Nuclear Behavior of the Cowpea Rust Fungus During the Early Stages of Basidiospore- or Urediospore-Derived Growth in Resistant or Susceptible Cowpea Cultivars

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ABSTRACT

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The relationship between fungal nuclear condition and growth of the two parasitic stages of the cowpea rust fungus was studied by light microscopy in living and fixed material. Uninucleate teliospores of the cowpea rust fungus germinated to form a promycelium that produced four basidiospores, each containing two nuclei as the result of a nuclear division during nuclear migration into the developing spore. Basidiospore-derived hyphae within an epidermal cell of a susceptible host cultivar rapidly became monokaryotic by (i) synchronous mitosis of the two basidiospore nuclei after they had migrated into the invasion hypha and the latter had grown to about 40 µm; (ii) the subsequent immediate division by septa of the invasion hypha into one binucleate and two uninucleate cells; and (iii) the disappearance of one of the nuclei in the binucleate cell. However, in the same host cultivar, the intercellular infection hyphae, secondary hyphae, and first two haustorial mother cells (HMCs) derived from dikaryotic urediospores of the same fungus had variable nuclear numbers due to nuclear degradation and asynchronous nuclear divisions. Nevertheless, by 3 days after inoculation, all subsequently formed intercellular hyphae, HMCs, and haustoria were binucleate. In the three resistant cultivars tested, the presence or absence of nuclear division in the basidiospore-derived, intracellular invasion hyphae depended on the degree of maximum fungal growth; growth inhibition was related to plant cell death in two of the cultivars and to callose encasement in cultivar Queen Anne. In urediospore-derived infections of these same cultivars, the intercellular infection hyphae and HMCs developed normally, but nuclear migration into the haustorium was reduced, often before callose encasement of the haustorium in Queen Anne. The data suggest that in Queen Anne, fungal encasement plays a greater role in resistance to the basidiospore-derived than to the urediospore-derived stage of infection.

Additional keywords: dikaryon, hypersensitive response, monokaryon, *Uromyces vignae*.

Rust fungi are economically important, biotrophic plant parasites that commonly have two parasitic stages in their life cycle: the monokaryotic stage, which is derived from basidiospore infection, and the dikaryotic stage, which is derived from aeciospore or urediospore infection. The uni- or binucleate status of cells in established mycelium from these two stages has long been known and has been demonstrated for many rust fungus species (23). However, there have been few investigations of nuclear behavior during the early stages of fungal development. Nuclei in infection structures produced in vitro from aeciospores or urediospores have been studied in a few species (11,18,21,24), and a few cytological investigations have examined nuclear behavior during the early stages of basidiospore or urediospore infection of a plant (1,3,23). However, the latter have provided conflicting data, particularly for basidiospore-derived infections.

For many rust fungus species, the two parasitic stages occur on different hosts, but for autoecious species such as the cowpea rust fungus, *Uromyces vignae* Barclay, both develop on the same plant. As a continuation of the studies into plant-parasite interactions at the cellular level, the investigation reported here compares early fungal growth and accompanying nuclear behavior of one race of *U. vignae* in four cultivars of cowpea that differ in susceptibility to the fungus. The results indicate how the monokaryotic and dikaryotic states of the fungus are established and provide further insight into plant-fungus interactions in resistant cultivars.

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MATERIALS AND METHODS

Plants and fungus. Cowpea (*Vigna unguiculata* (L.) Walp.) plants of resistant cultivars Dixie Cream (DC), Calico Crowder (CC), and Queen Anne (QA) and susceptible cultivar California Blackeye (CB) were grown from seed in a lighted growth room at 22°C, illuminated for 16 h/day at about 250 µM m^{-2} s^{-1} (4).

To produce the monokaryotic stage of rust infection, teliospores of *U. vignae* race 1 were incubated for about 2 days on 2% water agar to initiate germination and basidiospore production (4). Agar pieces were transferred, spore side down, to the upper surface of primary leaves of 9- or 10-day-old cowpea plants, which then were incubated for up to 24 h in the dark in a moist chamber. Inoculated plants harvested after this period had the agar removed and were placed in a lighted growth chamber under the same conditions as those used initially to grow the plants. This method of inoculation resulted in nonsynchronous infection, because teliospore germination, basidiospore formation, and infection by germinated basidiospores occurred throughout the 24-h incubation period.

The dikaryotic stage of the fungus was produced by brushing washed (12) urediospores onto the upper surface of primary leaves

of 9- or 10-day-old cowpea plants. Plants were sprayed with distilled water and incubated for up to 24 h in the dark at high humidity, as described for basidiospore-inoculated plants. If necessary, plants were subsequently incubated in the lighted growth chamber as described above.

Nuclear staining. Infected leaf pieces, about 1 to 4 cm^2, were decolorized in boiling 95% ethanol or vacuum infiltrated with either 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, or 4% freshly prepared paraformaldehyde in phosphate-buffered saline, pH 7.4. Ethanol-treated tissue was stored in 95% ethanol until required and stained for DNA by incubating the tissue for 1 to 3 days in 4 μg of propidium iodide per ml in 0.18 M Tris-HCl, pH 7.2, to which 1 drop of Tween 20 per about 10 ml of solution was added or in 100 μg of mithramycin (Pfizer, Pointe Clair, Dorval, QB, Canada) per ml in 0.067 M phosphate buffer, pH 7.0, containing 15 mM magnesium chloride (10).

In addition, some tissue was left overnight in 0.02 M sodium phosphate buffer, pH 7.2, containing per milliliter 5 μg of DAPI (4′,6-diamidino-2-phenylindole·2HCl, Sigma Chemical Company, St. Louis) to stain DNA and 0.001% diethanol (Uvitex 2B, Ciba Geigy Corp., Basel, Switzerland) to stain the plant and fungal walls. Leaf pieces fixed with aldehyde solutions were left in the fixative for 30 min, and whole pieces either were incubated overnight in the DAPI-diethanol solution described above or were stained for 10 min in 5 μg of DAPI per ml in phosphate buffer before being rinsed in water and stained for 5 min in 0.1% diethanol in 0.1 M Tris-HCl buffer, pH 8.5. These latter leaf pieces were washed in water for 2 to 3 min and left overnight in 50% glycerin. All leaf pieces were mounted on glass slides in water or 50% glycerin.

Some paraformaldehyde-fixed leaf pieces were sectioned before staining using a Lab-Line/Hooker plant microtome (Lab-Line Instruments, Inc., Melrose Park, IL), and the sections were mounted in phosphate-buffered DAPI. All leaf material was examined under epifluorescence illumination with a Reichert-Jung Polyvar microscope. Ultraviolet filter cube U1 (exciter filter BP 330-380, dichroic mirror DS 420, and barrier filter LP 418) was used for DAPI-stained nuclei, blue light filter cube B1 (BP 450-495, DS 510, and LP 520) was used for mithramycin-stained nuclei, and green light filter cube G2 (BP 520-580, DS 580, and LP 590) was used for propidium iodide-stained nuclei.

To examine nuclei of germinating teliospores and developing basidiospores, teliospores were surface-sterilized in 18% hydrogen peroxide for 1 min and washed in distilled water. The spores were distributed over the surface of 0.9% water agar on microscope slides and incubated in a humid chamber at 23°C in the dark for 48 to 72 h. DNA was stained with propidium iodide or DAPI, as described above, and fungal walls were visualized with diethanol or aqueous 0.01% Calcofluor White ST (American Cyanamid Co., Wayne, NJ). DAPI-stained material was examined as described above; propidium iodide-stained material was observed with a Zeiss (Oberkochen, Germany) axioskope fluorescence microscope using a G363 excitation filter, a FT 395 beam splitter, and a LP 420 emission filter.

Callose detection. To detect callose, leaf pieces decolorized in boiling ethanol were incubated overnight in 7×10^{-2} M K_2HPO_4, pH 8.9, containing 0.005% aniline blue (water-soluble, Sigma), mounted in water, and viewed under UV illumination with the U1 filter cube described above. Before observation, some leaf pieces of cultivar QA were soaked overnight in the DAPI solution.

Video-enhanced microscopy. Fresh, inoculated tissue containing a major vein was mounted on glass microscope slides in water (susceptible cultivar CB) or 5×10^{-4} M kinetin in 0.5% dimethyl sulfoxide (susceptible cultivar CB and resistant cultivar DC) (4). Living vein epidermal cells were observed with a Reichert-Jung Polyvar light microscope equipped with differential interference contrast (DIC) optics, and the image was computer-enhanced by a Hamamatsu C2400-77 video camera and control unit and an Image-1 image processing and analysis system (Universal Imaging Corporation, West Chester, PA). The process of infection was recorded with a Sanyo (Chatsworth, CA) TLS 2000 time-lapse video cassette recorder.

RESULTS

Features of basidiospore-derived infections in resistant and susceptible cultivars. As described previously (14,25), basidiospores of *U. vignae* germinate, producing a generally short germ tube that sequentially produces a small appressorium, a penetration peg that breaches the plant epidermal wall, and a spherical intraepidermal vesicle within the epidermal cell. The vesicle becomes isolated from the spore by a β-glucan–containing plug (26) and establishes tip growth to form a primary hypha. For convenience, we use the term "invasion hyphae" for the intraepidermal vesicle plus the primary hyphae, because once tip growth is established, it often is difficult to distinguish any clear junction between these two structures.

In susceptible cultivar CB and resistant cultivar QA, growth of the fungus was similar for the first 28 h, as shown by data from decolorized leaves (Table 1). Invasion hyphae in CB produced branches that eventually grew into neighboring epidermal and mesophyll cells and into the intercellular spaces of the mesophyll tissue. Intercellular hyphae produced hyphal-like M-haustoria (20) in adjacent mesophyll cells, as described previously (14). In QA, callose deposits began to develop around the fungus at about 20 h after inoculation. The final amount of fungal growth varied from infection sites with unbranched, aseptate, callose-encased invasion hyphae to hyphae with a small colony bearing callose-encased haustoria.

Data from ethanol-decolorized leaves showed that growth in resistant cultivars DC and CC usually ceased when the fungus was 10 to 20 μm long and always before 20 h after inoculation (Table 1). The walls of the invaded epidermal cell became autofluorescent (detectable under blue light irradiation), followed by autofluores-

TABLE 1. Number of nuclei and length of invasion hyphae of *Uromyces vignae* in resistant and susceptible cultivars of cowpea during the first 28 h after basidiospore inoculation

Cultivar	Time after inoculation	Number of nuclei in primary hyphae (%)					Mean primary hyphae length (μm)
		0	1	2	3	4	
CB[a]	20 h	0	0	79	8	13	28.0 ± 5.6[b]
	28 h	0	5	70	3	22	31.1 ± 5.4
QA	20 h	0	0	71	4	25	25.9 ± 5.7
	28 h	0	0	41	19	40	33.0 ± 5.2
CC[c]	20 h	19	4	77	0	0	20.7 ± 3.9
DC[c]	20 h	0	17	83	0	0	16.6 ± 1.0

[a] CB = susceptible cultivar California Blackeye; QA, CC, and DC = resistant cultivars Queen Anne, Calico Crowder, and Dixie Cream, respectively. All data were pooled from four experiments; n = 50 to 100 measurements per cultivar per time.
[b] Mean ± standard deviation.
[c] Fungal growth and number of nuclei could not be accurately measured after 20 h because of browning of the invaded cell.

cence of cell contents, and, finally, the entire fungus. Plant cell walls and contents became visibly brown shortly after they first became detectably autofluorescent. Video-enhanced microscopy of living DC tissue revealed that the fungus either stopped growing at about the time cytoplasmic streaming stopped in the plant cell or about 30 to 60 min later. Even in the latter situation, fungal growth ceased before the collapse of the plant protoplast, which usually occurred in video-taped cells 2 to 5 h after cessation of cytoplasmic streaming.

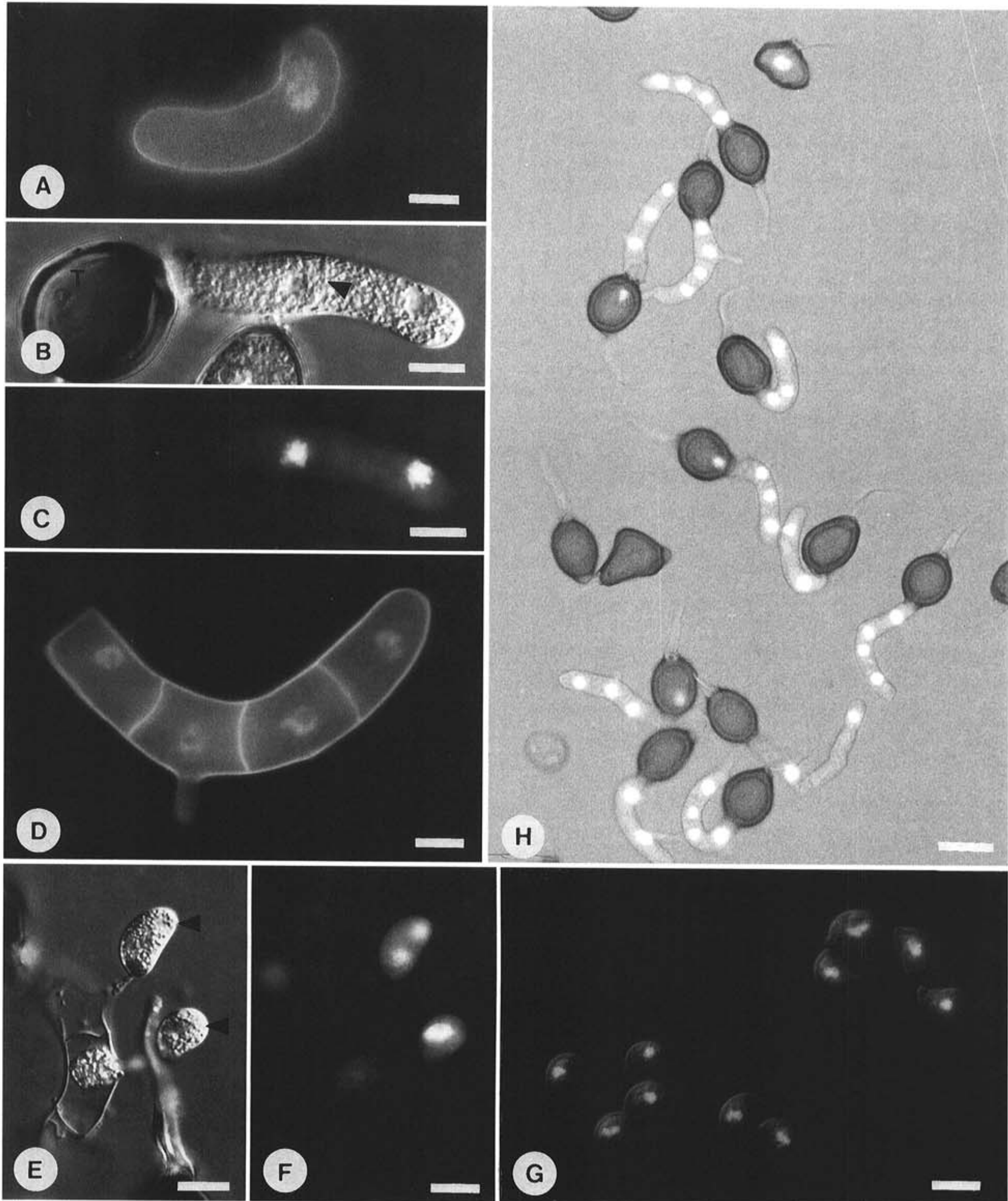

Fig. 1. Nuclear condition during *Uromyces vignae* teliospore germination and basidiospore formation. **A,** Germinating teliospore in which the single nucleus has just emerged from the spore. Stained with 4′,6-diamidino-2-phenylindole (DAPI) and diethanol and viewed under UV illumination. Bar = 10 µm. **B,** Differential interference contrast (DIC) image of a paraformaldehyde-fixed germinating teliospore that has produced a promycelium divided into two cells by a septum (arrowhead) that was clearly visible when followed at different levels of focus. Bar = 10 µm. **C,** The same promycelium as in **B**, stained with DAPI and viewed under UV illumination, showing the two nuclei. Bar = 10 µm. **D,** A mature promycelium with four cells, each containing one nucleus. Stained with propidium iodide and diethanol and illuminated with green light. Bar = 10 µm. **E,** A promycelium with two remaining basidiospores (arrowheads) viewed under DIC optics. Bar = 10 µm. **F,** The same basidiospores as in **E**, stained with DAPI and viewed under UV illumination; both spores have two nuclei. Bar = 10 µm. **G,** A group of binucleate and uninucleate basidiospores released from promycelia. Bar = 25 µm. **H,** Variability in nuclear condition in a field of germinating teliospores; promycelia generally have two or four nuclei, but in the latter case, one nucleus is sometimes in the teliospore. Stained with propidium iodide and illuminated with epifluorescence excitation and transmitted visible light. Bar = 50 µm.

Fungal nuclei in germinating teliospores and developing basidiospores. Teliospores contained a single nucleus (Fig. 1H) that migrated into the promycelium soon after it grew from the spore (Fig. 1A). Usually, a septum developed close to the teliospore, and the nucleus in the promycelium divided into two nuclei (Fig. 1B and C), presumably representing the first division of meiosis. A septum formed between the two nuclei (Fig. 1B), and the two nuclei divided again (presumably the second division of meiosis), and two more septa formed between the daughter nuclei (Fig. 1D). Each of these uninucleate cells developed a sterigma into which the nuclei migrated and immediately divided; as a result, two nuclei migrated into the developing basidiospore (Fig. 1E and F). On the agar slides, the basidiospores usually germinated while they were still attached to the sterigmata, but this was not the case when agar-bearing teliospores were placed on leaves during plant inoculation.

Although most fungal individuals followed the developmental sequence just described, there was some variation. The second meiotic division was sometimes asynchronous, and in some promycelia with four nuclei, one nucleus was sometimes seen just inside the spore (Fig. 1H). It could not be determined whether this latter condition was caused by the first meiotic division taking place before the nucleus had completely exited the teliospore or because the septum closest to the spore had not formed. In addition, some young basidiospores were uninucleate (Fig. 1G) because of the lack of nuclear division in the sterigmata; in such cases, nuclear division occurred in the basidiospore before germination.

Fungal nuclei in basidiospore-infected leaves. Studies of the number of nuclei in the fungus were initially performed with ethanol-decolorized tissue, because, although fungal DNA was artifactually slightly condensed, nuclei fluoresced well with propidium iodide or mythramycin and each invasion hypha could subsequently be observed by DIC optics. Some additional observations were made on DAPI- and diethanol-treated tissue in which both DNA and fungal walls fluoresced under UV illumination; this procedure allowed simultaneous observation of nuclei and fungal structures, although nuclei were not as easy to see because of the fluorescence of plant and fungal walls.

All types of DNA staining showed that germinated and ungerminated basidiospores contained two nuclei and that these migrated into the developing intraepidermal vesicle with the basidiospore cytoplasm, regardless of cultivar (Fig. 2A). In CC and DC, no fungal nuclear division or septation occurred prior to the death of the fungus and invaded cell, and after the collapse and browning of the latter, commonly only one or no nuclei could be detected in the invasion hypha (Table 1). In leaves of QA and CB, however, unbranched invasion hyphae with two, three, or four nuclei were observed by about 20 h after inoculation (Table 1). By 28 h after

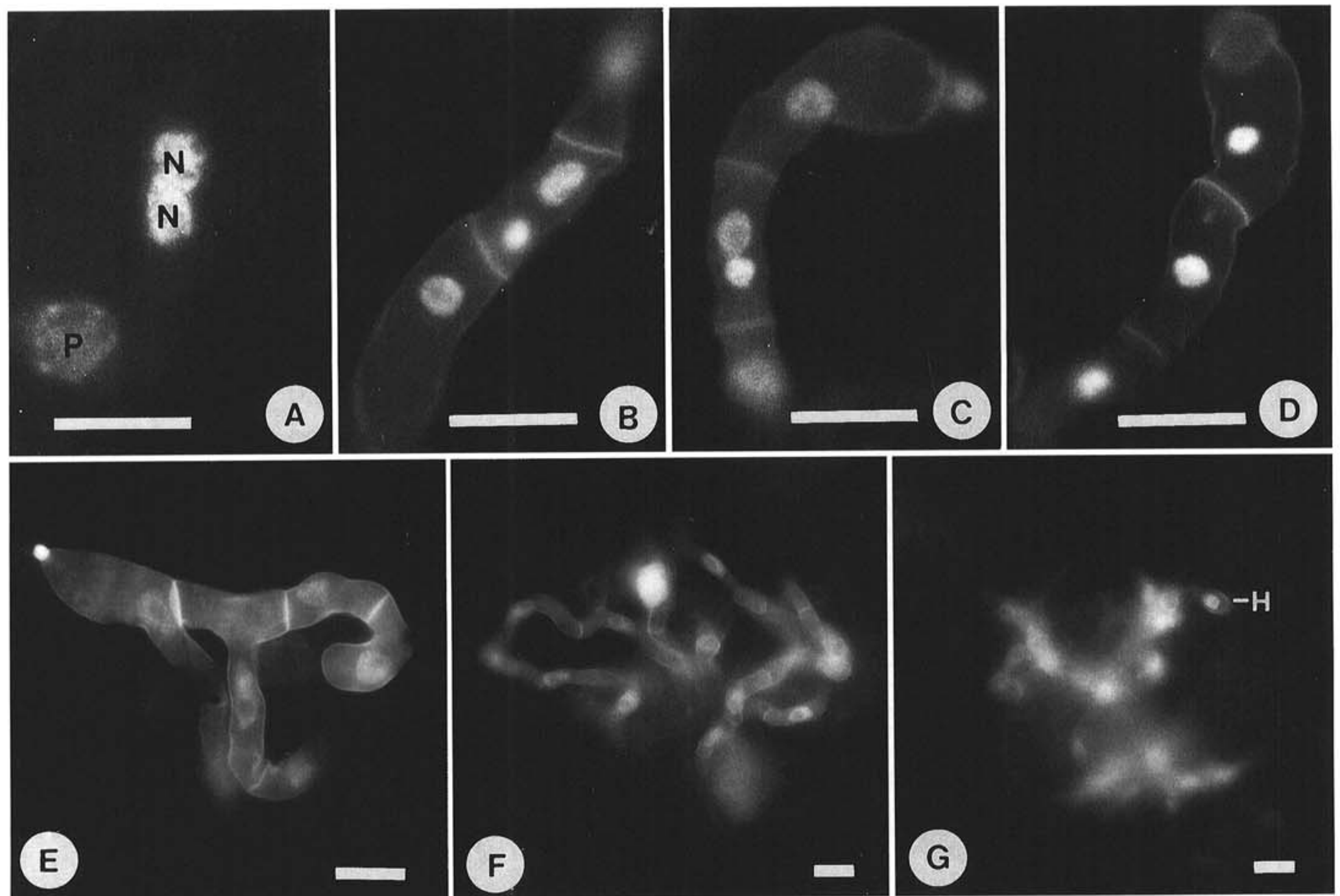

Fig. 2. Fungal nuclear condition during infection by *Uromyces vignae* basidiospores of susceptible cowpea cultivar California Blackeye. Tissue in **A** was fixed in ethanol, treated with propidium iodide, and viewed under green light. Tissue in **B through G** was fixed in paraformaldehyde, treated with 4′,6-diamidino-2-phenylindole and diethanol, and viewed under UV illumination. Bar = 10 µm. **A,** A young invasion hypha 12.5 h after inoculation that has two nuclei (N). The nucleus of the plant epidermal cell (P) typically is adjacent to the fungus. **B and C,** Older invasion hyphae divided into three cells by two septa. The central cell has two nuclei; one of the two nuclei in **B** looks smaller, in part because of the plane of the photograph, but the smaller nucleus in **C** is truly more condensed than the second nucleus. The fluorescent spot at the top end of the invasion hypha in **C** is the glucan plug that stains with diethanol. Fixed 28 to 32 h after inoculation. **D,** A three-celled invasion hypha with one nucleus per cell fixed 28 h after inoculation. **E,** A branched invasion hypha 32 h after inoculation. Compared with **D**, the tip of the hypha has elongated and produced another septum. The central cell also has produced a branch with a septum delimiting a tip cell. Each cell has only one nucleus. The fluorescent spot at the left of the invasion hypha is the glucan plug. **F,** Fungal mycelium 3 days after inoculation; all cells appear uninucleate. **G,** A fungal colony similar to the one in **F**, showing a uninucleate haustorium (H).

inoculation, a higher percentage of invasion hyphae had four nuclei in QA than in CB, suggesting that nuclear division occurred somewhat earlier in the former cultivar. In both cultivars, nuclear numbers greater than two were seen only in invasion hyphae longer than about 40 µm. In resistant QA at 28 h after inoculation, 40% ($n = 50$) of invasion hyphae with only two nuclei were partially or completely encased in callose. Only 6% ($n = 50$) of invasion hyphae with three or four nuclei were associated with callose, and the latter was usually a small pad at or near the tip of the hypha.

Nuclear division was observed by video-enhanced microscopy in CB in six living primary hyphae. In each case, mitosis of the two nuclei occurred when the primary hypha was about 40 µm long and was preceded by a short period of rapid movements of cytoplasmic particles. The two nuclei divided synchronously, and two developing septa became visible about 12 min later. These septa separated the most distal and proximal daughter nuclei from the rest of the invasion hypha. This left two nuclei in the central cell (Fig. 2B and C). However, when more than 190 primary hyphae that had four nuclei at 24 to 28 h after inoculation were surveyed in aldehyde-fixed tissue, the central cell was binucleate in only 58% of invasion hyphae, and the remaining

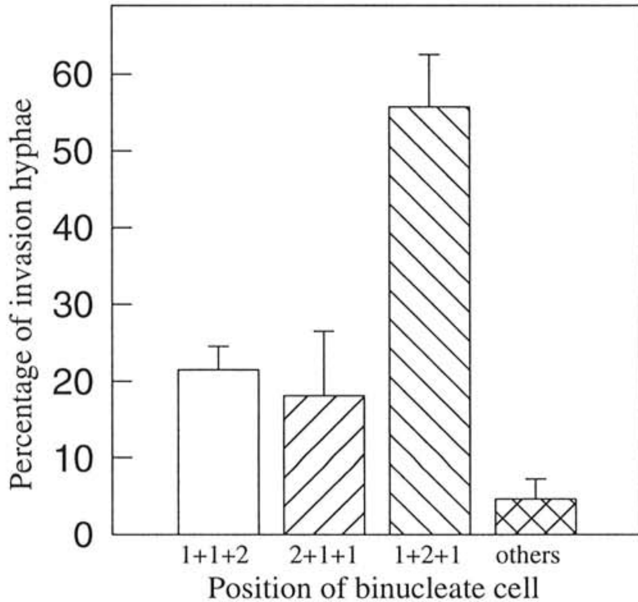

Fig. 3. Position of the binucleate cell in *Uromyces vignae* invasion hyphae with three cells and four nuclei seen in susceptible cowpea tissue harvested 24 to 28 h after inoculation. 1+1+2 indicates that the binucleate cell was the tip cell; 1+2+1 indicates that the middle cell was binucleate; 2+1+1 indicates that the first cell was binucleate. Invasion hyphae with only one or two cells are combined under "other." Data from 150 infection sites from three experiments. Vertical bars indicate the standard deviation.

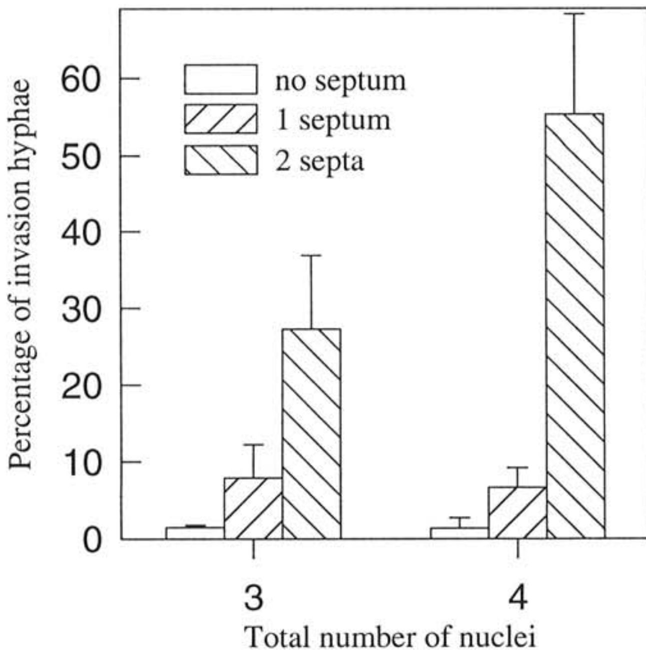

Fig. 4. Relationship between number of septa and number of nuclei in *Uromyces vignae* invasion hyphae in susceptible cowpea tissue harvested 28 to 32 h after inoculation. Data from 150 infection sites from three experiments. Vertical bars indicate the standard deviation.

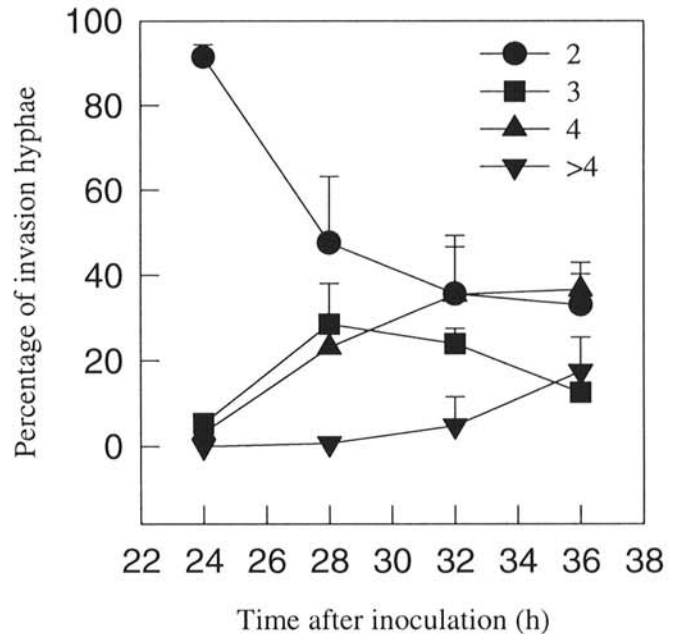

Fig. 5. Changes with time in the frequency of *Uromyces vignae* invasion hyphae with two, three, four, or more than four nuclei in susceptible cowpea cultivar California Blackeye. Vertical bars indicate standard deviation.

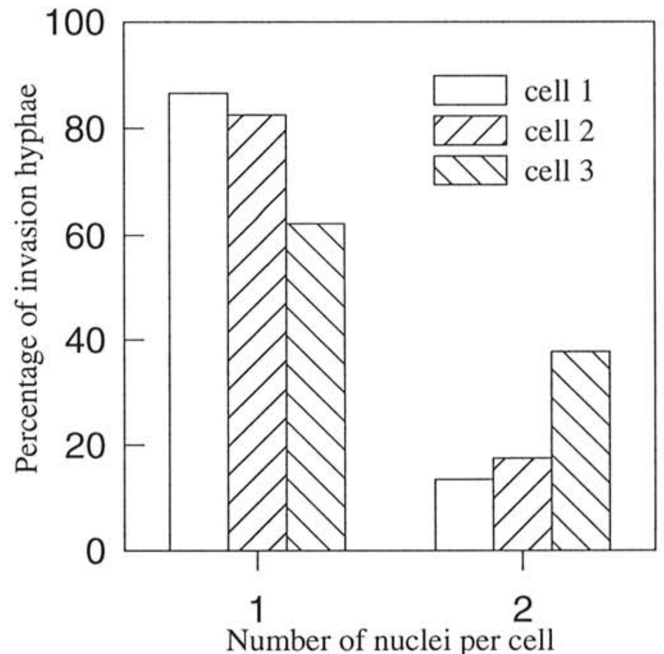

Fig. 6. Relationship between the frequency of branches produced by each *Uromyces vignae* cell in three-celled invasion hyphae in susceptible cowpea cultivar California Blackeye and their nuclear condition. Cell 1 is the cell nearest the penetration site; cell 2 is the middle cell; and cell 3 is the tip cell. The majority of cells plus their branches, regardless of position along the invasion hypha, have only one nucleus. Tissue was harvested 28 to 32 h after inoculation; $n = 138$.

invasion hyphae were binucleate in the first or the third cell (Fig. 3).

In leaves harvested and aldehyde-fixed at 28 to 32 h after inoculation, invasion hyphae usually had three or four nuclei and two septa (Figs. 2B through D and 4); therefore, those with three nuclei usually had one nucleus per cell (Fig. 2D). To determine the temporal relationship between the three- and four-nuclei stage, a more careful time-course study was performed with fixed leaves of CB. This revealed that as the percentage of invasion hyphae with two nuclei decreased during the first 36 h after inoculation, the percentage with three or four nuclei increased (Fig. 5).

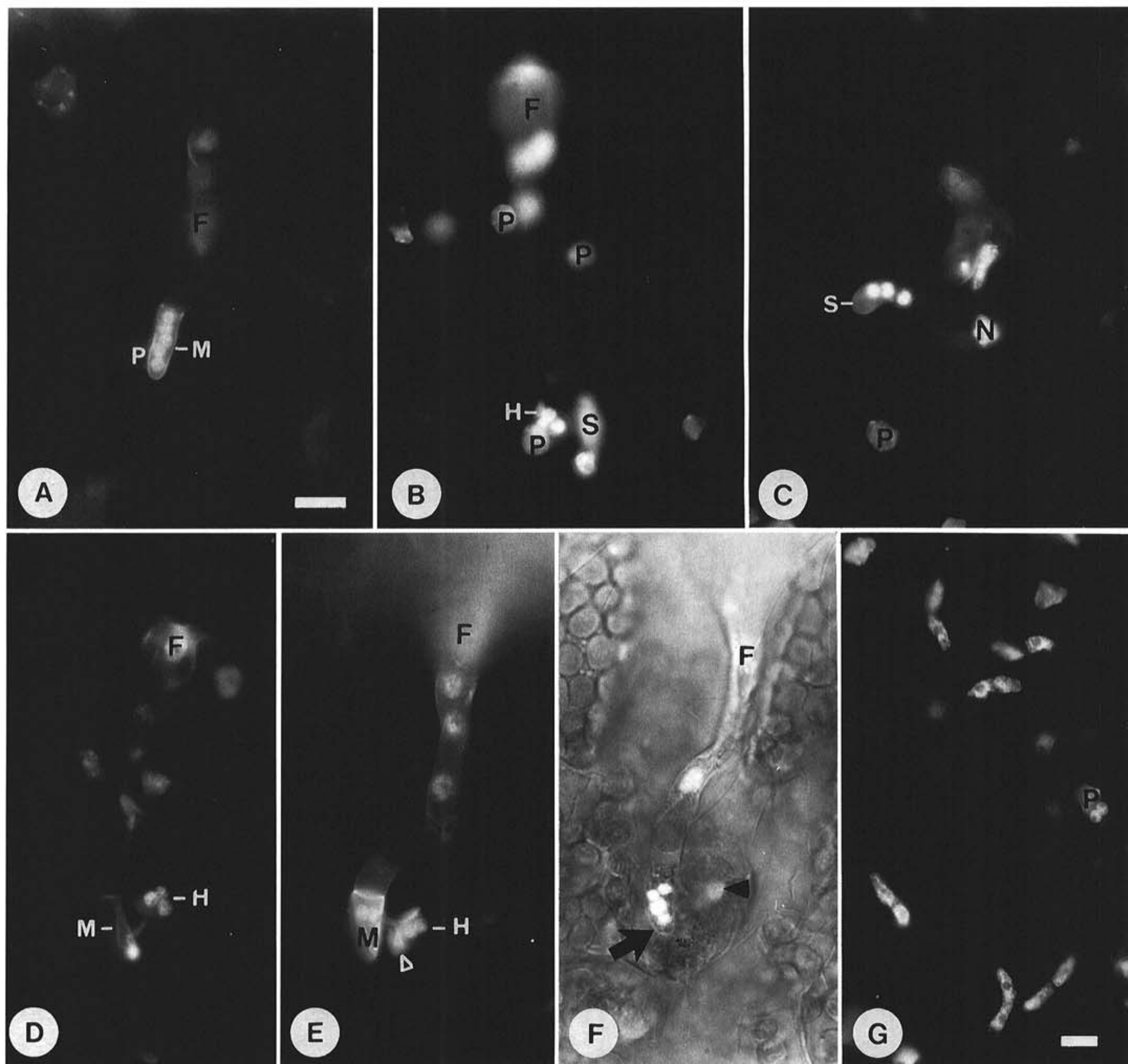

Fig. 7. Sections of paraformaldehyde-fixed, 4′,6-diamidino-2-phenylindole–stained, cowpea leaves inoculated with *Uromyces vignae* urediospores and viewed under UV illumination. Bars = 10 μm. **A,** An intercellular infection hypha (F) in resistant cultivar Queen Anne (QA) 17 h after inoculation. The fungus has formed a haustorial mother cell (HMC) (M), containing five nuclei, that has not yet formed a haustorium. A plant nucleus (P) is present in the adjacent mesophyll cell. **B,** An infection hypha (F) containing about four nuclei in susceptible cultivar California Blackeye (CB) 24 h after inoculation. The fungus has formed a haustorium (H) containing five nuclei in the adjacent mesophyll cell and a intercellular secondary hypha (S) containing several nuclei. The HMC has no nuclei and cannot be seen. Plant nuclei (P) are visible in mesophyll cells. **C,** A secondary hypha (S) in susceptible cultivar CB 24 h after inoculation. There is aggregation of small nuclei near its tip and a much larger fungal nucleus (N) further back along the hypha. P = plant nuclei in adjacent mesophyll cells. **D,** Infection hypha (F) with only one or two nuclei in the substomatal vesicle (under the letter F) in resistant cultivar QA 24 h after inoculation. The HMC (M) at the tip of the infection hypha contains one nucleus, and the adjacent haustorium (H) contains three. **E,** Infection hypha (F) in cultivar QA 24 h after inoculation that has formed a HMC (M) in which two nuclei can be seen. The adjacent haustorium (H) also has two nuclei and is beginning to be enveloped in a cuplike callose encasement (arrowhead). Three nuclei are visible in the infection hypha. **F,** Infection hypha (F) in resistant cultivar Dixie Cream 18 h after inoculation viewed under transmitted visible light as well as UV illumination. The HMC (large arrow) has five nuclei, and the small haustorium (undetectable in this micrograph) inside the plant cell has none. The nucleus (arrowhead) of the invaded cell is in close contact with the fungal haustorium. **G,** A heavily infected leaf of susceptible cultivar CB 5 days after inoculation. Hyphal nuclei are generally in pairs. Two nuclei inside a haustorium can be seen adjacent to a plant nucleus (P) within a mesophyll cell.

However, these data did not clarify the temporal relationship between the three- and four-nuclei stage, probably because of the nonsynchronous fungal penetration and development. Therefore, we examined developmentally older septate invasion hypha in which one or more fungal cells had produced a branch. Eighty percent (n = 138) of these branches were formed by the first (closest to the penetration site) or second (central) cell (Fig. 2E). Only 13 and 17%, respectively, of these cells were binucleate (Fig. 6), and the single nucleus was often in the branch rather than in the invasion hypha (Fig. 2E). This considerable reduction in the incidence of binucleate central cells compared with younger, unbranched invasion hyphae indicates that the three-nuclei stage arises from the disappearance, and presumed degradation, of one of the nuclei in the binucleate cells. Consistent with this conclusion, in about 40% of the unbranched invasion hyphae that had binucleate central or first cells, one of these nuclei was large, and the other was unusually small and condensed (Fig. 2C). This phenomenon was seen in both ethanol- and aldehyde-fixed tissue but was never observed when the two nuclei were in the third cell (at the growing tip of the hypha). In a few primary hyphae with three nuclei, small fluorescent particles were present in the first or middle cell, which could have been the remains of the degraded smaller nucleus.

Inoculated, aldehyde-fixed leaves also were harvested at 3 days after inoculation, by which time a small fungal colony had formed at each infection site (Fig. 2F and G). No nuclei were seen in the three cells of the original primary hypha, and the intercellular mycelium in the mesophyll predominantly had one nucleus per cell (Fig. 2F). M-haustoria contained a single nucleus (Fig. 2G).

Fungal nuclei in urediospore-inoculated leaves. The early stages of infection of CB, DC, CC, and QA by urediospore-derived infection structures of the cowpea rust fungus have been described in detail previously (14). In summary, after entering the leaf via a stoma, each fungal individual sequentially produces a substomatal vesicle, an infection hypha, and a terminal haustorial mother cell (HMC); the latter forms a D-haustorium (20) inside the adjacent mesophyll cell into which most of the cytoplasm from the HMC migrates. In susceptible cultivar CB, the infection hypha forms a secondary hypha, and more intercellular mycelium and haustoria develop until a small colony is formed and the fungus sporulates. In DC and CC, however, the first invaded cell dies, and no further intercellular fungal growth beyond the infection hypha occurs. Fungal growth usually is similarly limited in QA, but the first-formed haustorium often is encased in callose-containing material, and the invaded cell does not die. These sequences of events have been deduced from time-course studies of fixed and cleared leaves, because the formation of haustoria deep inside the leaf precludes any observation of the infection process in living tissue.

In the current study, nuclei were seen best in DAPI-stained sections of paraformaldehyde-fixed leaf pieces. In leaves harvested at 15 h after inoculation, infection structures that had short infection hyphae and had not formed HMCs all had eight small nuclei in the substomatal vesicle, as reported previously (18). Only eight infection structures were seen in QA and CB that had HMCs but no haustoria, and these contained from 5 to 12 nuclei. At sites at which haustoria had formed in CB, QA, CC, or DC, infection hyphae had 4 to 12 nuclei, regardless of cultivar, with 4, 5, or 6 being the most common numbers in different experiments. These nuclei usually appeared large and the DNA diffuse (Fig. 7B), but occasionally, one or more appeared small with condensed DNA; again, the frequency of these small nuclei varied between experiments and was not related to cultivar. The small nuclei were not always in pairs as might be expected if their size was an indicator of a recent mitosis (11).

At 15 to 17 h after inoculation, most HMCs had formed small haustoria in each cultivar, and other than the presence of partial encasements around haustoria in QA and reduced chlorophyll fluorescence under UV irradiation in about 28% of infection sites in DC, all invaded cells looked normal. Each HMC and its haustorium contained a total of three to six small nuclei, regardless of cultivar, with four or five being the most common number (Fig. 7A). In about one-third of the infection sites in CC and CB and in about half of those in DC and QA, at least one nucleus had migrated from the HMC to the haustorium or could be seen in the haustorium neck, apparently in the process of migration.

Table 2 presents more detailed data from tissue harvested at 24 h after inoculation, by which time the fungus had achieved its maximum growth in the resistant cultivars. Most infection hyphae without secondary hyphae had two to five nuclei, regardless of cultivar (Table 2; Fig. 7B). Infection hyphae with secondary hyphae were only seen in CB and contained two to three nuclei in the infection hypha and three to eight in the secondary hyphae. Secondary hyphae with more than three nuclei generally had a cluster of three or four smaller nuclei at the hyphal tip (Fig. 7C) that sometimes were separated from the rest of the hypha by a septum. These tip cells resembled HMCs. In some secondary hyphae, adjacent pairs of small nuclei had projections of DNA, suggesting they were products of recent mitosis.

In CB, nuclear migration from the primary HMC into the haustorium was complete by 24 h after inoculation, and HMCs contained no nuclei, whereas their corresponding haustoria contained three to five nuclei (Table 2; Fig. 7B). Haustoria in QA generally were fully encased and their nuclei were difficult to discern. Two to five nuclei were seen in some haustoria, whereas HMCs contained zero to three; incomplete nuclear migration was observed in unencased (Fig. 7D), partially (Fig. 7E), and fully encased haustoria. In DC and CC, cell death did not occur as rapidly as in previously published experiments, and haustorium-containing cells were only slightly autofluorescent by 24 h after inoculation. No nuclei had migrated into about 50% of the haustoria (Fig. 7F); in the remainder, the maximum number was one in DC and five in CC (Table 2). In all cultivars, the total number of nuclei observed in each HMC and its haustorium was three, four, or five.

Sections of susceptible CB tissue harvested 3 or 5 days after inoculation revealed extensive tissue colonization. Intracellular hyphae had two nuclei per cell, as did HMCs before they formed haustoria and all observed secondary haustoria (i.e., haustoria formed from hyphae other than the infection hypha) (Fig. 7G). The primary (first-formed) or second-formed haustoria were difficult to find in most infection sites, and only one primary haustorium was unambiguously identified; this haustorium had at least four nuclei.

TABLE 2. Number of nuclei in infection structures of *Uromyces vignae* observed in sections of resistant and susceptible cowpea cultivars harvested 24 h after urediospore inoculation[a]

	CB		QA		CC		DC	
Infection structure	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Infection hypha	2.9	2–5	3.7	3–4	3.5	2–4	3.5	3–5
HMC[b]	0	0	0.8	0–3	3.2	0–5	3.3	2–5
Haustorium	4.1	3–5	3.8	2–5	1.3	0–5	0.5	0–1
HMC + haustorium	4.1	3–5	4.4	4–5	4.5	3–5	3.8	3–6

[a] CB = susceptible cultivar California Blackeye; QA, CC, and DC = resistant cultivars Queen Anne, Calico Crowder, and Dixie Cream, respectively. Data were pooled from two experiments; n = 17 to 20 per cultivar.

[b] HMC = haustorial mother cell.

DISCUSSION

In susceptible cultivar CB, the early morphological development of *U. vignae* from basidiospores resembled that described previously for this fungus (14,25) as well as that of basidiospore-derived infections of other rust fungi (6,7,8,22). Basidiospore- and urediospore-derived fungal development in resistant cultivars DC, CC, and QA also was similar to that reported previously (4,14,16). However, none of the earlier studies of *U. vignae* accurately related basidiospore- or urediospore-derived development to nuclear condition.

Binucleate basidiospores, as seen here in *U. vignae*, are common among rust fungi (2), but cells of the fungal thallus established in susceptible plants are generally uninucleate (6), as was observed in the current study. However, we saw no indication that the fungus became uninucleate in the basidiospore germ tube, as suggested for *Gymnosporangium asiaticum* (19). Instead, our data suggest that the monokaryotic state typically is achieved in *U. vignae* by (i) the synchronous mitosis of the two basidiospore nuclei after they and the spore cytoplasm had entered the invasion hyphae and the latter had grown to about 40 µm long; (ii) the subsequent immediate division by two septa of the invasion hypha into one binucleate and two uninucleate cells; and (iii) the disappearance and probable degradation of one of the nuclei in the binucleate cell. Such a process suggests that the fungus becomes intolerant of binucleate cells once the invasion hypha becomes septate.

Although the originally binucleate cell of the septate invasion hypha usually was the middle of the three cells, in a significant number of hyphae it was the first or third (tip) cell. Interestingly, there was no sign of any degradation of nuclei in binucleate tip cells, suggesting either that the growing apex of the hypha was more tolerant than the two other cells to being binucleate or that the binucleate condition resulted from a second mitosis related to tip extension.

This variation in the initial partitioning of nuclei after septation of the invasion hypha is an indication that during the division of the two original nuclei the fungus does not have tight control over nuclear positioning in relation to septum development. Even the association of mitosis with septation was broken in a small, but significant, number of invasion hyphae that contained three or four nuclei with only one or no septa. If this variation in nuclear partitioning and hyphal septation is common among invasion hyphae of rust fungi, comparisons of our data with those in the literature becomes difficult, because it is unclear in most other studies how many infection sites were observed and whether what was described was typical of the fungal population.

Superficially, nuclear behavior in *U. vignae* seems most similar to that in *Endophyllum sempervivi*, because Ashworth (3) illustrates invasion hyphae with three cells, one of which is binucleate, and she mentions variation in distribution of nuclei and septation. However, if other reports are taken at face value, *U. vignae* differs substantially from other rust fungi. For *Puccinia sorghi*, septation is reported to follow one or more nuclear divisions, and trinucleate invasion hyphae lacking septa are illustrated as typical (23). For *P. malvacearum*, the invasion hypha is reported to be divided by a single septum into an anucleate cell and a binucleate cell, and the latter is reported to be subsequently divided by a septum into two uninucleate cells (1). For *P. xanthii*, nuclear condition has not been reported, but the invasion hypha is divided by a single septum, which separates the intraepidermal vesicle from the rest of the hypha (22). Finally, in *Cronartium quercuum*, electron microscopy suggests that the invasion hyphae may be nonseptate or variously septate (8). These reports suggest that the invasion hypha may establish monokaryotic mycelium differently in different rust fungus species.

In the two highly resistant cultivars, CC and DC, in which invaded cells rapidly died, basidiospore-derived fungal growth ceased before the invasion hypha had achieved the length at which mitosis normally occurred in the susceptible cultivar. Not unexpectedly, therefore, nuclei migrated from the basidiospore into the intraepidermal vesicle, but there was no subsequent nuclear division or fungal septation. In the less resistant cultivar, QA, in which invasion hyphae became encased in callose-containing material, nuclear division typically was seen in longer, as yet unencased, hyphae. Therefore, the presence or absence of nuclear division in basidiospore-derived invasion hyphae formed in resistant cowpea cultivars may reflect the extent of fungal growth rather than any direct effect of plant resistance.

On differentiation-inducing surfaces, urediospore-derived germ tubes of *U. vignae* sequentially form appressoria, substomatal vesicles, infection hyphae, and primary HMCs (the HMC formed at the tip of the infection hypha) that are morphologically identical to those formed in the plant (18,24). In living fungal individuals observed on oil-containing collodion membranes, synchronous division of the two nuclei originally present in the urediospore first occurs when the cytoplasm migrates into the appressorium, and the resulting four nuclei divide synchronously again after the fungal cytoplasm has migrated into the substomatal vesicle (11,18). In fixed infection hyphae of the same fungus formed on plastic surfaces, Stark-Urnau and Mendgen (24) reported a variable number of nuclei, up to nine. In the current study of sectioned leaves, short infection hyphae invariably had eight nuclei, but longer ones without HMCs often had more or less, although never as many as 16. Infection hyphae with a HMC initially had 4 to 12 nuclei, but the numbers decreased with age, regardless of cultivar. In combination, these observations suggest that, regardless of the presence of a host plant or its degree of susceptibility, nuclear division becomes asynchronized and variable after the round of synchronous division in the substomatal vesicle. Nuclear degradation also seems to be common, although it did not appear to be as consistent as that occurring in the binucleate cells of the basidiospore-derived, intracellular invasion hyphae.

Primary HMCs of *U. phaseoli* var. *typica* (=*U. appendiculatus*) formed on oil-containing collodion membranes have been reported to generally have two, sometimes up to five, nuclei (21). Stark-Urnau and Mendgen (24) reported that although three or four was the most common number of nuclei seen in primary HMCs of *U. vignae* on plastic surfaces, two was the most common number seen in the susceptible cultivar, and only a few HMCs contained three to five nuclei. However, in all four cowpea cultivars observed in the current study, the most prevalent number of nuclei in primary HMCs was four or five, with two being extremely rare. The small size of all of these nuclei suggests they were derived by one or more divisions of one or two nuclei in the infection hypha that moved to the terminal HMC as it formed.

Our observation of clusters of small nuclei at the tips of young aseptate, multinuclear secondary hyphae in susceptible cultivar CB suggests the second HMC formed by *U. vignae* also contained the products of recent mitoses and had more than two nuclei. It seems, therefore, that, unlike the basidiospore-derived invasion hypha in which nonapical cells with more than one nucleus are not tolerated beyond the first mitosis, multinuclearity and flexibility in nuclear number is the norm during the early stages of fungal development from urediosporelings.

Surprisingly, given the heterokaryotic nature of the urediospores (rust fungi generally are heterothallic), nuclei in infection and secondary hyphae were not arranged in pairs, yet by 3 days after inoculation with urediospores, all detected intercellular hyphae were binucleate, as were HMCs. Presumably, a binucleate, heterokaryotic hyphal tip is produced after the second HMC is formed, and the binucleate condition of hyphal cells and HMCs is tightly controlled thereafter. Such control seems to develop later in *P. striiformis*, which is unusual among rust fungi in that HMCs have two or four nuclei and multinucleate cells are common in young colonies but which produces binucleate hyphae adjacent to uredinia (5).

In susceptible cowpea cultivar CB, all of the nuclei in the HMC of *U. vignae* migrated into the attached haustorium as it matured. Therefore, the primary haustorium had up to five nuclei, and haustoria in established mycelium had two. In contrast, several *Puccinia* spp. have been reported to have only one nucleus in the mature haustorium, despite two nuclei in young HMCs (9). Whether the second nucleus remains in the HMC or disintegrates is unknown.

Nuclear migration into the primary haustorium of *U. vignae* was often not completed in resistant cultivar QA, in which the invaded cell commonly does not die but the extrahaustorial membrane appears abnormal and the haustorium becomes encased in callose-containing material (12,17). The fact that completed migration was delayed or absent in unencased or incompletely encased haustoria supports previous suggestions (17) that there are adverse effects on the haustorium prior to encasement. Because there were no obvious restrictions to the development of the invasion hyphae prior to encasement in this cultivar, it seems that callose encasement may play a more significant role in resistance to urediospore- than to basidiospore-derived infection.

In cultivars CC and DC in which the invaded cell died rapidly, nuclear migration was never completed, and only about half of the haustoria had any nuclei at all. Earlier ultrastructural studies suggested that the haustorium is not immediately killed in any of these resistant cultivars (13,17). Therefore, the lack of nuclear migration may be related to the similar absence of migration seen in living haustoria of *U. vignae* formed in vitro (15). Possibly, in all of these situations, the phenomenon is a sign of a lack of effective metabolic interaction between the haustorium and the plant cell.

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