

Protection of Carnation Against Fusarium Stem Rot by Fungi

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ABSTRACT

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Many species of fungi protected wounded carnation stem tissue from ingress by *Fusarium roseum* 'Avenaceum' when inoculation with the pathogen was made 24 hr after wounding. A nonpathogenic isolate of *F. roseum* 'Gibbosum' was used as the biocontrol agent in subsequent experiments to obtain more information on the protection phenomenon and to investigate the mechanism of biological control. Nine cultivars were protected by the biocontrol agent. Wounded carnation tissue without the agent gradually became resistant during 96 hr of incubation; however, application of *F. roseum* 'Gibbosum' to the infection court hastened this reaction so that comparable resistance was achieved in 24 hr of incubation. Among the potential mechanisms of biological control in this system, those involving antagonism

(antibiosis, competition, and exploitation) and hastening of morphologically identifiable host responses were eliminated systematically. Conidia of the pathogen did not germinate in the infection court when the biocontrol agent was present, but germination of the pathogen was profuse when the agent was absent. Washed agar disks were incubated for 24 hr in infection courts infested with the biocontrol agent. When these disks were removed, they contained a substance(s) inhibitory to germination of pathogen conidia. Metal salts ($HgCl_2$ and $CuCl_2$) and an autoclaved mycelial preparation of the biocontrol agent also protected carnation tissue. These treatments have been effective in inducing the phytoalexin response in other plants.

Fusarium stem rot of carnations (*Dianthus caryophyllus* L.) induced by cultivars of *Fusarium roseum* (Lk.) emend Snyder & Hans. has been responsible for extensive commercial losses in the industry and is a continuing threat in pathogen-free propagation programs (4). Penetration typically occurs only through wounds or senile tissue predisposed by extended periods of high humidity (9). Biological control was achieved when *Bacillus subtilis* (1) or a bacterium of Pseudomonadaceae (11) was introduced into the infection court of the host prior to inoculation with the pathogen. We investigated the potential for species of soilborne fungi to induce this kind of protection and attempted to determine the mechanism of biological control. A short report was published (3).

MATERIALS AND METHODS

All experiments were done in an analog system of the following description. Custom-made plastic stacking dishes 45 mm in diameter and 100 mm high containing Perlite® (to a height of approximately 25 mm) saturated with water were sterilized by autoclaving. When stacked, each dish fitted into the one above so that sterility and a high relative humidity were maintained within each

chamber. Leaves were stripped from carnation cuttings (cultivar CSU Pink Sim, unless otherwise noted) and stem segments cut 40 mm in length. A node was always included but the cut at the basal end was made just above a node. These segments were surface sterilized in 10% Clorox (a solution of sodium hypochlorite containing 0.52 percent available chlorine) for 10 min. Then a thin cross section of stem at the basal end was removed with a sterile razor blade to insure exposure of fresh noncontaminated wounded tissue (hereafter referred to as the infection court). The apical end of five to ten stem segments was uniformly inserted into each stacking dish so that the basal end was up.

Various candidate biocontrol agents were introduced onto the infection court (basal cut surface) immediately after insertion of stem segments into the plastic stacking dishes. Subsequently, conidia of a pathogenic isolate of *F. roseum* 'Avenaceum' (isolate 56-2) were applied to the infection court. Conidia were produced on 10- to 12-day-old cultures of the fungus growing on carnation dextrose agar (CDA) which contained a hot water extract of 200 g of chopped carnation tissue, 20 g glucose, and 20 g Difco agar/liter of water. Conidia were washed from the surface of cultures with sterile distilled water and the concentration adjusted to 1×10^4 conidia/ml of water. One drop of this spore suspension containing approximately 625 conidia (16 drops/ml) was applied to the infection court at the appropriate time in various treatments. Stacking dishes containing stem segments

were incubated in a chamber at 25 ± 2 C.

In subsequent experiments, a nonpathogenic isolate, *F. roseum* 'Gibbosum' obtained from P. E. Nelson (Pennsylvania State University), designated by him as No. 297, was used as the biocontrol agent in experiments designed to determine the mechanism of control. Conidium suspensions (1×10^4 conidia/ml of water) were introduced into the infection court as described for isolate 56-2.

Stem segments to which neither the biocontrol agent(s) nor pathogen were applied were always included in experiments as noninoculated, nontreated controls. In all cases no rot developed in these controls and thus results from these are not always included in the data.

Symptoms developed from the basal end of the stem segment in the internodal tissue as a reddish rot after inoculation. Discoloration was measured in mm from the cut basal end (originally the infection court) to the most advanced edge of the exterior rot 10-14 days after inoculation.

RESULTS

Protection of carnation against *Fusarium* stem rot by various fungi.—Species of fungi collected and classified by the late L. W. Durrell (Colorado State University) were screened for effectiveness in biological control of *Fusarium* stem rot.

The analog system was used for screening. Cultures of test fungi were grown on potato-dextrose agar (PDA) for 2-3 wk. Ten milliliters of sterile distilled water were added to the culture and one drop of the resulting spore suspension was applied aseptically to the cut surface of the carnation stem segments in the plastic dishes. A minimum of ten segments was used in each treatment. Twenty-four hr later, conidia of the pathogen were applied to the same surface. Nontreated surfaces of carnation segments cut at the same time also were inoculated to serve as controls. After 10-14 days, the extent of the lesions was measured and treatments recorded as percentage of rot relative to the inoculated control.

TABLE 1. Screening of fungi as potential biological control agents against *Fusarium* stem rot of carnations (cultivar CSU Pink Sim) induced in cut stem sections by *Fusarium roseum* 'Avenaceum'

Fungus ^a	Rot index relative to inoculated control ^b (%)	Fungus ^a	Rot index relative to inoculated control ^b (%)
<i>Penicillium frequentans</i>	2	<i>Penicillium diversum</i>	40
<i>Phialophora cinerescens</i>	2	<i>Penicillium purpurogenum</i>	40
<i>Diplodia</i> sp.	2	<i>Periconia</i> sp.	40
<i>Penicillium corymbiferum</i>	3	<i>Fusarium rigidiusculum</i>	41
<i>Aspergillus restrictus</i>	4	<i>Rhizopus nigricans</i>	41
<i>Phoma</i> sp.	4	<i>Verticillium lateritium</i>	44
<i>Monotospora</i> sp.	9	<i>Aspergillus tamaritii</i>	54
<i>Stysanus</i> sp.	9	<i>Syncephalastrum racemosum</i>	51
<i>Fusarium roseum</i>		<i>Chaetomium</i> sp.	54
'Gibbosum' (297)	10	<i>Penicillium granulatum</i>	54
<i>Mucor ramannianus</i>	19	<i>Thamnidium elegans</i>	54
<i>Aspergillus terreus</i>	17	<i>Aspergillus fumigatus</i>	59
<i>Trichurus</i> sp.	17	<i>Stemphylium</i> sp.	59
<i>Fusarium oxysporum</i>	17	<i>Thielaviopsis basicola</i>	59
<i>Aspergillus flavus</i>	20	<i>Botrytis cinerea</i>	66
<i>Curvularia tetramera</i>	20	<i>Stachybotrys</i> sp.	75
<i>Penicillium nigricans</i>	22	<i>Camarosporium</i> sp.	79
<i>Aureobasidium pullulans</i>	22	<i>Fusarium solani</i>	
<i>Sepedonium chrysospermum</i>	22	f. sp. <i>phaseoli</i>	79
<i>Mucor spinescens</i>	23	<i>Myrothecium verrucaria</i>	92
<i>Nematogonium humicola</i>	24		
<i>Penicillium amethystinum</i>	24	<i>Epicoccum nigrum</i>	144
<i>Aspergillus sulphureus</i>	28		
<i>Mucor varians</i>	39		
<i>Aspergillus clavatus</i>	35		
<i>Verticillium albo-atrum</i>	35		
<i>Fusarium oxysporum</i>			
f. sp. <i>lycopersici</i>	37		
<i>Arthrobotrys nematoda</i>	39		
<i>Aspergillus panamensis</i>	39		
<i>Cephalosporium</i> sp.	39		

^aFungi applied as conidium suspensions to cut surfaces of carnation stems and inoculated with *F. roseum* 'Avenaceum' 24 hr later.

^bRot index = $\frac{\text{average length of lesions (treatment)}}{\text{average length of lesions (inoculated control)}} \times 100$.

Data accumulated and averaged from repeated experiments in which seven to ten wounded carnation stem sections were used each time.

The results in Table 1 reflect typical data accumulated over 2 yr from a large number of repeated experiments. Results between these experiments were somewhat variable, apparently reflecting host factors associated with predisposition; however, comparison of the length of lesions in treatments with that in inoculated controls afforded a reasonably consistent pattern.

Most of the test fungi afforded an increment of protection against the pathogen. Rot was increased when *Epicoccum nigrum* was tested as a potential biological control agent. In this case the discoloration was atypical of that induced by *F. roseum* 'Avenaceum' alone, indicating some pathogenicity on the part of *E. nigrum*.

Characteristics of induced biological control.—Eight cultivars of carnation were screened in the analog system to determine response to the biocontrol agent. Isolate 297 was used in this and subsequent experiments as the test biocontrol agent. When isolate 297 and the pathogen both were applied to the cut surface of carnation stems immediately after wounding, there was little difference between treatment and the controls inoculated with the pathogen alone except for CSU White (Fig. 1-A). When isolate 297 was applied 24 hr before the pathogen biological control was observed in all cultivars, with the possible exception of Flamingo (Fig. 1-B). Differences in susceptibility among cultivars were apparent only when inoculations were delayed for 24 hr after wounding.

Isolate 297 was applied to carnation sections at the time of wounding and the pathogen was introduced on wounded surfaces 0, 24, 48, 72, and 96 hr later. Cut surfaces on carnation stem (CSU Pink Sim) segments not treated with isolate 297 but inoculated with the pathogen at the same times were maintained as controls. Protection was not obtained when isolate 297 was applied at the same time as the pathogen (Fig. 2). When the pathogen followed the biocontrol agent by 24 hr or more, reduction of rot occurred when compared with inoculated controls. Susceptibility also decreased in inoculated controls when inoculation was delayed after wounding by 24 hr or more, but the extent of lesions was greater than on surfaces to which isolate 297 was applied.

Visual observations of the infection court.—The possibility that wounds healed more quickly when the biocontrol agent was applied was investigated.

Isolate 297 was applied to cut surfaces of sections of carnation and the pathogen was introduced into the infection court 24 hr later. Inoculated and noninoculated sections also were included as controls. After 1, 3, 5, and 10 days incubation, sections of stem including the infection court and advancing lesion (if present) were excised and fixed in formalin-alcohol-acetic acid (FAA), dehydrated in an ethanol-xylene series, and radial paraffin sections stained with safranin O-fast green. No differences in host morphology of wound healing (12)

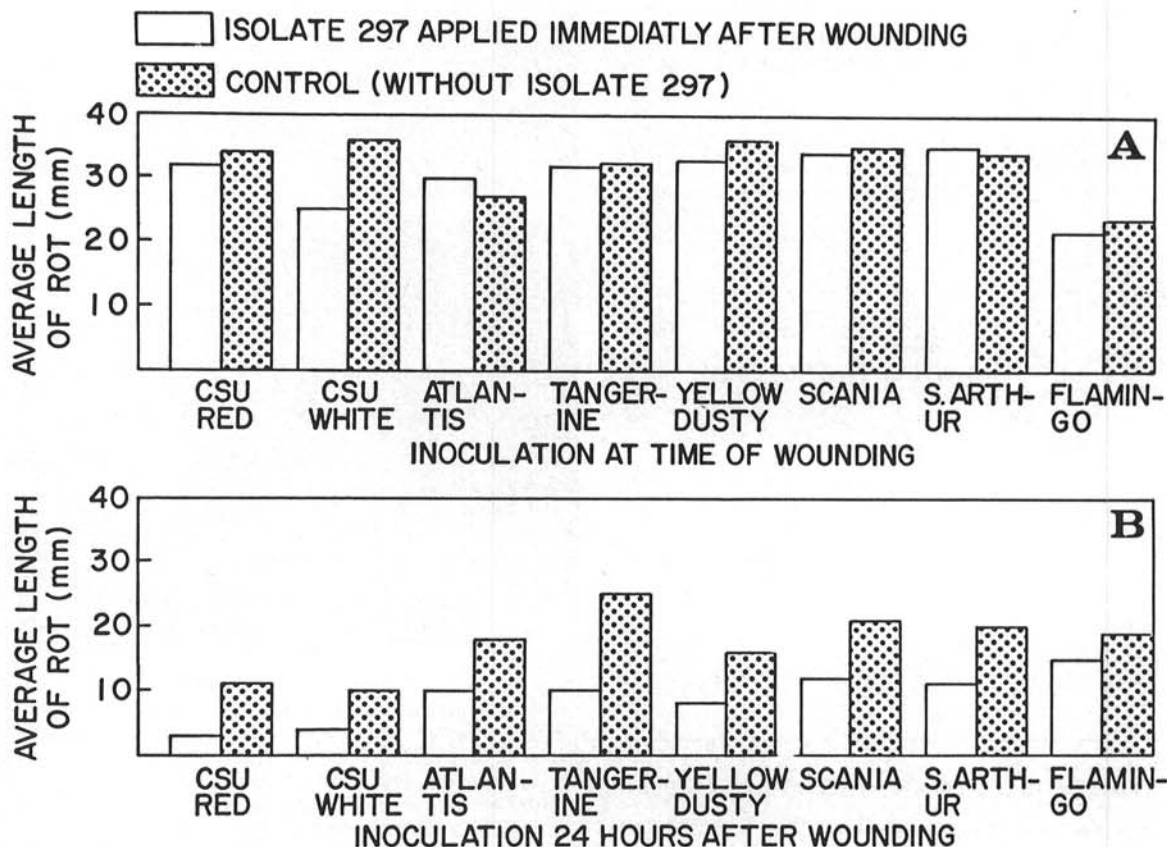


Fig. 1-(A, B). Response of carnation varieties when *Fusarium roseum* 'Gibbosum' (isolate 297) was placed in the infection court followed by inoculation with *F. roseum* 'Avenaceum' A) immediately or B) 24 hr after wounding.

were evident in comparisons between appropriate treatments and controls.

Germination of conidia in the infection court.—Mycelial mats of isolate 297 were harvested from 5-day-old liquid cultures growing on one-tenth strength potato dextrose broth (PDB). These mats were washed in sterile distilled water and placed on the cut surfaces of carnation stem segments. Conidia of *F. roseum* 'Avenaceum' were placed on these infection courts 24 hr later. Controls consisted of mycelial mats of the biocontrol agent placed on cut surfaces without subsequent inoculation with conidia of the pathogen and others inoculated 24 hr after wounding without intervening mycelial mats.

Twenty-four hr after inoculation, infection court surfaces were examined to determine germination of conidia of *F. roseum* 'Avenaceum' in the presence or absence of the biocontrol agent. This was accomplished by staining with cotton blue-lactophenol, supplying cellulose acetate, and stripping mycelia and/or conidia from the infection court after a suitable drying period. Alternatively, thin freehand cross sections of stem cut just below the infection court were placed on slides and stained with 1% cotton blue for examination. In repeated experiments, conidia germinated when applied to cut surfaces of tissue (Fig. 3-A) but no germination was detected in infection courts previously treated with the mycelial mat of the biocontrol agent (Fig. 3-B). No conidia were observed in treatments where only the mycelial mat had been applied (Fig. 3-C) indicating that isolate 297 had not sporulated and that the conidia seen in the latter case (Fig. 3-B) were from the pathogen and not the biocontrol agent.

Effect of introduced nutrients on biocontrol.—The possibility was explored that conidia of the pathogen did not germinate because competition for nutrients occurred in the infection court. Competition can be detected by

supplying candidate nutrients most likely to be limiting; in most cases these are carbon and/or nitrogen containing compounds (2). Accordingly, isolate 297 was placed on

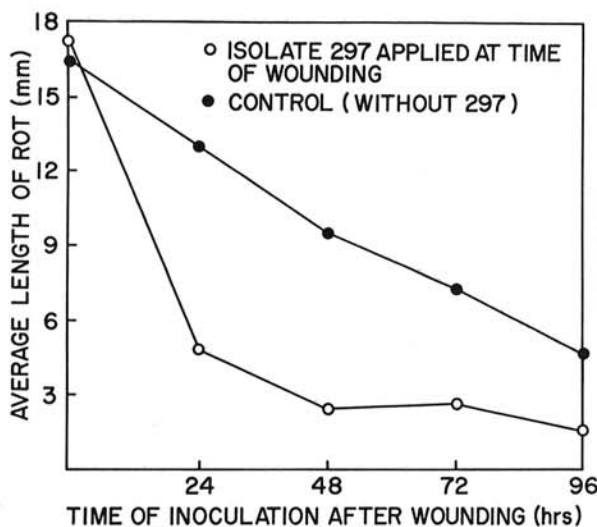


Fig. 2. Response of carnation cultivar CSU Pink Sim stem sections to infection by *Fusarium roseum* 'Avenaceum' introduced in to the infection court 0-96 hr after wounding with or without the presence of a biocontrol agent (isolate 297).

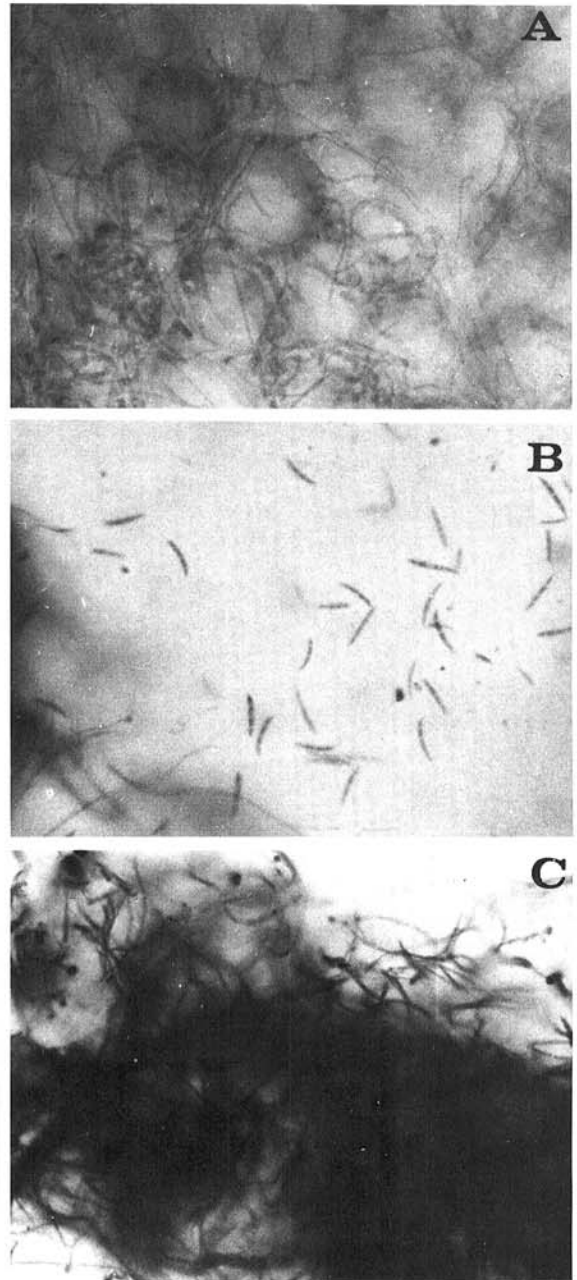


Fig. 3-(A to C). Germination of conidia of *Fusarium roseum* 'Avenaceum' in the infection court of cut carnation stems 24 hr after wounding. A) Conidia germinating in infection court in the absence of isolate 297. Walls of host parenchyma tissue may be seen in the background. B) Nongerminated conidia of the pathogen in the infection court infested with isolate 297. The dark areas in the background are portions of the mycelial mat of the biocontrol agent. C) Mycelial mat of isolate 297 in the infection court without addition of conidia of *F. roseum* 'Avenaceum'.

cut surfaces of carnation stems in the analog system and conidia of the pathogen applied 24 hr later with glucose (1,000 µg/ml of conidium suspension) and/or nitrogen in the form of ammonium nitrate (100 µg/ml of conidium suspension). Nutrients and/or conidia were applied to the infection court as a drop of liquid suspension or conidia were embedded with nutrients in an agar disk (5 mm diameter × 3 mm thick, 2% Difco Noble agar). The extent of stem rot was measured 10-14 days after inoculation.

In repeated experiments the biological control achieved with the addition of isolate 297 to the infection court was not inactivated when nutrients were introduced with inoculum of the pathogen (Table 2).

Host response to the biological control agent.—The washed agar disk method (14), which is a sensitive test for detection of volatile soil fungistatic factors, was employed to determine if a germination inhibitor was excreted by the host in response to the presence of the biocontrol agent.

Conidia of isolate 297 were applied to the cut surfaces of living or dead carnation stem segments (boiled for 8 min). Forty-eight hours later, 2% Difco Purified agar disks (6-7 mm in diameter and 4-5 mm thick) were washed to remove residual nutrients by methods previously described (7) and placed on Nuclepore® (Nuclepore Filter Corp., 7035 Commerce Circle, Pleasanton, CA 94566) filters. These filters were placed in contact with ends of the stem segments which separated the stems from direct contact with the agar disks. After 24 hr of incubation the disks were removed, placed in a moist chamber, and one drop of conidium suspension of the pathogen was placed on each. Stem segments prepared in the same manner without the application of isolate 297 served as controls. Percentage germination and germ tube length of conidia of *F. roseum* 'Avenaceum' were recorded 12 hr after these propagules were placed on the disks by methods previously described (10). Washed agar disks alone with conidium suspensions of *F. roseum* 'Avenaceum' on their

surfaces were also incubated in all experiments to serve as controls.

In two experiments there was a significant ($P = 0.05$) reduction in germination and germ tube length of conidia

TABLE 2. Effect of glucose and ammonium nitrate applied with inoculum (conidia of *Fusarium roseum* 'Avenaceum') to cut surfaces of carnation (cultivar CSU Pink Sim) stem sections to which isolate 297 (*F. roseum* 'Gibbosum') had been applied 24 hr previously

Treatment ^a	Average length of rot ^b (mm)
Control-noninoculated	0
Control-inoculated	33
Biological control agent 297 applied 24 hr before inoculation	
No nutrients with inoculum	6
Glucose (1×10^3 µg/ml) added with inoculum	4
Ammonium nitrate (100 µg/ml) added with inoculum	8
Glucose and ammonium nitrate added with inoculum	11

^aEach treatment consisted of five carnation stem sections in a plastic dish and treatments were replicated three times.

^bFigures represent average measurements of the extent of rot measured from the cut surface to the maximum advance of the lesion.

TABLE 3. Germination and average germ tube length of conidia of *Fusarium roseum* 'Avenaceum' on washed agar disks exposed to cut surfaces of carnation (cultivar CSU Pink Sim) stem sections

Treatment ^x	Response of conidia of pathogen ^{y,z}			
	Experiment 1		Experiment 2	
	Germination (%)	Germ tube length (µm)	Germination (%)	Germ tube length (µm)
Disk on stem section without isolate 297	91 a	0.291 c	97 eh	0.232 i
Disk on stem section with isolate 297	67 b	0.093 d	58 g	0.081 j
Disk on boiled stem section without isolate 297			98 h	0.178 k
Disk on boiled stem section with isolate 297			95 eh	0.200 i
Disk control	86 a	0.391 c	94 eh	0.184 ik

^xTwo percent washed Difco Purified agar disks on 'Nuclepore' filters placed over various treatments for 24 hr.

^yConidia placed on washed agar disks and incubated for 12 hr before germination and germ tube length recorded. There were four replications per experiment.

^zMeans followed by same letter are not statistically different ($P = 0.05$) by Wilcoxon's sum-of-ranks test.

of the pathogen on agar disks exposed to living stem sections in the presence of isolate 297 compared to sections not infested with the biocontrol agent (Table 3). When isolate 297 was applied to nonliving boiled stem segments, however, no inhibition of germination was observed in comparison with controls.

Effect of culture filtrates and chemicals on infection.—Various chemicals (Fig. 4) and autoclaved and nonautoclaved filtrates (filtered through Nuclepore filters) of 10-day-old cultures of isolate 297 growing in CDB were applied to cut surfaces of carnation stem sections and incubated for various periods before inoculation with the pathogen. Measurements of rot were made 14 days after inoculations. When inoculations were made 72 hr after wounding, isolate 297 induced biological control and reduction in rot was observed in all treatments, except Actinomycin D.

DISCUSSION

Many species of fungi found in soil and tested *in vitro* protected carnation stems from infection by *F. roseum* 'Avenaceum' (Table 1). This is also true for bacterial isolates naturally occurring in soil (1, 11). Thus,

protection afforded by biocontrol agents against *Fusarium* stem rot of carnations is nonspecific and can be elicited by many organisms not related either taxonomically or biochemically.

What is the mechanism of biological control of *Fusarium* stem rot induced by nonpathogenic fungi introduced into the infection court of carnations? Antagonism is usually thought to operate through antibiosis, exploitation, or competition (2). The first two mechanisms did not appear to operate; no antagonistic interactions in the form of antibiosis or actual mycoparasitism of the pathogen were observed between the biocontrol agent (isolate 297) and *F. roseum* 'Avenaceum' in plate culture. Moreover, it is not likely that so many fungi (listed in Table 1) would have antibiotic and/or mycoparasitic properties in common. Competition does not seem likely since addition of the principal nutrient candidates for limiting factors failed to nullify biological control induced by isolate 297 (Table 2). We conclude that antagonism between the biocontrol isolate and *F. roseum* 'Avenaceum' cannot explain the biocontrol obtained against stem rot.

The alternative mechanism for biological control in this system is that associated with host response. No obvious acceleration in wound healing was observed in

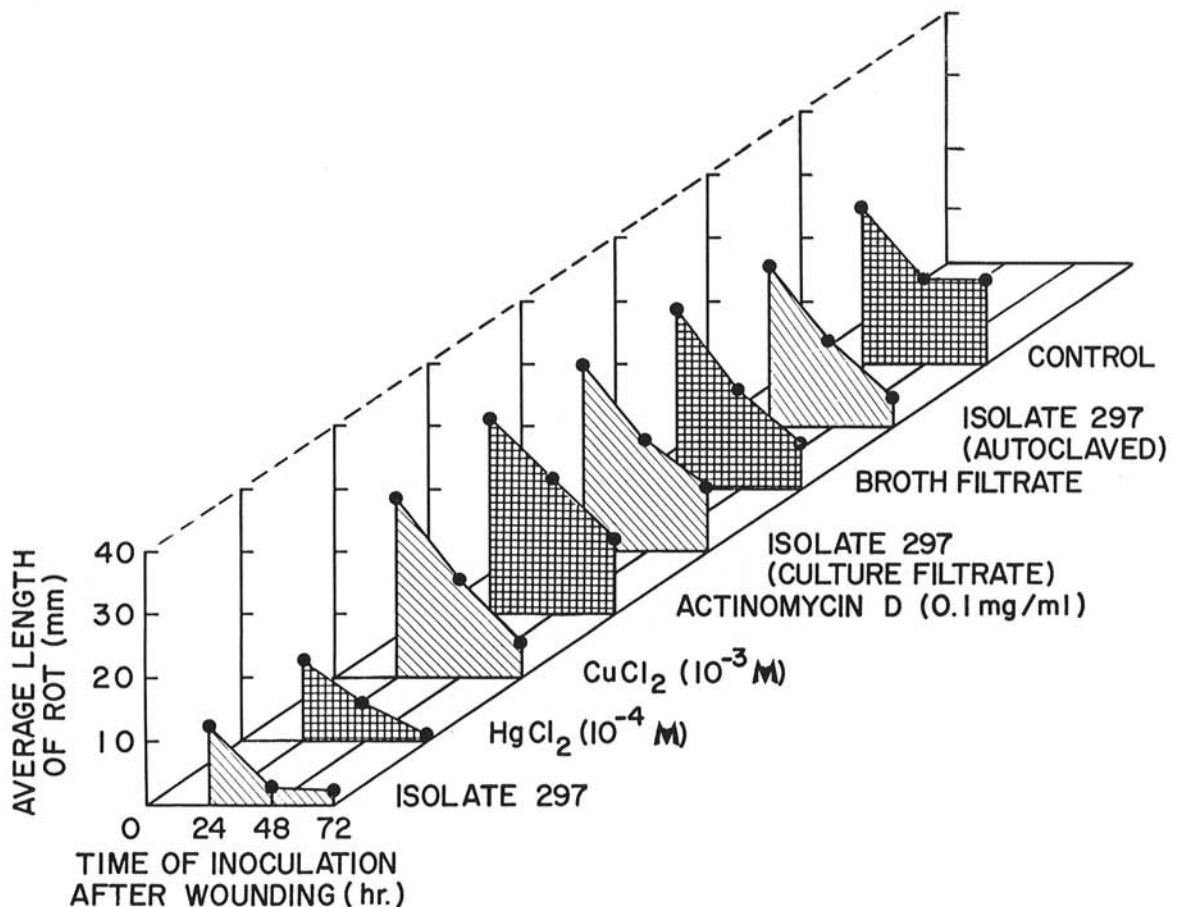


Fig. 4. Effect of culture filtrates and various chemicals introduced into the infection court 24-72 hr before inoculation with *Fusarium roseum* 'Avenaceum' on development of *Fusarium* stem rot.

host tissue treated with the biocontrol agent; however, conidia of the pathogen placed in the infection court 24 hr after application of the biocontrol agent did not germinate (Fig. 3-B). Competition could explain this effect but was ruled out (as discussed above); also, the conidia germinated on disks of purified agar (Table 3) suggesting that conidia of this isolate of *F. roseum* 'Avenaceum' do not require an exogenous source of nutrients for germination (15). The remaining alternative was that the host was induced to produce an inhibitory factor in the infection court in response to the presence of the biocontrol agent.

Positive evidence was obtained for host participation in the phenomena reported in this paper when washed agar disks were incubated on the infection court through an intervening sterile Nuclepore filter. Such disks became inhibitory to conidia of the pathogen when incubated over an infection court harboring the biocontrol agent (Table 3). When this agent was not present, conidial germination of the pathogen was comparable to that on agar disk controls (without host tissue present) or was even stimulated. However, living host tissue was a prerequisite for inhibitory factor production; there was no evidence of inhibition when the biocontrol agent was placed on carnation stem segments killed by boiling. We conclude that the biocontrol agent in the infection court induced the production of a factor by the host inhibitory to conidia of the pathogen.

As wounded tissue of CSU Pink Sim was incubated, it gradually became more resistant to the ingress of the pathogen (Fig. 2). Application of the biocontrol agent to the infection court immediately after wounding apparently hastened this reaction so that the extent of stem rot was small if inoculations were made 24 hr later. In contrast, nontreated controls required more than 96 hr after wounding to achieve comparable resistance. Apparently, resistance is also enhanced in many other carnation cultivars by application of isolate 297 (Fig. 1). These phenomena may be another example of the principle elaborated by Hammerschmidt et al (8) in which "some types of resistance may not be determined by the presence or absence of a genetic potential for resistance, but rather the ability of the potential to be quickly expressed with sufficient magnitude."

Mercuric chloride reduced infection when inoculation was delayed 48 hr (Fig. 4). Cupric chloride and an autoclaved mycelial preparation of isolate 297 were effective after 72 hr. It is unlikely that inhibition by the agents themselves was responsible since their effectiveness increased with time after application. The two metal salts are reportedly effective in inducing phytoalexins in other plants (6, 13). Bell and Presley (5) showed that heat-killed conidia of *Verticillium albo-atrum* induced gossypol accumulation and resistance in cotton. The possibility that these agents blocked or altered host sites necessary for pathogen invasion of carnation should also be considered. Actinomycin D was not an effective control agent at the concentration tested and, in fact, enhanced disease when tissue was challenged after a 48-hr

treatment. It may act by inhibiting normal wound healing or the production of fungitoxic materials or cause the release of stimulatory agents thus permitting increased pathogen growth.

Further studies will be necessary to ascertain the nature of the protective response described. Data are consistent with a phytoalexin(s) although isolation of such an agent is obviously required. Whether more than one mechanism of action is shown by diverse protective agents awaits further study.

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