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Pycnial nectar of rust fungi induces cap formation on pycniospores of opposite mating type[1]

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Abstract: Pycnial nectar (including pycniospores) transferred between pycnia of opposite mating type (as indicated by subsequent aecium formation) induced formation of a cap on one end of pycniospores. The polar caps developed with seven species of *Puccinia* and four of *Uromyces*, but not with *P. helianthi*, *Tranzschelia pruni-spinosae*, or *U. vignae*. Caps were induced equally in reciprocal transfers of nectar between pycnia of opposite mating type. The caps stained with India ink, or labeled with colloidal gold or wheat germ agglutinin conjugated with fluorescein isothiocyanate (WGA-FITC), but were not visible in unstained preparations. Cap formation started within 10 min of nectar transfer and was completed in 20–60 min. Nectar retained cap-inducing activity after pycniospores were removed by centrifugation, whereas pycniospores washed free of nectar did not induce caps. Pycniospores that were removed from a pycnium and induced to form caps by pycniospore-free nectar of opposite mating type did not induce aecia when returned to the original pycnium, showing that cap formation alone was not sufficient for completion of mating processes. Caps were removed by treatment with proteinase K, sodium dodecyl sulfate (SDS), or 0.01 N HCl, indicating the presence of protein. Labeling by WGA-FITC suggested the presence of polysaccharides or glycoproteins containing N-acetylglucosamine. The cap-inducing activity of nectar was lost if the nectar was boiled or autoclaved. Cap-inducing nectar contained a complex of high molecular weight proteins larger than 100 kDa as shown by native polyacrylamide gel electrophoresis analysis. This protein complex had cap-inducing activity as determined by placing pycniospores directly on gels after electrophoresis. In SDS gels, 6–7 polypeptides ranging in size from 14–70 kDa were observed, but bioassays of these polypeptides for cap induction were negative. The results indicate that pycnial nectar of several rust species contains high molecular weight cap-inducing proteins which are mating type-specific and induce pycniospore cap formation as an early event associated with processes leading to fertilization.

Key Words: aecia, aecium, fertilization, mating type, pycnia, pycnium, Uredinales

INTRODUCTION

Mating in rust fungi (Uredinales) takes place in haploid pycnia produced on pycnial host plants. In species investigated, pycniospores produced in pycnia of one mating type fertilize pycnia of a second mating type by fusion to a receptive hypha, transfer of a nucleus into the hypha, followed in most rust fungi by formation of dikaryotic hyphae that produce aecia (Allen 1933, Craigie 1927, 1931, 1933, Pierson 1933). The pycniospores are produced in pycnial nectar, a solution of high sugar concentration thought to encourage visits by insects, which then carry nectar and pycniospores from one pycnial cluster to another. In a comprehensive investigation of fertilization in rust fungi, Buller (1950) described formation of fusion tubes from the receptive hyphae. The tube grows toward a pycniospore, which in turn, produces a very short tiny papilla where fusion then takes place. Buller (1950) suggested that stimuli act at a distance to induce directed fusion tube growth and papilla formation. Since Buller (1950), little has been added to knowledge of the mode of attraction or details of the fusion process. Likewise, the possible role of nectar in fertilization processes other than as an attractant for insect vectors has not been investigated.

As part of a project to learn more about fertiliza-

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TABLE I. Species of *Puccinia, Tranzschelia* and *Uromyces* tested for nectar-induced pycniospore caps

Species	Collection[a]	Telial host	Pycnial host
Puccinia allii (DC.) Rud	8782, 8827, 8837	*Allium ampeloprasum* L.	*Allium ampeloprasum*
P. coronata Corda	8789, 8805, 8822	*Avena sativa* L.	*Rhamnus palaestinus* Boiss.
P. graminis Pers. f. sp. *tritici* Eriks. & E. Henn.	9522	*Triticum aestivum* L.	*Berberis vulgaris* L.
P. graminis f. sp. *secalis* Eriks. & E. Henn.	2969	*Agropyron repens* (L.) Pal.	*B. vulgaris*
P. helianthi Schwein.	2971[b], 2972[b], 2973[b], 2974[b], 8784, 8785, 8829	*Helianthus annuus* L.	*Helianthus annuus* L.
P. hordei G. Otth	1817, 1827, 1836, 1837	*Hordeum spontaneum* C. Koch.	*Ornithogalum* spp.
P. recondita Roberge ex Desmaz	9310, 9567, 9569	*Aegilops longissima* Schweinf. et Muschler emend Eig.	*Anchusa aggregata* Lehm.
P. recondita	9579, 9586, 9910	*Ae. ovata* L.	*Echium glomeratum* Poiret
P. recondita	2889[c]	*Secale cereale* L.	*Lycopsis arvensis* L.
P. recondita	77024, 77042	*Triticum turgidum* ssp. *durum* (Desf.) MK.	*An. italica* Retz.
P. sorghi Schwein.	8790, 8794	*Zea mays* L.	*Oxalis corniculata* L.
P. triticina Erikss.[d]	77025, 9675, 9583	*T. aestivum*	*Thalictrum speciosissimum* L.
Tranzschelia pruni-spinosae (Pers.) Dietel	8904	*Prunus amygdalus* Batsch.	*Anemone coronaria* L.
Uromyces hippomarathricola S. da Cam	8905	*Bilacunaria boissieri* Pimenov et Tichomirov	*Bilacunaria boissieri*
U. hordeastri Guyot	6422, 6437	*Hordeum bulbosum* L.	*Scilla autumnalis* L.
U. hordeinus (Arth.) Barth	77064[b], 77065[b]	*H. pusillum* Nutt.	*Nothoscordium* bivalve (L.) Britt.
U. reichertii Anikst. & Wahl	6385	*H. bulbosum*	*Scilla hyacinthoides* L.
U. vignae Barclay	CPR-1[e]	*Vigna unguiculata* L.	*Vigna unguiculata*

[a] Collections were from Israel, except as noted. Collections are as defined by Anikster et al (1997) to include aecial, pycnial, telial and uredinial progeny from selfing original teliospore collections.

[b] From USA.

[c] From Czech Republic (supplied by Z. Urban).

[d] *P. triticina* designates leaf rust of wheat (following Savile 1984) which is distinct from *P. recondita,* which includes leaf rusts of rye and wild wheats (Anikster et al 1997).

[e] From USA (supplied by K. Mendgen).

tion in rust fungi, we tested a number of staining procedures to help visualize fusion processes. Certain stains revealed that a cap was produced on one end of pycniospores when they were treated with nectar of opposite mating type. Here, we describe cap formation, demonstrate the correlation between cap formation and nectar mating type, and give preliminary evidence that the cap-inducing substances in nectar are proteins.

MATERIALS AND METHODS

Nectar and pycniospores were obtained from pycnial hosts infected by 14 rust species (TABLE I). For 10 of the rust species, pycnia were obtained with more than one collection (TABLE I) to increase the likelihood that a collection giving abundant nectar would be found. In some cases, nectar from two or three collections was combined to obtain enough nectar for experimental purposes. Within *Puccinia recondita,* collections were of four types differing in range of both telial and pycnial hosts (TABLE I) (Anikster et al 1997). The most abundant nectar was obtained with *P. recondita* collections from *Anchusa italica* and *Echium glomeratum.*

Pycnia were obtained in the greenhouse by inoculating pycnial host species with basidiospores from germinating teliospores following procedures of Anikster (1986) and Anikster et al (1997). If pycnial clusters originating from a single basidiospore were needed, plants were inoculated with low numbers of basidiospores. If nectar from multiple pycnial clusters was to be combined, plants were inoculated with higher numbers of basidiospores. To maximize volume of nectar, plants were placed in a cylindrical, covered, humidity chamber at the time pycnia began to develop, 8–15 d after inoculation. For experiments on cap induction within pycnial clusters, plants with pycnia were transferred from the greenhouse to a plant growth chamber (Karl Weiss) at 16 ± 2 C and a daily 12-h photoperiod.

Pycnia from individual basidiospore infections were

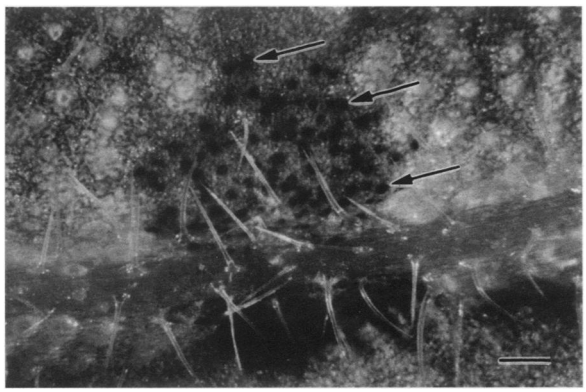

FIG. 1. Pycnial cluster of *Puccinia recondita* on leaf of *Anchusa italica*. The cluster is inferred to arise from a single basidiospore infection (see text). Arrows show examples of individual pycnia. Bar = 1 mm. Photograph taken with 35mm film.

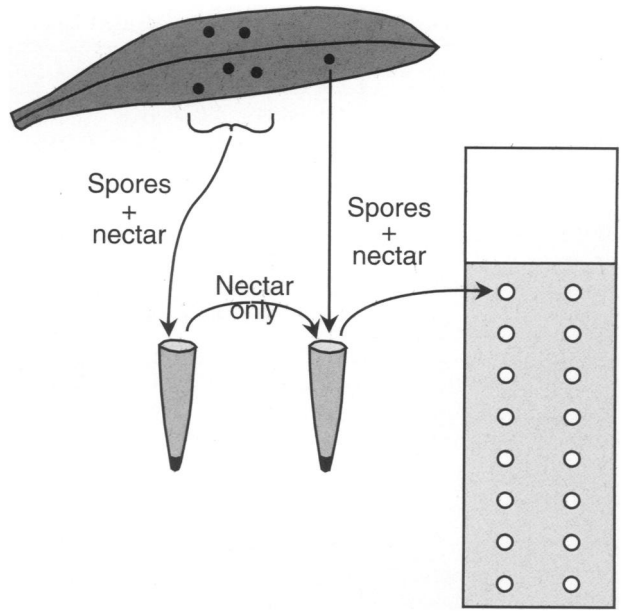

FIG. 2. Cap induction by pycniospore-free nectar. Nectar obtained by centrifugation from five (or more) pycnial clusters was added to pycniospores and nectar from a single pycnial cluster. After incubation, pycniospores and nectar were transferred to a test slide for staining and observation of caps.

formed in clusters (FIG. 1). A small sample of nectar containing pycniospores was taken from each cluster to be used experimentally. The pycniospores were stained to detect caps (as described later). Although clusters with caps were rarely found, only pycnial clusters free of caps were selected for use.

For experimental mixing of nectar and pycniospores from two or more pycnial clusters, the nectar and spores were transferred with water-wetted toothpicks, No. 0 or 1 artist's brushes, or 1 μL micropipettes (Vitrex Modulohm, Harlev, Denmark). Mixtures were incubated in pycnial clusters (on leaves), within circular scribed areas on glass slides (FIG. 2), or in Eppendorf tubes. Incubation time was 2 h except as noted. For experiments with pycniospore-free nectar, 5–20 μL of nectar from five or more pycnial clusters were transferred with micropipettes to a 0.2 mL Eppendorf tube. The tube was centrifuged for 6 min at 15 800 *g* and supernatant nectar (free of pycniospores) removed for experimental use. In the experiments of FIG. 2, the pycniospore-free nectar was transferred to a second Eppendorf tube for mixing with nectar and pycniospores from a single pycnial cluster or with washed pycniospores. The mixture was incubated for 10 min to 2 h; then samples were transferred to a test slide for observation of caps. If not used immediately, bulk nectar from multiple pycnial clusters (with or without pycniospores) was frozen and stored at −20 C. Thus stored, nectar retained cap-inducing activity at least one yr.

Crossing trials for production of aecia were conducted by the procedures of Anikster et al (1997). Nectar with pycniospores was transferred directly to receptor pycnia with a No. 0 or 1 artist's brush or with 1 μL micropipettes. After transfer, plants were incubated in the plant growth chamber described earlier. Plants were observed for aecia 10–14 d after nectar transfer.

For routine observation of caps, pycniospores were stained with India ink (Higgins Black Magic, Eberhard Faber, Lewisburg, Tennessee). Nectar with pycniospores on test slides was air-dried for about 5 min. Ink and washing treatments were applied to slides in droplets from Pasteur pipettes; excess water and dye were allowed to flow gently off the edge of the slide. Each nectar site on test slides (16 sites/slide, FIG. 2) was flooded with 10 μL of 1% India ink. Ink droplets from adjacent sites sometimes ran together but pycniospores remained attached to the slide at each site. After 15 min, the dye was washed from the slide. Specimens were covered with 50% glycerol and sealed under a cover glass with fingernail polish. Stained pycniospores were examined with a Zeiss Axioscope microscope using differential interference contrast (DIC) optics at magnifications of ×400–×1000. Photographs of spores were taken with T-Max 35-mm film (Kodak, Buffalo, New York) or a LIS-700 B&W video CCD camera (Applitec, Holon, Israel), coupled to an Apple Macintosh 8100/100 computer equipped with a Quick Capture DT 3155 frame grabber (Data Translation, Marlboro, Massachusetts).

To determine percentage of pycniospores with caps, the number of spores with and without caps was counted in each of three microscope fields (each field was 0.2 mm^2) within each of the circular scribed sites on test slides. Fields were selected in which pycniospores were not too crowded to see caps, but had 100 or more pycniospores so that a total of at least 300 spores was counted. Occasionally, more than three fields were required to obtain a total of 300 or more spores.

The percentage of pycniospores with caps was zero at the start of experiments since pycnial clusters showing caps were not used. At the end of experiments, however, the background percentage was sometimes greater than zero, although usually less than 0.5% and rarely as much as 2%. As will be described later, background capping percentages

TABLE II. Substances other than FITC-conjugated lectins tested for staining or labeling pycniospore caps

Stain[a]	Protein stained	Concentration	Solvent	Reference
Alcian blue	(−) charged	1.00%	3% acetic acid in water	Kuo and Hoch (1995)
Colloidal gold	(+) charged	0.01%[b]	Water	Jones et al (1995)
Coomassie blue	All	0.25%	50% methanol, 10% acetic acid in water	Kuo and Hoch (1995)
Cotton blue	(−) charged	0.05%	Lactophenol	Dhingra and Sinclair (1995); Kuo and Hoch (1995)
India ink	(+) charged[c]	1.00%	Water	Kuo and Hoch (1995)

[a] Stains and labeling agents were from Sigma, St. Louis, Missouri, except Coomassie blue, from Bio-rad, Hercules, California; and India ink (Higgins Black Magic), from Eberhard Faber, Lewisburg, Tennessee.

[b] Measured as $HAuCl_4$, particle diam 17–23 nm.

[c] Indirect evidence only, see Discussion.

were higher than usual in assays on polyacrylamide gels. In all cases, a treatment was judged to induce caps only if the capping percentage exceeded 10%.

For labeling of caps with wheat germ agglutinin conjugated with fluorescein isothiocyanate (WGA-FITC), pycniospores were placed on slides and dried as before, then fixed with 70% ethanol for 24 h at 4 C. Treatments were added with Pasteur pipettes as before. Spores were washed with water, then labeled with 15 μg/mL WGA-FITC (*Triticum vulgaris* lectin, #L4895, Sigma, St. Louis, Missouri) in phosphate saline buffer (PBS) for 30 min at room temperature in the dark. The WGA-FITC solution was removed and spores then stained with propidium iodide (PI) (Sigma) for nuclear DNA as described by Eilam et al (1994). The spores were then washed once with water and mounted in glycerine as described earlier. Spores were examined with a Zeiss LSM410 confocal laser scanning inverted microscope, using a ×100 objective lens, giving digital screen magnifications of ×1000–×10 000. The microscope has a 25 mW krypton-argon laser with emission lines at 488 nm (used for FITC) and 568 nm (used for PI). Images were printed with an Epson Stylus Color 600 printer at 1400 dpi. Procedures used for staining or labeling pycniospores with substances in addition to India ink, WGA-FITC or PI are given in TABLE II.

Tests for effects of enzymes, sodium dodecyl sulfate (SDS) or HCl on pycniospore caps were carried out in 0.2 mL Eppendorf tubes. A volume of 2–10 μL of nectar from 5–10 pycnial clusters was centrifuged to remove pycniospores. Spores and nectar from a single pycnial cluster were added to the nectar and incubated 2 h for cap formation. Spores were centrifuged and transferred to Eppendorf tubes with 20 μL solution of enzyme, SDS or HCl. Two h later, spores were centrifuged, transferred to a slide and stained with India ink. Treatments included cellulase (Fluka, Buchs, Switzerland, #22179), chitinase (Sigma, C6137), β-glucanase (Fluka #49101), laminarinase (Sigma L5144), Pronase E (Sigma P6911), proteinase K (Sigma P6556), trypsin, type I (Sigma T8003), SDS, 1% (Bio-Rad, Hercules, California), and 0.01 N HCl. Enzyme concentration was 1 mg/mL except for proteinase K at 0.2 mg/mL. Solutions were in 10 mM phosphate buffer, pH 6.5, except for Pronase E in HEPES buffer, pH 7.5, trypsin in MES buffer, pH 5.5 and proteinase K in water.

Protein content of nectar was determined spectrophotometrically at 595 nm using Bradford's reagent (Kruger 1994) calibrated with bovine serum albumin. Nectar (20 μL) was mixed with 500 μL of reagent.

Nectar was analyzed by polyacrylamide gel electrophoresis (PAGE) of both native and SDS-treated protein, following the general procedures of Walker (1994). The gels were prepared and run with minigel equipment (Mighty Small II, SE250, Hoefer, San Francisco, California). Two native gels were run simultaneously for each preparation of nectar: one for staining, the second for bioassay of cap induction. Native gels were usually run with 4% stacking and 10% separating polyacrylamide gels. SDS gels were run with 4% stacking and 10 or 15% separating gels. Pycniospore-free nectar (7–15 μL) was mixed with running buffer (containing bromphenol blue as marker) to a volume of 20 μL and loaded in wells, or 20 μL of running buffer was loaded without nectar. The nectar proteins were electrophoretically separated at 125 V until running buffer reached the bottom of the gel. Protein in gels was usually stained with Coomassie blue (Walker 1994). Silver stain (Walker 1994) gave virtually the same results (not shown). BSA was used as molecular standard for native gels; a 14–66 kDa standard kit (Sigma) was used for SDS gels.

Unstained protein in gels was assayed for induction of pycniospore caps by removing the glass cover from the gel and brushing each lane of the gel with nectar containing pycniospores from a single pycnial cluster using a No. 0 or 1 artist's brush. The gel with pycniospores was incubated 24 h in a closed plastic box within the Weiss plant growth chamber. Pycniospores were then removed with artist brushes and transferred to a test slide for staining with India ink and observation of caps. Stained gels were not used for cap induction assays because they had no cap-inducing activity.

RESULTS

The rust fungus species used in this investigation formed pycnia in clusters (FIG. 1). Nectar flowed to-

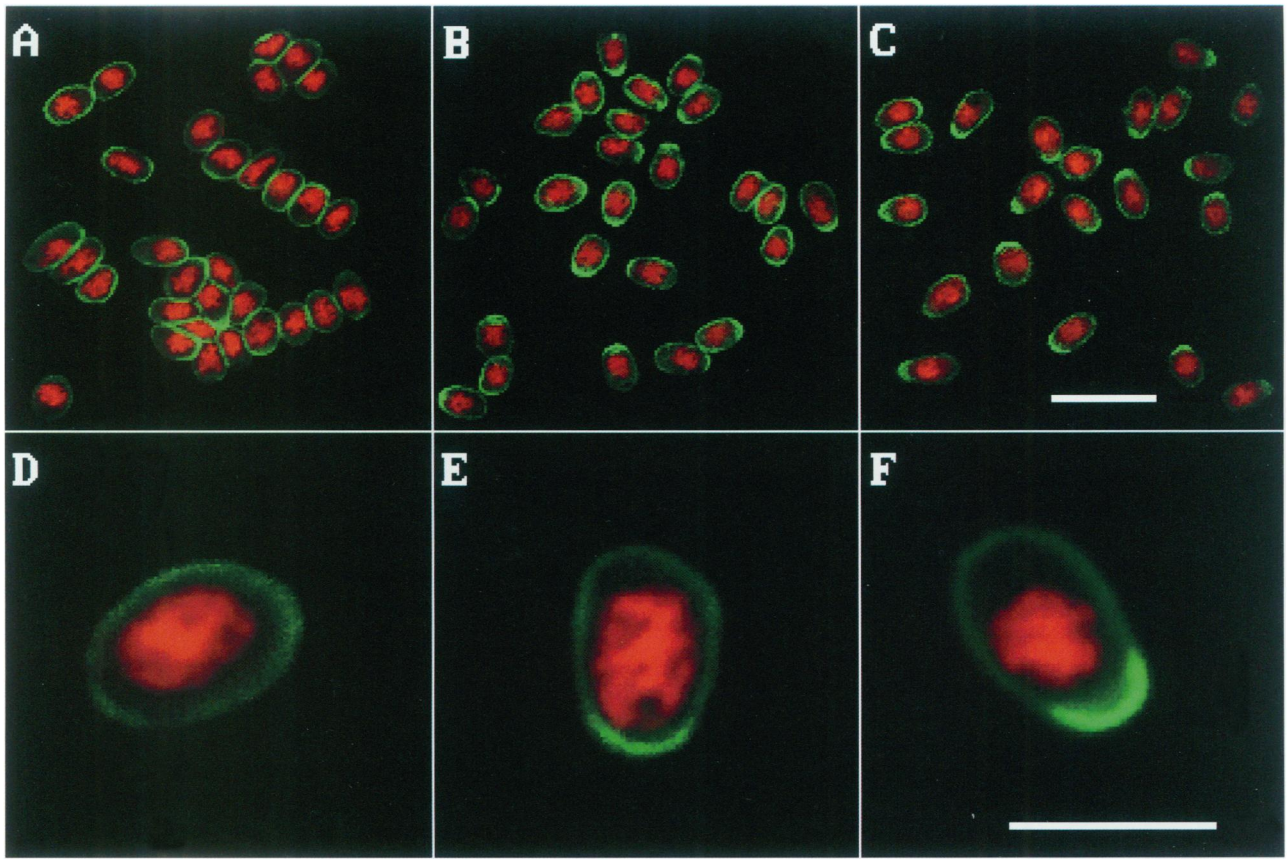

FIG. 3. Pycniospore caps as shown by fluorescence after spores of *Puccinia recondita* from *Anchusa italica* were labeled with WGA-FITC and viewed by confocal fluorescence microscopy. Spores were stained with propidium iodide to show nuclei. A, D. Pycniospores without caps in nectar from a single pycnial cluster, showing uniform fluorescence on outer spore walls. B, E. Pycniospores showing caps, 10 min after nectar from several pycnial clusters was mixed. C, F. Pycniospores showing caps 90 min after nectar was mixed. A, B, C, bar = 10 μm; D, E, F, bar = 5 μm.

gether, forming a single confluent drop, usually covering the entire cluster. In leaves with low numbers of clusters (1–5 per leaf or leaflet), aecia were produced on only rare occasions by individual clusters not receiving pycniospores from other clusters (about three aecia were produced in more than 1000 clusters used in this investigation). In leaves with higher numbers of pycnial clusters (50–100 per leaf or leaflet), 0–5 aecia per leaf or leaflet were produced. In contrast, 30–50% of pycnial clusters formed aecia if pycniospores were transferred from one pycnial cluster to another.

Caps were discovered after pycniospores and nectar from several pycnial clusters of *P. recondita* from *A. italica* were intermixed by transfer of nectar (with pycniospores) among the clusters, incubated for 90 min, transferred to a slide, fixed, stained with WGA-FITC, and examined by confocal fluorescence microscopy (FIGS. 3C, F). The caps were not present when pycniospores and nectar were taken from a single pycnial cluster (unmixed with nectar from other pycnia). Instead, spores from a single pycnial cluster exhibited a uniform, thin layer of FITC fluorescence on the spore surface (FIGS. 3A, D). Ten min after nectar from several pycnial clusters was mixed, many pycniospores had increased fluorescence at one end (FIGS. 3B, E). By 90 min, pycniospores had well-developed caps (FIGS. 3C, F), whereas the remainder of the spore surface remained fluorescent. Pycniospore caps did not fluoresce after treatment with other FITC-conjugated lectins including concanavalin A (Con A) and agglutinins from *Phaseolus vulgaris* (PHA), *Lens culinaris* (LCA), *Lotus tetragonolobus* (LTA) or *Arachis hypogaea* (PNA) (all from Sigma) (data not shown). Caps could not be seen by DIC microscopy if pycniospores were unstained.

We tested several stains or labels other than WGA-FITC (TABLE II) and found that India ink or colloidal gold revealed caps. As viewed by DIC microscopy, caps were evident at 10 min (FIGS. 4B, E) and were highly visible and darkly stained or labeled at 90 min after nectar from several pycnial clusters was mixed (FIGS. 4C, F). Unlike WGA-FITC, neither India ink nor colloidal gold consistently stained or labeled

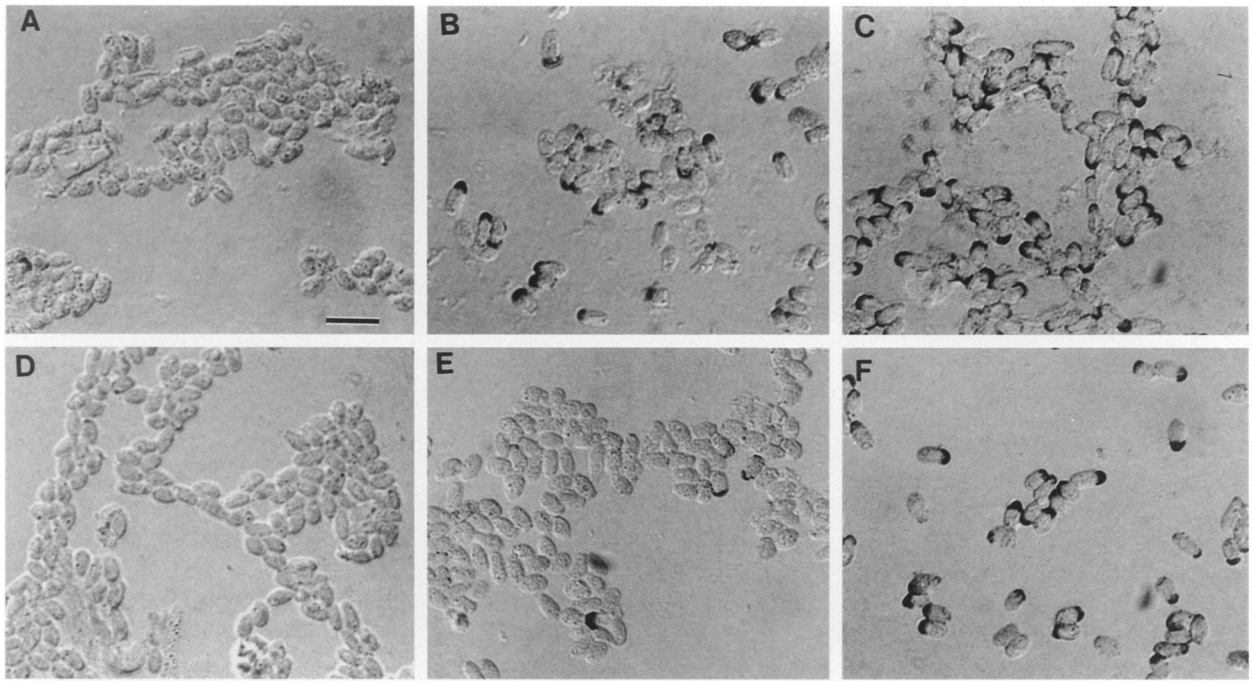

FIG. 4. Caps on pycniospores of *Puccinia recondita* from *Anchusa italica* as shown by staining with India ink (A, B, C) or labeling with colloidal gold (D, E, F) and viewed by DIC microscopy. A, D. Pycniospores without caps in nectar from a single pycnial cluster. B, E. Pycniospores showing caps, 10 min after nectar from several pycnial clusters was mixed; C, F. 90 min after nectar was mixed. Photographs taken with 35mm film. Bar = 10 μm.

spore wall regions other than the polar cap (FIG. 4), although these walls occasionally stained or labeled lightly (data not shown). As with WGA-FITC, pycniospores from a single pycnial cluster showed no caps when treated with ink or gold (FIGS. 4A, D). The other stains listed in TABLE II were ineffective.

Using the India ink staining method, we tested 14 rust species for cap formation after nectar and pycniospores from 5 or more pycnial clusters were mixed (TABLE I). Seven species of *Puccinia* and four of *Uromyces* formed caps, whereas *P. helianthi, U. vignae,* and *T. pruni-spinosae* did not. With *P. recondita,* collections from four different pycnial hosts (TABLE I) all formed caps. Furthermore, pairings among pycnial clusters from the four *P. recondita* hosts all gave caps. With *P. graminis,* caps were produced in all tests, both within and between each of two formae speciales.

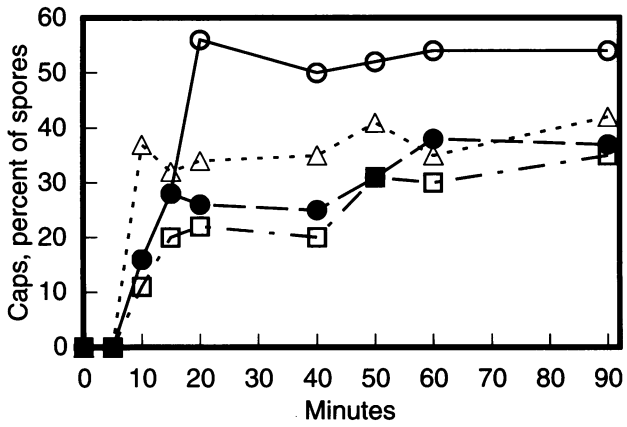

FIG. 5. Time of cap formation after nectar from 5–10 pycnial clusters was mixed. For each sampling time, a drop of nectar with pycniospores was stained with India ink for observation of caps. ○, *Puccinia recondita* from *A. aggregata*; □, *P. recondita* from *Anchusa italica*; ●, *P. recondita* from *Echium glomeratum*; △, *P. triticina* from *Thalictrum speciosissimum*.

Cap formation as viewed with India ink was followed at frequent intervals for 90 min after nectar from several pycnial clusters was mixed (FIG. 5). This was done with *P. triticina* from *Thalictrum speciosissimum* and *P. recondita* from three different pycnial hosts. In all cases, caps were evident in 12–37% of pycniospores within 10 min. By 20 min, 23–57% pycniospores had caps; by 90 min the percentage was 35% or more. In other experiments with both *P. recondita* and other species, higher percentages were sometimes obtained, but usually not exceeding 70%.

Seven enzyme preparations were tested for ability to remove caps (FIG. 6). Proteinase K (FIG. 6F) removed the caps completely, whereas cellulase (FIG. 6A), chitinase (FIG. 6B), β-glucanase (FIG. 6C), laminarinase (FIG. 6D), Pronase E (FIG. 6E), and trypsin (FIG. 6G) were inactive. Sodium dodecyl sulfate

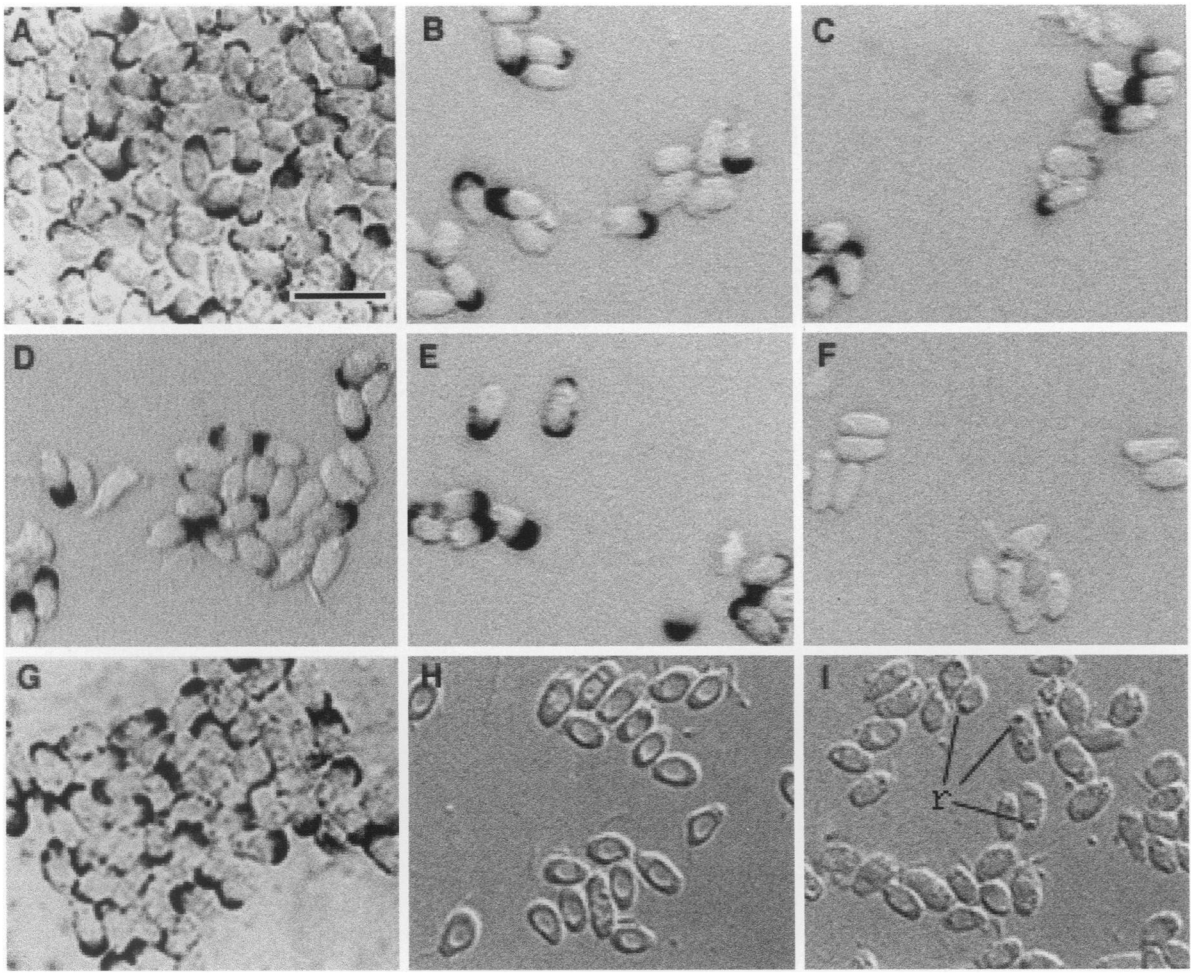

FIG. 6. Effects on pycniospore caps of treatment with enzymes, sodium dodecyl sulfate (SDS) or HCl. A. Cellulase. B. Chitinase. C. β-Glucanase. D. Laminarinase. E. Pronase E. F. Proteinase K. G. trypsin. H. SDS. I. 0.01 N HCl. Caps stained with India ink; r, darkly stained remnant of cap. Digitized images from a video camera. Bar = 10 μm.

(SDS) removed caps completely (FIG. 6H) whereas 0.01 N HCl partially removed them (FIG. 6I).

Mating type and cap formation.—To learn if cap formation relates to mating type in pairings between pycnial clusters, we did five experiments with *P. recondita* in which nectar (with pycniospores) from a single cluster was transferred to each of 10 individual pycnial clusters (FIG. 7A). Results for a typical experiment with *P. recondita* from *Anchusa italica* are shown in TABLE III. Four of the 10 receptor pycnial clusters showed cap formation and only these same pycnial clusters produced aecia. The six remaining pycnial clusters produced neither caps nor aecia. No aecia or caps were produced in five unfertilized control pycnial clusters. Similar results were obtained in two experiments with *P. recondita* from *Echium glomeratum,* and one experiment each with *P. recondita* from *A. aggregata* and *P. triticina* from *Thalictrum speciosissimum* (data not shown). In total (including the data of TABLE III), 25 receptor pycnial clusters formed both caps and aecia, whereas neither caps nor aecia were produced in 30 remaining receptor pycnial clusters. Furthermore, no caps or aecia were produced in a total of 25 unfertilized control pycnial clusters. These results are consistent with the hypothesis that cap formation results from mixing nectar and/or pycniospores from pycnia of opposite mating type.

A positive correlation between pycniospore cap formation and subsequent development of aecia also was found in other experiments with *P. recondita* from *A. italica* in which reciprocal transfers of nectar (with pycniospores) were made within pairs of individual pycnial clusters (FIG. 7B, TABLE IV). Of a total of 34 pairs, 16 gave both caps and aecia; the remaining 18 pairs gave neither caps nor aecia, indicating both members of the pair were of the same mating type. Results for reciprocal transfers within a pair were always the same.

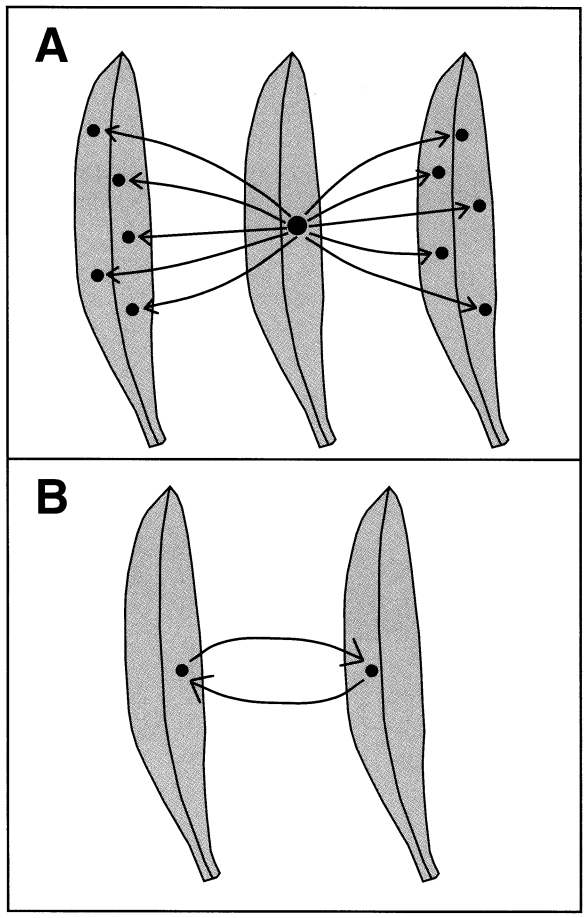

FIG. 7. Transfer of nectar with pycniospores to demonstrate correlation between cap and aecium formation. A. Transfer from a single pycnial cluster to ten individual clusters (results in TABLE III). B. Reciprocal transfers between two pycnial clusters (results in TABLE IV).

TABLE III. Example of correlation formation of pycniospore caps and acecia

Pycnial cluster[a]	Cap formation,[b] pycniospores %	Aecium formation[c]
Receiving nectar		
1	10	+
2	0	−
3	40	+
4	15	+
5	0	−
6	0	−
7	0	−
8	0	−
9	0	−
10	15	+
Not receiving nectar		
1	0	−
2	0	−
3	0	−
4	0	−
5	0	−

[a] Numbers designate individual pycnial clusters. Each cluster is produced from a single basidiospore infection of *P. recondita*. Ten pycnial clusters received nectar and pycniospores from a single pycnial cluster as shown in FIG. 7A; five (control) pycnial clusters did not receive nectar from *A. italica*.

[b] 4 h after pycnial cluster was fertilized.

[c] 10–14 days after fertilization: +, aecium present; −, no aecium present.

That aecia are not produced in the absence of nectar-induced capping was also indicated by placing nectar-free pycniospores of *P. recondita* (from *A. italica*) of one mating type on pycnial clusters of the other mating type from which nectar was first washed away with water. This was done with pycnial clusters at least 10 d old that had stopped producing nectar. Neither caps nor aecia resulted from the pycniospore transfers (data not shown).

Selfing experiments.—Two experiments were carried out to test the ability of capped pycniospores of a given mating type to fertilize pycnia of the same mating type (FIG. 8). In one experiment, spore-free nectar (tube 1, FIG. 8A) was obtained from five pycnial clusters, giving a high probability that nectar of both mating types was present, and used to induce caps on spores from a single pycnial cluster (cluster a, FIG. 8A). The capped spores were then returned to the cluster. In the second experiment, caps were induced

TABLE IV. Formation of pycniospore caps and aecia resulting from reciprocal transfer of nectar and pycniospores between individual pairs of pycnial clusters of *Puccinia recondita* from *Anchusa italica*

Experiment	No. of pairings[a]	Pairings with caps and aecia[b]
1	7	2
2	7	1
3	10	7
4	4	2
5	6	4
Total	34	16

[a] All pairings were tested with reciprocal transfers (see FIG. 7B), which always gave the same result. No pycnia were used to receive nectar that had any caps present before transfer.

[b] Aecia were formed in all pairings that gave pycniospore caps and vice versa.

in place in a single pycnial cluster (cluster b, FIG. 8B) by pycniospore-free nectar (tube 3, FIG. 8B) obtained from five pycnial clusters. Each experiment was repeated 3–6 times each, with *P. recondita* from *A. italica, P. recondita* from *A. aggregata* and *P. triticina* from *T. speciosissimum.* In no case were aecia produced. In contrast, aecia were always produced in concomitant control experiments in which nectar and pycniospores from pycnial clusters were placed on a single pycnial cluster. The experiments demonstrated that capping alone does not allow pycniospores to self-fertilize pycnial clusters.

Cap-inducing factor.—Results of several experiments indicated that cap-inducing activity was a property of nectar and not of pycniospores. Nectar with pycniospores removed (FIG. 2) had cap-inducing activity. Conversely, pycniospores of one mating type did not induce caps in pycniospores of the other type in the absence of nectar as shown by mixing nectar-free spores of opposite mating types (data not shown). Artificial nectar containing 10% glucose, 10% sucrose, and 10% fructose as found in nectar (Anikster unpubl) did not induce caps, indicating that inducing factors other than sugars were present in pycnial nectar.

Nectar was inactivated if boiled for 10 min or autoclaved for 15 min at 120 C, suggesting that active factors might be protein. Cap-inducing activity was measured in relation to total protein content over a series of 8 to 50-fold dilutions of nectar (FIG. 9). With nectar of *P. recondita* from *A. italica,* caps were induced on 30–47% of spores through dilutions down to 8 μg protein/mL. This nearly equaled the activity of undiluted nectar, which had 320 μg protein/mL (as calculated from diluted samples) and induced caps on 41% of spores. However, most activity was lost at a lower concentration (6.4 μg protein/mL). Likewise, nectar of *P. recondita* from *Echium glomeratum* induced caps on 30–41% of spores at 8.5 and 22 μg protein/mL, compared to 41% induced by undiluted nectar, which had 170 μg protein/mL. Again, activity was sharply reduced at 3.4–5.6 μg protein/mL. These results suggested that the concentration of cap-inducing factor was high enough in undiluted nectar to attempt bioassays for activity after PAGE separation of proteins.

In PAGE experiments with native gels, one gel was used for Coomassie blue localization of protein and a companion unstained gel was used for bioassay of cap induction on pycniospores (FIG. 10). For nectar from two cap-inducing species, *P. recondita* (from three pycnial hosts) and *P. triticina,* the gels showed a complex of protein larger than 100 kDa located near the origin (FIG. 11). In some cases, one or two

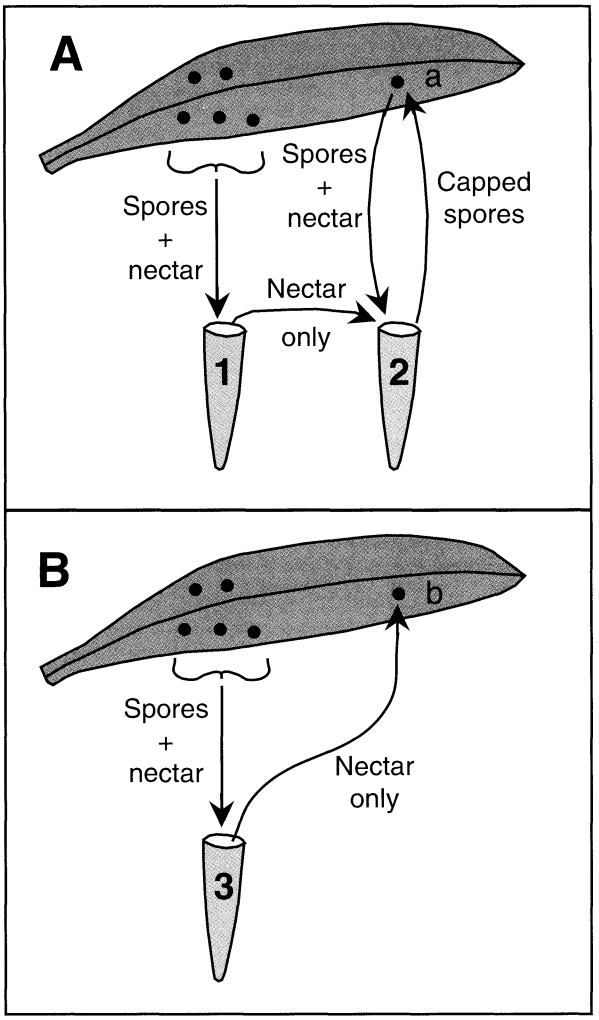

FIG. 8. Two experiments helped to determine if capped pycniospores of one mating type can induce aecia in a pycnial cluster of the same mating type. A. Pycniospores from a pycnial cluster (a) were treated with pycniospore-free nectar obtained from five other pycnial clusters, then returned to cluster (a) after caps were produced. B. Pycniospore-free nectar from five pycnial clusters (in tube 3) was transferred to a single pycnial cluster (b). Spores were separated from nectar by centrifugation.

faint bands (not visible in FIG. 11) were present near a 66 kDA marker. Nectar of *P. helianthi,* which did not induce caps, lacked the proteins larger than 100 kDa, although a faint band was present at 66 kDa gel (not shown).

For bioassay of cap-inducing activity, pycniospores were brushed onto each lane of the unstained gels (FIG. 10) and incubated for 24 h. Pycniospores were then transferred from each of eight zones of each lane to glass slides, stained with India ink, and examined for presence of caps. Caps were produced by 7–33% of pycniospores incubated on the two top zones of lanes containing nectar, corresponding in

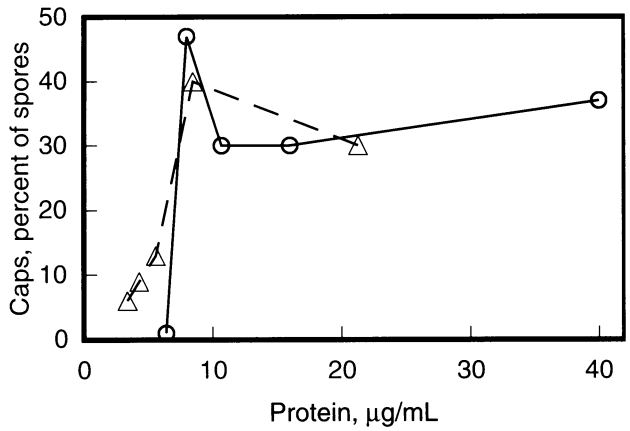

FIG. 9. Percentage of pycniospores forming caps in relation to total protein content of diluted pycnial nectar. Values are for nectar diluted ×8, ×20, ×30, ×40 and ×50, respectively, in water. ○——○ pycnial nectar of *Puccinia recondita* from *Anchusa italica*; △- - -△, pycnial nectar of *P. recondita* from *Echium glomeratum*.

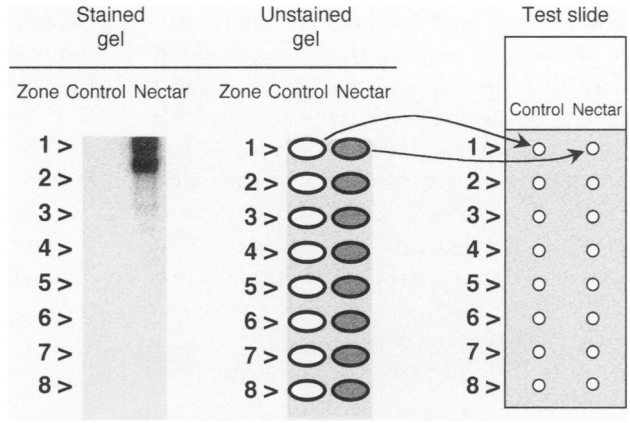

FIG. 10. Diagram of bioassay for pycniospore cap formation after separation of nectar components on PAGE gels. Position of protein shows in stained gel; cap formation by pycniospores placed on each of eight positions of each lane was assayed in unstained gel (shown are results for *Puccinia recondita* from *Anchusa italica*).

location to the high molecular weight protein complex (FIG. 11). Other zones had less activity except for the third zone from the top for nectar of *P. recondita* from *E. glomeratum* (FIG. 11, Lane 2). Pycniospores on lanes containing running buffer without nectar produced caps at rates of 0–5%, averaging 1.8% for all zones of the four lanes of FIG. 11. This was greater than background percentages obtained in other types of experiments.

In SDS PAGE gels, one to three bands were present at 60–70, 24–30, and/or 14–20 kDa in nectar from *P. recondita* (three types) or *P. triticina* (FIG. 12). No bands were present above 100 kDa either here or in other SDS gels run to resolve higher molecular weight proteins. No caps were produced when unstained SDS gels were assayed with pycniospores. Nectar from *P. helianthi* lacked bands at 66 kDa in SDS PAGE gels and gave relatively faint bands in other regions (FIG. 12). Results with nectar from ten individual pycnial clusters of *P. recondita* from *A. italica* run separately on SDS gels all gave identical bands (not shown), i.e., differences related to mating type were not detected.

DISCUSSION

Our results indicate that nectar of one mating type induces cap formation on one end of pycniospores of opposite mating type. This was true for 11 of 14 tested species of rust fungi. The 11 species exhibiting the cap phenomenon included 10 species with monocotyledonous hosts and one (*U. hippomarathricola*) with a dicotyledenous host. Pairings that gave caps also gave aecia (TABLES III, IV). Both occurred in

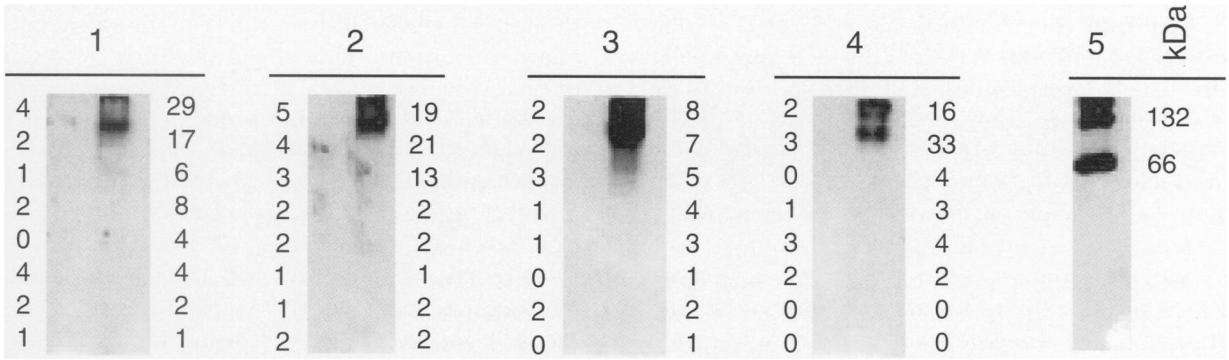

FIG. 11. Native PAGE analysis and bioassay for cap-inducing activity of pycnial nectar from four rust fungi. Left lane of each pair of lanes was run with buffer only; right lane was run with pycnial nectar in buffer. Numbers beside lanes indicate percentage of pycniospores forming caps in bioassay of corresponding unstained gels as shown in FIG. 10. Lane 1, *Puccinia recondita* from *Anchusa aggregata*; lane 2, *P. recondita* from *Echium glomeratum*; lane 3, *P. recondita* from *A. italica*; lane 4, *P. triticina* from *Thalictrum speciosissimum*. Lane 5, standards as indicated by kDa values.

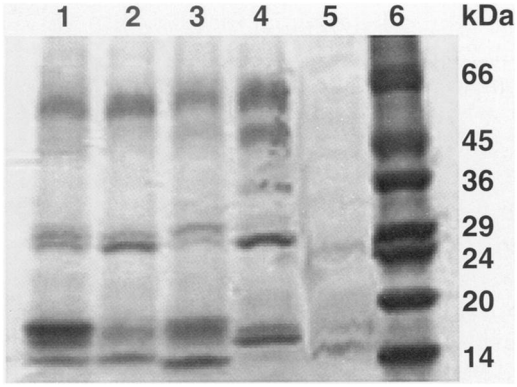

FIG. 12. SDS-PAGE analysis of pycnial nectar of five rust fungi. Lanes 1–4 are for four rust fungi in the order indicated for lanes 1–4 of the native PAGE gels of Fig. 11. Lane 5, nectar from *Puccinia helianthi* showing absence of major bands larger than 24 kD. Lane 6, standards as indicated by kDa values.

somewhat less than 50% of pairings, the maximum expected for simple bipolar mating type systems. Caps were rarely seen in pycniospores from a single pycnial cluster. That caps were induced by nectar and not by pycniospores was demonstrated by cap induction by spore-free nectar (FIG. 2) and absence of cap induction by nectar-free spores.

Pycnia were formed in clusters (FIG. 1) in all species investigated here. At low numbers of clusters per leaf or leaflet, aecia were rarely produced, indicating that each cluster contained pycniospores of only one mating type and probably was the result of an infection by a single basidiospore. Similar clusters were described by Buller (1950) for *P. graminis* and Craigie (1931) for *Puccinia helianthi,* who showed how nectar from individual pycnia flows together within the cluster, much as occurred in our experiments.

Pycniospore caps began to form within 10 min after inducing nectar was added to pycniospores and most were fully formed within 20 min (FIG. 5). The caps were labeled with WGA-FITC which also labeled a thin surface layer around the entire spore both before and after caps were formed (FIG. 3). This suggests that preformed cap materials may migrate to the cap along the spore surface. However, as viewed by light microscopy, the peripheral surface usually was not labeled with colloidal gold or stained with India ink, in contrast to the cap (FIG. 4), suggesting that caps may be the result of polar excretion from the pycniospores. We are now investigating patterns of colloidal gold distribution by electron microscopy to follow surface changes more precisely.

Staining by India ink or labeling by colloidal gold suggests that protein is a major component of pycniospore caps. Labeling with colloidal gold, which has affinity for positively charged protein, and lack of staining with alcian blue and cotton blue, which have affinity for negatively charged protein (TABLE II), are consistent with the cap protein being positively charged. In line with this, Kuo and Hoch (1995) reported that certain extracellular matrices of *Phyllosticta ampelicida* have an affinity for both colloidal gold and India ink, suggesting that India ink has affinity for positively charged protein. Labeling with WGA-FITC indicates caps contain polysaccharides or glycoproteins containing N-acetylglucosamine (Nagata and Burger 1974). In addition to staining properties, evidence for protein in caps was provided by their removal by proteinase K (FIG. 6F), SDS (FIG. 6H), HCl (FIG. 6I). However, neither Pronase E nor trypsin removed the caps (FIGS. 6E, G). The differential activity of proteases on caps needs further investigation. Proteinase K is specific for aliphatic and aromatic amino acid cleavage sites; trypsin is specific for arginine and lysine sites, whereas Pronase E is a mixture of eleven proteases with broad overall specificity (Keil 1992). Substances other than protein in caps may also influence protease activity.

In species exhibiting the capping phenomenon, cap formation correlated positively and consistently with aecium formation, and therefore may be a prerequisite for fertilization of pycnia by pycniospores. In these species, caps formed in all pairings of pycnia and pycniospores leading to aecium formation, i.e., with pycnia and pycniospores of opposite mating type. Conversely, neither caps nor aecia were produced in pairings of opposite mating types made with all nectar washed away. However, cap formation alone was not sufficient to allow pycniospores to fertilize pycnia as shown by the inability of capped pycniospores of one mating type to produce aecia when produced in, or transferred to pycnia of the same mating type (FIG. 8). Likewise, aecia were never produced in pairings between rust fungus species, although caps were induced in a mating-type specific manner by interspecific nectar transfers (results to be given elsewhere). Clearly, fertilization processes require species compatibility and factors related to mating type other than cap-inducing factor.

The interpretation that cap formation correlates positively with mating type rests on the assumption that the rust fungi investigated have bipolar mating systems. This is consistent with aecium production in somewhat less than half of transfers of pycniospores between any two pycnial clusters here (TABLES III, IV). We obtained similar results in a comprehensive investigation of hybridization in and among species of *Puccinia* and *Uromyces* (Anikster et al 1997). Furthermore, compatibility of members of a set of pycnial clusters always fall into two distinct classes. A giv-

en pycnial cluster is compatible with members of only one of the two classes, without overlap (TABLE III). Others have likewise found that somewhat less than 50% of pairings between individual pycnial clusters yield aecia, concluding that the rust fungi investigated have only (+) and (−) mating types (Craigie 1931, Allen 1934). However, a rigorous genetic study of mating in rust fungi has not been possible because the fungi are extremely difficult to culture axenically (MacLean 1982, Williams 1984). Furthermore, tetrad analysis is precluded because infection rates by individual basidiospores placed on host leaves are very low (Craigie 1931, Anikster unpubl). Nevertheless, the available evidence is consistent with the presence of bipolar mating systems in rust fungi.

Investigation of the cap-inducing factor was limited by the small amount of nectar produced by pycnial clusters (each cluster usually produced 0.5–5 μL of nectar); however, our results indicate that the active factors are proteins. Cap-inducing factor was inactivated by heat (boiling or autoclaving). Furthermore, the factor migrated on native PAGE minigels with protein as shown by Coomassie blue (FIG. 11) or silver (not shown) staining. The principal cap-inducing activity of nectar was located in regions of native gels containing proteins larger than 100 kDa. This region contained nearly all of the nectar protein except for a faint band at 66kDa. In denaturing SDS gels, on the other hand, no proteins were found greater than 100 kDa, but several bands were present at 14–70 kDa (FIG. 12). This indicates that the native protein complex above 100 kDa was dispersed by SDS treatment. However, we could not determine which, if any, of the bands on SDS gels had cap-inducing activity, as no caps were induced in several trials with pycniospores placed on the gels.

PAGE analysis of nectar from *P. helianthi,* a species which did not have nectar-induced capping of pycniospores, showed no protein greater than 100 kDa, a faint band at 66 kDa in native gels (not shown), and only faint bands of lower molecular weight in SDS gels (FIG. 12). Thus, the absence of proteins larger than 100 kDa correlated with lack of cap-inducing activity. The gels for *P. helianthi* were run with a nectar volume within the range of 7–14 μL used for other species. Protein concentration in *P. helianthi* nectar was not determined but the results of PAGE analysis indicate that protein concentration was lower than for cap-inducing species. In any case, the absence of cap induction in *P. helianthi* as well as in *T. pruni-spinosae* and *U. vignae* in our study indicates cap formation is not universally associated with fertilization in rust fungi.

The cap-inducing factors in rust fungi may relate to fungal pheromones, mating-type specific polypeptides shown to be essential for mating in several species of Basidiomycetes and said to be ubiquitous in the fungal kingdom (Bölker and Kahmann 1993, Kothe 1997, Vaillancourt and Raper 1996). These polypeptides contain 9–15 amino acid residues, so that pheromone size is about 1/50th to 1/100th that of cap-inducing protein complex (larger than 100 kDa as indicated by PAGE analysis). The fungal pheromones bind to pheromone receptors of opposite mating type (or nonself types in fungi with multiple mating types). The binding of pheromone to receptor initiates production of fusion tubes, fusion at tube apices, and transfer of the haploid nucleus of one tube to the other. The fusion tubes can grow directionally in a pheromone gradient toward fusion tubes of opposite mating type (Snetselaar et al 1996). In rusts, fusion tubes are produced only after a pycniospore adheres to a receptive hypha (Buller 1950). How the entire pycniospore is moved within nectar to a receptive hypha has not been determined. The pycniospore then adheres to the hypha and fusion processes are initiated. The receptive hypha produces a short fusion tube which extends either from the tip or lateral wall of the hypha and recurves backward so that the tip of the fusion tube encounters one end of the adherent pycniospore (Buller 1950). How the nectar-induced pycniospore cap relates to this sequence of events remains to be investigated. We speculate that the process of cap formation prepares the spore for adhesion to receptive hyphae, a possibility we are now investigating. In addition to the possible role of cap-inducing factors, it should be noted that pheromones have been implicated in adhesion by Miyakawa et al (1987) who showed that pheromone-induced alteration in surface proteins caused cells with fusion tubes to agglutinate in a mating-type specific manner.

Typically, 30–60% of pycniospores produced caps in response to cap-inducing nectar. The percentage generally did not exceed 70%. However, cap percentages varied considerably, especially if amounts of nectar were limited, such as when nectar from a single pycnial cluster was subdivided for transfer to ten receptor clusters (FIG. 7B), or in PAGE analyses (FIG. 11). Nevertheless, the concentration of cap-inducing factor was probably not the only limitation on the percentage of caps produced. Nectar could be diluted 20–40 fold without reduction in effectiveness (FIG. 9), indicating that undiluted nectar had an excess of inducing factor. Only at total protein concentrations below 8 μg/mL was the percentage of caps reduced. A sharp decline in activity below 8 μg/mL suggests that a ligand-receptor system may be operating. A meaningful investigation of putative binding sites will require purified factor. With undiluted nectar, phys-

iological factors related to age, viability and possibly genotype may limit the ability of a part of the pycniospore population to produce caps.

The establishment of a stable dikaryon after nuclear transfer has been shown in several Basidiomycetous fungi to be under control of genes in mating loci that code for homeodomain proteins (Kothe 1997). The stable dikaryon requires formation of heterodomain protein from proteins of unlike mating types. The possible participation of such proteins in establishment of rust fungus dikaryons remains to be investigated.

As nectar and pycniospores are transferred between pycnial clusters of opposite mating type by insects, rainwater or other factors, the cap-inducing factors in the nectar of receptor clusters rapidly induce caps on the incoming pycniospores. We need to determine if nectar-induced capping is required as an initial step leading to successful mating in the species in which caps are produced.

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Visualization of the Extracellular Matrix Surrounding Pycnidiospores, Germlings, and Appressoria of Phyllosticta ampelicida

KerChung Kuo; H. C. Hoch

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