Downy Mildew of Sunflower: Biology of Systemic Infection and the Nature of Resistance

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ABSTRACT

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Microscopic examination of fixed and stained serial sections of healthy and *Plasmopara halstedii*-infected (downy mildewed) sunflower (*Helianthus annuus*) seedlings revealed that zoospore encystment and infection occur primarily within or adjacent to the zone of elongation of the radicle. Up to 1,000 infection sites/mm of root length were observed. No infection was observed on the hypocotyl or cotyledons. Following infection of epidermal cells, mycelium entered into intercellular spaces and grew toward the apical meristem through the loosely packed inner cortical tissue. Mycelium progressed from the radicle to the apical meristem in 6-11 days in seedlings infected at 3 days of age. In older seedlings, the rate of progression was slower. Zoospores adhered to the surface but failed to penetrate the roots of sunflower lines possessing the PL_2 gene for downy mildew resistance; zoospores soon degenerated. Resistance apparently resulted from some physiological property or structural barrier that inhibited effective zoospore encystment or immediate post-encystment development.

Additional key words: hypersensitivity.

Downy mildew [caused by *Plasmopara halstedii* (Farl.) Berl. & de T.] is a destructive soil-, seed-, and airborne disease of sunflower (*Helianthus annuus* L.) (4, 5). This disease is characterized by distinct systemic and localized phases (6). Localized infection occurs primarily on leaves and seldom gives rise to systemic symptoms (6). Systemic infection, the most destructive phase of the disease, can reduce yields up to 50% (5). Sunflower seedlings are susceptible to systemic infection for only a short time after seed germination (6). Soilborne motile zoospores, produced either from oospores or from airborne sporangia, infect seedlings before or immediately after seedling emergence. The infection gives rise to intercellular hyphae and intracellular haustoria which ramify leaf and stem tissue (4). Systemic infection results when hyphae reach the apical meristem.

The exact site of penetration which results in systemic infection has not been conclusively established. Novotelnova (4) concluded that root hairs and root epidermal cells were the primary sites of entry. Cohen and Sackston (1) concluded that the hypocotyl was the primary site of entry which gave rise to systemic infection.

Secondary infection through apical meristems was shown by Cohen and Sackston (1) to culminate frequently in systemic symptoms. However, the low incidence of systemically infected plants in fields where 100% of the plants showed secondary infection led Zimmer and Kinman (7) to propose that secondary infection rarely culminated in systemic symptoms.

Cultural practices that restrict exposure of emerging seedlings to zoospores were the only effective means of minimizing losses from downy mildew until the introduction of resistant cultivars (7). Although resistance to the North American race(s) of *P. halstedii* is conditioned by a single dominate gene (5), little is known about the nature or mechanism of resistance. Cohen and Sackston (1), working with the less virulent European race, reported that resistance conditioned by the PL_2 gene resulted in failure of mycelium to spread sufficiently after infection to produce systemic infection.

The objectives of our light microscopy study were: (i) to establish the primary site of penetration which results in systemic infection; (ii) to trace post-penetration mycelial development in seedlings of a susceptible cultivar with special attention to the effect of seedling age; and (iii) to contrast host penetration, and post-penetration fungal development in seedlings of a susceptible cultivar to that of resistant seedlings in which resistance is conditioned by the PL_2 gene.

MATERIALS AND METHODS

Two sunflower cultivars, hybrid 896 and hybrid 894, susceptible and resistant to downy mildew, respectively, were used. Both hybrids share a common female parent, cms HA 89, but have different male parents. RHA 274, a downy mildew-resistant line with the universally effective resistance gene PL_2, is the male parent of hybrid 894. RHA 266, the male parent of hybrid 896, is susceptible to the North American races of *P. halstedii* but resistant to the European race(s).

To determine the primary site of infection, 3-day-old seedlings of both lines were placed in toto in an aqueous suspension of 1,000 - 4,000 zoospores/ml for 16 hr. Control seedlings were placed in water. Inoculum was prepared by allowing the pathogen in infected leaves (collected from the field) to sporulate overnight in a refrigerator. Sporangia could then be washed from the leaves and collected in water. Sporangia germinated readily to produce zoospores. After inoculation, seedlings were transplanted into sterile potting soil and grown for 2, 4, 6, 8, 10, and 12 days. At the end of each period, a representative sample of eight seedlings was selected; the hypocotyls were excised, and fixed in formalin-aceto alcohol (2) for 24 hr. Hypocotyls were dehydrated through 20% increments of tertiarybutyl alcohol, cleared through 20% increments of xylene, and embedded in Paraplast. A representative portion of each hypocotyl was sectioned at 10μm. Sections were affixed to glass slides, stained with Conant's Quadruple Stain (2), and mounted in Turtox Superior Resinous Mounting Media. In all subsequent histological work, tissue was prepared by the same procedure.

To establish more precisely the primary site of infection and to contrast the susceptible and resistant reaction, 3-day-old seedlings of both lines, each possessing radicles 10-20 mm long, were inoculated as described previously. Sixteen hours after inoculation, seedlings were fixed in toto and embedded. Starting at the root tip, and progressing into the hypocotyl, five embedded seedlings of each line were serially sectioned. One hundred sections, representing 1 mm of length were affixed to individual slides. Thus, entire seedlings were observed as microtomed sections in a sequence of slides, each slide representing 1 mm of tissue, progressing from root tip to the epicotyl.

Infection sites for each millimeter along the length of the seedlings were counted. Areas of susceptible seedlings, having the greatest number of infection sites, were compared with similar areas of resistant seedlings.

To monitor mycelium spread, 5-, 9-, and 18-day-old susceptible seedlings that had been grown in vermiculite were uprooted, washed, and inoculated as described previously. However, only the roots were susepended in the zoospore suspension. Seedlings were replanted in vermiculite after inoculation. Six, 11, and 16 days after inoculation, six seedlings were randomly selected from each age group. Seedlings were fixed and embedded. Transverse stem sections of 10 μm thickness were taken from each seedling at three locations: root-hypocotyl junction, midway along the hypocotyl, and immediately below the cotyledons. These sections were examined microscopically to determine if mycelium had progressed from the roots to the position on the seedling from which the sections were taken within the period from inoculation to fixation.

RESULTS AND DISCUSSION

Mycelium of *P. halstedii* was observed in hypocotyls of susceptible seedlings 4-12 days after inoculation.

TABLE 1. Number of infections caused by *Plasmopara halstedii* per millimeter of radicle length in susceptible sunflower seedlings (3 days old)

Distance from root tip (mm)	Seedling No.					Mean	Approximate position on seedling
	1	2	3	4	5		
22	0	0	0	0	0	0	hypocotyl
21	0	0	0	0	0	0	hypocotyl
20	0	0	0	0	0	0	hypocotyl
19	0	0	0	0	0	0	hypocotyl
18	0	0	0	0	0	0	hypocotyl
17	0	0	0	0	0	0	hypocotyl
16	0	0	0	0	0	0	maturation
15	0	0	0	0	0	0	maturation
14	0	10	22	0	0	6	maturation
13	0	28	96	0	0	25	maturation
12	0	106	224	0	0	55	maturation
11	0	292	259	1	0	110	maturation
10	0	582	587	1	25	239	maturation
9	2	...	459	7	40	127	maturation
8	6	770	1,009	24	24	366	maturation
7	54	771	928	72	17	368	maturation
6	106	920	647	228	22	384	matur./elong.
5	202	642	279	738	95	391	elongation
4	507	1,023	375	814	297	603	elongation
3	205	...	574	113	167	270	elongation
2	0	206	344	16	1	142	tip/elongation
1	0	34	3	8	0	9	tip

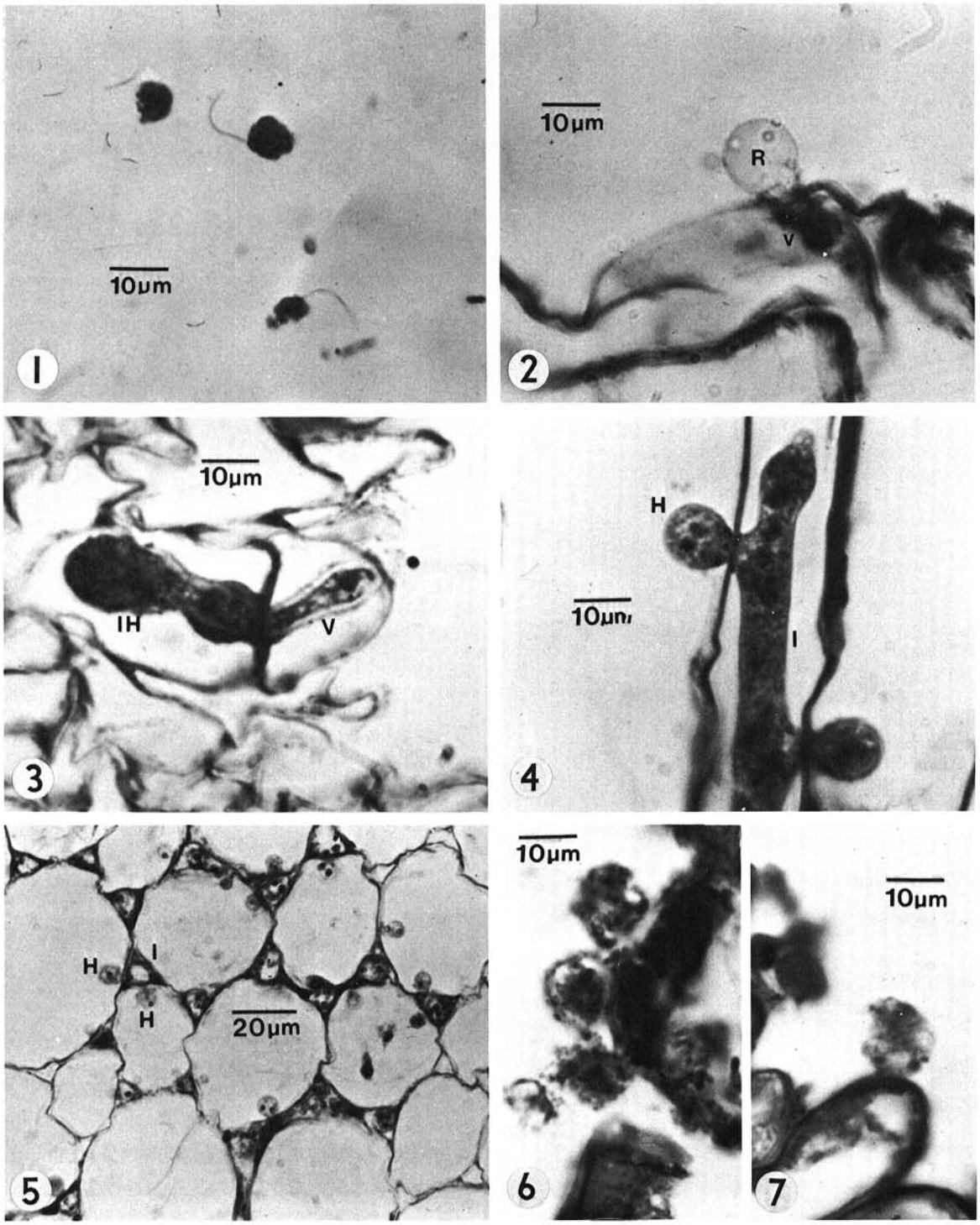

Fig. 1-7. Stages of infection of a susceptible sunflower cultivar by *Plasmopara halstedii*: **1)** Flagellated zoospores produced from sporangia; **2)** Penetration of hair root by encysted zoospore, remnant (R) of encysted zoospore devoid of cytoplasm, infection vesicle (V) within root hair cell 16 hr after commencement of inoculation period; **3)** Growth of pathogen (IH, intracellular hypha) into root cortical cell from the infection vesicle (V) in the epidermal cell, 48 hr after commencement of inoculation period; **4)** Intercellular hypha (I) and haustoria (H) in lower hypocotyl of host 2 days after removal of plants from inoculation to greenhouse (longitudinal section); **5)** Cross section of hypocotyl tissue showing congestion of intercellular spaces with intercellular hyphae (I), and haustoria (H); and **6 and 7)** Zoospores attached to root surface of resistant sunflower hybrid 894, 16 hr after commencement of inoculation period (host penetration did not occur).

Mycelium was observed only in the central cortex of the hypocotyl and not penetrating through the hypocotyl epidermis. Moreover, mycelial development in the peripheral region of the cortex was scant. This suggested that infection occurred most likely below the hypocotyl, and that mycelium progresses through the hypocotyl enroute to the apical meristem. These observations led us to focus on the radicle as the primary site of infection, and most likely the site of the resistance response.

Examination of serially sectioned seedlings of the susceptible cultivar revealed that zoospore encystment and subsequent penetration had occurred on the root surface within 16 hr after inoculation. The sequence of events which resulted in infection appeared as follows: zoospores (Fig. 1) swim to, adhere, and encyst on the root surface, and lose their flagellae. The encysted zoospores penetrated the host cell wall and gained entry (Fig. 2). Once inside the host epidermal cell, the fungus spread intracellularly. By 48 hr, the fungus had progressed through two or three cells (Fig. 3) and subsequently entered into the intercellular spaces.

The number of zoospore infection sites observed varied greatly among seedlings. However, the greatest number of infection sites occurred in the area of elongation of the primary root (Table 1), and tapered off through the zone of maturation. No infection sites were observed on either the hypocotyl or cotyledons. Peculiarities of the zone of elongation which made it the prime area of infection are not known. However, epidermal cells in this region are extremely thin-walled and may offer less resistance to penetration than cells of older tissue where continued deposition of wall materials may have strengthened the walls.

Once the invading organism apparently had established a nutritional link with the host, it produced an extensive intercellular network of hyphae and intracellular haustoria (Fig. 4 and 5). The pathogen grew in all directions but appeared to move upward through the loosely packed parenchymal cells of the cortical tissue more rapidly than inwardly through the more densely packed cells of the vascular or stelar region. In seedlings inoculated when 3 days old, mycelium reached the apical meristem 11 days after inoculation (Table 2). The rate of mycelial rise was slower in older seedlings. In seedlings inoculated at 9 days of age, 16 days were required for the mycelium to reach the apical meristem. Mycelium was not found in the hypocotyl in seedlings inoculated at 18 days of age. This suggested that either infection did not occur, or that the spread of the fungus following infection was suppressed.

Hypocotyls of the resistant hybrid fixed 2-12 days after inoculation showed no traces of mycelium. Zoospore infection sites were well established on the roots of susceptible seedlings within 16 hr after inoculation. Close examination of the zone of elongation of inoculated seedlings of the resistant hybrid, 16 hr after inoculation and at subsequent intervals thereafter, failed to reveal the presence of infection sites. Zoospores were observed adhering to the root surfaces (Fig. 6 and 7). Such observations suggested that the resistance mechanism was operative before the invading fungus could establish a nutritional relationship with the host. However, Montes and Sackston (3), working with a less widely virulent European race of *P. halstedii*, reported that resistance of HA 61, the original source of the PL_2 gene for resistance, was probably due to the inability of mycelium to reach the epicotyl following its establishment in the roots and hypocotyls. Our results suggest that the resistance to the North American race(s), conditioned by the PL_2 gene in hybrid 894, inhibits penetration or prepenetration processes during or soon after, zoospore encystment.

The lack of agreement between our results and those of Montes and Sackston (3) suggests that different mechanisms of resistance may be operative against the less virulent European race(s) and the more virulent North American race(s). The rapid and dramatic expression of resistance of hybrid 894 (possessing the PL_2 gene) to the North American race(s) as observed by light microscopy, suggests the presence of a structural barrier to penetration or the existence of a preformed diffusable chemical which inhibits penetration or prepenetration processes.

TABLE 2. Presence of *Plasmopara halstedii* mycelium in sunflower seedlings of different ages and days after inoculation

Seedling age at time of inoculation (days)	Position	Samples with mycelium/samples examined at days after inoculation		
		6 no.	11 no.	16 no.
3	top hypocotyl	0/6	6/6	6/6
	mid hypocotyl	0/6	6/6	6/6
	radicle/hypo.	0/6	6/6	6/6
9	top hypocotyl	0/6	2/6	6/6
	mid hypocotyl	0/6	3/6	6/6
	radicle/hypo.	0/6	3/6	6/6
18	top hypocotyl	0/6	0/6	0/6
	mid hypocotyl	0/6	0/6	0/6
	radicle/hypo.	0/6	0/6	0/6

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