

## Histology of Leaf Infection of Susceptible and Resistant Soybeans by *Peronospora manshurica*

J. H. Riggle and J. M. Dunleavy

Research Assistant, Department of Botany and Plant Pathology, Iowa State University; and Research Plant Pathologist, Agricultural Research Service, United States Department of Agriculture, and Professor of Plant Pathology Iowa State University, Ames 50010, respectively.

Joint contribution of the Agricultural Research Service, United States Department of Agriculture, and as Journal Paper No. J-7567 of the Iowa Agriculture and Home Economics Experiment Station, Ames 50010. Project 1179.

Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may be suitable.

We thank R. L. Bernard for the resistant isoline seed and for the fungus isolate.

Accepted for publication 29 October 1973.

### ABSTRACT

Detached leaves of a susceptible soybean cultivar ('Wayne') and a resistant isoline ('SL9') were inoculated with conidia of *Peronospora manshurica*. After 12, 24, 36, 48, and 51 h, the leaves were cleared, stained, and mounted on microscope slides for observation. There were no discernible differences in the formation of germ tubes, appressoria, or penetration pegs on the two cultivars. Penetration and early hyphal growth was as rapid on resistant tissue as on susceptible tissue, but by 24 h the hyphae had ramified farther

in the susceptible leaf than in the resistant leaf. At 36 h the fungus in the susceptible tissue had penetrated 40% farther than in the resistant tissue, and susceptible leaf tissue had a mean of 3.1 haustoria/penetration; whereas the resistant tissue had a mean of only 0.2 haustoria/penetration. After 36 h there were 14 haustoria/mm of hyphae in the susceptible tissue, but only 2.7/mm in the resistant tissue.

Phytopathology 64:522-526.

*Additional key words:* downy mildew.

Downy mildew caused by *Peronospora manshurica* (Naum.) Syd. ex Gaum. is one of the most common diseases of soybeans, *Glycine max* (L.) Merr. The fungus is readily disseminated by oospores that encrust seed coats of susceptible cultivars (3). Twenty-three races of the fungus have been described (2). Intercellular hyphae are small, and haustoria within cells are long and tenuous (8). Jones and Torrie (7) reported that systemic infections on upper leaves were connected by mycelia to original infections on lower leaves. Millikan et al. (10) reported less susceptibility to infection among older leaves and decreased RNA content of downy mildew diseased leaves vs. healthy soybean leaves. Millikan and Wyllie (9) compared the Ca and P content of cultivars 'Clark' (susceptible) and 'Kanrich' (resistant). Ca content of the two cultivars did not differ. P content was correlated with RNA content, but Millikan and Wyllie gave no data on comparative RNA content. Peyton and Bowen (11) described the ultrastructure of the interface between *P. manshurica* and infected host cells in a susceptible cultivar. They observed a collar of amorphous material at the haustorial penetration point. They reported possible secretory activity by the host cytoplasm into the "zone of apposition," which occurs between the haustorium cell wall and the invaginated host cell membrane.

No histological studies concerning infection by *P. manshurica* of resistant soybeans have been made. This study was conducted to quantitatively compare early infection by *P. manshurica* using susceptible and resistant near-isogenic lines of soybeans. The soybean-*Peronospora manshurica* host-parasite combination is well suited to the study of plant disease resistance because any different character associated with the resistant isolate is likely to be related to its resistance.

**MATERIALS AND METHODS.**—*Plants and pathogen.*—A susceptible soybean cultivar, Wayne, was compared with a near-isogenic resistant isolate, SL9, which is resistant to all known races of *P. manshurica* (1). The line SL9 is a homozygous selection from progeny of the ninth backcross of Wayne to Kanrich. The dominant resistance gene was derived from the cultivar Kanrich (1). The SL9 seed and the *Peronospora manshurica* isolate, race 12, used in this study were obtained from R. L. Bernard (U.S. Regional Soybean Laboratory, Urbana, Ill.). The fungus was maintained on the cultivar Wayne by the method of Dunleavy (2), except that temperature for inoculation was 20-25 C.

*Inoculation.*—Expanding unifoliate leaves (2-3 cm long) of Wayne and SL9 were inoculated by gently shaking them for 5 min in a suspension of  $10^5$  conidia/ml of 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20). After they were shaken, the wet leaves were placed in moist chambers. A microscope slide with three individual drops of the spore suspension on it also was placed in the chamber. After 12 h, percentage of germination was determined by microscopic examination.

*Histological methods.*—At intervals of 12, 24, 36, 48, and 51 h after inoculation, leaves were removed and cut into 1-cm<sup>2</sup> pieces and fixed in 95% (v/v) ethanol. Leaf pieces were cleared and stained by the method of Shobe and Lersten (12), except that the staining time was increased from a few seconds to 1-1.5 h.

*Observations.*—The slides were searched for germ tubes that had appressoria. Hyphal ingress and progress were observed mostly with the oil immersion lens. The lens field diam was 70  $\mu$  and was used to roughly measure the length of hyphal growth. The length of the germ tube, horizontal hyphal extension, depth of hyphal penetration, number of haustoria, number of stomatal penetrations, and number of collapsed hyphae, all were recorded. At least 10 observations were made for each time interval. Hyphae curving, branching, and vertically traversing the focal plane were difficult to measure. In such cases, estimates were made. The microscope fine focus graduations equalled 2  $\mu$ . Depth of hyphal penetration was measured by noting the difference between the level of the penetration peg and the deepest hypha that could be brought into focus.

**RESULTS.**—Germination of conidia in water drops ranged from 0 to 80%; 20-30% was most common. Spore germination was rapidly reduced by a self-inhibitor, which could be partly removed by one or two centrifugal washings. Young plants usually were infected, even when conidial germination on slides was zero. Apparently germination of conidia was host-stimulated and was essentially the same on leaves of both Wayne and SL9. The exact percentage of conidial germination on fixed leaves could not be determined because conidia without appressoria were washed off.

Within 12 h after inoculation, most germinated conidia had formed ovate appressoria about  $9 \times 12 \mu$ . Appressoria formed chiefly over adjacent cell walls of epidermal cells. Penetration pegs developed from the appressoria and forced their way between adjacent epidermal cells into the mesophyll intercellular air spaces. Some germ tubes bulged at cell wall junctures without penetrating the epidermis. Almost all appressoria formed on the leaf lower epidermis; about 10% of penetrations entered stomata without forming appressoria. Germ tube lengths on Wayne and SL9 were variable, but not significantly different except at 51 h (Fig. 1-A).

The infection process in Wayne (susceptible) and SL9 (resistant) was similar at 12 h, but by 24 h more hyphal growth and haustoria had formed in Wayne than in SL9 (Fig. 1-B, C, and D; 2-A, B). The fungus continued to make rapid progress in Wayne until at least 51 h after inoculation. Intercellular hyphae were about 5  $\mu$  in diam and ramified throughout mesophyll tissue, branching occasionally. Hyphae often grew vertically between palisade cells, putting haustoria into all four adjacent cells. The sheath parenchyma of larger veins often blocked further advance of the hyphae, resulting in the angular chlorotic areas characteristic of this disease. Very small vascular bundles were bypassed by hyphae that grew under the vein parenchyma sheath (Fig. 2-B). On SL9 the fungus made slow progress and formed an average of only one haustorium per penetration by 51 h (Fig. 1-D). Structure of the haustoria was the same on Wayne and SL9. The haustorial branches were about 2.5  $\mu$  in diam and were finger-like, curving, and branched up to five times within the cell (Fig. 3).

The depth of penetration on Wayne increased more rapidly than on SL9 from 12 to 36 h and leveled off at about 120  $\mu$  (Fig. 1-B). This was the maximum because the leaves were about 120  $\mu$  thick. There was an abrupt

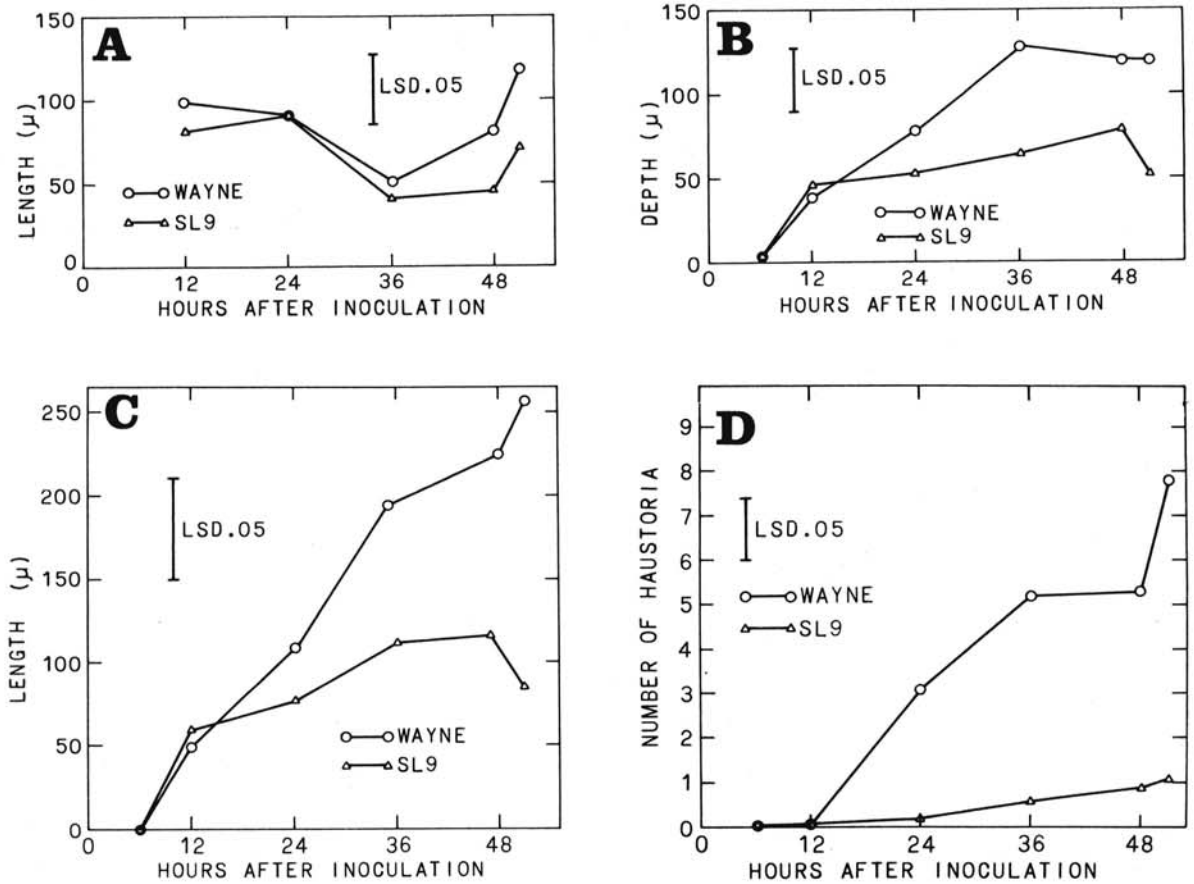


Fig. 1-(A to D). Hyphal growth of *Peronospora manshurica* on unifoliate leaves of cultivars Wayne (susceptible) and SL9 (resistant) soybean. A) Germ tube lengths from conidia to appressoria. B) Depth of penetration. C) Horizontal length. D) Number of haustoria/penetration.

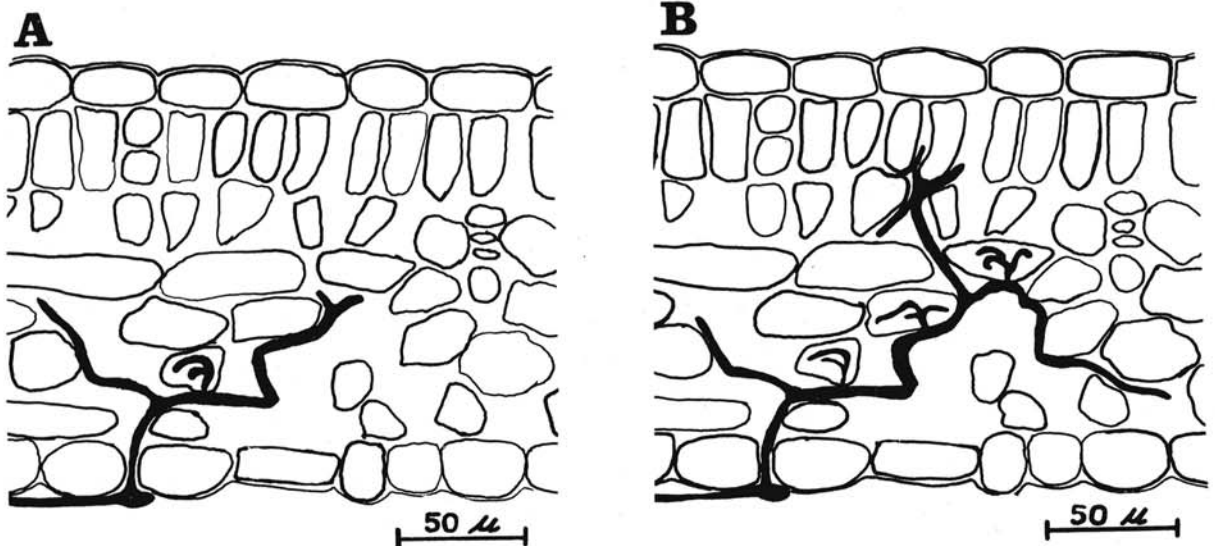


Fig. 2-(A, B). Diagram of typical invasion and haustoria formation of *Peronospora manshurica* on A) cultivars SL9 (resistant) and B) Wayne (susceptible) soybean leaves 24 h after inoculation.

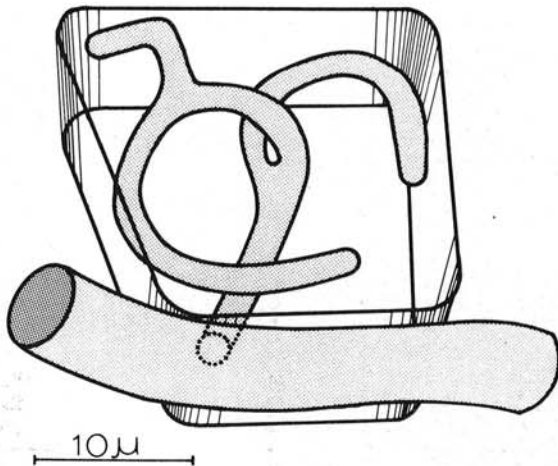


Fig. 3. Diagram of typical haustorium of *Peronospora manshurica* on the soybean cultivar SL9 51 h after inoculation.

decrease in length and depth on SL9 at 51 h (Fig. 1-B and C). The number of haustoria/mm of hyphae was markedly lower on SL9 than Wayne (Fig. 4).

DISCUSSION.—Germination, appressoria, penetrations, and sometimes haustoria have been reported previously for *Erysiphe graminis* [DC.] MÉRAT (13) and *Puccinia sorghi* Schw. (6) on resistant hosts. This was also the case in *Peronospora manshurica* infection of

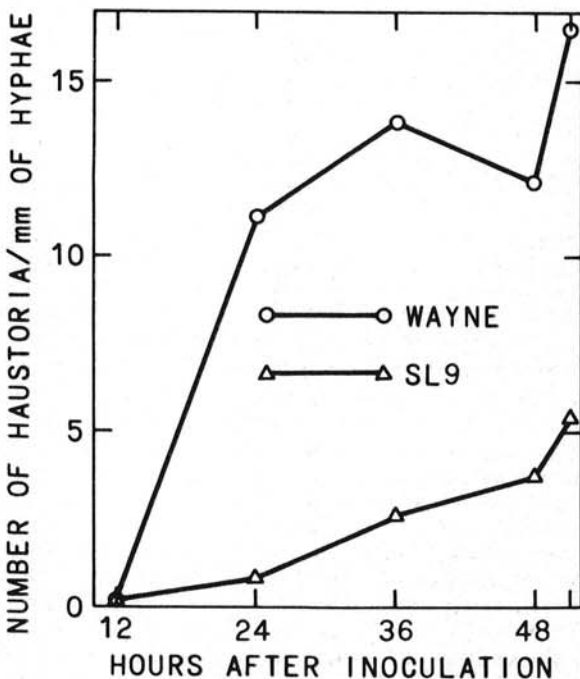


Fig. 4. Number of *Peronospora manshurica* haustoria/millimeter of hyphal length (combined hyphal and germ tube length) on the soybean cultivars Wayne (susceptible) and SL9 (resistant). No haustoria were observed prior to 24 h, and each point is a mean of 10 single penetrations.

the resistant soybean cultivar SL9.

Haustorium formation in resistant cowpea leaves infected by *Uromyces phaseoli* var. *vignae* resulted in either necrosis of the host cell or enclosure of the haustorium by a callose-containing sheath (4, 5). The fungus penetrated nonhost garden bean, but hyphal growth soon stopped. Only 10% of all infection hyphae formed haustoria on garden bean plants (5). A response by deposition of callose-like material occurred in all garden bean cells adjacent to hyphae. The inhibition of haustoria formation on SL9 could have been caused by similar host cell responses.

The abrupt decrease in mycelium length and penetration depth between 48 and 51 h on SL9 occurred because some hyphae had collapsed. Vertical branches appeared as thin flat lines under the microscope. Such hyphae were difficult to see, and many probably were missed. The decrease in lengths of germ tubes of conidia sampled at 36 h after inoculation may have been caused by late germinating conidia.

It is clear from the above data that resistance is expressed between 12 and 24 h. There was no difference at 12 h (Fig. 1-B, C) but the number of haustoria on the cultivar SL9 was significantly less than on Wayne by 24 h (Fig. 1-D) and the amount of hyphae on SL9 was significantly less at 36 h. Any search for chemical or induced-structural mechanisms for resistance should therefore concentrate on the period from 12 to 36 h after inoculation.

The techniques used here are well suited to the quantitative measurement of resistance of plants to fungi. These techniques could be used to measure the effect of individual genes in isogenic lines as well as the effect of environmental factors on early infection.

#### LITERATURE CITED

1. BERNARD, R. L., and C. R. CREMEENS. 1971. A gene for general resistance to downy mildew of soybeans. *J. Hered.* 62:359-362.
2. DUNLEAVY, J. M. 1971. Races of *Peronospora manshurica* in the United States. *Am. J. Bot.* 58:209-211.
3. DUNLEAVY, J. M., D. W. CHAMBERLAIN, and J. P. ROSS. 1966. Soybean diseases. U.S. Dep. Agric., Agric. Handb. 302. 38 p.
4. HEATH, M. C. 1971. Haustorial sheath formation in cowpea leaves immune to rust infection. *Phytopathology* 61:383-388.
5. HEATH, M. C. 1972. Ultrastructure of host and nonhost reactions to cowpea rust. *Phytopathology* 62:27-38.
6. HILU, H. M. 1965. Host-pathogen relationships of *Puccinia sorghi* in nearly isogenic resistant and susceptible seedling corn. *Phytopathology* 55:563-569.
7. JONES, F. R., and J. H. TORRIE. 1946. Systemic infection of downy mildew in soybean and alfalfa. *Phytopathology* 36:1057-1059.
8. LEHMAN, S. G., and F. A. WOLF. 1924. A new downy mildew of soybeans. *J. Elisha Mitchell Sci. Soc.* 39:164-169.
9. MILLIKAN, D. F., and T. D. WYLLIE. 1966. Some physiological changes associated with infection by, and resistance to, the downy mildew fungus, *Peronospora manshurica*, in soybean. *Phytopathology* 56:890 (Abstr.).
10. MILLIKAN, D. F., T. D. WYLLIE, and E. E. PICKETT. 1965. Some comparative biochemical changes associated

- with downy mildew infection in soybeans. *Phytopathology* 55:932.
11. PEYTON, G. A., and C. C. BOWEN. 1963. The host-parasite interface of *Peronospora manshurica* on *Glycine max*. *Am. J. Bot.* 50:787-797.
12. SHOBE, W. R., and N. R. LERSTEN. 1967. A technique for clearing and staining gymnosperm leaves. *Bot. Gaz.* 128:150-152.
13. SLESINSKI, R. S., and A. H. ELLINGBOE. 1970. Gene-for-gene interactions during primary infection of wheat by *Erysiphe graminis* f. sp. *tritici*. *Phytopathology* 60:1068-1070.