T1978PATH01

HISTOLOGY AND ULTRASTRUCTURE OF SUSCEPTIBLE AND RESISTANT REACTIONS OF SUNLFOWERS TO PLASMOPARA HALSTEDII [1,2]

by

Glenn Wehtje, Research Associate and Professor of Plant Pathology
North Dakota State University
Fargo, North Dakota 58102
and
David E. Zimmer, Area Director
Science and Education Administration
U.S. Department of Agriculture
Georgia Coastal Plains Experiment Station
Tifton, Georgia 31794

Abstract

Microscopic examination of serially sectioned sunflower seedlings (Helianthus annuus) inoculated with Plasmopara halstedii revealed that zoospore encystment and penetration are restricted to the zone of elongation of the primary radicle. Electron microscopy revealed that penetration by the pathogen is preceded by formation of a papilla and the accompanying invagination of the host plasma membrane. A primary infection vesicle forms in the penetrated epidermal cell, following localized host wall degradation. The infection vesicle elongates into a hypha which grows intracellularly through the epidermal cell into adjoining cortical cells. Host plasma membrane is continuous around the infection vesicle, however the continuity is often difficult to trace. Subsequently, the hypha becomes intercellular and grows upward through the loosely packed inner cortical tissues. Systemic infection results when hypha reach the apical meristem. The rate of hyphal growth is dependent on the age of the seedling. Host resistance is hypersensitive and is triggered prior to penetration of host cells by the fungus. Necrosis of the zoospore accompanies the hypersensitive reaction of the host.

Introduction

Downy mildew of sunflower (Helianthus annuus L.) caused by the phycomycetous fungi Plasmopara halstedii (Farl.) Berl. & de toni is a destructive disease. While infection may be localized on leaves, systemic infection is the most destructive phase of the disease. Yield losses of 50% are not uncommon (12). Sunflowers are susceptible to systemic infection only during the germination and emergence of seedlings. Soil borne motile zoospores, produced form zoospores of from airborne aporangia are the infecting agent. The infection gives rise to intercellular hyphae and intracellular haustoria which ramify leaf and stem tissue (7). Systemic infection results when hyphae reach the apical meristem.

---

[1] Mention of a trade of proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and nor does it imply approval to the exclusion of other products that may also be suitable.

[2] We thank V.D. Pederson for assistance in Preparation of photographs.

The exact site of penetration which results in systemic infection has not been conclusively established. Novotelnova (8) concluded that root hair and root epidermal cells were the primary sites on entry. Cohen and Sackston (2) concluded that the hypocotyl was the primary site of entry which gave rise to systemic infection.

Cultural practices that restrict the exposure of emerging seedlings to zoospores were the only effective means of minimizing losses from downy mildew until the introduction of resistant varieties (13). Although resistance to the North American race(s) of downy mildew is conditioned by a single dominate gene Pl_2, (13), little is known about the nature or mechanism of resistance. Cohen and Sackston (2), 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 microscope study were threefold: (i) establish the primary site of penetration which results in systemic infection; (ii) trace post-penetration mycelial development in seedlings of the susceptible cultivar with special attention to the effect of seedling age; (iii) contrast host penetration, and post-penetration fungual development in seedling of a susceptible cultivar to that of resistant seedlings in which resistance is conditioned by the Pl_2 gene, at both the light and electron microscope level.

Materials and Methods

Two sunflower (Helianthus annuus) 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. HA 274, a downy mildew resistant line with the universally effective resistant 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).

In order to determine the primary site of infection, 3-day old seedlings of both lines each possessing radicles 10-20 mm long were placed in downy mildew inoculum in toto. Downy mildew inoculum was prepared by allowing 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 readily germinated to produce zoospores. Zoospore concentration averaged between 1,000-4,000 during the inoculation period. Control seedlings were placed in water.

Seedlings were fixed in formalin-aceto alcohol (5) for 24 hours subsequent to the inoculation period. Seedlings were dehydrated through 20% increments of tertiarybutyl alcohol, cleared through 20% increments of xylene, and embedded in Paraplast. Starting at the root tip and progressing into the hypocotyl, five embedded seedlings of each line were serially sectioned. One hundred section, representing 1 mm of length were affixed to individual slides. Sections were stained with Conant's Quadruple Stain (5), and mounted in Turtox Superior Resinous Mounting Media. Thus, entire seedlings were observed as microtomed sections in a sequence of slides, each slide representing 1 mm of root length. Progress from root tip to the epicotyl. In all subsequent light

microscope histological work tissue was prepared by the same procedure. Infection sites for each millimeter along the length of the seedling were counted. Areas of susceptible seedlings, having the greatest number of infection sites were compared with the similar areas of the resistant seedlings.

In order to monitor mycelium spread subsequent to infection, 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 suspended 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 imbedded. Transverse stem sections of 10 um 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 mycellium had progressed from the roots to that position on the seedlings from which the sections were taken, within the period from inoculation to fixation.

For ultrastructural observation three-day-old seedlings of both the resistant and susceptible cultivars were inoculated for 16 hr as described previously. Control seedlings were similarly placed in water. In later work 500 ug/ml each of penicillin and streptomycin sulfate were added to the inoculum to control bacterial parasitism of zoospores (2).

Following inoculation the zone of elongation was excised from the primary root and cut into 1 mm lengths. Tissue was fixed at 5% glutaraldehyde in 0.1 M K_2PO_4 buffer, pH 7.4 for 2 hours. Following three 20 min. rinses in buffer tissue was postfixed in 2% OsO_4 solution of buffer for 4 hours at 4 C. It was then rinsed in buffer and dehydrated in a graded series of acetone. Tissue was held 4 hours in the 70% dehydrated solution which was saturated with uranyl acetate and eventually embedded in an Epon-Araldite mixture polymerized at 60 C for 24 hours.

Sections of the root were cut with a Dupont diamond knife on a Sorvall MT-2 ultramicrotome. Sections were mounted on copper slot grids covered with Parloidian support film and examined with an AEI Corinth 275 transmission electron microscope operated at 60 KV.

Results

Examination of serially sectioned seedlings of the susceptible cultivar revealed that zoospore encystment and subsequent penetration of root epidermal cells had occurred within 16 hours after the commencement of innoculation Figure 1. The number of zoospore infection sites observed varied among seedlings. However, the greatest number of infections 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 the hypocotyl nor the cotyledons.

Once the invading organism apparently had established a nutritional link with the host it penetrated through several cells, and eventually entered into the intercellular spaces. Mycelium appeared to move predominately upward

through the loosely packed parenchyma cells of the cortical tissue Fig. 2. Mycelium reached the apical meristem 11 days after inoculation in seedlings inoculated when three days old (Table 2). The rate of mycelial rise was slower in older seedlings. Sixteen days were required for the mycelium to reach the apical meristem in seedlings inoculated at nine days of age. Mycelium was not found in the hypocotyl in seedlings inoculated at 18 days of age.

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	Days after Inoculation 6	11	16
3	Top hypocotyl	0/6*	6/6	6/6
	Mid hypocotyl	0/6	6/6	6/6
	radicle/hypocotyl	0/6	6/6	6/6
6	Top hypocotyl	0/6	2/6	6/6
	Mid hypocotyl	0/6	3/6	6/6
	Radicle/hypocotyl	0/6	3/6	6/6
9	Top hypocotyl	0/6	0/6	0/6
	Mid hypocotyl	0/6	0/6	0/6
	Radicle/hypocotyl	0/6	0/6	0/6

* Number of samples with mycelium/number of samples examined.

Initial ultrastructural observations revealed that zoospores attached to the root surface often contained numerous, apparently parasitic bacteria when the inoculum had been prepared with plain water. Penicillin or streptomycin sulfate added to the inoculum markedly reduced bacterial infection without inhibiting penetration and infection of the host. The amount of infection was greater with the addition of antibiotics, thus the frequency of sectioning through an infection vesicle was enhanced.

The flocculent to electron dense materials between the cell walls of the encysted zoospore and the host suggest an adhesive substance(s) similar to that observed with zoospores of Olpidium (9), and penetration tips of Phytophthora infestans (3,4). Prior to penetration papillae form between the host plasma membrane and the host cell wall. Host penetration is accomplished by localized degradation of the host cell wall resulting in a pore Fig. 3. Cytoplasm flows from the encysted zoospore into the host cell resulting in an infection vesicle. During early stages of host penetration the papilla may surround the exapnding infection vesicle. Eventually the expanding infection vesicle penetrated the papilla and extend into the interior of the cell. The remnants of the papilla becomes a collar around the proximal end of the infection vesicle and may serve to prevent leakage of host cytoplasm. An infection vesicle contains an abundance of mitochondria, vacuoles and suspected lipid bodies. Continuity of the host plasma membrane is easily traced around the papilla in early stages of

Explanation of Figures: Fig. 1. Infection of root hair by encysted zoospore, light microscope (X 1,100). Fig. 2. Intercellular hyphae with intracellular haustoria, light microscope (X 1,100). Fig. 3. Developing infection vesicle; (N) nuclei; (V) vacuoles; (Z) encysted zoospore, electron microscope (X 50,000). Fig. 4. Hypersensitive reaction of cultivars of cultivars possessing the $P1_2$; note attached zoospores (Z), electron microscope (X 2,500).

development, prior to the rupture of the papilla. However, continuity of host plasma membrane around mature infection vesicles is difficult to trace.

Light microscope examination of sectioned radicles of inoculated seedlings of the resistant hybrid failed to reveal the presence of infection sites. However, zoospores were observed adhering to the root surface. Ultrastructural obersvations revealed resistance to be hypersensitive in nature Fig. 4. Attachment of zoospores to the root surface of the resistant cultivar is followed by necrosis and collapse of the contacted cell and one or more of the neighboring host cells. The incompatible response is initiated extremely rapidly prior to penetration of the epidermal cell. In none of the incompatible reactions examined did the fungus enter cells or intercellular spaces of the host. Necrosis of the challenged cell is accompanied by deterioration of the cytoplasm and organelles within the attached encysted zoospore.

Discussion

Infection of susceptible cultivars is strongly restricted to the zone of elongation of the primary radicle. Peculiarities of the zone of elongation which make 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 material may have strengthened the walls.

Systemic infection only results of mycelium reach the apical meristem. However, the rate of mycelial growth is supressed as seedling age. Thus, in effect, seedlings become resistant to systemic infection. Penetration in the *H. annuus*-*P. halstedii* system is typical to that described for other similar systems (3,4,11,14).

Resistance in the *H. annus*-*P. halstedii* system observed here is manifested by a hypersensitive response of such speed and intensity that penetration does not occur. Furthermore, in contrast to what has been described in other systems, papillae formation was never observed in the resistant reaction. The cell wall of neither the host nor the zoospore was broken during the hypersensitive reaction. Resistant responses to other phycomycetous pathogens occur only after the initiation of the pre-penetration process or more commonly after penetration by the pathogen (3,4,6,11). Montes and Sackston (7) 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 successful penetration in the roots and hypocotyls. We observed an unusually strong hypersensitive response. Thus we infer that a diffusible substance(s) emanating from the region of the hypersensitive reaction of the host has a toxic effect on the zoospore as well as neighboring host cells.

Literature Cited

1. AIST, J.R., and H.W. ISRAEL, 1977. Timing and significance of papillae formation during penetration of Olpidium brassicae. Phytopathology 67: 187-194.

2. COHEN, Y., and W.E. SACKSTON, 1973. Factors affecting infection of Plasmopara halstedii. Can. J. Bot. 51:15-22.

3. HOHL, H.R., and P. STOSSEL, 1976. Host-parasite interfaces in a resistant and a susceptible cultivar of Solanum tuberosum inoculated with Phytophthora infestans: tuber tissue. Can. J. Bot. 54:900-992.

4. HOHL, H.R., and E. SUTER, 1976. Host-parasite interfaces in a resistant and a susceptible cultivar of Solanum tuberosum inoculated with Phytophthora infestans: leaf tissue. Can J. Bot. 54:1956-1970.

5. JOHANSON, D.A., 1940. Plant Microtechnique. McGraw-Hill, New York 523 p.

6. JONES, D.R., W.G. GRAHAM, and W.E.B. WARD, 1975. Ultrastructural changes in peper cells in incompatible interaction with Phytophthora infestans. Phytopathology 65:1274-1285.

7. MONTES, F., and W.E. SACKSTON, 1974. Growth of Plasmopara within susceptible and resistant sunflower plants. In proceedings of the 6th International Sunflower Conference. Pages 623-629.

8. NOVETELNOVA, N.S., 1966. Downy mildew of sunflowers; taxonomy and biology of the pathogen, and pathogenesis of the disease. (In Russian) Moskva, Nauka, 14 p.

9. TEMINK, J.H.M., and R.N. CAMPBELL, 1969. The ultrastructure of Olpidium brassicae. III. Invection of host roots. Can. J. Bot. 47:421-424.

10. TIPPETT, J.T., A.A. HOLLAND. G.C. MARKS, and T.P. O'BRIEN, 1976. Penetration of Phytophthora cinnamoni into disease tolerant and susceptible Eucalpts. Arch. Microbiol. 108:231-242.

11. ZIMMER, D.E., 1971. A serious outbreak of downy mildew in the principal sunflower production area in the United States. Plant Dis. Rep. 55:11-12.

12. ZIMMER D.E., and M.L. KINMAN, 1972. Downy mildew resistance in cultivated sunflower and its inheritance. Crop Sci. 12:749-751.

TABLE 1. Average Number of Plasmopara halstedii infections per millimeter of radicle length in five susceptible sunflower seedlings (three days old).

Distance from root tip (mm)	Average number of infection sites	Approximate position on seedling
17	0*	hypocotyl
16	0	maturation
15	0	maturation
14	6	maturation
13	25	maturation
12	55	maturation
11	110	maturation
10	239	maturation
9	127	maturation
8	366	maturation
7	368	maturation
6	384	maturation/elongation
5	391	elongation
4	603	elongation
3	270	elongation
2	142	tip/elongation
1	9	tip

* No infection sites were found above this position.