THE MECHANISM OF HOST PENETRATION
BY THANATEPHORUS CUCUMERIS

By N. T. Flentje,* R. L. Dodman,* and A. Kerr*

[Manuscript received May 24, 1963]

Summary

Evidence is presented in support of the hypothesis that chemical stimuli control the development of infection structures on the host surface by T. cucumeris. On the stem of a young radish seedling there are discrete, susceptible areas which become fewer and eventually disappear as the seedling ages. Rubbing the stems of radish seedlings with or without an organic solvent increases the number of infection cushions formed by a crucifer-attacking isolate, and returns an older resistant seedling to the susceptible condition. No infection cushions form on strips of cuticle and epidermis removed from the host.

The susceptibility of several hosts to a number of isolates is closely correlated with the stimulation of these isolates by seedling roots enclosed in "Cellophane". In vitro investigations on "Cellophane" membranes show that root exudates from different hosts stimulate the growth of specialized and non-specialized isolates, and that under these conditions there is no correlation between specificity and stimulation.

Crucifer-attacking isolates form infection cushions on artificial membranes covering intact radish seedlings, stem, or cotyledon pieces. Using a bioassay technique with collodion membranes mounted on van Tieghem rings, it is shown that exudates from radish stems and cotyledons stimulate infection cushion formation. This is the first direct evidence that the stimulant is a natural constituent of the host plant and is not produced by an interaction between host and pathogen.

I. INTRODUCTION

The mechanisms by which fungi penetrate the surfaces of their hosts are still poorly understood and in recent years investigation of this aspect of pathogenicity has been neglected. Yet it is possible that in many interactions, resistance or susceptibility is determined at this early stage, prior to or at entry, rather than at a later stage in pathogenesis. It is also likely that there are important differences in penetration mechanisms between those fungi which are wound pathogens and those which penetrate through natural openings or intact host surfaces.

From the early work in this field, which has been reviewed by Flentje (1959), Dickinson (1960), and Wood (1960), arises the generally accepted concept that the stimulus for direct penetration of a host is contact with a solid surface. Recently Emge (1958), using Puccinia graminis var. tritici, and Pavgi and Dickson (1961), using Puccinia sorghi, presented evidence showing that infection-like structures developed on membranes without the addition of specialized nutrients. Chatterjee

* Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide.

HOST PENETRATION BY THANATEPHORUS CUCUMERIS

(1958) found that spores of *Ustilago hordei* germinated and penetrated both susceptible and resistant barley varieties and Dutta, Hall, and Heyne (1960) showed that several strains of *Colletotrichum lagenarium* penetrated a number of cucurbits, even though a differential resistance resulted after penetration had occurred. These results add further support to the hypothesis that penetration is dependent on a contact stimulus. However, Brown (1934) has pointed out that all fungi may not behave in this way and that the hypothesis may require modification.

Flentje (1957) has shown that specialized strains of *Thanatephorus cucumeris* (Frank) Donk. [= *Pellicularia filamentosa* (Pat.) Rogers] react differently on different hosts. A crucifer strain forms infection cushions on the stems of crucifer seedlings and penetrates directly through the cuticle from these structures, whereas the same strain fails to react on solanaceous hosts. Similarly, a solanaceous strain reacts on solanaceous hosts, but not on crucifers. None of the strains tested react on inert surfaces such as glass, gold leaf, or collodion. It was suggested that the theory of a contact stimulus was inadequate to explain this situation and that the development of infection cushions may be dependent on a chemical stimulus. Using various hosts, Kerr (1956) has shown that a marked aggregation of hyphae of *T. cucumeris* develops on the outside of "Cellophane" bags, directly opposite seedling roots enclosed within. Kerr and Flentje (1957), using several different hosts and strains, have briefly reported that by means of this "Cellophane" bag technique a close correlation between susceptibility and stimulation can be demonstrated. They also stated that a similar correlation can be demonstrated *in vitro* using a "Cellophane" bioassay technique. This paper presents a fuller account of these results and also provides further evidence in support of the hypothesis that *T. cucumeris* produces infection structures in response to chemical stimuli.

II. GENERAL METHODS

The source and pathogenic specificity of the isolates of *T. cucumeris* and *T. praticulos* (Kotila) Flentje used are listed in Table 1. Cultures were grown on potato-marmite-dextrose agar (P.M.D.A.) (Flentje 1956).

The following hosts were used: *Raphanus sativus* L. (radish, cv. Long White Iciele), *Lycopersicon esculentum* Mill. (tomato, cv. South Australian Early Dwarf Red), *Lactuca sativa* L. (lettuce, cv. Imperial Triumph).

Radish seedlings for pathogenicity tests and for exudate collection were grown for 10 days in washed coarse sand in a constant-environment cabinet with a day length of 12 hr, a light intensity of 2500 f.c., a day temperature of 22°C, and a night temperature of 19°C. The pots were watered daily with nutrient solution (Hoagland and Arnon 1950). For pathogenicity tests seedlings were removed from pots, washed free of sand in distilled water, laid on microscope slides (either one or two seedlings per slide), and secured by rubber bands about 1 cm from each end of the slide. Free water was removed from around the seedlings and slide by touching with absorbent tissue. Each slide was then placed on glass rods in a Petri dish with the seedling roots dipping into water in the bottom of the dish. A drop of melted "Vaseline" was placed over the base of each seedling to prevent water creeping up around the seedling stem from the bottom of the dish.
Each seedling was inoculated with an inoculum block 10 by 4 by 2 mm, cut from close behind the growing edge of a 3–4-day-old fungal culture, and placed about 3 mm from each seedling stem. Dishes containing seedlings were then incubated in the dark at 25°C. In preliminary experiments it was shown that the reaction of T. cucumeris was similar on radish seedlings incubated either in the dark or under day and night conditions.

In investigations of the reactions of T. cucumeris on artificial membranes, collodion membranes were made as follows: Collodion solution was prepared by dissolving 0.4 g of dried nitrocellulose powder (HP 3.50) in 85 parts by volume of ether (A.R.) and 15 parts by volume of absolute ethanol (A.R.). A sheet of membrane was made by spreading 1.5 ml of this solution on polished plate-glass 6 by 8 in. and allowing the solvents to evaporate. While on the glass the large sheet of collodion was cut into pieces which were floated off by slowly lowering the glass into a dish of distilled water. The outer edges were discarded and the remaining pieces separated from each other ready for use. The whole procedure was carried out in a constant-temperature laboratory to ensure that membranes were as uniform as possible.

“Cellophane” membranes were cut from sheets of moisture-proof “Cellophane” 20 μ thick, boiled in distilled water for 30 min, and autoclaved at 15 lb pressure for 20 min.
III. INVESTIGATION OF THE FUNGAL REACTION ON THE HOST SURFACE

(a) Formation of Infection Cushions on Discrete Areas of Seedlings

In previous experiments it was noticed that infection cushions were irregularly distributed over seedling stems. To investigate if the distribution was determined by the host, radish seedlings were mounted on microscope slides as described and a strip of squared paper mounted alongside each seedling. Isolate 68, which is pathogenic to crucifers, was used to inoculate six pairs of seedlings and after 24 hr the seedlings were examined at 12-hr intervals.

Hyphae from the inoculum plugs attached themselves firmly to the cuticle surface, grew along the length of the seedlings following the lines of junction of the epidermal cells, and then formed infection cushions freely. These were beginning to develop after 36 hr, and after 48 hr their distribution was mapped on the squared paper at the side of each seedling. Although the hyphae had covered the whole stem, infection cushions were not evenly distributed but were in patches scattered over approximately one-third of the area of the stem.

![Diagram](image)

Fig. 1.—Diagrammatic representation of the distribution of infection cushions on radish stems following consecutive inoculations with isolate 68.

Inoculum plugs, hyphae, and cushions were removed without damaging the stem surfaces and the few seedlings in which infection had already occurred were discarded. The seedlings were then reinoculated, incubated at 25°C for a further 48 hr, and the distribution of infection cushions plotted again (Fig. 1). There was a marked tendency for infection cushions to form on the same patches of the stems as previously. It is difficult to account for this reaction on the basis of a contact stimulus and it is thus suggested that there are discrete areas where the materials stimulating infection cushion formation are diffusing through the cuticle from the cells below.

(b) Effect of Seedling Age on Infection Cushion Formation

Damping-off diseases are usually important on young seedlings and as seedlings age they become resistant to infection. This is true for stem-attacking strains of *T. cucumeris*. The reaction of a crucifer strain of *T. cucumeris* on radish seedlings of different ages was examined to determine what changes occur as seedlings become resistant.
Radish seedlings, grown for 2, 4, and 6 weeks, were mounted on glass slides. The seedlings 2 weeks old had fully developed cotyledons, but no true leaves; those 4 weeks old had 1–2 leaves; those 6 weeks old had 2–3 leaves. From each age group, eight seedlings were inoculated with isolate 68 and after 1 day the seedlings were examined daily for 3 days. On seedlings of all ages, hyphae attached themselves to the surface and grew along the stems following the lines of junction of underlying epidermal cells. However, subsequent formation of infection cushions differed with seedlings of different ages (see Table 2, untreated) and it would seem that the most satisfactory explanation of these differences depends on a chemical stimulation of infection cushion formation.

**Table 2**

**REACTIONS OF T. CUCUMERIS ISOLATE 68 ON RADISH SEEDLINGS OF DIFFERENT AGES WITH DIFFERENT STEM TREATMENTS**

<table>
<thead>
<tr>
<th>Stem Treatment</th>
<th>Reaction of Isolate 68 on Seedlings:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 Weeks Old</td>
</tr>
<tr>
<td>(1) Untreated</td>
<td>After 36–48 hr, 10–20 infection cushions per seedling. No increase after 96 hr. Penetration and infection</td>
</tr>
<tr>
<td>(2) Rubbed with dry cotton-wool</td>
<td>After 24–36 hr, 25–35 infection cushions per seedling. No increase after 96 hr. Penetration and infection</td>
</tr>
<tr>
<td>(3) Rubbed with cotton-wool plus chloroform</td>
<td>After 98 hr, 1–3 infection cushions per seedling. Penetration and infection</td>
</tr>
<tr>
<td>(4) Rubbed with cotton-wool plus water</td>
<td>After 98 hr, 1–3 infection cushions per seedling. Penetration and infection</td>
</tr>
</tbody>
</table>

(c) *Effect of Surface Treatment of Seedlings on Infection Cushion Formation*

The results of the previous experiment suggested that as seedlings age the materials stimulating the formation of infection cushions are no longer present on the stem surface. Since it had been shown that on young seedling stems there are discrete areas where infection cushions form, it seemed possible that these areas are due to the uneven secretion of wax on the cuticle surface and that in older seedlings the secreted wax forms a continuous impermeable layer which prevents exudation.
Several stem treatments were used to determine their effect on the reaction of the fungus to the host. Radish seedlings, grown for 2, 4, and 6 weeks, were separated at random into four groups for each seedling age and subjected to one of the following treatments:

(1) The first group was left untreated.
(2) The stems of the second group were drawn once through a pad of dry cotton-wool held firmly in the fingers.
(3) The stems of the third group were similarly rubbed with a pad of cotton-wool to which several drops of chloroform had been added.
(4) The stems of the fourth group were rubbed with a pad of cotton-wool moistened with water.

The seedlings were then fixed to slides and eight seedlings from each of the above treatments from each age group were inoculated with isolate 68. After 24 hr the seedlings were examined at 12-hr intervals for 72 hr.

On seedlings of all ages, and with all treatments, hyphae attached themselves to the surface and grew along the stems following the lines of junction of underlying epidermal cells. Subsequent formation of infection cushions differed for different treatments with different seedling ages and these results are given in Table 2.

It is known that surface waxes are easily removed (Hall and Jones 1961) and thus it seems likely that treatment of stems with cotton-wool, either dry or moistened with chloroform, removes some of the wax deposit from the cuticle surface, allowing a more rapid development of a greater number of infection cushions on seedlings 2 and 4 weeks old and also causing seedlings 6 weeks old to become susceptible again. Resistance of older seedlings would thus seem to be due to the development of this waxy layer, rather than to the cessation of production of materials stimulating the formation of infection cushions.

The results following treatment (4) are not readily explained. It is possible that moist cotton-wool, instead of removing the cuticular wax, spreads it over the surface of the stem, thereby covering many of the potential infection sites. In addition, moisture left on the seedling from the cotton-wool may lead to dilution of materials stimulating the formation of infection cushions.

\[(d) \ \textbf{Effect of Tissue Thickness on Infection Cushion Formation}\]

To determine the effect of removing the cells underlying the epidermis, radish seedlings were subjected to two treatments. Firstly stems were cut along their length so that the section increased in thickness from the epidermis and cuticle at one end to the full diameter of the stem at the other end. These sections were mounted on slides and inoculated with isolate 68. Secondly, strips of cuticle and epidermis approximately 30 by 2.5 mm were removed from radish stems and floated on water on long rectangular coverslips. From the coverslips they were drawn, cuticle side up, on to distilled water-agar blocks 10 by 2 by 2 mm so that each strip covered most of the top of a block but folded down 0.5–1 mm over one side. The blocks were then placed singly on microscope slides and inoculated with isolate 68 with the inoculum nearest
the side over which the strip of cuticle and epidermis was folded. The stem sections and epidermal strips were incubated and examined as previously described.

On the strips of cuticle and epidermis hyphae became attached and grew along the lines of junction of epidermal cells, but no infection cushions developed. On the stem sections hyphae became attached and followed the lines of cell junction and after 48–60 hr infection cushions developed where the underlying tissue was four to five cells or more thick, whereas none formed at the thinner end of the section. Penetration occurred only below infection cushions. These results strongly suggest that infection cushion formation is dependent on a chemical stimulus and indicate that more than a single layer of cortical cells is required to provide sufficient material to stimulate the development of infection cushions.

These results together with the previous work (Flentje 1957; Kerr and Flentje 1957) provide strong but indirect evidence that the formation of infection cushions by *T. cucumeris* is stimulated by materials diffusing through the cuticle from underlying cells. This hypothesis was further developed by an investigation of the reactions of various isolates of *T. cucumeris* to a number of different seedlings and exudates covered with artificial membranes.

IV. Investigation of the Fungal Reaction on Artificial Membranes

Kerr (1956) has shown that lettuce and radish seedlings enclosed in “Cellophane” bags and planted in soil inoculated with *T. cucumeris* cause a marked stimulation of the fungus on the outside of the “Cellophane” directly adjacent to the roots growing within the bags. This stimulation was investigated to determine if it is relevant to infection.

(a) “Cellophane” Bag Experiments

Different isolates of *T. cucumeris* and *T. praticolus* were tested against radish, lettuce, and tomato using the “Cellophane” bag technique. The results are given in Table 3 along with data on the pathogenicity of the strains to the test plants. A quantitative measurement could not be devised and an arbitrary scale was adopted for measuring stimulation.

There were 18 combinations of pathogenic fungus and susceptible host and in 13 of these the fungus was stimulated. The only other case of stimulation was that of isolate 79 which is non-pathogenic on radish. This demonstrates a close correlation between susceptibility of seedlings to the various isolates and stimulation of the isolates by seedlings. Whenever marked fungal stimulation occurred, the seedling roots directly opposed to the areas of stimulation were damaged, radish roots being severely blackened, and lettuce and tomato roots becoming brown and necrotic. It was also observed that the hyphae aggregated on the “Cellophane” in a manner resembling infection cushion development.

As there was no physical contact between seedlings in the “Cellophane” bags and mycelium in the surrounding substrate, the specificity of the reactions must have been due to diffusible materials. Either the isolates responded differently to exudates from roots of the three test plants, or the seedlings reacted differently to the metabolic products of the fungi.
(b) In vitro Reaction to Root Exudates on “Cellophane”

The reactions of various isolates of *T. cucumeris* to exudates from radish and lettuce were investigated to determine the importance of exudates in the “Cellophane” bag interactions.

Exudates from radish and lettuce seedlings were collected as follows: Seeds were surface-sterilized with equal parts by volume of ethanol (95%) and hydrogen peroxide (20 vols.), washed in several changes of sterile distilled water, and plated out on distilled water-agar. After 2 days, uncontaminated seedlings were transferred to sterile petri dishes containing 10 ml sterile glass-distilled water (20–25 seedlings per dish and a total of 200 radish and 1000 lettuce seedlings). After incubation for 5 days in the dark at 25°C, the seedlings and the liquid in which they had been growing were transferred to a sterile beaker and left for 1 hr. In this way, all seedlings were covered with liquid and exudate was washed off any roots which had not been covered with liquid while in the dishes. When this liquid was poured off, the seedlings were rinsed with sterile glass-distilled water which had been previously transferred from dish to dish to rinse out any remaining exudate. This liquid was combined with the rest of the exudate, which was then dried by rotary evaporation under vacuum at temperatures below 40°C. The dried material was dissolved in a small volume of water and the solution passed through an “Oxoid” bacterial membrane filter into a weighed, sterile tube. The sterile exudate was then re-dried in a vacuum desiccator, and its dry weight determined. Sterile glass-distilled water was then added to give a 10% (w/v) solution.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate No.</th>
<th>Radish</th>
<th>Lettuce</th>
<th>Tomato</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Patho-</td>
<td>Root</td>
<td>Patho-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>genicity</td>
<td>Exudate</td>
<td>genicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>of Isolate</td>
<td>Stimulation</td>
<td>of Isolate</td>
</tr>
<tr>
<td><em>T. cucumeris</em></td>
<td>16</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>68</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>78</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>T. pratensis</em></td>
<td>81</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>
The reaction of various isolates of *T. cucumeris* to these exudates was determined as follows: Concentric rings were cut in 2% distilled water agar in 9-cm petri dishes (15 ml agar per dish), using cork borers of internal diameters 7 and 12 mm. The agar between the cuts was removed, leaving an "island" with a volume of approximately 0·1 ml separated from the rest of the agar by a "moat" 2·5 mm wide. Using an "Agla" micrometer syringe, 5-ml aliquots of the material to be tested were added to sterile filter paper disks (6 mm in diameter) and one disk placed on top of each agar island. The agar in each petri dish was then covered with a "Cellophane" membrane (8 cm in diameter). Using a cork borer with a diameter of 7 mm, circular plugs of inoculum were cut from just behind the advancing edge of cultures growing on P.M.D.A., and placed on top of the "Cellophane" in the centre of each petri dish. The technique is illustrated in Figure 2.

![Diagram of the technique used for the bioassay of root exudates on "Cellophane".](image)

Isolates 56, 68, and 81, which had responded differently to radish and lettuce seedlings in the "Cellophane" bag experiments, were each used to inoculate three dishes, each dish containing two replicates of radish exudate, lettuce exudate, and sterile glass-distilled water. The dishes were incubated at 25°C until the "Cellophane" membranes were covered with mycelium. Each membrane was then lifted off and a comparison made, both before and after staining with lactophenol cotton blue, between the amount of fungal growth over the exudates and that over distilled water.

It was found that growth of all three isolates was stimulated by both radish and lettuce root exudates. These results do not agree with those reported by Kerr and Flentje (1957) for a similar experiment where only one of the three strains tested responded to radish root exudate. In the earlier experiment root exudate was collected by growing seedlings in sterilized sand and expressing the solution from the sand through "Visking" membrane in a pressure membrane apparatus (Richards 1941). It was later found that "Visking" membrane contained material which stimulated, inhibited, or had little effect on the growth of various isolates of *T. cucumeris*. At least some of the specificity reported in the earlier paper could be attributed to this material.

The results with the "Cellophane" bag experiments and the *in vitro* testing of root exudates were conflicting in regard to specificity. Furthermore, although the
reaction on "Cellophane" over root exudates and actual infection cushions were sufficiently alike to suggest a common stimulus, they were sufficiently different to indicate that either the nature of the "Cellophane" surface was unsatisfactory for infection cushion development or that root exudates contained materials not present in stem exudates.

Further investigation was directed toward finding a membrane suitable for infection cushion formation using stem and cotyledon exudates in preference to root exudates.

(c) Reaction to the Stems of Whole Radish Seedlings covered with "Cellophane" or Collodion

Kerr and Flentje (1957) developed a bioassay technique, using epidermal strips from radish stems, and showed that a crucifer strain formed infection cushions on these strips only in the presence of radish exudates. The use of this technique was discontinued because of difficulties associated with obtaining large numbers of strips and keeping them free of bacterial contamination. It was also thought that a membrane of non-living material would be preferable to the epidermal strips. Thus further testing with "Cellophane" was continued and the suitability of collodion investigated.

"Cellophane" and collodion membranes were prepared as described. Radish seedlings were covered with membranes by dipping a seedling into water on which membranes were floating and slowly lifting the seedling beneath a membrane so that it folded around the seedling in a smooth single layer, covering approximately

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Reaction of Radish Seedlings which were:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uncovered</td>
<td>&quot;Cellophane&quot;-covered</td>
</tr>
<tr>
<td>48</td>
<td>Infection cushions and penetration</td>
<td>Increased branching and curling of side-branches, some tendency to form loosely woven structures resembling infection cushions. No penetration</td>
</tr>
<tr>
<td>68</td>
<td>Infection cushions and penetration</td>
<td>Increased branching and curling of side-branches. No infection cushions</td>
</tr>
<tr>
<td>69</td>
<td>Infection cushions and penetration</td>
<td>Increased branching and curling of side-branches. No infection cushions</td>
</tr>
</tbody>
</table>
4 cm of the length of the stem. The seedling was then mounted on a microscope slide, and excess water removed with absorbent tissue. Isolates 48, 68, and 69, which are all pathogenic to crucifers, were each used to inoculate 12 seedlings, 4 being covered with “Cellophane”, 4 with collodion, and 4 being left uncovered as controls. The seedlings were incubated and examined as previously described. The reactions of the various isolates are given in Table 4.

The formation of infection cushions on collodion provided the first demonstration of this reaction on an artificial membrane. Penetration occurred directly from these cushions, suggesting that they were identical with those formed on uncovered seedlings. Although no infection cushions formed on “Cellophane” under these conditions, testing was continued because it seemed possible that the failure to obtain infection cushion formation may have been due to poor contact between the “Cellophane” and seedling stems.

(d) Reaction to Pieces of Radish Stem and Cotyledon covered with “Cellophane” or Collodion

Since it is difficult to obtain stem exudates separate from cotyledon exudates, it was necessary to determine if the materials stimulating infection cushion formation are present in cotyledons as well as stems. A bioassay technique, which was suitable for testing both host tissue and exudates collected from the host, was developed using artificial membranes (Fig. 3).

Collodion membranes were mounted on van Tieghem rings as follows: A van Tieghem ring, supported on a microscope slide, was dipped into water beneath a membrane and then slowly raised under the membrane until clear of the water. The ring was then lifted slowly from the slide, thus allowing the trapped water to escape. Any remaining water was removed with absorbent tissue. “Cellophane” membranes were secured to van Tieghem rings with rubber bands and then autoclaved before use.

Radish stem sections 10 mm long and cotyledon pieces 10 by 3 mm were placed on the underside of “Cellophane” or collodion membranes (as illustrated in Fig. 3), and isolates 48, 68, and 69 allowed to grow from P.M.D.A. inoculum plugs across the upper surface of the membranes. The results are given in Table 5.

Infection cushions formed on cotyledon and stem pieces covered with membranes, indicating that the stimulating materials are exuded from both stems and
cotyledons. On collodion infection cushions formed only over the tissue and penetration occurred from these infection cushions. On "Cellophane" the infection cushions appeared to be identical with those formed on the uncovered host. However, no penetration of the "Cellophane" was observed and also the reaction was not confined to the area over the tissue. For these reasons further testing with "Cellophane" membranes was discontinued.

**Table 5**

**Reactions of T. Cucumeris Isolates on Pieces of Radish Stem and Cotyledon Covered with "Cellophane" or Collodion**

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>&quot;Cellophane&quot;-covered</th>
<th>Collodion-covered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stem</td>
<td>Cotyledon</td>
</tr>
<tr>
<td>48</td>
<td>Curling and clumping. Structures similar to cushions but less tightly woven</td>
<td>Structures very similar to cushions. No penetration</td>
</tr>
<tr>
<td>68</td>
<td>Curling and clumping. No cushions</td>
<td>Slight stimulation of growth. No cushions</td>
</tr>
<tr>
<td>69</td>
<td>Marked curling and clumping. Structures very similar to cushions, but smaller and more loosely woven. No penetration</td>
<td>Typical cushions formed. No penetration</td>
</tr>
</tbody>
</table>

It is possible that the different reactions shown by isolates 48, 68, and 69 on "Cellophane" and collodion (Table 5) are due to these isolates being heterocaryotic. To reduce such variability it seemed advisable to use homocaryotic isolates derived from single nuclei. Flentje, Stretton, and Hawn (1963) and Flentje (unpublished data) have shown that 99 and 96% of basidiospores from isolates 48 and 69, respectively, contain only one nucleus. Thus single-basidiospore isolates collected from fructifications of isolates 48 and 69 were tested on uncovered seedlings, on seedlings covered with collodion membranes, and on collodion membranes mounted on van Tieghem rings with either stem or cotyledon pieces attached below. Single-spore isolates 69-11 and 69-35 were selected for use in future investigations as they were quick-growing and reacted consistently on the host and on the host covered with collodion membranes.
(e) In vitro Reaction on Colloidion to Radish Stem and Cotyledon Exudates

Exudates were collected from radish seedlings by three methods. In the first, seedlings were removed from pots of sand, with as little damage as possible, assembled in a bundle, and suspended upside down with stems and cotyledons immersed in water for 6 hr (500–600 seedlings per 200 ml glass-distilled water).

In the second method seedlings were grown in pots of sand for 8 days, the seedlings then being about 2 in. high. The surface of the sand in the pots was then covered with “Silastic RTV 502” (Stotzky, Culbreth, and Mish 1961) which when set provided an impermeable barrier between stems and roots. Watering was carried out through holes in the bottoms of the pots and after 2 days the pots were inverted with the stems and cotyledons immersed in water. Three pots, each containing 275 seedlings, were immersed in succession for 2 hr in 1 litre of glass-distilled water.

In the third method, radish seedlings were mounted on microscope slides and placed in an upright position in glass jars, with the seedling roots dipping in water. Agar blocks 10 by 3 by 1 mm cut from 1% distilled water-agar (“Oxoid Ionagar No. 2”) were placed on the stems or cotyledons, the jars covered with loose-fitting glass lids, and placed in the constant-environment cabinet for 48 hr.

Batches of exudate collected by the first two methods were evaporated to approximately 5 ml as previously described. In some cases the material was freeze-dried to complete dryness and the residue then dissolved in a small volume of water. The concentrated liquid was passed through an “Oxoid” bacterial membrane filter and finally evaporated to a standard volume in a vacuum desiccator.

The exudates collected by these three methods were assayed on collodion, using isolates 69–11 and 69–35. With exudates collected by the first two methods, agar blocks, similar to those previously described, were suspended from the underside of collodion membranes mounted on van Tieghem rings, and 10-μl aliquots of exudate added to each block with an “Aga” micrometer syringe. Sterile glass-distilled water was added to control blocks. With exudate collected by the third method, agar blocks were removed from stems or cotyledons and suspended from collodion membranes as described. Controls consisted of untreated distilled water-agar blocks.

Consistent stimulation of infection cushion formation could not be obtained with several different batches of exudate collected by the first two methods, whereas agar blocks, from either stems or cotyledons, stimulated excellent infection cushion formation in most cases. No infection cushions formed on controls. This provided the first unequivocal evidence that exudates collected from the host stimulated the production of infection cushions. In Plate 1 infection cushions on the host surface and infection cushions on collodion stimulated by radish exudate are illustrated.

Owing to plant variability, a uniform concentration of active material was not obtained in each block and thus some variation occurred in the reaction from one replicate to another. This variability was overcome by collecting 50–100 agar blocks from radish cotyledons, placing them in a test tube, and heating in a bath of boiling water for 1–2 min until the agar melted. The liquid was thoroughly mixed and 40-μl aliquots were then dispensed into “Perspex” moulds 10 by 3 by 1 mm. When the
resolidified blocks were assayed on collodion with isolates 69-11 and 69-35, a consistent reaction was obtained. These results indicated that the active materials were not destroyed by heating at 100°C for 1–2 min. Further heating at 100°C for 15 min was carried out with no apparent loss of activity.

Using the above method of collecting, melting, and resolidifying, agar blocks were left on cotyledons for 6, 12, 24, 36, 48, and 60 hr and then assayed on collodion. Many infection cushions developed on the blocks following 24-, 36-, 48-, and 60-hr collection periods, whereas very few developed following 12 hr and none after 6 hr. This suggested a possible relation between the number of infection cushions developing and the concentration of active material. It is hoped that a quantitative relationship can be established and that this will aid in further investigations concerning the nature of the active material.

V. Discussion

Flentje (1957) and Kerr and Flentje (1957) suggested from circumstantial evidence that the hypothesis of a contact stimulus as suggested by Brown and Harvey (1927) was not adequate to explain the reactions of T. cucumeris on various hosts. Brown (1934) had pointed out the possibility of finding exceptions to the hypothesis and the additional circumstantial evidence now presented, together with the direct evidence that infection cushions develop on artificial membranes over exudate from the host, indicate that chemical stimuli control the development of infection structures by T. cucumeris.

It is now well established that there are some strains of T. cucumeris with a wide host range and other strains which are more specialized. Flentje (1957) has described the reactions of various strains of T. cucumeris on several hosts, and has indicated several stages where the infection process may be interrupted, leading to host resistance. One of these stages is concerned with the development of infection cushions and it was shown that in some cases specialized strains form infection cushions on a susceptible host, but not on a resistant host. It was thus suggested (Flentje 1957) that a susceptible host exudes materials which stimulate infection cushion formation, whereas a resistant host does not. It has now been shown that materials exuded by a susceptible host stimulate infection cushion development, but until the nature of these materials is known it is difficult to show conclusively that the same materials are not exuded by resistant hosts. An alternative explanation of resistance is that both susceptible and resistant hosts exude the same stimulatory materials, but that on a resistant host infection cushion development is suppressed by inhibitory materials. Again it is difficult to obtain direct evidence that such inhibitors are exuded and this awaits purification and identification of the materials stimulating infection cushion formation. The fact that infection cushions will develop on collodion over exudate collected from the host indicates that this reaction is determined by materials present in the host prior to inoculation and is not dependent on an interaction between pathogen and host.

Although it has been shown that on radish stems there are discrete areas where infection cushions form, it is clear that this is determined by the host. With
a uniform presentation of material as occurs with agar blocks, infection cushions are regularly distributed over the entire area of the block. The actual morphogenetic changes occurring within the hyphae at the time of infection cushion formation are of considerable interest and it is hoped to get a greater understanding of these processes when the stimulatory materials are identified.

The formation of infection cushions on “Cellophane” indicates that it allows the stimulatory materials to diffuse through, and that it is a suitable surface for infection cushion formation. Poor contact between stems and “Cellophane” membranes is probably responsible for the failure of infection cushion development when whole seedlings are used. Although hyphal clumps somewhat similar to infection cushions are formed on “Cellophane” enclosing roots, it would seem that either the materials exuded by roots are different from those exuded by stems, or that other materials are exuded by roots which interfere with the reactions leading to typical infection cushion formation. No satisfactory explanation of the differences between “Cellophane” bag and in vitro tests has as yet been found. It is possible that in the “Cellophane” bag tests, a susceptible host stimulates all isolates to some extent, but that pathogenic strains produce toxins which damage the roots, thereby leading to a greater release of exudate and more stimulation. This reaction could not occur in vitro.

It has been noticed that isolates 48 and 69 form many more infection cushions on collodion covering cotyledon pieces than on collodion covering stem pieces; also isolate 68 develops structures very similar to infection cushions on collodion covering cotyledon pieces but not on collodion covering stem pieces. This suggests either that more materials are exuded by cotyledons than by stems or that different materials are exuded from cotyledons than from stems. It may also suggest that the materials are synthesized in the cotyledons and then translocated down the stems. It is hoped that definite evidence on these aspects will be available following the identification of the active materials.

A major factor hindering identification of the active materials has been the difficulty of collecting material for bioassay and purification. The material readily diffuses through agar, but attempts to collect active materials by immersing stems and cotyledons in water have been generally unsuccessful. It is possible that the materials are held on the surface of stems and cotyledons by some type of chemical bond and that agar has properties which overcome these forces, thereby allowing active material to collect in agar blocks placed on stems or cotyledons. Further work on the purification and identification of the active materials is continuing and should provide information on these problems.

VI. Acknowledgments

We wish to thank Mrs. T. Wickman and Miss H. Hughes for their able technical assistance and also Miss H. Stretton for providing several of the isolates used. One of us (R.L.D.) was supported in this work by grants from the University of Adelaide and the Wheat Industry Research Council of Australia.
Infection cushions formed by crucifer-attacking isolate 69–35 on radish stem (Fig. 1, ×160) and on collodion over radish exudate (Figs. 2 (×50) and 3 (×130), respectively).

VII. References


