Cytology and Morphological Development of Basidia, Dikaryons, and Infective Structures of *Urocystis agropyri* from Wheat

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ABSTRACT

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The morphology and cytology of basidia, dikaryons, and infective structures of *Urocystis agropyri* from wheat were studied in vitro and in vivo. The blastogenous basidiospores developed synchronously, but remained adnate to the promycelial apex, despite attempts to remove them by micromanipulation. Meiosis, which apparently occurred in the teliospores, was followed by mitoses; thereafter, haploid nuclei migrated to the basidiospores. Nuclear migration was correlated with the completion of basidiospore elongation. Generally, one nucleus migrated to each basidiospore, although occasionally two migrated into some of them. Plasmogamy occurred in situ between mononucleate basidiospores during which the nucleus of one basidiospore migrated into the protoplast of the other. In most of these cases, fusions occurred between two hyphal pegs, one at the base of each basidiospore, although apical fusions also were seen. Some dikaryons also formed following fusions between promycelia and basidiospores, or by the ingress of two nuclei into single basidiospores, or from the promycelium itself when teliospores germinated directly. On wheat coleoptiles, the apical cells of dikaryotic infection hyphae formed appressoria with penetration pegs which directly penetrated the epidermis and emptied their contents into a system of intracellular hyphae.

Additional key words: conjugation tubes, cytoplasmic migration, *Triticum aestivum*.

Urocystis agropyri (Preuss) Schroet. (=*Urocystis tritici* Koern.) causes flag smut of wheat (*Triticum aestivum* L.) in the Pacific Northwest (PNW). The disease is destructive in localized areas of southcentral and southeastern Washington state. Some research has dealt with factors that influence teliospore germination and infection under field conditions (2,34), but little is known about the genetics of the fungus including the incompatibility system, pathogenicity, and cytological aspects. Such investigations are contingent on adequate understanding of basidial ontogeny, cytology, the origin of the dikaryophase, and how to manipulate the fungus for experimental purposes. Because there are conflicting reports on these aspects of the biology of *U. agropyri* (6,14,28,32,40), experiments were designed to: demonstrate the morphological and cytological events which result in the formation of basidia and dikaryons, isolate the haplophase and characterize the infective structures.

In interpreting the results, it was assumed that teliospores were diploid and mononucleate (30), and that (as with other smut fungi [17]) this fungus had a diplo-, haplo-, and dikaryophase.

MATERIALS AND METHODS

Teliospore germination and nuclear staining of basidia. To enhance germination, teliospores were presoaked in distilled water for 3 days at 20 C (2,3). Bacterial contamination promoted by the soaking was reduced by washing the spores for 10 sec in 0.5% sodium hypochlorite. Following washing, the spores were rinsed in sterile water, sown on 1.5% water agar, and incubated in darkness at 20 C.

Agar blocks (1 × 2 cm) with basidia in various developmental stages were inverted onto microscope slides (16), flooded with a mixture of Haupt's adhesive and formalin (7), and dried on a slide warmer at 40–45 C for 24 hr. Thereafter, the slides were placed in a 60 C water bath for 5 min to hydrate the agar and then in a 50% solution of HCl at 60 C for 5 min to remove the agar and hydrolyze the specimens. The specimens were rinsed in distilled water and Sorenson's phosphate buffer, pH 6.5, then stained for 30–60 min in fresh solutions of two parts Giemsa stock solution (41) and 25 parts phosphate buffer. After staining, the specimens were immersed briefly in a surfactant (one drop of Tween-20 per liter of H_2O). This was followed with an acetone-xylene series to differentiate nuclei and dehydrate the specimens (12). After mounting them in Coverbond synthetic resin (Scientific Products, McGaw Park, IL 60085), the specimens were examined with bright-field microscopy and photographed with Kodak high contrast copy film.

Presoaked teliospores also were germinated on coleoptiles of Springfield wheat. Coleoptiles of 24- to 36-hr-old seedlings were coated with teliospores and incubated on moist filter papers in petri dishes for 8–36 hr at 20 C. When the teliospores germinated, basidia in various developmental stages were appressed or washed onto water agar and stained as described above.

Observations of basidial development. Presoaked teliospores

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were streaked in a film of water on #1 coverglasses. These were subsequently inverted over culture microslides containing a drop of water in each well and coated with petrolatum to maintain consistent moisture. Germination was observed with bright-field microscopy at both low and high magnifications (×100–1,250). Basidia in various stages of development were photographed with Kodak 35-mm Panatomic X film.

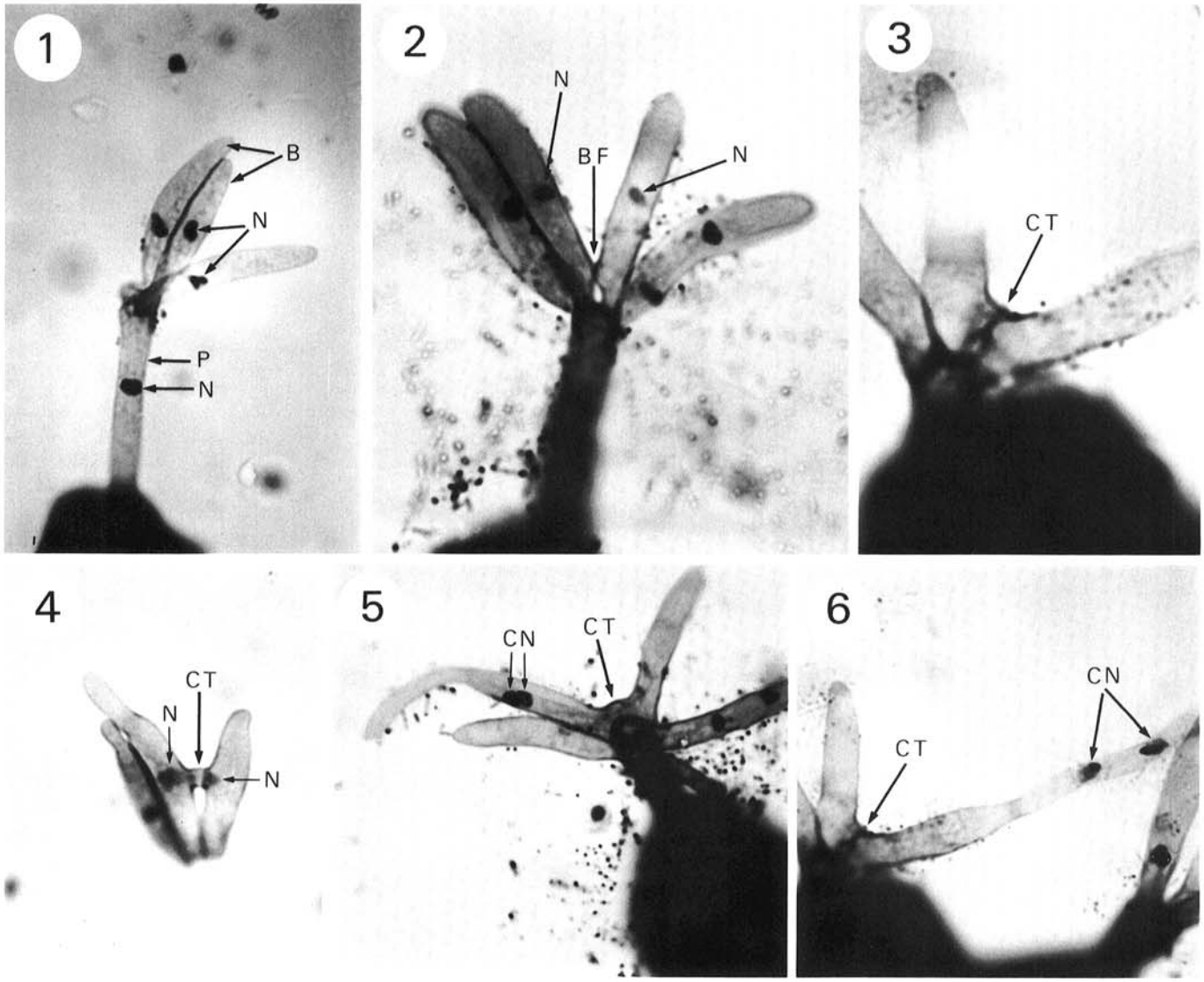
Time-lapse cinephotomicrography using a Bolex 16 mm camera and Kodak TRI-X reversal film also was used to record basidial development. The camera was mounted on a Nikon model L-ke trinocular microscope.

Identification and characterization of infective structures. Seedlings 24–36-hr old were inoculated by coating coleoptiles with presoaked teliospores and then incubating them for 1–3 days at 20 C. The infected coleoptiles were fixed in a solution of 0.5% chromic acid, 3.5% glacial acetic acid, 15% commercial formalin, and distilled water for 24 hr, rinsed in water and stained in 0.06% aniline blue for 12–24 hr at 35–40 C. The coleoptiles were destained and mounted in 85% lactic acid and examined with bright-field microscopy. Infective structures were photographed with Kodak 35-mm Panatomic X film. For scanning electron microscopy, the coleoptiles were fixed in 3% glutaraldehyde and 3% acrolein in 0.1 M sodium cacodylate buffer for 12 hr, dehydrated through a graded ethanol series, critical-point dried, and coated with gold in a Technics Hummer 2 sputter coating apparatus. The structures were viewed and photographed with an Etec Autoscan U-1 scanning electron microscope at 20 kV.

RESULTS

Cytology and morphological development of basidia and dikaryons. Presoaked teliospores germinated in 8–12 hr. Promycelia about 3–4 μm in diameter and about 4–20 μm long usually split the spore balls at the point of emergence. Basidiospore formation began as rounded protrusions at the apex of the promycelium. The basidiospores formed blastogenously, developed synchronously, and required 4–5 hr to elongate fully. Usually two to eight formed per basidium (Fig. 1). Fully developed basidiospores ranged from 2.5–4.0 μm thick and 15–22 μm long. Secondary basidiospores were not seen and apparently did not form.

Migration of nuclei from teliospores to basidiospores began only



Figs. 1–6. Basidia of *Urocystis agropyri* stained with Giemsa. **1,** Mononucleate basidiospores and apparently a supernumerary nucleus in the promycelium (×1,480). **2,** Initiation of a basal fusion (center arrow) between two mononucleate basidiospores (×1,480). **3,** Conjugation tube joining basidiospores. The dark line in the center indicates where the hyphal pegs fused (×2,100). **4,** Nuclear migration in fused basidiospores. The nucleus on the right is entering the conjugation tube to join the nucleus in the basidiospore on the left which has germinated at the apex (×1,480). **5,** Fused basidiospores showing early formation of dikaryon. The dikaryotic basidiospore has germinated (×1,480). **6,** Migration of conjugately associated nuclei from fused basidiospores into a young infection hypha (×1,480). B = basidiospore, BF = basal fusion, CN = conjugately associated nuclei, CT = conjugation tube, N = nucleus, and P = promycelium.

when the latter were fully elongated. In fact, nuclei were not observed in promycelia until that time. Nuclei characteristically appeared in basidiospores within 30 min following the completion of basidiospore elongation. Generally one nucleus entered each basidiospore (Fig. 1), but occasionally two nuclei migrated into a single basidiospore. Supernumerary nuclei frequently were observed in promycelia (Fig. 1). Apparently, meiosis and postmeiotic divisions occurred only in the teliospores, since nuclear divisions were not seen in either promycelia or basidiospores. Basidia generally contained four, six, or eight nuclei, but five to seven occasionally were observed, indicating that postmeiotic divisions were sometimes asynchronous. Most nuclei were about 1 μm in diameter.

Dikaryons usually formed following basal fusions between mononucleate basidiospores. The fusion process was preceded by formation of a peglike hyphal projection from the base of each basidiospore (Fig. 2). The pegs formed perpendicularly to the axes of the basidiospores and were directed toward each other. As they grew, the pegs touched end-to-end, elongated, and tended to push the basidiospores apart (Figs. 2 and 3). The point at which the hyphal pegs actually fused and formed conjugation tubes was discernible in stained preparations (Fig. 3). Fusions always occurred in situ since basidiospores at no time abstricted from the promycelia.

Conjugation tubes began to form in about 0.5–3.0 hr after basidiospores were fully elongated, and then development was relatively rapid. After 10–30 min, the conjugation tubes were fully elongated, but it was impossible to determine precisely when plasmogamy occurred. Basidiospores germinated about 10 min to 3 hr after conjugation tubes formed, and germination seemed to be correlated with the migration of one nucleus into the other basidiospore (Fig. 4). Nuclear migration was interpreted to indicate that plasmogamy was either in progress or completed. These observations suggested that plasmogamy was completed soon after conjugation tubes formed.

While the nucleus from one basidiospore migrated through the conjugation tube, the nucleus in the other basidiospore appeared stationary (Fig. 4). Dikaryotic nuclei often were positioned centrally in one basidiospore (Fig. 5), but as germination proceeded, both simultaneously migrated into the developing germ tube (Fig. 6). Mostly, dikaryons germinated by a germ tube at the apex of one basidiospore, but some germinated at the base.

Apical fusions between basidiospores also were observed. In these instances, germ tubes developed from the conjugation tubes where the hyphal pegs had fused. Then, the nuclei migrated into the conjugation tubes, became conjugately associated, and passed into the germ tubes.

Dikaryons also frequently formed following fusions between basidiospores and promycelia. Hyphal pegs at the base of the basidiospores fused with others which developed at the apices of promycelia. In terms of shape and size, the promycelial pegs were similar to those of basidiospores. Both usually were juxtaposed and fused side by side. Others that were more distant from one another fused end to end. Dikaryons formed when a promycelial nucleus migrated through the conjugation tube into the basidiospore, or when both promycelial and basidiosporic nuclei simultaneously migrated into the conjugation tube from which germ tubes developed. Two other methods of dikaryon formation were observed. In one, nuclei, conjugately associated, migrated into germ tubes formed by the promycelium and, in the other, two nuclei migrated from promycelia into single basidiospores.

Although germ tubes formed by basidiospores and promycelia mostly were dikaryotic (Fig. 6), some were observed with three to four nuclei. Apparently, when there were supernumerary nuclei in basidia, some germ tubes received additional nuclei. All nuclei in basidia ultimately entered germ tubes, but did not divide in them. The multinucleate germ tubes appeared to have resulted from migration rather than from postmigration nuclear divisions. At no time were mononucleate germ tubes observed. Germ tubes developed infection hyphae typical of other smut fungi, each of which consisted of a normal dikaryotic apical cell (Fig. 7) subtended by a chain of empty cells.

Basidiospores did not abstrict from promycelia, and it was not possible to remove them by micromanipulation. In fact, they remained adnate to the promycelia even after basidia were devoid of cytoplasm. With time lapse cinephotomicrography, it was possible to see that cytoplasm of whole basidia moved from promycelia into and out of germinating basidiospores, indicating that septa did not form between promycelia and basidiospores.

Nuclei migrated from teliospores to basidiospores after cytoplasm had moved into the developing basidiospores. Evidence to support this conclusion was based on critical examination of whole basidia, stained with Giemsa, and by observing live basidia in which promycelia and basidiospores remained anucleate until basidiospores were fully elongated and engorged with cytoplasm. Only then were nuclei seen in promycelia migrating toward the basidiospores. In fused basidiospores, the nuclei migrated from one basidiospore to the other and became conjugately associated before the entire cytoplasm migrated into the germ tubes. There was no evidence of cytoplasmic streaming associated with nuclear migration.

From the promycelia the cytoplasm migrated either into promycelial germ tubes or into and out of germinating basidiospores. In fused basidiospores, the cytoplasm flowed through the conjugation tubes and entered the germ tubes en masse, usually within 2–6 hr. Septa commonly formed during this process leaving behind empty, two-celled basidiospores. Occasionally bits of cytoplasm remained in promycelia and basidiospores. The rate of cytoplasmic flow was calculated to be about 17 μm/hr at 22 C.

The cytology and morphological development of basidia and dikaryons was the same on water agar, wheat coleoptiles, and in the culture microslides used to observe live basidia.

Repeated attempts to remove basidiospores from promycelia by micromanipulation failed, and, hence, the haplophase could not be isolated. During micromanipulation, the micromanipulator needle frequently separated promycelia and teliospores, but never basidiospores from promycelia. Dikaryotic germ tubes (ie, infection hyphae), however, were routinely isolated by breaking the long chain of empty cells which formed within 2–3 days following teliospore germination, and moving the apical cell to nutrient media where they grew for several days.

Identification and characterization of infective structures. On wheat coleoptiles, dikaryons formed infection hyphae, the tips of which swelled and formed appressoria (Fig. 8). The appressoria were clubshaped, hyaline, 3–6 μm wide at the swollen end, and formed penetration pegs (Figs. 9–13). These pierced the epidermal wall, after which a system of branched intracellular hyphae about 2–3 μm in diameter developed in the epidermis (Fig. 11). Formation of appressoria began about 30 hr following inoculation, and infection occurred within about 54 hr. Infection hyphae were easily removed from the coleoptiles and were consistently dikaryotic; however, once appressorium formation began, they

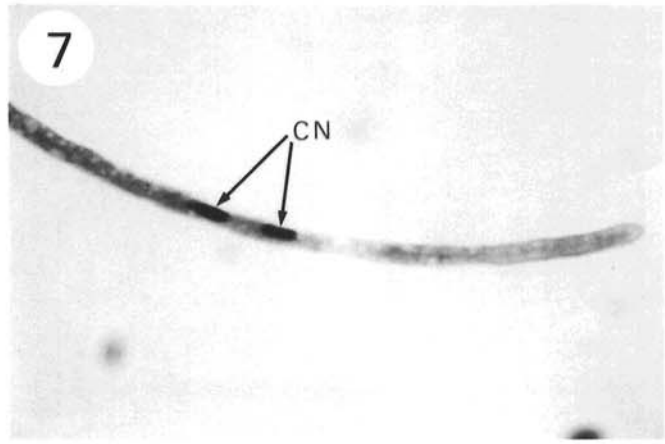


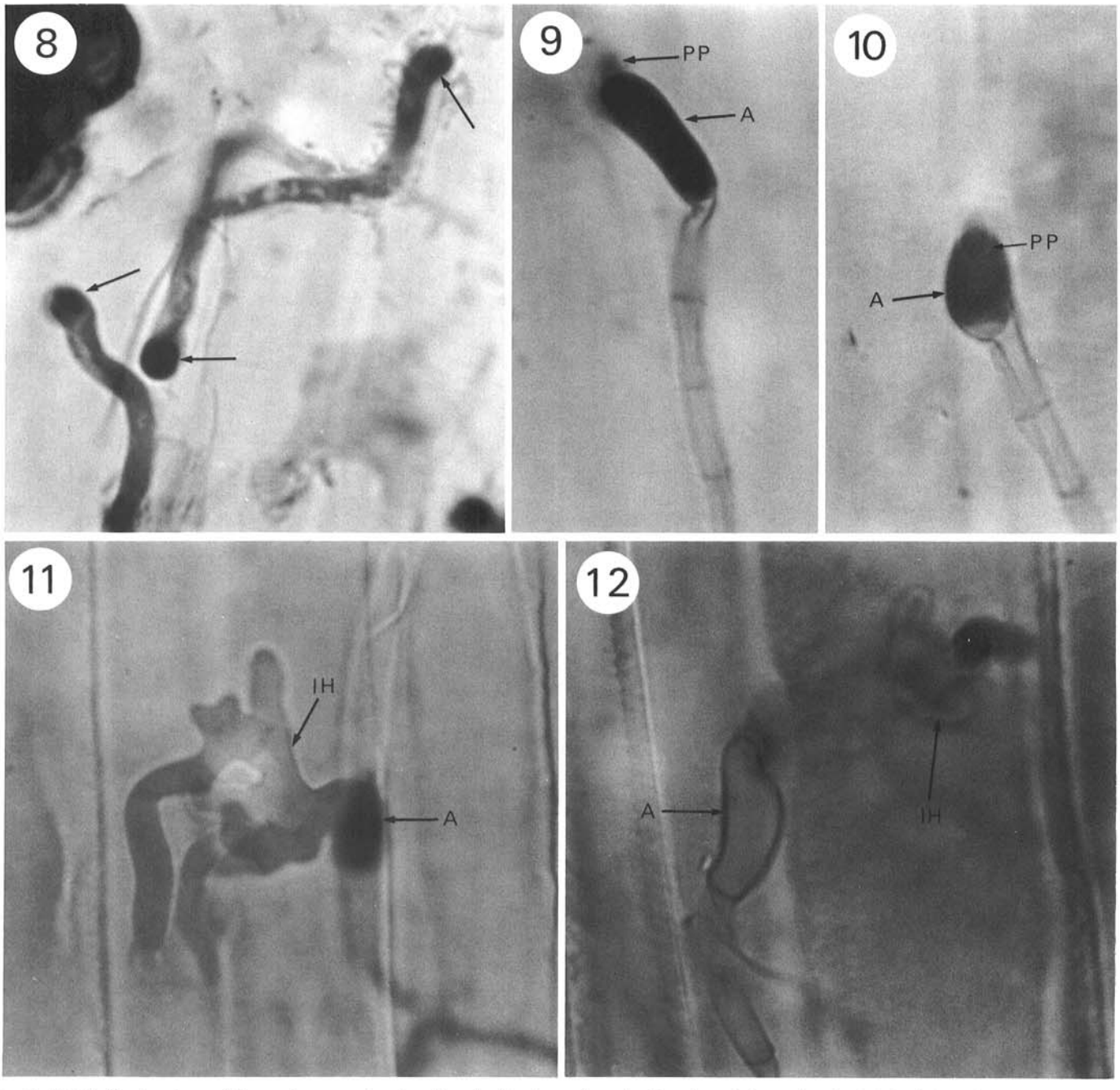
Fig. 7. The dikaryotic apical cell of an infection hypha of *Urocystis agropyri* stained with Giemsa (×1,480). CN = conjugately associated nuclei.

could not be removed from coleoptiles. Thus, nuclear condition of appressoria was not determined. Cytoplasm in appressoria moved into the intracellular hyphae leaving the empty appressoria attached to the epidermis (Fig. 12).

DISCUSSION

U. agropyri apparently is heterothallic, although secondary homothallism sensu Ainsworth et al (1) occasionally was suggested when basidiospores received two nuclei. Basidiospore fusions and formation of dikaryons following nuclear migration provided strong evidence of heterothallism. This sequence of events is well known in the Ustilaginales (17). However, heterothallism could not be conclusively demonstrated by incompatibility studies and pathogenicity tests because the haplophase was not isolable.

Our results contrast with those of El-Khadem et al (14) who isolated primary and secondary basidiospores of the fungus from wheat in Egypt. They determined that the fungus was bipolar with multiple alleles controlling heterothallism. We were unable to remove basidiospores from promycelia at any time during their development and saw nothing to indicate that they abstricted either on wheat coleoptiles or water agar. Also, secondary basidiospores were never observed on coleoptiles or water agar. Nor did they form when teliospores were germinated on various media (eg, PDA, soil extract agar, or in the presence of crushed coleoptile and leaf tissue). Although failure of basidiospores to abstrict may in part have been due to environmental factors, it seemed more to be an innate characteristic of the fungus. This peculiarity was not unique to specific teliospore collections, since others from the PNW which also were germinated behaved similarly. Moreover,



Figs. 8–12. Infective structures of *Urocystis agropyri* on the epidermis of a wheat coleoptile. Cytoplasm in fungus is stained dark with analine blue (×1,990). **8,** Initiation of appressorial formation by swelling of the apical cells of infection hyphae (arrows). **9,** A darkly stained appressorium and a penetration peg developing at the tip. Note chain of empty cells subtending appressorium. **10,** Appressorium showing outline of developing penetration peg. Photographed from the ventral side of the appressorium by focusing through the epidermis. **11,** Intracellular hyphae and an appressorium (out of focus). **12,** Empty appressorium resulting from migration of cytoplasm into the intracellular hyphae. A = appressorium, IH = intracellular hyphae, and PP = penetration peg.

researchers from other parts of the world have reported similar results (6,21,32). Thus far, apparently, only *U. agropyri* from Egypt is known to abstrict basidiospores and form secondary basidiospores (14).

Whether the pattern of heterothallism in *U. agropyri* from the PNW is similar to that of the fungus in Egypt (14) is a moot question. Since haplonts could not be isolated from PNW collections by conventional techniques, unconventional methods may be required. For example, since dikaryons dissociate on artificial media (30), it might be possible to separate monokaryotic cells by microsurgery, culture such cells, and use them in pathogenicity tests. However, our axenic cultures of the fungus were strictly mycelial and dikaryons did not form. Also, numerous attempts were made to reproduce flag smut on susceptible wheat using mycelium from axenic cultures, but without success (30). Therefore, studying the genetics of this fungus using axenic cultures may not be feasible.

Direct migration of nuclei from promycelia into basidiospores to establish dikaryons indicated secondary homothallism. Similar nuclear behavior was observed in *U. agropyri* from *Agrostis alba* (39) and in *U. occulta* from rye (38). Recent research (13) has shown that primary basidiospores of other Tilletiaceae are multinucleate and have both mating-type nuclei. Obviously, the presence of nuclei of both mating types in single basidiospores has important cytogenetic implications in terms of incompatibility and pathogenicity. Unfortunately, neither mono- nor binucleate basidiospores were isolable to test their pathogenicity. On the other hand, El-Khadem et al (14) reported no evidence of solopathogenicity from Egypt.

Noble (32) and Verwoerd (40) reported that some dikaryons originated from basidiospores in which a single nucleus underwent a series of mitotic divisions, thus implying primary homothallism sensu Ainsworth et al (1). Perhaps these researchers actually dealt with secondary homothallism rather than primary, because they may have failed to note the easily overlooked basal fusions between basidiospores. In any event, we found no evidence of primary homothallism.

Conjugation tubes formed between basidiospores in a manner similar to that described by Biraghi (6) for *U. agropyri* and by others for other species of smut fungi (8,24,25). However, Biraghi (6) described fusions in which one basidiospore produced a hyphal peg that fused with the wall of the other basidiospore. In every case, we observed that both basidiospores had formed a hyphal peg. Also, after the pegs established contact and elongated, the basidiospores often were forced apart at angles exceeding 90 degrees. Holton (22) also described this curious phenomenon in *U. avenae*. The precise end-to-end contact between the hyphal pegs of conjugating basidiospores suggested that forces of attraction drew them together well before plasmogamy was consummated. Kollmorgen et al (25) made a similar suggestion to explain this apparent phenomenon in *Tilletia caries*. For smut fungi and other basidiomycetes, there is evidence that fimbriae and/or hormones play a role in attraction between basidiospores of opposite polarity (4,11,19,26).

Migration of nuclei from teliospores to the promycelium and then to the basidiospores apparently was stimulated by the stage of basidiospore development. Not until basidiospores were fully developed did the nuclei begin exodus from the teliospores toward the basidiospores. Since nuclei could not be seen in the pigmented teliospores, it was impossible to count them, and, hence, the number present could not be correlated with the onset of migration. In contrast, nuclear migration between fused basidiospores always was associated with the formation of conjugation tubes, which suggested that the stimulus to migrate most likely resulted from the amalgamation of protoplasts. Regardless of the initiating forces, nuclear migration mostly resulted in formation of dikaryons. Rarely, promycelia formed germ tubes into which supernumerary nuclei migrated, but which nevertheless appeared to be functional dikaryons in a morphological sense.

Nothing was seen to indicate that nuclear migration from promycelia to basidiospores, or between basidiospores, was correlated with cytoplasmic flow. This interpretation is consistent with the results of other research (20,29,31,35,37) which indicates that nuclear migration in fungi is autonomous. Forer (18) suggested that actinlike filaments generate forces for chromosome movement during nuclear divisions and implied that microtubules may regulate the rate of movement. Perhaps similar forces influence migration of nuclei in basidial components. Poon and Day (33), for example, have shown that microtubules attached to the spindle pole body of the nucleus of *Ustilago violaceae* function in nuclear movement in budding sporidia.

Dikaryon formation by *U. agropyri* is highly variable, which is consistent with the heterogeneity of basidia in the heterobasidiomycetes (36). In nature, environmental factors probably affect the mode of formation. Excess moisture, for example, promotes direct germination of teliospores (36). This flexibility is common among smut fungi (17,23) and merits no special mention except to indicate that it most likely is under genetic control and probably confers survival value.

Appressoria observed were of the simple hyaline type, according to criteria of Emmett and Parbery (15), which predominates in the Erysiphales and Uredinales and were similar to those described for *Tilletia caries* (10) and *Ustilago nuda* (5). Mostly, appressoria emptied their cytoplasm into the intracellular hyphae in a manner similar to that described for urediospores (27). Although the nuclear condition of appressoria was not demonstrated, presumably they were dikaryotic since they developed directly from apical cells of infection hyphae which are characteristically dikaryotic. Furthermore, no evidence of nuclear dissociation was seen to suggest monokaryotic infections similar to those caused by *U. maydis* in corn (9).

In general, the pattern of nuclear and cytoplasmic migration that we observed indicated that *U. agropyri* utilizes all cytoplasm and organelles in basidia to establish infection. Basidia directly established dikaryons, secondary basidiospores did not form, and all nuclei and cytoplasm in both basidiospores and promycelia (with few exceptions) were utilized in dikaryon formation and to form appressoria. As dikaryons (ie, infection hyphae) grew, the

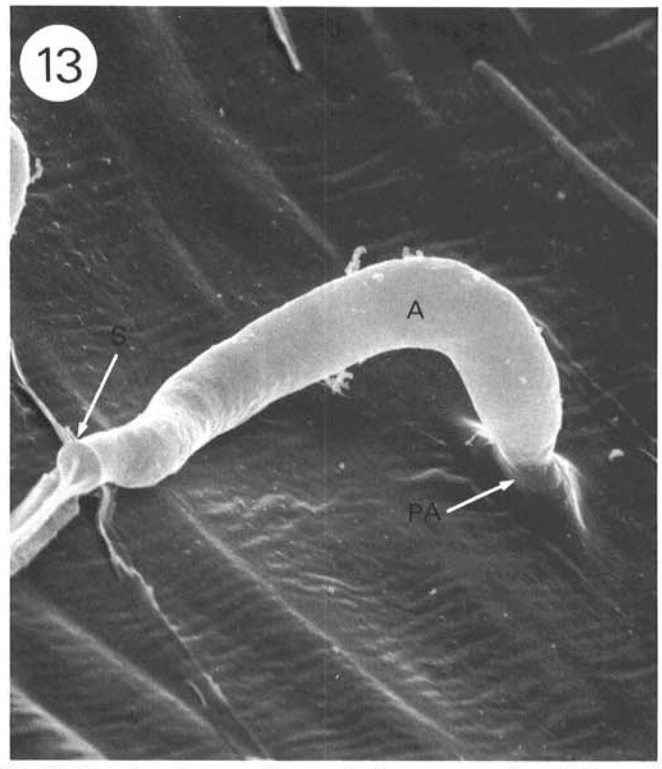


Fig. 13. Scanning electron micrograph of an appressorium of *Urocystis agropyri* on a wheat coleoptile. Note the point of attachment to the epidermis and prominent septum between appressorium and subtending chain of empty cells (×2,770). A = appressorium, PA = point of attachment, and S = septum.

conjugately associated nuclei therein did not divide, the protoplasts were always confined to the apical cells, and the entire cells were consumed in the infection process. These characteristics imply mechanisms to conserve energy and use food reserves strictly to locate and infect the host.

Thus far, the flag smut fungus from the PNW has shown little pathogenic variation. Only one race is reported (34), and screening wheat for resistance for many years has not indicated new pathogenic variability (R. L. Line, *personal communication*). Basidial characteristics may be partly responsible. The production of few basidiospores, the absence of secondary basidiospores, fusions in situ, and the fact that dikaryons often form directly from promycelia or by migration of two nuclei into basidiospores, would seem to restrict outcrossing and promote inbreeding. Obviously, there are other feasible explanations for lack of variability. Interestingly, basidial characteristics of *U. occulta*, the causal organism of stem smut of rye, are similar to those of *U. agropyri* and it, too, has thus far shown little variation (38).

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