Factors Affecting Infection Cushion Development by *Rhizoctonia solani* on Cotton

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R. H. Garber, U. S. Cotton Research Station, Shafter, CA, contributed a generous supply of cotton seeds for use in this study. S. T. Nameth performed the gnotobiotic experiment. The authors thank L. Blakely and F. Roth for reading the manuscript. The Cal Poly Unit Kellogg Foundation, Inc., the Faculty Development Program of California State Polytechnic University, Pomona, and the California Planting Cotton Seed Distributors provided funds.

Accepted for publication 1 October 1986.

ABSTRACT

Armentrout, V. N., Downer, A. J., Grasmick, D. L., and Weinhold, A. R. 1987. Factors affecting infection cushion development by *Rhizoctonia solani* on cotton. Phytopathology 77: 623-630.

Infection cushions were produced by *Rhizoctonia solani* AG-4 on the excised hypocotyls of cotton seedlings grown in the light, but not on those of seedlings grown in the dark. Hypocotyls of dark-grown seedlings bathed in sucrose solution supported cushion formation. Cushions formed on intact light-grown seedlings under gnotobiotic conditions. Internodes (distances between lateral branches) were much shorter in hyphae growing on light-grown seedling hypocotyls than in hyphae growing in agar culture. This internode shortening was not elicited in agar culture by hypocotyl exudate, whether from light- or dark-grown plants. Internode shortening did occur on replicas of the hypocotyl surface. Hyphal alignment, foot formation, and branching to form a cushion-like structure also took place on hypocotyl replicas of either light- or dark-grown plants in the presence of exudate or sucrose. Hyphal aggregations that formed on cellophane appressed to hypocotyls did not resemble cushions. These results were interpreted as signifying that the principal stimulant in infection cushion formation by *R. solani* is the topography of the hypocotyl surface. Methyl glucose (MEG) inhibited cushion formation, but not if applied after hyphae had adhered to the hypocotyl surface. MEG caused a depression in disease severity similar to that resulting from the use of low-nutrient inoculum, but did not inhibit growth of *R. solani* in culture. The MEG effect may be attributed to interference with mucilage formation and adherence, so that physical contact with the hypocotyl surface is inadequate to stimulate cushion formation.

Additional key words: chemodifferentiation, exudates, infection structure, mucilage, thigmodifferentiation.

Rhizoctonia solani Kühn (= *Thanatephorus cucumeris* (Frank) Donk) AG-4 penetrates cotton seedlings by means of an infection cushion. The cushion is a complex structure that is highly branched and that results in penetration of the hypocotyl surface via many hyphal tips within 24 hr after inoculation (2,33). It resembles the typical dome-shaped cushion produced by many different isolates and anastomosis groups of *R. solani* on various hosts and under a variety of conditions.

The ubiquity of the fungus and the intricacy of this infection structure have elicited numerous studies of the factors influencing development of the infection cushion. Most of these studies have examined the traditional dichotomy between two types of stimulants to infection structure formation, i.e., whether the infection cushion is produced by the fungus upon recognition of the host surface by chemical stimuli (exudates) or whether the stimulus for infection cushion formation is the physical nature, especially the topography, of the surface. The most common conclusion is that plant exudates serve as the stimulus for infection cushion formation (13,22,29). There has also been support, however, for a contact stimulus for this process (8,15,23).

Evidence has been accumulating for the importance of contact with the plant surface in production of appressoria by fungi. Although host exudates alone apparently induce appressoria in *Colletotrichum piperatum* E & E (E & H) (18), most *Colletotrichum* spp. require a contact stimulus as well as a nutrient source for appressorium formation (see review by Staples and Macko, 27). Contact stimuli have been shown to elicit appressorium formation in *Uromyces phaseoli* (Pers.) Wint (35) and in *Puccinia graminis* f. sp. *tritici* Eriks & E. Henn (26), although chemical stimuli have been shown to be effective in induction of appressorium formation by these two rust fungi under experimental conditions (17,25). Most rust fungi produce appressoria on membranes in a fashion similar to *U. phaseoli* (27). After a review of current evidence regarding preinfection development by fungi, Wynn and Staples (36) concluded that

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surface contact is the major stimulus. Resolution of the relative importance of such stimuli and of plant exudates to infection cushion formation by *R. solani* is desirable in the light of these recent findings.

In a previous study (2), we described the events occurring during the development of the infection cushion of *R. solani* on the cotton hypocotyl. The present study was undertaken to determine whether a stimulant for cushion formation was present in exudates of uninoculated cotton plants. In addition, we examined the behavior of *R. solani* on replicas of the plant surface. The inhibitory effect of 3-*O*-methyl glucose on infection cushion formation by *R. solani*, as described by Weinhold and Bowman (31), was also reexamined.

MATERIALS AND METHODS

R. solani AG-4 (ATCC 60734) was maintained on potato-dextrose agar (Difco Laboratories, Inc., Detroit, MI) at 25 C. Inoculum for experiments consisted of disks of mycelium cut from cultures grown in still culture on liquid Medium A (32), as previously described (3). For some experiments, the final glucose concentration in Medium A was 0.11 M (20 g of glucose per liter); however, for most experiments, the concentration was 0.1 M glucose. Agar was added (1.5% w/v) for slide culture assays.

Cotton, *gossipium hirsutum* L., cultivar Acala 4-42, was used for the methyl glucose experiments. For all other studies, the cotton cultivar SJ-2 was used. No differences were observed in the behavior of *R. solani* on these two cultivars. Seedlings were generally grown in a growth chamber with fluorescent illumination (G.E. cool-white bulbs; 60 μ Em^{-2} sec^{-1}). These light-grown plants were used for most experiments unless otherwise stated. For some experiments, seedlings were grown without illumination (dark-grown plants) but were otherwise treated similarly. Seven-day-old seedlings were used for all experiments.

Five replicates were prepared for each treatment, and most experiments were repeated once. Results were analyzed by means of an analysis of variance (one-way) or Student's *t* test, with means compared by Fisher's lsd at $P = 0.05$.

Hypocotyl and seedling experiments. Detached cotton hypocotyls were prepared and inoculated with *R. solani* as previously described (2). All five replicates for an experiment were aseptically placed in a sterile moist chamber (moistened Whatman No. 1 filter paper in 14.5-cm petri plates) and inoculated. The chambers were sealed with Parafilm (American Can Co., Greenwich, CT) and incubated for 24 hr at 25 C without illumination.

Infection cushion formation by *R. solani* was compared on hypocotyls of light- and dark-grown plants inoculated and incubated in the presence of sterile water only and on hypocotyls of dark-grown plants to which sterile sucrose solution (0.1 M) was applied at the time of inoculation. Infection cushion rating was scored as follows: 0 = no cushions; 1 = 1–2 cushions; 2 = 3–10 cushions; 3 = extensive cushion development (more than 10 cushions present on one hypocotyl). The hypocotyls were immersed in 0.5% trypan blue in 50% acetic acid for 10 min in preparation for observation. They were photographed by reflected light with a Zeiss Standard 14 compound microscope. An external lamp was used to illuminate the surface of the hypocotyl.

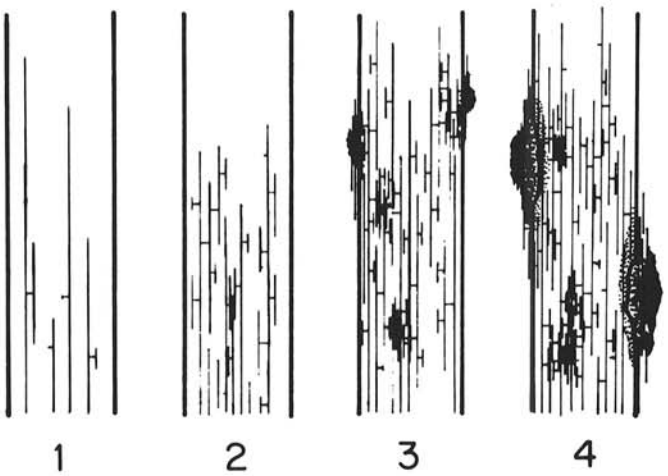


Fig. 1. Disease Severity Index used in rating methyl glucose experiments. **1,** Hyphae are present but with little branching evident. **2,** Characteristic branching of hyphae is observed. **3,** Extensive branching and infection cushion initials are present. **4,** Fully formed infection cushions subtended by a lesion are observed.

Detached hypocotyls were inoculated after wrapping in cellophane. Cellophane was sterilized by irradiation with an ultraviolet lamp (Westinghouse 630T8; λ_{max} = 254 nm) for approximately 10 min. The cellophane was pressed snugly over detached hypocotyls, which were placed on 1.5% water agar in a petri plate. Inoculum plugs of *R. solani* were placed directly on the cellophane over the hypocotyl and incubated for 24 hr. Detached hypocotyls without cellophane coverings were inoculated as a check. The cellophane was lifted from the hypocotyl, immersed in trypan blue stain for 10 min, then placed on a microscope slide for observation.

Hypocotyls were wounded by piercing them with sterile 26-gauge hypodermic needles after surface sterilization, then were inoculated and incubated.

Gnotobiotic cotton plants were grown from surface-sterilized seeds planted aseptically in sterile UC mix (4); this was in a clay pot enclosed in a Nalgene tub and covered with another inverted tub, all of which were autoclaved before the seeds were planted. The entire system was placed in an illuminated growth chamber; the translucent Nalgene tub transmitted enough light for normal development of the seedlings. The seedlings were inoculated with *R. solani*, the system resealed, then incubated in the growth chamber for 24 hr. Portions of the hypocotyl were removed aseptically to verify the lack of bacterial contamination, then the seedlings were examined for infection cushions.

Methyl glucose studies. The standard treatment to examine the effects of 3-*O*-methyl glucose (MEG) on infection cushion development by *R. solani* consisted of the application of a solution of 25 mM MEG and 5 mM glucose to the hypocotyls of intact cotton seedlings in individual styrofoam cup sand cultures (2), using a syringe with spray nozzle attachment, just before inoculation. Inoculum consisted of disks of *R. solani* grown on Medium A with 0.11 M glucose. For each experiment, some seedling hypocotyls were sprayed with deionized water for the control treatment. The inoculated seedlings were incubated for 24 hr in a growth chamber at 28 C and 95% relative humidity and under fluorescent illumination.

Inoculated hypocotyls were stained with trypan blue and rated according to the following disease severity index (DSI): 0 = no contact of the hypocotyl by hyphae of *R. solani*; 1 = hyphae present but with little branching evident; 2 = characteristic branching observed; 3 = extensive branching and infection cushion initials present; and 4 = fully formed infection cushions subtended by a lesion (Fig. 1).

Scanning electron microscopy (SEM) was performed as previously described (2).

The development of *R. solani* infection cushions when inoculum was grown on Medium A containing varied amounts of glucose (5, 10, 15, and 20 g of glucose per liter) was compared with the MEG effect; MEG was applied to hypocotyls of seedlings that were inoculated with mycelium grown on Medium A at 20 g of glucose per liter (0.11 M).

The effect of 3-*O*-methyl glucose on growth of *R. solani* in vitro was examined by comparing dry weights of mycelium from liquid Medium A cultures in which various concentrations of MEG plus glucose were substituted for the standard (0.11 M) glucose concentration (Table 1). Cultures were incubated for 5 days at 25 C; mycelial mats were oven dried for 48 hr at 100 C and weighed.

The effect of delaying application of MEG until various intervals after inoculation was examined. Controls, in which hypocotyls were sprayed with deionized water at the same intervals after inoculation, were designed to detect the effect of forceful washing

of hyphae by spray applications.

Exudates and replicas. Hypocotyl exudates were collected from both light- and dark-grown plants. To collect the exudates, the seedlings were rinsed free of soil and the hypocotyls only immersed in 280 ml of glass-distilled water in a glass finger bowl. The cotyledons and radicles were held out of solution by two supporting Dalron (Cadillac Plastic Co., Chino, CA) rods on each end of the finger bowl (Fig. 2). The entire apparatus was placed in a glass moisture chamber and on a magnetic stirrer, under a bank of fluorescent lights (light-grown plants) or in a dark enclosure (dark-grown plants) for 3 hr at 22–25 C. The solution with exudate was then filtered (Whatman glass microfibre No. 934-AH), concentrated under reduced pressure, and frozen. Pooled exudate collections were filter-sterilized (Millipore No. 047, 0.45 μm, Millipore/Continental Water Systems, Bedford, MA) and the carbohydrate concentration determined by the anthrone procedure (19). The pooled exudates were each adjusted to a final concentration of 58 glucose equivalents per liter with distilled water.

Replicas of the hypocotyl surface of light- or dark-grown plants, or microscope slides ("flat replicas") were prepared by the method of Wynn (35), using Silastic 382 Medical Grade Elastomer (Dow Corning Corp., Midland, MI) to produce negative replicas. A few drops of the hardener was added to the Silastic, with rapid mixing; then detached hypocotyls, prepared as previously described, were pressed into the elastomer. The resulting negative images were washed with deionized water for 5 min, then with 95% ethanol, 100% acetone, and finally with deionized water. Multiple positive replicas were prepared by brushing on a polystyrene solution (5 g of polystyrene petri plate fragments dissolved in 25 ml of chloroform). Several positive polystyrene replicas were then made and discarded. The replicas used for experiments could thus be considered to be free of contamination from the hypocotyl surface. Replicas were surface sterilized by UV irradiation for 5 min before use. Hypocotyl replicas of both light- and dark-grown plants were affixed to stubs for SEM and observed after sputter-coating as previously described (2). A millimeter rule was used to measure the width of epidermal cells from the negatives.

To study the effects of various substances on *R. solani* on replica surfaces, five identical hypocotyl replicas (made from the same negative mold) were placed in a sterile moist chamber; 3 ml of the test substance was pipetted over the surfaces of the replicas. An inoculum plug was placed directly on each replica. The replicas were incubated for 24 hr at 25 C, then bathed in trypan blue for 5 min and placed on a microscope slide for observation. Photomicrographs were taken by transmitted light. Hyphae distal to the inoculum plug were selected for study. The effects on hyphal alignment, foot formation, and hyphal aggregation of *R. solani* of applying exudate from either light- or dark-grown plants to hypocotyl replicas of either plant were compared with the effects of liquid Medium A or sterile distilled water on similar replicas.

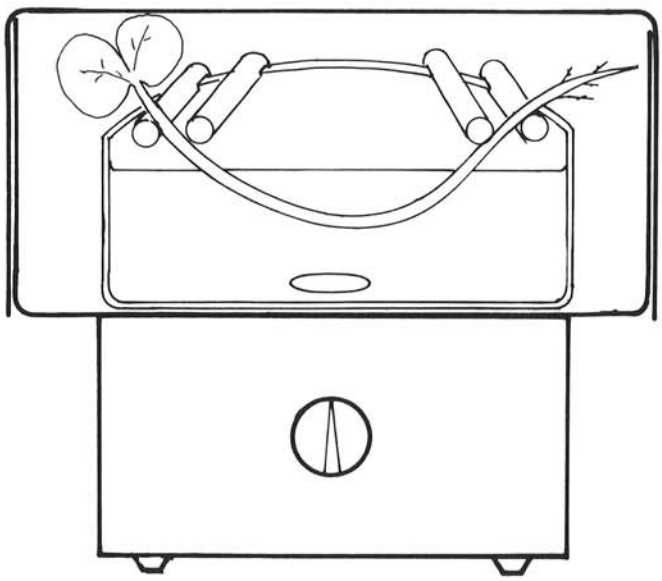


Fig. 2. Apparatus used to collect exudate from cotton hypocotyls. The cotyledons and radicles are held out of the distilled water in which exudate is collected by the Dalron rods on each end of the finger bowl. The entire apparatus is placed in a glass moisture chamber and on a magnetic stirrer, either under a bank of fluorescent lights (light-grown plants) or in a dark enclosure (dark-grown plants). Exudate is collected for 3 hr at 22–25 C.

Branching assays. An assay for the effects of plant exudates on the branching of *R. solani* in culture was performed with slide cultures, as previously described (3). The internode lengths (μm), or distances between branch junctions were measured directly with an ocular micrometer and the Internode Length Unit (ILU), the mean of 10 internodes for each hypha, was used to express the degree of branching (production of lateral branches) quantitatively (3). Similar measurements were made of selected hyphae of *R. solani*, which adhered to the surface of detached hypocotyls of both light- and dark-grown cotton seedlings. The ILU of *R. solani* on hypocotyl replicas (light-grown plants) or on replicas of glass slides (flat replicas) were measured in the presence of exudate from light-grown plants, sucrose (1×10^{-4} M), or sterile distilled water. Hyphae oriented with the grooves of the replica were selected for measurement. Where no grooves were present (flat replicas), major hyphae were measured.

RESULTS

Development of *R. solani* on detached hypocotyls from light-grown plants was similar to that observed when intact cotton seedlings were inoculated with the fungus (2). Hyphal alignment with anticlinal grooves and foot formation led to patterned branching and cushions by 24 hr (Fig. 3). On detached hypocotyls from dark-grown plants, however, hyphal alignment, foot formation, and infection cushions were not observed (Fig. 4). This "dark-grown effect" was reversible by addition of sucrose (Fig. 5); numerous infection cushions formed when 0.01 M sucrose was applied to the surface of detached hypocotyls of dark-grown plants before inoculation with *R. solani* (infection cushion rating = 2.1±1.0), significantly greater than that on dark-grown hypocotyls without sucrose (infection cushion rating = 0.6±0.9).

Wounded, inoculated hypocotyls had prominent cushions surrounding each wound after 24 hr. The area covered by these cushions appeared to be more extensive than that covered by the scattered cushions on nonwounded hypocotyls.

Typical infection cushions were formed by *R. solani* on cotton plants grown and inoculated aseptically. Test cultures confirmed that this gnotobiotic system did not include bacteria on the hypocotyl surface.

Cushions did not form on cotton seedlings inoculated with *R. solani* in the presence of MEG. Instead, a number of relatively unbranched hyphae grew over the surface of the hypocotyl in a

TABLE 1. Effects of 3-*O*-Methyl glucose on growth of *Rhizoctonia solani* in culture

Treatment[z] MEG:Glucose (mM)	Dry weight[y] Experiment I	Experiment II
0:0	2.7 ± 0.8 a	···
25:0	4.1 ± 2.6 b	···
25:5	6.4 ± 0.5 c	···
0:5	7.1 ± 0.5 c	···
0:111	···	71.3 ± 2.5 a
25:111	···	84.5 ± 3.2 b
0:222	···	114.6 ± 5.5 c
111:111	···	115.6 ± 10.5 c

[y] Figures represent the mean and standard deviation of colony dry weights ($g \times 10^3$) of five replicates for each experiment. Values followed by the same symbol within a column are not different according to a one-way analysis of variance and Fisher's lsd ($P = 0.05$).

[z] Treatments consist of varying concentrations (mM) of 3-*O*-methyl glucose: (and) glucose added to the basal salts mixture of Medium A for culture of *R. solani*.

random fashion (Fig. 6). These hyphae were not oriented along the anticlinal grooves of epidermal cells as was invariably observed in non-MEG (control) treatments (Fig. 7); furthermore, they often appeared to adhere very loosely to the plant surface and were easily washed off during staining. The mean DSI for MEG treatments over many trials was 1.8, compared with a mean DSI of 3.9 for control treatments.

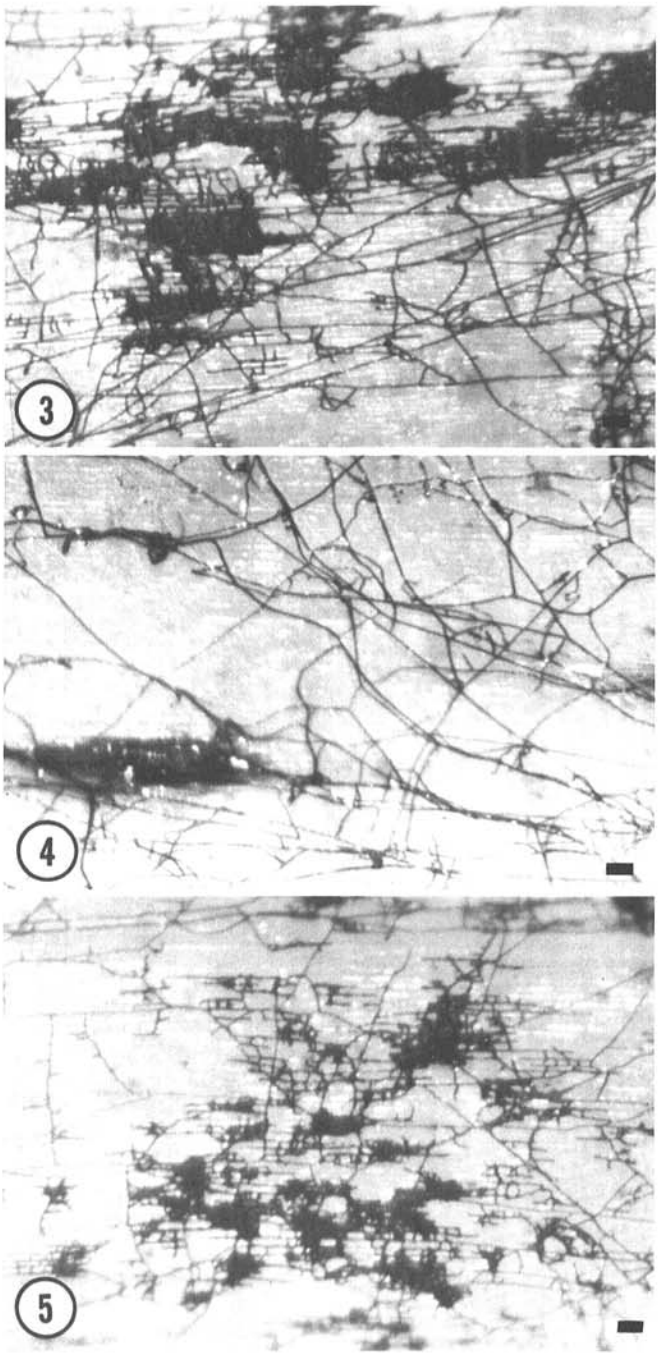
Delay in addition of MEG to inoculated plants reduced the depression in DSI observed when MEG is added at inoculation. Addition of MEG at 10, 12 and 14 hr after inoculation resulted in a lower DSI in comparison with water-washed controls, but addition at 16 hr and thereafter did not (Fig. 8).

Inoculum nutrient level affected the ability of *R. solani* to form cushions on intact seedlings. A lower DSI was observed when plants were inoculated with *R. solani* from cultures grown with less than 2 g of glucose per liter (Table 2). The DSI of plants inoculated with mycelium grown on medium containing 0.5 g of glucose per liter was similar to the DSI of plants that received the MEG treatment but were inoculated with mycelium from a medium containing 2 g of glucose per liter.

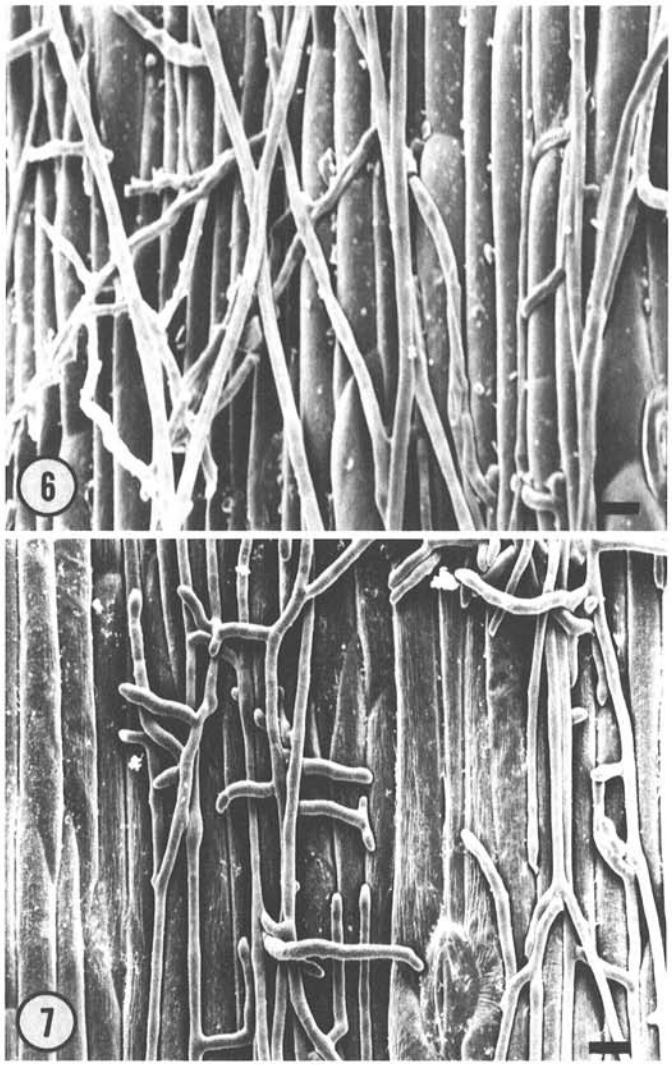
Growth of *R. solani*, as measured by mass, was not inhibited by the presence of MEG in vitro. Cultures grown with MEG in the culture medium either had a slightly higher mass than those grown without MEG, or there was no detectable difference (Table 1).

Branching frequencies (expressed as ILU's) of hyphae of *R. solani* adhering to hypocotyls of light-grown cotton seedlings were much greater than those of hyphae growing over the hypocotyls of plants grown in the dark. Hyphae aligned with the grooves formed by the anticlinal walls of epidermal cells on light-grown plants had ILU's of 109±25 and 119±21 in two experiments, significantly different from ILU's of 175±28 and 204±18 for major hyphae (hyphal alignment was rarely observed on these plants) on dark-grown plants.

Hypocotyl exudates had no special effect on branching behavior of *R. solani* in culture. Branching frequencies (ILU's) of the fungus grown on exudates of light-grown plants were 234±27 and 235±46 in two experiments, while those of the fungus grown on exudates of dark-grown plants were 226±17 and 216±36. These values were not different from one another nor from those obtained by growing the fungus on Medium A (243±12, 262±47). Furthermore, exudate



Figs. 3–5. Cotton seedling hypocotyls 24 hr after inoculation with *Rhizoctonia solani*; bar = 20μm. **3,** Hypocotyl of light-grown plant, note infection cushions. **4,** Hypocotyl of dark-grown plant, infection cushions are absent (the dark area in the left-hand corner is a gland). **5,** Hypocotyl of dark-grown plant to which sucrose (0.1 M) was added at inoculation; infection cushions are present.



Figs. 6 and 7. Cotton seedling hypocotyls (scanning electron microscope) inoculated with *Rhizoctonia solani*; bar = 10 μm. **6,** Methyl glucose-treated hypocotyl, 18 hr after inoculation. Hyphae do not adhere to surface. **7,** Untreated hypocotyl 18 hr after inoculation, showing typical hyphal alignment, branching, and foot formation.

was no more effective than sucrose in eliciting internode shortening on replicas of the hypocotyl surface (Table 3).

Hyphal orientation and foot formation, characteristic of early stages of cushion development, were observed on replicas of the cotton hypocotyl surface inoculated with *R. solani* (Figs. 9 and 10). These behaviors were observed on hypocotyl replicas of either light- or dark-grown plants in the presence of a growth medium, either sucrose or the exudate from light- or dark-grown plants. When *R. solani* was placed on replicas with water only, the fungus grew loosely over the surface of the replica and displayed relatively little orientation in anticlinal grooves (Fig. 11). This behavior of the fungus in the absence of exogenous substrates varied from experiment to experiment. On occasion, some hyphal orientation and foot formation was observed in the presence of water alone on a replica.

There was no difference in mean widths of 10 epidermal cells as measured from hypocotyl replicas of light- (13.6 ± 1.8 μm) or dark-grown plants (14.4 ± 2.5 μm).

The branching frequency was greater for hyphae of *R. solani* that were aligned with the grooves in hypocotyl replicas than for those on flat (glass slide) replicas (Table 3). The presence of an exogenous nutrient source (sucrose or hypocotyl exudate) generally promoted this increased branching frequency. Several repetitions of the experiment shown in Table 3 yielded inconsistent results, as seen by the significantly lower branching frequency (higher ILU) for the water-only treatment (Experiment I) or the similar branching frequency for water and nutrient treatments (Experiment II). A higher branching frequency (lower ILU) was generally observed when hyphae aligned with the grooves of replicas in water-only treatments.

Cushion-like structures sometimes formed when *R. solani* was incubated on hypocotyl replicas of light-grown plants in the presence of exudate or Medium A for 48 hr. Such structures displayed hyphal alignment and foot formation, together with some elaboration of branches (Fig. 12).

Hyphal aggregations formed when *R. solani* was grown on cellophane placed over detached hypocotyls. There was no hyphal alignment or foot formation; however, these aggregations were often characterized by many short branches (Fig. 13).

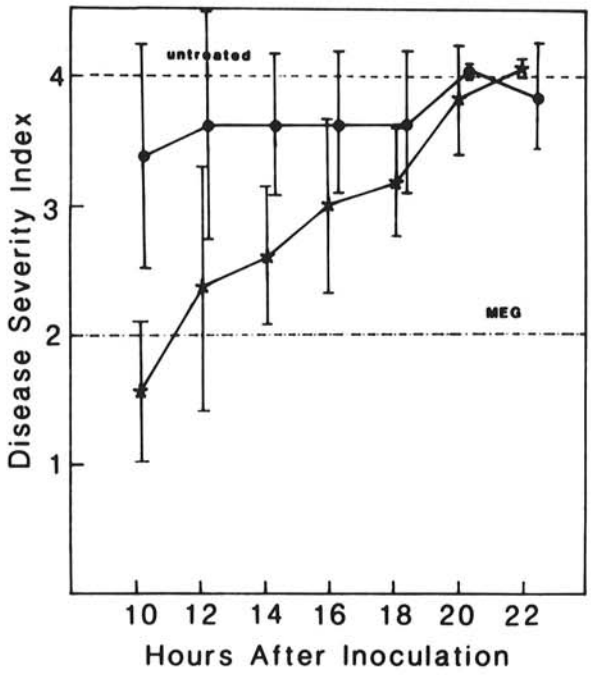


Fig. 8. Graph comparing the effect on disease severity (DSI) of delayed addition of methyl glucose (MEG) (*) to cotton hypocotyls, compared with controls water-washed at the same time intervals (•) and to the standard MEG treatment and untreated control (dashed lines). DSI values for MEG or water-washed treatments were not significantly different at 16 hr and thereafter, by a one-way analysis of variance and Fisher's lsd ($P = 0.05$).

DISCUSSION

There is much recent interest in the factors that regulate the formation of infection structures by fungi (27,36). Many studies have emphasized the relative importance of two possible stimuli to infection structure formation: physical factors, such as contact with features of the plant surface, and chemicals available on the surface. Responses of fungi to contact stimuli have been termed "thigmodifferentiation," and to chemical factors, "chemodifferentiation" (28). Infection cushion formation by *R. solani* has largely been considered to be chemodifferentiation, attributed to chemical stimulants present in plant exudates: "Infection cushion formation is stimulated by chemical, not topographical, factors" (29). In contrast, thigmodifferentiation of infection structures of many fungi has been described recently (26,27,36). The present study examined the separate importance of contact and chemical stimuli to infection cushion development.

There is a voluminous literature that supports the role of plant exudates in infection cushion formation by *R. solani*. The production of "cushions" on membranes overlying plant roots or hypocotyls has been strong presumptive evidence that infection cushions are formed by *R. solani* in response to host exudates (11,13,22,29). This stimulation of cushion formation on membranes was not correlated, however, with susceptibility of the plant cultivar used (11), although a cellophane membrane technique has been used to assay susceptibility in reference to exudate loss by older seedlings (29). The cushions formed, where illustrated (11,13,22), appear to be hyphal aggregations without the orderly form characteristic of infection cushions. In the present study, we also observed hyphal aggregations in *R. solani* placed on cellophane overlying cotton hypocotyls (Fig. 13), but the aggregations did not resemble infection cushions (2). There was

TABLE 2. Effects of inoculum nutrition level on disease severity of *Rhizoctonia solani* on cotton seedlings

Treatment[y]	Disease severity index[x]	
	Experiment I	Experiment II
0.5	1.6 ± 0.9 a[z]	2.0 ± 1.2 ab
1.0	2.8 ± 0.8 b	2.4 ± 0.5 b
1.5	3.0 ± 1.0 b	3.4 ± 0.9 c
2.0	3.4 ± 0.5 b	4.0 ± 0.0 c
2.0/MEG	1.4 ± 0.5 a	1.2 ± 0.4 a

[x] Disease severity index: 0 = no contact of the hypocotyl by hyphae of *R. solani*; 1 = hyphae present but with little branching evident; 2 = characteristic branching observed; 3 = extensive branching and infection cushion initials present; and 4 = fully formed infection cushions subtended by a lesion.

[y] Treatments indicate varying levels (g/L) of glucose in Medium A. In addition, a solution of 25 mM 3-*O*-methyl glucose (MEG) and 5mM glucose was applied at the time of inoculation for the last treatment.

[z] Figures represent the means and standard deviations of five replicates. Values followed by the same symbol within an experiment are not different at $P = 0.05$.

TABLE 3. Internode lengths[x] of *Rhizoctonia solani* on polystyrene replicas

Treatment[y]	Experiment I		Experiment II	
	Ridged[z]	Flat	Ridged	Flat
Sucrose	114 ± 27 a	163 ± 25 ab	157 ± 21 a	256 ± 27 b
Exudate	132 ± 40 a	235 ± 39 bc	124 ± 27 a	256 ± 37 b
Water	202 ± 27 b	278 ± 26 c	161 ± 17 a	326 ± 46 c

[x] Figures represent the mean ILU (Internode Length Units = mean in μm of 10 internodes for one hypha) and their standard deviations for two experiments. Values followed by the same symbol within an experiment are not different according to a one-way analysis of variance and Fisher's lsd ($P = 0.05$).

[y] Treatments consist of solutions of 0.1 mM sucrose, exudate from hypocotyls of light-grown cotton plants, or distilled water, applied to replicas just before inoculation with *Rhizoctonia solani*.

[z] Polystyrene replicas, either of cotton hypocotyls (ridged) or of microscope slides (flat).

neither hyphal alignment, foot formation, nor any indication of penetration pegs, although there were numerous short side branches (Fig. 13); a similarly increased production of lateral branches is observed when the fungus is grown on cellophane overlaying a nutrient medium (3). Flentje et al (13) commented that no penetration occurred from the cushions formed on cellophane in their experiments. It may be that the cushions described in many studies as forming on membranes are simply aggregations resulting from the increased production of lateral branches (a thigmodifferentiation by the fungus in response to the flat surface).

Formation of cushions on membranes over exudate collected from plants was the major basis for the hypothesis that exudates contain a stimulant to infection cushion formation by *R. solani* (13). In those experiments, collodion was placed over agar blocks containing exudate. Formation of hyphal aggregations was taken as positive evidence for the stimulant. Controls consisted of water agar blocks. There were no other controls, such as a nutrient medium incorporated into the blocks. Further, these tests did not demonstrate the appropriate specificity between isolate and plant species (13). This lack of such specificity in plant exudates was noted by Da Silva and Wood (8) and does not agree with the concept of a specific stimulus.

Much of the difficulty in resolving the importance of exudates in infection cushion formation has resided in the definition of this structure. In many previous studies, it has simply been reported that cushions formed under a given set of conditions; apparently any aggregation of hyphae that formed a discrete clump was considered to be an infection cushion. The steps leading to the development of the infection cushion on the cotton hypocotyl were described in a previous study (2). We have defined cushion formation for experimental purposes as a series of special behaviors based on those steps, i.e., alignment of hyphae on the hypocotyl, internode shortening, short branch and foot formation, elongation of feet to form new axial hyphae, and formation of an orderly aggregation of hyphae by repetition of the previous steps. Each of these was then used as a testable quality in the study of effects of exudates or host surface on cushion morphogenesis. Internode shortening was assayed quantitatively. Shorter internodes, shown as ILU's (3), are an indication of increased branching frequency and are characteristic of cushion formation.

We found no evidence that a stimulant with a specific morphogenetic effect on *R. solani* is present in the exudate of heathy cotton plants. Exudate did not promote internode shortening in culture and was no more effective than sucrose in eliciting any of the test behaviors on replicas of the hypocotyl surface, including internode shortening (Table 3).

The lack of development of infection cushions on plants grown in the dark might be attributed either to a change in the quality or quantity of the exudate from these plants, or to an alteration in the physical characteristics of the surface, as compared with plants

Figs. 9–13. Various surfaces 24 hr (unless otherwise described) after inoculation with *Rhizoctonia solani*; bar = μm. **9,** Polystyrene replica of the cotton hypocotyl, to which sucrose solution was added before inoculation (note hyphal alignment). **10,** Polystyrene replica of the cotton hypocotyl, to which sucrose solution was added before inoculation; note foot formation. **11,** Polystyrene replica with water only; note lack of adherence and hyphal alignment. **12,** Polystyrene replica with concentrated cotton hypocotyl exudate added, 48 hr after inoculation (cushion-like structure shows alignment, branches, feet). **13,** Cellophane removed from a hypocotyl, showing hyphal aggregation and short lateral branches.

grown in the light. The internode-shortening abilities of exudates of equal concentrations from light- and dark-grown plants were identical, which indicates that a stimulant of branching is not present in the exudates of light-grown plants. Because hyphal alignment and foot formation were observed on hypocotyl replicas of both light- and dark-grown plants when a substrate (including hypocotyl exudates of either light- or dark-grown plants) was present, it appears that the lack of cushion development on these plants cannot be attributed to altered surfaces on dark-grown plants. It seems a reasonable assumption that exudates from cotton seedlings that do not undergo photosynthesis would not be as rich in nutrients (sugars and amino acids) as those from light-grown plants. The hypothesis that the plant exudate has only a nutritional (not a stimulatory) role is further supported by the observation that cushions will form on dark-grown hypocotyls bathed in sucrose (Fig. 5).

A chemical stimulant could possibly be produced by microflora on the hypocotyl surface. Exudates serve as a substrate for the growth of bacteria, which we observed in micrographs of the plant surface. Appressorium formation in some *Colletotrichum* spp. has been shown to be enhanced by the presence of phylloplane bacteria (24). The results of the gnotobiotic experiment, in which infection cushions formed on plants grown and inoculated aseptically, indicate that such interaction with microflora is not responsible for infection structure formation by *R. solani*.

Alignment of hyphae with the grooves formed by the anticlinal walls of epidermal cells appears to be facilitated by the production of mucilage, which enables the hyphae to adhere to the surface of the hypocotyl (12). Mutants of *R. solani* unable to adhere to the hypocotyl, therefore unable to form infection cushions, have been isolated (1,44). A mucilage-like material has been described by microscopy (2,12,20) and has also been inferred in this and other studies by the tenacity with which hyphae of *R. solani* cling to the plant surface. In treatments where such tight adherence did not occur (such as with MEG and on dark-grown plants) hyphae did not align with topographical features of the surface, but wandered over it.

Mucilage production by *R. solani* may be dependent on a local exogenous source of nutrients. The methyl glucose effect, in which infection cushion formation was inhibited, but growth on the hypocotyl (31) or in culture (Table 1) was not, could be explained by an interference by MEG of the uptake of nutrients (plant exudates) available locally so that mucilage production was prevented. MEG is known to have the same transport system as glucose in some fungi (30). The MEG effect was not observed when MEG is added after hyphae of *R. solani* have adhered to the surface and branching was well under way (Fig. 8). Addition of sucrose to the hypocotyl surface of dark-grown seedlings permitted alignment and cushion formation to occur. Hyphal alignment was generally observed on hypocotyl replicas only where a nutrient source (sucrose or exudate) was supplied, although this was not consistent. The effects of nutrients in promoting internode shortening on replicas (Table 3) were also variable. Weinhold et al (32) have shown the nutrition available from the inoculum itself to be important in infection of cotton by *R. solani*, although they used lesion area, not cushion development, as a gauge. Our results with development measured as DSI were similar (Table 2). The inhibition of cushion development after addition of MEG also resembled the effect of growing inoculum on a low-nutrient medium (Table 2). It is possible that nutrients in the inoculum and local exogenous nutrients are additive; repeated attempts to determine the effective concentration of sucrose or glucose required for adherence yielded variable results (10). We attributed this to a variation in the amount of medium remaining in the inoculum plug.

Alignment with grooves promotes branching (internode shortening) in *R. solani*. Internodes were dramatically shorter on hypocotyls of light-grown seedlings than in culture, although branching frequency in agar culture is consistent over a range of carbon source or nutrient concentration (3). Grooves appear to be a highly effective stimulus to internode shortening: internodes were shorter on replicas of the hypocotyl surface than on flat polystyrene replicas (Table 3). A similar result was obtained with hyphae of *R. solani* aligned with the grooves on scratched Parafilm (3).

Other behaviors characteristic of early cushion development (2) were observed on replicas, including the "feet," T-shaped short branches (Fig. 11). A further elaboration of branches to form infection cushions was rarely observed on replicas in our study, although sufficient branching to suggest a cushion was sometimes observed after a 48-hr incubation of *R. solani* on replicas (Fig. 12).

One aspect of the host-parasite interaction that has not been taken into account in studies on the stimulation of infection cushion formation is the possible effect of polygalacturonase produced by *R. solani* during the prepenetration period on cotton (6,7). This enzyme is known to cause damage to plant protoplasts and a resulting permeability increase (34). Leakage of electrolytes from bean hypocotyls has been reported within 12 hr of treatment with the polygalacturonase from *R. solani* (21), and loss of pectic substances from walls of cotton hypocotyls has been detected within 15 hr after inoculation (33). This early leakage of materials from plant cells could have an influence on the proliferation of branches and the further development of the infection cushion. Wounding has been found to cause formation of massive cushions (13), as we also observed. Flentje et al (13) noted further that cushions formed more readily on areas where infection cushions had already been formed and removed.

Resolution of the role of exudates in infection cushion formation by *R. solani* has been complicated by the lack of information about them. Few studies on this question have included a characterization of the exudate. Whole plants or hypocotyls with roots and/or seeds or cotyledons attached have been used (7,8,13) for collection of exudate rather than the tissue (hypocotyls) on which infection cushions are formed. We used intact cotton plants (held [Fig. 2] so that cotyledons and roots could not make a contribution to the exudate) and illuminated them during the collection period, minimized collection time to retard bacterial growth, and filter-sterilized the concentrated exudate. The concentration of the exudate used should, ideally, be the same as that at the plant surface. Flentje et al (13) obtained their most dramatic results by using blocks of agar that had been placed alongside the plant surface, which presumably yielded a higher concentration of materials than other (washing) methods. We did not characterize the components of the exudate, nor determine the concentration of exudate per hypocotyl, although a standardized concentration (as determined by anthrone assay) was used in all tests.

The importance of the plant surface to development of infection cushions by *Rhizoctonia* spp. has been indicated by some other studies. Infection cushions did not form when *R. tuliparum* Whetzel & Arthur (15) or *R. solani* (23) were inoculated onto roots, which lack a cuticle. Infection cushions of *R. tuliparum* (16) or *R. solani* (9) took on a different form, according to the plant species on which they were produced. Removal of "wax deposits" from resistant rice cultivars allowed formation of infection cushions by *R. solani* (22). Only appressoria were formed on the surface of tomato fruit resistant to *R. solani* (5), whereas cushions were formed on fruits of a susceptible cultivar. Results of these last two studies could be interpreted to be a consequence of a thick cuticle, which either prevented exudates from reaching the fungus or obscured the topography of the surface, so that the appropriate contact stimulus was lacking. Studies with replicas should be less ambiguous; Marshall and Rush (22) found that cushions did not form on replicas of susceptible rice cultivars. However, they did not supply nutrients. We found that a nutrient medium was generally necessary to elicit alignment, internode shortening, and foot formation by *R. solani* on replicas. Da Silva and Wood (8) found that infection cushions formed on epidermal strips of the specific plant host to which each of three isolates were pathogenic. Exudates of any of the plants were equally effective at supporting this cushion formation.

Our data are consistent with the following model of the interaction of *R. solani* and the cotton plant to produce an infection cushion. Contact with the plant surface, especially with

its ridges and grooves, stimulates internode shortening, production of short side branches, and the formation of feet. The grooves also support the further elongation of feet. Plant exudates may provide the nutrient necessary for production of mucilage with which the fungus can adhere tightly enough to the surface for a contact stimulus to be effective. The early development of the infection cushion is thus an example of thigmodifferentiation, not of chemodifferentiation as has been believed (13,22,29).

LITERATURE CITED

1. Anderson, N. A., and Stretton, H. M. 1978. Genetic control of pathogenicity in *Rhizoctonia solani*. Phytopathology 68:1314-1317.
2. Armentrout, V. N., and Downer, A. J. 1987. Infection cushion development by *Rhizoctonia solani* on cotton. Phytopathology 77:619-623.
3. Armentrout, V. N., Downer, A. J., and Nameth, S. T. 1986. A simplified branching assay for *Rhizoctonia solani*. Mycologia 78:657-663.
4. Baker, K. F. 1957. The U.C. System for Producing Healthy Container-Grown Plants. Calif., Agric. Exp. Stn. Serv. Man. 23. 332 pp.
5. Bassi, A., Jr., Moore, E. L., and Batson, W. E., Jr. 1979. Histopathology of resistant and susceptible tomato fruit infected with *Rhizoctonia solani*. Phytopathology 69:556-559.
6. Brookhouser, L. W., Hancock, J. G., and Weinhold, A. R. 1980. Characterization of endopolyglacturonase produced by *Rhizoctonia solani* in culture and during infection of cotton seedlings. Phytopathology 70:1039-1042.
7. Brookhouser, L. W., and Weinhold, A. R. 1979. Induction of polygalacturonase from *Rhizoctonia solani* by cotton seed and hypocotyl exudates. Phytopathology 69:599-602.
8. Da Silva, R. L., and Wood, R. K. S. 1964. Infection of plants by *Corticium solani* and *Corticium praticola*–Effect of plant exudates. Trans. Br. Mycol. Soc. 47:15-24.
9. Dodman, R. L., Barker, K. R., and Walker, J. C. 1968. A detailed study of the different modes of penetration by *Rhizoctonia solani*. Phytopathology 58:1271-1276.
10. Downer, A. J. 1983. Factors affecting infection cushion formation in *Rhizoctonia solani*. M.S. thesis. California State Polytechnic University, Pomona.
11. El-Samra, I. A., El-Faham, Y. M., and Kamara, A. M. 1981. Selective induction of infection cushions by *Rhizoctonia solani* in relation to host responses. Phytopathol. Z. 102:122-126.
12. Flentje, N. T. 1957. Studies on *Pellicularia filamentosa* (Pat.) Rogers. III. Host penetration and resistance, and strain specialization. Trans. Br. Mycol. Soc. 40:322-336.
13. Flentje, N. T., Dodman, R. L., and Kerr, A. 1963. The mechanism of host penetration by *Thanatephorus cucumeris*. Aust. J. Biol. Sci. 16:784-799.
14. Flentje, N. T., Stratton, H. M., and McKenzie, A. R. 1967. Mutation in *Thanatephorus cucumeris*. Aust. J. Biol. Sci. 20:1173-1180.
15. Gladders, P., and Coley-Smith, J. R. 1977. Infection cushion formation in *Rhizoctonia tuliparum*. Trans. Br. Mycol. Soc. 68:115-118.
16. Gladders, P., and Coley-Smith, J. R. 1979. Host infection by *Rhizoctonia tuliparum*. Trans. Br. Mycol. Soc. 72:251-260.
17. Grambow, H. -J., and Riedel, S. 1977. The effect of morphogenically active factors from host and non-host plants on the in vitro differentiation of infection structures of *Puccinia graminis* f. sp. *tritici*. Physiol. Plant Pathol. 11:213-224.
18. Grover, R. K. 1971. Participation of host exudate chemicals in appressorium formation by *Colletotrichum piperatum*. Pages 509-518 in: Ecology of Leaf Surface Micro-Organisms. T. F. Preece and C. H. Dickinson, eds. Academic Press, London.
19. Hassid, W. Z., and Abraham, S. 1957. Chemical procedures for analysis of polysaccharides. Pages 34-50 in: Methods in Enzymology, V. III, S. P. Colowick and N. 0. Kaplan, eds. Academic Press, New York, NY.
20. Kenning, L. A., and Hanchey, P. 1980. Ultrastructure of lesion formation in *Rhizoctonia*-infected bean hypocotyls. Phytopathology 70:998-1004.
21. Lai, M. -T., Weinhold, A. R., and Hancock, J. G. 1968. Permeability changes in *Phaseolus aureus* associated with infection by *Rhizoctonia solani*. Phytopathology 58:240-245.
22. Marshall, D. S., and Rush, M. C. 1980. Infection cushion formation on rice sheaths by *Rhizoctonia solani*. Phytopathology 70:947-950.
23. Murray, D. I. L. 1982. Penetration of barley root and coleoptile surfaces by *Rhizoctonia solani*. Trans. Br. Mycol. Soc. 79:354-360.
24. Parbery, D. G., and Blakeman, J. P. 1978. Effect of substances associated with leaf surfaces on appressorium formation by *Colletotrichum acutatum*. Trans. Br. Mycol. Soc. 70:7-19.
25. Staples, R. C., Grambow, H. -J., and Hoch, H. C. 1983. Potassium ion induces rust fungi to develop infection structures. Exp. Mycol.7:40-46.
26. Staples, R. C., Grambow, H. -J., Hoch, H. C., and Wynn, W. K. 1983. Contact with membrane grooves induces wheat stem rust uredospore germlings to differentiate appressoria but not vesicles. Phytopathology 73:1436-1439.
27. Staples, R. C., and Macko, V. 1980. Formation of infection structures as a recognition response in fungi. Exp. Mycol. 4:2-16.
28. Staples, R. C., Macko, V., Wynn, W. K., and Hoch, H. C. 1983. Terminology to describe the differentiation response by germlings of fungal spores. Phytopathology 73:380.
29. Stockwell, V., and Hanchey, P. 1983. The role of the cuticle in resistance of beans to *Rhizoctonia solani*. Phytopathology 73:1640-1642.
30. Teh, J. S. 1975. Glucose transport and its inhibition by short-chain *n*-alkanes in *Cladosporium resinae*. J. Bacteriol. 122:832-840.
31. Weinhold, A. R., and Bowman, T. 1974. Repression of virulence in *Rhizoctonia solani* by glucose and 3-*O*-methyl glucose. Phytopathology 64:985-990.
32. Weinhold, A. R., Bowman, T., and Dodman, R. L. 1969. Virulence of *Rhizoctonia solani* as affected by nutrition of the pathogen. Phytopathology 59:1601-1605.
33. Weinhold, A. R., and Motta, J. 1973. Initial host responses in cotton to infection by *Rhizoctonia solani*. Phytopathology 63:157-162.
34. Wood, R. K. S. 1976. Killing of protoplasts. Pages 105-116 in: Biochemical Aspects of Plant-Parasite Relationships, J. Friend and D. R. Threlfall, eds. Academic Press, London.
35. Wynn, W. K. 1976. Appressorium formation over stomates by the bean rust fungus: Response to a surface contact stimulus. Phytopathology 66:136-146.
36. Wynn, W. K., and Staples, R. C. 1981. Tropisms of fungi in host recognition. Pages 45-69 in: Plant Disease Control. R. C. Staples and G. H. Toenniessen, eds., John Wiley & Sons, New York.