Fig. 1a). Five plants developed primarily gray discoloration (Fig. 1c) and seven plants developed gray discoloration intermixed with black-to-brown discoloration. *A. alternata, F. solani, M. phaseolina, P. macdonaldii*, and *R. arrhizus* were isolated from 40, 49, 30, 21, and 26%, respectively, of the 43 black-to-brown discolored sunflower plants.

The 20 sunflower plants parasitized with nonsurface-sterilized larvae and inoculated with *M. phaseolina* developed black-to-brown discoloration (12 plants), gray discoloration either intermixed with or growing above the black-to-brown discoloration (five plants, Fig. 2c), and charcoal rot symptoms with black-to-brown discoloration near the sites of infestation and inoculation only (three plants). *M. phaseolina* was reisolated from the inoculated sites and the gray discolored areas. *A. alternata, F. solani, P. macdonaldii*, and *R. arrhizus* were isolated from the black-to-brown discolored tissues.

**Inoculation of sunflower with fungi.** Sunflower plants inoculated separately with isolates of each fungal species (*A. alternata, F. roseum, F. solani, P. macdonaldii*, and *R. arrhizus* from adult and larval weevils and from larva-parasitized plants) developed black-to-brown discoloration of the inoculated stalks.

TABLE 1. Fungi isolated from nonsurface-sterilized adults and larvae of *Cylindrocopturus adspersus* on potato-dextrose agar amended with penicillin and streptomycin

| | Frequency of isolation (%) from collections of | | |
|---|---|---|---|
| | 133 adults | 75 larvae | |
| Species isolated | June 1981 | 3 Sept 1981 | 14 Oct 1981 |
| *Alternaria alternata* | 32 | 47 | 64 |
| *Aspergillus niger* | 11 | 3 | 7 |
| *Cladosporium cladosporioides* | 4 | 1 | 0 |
| *Fusarium roseum* | 0 | 3 | 0 |
| *F. solani* | 43 | 71 | 73 |
| *Macrophomina phaseolina* | 3 | 17 | 37 |
| *Penicillium* spp. | 29 | 11 | 7 |
| *Phoma macdonaldii* | 2 | 9 | 7 |
| *Rhizopus arrhizus* | 21 | 35 | 40 |

TABLE 2. Isolation of fungi on potato-dextrose agar, amended with penicillin and streptomycin, from surface-sterilized and nonsurface-sterilized sunflower stem tissues

| | Frequency of isolation (%) from 25 sunflower stalks collected on | | | |
|---|---|---|---|---|
| | 3 Sept 1981 | | 14 Oct 1981 | |
| Species isolated | NS[a] | SS[a] | NS | SS |
| *Alternaria alternata* | 60 | 56 | 64 | 64 |
| *Aspergillus niger* | 4 | 0 | 8 | 0 |
| *Cladosporium cladosporioides* | 32 | 4 | 4 | 0 |
| *C. sphaerospermum* | 24 | 4 | 20 | 0 |
| *Curvularia lunata* | 0 | 4 | 0 | 0 |
| *Fusarium roseum* | 4 | 0 | 0 | 0 |
| *F. solani* | 72 | 80 | 68 | 84 |
| *Macrophomina phaseolina* | 56 | 48 | 64 | 36 |
| *Phoma macdonaldii* | 64 | 60 | 56 | 64 |
| *Penicillium* spp. | 4 | 0 | 4 | 0 |
| *Rhizopus arrhizus* | 44 | 20 | 52 | 12 |

[a] NS = nonsurface-sterilized and SS = surface-sterilized.

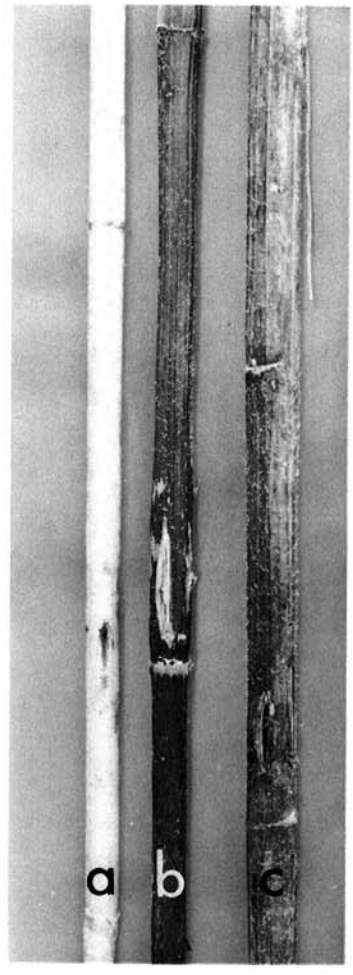


**Fig. 1.** Stalks of sunflower plants (TT894A) artificially infested with larvae of *Cylindrocopturus adspersus* in the greenhouse. Stalk a, control, noninfested, showing black discoloration near the site of the wound. Stalk b, black-to-brown discoloration. Stalk c, gray discoloration above the black-to-brown discoloration. Photographed 8 wk after initial infestation.

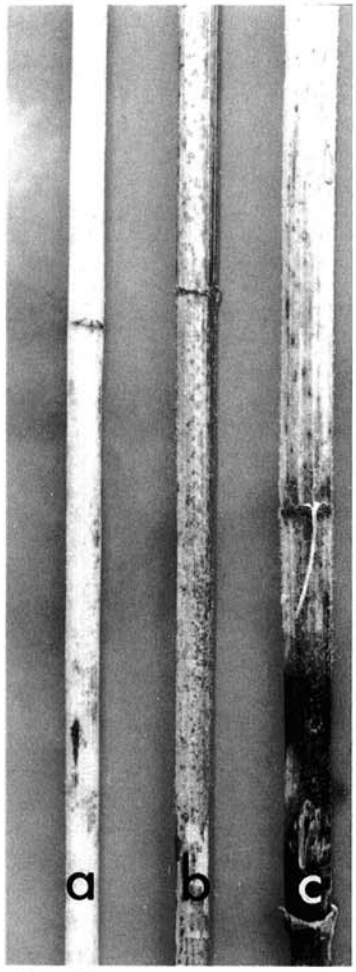


**Fig. 2.** Stalks of sunflower plants (TT894A) inoculated with *Macrophomina phaseolina* and artificially infested with larvae of the stem weevil, *Cylindrocopturus adspersus*. Stalk a, control, no weevil larvae, no fungi, black discoloration only near the site of the wound. Stalk b, *M. phaseolina*, gray discoloration on the epidermis of inoculated stalk. Stalk c, *M. phaseolina* and larvae of *C. adspersus*, gray discoloration intermixed with and growing above the black-to-brown discoloration. Photograph was taken 8 wk after inoculation.

Sunflower plants inoculated with *M. phaseolina* alone (Fig. 2b) developed the typically gray charcoal-rot symptoms. Isolates of the other fungi listed in Tables 1 and 2 did not discolor the inoculated stalks.

Forty sunflower plants were inoculated with *M. phaseolina* in combination with a virulent isolate of *A. alternata, F. solani, P. macdonaldii*, or *R. arrhizus* (10 plants for each combination). Thirty plants developed black-to-brown discoloration; four, gray discoloration spreading from black-to-brown discoloration; three, gray discoloration intermixed with the black-to-brown discoloration; and three, typical charcoal rot symptoms with black-to-brown discoloration near the site of inoculation. *M. phaseolina* was isolated from the sites of inoculation and also from the gray discolored areas. The other inoculated fungi were also isolated from the discolored tissues of inoculated plants.

## DISCUSSION

*A. alternata, F. roseum, F. solani, P. macdonaldii*, and *R. arrhizus* were isolated from sunflower plants parasitized with larvae of *C. adspersus*. Sunflower plants inoculated with these fungi alone developed black-to-brown discoloration on sunflower stalks. *A. alternata* causes leaf blight of sunflower in India (2) and Iran (5), and *P. macdonaldii* causes black stalk of sunflower (1,6,14). The fungi, *A. alternata, F. roseum, F. solani*, and *R. arrhizus*, are reported here for the first time as etiologic agents of black stalk in sunflower. All fungi were also recovered from non-surface-sterilized adults and larvae of *C. adspersus* (Table 1). These soil fungi enter sunflower stalks either by means of the insects or through wounds caused by either ovipositing female or by feeding larvae.

During oviposition, the female chews a small hole through the stalk epidermis, mostly at the first and second internodes from the base (glabrous regions) of the stalk, deposits an egg in the hole, and then seals the hole with an anal secretion (13). Adults of *C. adspersus* may also transmit soil fungi to the egg hole during oviposition. Transmission of *M. phaseolina* to sunflower via the egg hole by adults of *C. adspersus* has been reported (13). About 1 to 2 weeks after oviposition, the larvae emerge from the eggs and feed on subepidermal tissues (9). Injuries extend from the epidermis to the fibrovascular bundles. The soil fungi may reach the wounds from the soil by wind or splashing rain drops. As the larvae mature, they tunnel up and down the stalk, injuring and destroying the tissue inside (9). Stalk injuries by the larvae prior to inoculation with *A. alternata, F. solani*, and *R. arrhizus* hastened the development of the black-to-brown discoloration of stalks (11). Injury and damage apparently enhance the growth of the weak wound pathogens inside the stalks and result in the development of black-to-brown discoloration of the stalk.

On the Texas High Plains, the majority of sunflower plants showed symptoms atypical of charcoal rot in the form of black-to-brown discoloration in these stalks parasitized with larvae of the stem weevil (12). The result indicates that the development of atypical symptoms of charcoal rot on sunflowers, which are both infected by *M. phaseolina* and parasitized by the larvae of *C. adspersus*, result from the presence and enhanced growth of wound pathogens associated with injury of the tissue by the feeding larvae.

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