Characterization and Quantification of Fungal Colonization of *Phakopsora pachyrhizi* in Soybean Genotypes

R. Vittal, C. Paul, C. B. Hill, and G. L. Hartman

All authors: Department of Crop Sciences; University of Illinois, Urbana 61801; and fourth author: United States Department of Agriculture–Agricultural Research Services, Urbana, IL 61801.
Accepted for publication 25 July 2013.

ABSTRACT

Vittal, R., Paul, C., Hill, C. B., and Hartman, G. L. 2014. Characterization and quantification of fungal colonization of *Phakopsora pachyrhizi* in soybean genotypes. Phytopathology 104:86-94.

Soybean rust, caused by the fungus *Phakopsora pachyrhizi*, is an economically important disease of soybean with potential to cause severe epidemics resulting in significant yield losses. Host resistance is one of the management tools to control this disease. This study compared soybean genotypes exhibiting immunity, complete and incomplete resistance, and susceptibility to an isolate of *P. pachyrhizi* based on visual assessment of reaction type, other visual traits such as sporulation, quantitative measurements of the amount of fungal DNA (FDNA) present in leaf tissues, and data on infection and colonization levels. Soybean genotype UG5 (immune), and plant introduction (PI) 567102B and PI 567104B (complete resistance) had lower quantities of uredinia and FDNA than four other genotypes with incomplete resistance. Based on microscopic observations, early events of spore germination, appressorium formation, and fungal penetration of the epidermis occurred within 24 h postinoculation and were similar among the tested soybean genotypes. Differences in infection among the genotypes were evident once the hyphae penetrated into the intercellular spaces between the mesophyll cells. At 2 days after inoculation (dai), soybean genotype Williams 82 had a significantly ($P < 0.05$) higher percentage of hyphae in the mesophyll tissue than other soybean genotypes, with UG5 having significantly ($P < 0.05$) lower percentages than all of the other soybean genotypes at 3, 4, and 5 dai. The percentage of interaction sites with mesophyll cell death was significantly ($P < 0.05$) higher in UG5 than other genotypes at 3, 4, and 5 dai. There was a significant positive correlation ($r = 0.30$, $P < 0.001$) between quantities of hyphae in the mesophyll cells and FDNA. These results demonstrated that incompatible soybean–*P. pachyrhizi* interaction results in restricted hyphal development in mesophyll cell tissue, likely due to hypersensitive apoptosis.

Soybean rust, caused by the fungus *Phakopsora pachyrhizi* Syd. & P. Syd., is an economically important disease of soybean with the potential to cause severe epiphytotics resulting in significant yield losses (26,37). The pathogen was initially described in Japan in 1902 (11) and, since then, has been found in most soybean-producing countries throughout the world (9). Soybean rust was first found in the continental United States in 2004 (34). Symptoms of rust infection include small lesions that increase in size and change from gray to tan or red-brown (RB) on the undersides of the leaves, depending on the soybean genotype (10,18). Fungicide application and resistant cultivars are two important components for effective management of soybean rust (9).

Epiphytotics are the result of multicyclic production, release, and spread of urediniospores from sporulating uredinia of *P. pachyrhizi*. Telia have been found in nature (8) and have been induced in the laboratory to form teliospores that germinate to produce probasidia and basidiospores (32); however, basidiospores have not been shown to be infectious because no susceptible alternate host has been found.

Resistance to *P. pachyrhizi* has been characterized by visually describing the signs and symptoms observed in the response to rust infection of soybean leaves. The responses described most frequently include the (i) immune reaction (IM) without visible lesions, indicating an incompatible host–parasite interaction; (ii) RB lesions without sporulation (complete resistance); (iii) RB lesions with varying levels of sporulation (incomplete resistance); (iv) tan-colored lesions (TAN) with limited sporulation (partial resistance); and (v) TAN lesions with abundant urediniospore production (susceptible reaction) (3,25,30). Based on genetic analyses of phenotypes within segregating populations developed from crossing parents having IM or RB resistant phenotypes with TAN susceptible parents, six soybean rust resistance loci (*Rpp1-Rpp5*, some multi-allelic) have been identified and mapped in the soybean genome, and the interaction of these loci with *P. pachyrhizi* pathotypes has been summarized (9). The resistant gene *Rpp6*, from plant introduction (PI) 567102B, expressing an RB response to some rust pathotypes, was recently named (20). These genes confer pathotype-specific resistance, controlled by the interaction of *Rpp* genes in soybean with virulence genes in *P. pachyrhizi* pathotypes (9,31,36).

Infection of *P. pachyrhizi* in soybean was shown to be initiated when a urediniospore germinates to form a single germ tube of ≈3 μm in width and of varying length of ≥100 μm before terminating by the formation of an appressorium, approximately the same size as a urediniospore (13,17). An appressorial cone initiated penetration into the epidermal cell by a penetration hypha, which grew through the epidermal cell and intercellular space, first forming primary invading hyphae, then secondary hyphae populating intercellular spaces (1,5,6,17). In compatible interactions, primary haustoria formed in mesophyll cells and, with continued hyphal growth, secondary haustoria formed within 12 days. Ultimately, in a compatible interaction, a dome erupted in the epidermis from the formation of uredinia. In an incompatible interaction, branching of fungal hyphae in the mesophyll was less common and was associated with mesophyll cell necrosis without organized haustoria and uredinia (24). In the same study, differences in fungal development in susceptible and

Corresponding author: G. L. Hartman; E-mail address: ghartman@illinois.edu

http://dx.doi.org/10.1094/PHYTO-12-12-0334-R

resistant hosts were not observed until 5 days after inoculation (dai). In the resistant genotype, fungal growth ceased and no uredinia developed, whereas fungal growth continued in susceptible genotypes, leading to sporulation (24). In another study comparing the susceptible 'Dare' with the resistant 'Komata', differences in fungal growth were observed as early as 2 dai. In Komata, there was less hyphal growth and branching compared with Dare. No uredinia developed in Komata, whereas there was full sporulation in Dare (16).

In addition to histological research on the interaction between soybean and *P. pachyrhizi*, there have been two studies on *P. pachyrhizi* interactions in the nonhost plants *Arabidopsis thaliana* (22) and *Hordeum vulgare* (barley) (12). Seven pre- and post-epidermal penetration events were defined as interaction sites when *A. thaliana* leaves were inoculated with *P. pachyrhizi*; in this case, the urediniospores germinated and penetrated the epidermal cells but did not grow either inter- or intracellularly into the mesophyll tissue layer (22). In barley, the urediniospores germinated but mostly failed to penetrate through the cuticle and the epidermal cells; however, when the fungus successfully penetrated, the epidermal cells died and colonization was arrested by the hypersensitive collapse of the mesophyll cells (12).

A quantitative polymerase chain reaction (Q-PCR) assay has been used to measure the levels of *P. pachyrhizi* colonization in soybean (30). Quantification of fungal DNA (FDNA) demonstrated differences in fungal colonization among soybean genotypes, even when the visual differences in signs and symptoms were not evident (30). However, it was not clear when soybean genotypes began to show differences in fungal colonization. To our knowledge, no studies have compared the histological development of *P. pachyrhizi* in soybean with FDNA quantities. A study associating the microscopic events and the amount of *P. pachyrhizi* DNA during the initial stages of infection would provide a better understanding of the differences among the various rust reaction phenotypes and how resistance expression affects *P. pachyrhizi* colonization.

The primary objective of this research was to quantify levels of *P. pachyrhizi* colonization in soybean genotypes having different visual rust reaction types, using FDNA quantification, with microscopic examination of resistance expression within leaf tissues. To achieve this, the quantity of FDNA and histological observations were compared at predetermined intervals during early stages of infection.

MATERIALS AND METHODS

Two experiments were performed. The first experiment evaluated eight soybean genotypes inoculated with *P. pachyrhizi* for visual assessment of reaction type, other visual traits such as sporulation, and measurements of FDNA. Five of the eight soybean genotypes used in experiment one were selected for evaluation in a second experiment. After these genotypes were inoculated, leaf tissues were sampled daily for 5 days to measure fungal colonization, as FDNA determined by Q-PCR, for comparison with histological observation of the infection in detached soybean leaves. Each of the experiments was repeated once, with different treatment randomization in each of the trials for both experiments.

Experiment 1: Evaluation of signs, symptoms, and FDNA in eight soybean genotypes differing in soybean rust resistance. Eight soybean genotypes, representing different levels of resistance, were selected based on a priori knowledge of their reaction to *P. pachyrhizi* isolate FL07-1 (20,27,29,31) (Table 1). Seed of soybean genotype UG5 was obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Seed of DT2000 was obtained from Vietnam (Plant Protection Research Institute, Hanoi, Vietnam). Seed of the other PIs and 'Williams 82' was obtained from the United States Department of Agriculture Soybean Germplasm Collection at Urbana, IL.

Soybean plants of each of the eight genotypes were grown in multi-pot trays (52 by 26 cm) that consisted of 18 pots (8 by 8 cm) filled with soilless mix (Sunshine Mix, LC1; Sun Gro Horticulture Inc., WA) and fertilized with 5 cm^2 of 14-14-14 Osmocote (Scotts Miracle-Gro Co., OH) at planting. Trays were placed in a growth chamber (Percival Scientific, Inc., IA) maintained at 65 to 70% relative humidity with 14 h of light (500 µmol m^{-2} s^{-1} photosynthetically active radiation) and 10 h of darkness, and with alternating day and night temperatures of 24 and 20°C, respectively. Excised leaflets from the youngest, fully expanded trifoliolates of 19-day-old plants were rinsed twice in sterile, distilled water and placed with the adaxial side up on 1.5% (wt/vol) water agar amended with 6-benzylaminopurine (BAP) at 1.5 mg/liter (35).

Urediniospores were produced from a *P. pachyrhizi* isolate collected in Florida (FL07-1) in 2007. A single-spore isolate was obtained by spreading urediniospores on 1.5% water agar, teasing out single spores with a fine dissecting needle, and transferring a single spore to a 10-µl drop of water on a detached leaflet of Williams 82. After 3 weeks, urediniospores resulting from a single uredinium that developed from the single spore were harvested and multiplied for use as inoculum for the detached-leaf assays (35) at a concentration of 2.5×10^4 spores/ml of sterile distilled water and 0.01% polysorbate surfactant Tween 20.

Each leaflet of the test genotypes was inoculated by placing three 15-µl drops of the urediniospore suspension and a control (water droplet) equidistantly on each side of the leaflet midrib, making a total of eight droplets applied per leaflet. Petri dishes containing the inoculated leaflets on the BAP-amended water agar were placed in plastic zip-lock bags (0.9-liter Zipper; Webster Industries, MA) (two dishes per bag) and incubated in 12 h of darkness and 12 h of light (380 µmol m^{-2} s^{-1}) inside a plant tissue culture chamber (Percival Scientific, Inc.) maintained at 23°C.

Single detached leaflets of each soybean genotype were the experimental units. The petri dishes containing detached leaflets were arranged inside the chamber in a completely randomized design with four replications. At 6 dai and daily up to 16 dai, the number of days to first lesion appearance and sporulation from each inoculation point was recorded. At 16 dai, the number of lesions and sporulating uredinia per square centimeter at each inoculation point was counted with the aid of a stereoscope at ×100 magnification (Olympus SZX16; Olympus Corp., Tokyo), and a 1.7-cm-diameter cork borer was used to excise leaf tissue surrounding each of the six inoculation points. After taking the data on visual signs and symptoms, the excised leaf disks were bulked and placed into 2-ml Fast DNA extraction tubes (MP

TABLE 1. Name, origin, and the description of soybean genotypes with phenotypic reactions produced in response to *Phakopsora pachyrhizi* isolate FL07-1

Genotype[a]	Geographic origin	Seed source[b]	Reaction type[c]	Reference
DT 2000	Taiwan	PPRI	RB	31
PI 224268	Australia	USDA-ARS	RB	29
PI 561377	Japan	USDA-ARS	RB	29
PI 567102B	Indonesia	USDA-ARS	RB	19
PI 567104B	Indonesia	USDA-ARS	RB	25
PI 203398	Brazil	USDA-ARS	RB	25
UG5	Uganda	IITA	IM	29
Williams 82	Illinois, United States	USDA-ARS	TAN	25

[a] All of the genotypes were used in experiment 1. Plant introduction (PI) 224268, PI 561377, PI 567102B, UG5, and Williams 82 were used in experiment 2.

[b] PPRI = Plant Protection Research Institute, Hanoi, Vietnam; USDA-ARS = United States Department of Agriculture–Agricultural Research Service, Soybean Germplasm Collection, Urbana, IL; and IITA = International Institute of Tropical Agriculture, Ibadan, Nigeria.

[c] IM = immune, RB = red-brown lesions (complete or incomplete resistance), and TAN = tan lesions (susceptible or partial resistance).

Biomedicals, CA) for DNA extraction following the manufacturer's instructions. The Q-PCR assay was performed following the protocol previously described (30).

Experiment 2: Evaluation of fungal growth and histological changes in five soybean genotypes. Five of the eight genotypes from experiment 1 were selected. The inoculation method was similar to experiment 1 except that 14 inoculation points per leaflet from the youngest fully expanded trifoliolates of 21-day-old plants (12 points inoculated with the urediniospore suspension and 2 controls) were used on each detached leaflet.

The experimental design was a repeated measures experiment with detached leaflets as the experimental units arranged in a completely randomized design with four replications. Treatments were soybean genotypes and days after inoculation. From each experimental unit, 1-cm-diameter leaf disks from 6 of the 12 points inoculated with the urediniospore suspension were randomly excised at 8 h after inoculation (hai) and 1, 2, 3, 4, and 5 dai. The disks were placed in 2-ml DNA extraction tubes, and Q-PCR assay was performed as in experiment 1. Assuming variability among leaf disks in each experimental unit was negligible, FDNA measurement of each leaf disk was considered a repeated measurement of the whole experimental unit.

To monitor fungal development and microscopic infection events in the detached leaflets, the six remaining disks from each detached leaflet not used for FDNA measurements were collected in succession on the same days as the disks sampled for FDNA determination. Individual leaflet disks were stained and fixed by submerging them into a solution of 0.05% trypan blue in 1:2 (vol/vol) lactophenol/ethanol, boiled for 1 min in the staining solution, and incubated at room temperature overnight. Following stain fixation, each of the leaf disks was destained in clean petri dishes with lactophenol/ethanol (1:2, vol/vol), rinsed several times with distilled water, and then submerged in saturated chloral hydrate (2.5 g ml^{-1}) for 7 days to clear the tissue. Stained leaf disks were examined using an Olympus BX51 compound microscope at ×400 (Olympus Corp.) using differential interference contrast microscopy and bright-field microscopy. In addition, images of each disk were recorded using QCapture software with a digital camera (QImaging, Inc., BC, Canada) mounted on the compound microscope for assessment of signs and symptoms.

Germination percentage and appressoria development of 30 randomly selected urediniospores were monitored and recorded at 8 hai and 1 dai at each of the inoculation sites. The number of interaction sites, defined as either the site of infection or the site of epidermal cell death, was recorded at 8 hai and 1, 2, 3, 4, and 5 dai for each of 30 randomly selected interacting urediniospores by focusing downward through the cleared leaflet tissue following the penetrating hyphae from each of the selected urediniospores. An infection site was defined as the site of interaction associated with a single urediniospore, including pre- and post-penetration events, epidermal cell browning, hyphae in the mesophyll cell, mesophyll cell death, and either hyphal or cell death. Three kinds of interaction sites in the mesophyll cell layer were distinguished from one another by (i) the presence of hyphae without mesophyll cell mortality; (ii) the presence of hyphae with mesophyll cell mortality, as indicated by the retention of trypan blue stain; and (iii) the lack of hyphae present with mesophyll cell mortality.

Statistical analyses. For experiment 1, data were combined from the two trials. Arithmetic means and standard errors for each genotype were calculated for FDNA and the other quantitative traits measured using JMP 10 (SAS Institute Inc., Cary, NC). For experiment 2, FDNA data were log transformed and percentage values recorded from the histological observations were transformed using the arcsine-square root transformation prior to analysis to correct for nonconstant variance for each parameter among the soybean genotypes. Data from the two trials were pooled in the experiment for combined analysis if Bartlett's test was nonsignificant ($P > 0.05$). Multivariate analysis of variance (MANOVA) using the repeated measures procedure (JMP 10) was performed on FDNA data to analyze the effects between soybean genotypes, within soybean genotype effects of days after inoculation (repeated measurements), and the interaction between days and genotypes. Effects of soybean genotypes on FDNA and histological observations on each day sampled were analyzed by analysis of variance (JMP 10), with Y = transformed percentages and X = soybean genotypes. For both experiments, simple linear correlations (r) among quantitative traits and FDNA were computed using JMP 10. Means of the soybean genotypes for each parameter on each day were separated by least significant difference ($\alpha = 0.05$) if differences among the genotypes were significant.

RESULTS

Evaluation of signs, symptoms, and FDNA in eight soybean genotypes differing in soybean rust resistance. Reaction types of each of the eight genotypes tested were consistent with previous reported results (Table 1). The three rust reaction types observed were IM, RB, and TAN. The earliest visual appearance of the TAN reaction type occurred at 6 dai on Williams 82, earlier than all the other genotypes. The RB reaction type appeared 7 to 9 dai, depending on the genotype. UG5 produced no visible signs or symptoms of rust, indicating an immune reaction type, and a zero was recorded for each of the rust traits measured for this genotype.

Rust lesion development on Williams 82 occurred approximately a day earlier than on PI 203398, PI 567102B, and PI 567104B; 2 days earlier than on DT 2000; and 3 days earlier than on PI 224268 (Table 2). No rust lesions were observed on UG5. The number of lesions per square centimeter varied from none for UG5 to 5.2 for PI 567104B. PI 567102B and PI 567104B had more rust lesions per square centimeter than Williams 82, which had significantly more than DT 2000. All genotypes, except UG5, had uredinia 16 dai. Williams 82 had a higher number of uredinia per square centimeter than all of the other genotypes. Uredinia on PI 224268 took 13 days to develop compared with only 3 days on

TABLE 2. Mean ± standard error for number of days to first lesion and first uredinia eruption, lesion per square centimeter, uredinia per square centimeter, and fungal DNA (FDNA) quantified from a 1.7-cm-diameter leaf disc from detached leaflets of eight soybean genotypes after inoculation with *Phakopsora pachyrhizi* isolate FL07-1 from combined data of two trials

Soybean genotype	Reaction[a]	Number of days to first lesion appearance	Number of days to first uredinium	Number of lesions (cm^2)	Number of sporulating uredinia (cm^2)	Fungal DNA (pg)
DT 2000	RB	8 ± 0.3	9 ± 2.0	3 ± 0.5	2 ± 0.1	31 ± 6.7
PI 203398	RB	7 ± 0.2	8 ± 1.7	4 ± 0.1	7 ± 2.4	211 ± 58.0
PI 224268	RB	9 ± 0.2	13 ± 0.3	5 ± 0.3	2 ± 0.2	15 ± 3.0
PI 561377	RB	7 ± 0.1	9 ± 0.1	5 ± 0.2	9 ± 0.7	83 ± 9.8
PI 567102B	RB	7 ± 0.0	3 ± 2.1	5 ± 0.3	0 ± 0.0	53 ± 12.4
PI 567104B	RB	7 ± 0.1	3 ± 2.1	6 ± 0.4	0 ± 0.0	56 ± 7.3
UG5	IM	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.1
Williams 82	TAN	6 ± 0.0	8 ± 0.1	4 ± 0.4	21 ± 5.2	308 ± 42.3

[a] IM = immune, RB = red-brown lesions (incomplete resistance), and TAN = tan lesions (susceptible or partial resistance).

PI 567102B and PI 567104B. No sporulating uredinia were observed on PI 567102B or PI 567104B. The quantity of FDNA in Williams 82 was much higher than in the other genotypes whereas, in contrast, FDNA in UG5 was nearly zero. PI 203398 had lower FDNA than Williams 82, but it was much higher than in PI 567377, PI 224268, PI 567102B, and PI 567104B at 16 dai.

Significant positive correlations were found between number of days to first lesion appearance and the number of days to first appearance of a sporulating uredinium ($r = 0.52$, $P < 0.001$), as well as between number of days to first lesion appearance and lesions per square centimeter ($r = 0.61$, $P < 0.001$). However, no significant correlations were found between number of days to first lesion appearance and FDNA or number of sporulating uredinia per square centimeter. Number of days to first sporulating uredinium was not significantly correlated with either lesion or uredinia numbers per square centimeter.

Experiment 2. Data were pooled from the two trials for combined analysis, based on the results of Bartlett's test for homogeneity of variance, indicating that the variances between the two trials of the experiment were nonsignificant ($P > 0.05$) for all of the parameters (percentage of uredinial infection sites with hyphae entering into mesophyll cells, mesophyll cell death, hyphae present in mesophyll associated with cell death, and FDNA). Repeated measures MANOVAs determined there were significant ($P < 0.01$) differences between the genotypes across the 5 days of the experiment for all of the parameters assessed. Significant genotype–day interaction within the genotypes was found for percentage of uredinial infection sites with hyphae entering into mesophyll cells, mesophyll cell death, hyphae present in mesophyll associated with cell death, and FDNA, indicating that the levels of these parameters measured on the soybean genotypes were dependent on the day of measurement. The genotype–day interaction within the genotypes for the percentage of infection sites with epidermal cell browning was nonsignificant ($P > 0.05$).

At 8 hai (0 dai) and at 1 dai, FDNA among the genotypes was not significantly different, indicating that initial fungal colonization was similar in all of the genotypes; however, FDNA was significantly ($P < 0.05$) different among the genotypes at days 2, 3, 4, and 5, when expression of rust resistance apparently began to have a negative impact on fungal colonization (Fig. 1). Williams 82 and PI 561377 had significantly ($P < 0.05$) greater quantity of FDNA than PI 567102B (at 4 and 5 dai), PI 224268 (at 3, 4, and 5 dai), and UG5 (at 2, 3, 4, and 5 dai), indicating that those two genotypes were the most compatible with *P. pachyrhizi* isolate FL07-1. Williams 82 had a significantly ($P < 0.05$) greater quantity of FDNA than PI 561377 by 5 dai. Based on having the lowest FDNA throughout the 5 days, UG5 appeared to be the least compatible genotype with *P. pachyrhizi* isolate FL07-1.

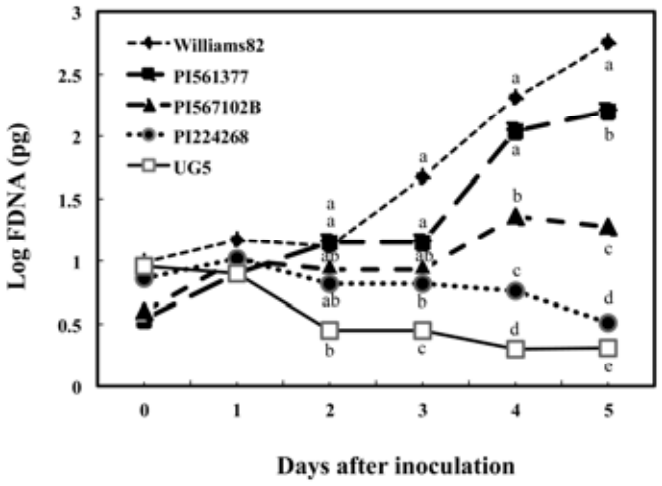

Fig. 1. Log of fungal DNA (FDNA) in leaflets of five soybean genotypes inoculated with *Phakopsora pachyrhizi* isolate FL07-1 measured by quantitative polymerase chain reaction 0 to 5 days after inoculation. Common letters above the points for each day indicated that the means were not significantly different ($P > 0.05$) using least significant difference, which was performed to separate the means each day and not across all 5 days.

Analyses of microscopic data of rust infection across all soybean genotypes indicated that >90% of urediniospores applied germinated, and appressoria formed at the tips of >85% of the germ tubes by 8 hai. Browning and granulation of the cytoplasm of the epidermal cell matrix indicated successful penetration into the epidermal cell lumen by infection pegs originating from appressoria (Fig. 2A and B). By 1 dai, the primary hyphae grew through the epidermis and entered into the intercellular spaces between the mesophyll cells, eventually developing haustorial mother cells in all the genotypes except UG5 (Fig. 2C and D).

In Williams 82, the haustoria formed in the mesophyll cells by 1 dai (Fig. 3A). Hyphal growth expanded into four to seven mesophyll cells by 2 dai (Fig. 3B). Colonization of mesophyll cells continued through 3 and 4 dai (Fig. 3C and D) and, by 5 dai, extensive colonization involving 30 to 40 mesophyll cells was observed (Fig. 3E).

Fungal colonization in PI 561377 was similar to that occurring in Williams 82, although only 10 to 15 mesophyll cells were colonized. Primary hyphae and haustoria were observed in mesophyll cells by 2 dai (Fig. 4A and B). Hyphal branching increased 3 to 5 dai (Fig. 4C to E).

In PI 224268, fungal growth was sparse and gradual, expanding to five to six mesophyll cells by 3 dai (Fig. 5A to C). At 4 dai, two to four mesophyll cells died with hyphae present at those

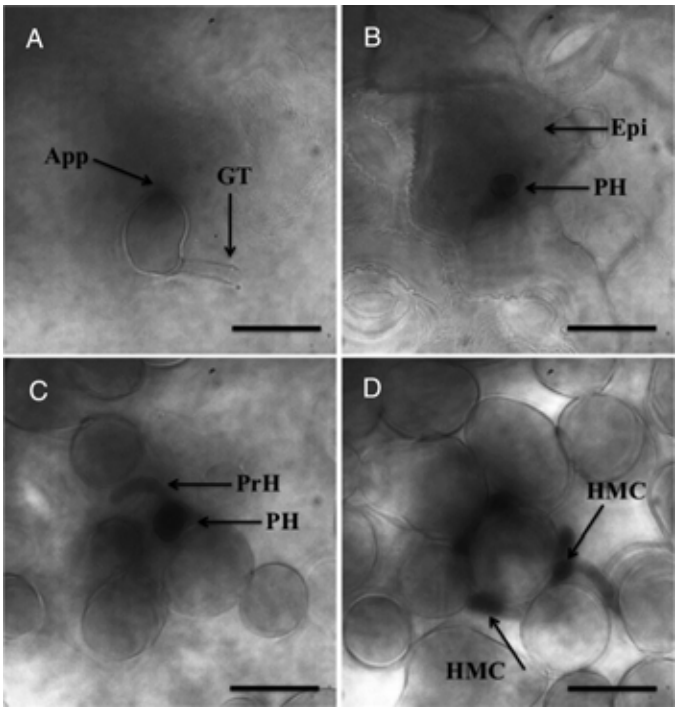

Fig. 2. Formation of infection structures of *Phakopsora pachyrhizi* isolate FL07-1 on soybean genotype 'Williams 82' at 1 day after inoculation. **A,** Germ tube of a urediniospore with an appressorium. **B,** Penetration hypha entering into the epidermal cell causing the browning and death of the epidermal cell. **C,** Penetration hypha entering the intercellular spaces of mesophyll and forming a primary hypha. **D,** Haustorium mother cells on the mesophyll cell. Leaves were stained with trypan blue and analyzed with differential interference contrast microscopy using an Olympus BX 51 microscope. Series of consecutive optical cuttings were made across particular infection site starting with the focus on urediniospore (not shown), then moving stepwise through the appressorium (A), epidermis (B), and finally to the mesophyll (C and D). GT = germ tube, App = appressorium, PH = penetration hypha, Epi = epidermal cell, PrH = primary hypha, HMC = haustorium mother cell. Scale bar represents 20 µm.

infection sites (Fig. 5D). By 5 dai, there was extensive mesophyll cell death, spreading between 10 and 15 cells per leaf disk, that appeared to arrest fungal colonization (Fig. 5E).

In PI 567102B, the fungus entered the mesophyll cells by 1 dai (Fig. 6A). The death of mesophyll cells first appeared at 2 dai (Fig. 6B). At 3 dai, hyphal colonization expanded to five or six mesophyll cells (Fig. 6C) but hyphal growth ceased by 4 to 5 dai (Fig. 6D), accompanied by extensive mesophyll cell death (Fig. 6E).

Limited colonization was observed in UG5. Colonization was restricted to one or two mesophyll cells without any haustoria formation (Fig. 7A). At 2 dai, death of mesophyll cells was evident and, by 3 dai, colonization in the mesophyll tissue appeared to be completely halted (Fig. 7A to C).

The percentage of interaction sites with hyphae in the mesophyll cells increased dramatically from 8 hai (0 dai) to 1 dai in all soybean genotypes (Fig. 8A), supporting observations that FL07-1 infection was able to penetrate through the epidermis and into the mesophyll layers in all of the genotypes. At 2 dai, Williams 82 had a significantly ($P < 0.05$) higher percentage of interaction sites with hyphae in the mesophyll cells than the other four genotypes and this trend continued until 5 dai, when the percentage for PI 561377 rose to a level not significantly ($P > 0.05$) different from Williams 82, indicating that the extent of fungal colonization appeared to be equal in both genotypes. A trend of declining percentage of interaction sites with hyphae in the mesophyll after day 2 was found in PI 567102B, PI 224268, and UG5, indicating that rust resistance expressed in those genotypes began to reduce the extent of fungal colonization that had occurred up until that point in time. From day 2 to day 3, the percentage of interaction sites with hyphae in the mesophyll cells in immune UG5 declined dramatically from 80% to 7%, while a

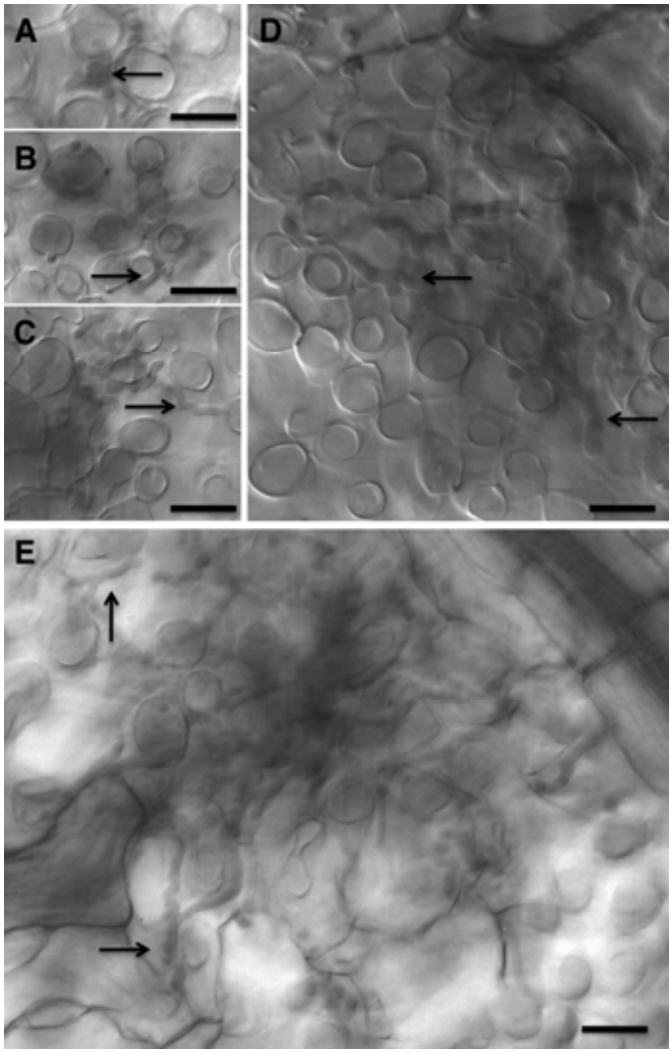

Fig. 3. Infection of *Phakopsora pachyrhizi* isolate FL07-1 on susceptible soybean genotype 'Williams 82' observed 1 to 5 days after inoculation (dai). The fungus spread between the intercellular spaces of the mesophyll cells and the number of mesophyll cells infected with the hyphae (see arrows) increased greatly over time. **A, B, C, D, and E,** Hyphae in the intercellular spaces of the mesophyll at 1, 2, 3, 4, and 5 dai, respectively. Leaves were stained with trypan blue and observed with differential interference contrast microscopy using an Olympus BX 51 microscope. Scale bar represents 20 µm.

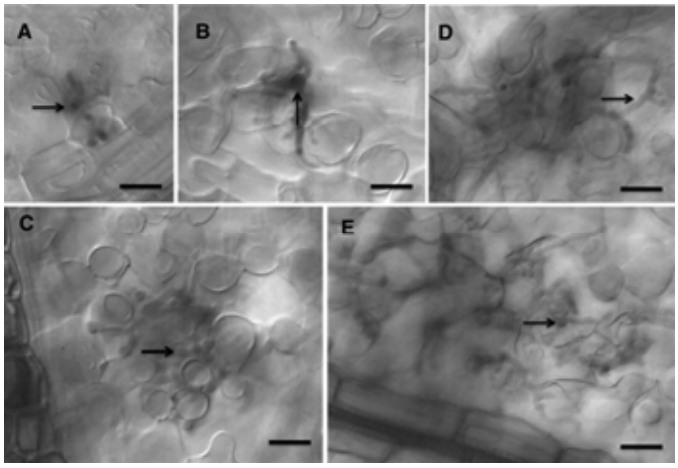

Fig. 4. Infection of *Phakopsora pachyrhizi* isolate FL07-1 on resistant soybean genotype PI 561377 observed 1 to 5 days after inoculation (dai). **A, B, C, D, and E,** Hyphae in the intercellular spaces of the mesophyll (see arrows) at 1, 2, 3, 4, and 5 dai, respectively. Leaves were stained with trypan blue and observed with differential interference contrast microscopy using an Olympus BX 51 microscope. Scale bar represents 20 µm.

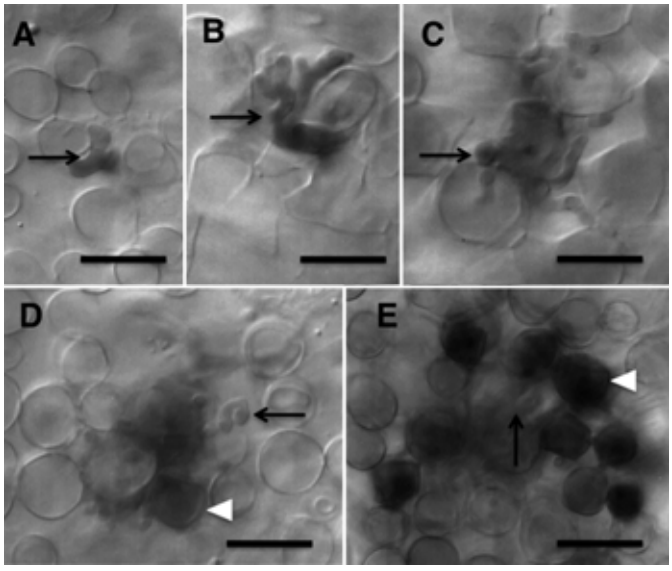

Fig. 5. Infection of *Phakopsora pachyrhizi* isolate FL07-1 on resistant soybean genotype PI 224268 observed 1 to 5 days after inoculation (dai). The fungus entered the intercellular spaces of the mesophyll and gradually grew (see arrows) until 3 dai, after which the growth was arrested by dying mesophyll cells, visualized by their retention of trypan blue stain (see arrowheads). **A, B, and C,** Hyphae in the intercellular space of the mesophyll at 1, 2, and 3 dai, respectively. **D,** Slight discoloration of the mesophyll cells at 4 dai indicating the initiation of defense to arrest the spread of the hyphae. **E,** Increased discoloration of several mesophyll cells around and near the fungus at 5 dai. Leaves were stained with trypan blue and observed with differential interference contrast microscopy using an Olympus BX 51 microscope. Scale bar represents 20 µm.

steep decline in resistant genotypes PI 567102B and PI 224268 did not occur until 4 dai. The percentage of interaction sites with hyphae in mesophyll cells was lowest in UG5 at day 3 and day 5, showing the strongest expression of resistance among the genotypes.

The percentage of interaction sites with mesophyll cell death did not increase in any of the genotypes until 2 dai (Fig. 8B), indicating that hyphae entered into the mesophyll layer (Fig. 8A) before hypersensitivity occurred. The most rapid increase in mesophyll cell death occurred in immune UG5, increasing to levels significantly ($P < 0.05$) higher than all of the other genotypes from day 3 through day 5. Both Williams 82 and PI 561377 had a significantly ($P < 0.05$) lower percentage of interaction sites with mesophyll cell death than all the other genotypes by 4 and 5 dai, indicating that they had a lower level of hypersensitivity against rust isolate FL07-1 than the other genotypes.

Differences among the soybean genotypes for the percentage of interaction sites with hyphae and with mesophyll cell death began to become significant from day 3 through day 5 (Fig. 8C), when hypersensitive resistance expression began to reduce the presence of hyphae in the mesophyll. The percentage in Williams 82 began to increase slowly after day 3, whereas the percentage in UG5 reached its highest point on day 3 and steeply declined after that.

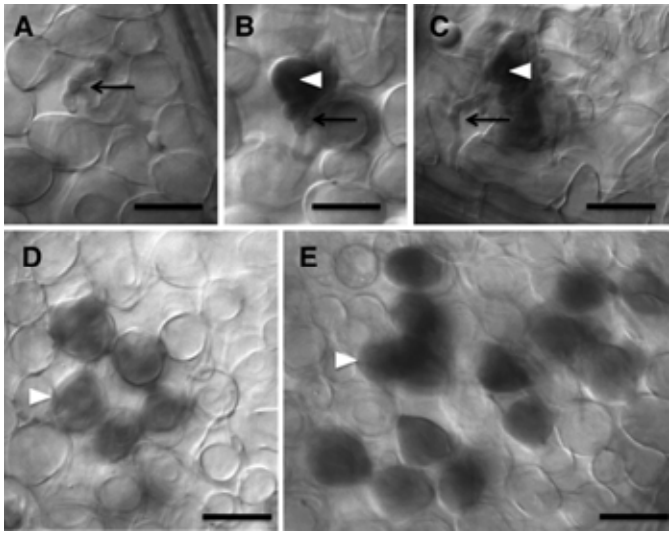

Fig. 6. Infection of *Phakopsora pachyrhizi* isolate FL07-1 on resistant soybean genotype PI 56102B observed 1 to 5 days after inoculation (dai). The fungus entered the intercellular spaces of the mesophyll and formed hyphae (see arrows) but the growth was arrested by dying mesophyll cells (see arrowheads) visualized by their retention of trypan blue stain. **A,** Hyphae in the intercellular space of the mesophyll at 1 dai. **B and C,** Hyphae in the intercellular spaces of mesophyll cells and blue discoloration of the infected mesophyll cells at 2 and 3 dai, respectively. **D and E,** Blue discoloration of a group of mesophyll cells resulting in a complete arrestment of hyphal growth at 4 and 5 dai, respectively. Leaves were stained with trypan blue and observed with differential interference contrast microscopy using an Olympus BX 51 microscope. Scale bar represents 20 µm.

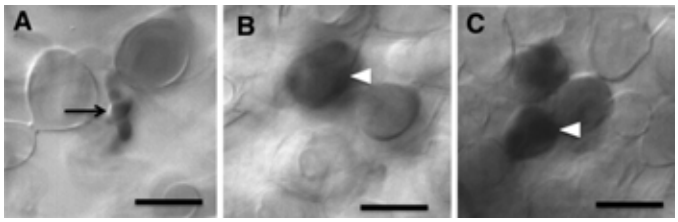

Fig. 7. Infection of *Phakopsora pachyrhizi* isolate FL07-1 on immune soybean genotype UG5 observed 1 to 3 days after inoculation (dai). The fungus entered the intercellular spaces of the mesophyll, but the growth was arrested by dying mesophyll cells, as indicated by their retention of trypan blue stain. **A,** Hyphae in the intercellular space of the mesophyll at 1 dai (see arrows). **B and C,** Mesophyll cell death resulting a complete arrestment of the fungal growth at 2 and 3 dai, respectively (see arrowheads). Leaves were stained with trypan blue and observed with differential interference contrast microscopy using an Olympus BX 51 microscope. Scale bar represents 20 µm.

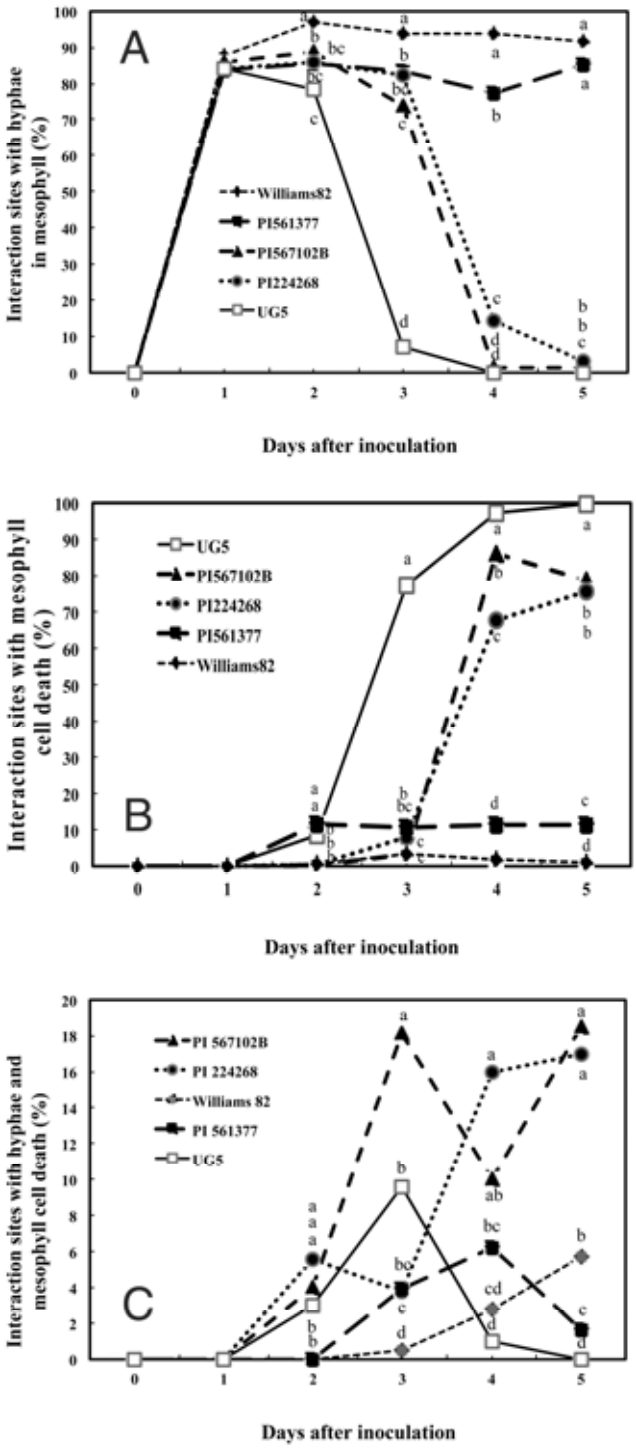

Fig. 8. Infection of *Phakopsora pachyrhizi* isolate FL07-1 on the leaflets of five soybean genotypes observed 1 to 5 days after inoculation (dai). **A,** Percentage of interaction sites with hyphae in the mesophyll. **B,** Percentage of interaction sites with mesophyll cell death. **C,** Percentage of interaction sites with hyphae in the mesophyll and mesophyll cell death. Leaflets were stained with trypan blue and the percentages were determined using light microscopy based on the observations of 30 urediniospores on one 1-cm-diameter leaf disk per replication. Common letters above the points for each day indicated that the means were not significantly different ($P > 0.05$) using least significant difference, which was performed to separate the means each day and not across all 5 days.

By day 4, differences between UG5 and PI 567102B or PI 224268 were significant, supporting the microscopic observations that hypersensitive-type resistance expressed in UG5 occurred earlier than in the other two genotypes. These results also confirmed the observations that, by day 4, fungal hyphae were not observed in the mesophyll tissue of immune UG5, which had considerable mesophyll cell mortality by then, and showed that mesophyll death rose dramatically after day 2, while the percentage in PI 224268 continued to increase through day 5, indicating that fungal colonization in UG5 declined more rapidly than in PI 224268 due to apparent apoptosis. The percentage in PI 567102B also tended to increase to day 5 after a slight decline 4 dai. The percentage of interaction sites with mesophyll death in PI 561377 increased earlier than Williams 82 but was significantly lower than Williams 82 by day 5, suggesting that PI 561377 expressed a level of partial resistance or reduced susceptibility to FL07-1 comparable with Williams 82.

Overall, the quantity of FDNA and the percentage of interaction sites with hyphae in mesophyll cells were positively correlated ($r = 0.30$, $P < 0.001$), indicating that higher fungal colonization occurred as infection progressed into the mesophyll tissue, which tended to be higher in the more compatible rust–soybean genotype interactions. The quantity of FDNA was negatively correlated ($r = –0.23$, $P < 0.0004$) with percentage of interaction sites with epidermal cell browning and with percentage of interaction sites with mesophyll cell death ($r = –0.14$, $P < 0.03$), suggesting that cellular apoptosis restricted fungal colonization, especially in the most incompatible genotypes. The percentage of interaction sites with epidermal cell browning was positively correlated ($r = 0.36$, $P < 0.0001$) with percentage of interaction sites with hyphae in mesophyll cells, but the percentage of interaction sites with epidermal cell browning was negatively correlated ($r = –0.25$, $P < 0.0002$) with the percentage of interaction sites with mesophyll cell death. It appeared that infection events occurring in the two different leaf tissues were inversely related, with higher colonization and epidermal cell browning tending to increasingly occur in the more compatible interactions, and lower colonization and higher mesophyll cell death occurring in the more incompatible interactions. The percentage of interaction sites with hyphae in mesophyll cells and percentage of interaction sites with mesophyll cell death were negatively correlated ($r = –0.54$, $P < 0.0001$), which indicated the two opposing responses were occurring in compatible and incompatible interactions, respectively.

DISCUSSION

The initial infection of soybean genotypes by *P. pachyrhizi*, regardless of their reaction type, was similar with successful urediniospore germination and appressorium formation, indicating that resistance expression occurred after fungal penetration through the epidermis. Once the fungus entered the mesophyll tissue layer, there was extensive fungal colonization with low mortality of mesophyll tissue in the susceptible genotype Williams 82, which was a fully compatible interaction. In the immune reaction expressed by genotype UG5, fungal colonization appeared to be restricted by apoptosis, evident by extensive mesophyll mortality surrounding the infection sites. Between this fully compatible reaction and the incompatible interaction was a range of responses in the mesophyll tissue that corresponded to reaction types of complete, incomplete, and partial resistance.

Results of FDNA quantification were in agreement with the microscopic observations of *P. pachyrhizi* colonization. Both methods revealed differences in colonization levels among the soybean genotypes before visible signs and symptoms appeared on the leaves. Whereas FDNA was considerably higher in rust-susceptible Williams 82 than in rust-resistant PI 561377 16 dai, the increase in FDNA with increase in percentage of interaction sites with hyphae in mesophyll tissue over the first 5 days of infection appeared to be similar in both genotypes, showing that the rust response in PI 561377 appeared to resemble a partially resistant response (30), even though it had an RB reaction type with relatively low levels of signs and symptoms of rust infection when challenged with soybean rust FL07-1. In contrast, levels of fungal colonization and cellular mortality occurring in mesophyll tissue appeared to differentiate the different types of resistance expression found among PI 567102B, PI 224268, and UG5. Expression of resistance in UG5 was confirmed to produce an immune interaction phenotype (30) both macro- and microscopically, and was supported by the nearly complete lack of colonization 5 to 16 dai from FDNA evidence. Resistance expressed in PI 567102B and PI 224268 appeared to be similar to UG5 after macroscopic examination; however, FDNA and microscopic evidence showed that resistance expression in both genotypes was slower to develop than in UG5 but stronger in PI 224268 compared with PI 567102B. This is the first report of comparing macroscopic, microscopic, and FDNA evidence collected after infecting soybean genotypes differing in resistance expression to a specific soybean rust pathotype. The resistance expression differences that were revealed with the approach used in this study may be similar in other comparable host–pathogen interactions.

The interactions between other host plant species and their fungal pathogens, including anthracnose of olive caused by *Colletotrichum acutatum* (7), barley stem rust caused by *Puccinia graminis* f. sp. *tritici* (21), and wheat stripe rust caused by *P. striiformis* f. sp. *tritici* (23). For example, we found the initial stages of infection in both compatible and incompatible interaction phenotypes were identical until further colonization of the mesophyll tissue occurred. For the interaction of olive with *C. acutatum,* the rates of conidial germination and appressorial formation were identical in both susceptible and tolerant olive cultivars (7); however, as in our study, differences in *C. acutatum* colonization rates in susceptible and tolerant cultivars were observed only after the pathogen penetrated into the mesocarp and mesophyll tissues, respectively. In barley, *P. graminis* f. sp. *tritici* urediniospore germination, appressorium formation, and substomatal penetration were similar in a panel of genotypes differing in resistance (21). Increased expression of resistance was associated with higher frequencies of post-penetration abortion of infection, reduced colonization, and higher incidence of host-cell necrosis. In the interaction between wheat and the striped rust pathogen, *P. striiformis* f. sp. *tritici* extensively colonized the leaves of a susceptible cultivar (23). The hypersensitive response (HR) in resistant and partially resistant wheat genotypes was similar but mesophyll host cells died later and there were fewer dead cells in partially resistant slow-rusting genotypes compared with pathotype-specific resistant genotypes when observations were made at 5 and 7 dai. In our study, *Phakopsora pachyrhizi* extensively colonized the leaves of susceptible Williams 82, and the growth was arrested by relatively rapid hypersensitive mesophyll tissue death in the UG5. In barley, necrotic host cells often contained haustoria in the resistant lines (21) whereas, in our study, haustoria were not observed in both live and dead mesophyll cells in the immune UG5. None of the soybean genotypes in this study appeared to express a slow-rusting phenotype. Differences in the rate of mesophyll cell death and mesophyll death with hyphae still present suggested that resistance expression in PI 561377 resembled a partial resistance phenotype because early colonization and mesophyll mortality were similar to susceptible Williams 82 but sporulation and FDNA in PI 561377 were much lower than Williams 82 16 dai. In contrast to results in the present study, in which appressoria development was similar in all of the genotypes, lower frequencies of stomatal appressoria, nonstomatal appressoria, and substomatal vesicles, developing as early as 6 hai in different resistant wheat genotypes infected with the leaf rust pathogen *Puccinia recondita* f. sp. *tritici*, were associated with higher prehaustorial resistance (14).

Arabidopsis thaliana (22) and barley (12) have been inoculated with *P. pachyrhizi* to study microscopic events of soybean rust infection in nonhosts. Results of those studies revealed that pre- and post-penetration infection events in those hosts were similar to those in soybean, but mesophyll colonization by the fungus did not occur due to restriction by the formation of papillae encasing pathogen hyphae at the site of penetration in the epidermis, halting further rust penetration. In contrast, in soybean, even in immune UG5, no papillae formed at the site of penetration; therefore, expression of host resistance occurred only after successful penetration into the mesophyll tissue and did not occur in the epidermis. Fungal growth in soybean was only restricted in the mesophyll cells and not in the epidermis. In barley, the HR was characterized by callose deposition on the epidermal cell walls; however, at sites where *P. pachyrhizi* succeeded in penetration, epidermal cells died, along with the collapse of neighboring mesophyll cells, arresting colonization (12). The nonhost HR in both *A. thaliana* and barley occurred more rapidly than what was observed in the host soybean genotype UG5, indicating that nonhost HR was more rapid than host HR, as would be expected.

In another *P. pachyrhizi* host, kudzu (*Pueraria lobata*), *P. pachyrhizi* colonization in an immune genotype was limited by early onset of multicellular HR and by cell wall depositions in infected epidermal cells in combination with HR (15). In contrast, colonization of *P. pachyrhizi* in UG5 was completely arrested by 2 dai due to HR caused by mesophyll cell death, but callose deposition in the infected epidermal cells was not observed.

Recently, Q-PCR was used to determine the extent of *P. pachyrhizi* colonization in different soybean (30) and kudzu (15) genotypes. Clear differences in FDNA in genotypes with different resistance expression levels were found in both studies similar to our results. In our study, even though the quantity of FDNA in PI 567102B was higher than in PI 224268 at 16 dai, no sporulation developed in PI 567102B, unlike in PI 224268. This is in agreement with the microscopic observations in which PI 567102B had a significantly lower number of sites with hyphae in mesophyll and significantly higher number of sites with mesophyll cell death than PI 224268, indicating that PI 567102B had a stronger level of HR-type resistance expression compared with PI 224268, suggesting that differences in FDNA level at 16 dai did not affect sporulation.

Based on the quantities of FDNA, cytological observations, and quantitative measurements of signs and symptoms, most of the soybean genotypes appeared to fall into four categories of resistance expression: (i) susceptible or fully compatible soybean–*P. pachyrhizi* interaction, as observed in Williams 82, with TAN reaction type, maximum sporulation, the highest level of fungal colonization 16 dai and little or no mesophyll hypersensitivity; (ii) incomplete resistance, expressed in PI 224268, with RB rust reaction type with sporulation and FDNA levels much lower than Williams 82 with moderate to high mesophyll hypersensitivity; (iii) complete resistance, expressed in PI 567102B, with an RB reaction type without sporulation with low FDNA and high mesophyll hypersensitivity; and (iv) immune, with no visible lesions, only a trace of FDNA evident at 16 dai, and extensive, rapidly developing mesophyll hypersensitivity, as observed in UG5. Resistance in PI 561377 did not fit clearly into any of those categories. Although it had an RB reaction type and much lower FDNA and sporulation than Williams 82 16 dai, microscopic observations of early infection up to 5 dai were similar to Williams 82, suggesting that it had features of partial resistance or reduced susceptibility.

An analysis of soybean transcriptome changes during soybean rust infection showed complex gene expression changes at 12 h and 3 dai (33), including an early burst of gene expression at 12 h, in both compatible and incompatible interactions (33). This timing corresponded to the progression of urediniospore germination and appressoria formation in our study, suggesting that these infection events may induce dramatic increases in gene expression. A quiescent period of gene expression occurred 1 to 2 dai, as previously reported (33), which corresponded to the period when hyphae entered into the mesophyll cells in all the genotypes observed in our study. Major differences in gene expression between compatible and incompatible genotypes were observed at 3 dai, which were postulated to be due to host responses to successful haustoria formation (33). Observations in our study indicated that higher mesophyll cell mortality, which occurred without the formation of haustoria, was associated with lower fungal colonization, suggesting that genes expressed at 3 dai were involved in resistance-gene-mediated defense responses.

Mur et al. (28) defined the HR as an area of cell death that forms at the point of attempted pathogen ingress and which correlates with the exhibition of resistance expression. A previous study reported that an immune reaction in PI 224268 and four other soybean lines occurred when inoculated with *P. pachyrhizi* isolate Q-1, with the cessation of fungal growth and appearance of collapsed host cells occurring at the same time (within 1 dai) in all the lines. However, PI 224268 had a slower rate of host cell collapse than the other immune lines, similar to the rate found in the susceptible 'Will' (24). In our study using *P. pachyrhizi* isolate FL07-1, colonization in PI 224268 increased until 3 dai, and it was not until 5 dai that mesophyll cell necrosis was observed, which was also slower than the immune response produced by UG5 but similar to PI 567102B. In a recent study of rust resistance in PI 567102B, found to be mediated by *Rpp6* (19), both incompletely resistant RB with sporulation and immune reactions were observed, depending on the rust isolate used in the inoculations. In addition, near-immune reactions with pinhead-like dark specks at the inoculation points were reported with some isolates. In our study with *P. pachyrhizi* isolate FL07-1, PI 567102B response (30) produced RB lesions without any sporulation, and microscopic examination confirmed an extensive HR-like response at 4 dai, whereas the immune response produced by UG5 elicited earlier and more localized HR by 2 to 3 dai.

Choi et al. (4) reported a close association between upregulation of peroxidases in the soybean genotype PI 200492, which produced an immune reaction to an isolate of *P. pachyrhizi* named HW94-1, and an oxidative burst in infected cells that triggered programmed cell death or apoptosis. Further work is needed to ascertain whether the mesophyll cell death produced by the immune reaction in soybean UG5 was caused by a similar hydrogen peroxide accumulation.

Over the last several years, parameters such as reaction phenotypes (2), sporulation levels (30), histological observations (16), and FDNA levels (30) have been evaluated to characterize rust resistance in soybean. We found clear associations between histological observations of the infection process with FDNA measurements that indicated rust colonization level, with the exception of PI 561377. This information will be valuable to soybean breeders interested in immproving soybean rust resistance, because they can prioritize their efforts according to the type and level of resistance expression desired, which might indicate different durability against rust populations with variable virulence. In addition, the type of resistance expression could suggest the mode of inheritance of resistance: whether it is qualitative, controlled by *Rpp* genes, or quantitative, controlled by quantitative trait loci.

ACKNOWLEDGMENTS

We thank the United Soybean Board for partial financial support of this research and T. Herman for critical review of this manuscript. Trade and manufacturers' names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

LITERATURE CITED

1. Bonde, M. R., Melching, J. S., and Bromfield, K. R. 1976. Histology of the suspect-pathogen relationship between *Glycine max* and *Phakopsora pachyrhizi*, the cause of soybean rust. Phytopathology 66:1290-1294.
2. Bonde, M. R., Nester, S. E., Austin, C. N., Stone, C. L., Frederick, R. D., Hartman, G. L., and Miles, M. R. 2006. Evaluation of virulence of *Phakopsora pachyrhizi* and *P. meibomiae* isolates. Plant Dis. 90:708-716.
3. Bromfield, K. R. 1984. Soybean Rust, Monograph No. 11. American Phytopathological Society, St. Paul, MN.
4. Choi, J. J., Alkharouf, N. W., Schneider, K. T., Matthews, B. F., and Frederick, R. D. 2008. Expression patterns in soybean resistant to *Phakopsora pachyrhizi* reveal the importance of peroxidases and lipoxygenases. Funct. Integr. Genomics 8:341-359.
5. Deverall, B. J., Keogh, R. C., and McLeod, S. 1977. Responses of soybean to infection by, and to germination fluids from, urediniospores of *Phakopsora pachyrhizi*. Trans. Br. Mycol. Soc. 69:411-415.
6. Edwards, H., and Bonde, M. R. 2011. Penetration and establishment of *Phakopsora pachyrhizi* in soybean leaves as observed by transmission electron microscopy. Phytopathology 101:894-900.
7. Gomes, S., Prieto, P., Lopes, P. M., Carvalho, T., Martin, A., and Pinto, H. G. 2009. Development of *Colletotrichum acutatum* on tolerant and susceptible *Olea europaea* L. cultivars: A microscopic analysis. Mycopathologia 168:203-211.
8. Harmon, C. L., Harmon, P. F., Mueller, T. A., Marois, J. J., and Hartman, G. L. 2006. First report of *Phakopsora pachyrhizi* telia on kudzu in the United States. Plant Dis. 90:380.
9. Hartman, G. L., Hill, C. B., Twizeyimana, M., Miles, M. R., and Bandyopadhyay, R. 2011. Interaction of soybean and *Phakopsora pachyrhizi*, the cause of soybean rust. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 6. Online publication. doi:10.1079/PAVSNNR20116025
10. Hartman, G. L., Wang, T. C., and Tschanz, A. T. 1991. Soybean rust development and the quantitative relationship between rust severity and soybean yield. Plant Dis. 75:596-600.
11. Hennings, V. P. 1903. A few new Japanese Uredinaceae. Hedwigia 42:S107-108.
12. Hoefle, C., Loehrer, M., Schaffrath, U., Frank, M., Schultheiss, H., and Huckelhoven, R. 2009. Transgenic suppression of cell death limits penetration success of the soybean rust fungus *Phakopsora pachyrhizi* into epidermal cells of barley. Phytopathology 99:220-226.
13. Hoppe, H., and Koch, E. 1989. Defense reactions in host and nonhost plants against the soybean rust fungus (*Phakopsora pachyrhizi* Syd.). J. Phytopathol. 125:77-88.
14. Jacobs, A. S., Pretorius, Z. A., and Coutinho, T. A. 2000. Quantification of early infection structures of Puccinia *recondita* f. sp. *tritici* in wheat with leaf rust resistance derived from *Triticum monococcum*. S. Afr. J. Sci. 96:86-90.
15. Jordan, S. A., Mailhot, D. J., Gevens, A. J., Marois, J. J., Wright, D. L., Harmon, C. L., and Harmon, P. F. 2010. Characterization of kudzu (*Pueraria* spp.) resistance to *Phakopsora pachyrihizi*, the causal agent of soybean rust. Phytopathology 100:941-948.
16. Keogh, R. C., Deverall, B. J., and McLeod, S. 1980. Comparison of histological and physiological responses to *Phakopsora pachyrhizi* in resistant and susceptible soybean. Trans. Br. Mycol. Soc. 74:329-333.
17. Koch, E., Ebrahim Nesbat, F., and Hoppe, H. H. 1983. Light and electron microscopic studies on the development of soybean rust (*Phakopsora pachyrhizi* Syd) in susceptible soybean leaves. Phytopathol. Z. 106:302-320.
18. Kumudini, S., Godoy, C., Board, J., Omielan, J., and Tollenaar, M. 2008. Mechanisms involved in soybean rust-induced yield reduction. Crop Sci. 48 48:2334-2342.
19. Li, S. 2009. Reaction of soybean rust-resistant lines identified in Paraguay to Mississippi isolates of *Phakopsora pachyrhizi*. Crop Sci. 49:887-894.
20. Li, S., Smith, J. R., Ray, J. D., and Frederick, R. D. 2012. Identification of a new soybean rust resistance gene in PI 567102B. Theor. Appl. Genet. 125:133-142.
21. Liu, J. Q., and Harder, D. E. 1996. Components of resistance in barley to stem rust: Pre- and post-penetration development in seedling and adult plant leaves. Can. J. Bot. 74:1305-1312.
22. Loehrer, M., Langenbach, C., Goellner, K., Conrath, U., and Schaffrath, U. 2008. Characterization of nonhost resistance of *Arabidopsis* to the Asian soybean rust. Mol. Plant-Microbe Interact. 21:1421-1430.
23. Ma, Q., and Shang, H. S. 2009. Ultrastructure of stripe rust (*Puccinia striiformis* f. sp. *tritici*) interacting with slow-rusting, highly resistant, and susceptible wheat cultivars. Plant Pathol. 91:597-606.
24. McLean, R. J. 1979. Histological studies of resistance to soybean rust, *Phakopsora pachyrhizi* Syd. Aust. J. Agric. Res. 30:77-84.
25. Miles, M. R., Bonde, M. R., Nester, S. E., Berner, D. K., Frederick, R. D., and Hartman, G. L. 2011. Characterizing resistance to *Phakopsora pachyrhizi* in soybean. Plant Dis. 95:577-581.
26. Miles, M. R., Hartman, G. L., and Frederick, R. D. 2003. Soybean rust: Is the U.S. soybean crop at risk? APS*net* feature, June 2003. American Phytopathological Society, St. Paul, MN. Online publication. http://www.apsnet.org/publications/apsnetfeatures/Pages/SoybeanRust.aspx
27. Miles, M. R., Morel, W., Ray, J. D., Smith, J. R., Frederick, R. D., and Hartman, G. L. 2008. Adult plant evaluation of soybean accessions for resistance to *Phakopsora pachyrhizi* in the field and greenhouse in Paraguay. Plant Dis. 92:96-105.
28. Mur, L. A., Kenton, P., Lloyd, A. J., Ougham, H., and Prats, E. 2008. The hypersensitive response; the centenary is upon us but how much do we know? J. Exp. Bot. 59:501-520.
29. Paul, C., and Hartman, G. L. 2009. Sources of soybean rust resistance challenged with single-spored isolates of *Phakopsora pachyrhizi* collected from the USA. Crop Sci. 49:1781-1785.
30. Paul, C., Hill, C. B., and Hartman, G. L. 2011. Comparisons of visual rust assessments and DNA levels of *Phakopsora pachyrhizi* in soybean genotypes varying in rust resistance. Plant Dis. 95:1007-1012.
31. Pham, T. A., Miles, M. R., Frederick, R. D., Hill, C. B., and Hartman, G. L. 2009. Differential responses of resistant soybean genotypes to ten isolates of *Phakopsora pachyrhizi*. Plant Dis. 93:224-228.
32. Saksirirat, W., and Hoppe, H. H. 1991. Teliospore germination of soybean rust fungus (*Phakopsora pachyrhizi* Syd.). J. Phytopathol. 132:339-342.
33. Schneider, K. T., Van de Mortel, M., Bancroft, T. J., Braun, E., Nettleton, D., Nelson, R. T., Frederick, R. D., Baum, T. J., Graham, M. A., and Whitham, S. A. 2011. Biphasic gene expression changes elicited by *Phakopsora pachyrhizi* in soybean correlate with fungal penetration and haustoria formation. Plant Physiol. 157:355-371.
34. Schneider, R. W., Hollier, C. A., H.K., W., Palm, M. E., McKemy, J. M., Hernandez, J. R., Levy, L., and DeVries-Paterson, R. 2005. First report of soybean rust caused by *Phakopsora pachyrhizi* in the continental United States. Plant Dis. 89:774.
35. Twizeyimana, M., and Hartman, G. L. 2010. Culturing *Phakopsora pachyrhizi* on detached leaves and urediniospore survival at different temperatures and relative humidities. Plant Dis. 94:1453-1460.
36. Twizeyimana, M., and Hartman, G. L. 2012. Pathogenic variation of *Phakopsora pachyrhizi* isolates on soybean in the United States from 2006 to 2009. Plant Dis. 95:75-81.
37. Yorinori, J. T., Paiva, W. M., Frederick, R. D., Costamilan, L. M., Bertagnolli, P. F., Hartman, G. L., Godoy, C. V., and Nunes, J., Jr. 2005. Epidemics of soybean rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001-2003. Plant Dis. 89:675-677.