Etiology of a Late Season Wilt in *Helianthus annuus*

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ABSTRACT

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Two types of bacteria and a fungus were isolated from lesions on *Helianthus annuus* showing wilt symptoms. One bacterial isolate was *Erwinia carotovora* var. *carotovora*, the other *Xanthomonas campestris*. The morphology of the fungus was similar to that of *Cephalosporium*. Suspensions of spores or cells of the isolates were used to inoculate seeds, seedlings, and growing plants at 2-wk intervals until head set. Healthy young plants were resistant to the isolates. As plants matured, they became more susceptible to *E. carotovora* and *Cephalosporium*, which may produce necrosis on the stems and consequently weaken them. *Xanthomonas* appeared to be opportunistic, acting secondarily to contribute to the wilt syndrome.

The oil-producing sunflower, *Helianthus annuus* L., has the potential for increased economic importance in the United States. The discovery of wilt in a commercial field near Auburn, IN, in the fall of 1979 initiated this study. Diseased plants had externally visible, brown lesions; interior to the lesions, the stem was hollow. As a result, the weakened stems were bent and collapsed under the weight of the maturing head. Because an erect plant is necessary for mechanical harvesting, the wilt resulted in significant crop losses associated with breaking and lodging of heads.

Two types of bacteria and a fungus were isolated from tissue removed aseptically from the interior of the diseased stem. In this report, the morphological, cultural, and biochemical properties of the isolated organisms are described together with experiments to determine etiology of the disease.

Diseases of sunflower have been investigated in several countries. A bacterial leaf spot was reported on sunflowers in Canada in 1976, and the causative organism was tentatively identified as *Pseudomonas syringae* van Hall (8). Symptoms included angular brown leaf spots, lesions on the stems, and discolored vascular system. An unusual bacterial soft rot of sunflower appeared in Yugoslavia in 1970 following damage by hailstones (1). The disease was evident as greenish black spots on the stalk, which coalesced and covered most of the surface and caused the stalk to soften and affected parts to desiccate. Flowers and seeds also showed symptoms. A bacterial isolate was identified tentatively as *Pectobacterium carotovorum* (Jones) Waldee (*Erwinia carotovora* (Jones) Bergey, Harrison, Breed, Hammer and Huntoon) (11), and its pathogenicity was confirmed experimentally.

Incidence of *Erwinia* soft rot in sunflower has been reported elsewhere outside the United States (6). A survey of diseases observed on sunflowers in California in 1976 revealed a large number of pathogens, primarily fungi (3). The list did not include *E. carotovora* or any of the organisms identified in this study.

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MATERIALS AND METHODS

Isolation and identification of isolates. Primary isolation was achieved by washing the diseased stem with a 1:600 aqueous solution of benzalkonium chloride (Zephiran Chloride; Sigma Chemical Company, St. Louis, MO 63178) and dissecting out tissue interior to the lesions with sterile instruments. Pieces of diseased tissue were placed on trypticase soy agar (TSA) enriched with 5% sucrose and 0.3% yeast extract for bacterial isolates and on Sabouraud's agar for fungi. Inoculated TSA plates were incubated at room temperature for 2 days, after which ample growth of the two bacteria allowed differentiation by morphological characteristics of the colonies. Well-isolated, individual colonies of the two bacteria were restreaked to ensure pure culture. The isolates were transferred to fresh medium every 2 wk and maintained at 4 C.

Sabouraud's agar plates were incubated at room temperature for 1 wk. Only one type of fungus was isolated, and it was transferred to and maintained on potato-dextrose agar amended by the addition of 30 mg of tetracycline, 30 mg of streptomycin, and 50 mg of minocycline per 200 ml of medium. Cell morphology, staining reactions, motility, biochemical characteristics, and temperature and oxygen optima were determined by standard microbiologic procedures. Authority for determining the identity of the bacteria was "Bergey's Manual of Determinative Bacteriology," 8th edition. Microscopic fungal morphology was observed on slide cultures. Cultural morphology, characteristics of vegetative and reproductive mycelia, and asexual spore morphology were used as criteria in identification.

Etiologic determinations. Sunflowers were grown in 10-cm pots and maintained in Sherer controlled-environment chambers (Model CEL4-4; Warren Sherer, 9101 Industrial Road, Marshall, MI 94068). Each chamber was provided with eight standard 24-in., 20-watt fluorescent lamps; day length was set at 14 hr, night length at 10 hr. Day temperature was 22 C and night temperature was 20 C; the humidity was set at 60%.

Plants were spray, scratch, or needle inoculated with aqueous cell suspensions from 18- to 24-hr bacteria cultures or 5-day fungus cultures. Concentration was 10^8 cells per milliliter for bacterial suspensions and 10^6 spores per milliliter for fungal spore suspensions. Needle

Table 1. Characteristics of an unknown sunflower bacterial pathogen as compared with the groups of genus *Erwinia*[y]

Characteristic	Amylovora group (six species)	Herbicola group (four species)	Carotovora group (six species)	Unknown
Nitrate reduction	−[z]	D	+	+
Yellow pigment	−	+	−	−
Hydrogen sulfide	D	D	+	−
Gas from glucose	−	−	D	+
Acid from lactose	−	−	D	+
Acid from mannitol	−	−	+	+
Acid from xylose	−	+	+	+

[y] Data from "Bergey's Manual of Determinative Bacteriology," 8th ed.

[z] − = 20% or less of strains positive, + = 80% or more of strains positive, and D = different reactions given by different species.

inoculations were approximately 100 µl. Sunflower seeds were exposed to the organisms by being wrapped in paper towels that had been saturated with spore or bacterial cell suspensions. Seeds were allowed to soak for 24 hr before being planted in individual pots in autoclaved topsoil. Plants to be spray-inoculated were wetted thoroughly on all surfaces with a mist of cell or spore suspensions. For scratch inoculations, the plant epidermis was lacerated with a needle tip in two or three parallel lines about 3–4 mm long. For needle inoculations, the suspension of cells or spores was injected or punctured into the plant tissues at various sites, including nodes, internodes, peduncles, and leaves.

Because age of the host plant at the time of inoculation might affect resistance, plants were exposed to infection as seeds, as young plants, and at 2-wk intervals until maturity. The diseased areas of infected plants were excised and placed on TSA-sucrose-yeast agar (for bacteria) or potato-dextrose agar with antibiotics (for fungi) to determine the presence of microorganisms. The organisms were isolated, grown in pure culture, and identified. Healthy plants were similarly dissected and tissues incubated to determine whether bacteria or fungi were present.

RESULTS AND DISCUSSION

Two types of bacteria and one filamentous fungus were isolated from naturally infected plants. Both bacterial pathogens were motile, Gram-negative, asporogenous rods with a temperature growth optimum of 20–25 C. Both isolates produced large, domed, mucoid colonies on nutrient agar containing 5% sucrose. Both types had encapsulated cells. The two bacterial isolates were identified as *E. carotovora* and *Xanthomonas campestris*.

The *Erwinia* are facultative anaerobes of the family Enterobacteriaceae. They produce acid and gas from glucose but are otherwise fermentatively erratic. The *Erwinia* isolate was oxidase negative, catalase positive, and produced large amounts of levan when grown on 5% sucrose agar, a characteristic more typical of *E. amylovora* but a variable trait in *E. carotovora* as well. The sunflower isolate hydrolyzed casein, also more typical of *E. amylovora*. However, positive lactose and mannitol reactions place the isolate well within the *E. carotovora* group (Table 1). The *Erwinia* isolate was similar to *E. carotovora* var. *carotovora* Dye in 19 of 22 physiologic and cultural criteria (Table 2).

The second bacterial isolate was a strict aerobe, weakly catalase positive and oxidase negative. The organism produced a distinct yellow pigment. These and other characteristics place the unknown bacterium in the Pseudomonadaceae. Organisms resembling *Pseudomonas* that produce yellow colonies and are normally plant pathogens are included in the genus *Xanthomonas*. The non-diffusible, carotenoid pigment of *Xanthomonas* helps differentiate it from *Pseudomonas fluorescens*, which it closely resembles (7).

All species of recognized *Xanthomonas* are known plant pathogens and are found in association with plants or plant materials (2). More than 100 nomen species are reported that can be distinguished from the type species *X. campestris* by host reactions only. Although *Xanthomonas* may be regarded as essentially one species, five are

Table 2. Biochemical, physiologic, and cultural characteristics of *Erwinia carotovora* group[x] and unknown sunflower pathogen

Characteristic	*E. carotovora* var. *carotovora*	*E. carotovora* var. *atroseptica*	*E. chrysanthemi*	*E. cypripedii*	*E. rhapontici*	Unknown
Hydrogen sulfide	+[y]	+	+	+	+	−
Urease	−	−	−	−	−	−
Growth at 36 C	+	−	+	+	−	+
Mucoid growth on sucrose	d	−	d	d	d	+
Nitrate reduction	+	+	+	+	+	+
Indole	−	−	+	−	−	−
Blue pigment	−	−	+	−	−	−
Pink diffusible pigment	−	−	−	−	+	−
Acetoin	+	+	+	−	+	+
Casein hydrolysis	+	d	d	−	−	−
Starch hydrolysis	−	−	−	−	−	−
Growth at 38 C	+	−	+	+	−	+
Growth in 5% sodium chloride	+	+	−	+	+	+
Acid from mannitol	+	+	+	+	+	+
Acid from lactose	+	+	−	−	+	(+)[z]
Acid from arabinose	+	+	+	+	+	+
Acid from sucrose	+	+	+	+	+	+
Acid from xylose	+	+	+	+	+	+
Gas from glucose	d	d	+	+	−	+
Lipase	d	−	d	+	d	−
Sensitivity to erythromycin	−	−	+	+	+	−

[x] Data from "Bergey's Manual of Determinative Bacteriology," 8th ed.
[y] + = 80% or more of strains positive, − = 20% or less of strains positive, and d = 21–79% of strains positive.
[z] (+) = delayed.

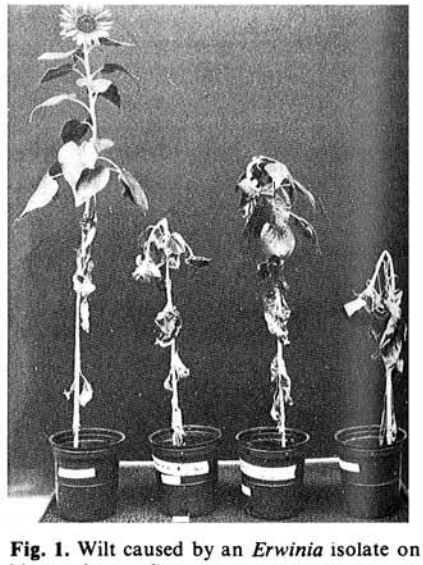

Fig. 1. Wilt caused by an *Erwinia* isolate on blossoming sunflowers.

Table 3. Biochemical, physiologic, and cultural characteristics of *Xanthomonas*[x] species and unknown sunflower pathogen

Characteristic	*X. campestris*	*X. fragariae*	*X. albilineans*	*X. axonpodis*	*X. ampelina*	Unknown
Growth at 35 C	+[y]	+	+	+	−	+
Mucoid growth on nutrient glucose agar	+	+	−	−		−
Proteolysis of milk	+	−	−	−	−	+
Hydrogen sulfide from peptone	+	−	−	+	d	+
Urease	−	−	−	−	+	−
Tolerance of sodium chloride, %	2.0–5.0	0.5–1.0	0.5	1.0	1.0	2.0–5.0
Acid production from glucose	+	+	+	+	−	(+)[z]
Gelatin liquefaction	+	+	d	−	−	+

[x]Data from "Bergey's Manual of Determinative Bacteriology," 8th ed.
[y]+ = 80% or more of strains positive, − = 20% or less of strains positive, and d = 21–79% of strains positive.
[z](+) = weak reaction.

currently differentiated. Characteristics that differentiate the species of the genus *Xanthomonas* were similar to those of the unknown aerobic isolate shown in Table 3. The isolate was identical to *X. campestris* in 23 basic biochemical and cultural traits.

Seeds inoculated with *E. carotovora* or *X. campestris* produced healthy seedlings. Ten-day-old plants that were scratched, sprayed, or punctured with suspensions of the two bacteria were unaffected. Inoculation of sunflowers with any one or combinations of the three microorganisms did not produce disease in 10-day-old plants. Finally, two series of 10 seedlings were punctured at the nodes and internodes at 2-wk intervals until maturity. The plants remained healthy until heads began to set. At that time, 30% of the plants inoculated with *E. carotovora* developed weakened stems and bent under the weight of the developing heads (Fig. 1). The plant parts above the collapse site died. All samples of the internal stem area from beneath the collapsed area produced large numbers of *E. carotovora*.

Plants inoculated with *X. campestris* at 2-wk intervals until maturity remained healthy through maturity except for 20% of the plants, which showed temporary collapse. However, the infected parts of the plants did not die. Internal stem tissues from the infected areas when incubated produced only a few colonies of *X. campestris*.

The fungal isolate was similar in morphology and cultural traits to *Cephalosporium acremonium* Corda. Isolated colonies were mature within 5 days. The colony was at first compact, with white, cottony mycelium that later became pale peach in color. The hyphae were septate, and the conidia formed easily disturbed clusters at the tips of unbranched conidiophores.

The pathogenicity of *Cephalosporium* in cereals has been known for some time (4). Both *C. maydis* (9) and *C. acremonium* (10) are known to attack maize; *C. gramineum* infects winter wheat (5). Studies with downy mildew on *Helianthus* suggest that this pathogen may spread by root contact (12). Seeds were therefore planted in sterile soil that had been infested with 50 ml of a densely turbid suspension of *Cephalosporium* spores. Sunflower plants with no symptoms of disease grew in the infested soil. Ten-day-old seedlings scratched, sprayed, or injected with suspensions of *Cephalosporium* spores at 2-wk intervals remained healthy. Plants injected with *Cephalosporium* spores at the onset of senescence developed black necrotic areas covered with mycelium.

The evidence indicates that the three isolated organisms are weakly pathogenic on young, healthy, growing sunflowers. As the plant matures and heads form, it becomes more susceptible to infection by *E. carotovora* and *Cephalosporium*. These organisms may invade alone or simultaneously and must in any case be injected into the plant to produce disease. Under natural conditions, the pathogens could be introduced into the plant by piercing insect bites or possibly by mechanical injury from wind or hail or through cultivation. The stem is weakened as a result of the infection. *X. campestris* appears to be an opportunistic pathogen that in the event of existing disease may act secondarily to increase the symptoms of the wilt syndrome.

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