Uniformity Among Races of *Uromyces appendiculatus* in Response to Topographic Signaling for Appressorium Formation

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ABSTRACT

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Forty pathogenic races of *Uromyces appendiculatus* from diverse origins were tested for thigmotropic sensing of surface topography in the formation of appressoria. Defined topographic signals (ridges) ranging from 0 to 1.24 μm high were tested. All races responded similarly, with minimal levels of appressorium formation occurring on ridges less than 0.18 μm high and greater than 1.24 μm high. Development of appressoria occurred optimally (>80%) on ridges 0.4–0.8 μm high. The uniform response for appressorium formation on the different topographies was observed among test races and among repeated tests of a standard race. The similarity in response for appressorium development among these bean rust races has implications for development of thigmotropic-based race nonspecific resistance.

The rust disease caused by *Uromyces appendiculatus* (Pers.:Pers.) Unger (*U. phaseoli* (Pers.) G. Wint.) is a serious problem on bean (*Phaseolus vulgaris* L.) in many regions of the world where it can cause severe epidemics (8). The disease can be kept below economic threshold levels primarily through pesticide applications and the use of resistant cultivars. Although the planting of resistant cultivars is the most widely successful of these strategies, such resistance is often race-specific and frequently short-lived because *U. appendiculatus* shows wide variation in pathogenicity and adaptability (2,7,9). The identification and incorporation of race nonspecific resistance has been proposed as a means of developing a more stable form of disease control (4,10,13). For example, Shaik (4) suggested that leaf pubescence could confer race nonspecific resistance by reducing the number of spores reaching and germinating on the leaf surface. Hoch and co-workers (3) showed that the bean rust fungus is sensitive to specific leaf surface topographies. Leaf characters such as epidermal cell shape and cell orientation affect germ tube growth, and features of stomatal architecture trigger the formation of appressoria (12). Hoch and colleagues (3), in studying the growth of the fungus on plastic membranes bearing microfabricated ridges, identified the signal necessary for induction of appressorium formation as a sharp change in substrate elevation, in the order of 0.5 μm. Ridge heights less than 0.25 μm or greater than 1.0 μm were significantly less inductive. Subsequent examination of bean stomata showed that some morphological components (e.g., stomatal guard cell lips) fell within this size range, and it was proposed that such features constitute the inductive signal (3,11).

Considering the apparently high specificity of *U. appendiculatus* urediniospore germlings for thigmotropic sensing, it may be plausible to reduce the number of appressoria formed by modifying the stomatal feature(s) responsible for signal induction or by providing confusing leaf topographies that cause the fungus to differentiate at sites other than over stomata. Either approach would prevent fungal entry into the leaf, thus reducing the probability of successful infection. To determine if such strategies have potential to confer race nonspecific resistance, it is imperative that we first determine whether or not different races of *U. appendiculatus* respond similarly to inductive topographies. The purpose, then, of this study was to assess the signaling parameters for appressorium formation among a diverse selection of *U. appendiculatus* races.

MATERIALS AND METHODS

Preparation of test materials. Forty races of *U. appendiculatus* from Central American, Caribbean, and North American collections were chosen for testing to represent a diverse range of pathogenic variability and were identified by testing known differential bean cultivars (Table 1). The numbered races have been previously described (7). Races identified by letters and numbers were isolated from collections made between 1982 and 1987 from bean production regions in the Dominican Republic, Honduras, and the high plains of the United States. The Mexican race was obtained from J. V. Groth, University of Minnesota. All rust collections were inoculated, by a handheld sprayer, onto primary leaves of the 19 differential cultivars/lines used for U.S. race identification (7) and Mountaineer White Half Runner. After incubation in a mist chamber for 14 h at 20 C, the plants were placed in a greenhouse at 23 ± 2 C for uredinia development. After repeated single uredinia isolations, individual races were inoculated onto the 20 differential host indicators and rated for rust reaction after 15 days with Stavely's grading scale (7). Samples of test races were frozen and maintained at −85 C until used. Race 0 is not a race recognized as currently being widespread in the United States. Staples and Wynn originally obtained it from C. E. Yarwood and designated it race 0 (12). During the course of this investigation one of us (J. R. Stavely) tested it on the standard differential cultivars for races of the bean rust fungus and found that it formed large uredinia on Pinto 650, Kentucky Wonder (KW) 780, and other dry bean cultivars; moderate size uredinia on Redlands Pioneer, Brown Beauty, 51051, Early Gallatin, and many other snap bean cultivars; small uredinia (predominantly less than 0.3 mm in diameter) on US 3, KW 765, KW 814, Ecuador 299, Mexico 235 and 309, AXS 37, NEP-2, Aurora, and CNC; and nonsporulating necrotic spots on CSW 643, Golden Gate Wax, and Olathe. These results indicate

race 0 is different on the standard differential cultivars from all other races described on them or on a portion of them from the United States, Brazil, and other locations (7–9). Therefore, we are continuing to designate it as race 0. This race has been used in one of our laboratories (HCH) for many years as the standard test isolate, including earlier studies concerned with thigmotropic sensing (3,12). For this study, urediniospores of race 0 were obtained from greenhouse-inoculated plants of *P. vulgaris* 'Pinto 111,' and were used either fresh or after storage at 5 or −85 C.

Test membranes were produced by casting a thin film of dissolved polystyrene (Styron 685D, Dow Chemical, Midland, MI) (20% w/v in ethyl acetate) onto silicon wafer templates fabricated by either optical or electron beam lithography (3). Once the solvent had completely evaporated from the polystyrene, the resulting membranes were floated off the templates in a 45 C water bath. Each membrane had a uniform pattern of a single height ridge, 2 μm wide, and spaced 60 μm apart in a grid pattern. For most tests, 10 different ridge heights, 0.11, 0.18, 0.23, 0.31, 0.36, 0.42, 0.5, 0.7, 0.9, and 1.24 μm were used. In some tests, two additional ridge heights, 2.27 and 6.7 μm, were used. The heights of the ridges were confirmed by scanning electron microscopic (SEM) examination of test membranes evaluated with a measuring program of an integrated image analysis system (FD-5000, Gould/DeAnza, San Jose, CA). Smooth test surfaces were created by treating glass coverslips with 1.5% dimethyldichlorosilane (Petrarch Systems, Bristol, PA) in methylene chloride. Coverslips were soaked in the silane solution for 5 min, rinsed in methylene chloride, allowed to air-dry, and baked at 100 C for 60 min. Silane treatment was necessary to render the surface hydrophobic, a physical property necessary for urediniospore germling adhesion.

Urediniospores of the test races were treated with 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one (β-ionone, Sigma

TABLE 1. Percentage of appressorium formation by race 0 and test races on 10 different ridge heights

Race	Ridge height (μm)										Mean difference[a]	R^b	Virulence rating[c]	
	0.12	0.18	0.23	0.31	0.36	0.42	0.50	0.70	0.90	1.24			A	B
0	2.13	21.74	35.18	64.89	75.83	79.92	80.01	91.06	61.08	13.37	0.00	1.00	84	32
38	0.00	50.67	64.67	71.83	91.67	93.33	82.17	99.00	89.33	N/A	−14.54	0.92**	11	11
40	1.00	9.50	4.00	26.17	48.00	54.00	47.00	42.67	22.33	2.50	26.80	0.93**	37	32
41	0.83	28.17	26.00	54.17	75.67	70.67	72.83	64.50	28.00	5.50	9.79	0.93*	47	37
48	0.33	15.00	39.00	59.00	83.83	80.00	84.33	83.17	54.17	4.83	2.15	0.99	84	53
49	1.83	35.17	61.67	88.33	88.50	92.17	93.17	94.17	79.00	3.00	−11.18	0.96*	79	53
H87 DA3-12B	0.50	22.83	51.17	68.00	83.83	84.33	83.50	89.67	70.33	N/A	−4.70	0.98*	95	75
H87 DA3-8D	0.67	29.83	53.67	68.83	90.67	96.33	87.83	85.17	82.67	N/A	−9.31	0.96*	85	80
H87 DA3-15D	0.33	40.17	22.67	50.67	66.17	73.50	69.17	75.67	51.67	N/A	6.87	0.95	95	75
H87 DA3-12D	0.33	57.33	34.00	53.50	71.83	71.67	69.00	76.83	40.67	N/A	4.07	0.85	95	85
H87 DA3-13D	1.00	84.50	53.17	81.67	98.33	95.33	92.17	90.50	71.00	N/A	−17.31	0.81*	100	75
H87 DA3-4C	0.67	25.50	66.67	85.00	87.17	94.83	90.83	95.00	55.67	6.50	−8.26	0.95	100	90
H87 DA2-18E	5.00	5.67	77.67	84.83	85.33	87.33	85.67	90.17	79.50	0.33	−10.63	0.91*	100	90
H87 DA3-10C	2.50	42.67	73.50	83.33	85.50	91.83	88.67	84.00	47.00	3.83	−7.76	0.89	N/A	N/A
H87 DA3-15B	5.00	36.33	64.33	90.17	92.00	93.33	93.50	95.67	77.83	20.00	−14.30	0.97**	100	70
H87 DA3-4A	4.00	34.83	23.17	69.50	85.33	90.50	89.17	95.67	74.83	8.67	−5.05	0.98	95	90
H87 DA3-10A	6.83	46.67	39.50	86.00	93.33	93.17	92.17	88.17	56.67	4.17	−8.15	0.95	100	100
H87 EAP-21F	0.00	51.83	38.67	68.67	80.83	84.17	81.67	86.83	70.50	12.33	−5.03	0.95	60	60
H87 ZA3-5A	10.67	41.17	60.83	67.33	79.33	84.67	82.50	81.67	51.00	6.67	−4.06	0.93	100	100
H87 DA4-16E	1.17	6.83	30.67	57.83	76.17	74.83	79.00	88.33	56.83	8.50	4.50	0.99**	85	85
H87 EAP1-4G	0.17	8.83	41.33	47.33	78.83	81.67	71.33	78.67	76.67	23.83	1.65	0.94	60	55
H87 03	1.17	59.33	68.17	82.50	61.83	88.83	84.67	86.83	82.83	16.83	−10.78	0.86	70	65
D82 VC74F-6	0.50	4.50	17.00	23.17	38.50	57.00	47.00	62.17	36.50	8.67	23.02	0.95**	8	65
D85 C1-7	1.67	9.33	27.50	45.00	60.83	71.83	89.33	86.83	73.67	21.33	3.79	0.94	35	30
D85 C1-8	1.33	8.33	19.00	24.50	51.00	64.83	51.67	71.00	70.17	6.17	15.72	0.90**	40	40
D85 C1-5	3.83	49.17	61.67	67.83	84.83	91.00	85.50	90.33	61.00	7.83	−7.78	0.94	65	50
US81M36BDAA	2.00	39.33	62.00	75.17	89.17	98.83	87.67	97.17	93.00	10.67	−12.98	0.96**	79	47
H87 DA4-21D	1.83	58.50	65.50	78.17	84.00	93.17	93.17	95.50	84.67	6.17	−13.55	0.92*	100	100
US86 NE6-1	12.50	40.00	56.67	66.00	87.50	90.00	86.33	96.33	92.67	14.67	−11.75	0.96**	65	60
US85 NP14-1	1.67	74.00	80.50	86.33	89.83	95.83	91.00	98.33	96.83	11.33	−20.05	0.98	50	50
US86 NE5-1	0.17	24.50	36.33	47.67	64.17	54.17	73.83	73.67	67.67	29.67	5.34	0.93	0	70
H87 EAP4-15E	4.83	21.67	57.00	78.50	77.17	86.50	87.83	94.83	80.67	N/A	−8.57	0.97*	95	95
D85 SJ4-1	0.83	11.83	51.83	58.50	68.83	75.67	93.50	85.67	61.00	27.17	−0.96	0.95	95	84
D85 SJ13-1	2.17	24.83	44.17	64.17	79.17	80.50	87.33	95.17	73.67	20.67	−4.66	0.99**	79	26
US84MV2SP6	0.00	29.50	57.50	66.00	89.33	88.83	95.33	93.50	75.33	22.00	−9.21	0.98**	42	26
US85 NP11-1	17.17	70.33	52.50	70.17	89.67	96.83	90.83	97.50	92.17	20.50	−17.25	0.91**	47	42
H87 DA4-21D	14.67	48.83	63.33	70.83	87.00	88.83	85.83	88.00	82.00	20.00	−12.41	0.95**	100	100
MEX 3-1	6.83	65.50	72.67	78.50	93.83	98.17	96.67	96.83	83.33	13.00	−18.01	0.91**	75	75
US85 NP10-1	4.67	19.67	6.67	30.17	31.00	59.50	73.50	79.33	76.00	5.00	13.97	0.83*	58	32
US82 NP10-4	7.33	39.17	58.83	86.00	70.83	89.00	92.33	93.83	86.67	1.17	−10.00	0.94*	35	30
Average of test races	3.31	35.94	48.34	65.68	77.97	83.51	82.79	86.37	69.37	12.77	−3.86	0.98		

[a] Mean difference of specified test race and race 0 over all ridge heights.
[b] Pearson correlation coefficient between test races and race 0 ($P \leq 0.05$, $P \leq 0.01$).
[c] Virulence ratings signify the percentage of differential *Phaseolus vulgaris* lines/cultivars with: A, pustules as the primary reaction (i.e., spore production from any size uredinia); and B, pustules greater than 300 μm diameter (i.e., intermediate to large pustules). Identification of races 38, 39, 40, 41, 48, and 49 were published previously (7). All other races, including race 0, were identified as described in Materials and Methods.

Chemical Co., St. Louis, MO) vapor for 20 min in covered plastic petri dishes to counteract the germination self-inhibitor. Urediniospores were deposited at a density of about 700 spores per squared centimeter onto test membranes in a spore settling tower. To improve spore adhesion, the spore-laden membranes were atomized lightly with distilled water, left at high humidity for 10 min, and air-dried. The membranes were then placed spore side down on distilled water in plastic petri dishes, incubated at 18 C in the dark for 6 h, and mounted in 0.01% toluidine blue in glycerol on glass slides. The membranes were examined and photographed with either phase contrast or differential-interference contrast microscopy with a Zeiss Photomicroscope III (Zeiss, Carl, Inc., Thornwood, NY). For SEM examination, the membranes with germlings were fixed in 2% aqueous osmium tetroxide overnight at 5 C, dehydrated in a graded ethanol series, critical point-dried, and coated with gold palladium. Specimens were observed with a Hitachi S-530 SEM (Hitachi America Ltd., Tarrytown, NY) operated at 25 kV.

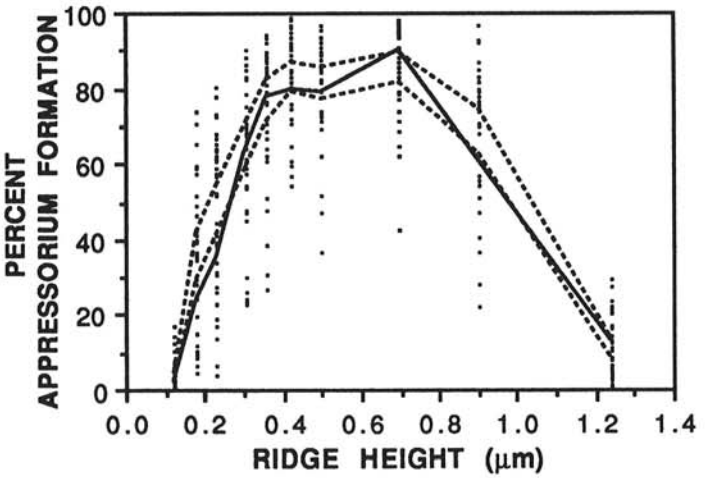


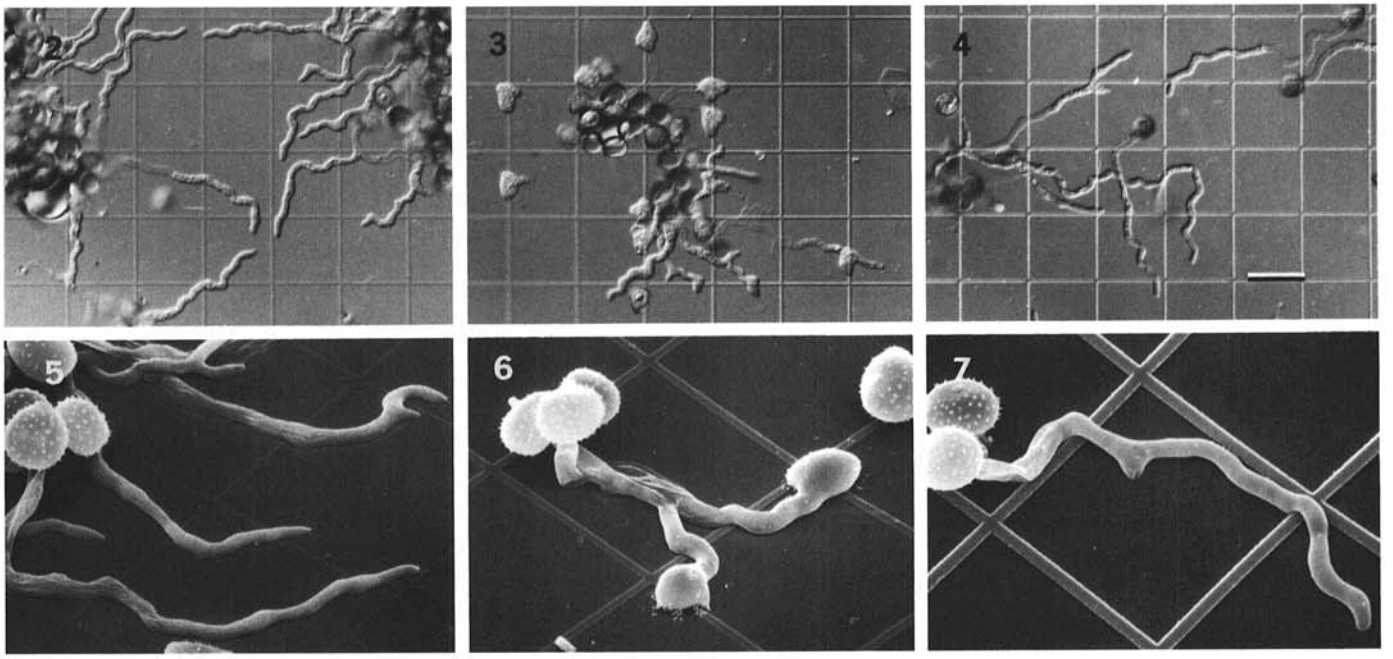
Fig. 1. Appressorium formation by 40 races of *Uromyces appendiculatus* on 10 different ridge heights. Points represent mean values for individual races. Dotted lines show 95% confidence intervals for the mean response of all test races. Solid line represents the mean of repeated tests of race 0.

Experimental design. Six different races, including a sample of race 0, were tested on each test day. Three replicates of each of the 10 ridge heights were included for each race tested. On each replicate membrane 200 germlings were examined, and the percentage of differentiation (appressorium formation) on the ridges was recorded. Means and standard deviations were calculated. Raw data was tested with the Wilk-Shapiro test for normality (5) and was determined to be normal in most cases. Non-normality generally was due to replicate counts being very similar or identical. Paired Student's t tests were computed between the average value of repeated tests of race 0 and averages of test races. Pearson correlation coefficients were obtained for each test pair. Correlations were also calculated between appressorium formation at each ridge height and virulence ratings for each race. Levene's test of homogeneity of variance (6) was used to compare the variation observed within test races with that observed in repeated tests of race 0.

RESULTS

Germination of urediniospores was observed on the full range of test membranes. Some test races exhibited low or no germination and were excluded from the study. Germ tubes adhered closely to the polystyrene substrate and exhibited random growth orientation on smooth areas of the plastic membranes. On membranes bearing 2.25 and 6.7 μm high ridges, germ tubes exhibited close adherence to the membrane except on the front side of the ridge where a gap was formed between the germ tubes and the substrate. Appressorium formation was only observed in association with ridges of appropriate heights. This was further confirmed in subsequent tests on a random selection of 10 races grown on silane-treated glass coverslips where no appressorium formation was observed. Glass coverslips were used as a control surface because they were smooth, and they lacked a topography that might trigger appressorium formation.

The responses of all test races and that of race 0 were similar in overall response to the various ridge heights (Fig. 1). The general trend of the differentiation response after 6 h of growth showed few appressoria formed on 0.12 μm high ridges (Figs. 2 and 3), increasing to a maximum of about 80% on ridges between 0.4 and 0.8 μm high (Figs. 4 and 5). As ridge height increased further, the percentage of differentiation decreased to levels averaging



Figs. 2–7. Light (Figs. 2–4) and scanning electron microscopic (Figs. 5–7) micrographs of *Uromyces appendiculatus* urediniospore germlings grown for 6 h on microfabricated polystyrene membranes. **2 and 5,** germling growth over 0.12-μm-high ridges. Appressoria were not induced by this topography. **3 and 6,** germling growth and appressorium formation on 0.7-μm-high ridges. **4 and 7,** germling growth, without appressorium formation over 1.24-μm-high ridges. Figs. 2–4, magnification bar = 50 μm; Figs. 5–7, magnification bar = 20 μm.

12% on 1.24 μm high ridges (Figs. 6 and 7). In subsequent tests with randomly selected races (chosen for having demonstrated relatively high levels of appressorium formation on 1.24 μm high ridges), no appressorium formation was observed on membranes with ridges 2.24 and 6.7 μm high.

The mean values of race 0 tests were highly correlated with the means of the test races, although variation was observed in the mean differences between the race responses and that of race 0 (Table 1). Overall, the response of race 0 was slightly lower (mean difference of −3.86%) than the average of all test races.

Variation was observed in the response of both the test races and repeated tests of race 0 to specific ridge heights (Figs. 1 and 8). The greatest range of variation was evident on suboptimal ridges (e.g., 0.18, 0.23, and 0.9 μm). Race D85 C1-5 and race 49 exemplified extreme differences in response to the test ridges (Fig. 8). However, in view of the variation in the responses observed in multiple tests of a single race (race 0), these extremes would likely diminish if races D85 C1-5 and 49 were also tested repeatedly. Tests for homogeneity of the variance observed within test races and that observed in repeated tests of race 0 showed no significant differences at the 95% level. Where extreme variation was observed, outlying points could usually be attributed to faulty experimental conditions. Spot checks of test membranes generally showed the polystyrene ridges to be accurately replicated and of the proper height. Occasionally, however, flawed ridges or otherwise poor membrane surfaces affected appressorium formation. Membrane flaws usually caused an increase in ridge heights or created inductive imperfections resulting in aberrant appressorium formation away from the ridges. Appressoria that formed on such flaws were not recorded.

The correlation between the two virulence ratings (Table 1) and the level of appressorium formation on the 10 ridge heights were all very low (Table 2) indicating a poor relationship, if any, between these factors.

DISCUSSION

The results of this study show that 39 races of *U. appendiculatus* sense and respond similarly to topographic features inductive for appressorium formation. Because the races tested in the study were collected from diverse geographic locations over a period of more than 20 yr, and represent varying levels of pathogenicity, it is likely that most, if not all races of *U. appendiculatus*, would respond similarly. *U. appendiculatus* does not form appressoria on smooth surfaces, but requires an abrupt change in substrate elevation, either up or down, to induce appressorium formation (3). The magnitude of the change relates directly to the effectiveness of the signal to induce the development of appressoria. The results of this study indicate that the optimal height for *U. appendiculatus* ranges from 0.4 to 0.8 μm; ridges above and below this height range are less inductive. These results show a slightly expanded optimal height range compared to that reported by Hoch and co-workers (3), likely resulting from the greater number of ridge heights tested in this investigation.

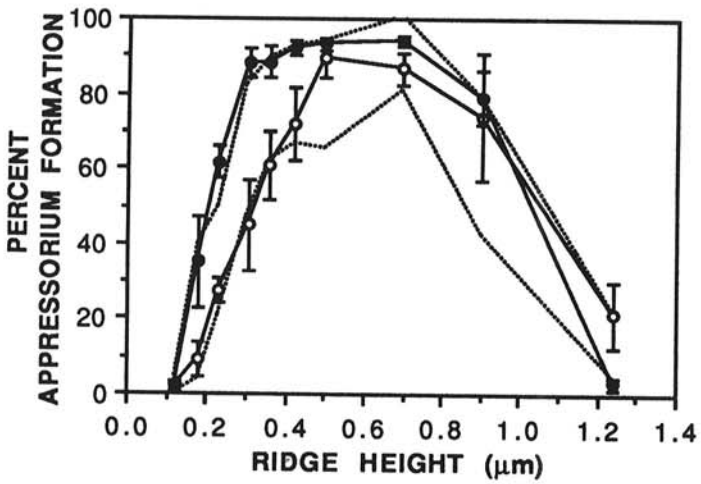


Fig. 8. Appressorium formation by two *Uromyces appendiculatus* races (○, race D85 C1-5; ●, race 49) on 10 different ridge heights representing response extremes. Dotted lines show the standard deviation of repeated tests of race 0.

The responses of the test races were highly correlated with the average response of repeated tests of race 0. The differences observed among the test races were largely quantitative, affecting the overall magnitude of the differentiation response curves. These differences may have been due to experimental variables such as spore age or storage conditions, or to flaws in the test membranes, because the variability that did occur among the test races fell within the range of responses exhibited by race 0. As a result of this common variation, it would be difficult to separate an unknown race of *U. appendiculatus* from race 0 on the basis of its thigmotropic response alone.

The slightly lower level of appressorium formation exhibited by race 0 is likely indicative of the natural variation in thigmotropic sensitivity inherent in *U. appendiculatus*. The choice of race 0 as a standard was not made with any previous information regarding its sensitivity to ridge height. Any other race could have been used and would likely have demonstrated its own variability and overall level of response. The biological significance of this variation is currently unknown. Observations of germ tube growth by *U. appendiculatus* races on bean leaf surfaces reveal no apparent differences in their ability to locate and form appressoria over stomata in spite of differences in stomatal and epidermal cell characteristics (E. A. Allen, *unpublished*). Although the inductive signal associated with stomata has not been conclusively identified, there is increasing evidence that it involves the stomatal guard cell lip and/or ledge (3,11,12). The relationship between thigmotropic sensitivity and infection success could be logistically tested further when host material exhibiting consequential differences in stomatal architecture has been identified.

Many other genera of rust fungi demonstrate quite different responses to the topographical signals used in this study (1). In addition to quantitative differences as observed with *U. appendiculatus*, other genera exhibited qualitative differences with significant variation in the upper and lower limits of inductive ridge heights. By comparison, the responses of all of the *U. appendiculatus* races were very uniform.

In summary, a variety of races of *U. appendiculatus* that were from diverse geographic locations and that exhibited varying degrees of virulence on *P. vulgaris* hosts showed a uniform response to signal height for appressorium formation. This finding is a necessary prerequisite if we are to logically seek *Phaseolus* material for breeding purposes in which stomatal lips are of heights that do not induce appressorium formation. The results of this study are also relevant in the identification of inductive topographies, other than stomata, that could provide a disruptive prepenetration signal, preventing the fungus from entering the leaf and establishing infection. A number of important questions pertaining to the development of race nonspecific resistance to

TABLE 2. Correlation (Pearson *R* coefficients) between virulence ratings and *Uromyces appendiculatus* test race responses on 10 ridge heights

Ridge height (μm)	Virulence rating	
	A	B
0.12	0.105	0.222
0.18	0.086	0.231
0.23	0.335	0.346
0.31	0.421	0.368
0.36	0.374	0.337
0.42	0.320	0.291
0.5	0.316	0.242
0.7	0.319	0.193
0.9	−0.020	−0.001
1.24	−0.052	−0.070

bean rust arise from this study. Foremost is a greater understanding of the precise parameters of the inductive signal on the leaf, their variation in bean germ plasm, and their relationship with the in vitro model system used in this study.

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