Appressorium Formation over Stomates by the Bean Rust Fungus: Response to a Surface Contact Stimulus

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ABSTRACT

Germ tubes of bean rust (*Uromyces phaseoli*) formed appressoria in response to two types of topographical features: the surfaces of artificial membranes, and the stomates of bean leaves. The topography of collodion membranes together with their capacity to induce appressoria was altered by varying the procedures of membrane preparation. Appressoria were also induced specifically over wrinkles, craters, and scratches on membranes. Bean rust formed comparable numbers of appressoria on leaves and on plastic replicas of the leaves. On five bean cultivars, at least 92% of the appressoria were formed over stomates on both leaves and corresponding leaf replicas. On nonhosts, appressorial counts on replicas decreased similarly to those on leaves; total appressorium formation was less than 5% on leaves and replicas of two gramineous plants. The use of serial replicas demonstrated that no chemical stimulants were carried from leaves to replicas. Germ tubes were directed toward the stomates on bean leaf replicas since growth was predominantly at right angles to the ridges of epidermal cells and these ridges generally encircled the stomates. Evidence from scanning electron microscopy showed that the specific surface feature which induced appressorium formation on leaves was apparently the stomatal lips.

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The initiation of penetration by rust fungi involves a remarkable sensory process: after uredospores germinate on a leaf surface, the germ tubes "find" stomates over which they form appressoria. Once anchored, the appressoria produce infection hyphae which penetrate the stomatal openings. Since germinating uredospores of rusts cannot penetrate epidermal cells directly, successful invasion depends upon the capacity to form appressoria specifically over stomates. The formation of appressoria over stomates is not at random. For example, Allen (2) observed that germ tubes of wheat stem rust grew toward and formed appressoria specifically over stomates on both susceptible and resistant cultivars of wheat.

Why do germ tubes form appressoria over stomates? The question has been debated since the turn of the century (for references, see 13, 18); and theories have covered a gamut of stimuli, including contact, nutrition, specific chemicals, moisture, light, and temperature. Effective stimuli apparently can vary with different rusts. Consistent appressorium formation by wheat stem rust away from the host can be induced by temperature shock, and volatile chemicals (1, 10, 14). Experiments with artificial membranes indicate that at least eight species of rust fungi can form appressoria in response to a surface contact stimulus (6, 8, 9, 14). At present, the most important unsolved problem is the induction of appressoria over stomates of leaves in nature.

The purpose of the present paper is to offer evidence, from experiments with artificial membranes and leaf replicas, that the germ tubes of the bean rust fungus form appressoria in response to specific physical topography, and that stomates provide this topography on the leaf surface.

MATERIALS AND METHODS.—*Inoculation and germination.*—The isolate of the bean rust fungus, *Uromyces phaseoli* var. *typica* (Pers.) Wint., was obtained from Richard C. Staples, Boyce Thompson Institute, Yonkers, N.Y. We have, for convenience, designated it as race 0 since it does not conform to any of Zaumeyer's 35 races of bean rust as determined by reactions on standard bean differentials (W. J. Zaumeyer, *personal communication*). Uredospores were produced on infected leaves of bean (*Phaseolus vulgaris* L. 'Pinto') plants in a growth chamber (19). The spores were collected 12 days after inoculation, stored at −25 C, and used within 1 month. The procedure for germination was modified slightly from that reported previously (21). Fifty milligrams of spores were floated on 100 ml water at 4 C for 16 hours to remove self-inhibitor, recovered by filtration, dispersed in a settling tower to give a density of 8,000 spores/cm^2 on each surface used for germination, atomized with glass-distilled water to give a uniform layer of small droplets over the surface, and then incubated at 20 C in a saturated atmosphere. Germination ranged from 70 to 90% when measured at 4 hours. Neither floating, nor spore density, affected appressorium formation per se; however, floating was necessary to obtain good germination at densities above 5,000 spores/cm^2. Appressorium formation was not reduced at densities as high as 30,000 spores/cm^2 as long as the germ tubes had emerged and were growing actively.

Artificial membranes.—Two types of collodion membranes, prepared similarly to the method described earlier (21), were used as controls: (i) Membrane which did not induce appressorium formation (*standard membrane without hydrocarbon*). Collodion was diluted

with ethyl ether-ethanol (3:1, v/v), pipetted evenly over the bottom of a glass petri dish, and the solvent was completely evaporated before floating the membrane on distilled water. (ii) Membrane which induced appressorium formation (*standard membrane plus oil*). Preparation was the same as (i), except that light paraffin oil (Fisher Scientific Co.; 4 mg/ml diluted collodion solution, equivalent to 46 μg oil/cm^2 membrane) was added before pipetting the solution over the glass surface. The modified membranes tested in this study were prepared basically as the two control membranes except for variations in solvents, evaporation time, amount and type of hydrocarbon, formation on surfaces other than glass, and atomizing with ethyl acetate. All collodion membranes were prepared by diluting commercial collodion solution with the desired solvent (1:4, v/v) to give a final cellulose nitrate concentration of 8 mg/ml (equivalent to 92 μg/cm^2 membrane).

Percentage appressorium formation (germ tubes with appressoria ÷ total germ tubes × 100) was counted after 4.5 hours of germination. Two squares were cut from each membrane, stained with 0.02% trypan blue in lactophenol, and 100-200 germinated spores were counted in each square. Final data are presented as appressorium counts on test membranes divided by equivalent counts on controls (standard membranes, plus oil).

Leaves and leaf replicas.—Plants were grown in a growth chamber at 20 C with a photoperiod of 16 hours and an illumination of 10,800 lux. Primary leaves of legumes and third or fourth leaves of grasses were excised from 10- to 20-day-old plants, and immediately inoculated or used to prepare replicas which were inoculated. For each experiment, comparable leaves from the same group of plants were used for direct inoculation and for preparing replicas. The only difference was that the leaves for replicas were excised 1 day earlier so that germination on leaves and their corresponding replicas could be simultaneous.

For direct inoculation, leaves were washed in distilled water, gently dried, placed in petri dishes containing a film of water, and then inoculated and incubated as described above. After 6-, 12-, or 24-hours of germination, leguminous leaves were fixed and cleared in Carnoy's solution (ethanol-acetic acid-chloroform, 3:1:2, v/v) and then stained with trypan blue in lactophenol. Gramineous leaves were fixed and stained directly with trypan blue in lactophenol since Carnoy's solution removed some of the germ tubes from these leaves. Counts were made of percentage appressorium formation (as with the collodion membranes) and percentage appressoria over stomates (appressoria over stomates ÷ total appressoria × 100). Counts of 100 germinated spores each were replicated twice on the same leaf, and several times on different leaves.

The overall process for the preparation of leaf replicas included making a negative replica of part of the leaf surface with silicone rubber, and then using this replica to form a positive replica of polystyrene which was used as the surface for spore germination. Rubber-based negative replicas were made by modifying the skin replication procedure of Bernstein and Jones (4), using 'Silastic 382' (Medical-Grade Elastomer), '200 Fluid' (dimethylpolysiloxane thinner for the Silastic), and 'Catalyst M' (stannous octoate), all obtained from Dow-Corning Corp., Medical Products Division, Midland, Mich. Five grams of Silastic was mixed with 0.5 ml of 200 Fluid in a small dish. Immediately before use, 0.035 ml of Catalyst M was added and mixed for 40 seconds. The rubber preparation was spread over portions of freshly excised leaves with a spatula, and the leaves were placed in a container under reduced pressure from an aspirator [150 mm (6 inches) Hg] until the bubbles which formed in the rubber began to recede (approximately 1 minute). After deaeration, the leaves were placed on a flat surface with the rubber up, a glass microscope slide was pressed firmly over each dab of rubber, a 100-g weight was placed on each slide, and the preparations were allowed to set up for 30 minutes. The negative replicas, which remained attached to the glass slides, were removed from the leaf surfaces and rinsed twice in chloroform for 10 seconds each time to remove adhering cuticle waxes. They were then washed 2 hours in distilled water, and cured for 1 hour at 180 C.

Polystyrene squares for the positive replicas were prepared as follows: The flat surfaces were cut out of 9-cm diameter disposable petri dishes (Falcon Plastics, Division of Bioquest, Oxnard, Calif.). Double layers of these pieces of polystyrene were put between two glass plates in a cold oven, heated slowly to 180 C, and kept at this temperature for 3 hours until the disks had melted together and shrunk to a constant size. The slow heating was necessary to prevent entrapment of air bubbles in the plastic. After cooling, the preshrunk disks were cut into 2- by 2-cm squares, washed in methanol, and dried.

The final positive leaf replicas were made from the cured rubber negative replicas. Several rubber replicas were put replica-side up on a glass plate in an oven, a polystyrene square was placed on the surface of each replica, a glass plate was added to the top of the squares for weight, and all materials were heated for 30 minutes at 180 C. After cooling, the first set of newly formed polystyrene positive replicas was removed and discarded in order to eliminate any leaf-surface impurities which might have been carried over on the rubber replicas. Additional sets of positive replicas were made from the same negative replicas by reheating for 60 minutes at 180 C with new squares of plastic. Several sets of good polystyrene replicas have been made from a single rubber replica without apparent damage to that replica. The second and successive sets of polystyrene positive replicas were washed in ethanol and water and used for germination as described for excised leaves. At the end of the germination periods, appressoria were stained with trypan blue in lactophenol, the excess stain was removed by rinsing with water and methanol, and the replicas were air-dried before observation. Counts of percentage appressorium formation and percentage appressoria over stomates were made the same as with leaves. Since the polystyrene replicas were clear and rigid and since uredospore germ tubes adhered to them, they were easily handled during germination and observation. Control replicas, prepared by making replicas of smooth glass surfaces instead of leaves, were included in several experiments.

Scanning electron microscopy.—Leaf pieces were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 16 hours, and postfixed in 1.5% osmium tetroxide in

TABLE 1. Appressorium formation by germ tubes of *Uromyces phaseoli* on four hydrocarbons incorporated into, applied over, and in the absence of standard collodion membranes without oil

Hydrocarbon		Appressoria at 4.5 hours (% of control)[a]		
Compound or mixture	Concentration (mg/ml)[b]	Incorporated into membrane	Applied over membrane[c]	Applied without membrane[d]
Paraffin oil	4	100[e]	0	0
	8	102	0	0
Hexadecane	4	< 1	0	0
	8	67	0	0
Paraffin wax	4	2	89	0
	8	89	83	0
Octacosane	4	9	60	0
	8	19	29	0

[a]Appressorium formation of controls during course of experiments = 32-72%.
[b]Concentration of hydrocarbon was based on amount used in collodion solution to prepare membranes containing hydrocarbons. Each concentration gave approximately the same amount per surface area when applied the three different ways.
[c]Hydrocarbon dissolved in ethyl ether, and atomized or pipetted over preformed membranes containing no hydrocarbons.
[d]Hydrocarbon dissolved in ethyl ether, and atomized or pipetted over glass or water surface.
[e]Control (standard membrane plus oil).

the same buffer for 1 hour. They were washed in cacodylate buffer containing 5% sucrose three times for a total of 30 minutes after each fixation. Tween-20 at a final concentration of 0.005% was used in all fixative and washing solutions. The specimens were dehydrated through an ethanol series and dried by the critical point method (3) using absolute ethanol as the intermediate fluid and carbon dioxide as the transition fluid. Temperature in the bomb was not allowed to exceed 38 C. Specimens were then mounted on stubs with silver paint, coated three times with gold in a vacuum evaporator, and stored in a desiccator.

Replicas, collodion membranes, and polyethylene films were fixed for 8 minutes in modified Parducz' reagent (17) containing 3% osmium tetroxide, 1.85% mercuric chloride, and 0.005% Tween-20. They were washed in 0.01% Tween-20, and then in distilled water. Dehydration, drying, mounting, and coating were the same as for leaves. In order to facilitate handling, the collodion membranes were prepared on 12-mm diameter glass cover slips prior to use.

All specimens were examined and photographed in a Cambridge Stereoscan, Model Mark 2A, scanning electron microscope.

RESULTS.—*Artificial membranes.*—Since germ-tube differentiation can be induced on collodion membranes by the addition of hydrocarbons (6, 14), experiments were designed to examine the function of hydrocarbons in relation to appressorium formation and membrane surfaces.

TABLE 2. Appressorium formation by germ tubes of *Uromyces phaseoli* on collodion membranes containing paraffin oil (4 mg/ml collodion solution) prepared with various solvents

Solvent for collodion	Appressoria at 4.5 hours (% of control)[a]
Ethyl ether-ethanol, 3:1	100[b]
Ethyl ether-ethanol, 3:1 (partial evaporation)[c]	0
Ethyl ether-methanol, 3:1	95
Ethyl ether-propanol, 3:1	0
Ethyl acetate	125
Butyl acetate	8
Acetone	13

[a]Percentage appressorium formation of controls during course of experiments = 56-85.
[b]Control (standard membrane plus oil).
[c]Evaporation of ethanol was incomplete before the membrane was floated up on water.

On standard membranes without hydrocarbon, surface features were absent and percentage appressorium formation at 4.5 hours was always 0 (Fig. 1); at 24 hours, occasional (<1%) differentiated germ tubes were present. The incorporation of paraffin oil into collodion membranes created a rough surface, and effectively induced appressoria (Fig. 2; Table 1). On standard

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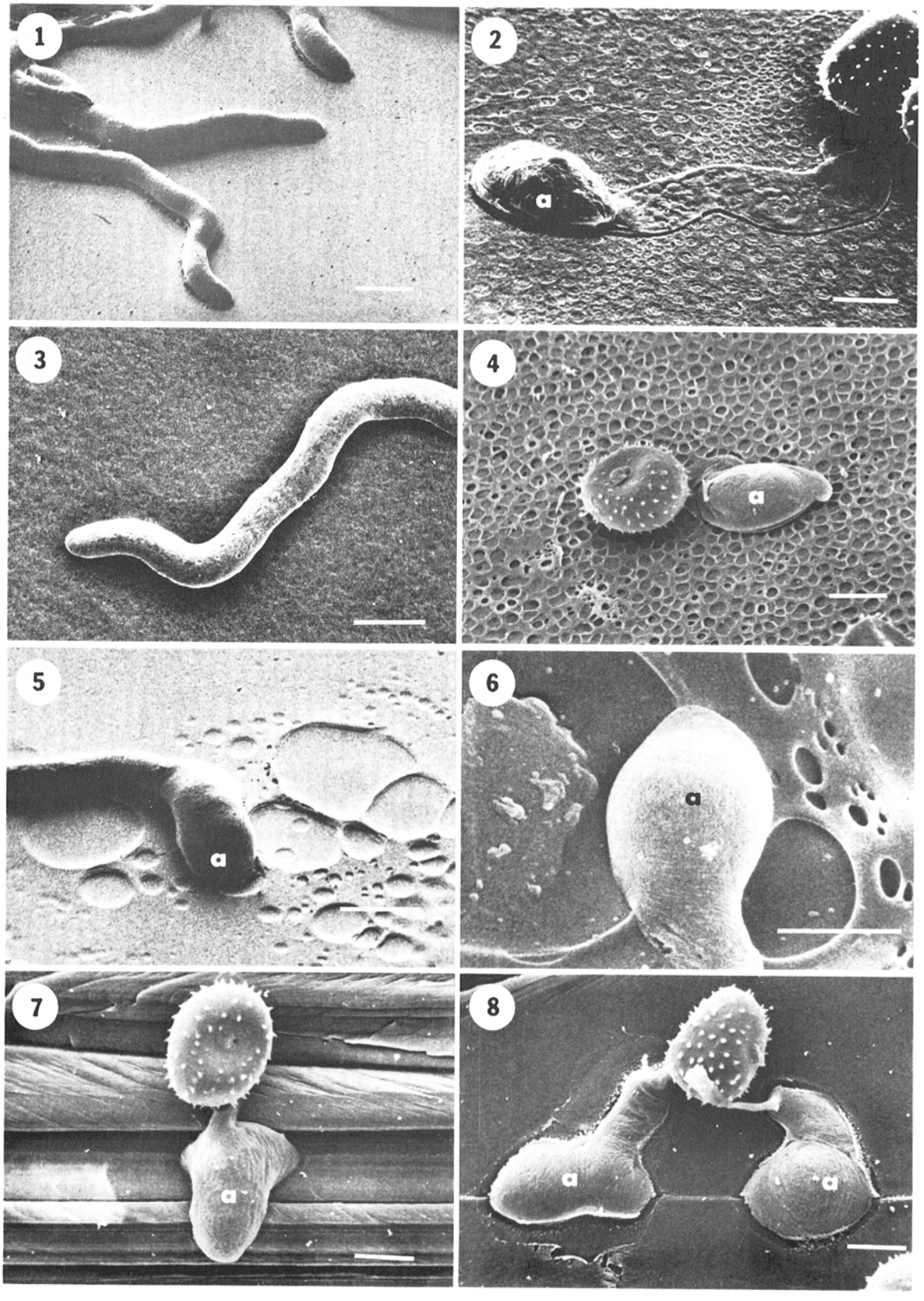
Fig. 1-8. Scanning electron micrographs of *Uromyces phaseoli* after germination for 4.5 hours on artificial membranes; a = appressorium; each scale line = 10 μm. **1)** Germ tube tips on standard collodion membrane without hydrocarbon. **2)** Uredospore, germ tube, and appressorium on standard collodion membrane plus paraffin oil. **3)** Germ tube tip on collodion membrane plus paraffin oil prepared by partial evaporation of the ethyl ether-ethanol solvent. **4)** Uredospore and appressorium on collodion membrane plus paraffin oil prepared with ethyl acetate solvent. Germ tube is too short to see. **5)** Germ tube and appressorium on collodion membrane without hydrocarbon which was atomized with ethyl acetate. Appressorium has formed over craters produced by the ethyl acetate. **6)** Higher magnification of the same type of membrane as in Fig. 5, showing appressorium formation over the raised rim of a crater. **7)** Appressorium over scratch marks produced by rubbing polyethylene film with fine sandpaper. **8)** Uredospore with two appressoria over ridge on polyethylene film scratched with sandpaper.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membranes plus paraffin oil, appressorium formation at 4.5 hours ranged from 29% to 87% throughout all the experiments; at 24 hours, it was usually greater than 80% (although accurate counts at this time were not possible because germ tubes would not stain, and the infection hyphae became enmeshed). Different viscosities and sources of paraffin oil did not affect differentiation.

When eight hydrocarbons and 16 lipids were substituted for paraffin oil in standard membranes at concentrations ranging from 7-14 mg/ml collodion solution, they fell into three groups, based on appressorium formation at 4.5 hours as a percentage of that on the control (standard membrane plus oil): (i) fully effective materials (80 to 130% of control) were pentadecane, hexadecane, paraffin wax (mp, 55 C), triolein, cottonseed oil, and safflower oil; (ii) partially effective materials (10-60% of control) were octacosane, palmitate, stearate, methyl heptadecanoate, trilinolein, and cholesterol; (iii) noneffective materials (<1% of control) were decane, tridecane, tetradecane, heptadecane, laurate, methyl nonanoate, methyl undecanoate, methyl tridecanoate, methyl palmitate, methyl oleate, dipalmitin, and lecithin. Each group contained both hydrocarbons and lipids. Only paraffin wax, cottonseed oil, and safflower oils are mixtures; the remainder are compounds. Of the 12 fully and partially effective materials, six (pentadecane, hexadecane, triolein, trilinolein, cottonseed oil, and safflower oil) are liquids at 20 C; the other six are solids. The chemical dissimilarity among the effective materials indicated that no single compound was necessary for induction of appressoria.

The concentration of the hydrocarbons and lipids was often critical (Table 1). When incorporated into the membranes at the standard concentration (4 mg/ml collodion solution), paraffin oil was more effective than any hydrocarbon or lipid tested. However, at higher concentrations, cottonseed and safflower oils (7 mg/ml), triolein (10 mg/ml), and pentadecane and hexadecane (14 mg/ml) were superior to paraffin oil. The variations in amounts of oil necessary for appressorium induction are illustrated by the following examples from two experiments: at 2, 4, and 8 mg/ml collodion solution, paraffin oil gave 8, 64, and 65% appressorium formation, respectively, at 4.5 hours; safflower oil gave 0, 26, and 73%; and hexadecane gave 0, 0, and 36%.

The data in Table 1 show that the two liquid materials, paraffin oil and hexadecane, had to be incorporated into collodion membranes to induce differentiation. The two solid materials, paraffin wax and octacosane, stimulated appressorium formation when associated with collodion membrane, but not when they were used alone. Although solid hydrocarbons differed from liquids in their effectiveness when applied over preformed collodion membranes, none of the hydrocarbons were effective in the absence of collodion.

The properties of membranes containing paraffin oil were altered by two methods: (i) When membranes were prepared by stopping the ethyl ether-ethanol evaporation before it was complete, no appressorium formation occurred (Fig. 3, Table 2); the germ tubes behaved as if no oil were present. These partially evaporated membranes had a slightly uneven surface (Fig. 3) which was distinctly smoother than the pockmarked surface of the corresponding completely evaporated membranes (Fig. 2). (ii) Changing the solvents used for diluting the collodion resulted in different types of membranes (Fig. 4, Table 2). Membranes prepared by dilution with ethyl acetate and ethyl ether-methanol effectively induced appressoria, whereas those prepared with ethyl ether-propanol, acetone, and butyl acetate were ineffective. The surface of the ethyl acetate membranes had a uniform topographical pattern resembling honeycomb (Fig. 4).

Two methods were found to produce membranes without oil, which routinely stimulated appressorium formation at 4.5 hours: (i) Collodion membranes were highly wrinkled when they were formed over 40 C water instead of over glass as in the standard procedure. Twenty percent appressorium formation occurred on these membranes. (ii) When standard membranes were atomized lightly with ethyl acetate, "craters" were formed where the ethyl acetate had partially dissolved the cellulose nitrate before it evaporated (Fig. 5, 6). The larger craters had raised rims around them. As germ tubes contacted the craters, they formed appressoria immediately (Fig. 5, 6). No appressoria were produced over the smooth surface between craters.

A pattern of appressorium formation similar to that on ethyl acetate-sprayed membranes occurred when spores were germinated on polyethylene film scratched with fine sandpaper [104-68 μm (grades no. 150-220) aluminum oxide]. The germ tubes produced appressoria as they contacted the smaller scratch lines (Fig. 7, 8). Appressorium formation ranged from 20% to 30% at 4.5 hours; no appressoria formed between scratches.

Leaves and leaf replicas.—In order to obtain an accurate picture of appressorial response, it was necessary to use counts of percentage appressorium formation in conjunction with percentage appressoria over stomates. For example, percentage appressoria over stomates on wheat and oats (Table 4) was not especially low; however, since percentage appressorium formation was negligible, the few appressoria that formed over stomates were biologically insignificant.

Table 3 shows both measurements at three time intervals on lower and upper surfaces of Pinto bean leaves and corresponding replicas. The most outstanding result was the similarity of appressorial counts on the replicas as compared to the leaves. Only three pairs of comparable leaves and replicas showed statistically significant differences, and in two of these the counts on the replicas were higher. After 6 hours of germination on the lower surface, percentage appressorium formation was 50% on leaves and 41% on replicas; after 24 hours, when germ tube growth was complete, these values had increased to 85% and 77%, respectively. Percentage appressoria over stomates on the lower leaf surface was 92% or greater at all germination times; on replicas this value was 96-98%. Percentage appressorium formation on both leaves and replicas increased with time since the opportunity of germ tubes to contact stomates was greater as they elongated. In contrast, percentage appressoria over stomates was essentially independent of time, since the ability of germ tubes to recognize stomates did not increase as they elongated. Production of appressoria on the upper surface was similar to that on the lower in that counts on replicas were close to those on leaves. However, the counts were less on the upper surface. For percentage

TABLE 3. Appressorium formation by germ tubes of *Uromyces phaseoli* on primary leaves and corresponding leaf replicas of Pinto bean plants

Surface of leaf	Germination time (hours)	Appressorium formation[a]		Appressoria over stomates[b]	
		Leaves (%)	Replicas (%)	Leaves (%)	Replicas (%)
Lower	6	50 ± 2.9*	41 ± 2.1*	92 ± 1.9*	96 ± 0.6*
	12	68 ± 2.4	61 ± 2.9	98 ± 0.5	97 ± 0.7
	24	85 ± 2.7	77 ± 2.7	98 ± 0.9	98 ± 0.4
Upper	6	20 ± 1.8	15 ± 1.5	91 ± 1.6	94 ± 1.3
	12	26 ± 0.6	29 ± 2.9	88 ± 1.0	93 ± 1.7
	24	35 ± 2.3	38 ± 4.9	83 ± 1.8**	94 ± 1.2**

[a]Germ tubes with appressoria ÷ total germ tubes × 100.
[b]Appressoria over stomates ÷ total appressoria × 100.
Asterisks indicate (*/**) significant at $P = 0.05, 0.01$, respectively, by analysis of variance in which percentage appressorium formation and percentage appressoria over stomates were compared between leaves and replicas at each germination time. No asterisk indicates no significant difference between comparable leaves and replicas. Each mean was determined from 4-16 replications of 100 counts each.

appressorium formation, the low counts were correlated with fewer stomates on the upper surface (30/mm^2 leaf area) as compared to the lower surface (130/mm^2 leaf area). For percentage appressoria over stomates, the fewer counts on the upper surface reflected more mistakes; on the leaves these mistakes increased with time. The number of infections on leaves was proportional to the appressorium counts on each surface: 14 pustules/cm^2 leaf area were produced from inoculation of the upper surface versus 35/cm^2 from inoculation of the lower surface with the same spore density.

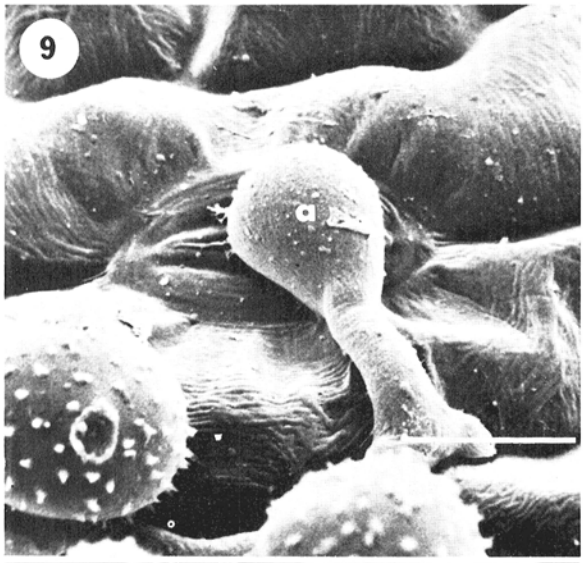
Appressoria over stomates on the lower surfaces of Pinto leaves after 6 hours of germination are shown in Fig. 9 and 12. At this stage, the appressoria were filled with cytoplasm, and therefore turgid. Appressoria over stomates on replicas of the lower surfaces of Pinto leaves after 4.5-12 hours of germination are shown in Fig. 10, 11, 13, and 14. Fully developed appressoria usually covered the stomates completely (Fig. 11, 13, 14). After 12 hours of germination, the infection hyphae were unable to grow into the replica, and therefore grew laterally on the replica surface (Fig. 14). At this stage, the cytoplasm moved into the infection hyphae, and the appressoria collapsed.

Table 4 shows the percentages of appressoria formed on leaves and corresponding leaf replicas of several leguminous and gramineous plants, including five cultivars of common bean, which were either susceptible, resistant, or immune to the race of bean rust used in these studies. As with Pinto leaves, both types of appressorial counts on various replicas were similar to those on the original leaves. Major exceptions among the legumes were lower percentages of appressorium formation on leaf replicas than on the leaves of Bountiful bean and Early Ramshorn cowpea. Replicas from these cultivars did not preserve surface detail as well as those from the other plants. It was particularly hard to make clear-cut replicas from cowpea leaves, apparently because of the type of cuticle. Within the limited number of plants tested, there was no difference in appressorium counts on susceptible and resistant cultivars of bean. However percentage appressorium formation was lower on the immune Early Ramshorn cowpea and Bragg soybean, both of which are nonhosts; percentage appressoria over stomates was lower on the cowpea, but not on the soybean. Differences in stomatal density (100 and 140/mm^2 lower leaf surface of cowpea and soybean, respectively; 120-150/mm^2 lower leaf surface on the bean cultivars) were not enough to account for the differences in appressorium formation on the nonhost legumes. The lower percentage appressorium formation on soybean was correlated with a high density of leaf hairs: germ tubes often grew along these trichomes, and therefore did not come in contact with the leaf surface.

The major difference in appressorium production was on the gramineous plants; i.e., wheat, and oats. The most striking distinction between germ tube behavior on leaves and replicas of wheat and oats as compared to the leaves and replicas of the legumes was the lack of response to the stomates. The vast majority of the germ tubes passed over the stomates without forming appressoria (Fig. 15). This resulted in percentage appressorium formation less than 5% (Table 4). When appressoria did occasionally form, less than two-thirds of them were over stomates. The statistically significant differences between percentage appressorium formation on leaves and replicas appear to be unimportant biologically since so few appressoria were formed on either surface.

Surface details of stomates, guard cells, and surrounding epidermal cells were examined in order to help explain the differences in appressorium response to stomates of bean and wheat leaves. Stomates of bean leaves and replicas were surrounded by prominent stomatal lips on the guard cells (Fig. 9, 10, 16, 17). No other distinctive features were present in the stomatal areas. The gramineous type stomates on wheat leaves and replicas had inconspicuous lips which were mostly concealed at the inner edges of the guard cells (Fig. 18, 19). Guard, and subsidiary, cells had no prominent surface features.

In order to determine if substances associated with stomates and guard cells on Pinto bean leaves were transferred to the replicas and induced appressorium formation on the replicas, two types of experiments were set up. (i) A single rubber negative replica of the lower leaf surface was used to make 12 consecutive positive replicas.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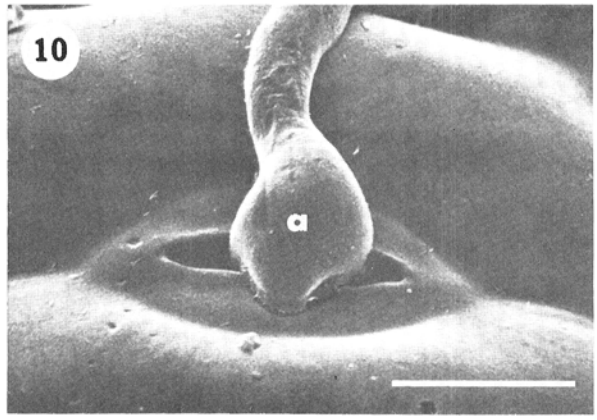
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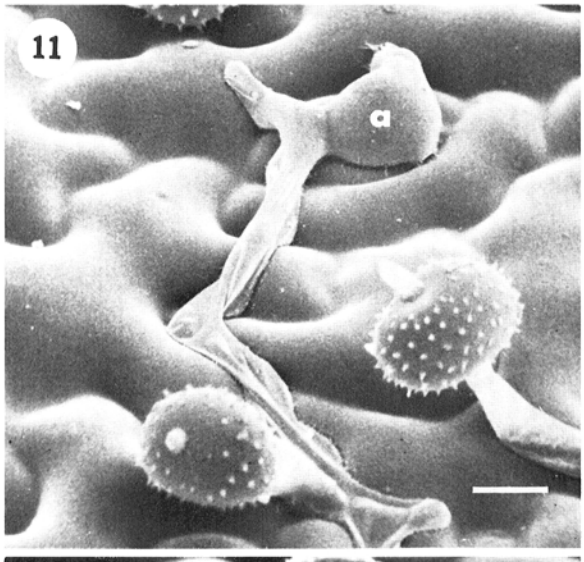
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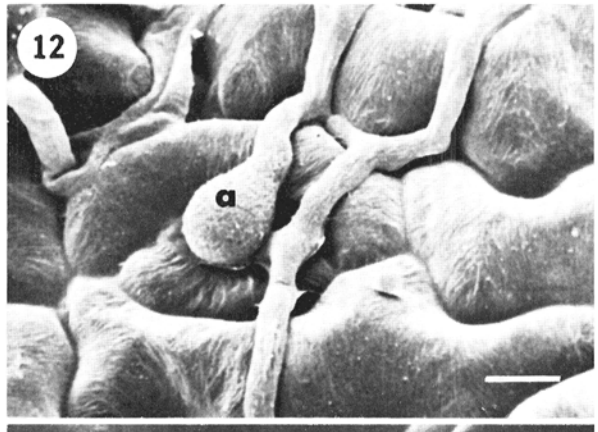
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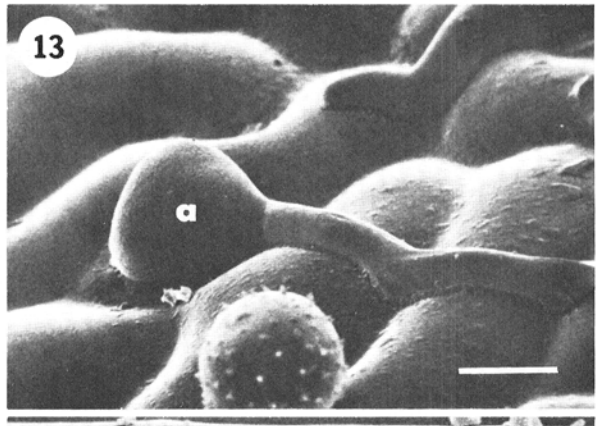
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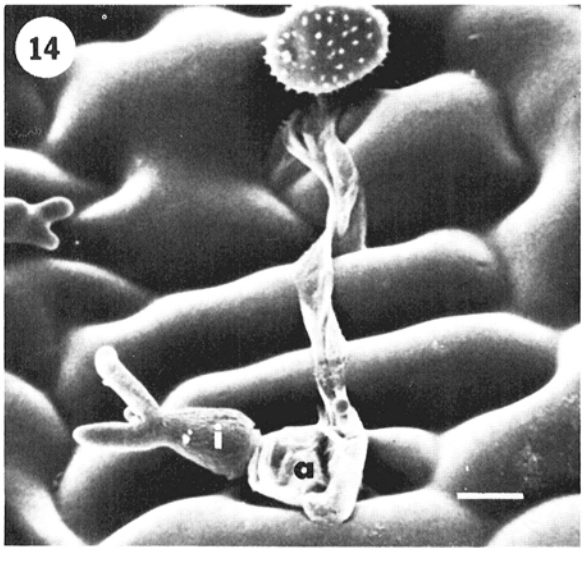
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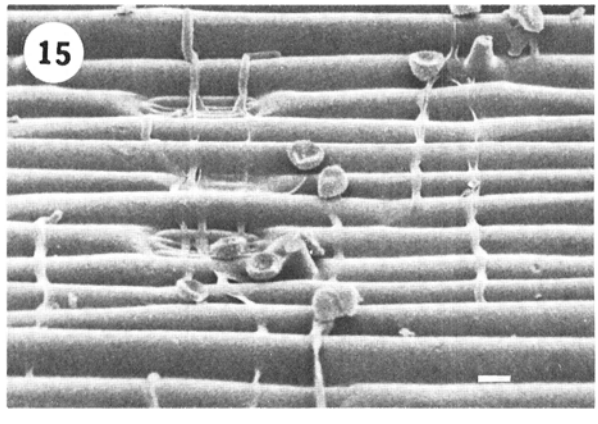
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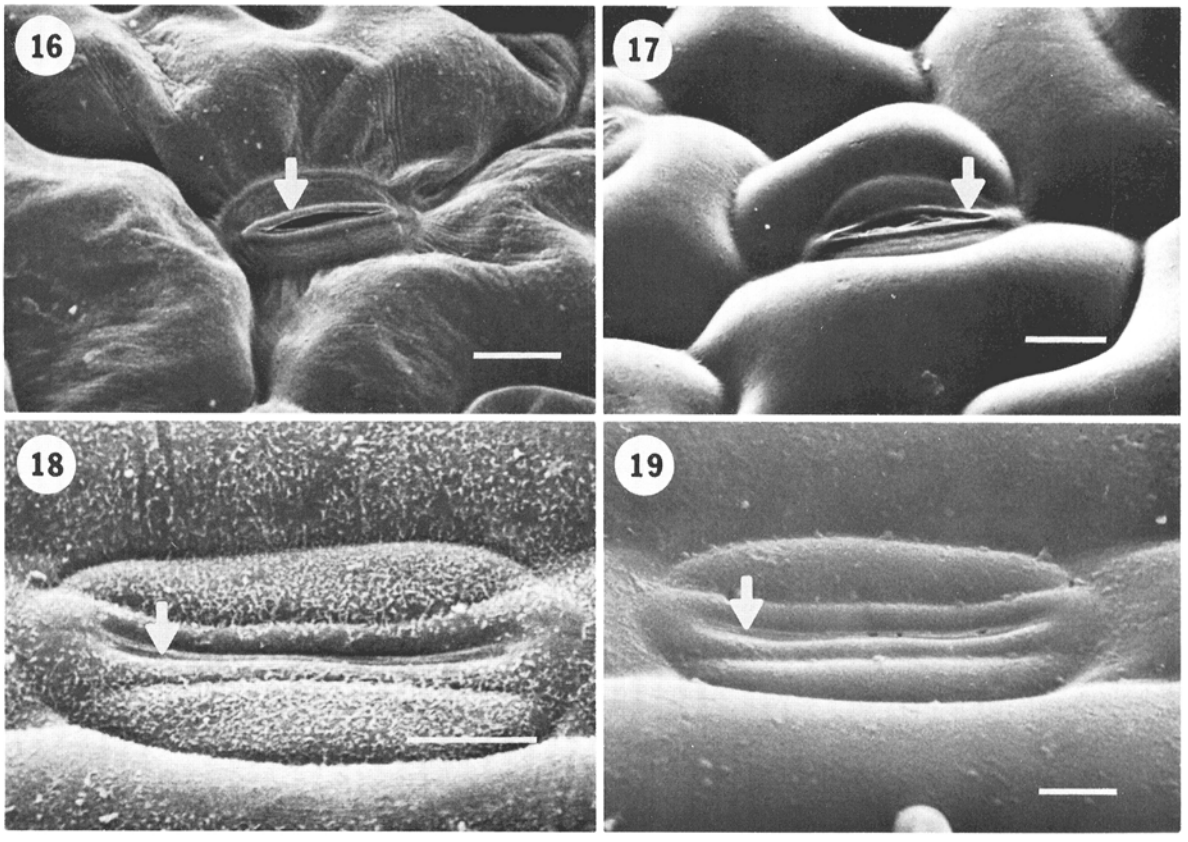

Fig. 16-19. Scanning electron micrographs of stomates on the lower surface of leaves and corresponding leaf replicas; arrows point to stomatal lips; each scale line = 10 μm. **16)** Pinto bean leaf. **17)** Replica of Pinto bean leaf. **18)** McNair 2203 wheat leaf. **19)** Replica of McNair 2203 wheat leaf.

Appressorium counts on the last of these replicas were the same as those on the first. (ii) Five rubber replicas of lower leaf surfaces were used to make positive replicas. These positive replicas (R_1) were then used in place of leaves to make another set of five rubber replicas which were used in turn to make positive replicas (R_2). The entire procedure was repeated again, using R_2 in place of leaves to make a third set of positive replicas (R_3). The final result was three sets of Pinto bean replicas: R_1, original replicas of leaves; R_2, replicas of the original replicas; R_3, replicas of the second replicas. Percentage appressoria over stomates averaged 95% for R_1, 91% for R_2, and 90% for R_3. Thus, there was no evidence that chemical stimulants of appressorium formation were carried over from leaf to replica.

Two types of mistakes in appressorium formation were observed on leaves and replicas. (i) Appressoria formed over interstomatal areas instead of over stomates. After 24 hours of germination, only 2% of the appressoria were not over stomates on the lower surface of Pinto leaves, whereas 36% were not over stomates on the lower surface of wheat leaves (Table 3, 4). (ii) Germ tubes failed to form appressoria as they passed over stomates (Fig. 14, 15). Counts were made of 12-hour germ tubes which grew over one or more stomates without forming an appressorium on lower leaf surfaces. Based on total germ tubes, 4% of those on Pinto and 44% of those on wheat failed to form appressoria when contacting stomates.

Three additional observations were made on appressorium formation on leaves and replicas. (i) When

Fig. 9-15. Scanning electron micrographs of *Uromyces phaseoli* after germination on the lower surface of leaves and on replicas of the lower surface of leaves; a = appressorium; each scale line = 10 μm. **9)** Appressorium formed after 6-hr germination over closed stomate on Pinto bean leaf. **10)** Young appressorium after 4.5 hours of germination over open stomate on replica of Pinto bean leaf. **11)** Uredospore, germ tube, and appressorium after 6 hours of germination on replica of Pinto leaf. Appressorium is covering stomate. **12)** Appressorium after 6 hours of germination over stomate on Pinto leaf. Another germ tube could not grow directly over the occupied stomate and did not form an appressorium. **13)** Germ tube growing across epidermal cell ridges and appressorium covering stomate after 6-hr germination on replica of Pinto leaf. **14)** Germination for 12 hours on replica of Pinto leaf. Germ tube failed to form appressorium over stomate beside uredospore, but it continued to grow toward a second stomate which it is covering with an appressorium. The branched infection hypha (i), unable to penetrate the replica, is on the surface, **15)** Germ tubes growing across epidermal ridges after 6 hours of germination on replica of McNair 2203 wheat leaf. No appressoria formed over the stomates.

TABLE 4. Appressorium formation by germ tubes of *Uromyces phaseoli* after 24 hours of germination on the lower surface of leaves and corresponding leaf replicas of 10 leguminous and gramineous plants

Plants	Reaction type[a]	Appressorium formation[b]		Appressoria over stomates[c]	
		Leaves (%)	Replicas (%)	Leaves (%)	Replicas (%)
Phaseolus vulgaris (bean)					
'Pinto'	S	80 ± 2.6*	71 ± 2.5*	99 ± 0.5	97 ± 1.4
'Bountiful'	S	80 ± 1.5**	51 ± 0.9**	99 ± 0.4	95 ± 1.7
'Topcrop'	S	79 ± 1.6	68 ± 5.1	98 ± 0.7	99 ± 0.5
'USDA No. 643'	R	77 ± 0.8	72 ± 2.3	99 ± 0.7	98 ± 0.9
'USDA No. 765'	R	80 ± 1.8*	73 ± 2.8*	99 ± 0.9	99 ± 0.3
P. limensis (lima bean)					
'Fordhook'	S	74 ± 2.5	68 ± 2.2	97 ± 1.0	97 ± 0.9
Vigna sinensis (cowpea)					
'Early Ramshorn'	I	49 ± 1.7**	17 ± 3.2**	80 ± 4.1	81 ± 5.0
Glycine max (soybean)					
'Bragg'	I	38 ± 3.1	35 ± 2.4	89 ± 2.9	97 ± 2.5
Triticum aestivum (wheat)					
'McNair 2203'	I	4 ± 0.4**	2 ± 0.4**	64 ± 5.6	53 ± 14.8
Avena sativa (oats)					
'Markton'	I	5 ± 0.7**	3 ± 0.5**	32 ± 5.7	37 ± 9.1

[a]Reactions on intact plants 10 days after inoculation. S = susceptible (pustules formed); R = resistant (necrotic flecking without sporulation); I = immune (no macroscopic symptoms).

[b]Germ tubes with appressoria ÷ total germ tubes × 100.

[c]Appressoria over stomates ÷ total appressoria × 100.

(*/**) Significant at $P = 0.05, 0.01$ by analysis of variance in which percentage appressorium formation and percentage appressoria over stomates were compared between leaves and replicas from each variety. No asterisk indicates no significant difference between comparable leaves and replicas. Each mean was determined from 3-26 replications of 100 counts each, except for percentage appressoria over stomates on wheat and oats where individual samples were less than 100 counts.

Pinto leaves were dipped in ethyl ether for 5 seconds to alter the cuticle, and then replicas were made of the lower surfaces, percentage appressorium formation was 33 and percentage appressoria over stomates was 32 after 24-hour germination on these replicas. Compared to replicas of nontreated leaves (Table 3), the ether treatment decreased appressorium production and increased mistakes. Surface features (stomates, epidermal cell surfaces, and cell wall lines) were indistinct and irregular on replicas from treated leaves; some areas were distorted to the extent that no surface features were recognizable. (ii) The procedure for preparing replicas from bean leaves normally gave replicas with some of the stomates open, and some closed. However, counts of appressoria over open stomates on replicas were identical to counts of appressoria over closed stomates. Since germination was carried out in the dark, stomates on the inoculated leaves were always closed. (iii) Appressorial counts were made on excised and intact primary Pinto leaves from plants 10-18 days old. There were no significant differences in percentage appressoria over stomates on intact and excised leaves, and on different-aged leaves during the period tested.

Counts of appressoria on leaves and replicas indicate the relationship between stomates and appressorium formation. However, these data show nothing about directional growth of germ tubes. It appears obvious that if germ tube growth were completely random on the leaf surface, percentage appressorium formation could not be as high as shown in Tables 3 and 4 since less than 4% of the surface area is covered by the stomates plus the guard cells. Therefore, in an attempt to answer the question of directional growth, spores were germinated for 4-8 hours on replicas of the lower surface of Pinto leaves. Counts were made of epidermal cells with germ tubes perpendicular to their ridges, and expressed as a percentage of epidermal cells which were contacted by germ tubes at any point. In three experiments with a total of 29 replications of 100 counts each, the epidermal cells with germ tubes perpendicular to ridges averaged 83 ± 0.7%. This growth primarily across ridges is shown in Fig. 11, 12, 13, and 14. The pattern of the epidermal ridges on a bean leaf is not random, since, in general, the cells are arranged so that their ridges form broken concentric rings around the stomates (Fig. 10, 11, 12, 14, 16, 17). Other observed examples of germ tube growth across ridges are: circular-type growth perpendicular to the epidermal ridges radiating out from a trichome; linear growth perpendicular to the parallel ridges formed by the epidermal cells covering the vascular bundles; and linear growth perpendicular to the parallel ridges of epidermal cells of gramineous plants (Fig. 15).

DISCUSSION.—*Topography and directional germ tube growth.*—Evidence for the control of directional growth of germ tubes of several rusts by contact stimulus is convincing. Germ tubes of *P. graminis* f. sp. *tritici* and *P. antirrhini* have been shown to grow perpendicular to the cuticular ridges on epidermal cells of wheat and

snapdragon leaves, respectively (11, 12, 15). Growth at right angles to the structural lines of artificial membranes has been clearly demonstrated for five species of *Puccinia* and one of *Uromyces;* this growth is directed by physical contact of the growing tips with the membrane surfaces (7, 9). As pointed out previously (12), a germ tube of wheat stem rust fungus whose growth is directed across a wheat leaf can hardly miss a stomate if it grows far enough because of the arrangement of stomates in staggered rows. On bean leaves germ tube growth at right angles to the epidermal ridges encircling the stomates directs the fungus to the stomates by the same mechanism as growth across the regularly parallel ridges on gramineous leaves and the more irregular ridges on snapdragon.

Close adherence of germ tubes to the surface on which they are growing is essential for controlled directional growth (7). This is supported by observations associated with the present study in which wheat stem rust, but not bean rust, grew consistently at right angles to the ridges on wheat leaves. The germ tubes of wheat stem rust were tightly appressed to the leaf surface; those of bean rust were not. In comparison, germ tubes of bean rust grew directionally on bean leaves, bean leaf replicas, and wheat leaf replicas; and they grew tightly appressed to these surfaces. A reasonable interpretation of the different responses of bean rust is that the cuticle waxes on wheat leaves prevented germ tube adherence. These wax extrusions were not present on replicas and on bean leaves (Fig. 16-19).

Topography and appressorium formation.—Published evidence for induction of appressoria by physical contact is not as convincing as for directional growth. To my knowledge, the only hard data showing that appressoria of rusts are consistently induced in response to surface contact alone are from Dickinson's work with *P. coronata* and *P. recondita* on specific artificial membranes (8, 9). No rigorous evidence exists for a topographical stimulus of appressorium formation on host leaves.

Maheshwari et al. (14) demonstrated for three rust fungi that high numbers of appressoria could be consistently induced on collodion membranes when hydrocarbon was added. Scanning electron microscopy showed that paraffin oil in collodion membranes produced a rough surface (Fig. 2,4). Membranes without oil had a smooth surface (Fig. 1); those prepared with oil in a modified procedure had a nearly smooth surface (Fig. 3). The exact oil-membrane topographies shown in Fig. 2, 3, and 4 may be artifactual, since the paraffin oil probably volatilized under the high vacuum used for electron microscopy. When oil-containing membranes were examined by light microscopy, the surfaces appeared bumpy; the size and arrangement of bumps varied with membrane preparation. But, regardless of the method of viewing, it is apparent that the effect of hydrocarbon on germ tube differentiation is due to its effect on the collodion surface.

Better evidence for the dependence of appressorium induction on membrane topography is provided by hydrocarbon-free membranes with specific surface features (Fig. 5-8). Appressoria formed only over the edges of craters and scratches on the membranes.

The argument behind the experiments with leaf replicas was that if appressoria form over stomates of leaves in response to physical contact, then they should also form over the "stomates" of inert leaf replicas which are faithful reproductions of the leaves. When spores were germinated on leaves and their corresponding replicas, most appressorial counts on replicas correlated well with analogous counts on leaves (Tables 3, 4). These correlations were maintained with different germination times, upper and lower leaf surfaces, and susceptible, resistant, and immune plants. It was conceivable that an appressorium-inducing chemical could have been carried over from stomates on leaves to corresponding areas on replicas. This possibility was ruled out when counts of percentage appressoria over stomates did not diminish on multiple positive replicas from a single negative replica, and on a series of replicas prepared from other replicas instead of from leaves. Thus, germ tubes of bean rust appear to form appressoria over stomates solely in response to a contact stimulus.

The close adherence of germ tubes to surfaces was as essential for appressorium formation as it was for directional growth. Wide variations in appressorial counts, such as percentage appressoria over stomates on wheat and oat leaves (Table 4), were usually due to poor adherence. Since germ tubes of the bean rust fungus did not grow appressed to surfaces of gramineous leaves, the occasional appressoria were formed at random in the same way that germ tube growth was random.

Germ tubes on bean leaf replicas sometimes failed to form appressoria as they grew over stomates. However, they usually did flatten out and enlarge over the stomates. This indicated a partial response, but one insufficient to induce differentiation, and suggests that the contact stimulus may be quantitative. Others have observed similar partial responses of germ tubes to surface features (6, 11, 12).

The specific appressorium stimulus.—The stomatal lips are the most logical candidate for the specific stimulus of appressorium formation by bean rust for the following reasons: (i) Stomatal lips on bean leaves project upward at an angle over the stomatal pores to form prominent ridges which surround the pores (16). Because of their position on the guard cells, the lips project when the pores are either open or closed. From scanning electron micrographs (Fig. 9, 10, 16, 17) of bean leaves and replicas, the only morphological surface features that distinguish the stomates from the remainder of the leaf surface are the stomatal lips. (ii) Stomatal lips on wheat and oat leaves are distinctly smaller and more poorly developed than those on bean and related dicotyledonous leaves (5, 16, 20; Fig. 18, 19); bean rust does not form significant numbers of appressoria over stomates on wheat and oat leaves (Table 4). Corn, another gramineous plant, has larger stomatal lips than wheat and oats (5, 20); appressorium counts of the bean rust fungus on corn leaves [34% appressorium formation, and 90% appressoria over stomates–(Wynn, *unpublished*)] are higher than wheat and oats. (iii) Collodion membranes sprayed with ethyl acetate and polyethylene film scratched with sandpaper had ridges which induced appressorium formation (Fig. 6, 8). These ridges resemble the shape and size of stomatal lips of bean.

Considering the evidence for both directional growth and appressorium induction, it seems clear that the germ tubes of the bean rust fungus are capable of two separate

contact responses to two different stimuli. When a germ tube begins to grow on a leaf, first it must translate the cuticular ridge pattern into a message dictating direction of elongation and then it must translate the stomatal lip stimulus into a message dictating that elongation be stopped and appressorium formation be initiated. It is reasonable that components in the germ tube wall or associated with the wall are receptors for both stimuli.

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