

Histology of the Suscept-Pathogen Relationship Between *Glycine max* and *Phakopsora pachyrhizi*, the Cause of Soybean Rust

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ABSTRACT

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Uredospores of *Phakopsora pachyrhizi* germinated on soybean plants 1-2 hours after inoculated plants were placed into a dew chamber at 20 C in the dark. Appressoria began developing within 2 hours, and within 5 hours many had grown to nearly the size of their parent spores. Many appressoria were sessile to their parent spores; measured germ tubes varied from a few μm to at least 320 μm in length. Eighty-five percent of the appressoria developed over anticlinal walls of epidermal cells. Penetration into the leaf epidermis always occurred directly through the cuticle; the earliest that penetration was observed was 7 hours after plants were placed into the dew chamber. Penetration was by

means of a structure, the transepidermal vesicle, which transversed the lumen of the invaded epidermal cell. Transepidermal vesicles initially were 3 μm in diameter and eventually grew to form cylindrical structures with an average maximum diameter of 8 μm . By 22 hours a hypha often had grown from the distal end of the transepidermal vesicle, had emerged from the invaded epidermal cell, and had formed an intercellular primary hypha in the leaf mesophyll tissue. From the primary hyphae, intercellular secondary hyphae grew, branched, and within 8 days many had extended to at least 400 μm .

Additional key words: fungal penetration, fungal colonization.

Rust of soybean [*Glycine max* (L.) Merr.], caused by the fungus *Phakopsora pachyrhizi* Sydow and known in the Orient for several decades, has been reported from numerous countries within an extensive area including and bounded by Japan and Taiwan to the east, India to the west, Manchuria in the north, and Australia in the south (3). The disease is considered a dangerous threat to soybean production throughout the Eastern Hemisphere (3). Crop losses of 70-80% in individual fields have been reported (8), and Liu (14) estimated that the average annual loss in Taiwan due to rust was 20-30%. Soybean rust has not been reported outside the Eastern Hemisphere.

Kitani and Inoue (11) in Japan and Keogh (10) in Australia described the development of local isolates of *P. pachyrhizi* on soybean. The work reported here describes detailed investigations of the pathogen-suscept relationship in soybean rust, particularly morphological development and establishment of the pathogen during early stages of pathogenesis on an economically important U.S. cultivar.

MATERIALS AND METHODS

The rust culture (Taiwan 72-1) used was isolated originally from rusted leaves sent to our laboratory from Taiwan. The fungus was maintained in the greenhouse on soybean plants (cultivar Wayne). In all experiments, freshly-harvested uredospores were used as inoculum, and the studies were conducted under strict quarantine

conditions within our containment facility.

Soybeans (cultivar Wayne) grown in 10.2-cm (4-inch) diameter clay pots, one plant per pot, were inoculated at the two- to four-trifoliolate leaf stage in a turntable tower (16). A total of 45-50 mg of dry spores was applied as either two or three equal spore releases per set of eight plants. The two lowermost trifoliolate leaves were held in an inverted position during inoculation by wooden splints attached horizontally to a stake so that the spores, settling under the influence of gravity, were deposited on the lower (abaxial) surface of the leaves. Immediately after inoculation, the plants were placed in a dew chamber (12) at 20 C in the dark. Previous studies at constant air temperatures during dew had shown these conditions to be optimum for infection (15, 17).

Prepenetration development of the pathogen was determined on leaflets collected at 0-5 hours after inoculation and supported over 38% formaldehyde in a desiccator for at least 1 hour to halt fungal development. Following the formaldehyde treatment, leaf segments (1-3 cm^2) were placed on glass microscope slides with the abaxial surface facing up, and one or two droplets of 0.1% cotton blue in lactophenol were applied. The cotton blue solution was distributed across the leaf surface by gently lowering a coverslip onto each leaf segment. The amount of staining solution and the manner in which the stain was spread were controlled so that none of the stain or spores flowed off the leaf section. This enabled quantitative determination of spore density and percentage germination on the leaves. The leaf surface was observed microscopically at $\times 100$ and $\times 473$ magnification.

Microscopic observation of development of the pathogen following penetration required fixing, clearing

of pigments, and staining leaf tissue with cotton blue in lactophenol. Plants were removed from the dew chamber at various times from 5-16 hours after inoculation (Fig. 1), and one lateral leaflet immediately was excised from

each of the two lowermost trifoliolates. The detached leaflets were cut into 1-3 cm² pieces and fixed in absolute ethanol-acetic acid (2:1, v/v). After at least 48 hours in the fixing solution, the leaf pieces were cleared for 48 hours in lactophenol, stained 15-18 hours in 0.1% cotton blue in lactophenol, and microscopic observations were made of the whole-leaf mounts. After collection of leaflets, each plant was placed in the greenhouse; 10-17 days after inoculation, the number of lesions that developed per cm² of leaf area was determined.

The extent of pathogenesis and of morphological development of the fungus at times longer than 16 hours were determined on leaflets collected from plants that had received 12 or 16 hours of dew treatment (depending on the particular experiment) before being placed in the greenhouse. These leaflets were fixed, cleared, and stained as described previously.

RESULTS

On leaflets held in a formaldehyde-saturated atmosphere after collection, observations revealed that germination initially occurred between 1 and 2 hours after inoculation and placement of plants in dew. Appressoria began developing within 2 hours, and within 5 hours many had expanded and were nearly the size of their parent spores (Fig. 2). Many appressoria were sessile to their parent spores. When germ tubes were present, their lengths varied from a few to more than 320 μm.

Leaflets collected primarily for determining fungal development following penetration were excised from inoculated plants after 5, 6, 7, 8, 9, 10, 12, or 16 hours in

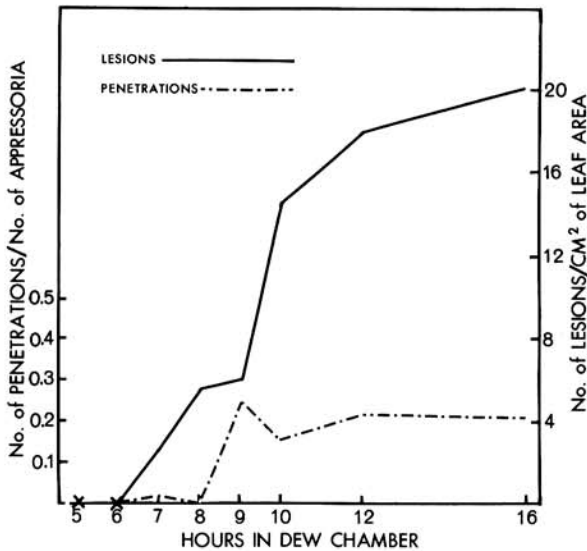


Fig. 1. Number of penetrations in relation to number of appressoria formed following incubation of inoculated plants in a dew chamber for 5-16 hours, and the density of soybean rust lesions which subsequently developed on plants which were removed from the dew chamber at times indicated.

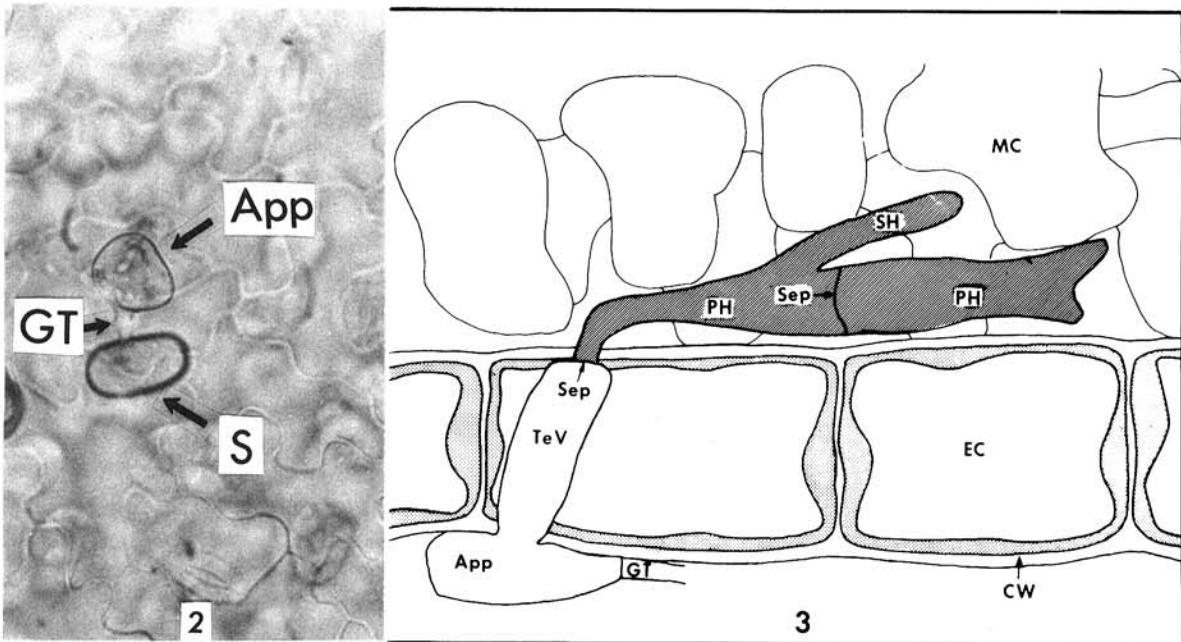


Fig. 2-3. Development of *Phakopsora pachyrhizi* on soybean leaf. 2) Germinated spore and appressorium on leaf surface. Penetration has occurred and cytoplasmic contents of spore and appressorium have passed into the leaf. 3) Diagram depicting early morphological development of pathogen following penetration of lower leaf surface. Contents of transepidermal vesicle have passed into the primary hypha. Legend: S = spore; GT = germ tube; App = appressorium; TeV = transepidermal vesicle; Sep = septum; PH = primary hypha; SH = secondary hypha; EC = epidermal cell; and CW = cell wall.

dew (two leaflets from one plant for each sampling). In a second test, samples were collected after 4, 5, 6, 7, 8, 10, or 12 hours in dew (four leaflets, two from each of two plants for each sampling). In a third experiment, collections were made after 22 hours, 50 hours, or 10 days, and in a fourth, after 16, 28, 40, 90 hours, or 6, 8, or 12 days.

The number of lesions that developed on plants removed from dew after 5-16 hours and the number of penetrations in relation to appressoria present at the time of their removal are presented for the first experiment (Fig. 1). Increasing the dew period from 7 to 9 hours increased the number of penetrations; no additional increase in penetrations resulted from further increases in length of dew up to 16 hours, but large, progressive increases in numbers of lesions did occur. Apparently, the increases in lesions observed on plants exposed to 10-16 hours of dew were mostly from sites of penetration already present at 9 hours of dew and not from penetrations that occurred later. Similar results were obtained when the experiment was repeated.

About 85% of the appressoria developed over anticlinal walls separating adjacent epidermal cells. The average length and width for 39 fully-developed appressoria were 20 and 17 μm , respectively. These dimensions were slightly less than the average length and width (24 and 18 μm) of 40 parent spores measured.

Penetration of the leaf always occurred directly through the cuticle. The earliest this was observed was 7 hours after plants had been inoculated and placed in the dew chamber (Fig. 1). Penetration from appressoria that formed over anticlinal cell walls was of two types. More frequently a hypha-shaped structure penetrated the outer epidermal cell wall directly into the cell lumen (Fig. 3). Occasionally, the hypha-shaped structure penetrated between adjacent epidermal cells and then entered one of the epidermal cells through its anticlinal wall. Regardless of the mode of entry, the structure initially was approximately the same diameter as the germ tube (3 μm). By 9 hours, epidermal cells containing these structures were common, and by 12 hours the extent of fungal growth in the invaded epidermal cells was as great as 36 μm , although usually considerably less. There was little increase in extent of growth between 12 and 16 hours; the fungus usually had not penetrated beyond the first cell, but the penetration structures had begun to increase in diameter. After 40 to 90 hours, they were frequently about 8 μm in diameter and were always aseptate (Fig. 3). Apparently these structures are the same as those observed and termed "transepidermal vesicles" by Keogh (10).

By 22 hours a primary hypha had often grown from the distal end of the transepidermal vesicle (Fig. 3). The distal end of the vesicle was in contact with or nearly touching the inner wall (bordering the mesophyll tissue) of the invaded epidermal cell, and a septum had formed which separated the transepidermal vesicle from the primary hypha (Fig. 3). The end of the primary hypha proximal to the transepidermal vesicle remained relatively narrow (3 μm diameter). The primary hypha had emerged from the epidermal cell, and immediately beyond the point of exit had formed an intercellular, elongated, "bag-like" structure (average maximum diameter, 8 μm) in the leaf mesophyll tissue. Secondary hyphae (3-5 μm diameter) had begun to grow out from the primary hyphae (Fig. 3)

and by 28 hours were common; by 90 hours they had extended intercellularly at least 75 μm from the penetration site. This distance increased to 300 μm by 6 days and to 450 μm by 8 days, when the pathogen occupied areas up to 0.6 mm^2 .

The epidermal cell initially invaded by the fungus frequently became yellow or brown. This was rare at 28 hours but later became more common.

Collapse of a few mesophyll cells near some penetration sites was first evident at 28 hours and was relatively common within 40 hours after inoculation. At 6 days, mesophyll cells near many penetration sites had become hypertrophied. Hypertrophied cells also were observed occasionally near hyphae at a distance from the penetration site. Haustoria were not observed.

DISCUSSION

There are numerous reports (e.g., 4, 5, 6, 13, 21) of rust fungi entering leaves through stomates via penetration structures arising from uredospores. Reports of direct penetration of the leaf cuticle from uredospores have been rare; we know of only three rust-causing fungi reported to do so: *P. pachyrhizi* (10, 11), *Puccinia psidii*, and *Ravenelia humphreyana* (9). Recent studies (Bonde et al., unpublished), however, demonstrated that *Physopella zaeae*, a pathogen of corn, also penetrates from uredospores directly through the cuticle.

Puccinia psidii, a pathogen of rose apple (*Syzygium jambos*), produces an appressorium at the tip of a short germ tube, and from it a narrow penetration peg develops and penetrates the leaf surface between epidermal cells to establish an intercellular mycelium with haustoria in the mesophyll cells. The fungus does not colonize the epidermis. *Ravenelia humphreyana*, on *Caesalpinia pulcherrima*, produces an appressorium that most often is sessile to the uredospore. From the appressorium a penetration peg penetrates a leaf epidermal cell and within the cell enlarges to form a "vesicular haustorium." This fungus subsequently colonizes adjacent epidermal and mesophyll cells by formation of intracellular mycelium (9).

The development of *P. pachyrhizi* in its susceptible differs from that of either *Puccinia psidii* or *R. humphreyana* in their respective hosts. The soybean rust pathogen is the only one of the three that becomes established initially in an epidermal cell and then proceeds to colonize the mesophyll tissue initially as intercellular hyphae. This mode of penetration and subsequent early colonization is similar to these processes as they occur with sporidia of *Puccinia graminis tritici* on leaves of barberry (1).

Three of these four rust-causing fungi (known to us) that penetrate directly from uredospores are pathogens of dicotyledonous plant species. Perhaps as more rusts on nongrass susceptibles are studied in detail, it will be found that direct penetration from uredospores is not so rare among these species as it is on monocot susceptibles.

In his study of the development of *P. pachyrhizi* on soybean, prior to and following penetration, Keogh (10) concluded that the transepidermal vesicle was a structure with "no close counterpart in the structures elaborated in penetration by commonly studied rust species." There appears to be no advantage at this stage in the study of soybean rust to coin a new term for this initial penetration

structure. We have accepted it tentatively, therefore, with the recognition that there is no clearly-defined penetration peg at the site of entry that remains morphologically distinct from the rest of the transepidermal vesicle.

We have called the hypha arising from the transepidermal vesicle the primary hypha, a term which previously has been used for similar structures formed by other fungi [e.g., *Puccinia malvacearum* (2), *Gymnosporangium juniperi-virginianae* (18), *Stemphylium botryosum* (7, 20), and *Helminthosporium victoriae* (19)]. Secondary hyphae (3-5 μ m diameter) begin to develop by 22 hours, branch extensively, and eventually extend several millimeters from the primary hypha. The morphological differences in the two types of hyphae are readily apparent and therefore, we believe, warrant separate terms.

Our description of the primary hyphae and early secondary hyphae near the penetration site was based on observations made at 28 or fewer hours after inoculation because of subsequent disorganization of the leaf tissue. Secondary hyphae that formed later than 28 hours, and at a distance from penetration sites, appeared morphologically identical to those that initially grew from the primary hyphae.

Our observations were made almost exclusively of the development of the fungus following inoculation of the uredospores onto the lower leaf surface. This particular placement of spores was used because earlier investigations had revealed that it was considerably easier to observe early development of the fungus within the leaf on whole-leaf mounts when the optically obstructive compact palisade cell layer was not in the same focal plane as the pathogen. Limited observations of infections on the adaxial (upper) surface indicated that there were no essential differences in infection of the upper and lower leaf surfaces. In our studies, haustoria were not observed. This finding was similar to the observations of Kitani and Inoue (11). Keogh (10), however, did observe and photograph haustoria in both epidermal and mesophyll cells. Perhaps differences in histological technique may account for the apparent discrepancy.

Under our experimental conditions, which were near optimum for infection, penetration of the leaf required 7 or more hours in the dew chamber. During this time (plus the drying off period after removal from the chamber) there was sufficient surface moisture to permit establishment of the pathogen, which was proven by the subsequent development of lesions in the absence of additional periods of surface wetness.

It was difficult to determine precisely the minimal length of dew period that permits penetration and lesion development. In the dew chamber, nature is simulated in that the surface temperature of the plants drops below the dew point temperature of the air and condensation occurs. There is a lag period after placement of plants in the chamber before visual dew appears; furthermore, the initiation and disappearance of dew is never exactly the same on different leaves or even across the same leaflet. Under our conditions, we determined that 0.5 - 1.0 hour was required for visible dew to form after placing plants in the chamber, and 1-2 hours for dew to disappear completely from most leaflets after removal from the chamber. A good estimate of the minimum period of leaf

wetness necessary for penetration and lesion development is 7-8 hours.

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