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STUDIES ON THE BIOLOGICAL CONTROL
OF VERTICILLIUM WILT OF OKRA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

by

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To

MY PARENTS

This piece of work is humbly dedicated.

P.S.B.

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INTRODUCTION

Verticillium wilt of okra (*Hibiscus esculentus* L.) due to *Verticillium albo-atrum* Reinke and Berth. is of common occurrence in the Punjab State of India, especially in the warmer sections. The conventional control measures like crop rotation and soil sterilization are not economically feasible. Resistant varieties of agro-commercial value are not available. Losses due to the disease continue to increase every year and it is feared that in a course of time the cultivation of the crop in certain areas may not be profitable. Under the circumstances, it appears attractive to investigate the possibility of applying some kind of biological approach to the problem.

The intensive research work at The Ohio State University extending from 1954 has shown that under certain conditions, a species of *Cephalosporium* Corda can inhibit the incidence of fusarial wilts in tomato, cotton, and cowpeas (6, 15, 25). It was, therefore, considered desirable to study the effects of *Cephalosporium* on the incidence of *Verticillium* wilt in okra also. In order to achieve this, a study was undertaken to investigate: (1) the effect of *Cephalosporium* preinoculation of okra roots and seeds on the incidence of *Verticillium* wilt; (2) the conditions under which the inhibition of *Verticillium* wilt by *Cephalosporium* occurs; and (3) the mechanism of wilt reduction.

The attention was mainly focused on the rhizosphere, rhizoplane, and spermatosphere of okra plants as these three zones seem to play an important part in the successful establishment of root pathogens in plants.

LITERATURE REVIEW

Since the beginning of the twentieth century, there have been many reports regarding widespread occurrence of antagonism among soil-borne microorganisms both in the rhizosphere and on the rhizoplane of the plants (2, 9, 10, 11, 12, 16, 17, 22, 32, 26, 27, 28, 32, 34, 35). Recently many workers have reported that preinoculation of plant roots with certain pathogens and non-pathogens induces some kind of resistance in them against infection by pathogens. Begga (1) in 1954 reported the control of sweet potato wilt caused by *Fusarium oxysporum* f. *batatas* (Wr.) Snyd. and Hansen by dipping freshly cut sprouts in a spore suspension of *Fusarium solani* f. *batatas* McClure before planting in the field. Buxton (4) noticed that the inoculation of peas with both pathogenic and nonpathogenic cultures of *F. solani* (Mart.) Appel and Wr., made them resistant to *F. oxysporum* f. *pisi* (Linford) race 1 Snyder and Hansen. Kuc *et al.* (13) in 1956 and Kuc (14) in 1957 reported that plants form chemical compounds in response to invasion by pathogens and non-pathogens which not only check the invading pathogens but also other fungi. Boller *et al.* (3) were able to isolate an antifungal principle from orchid tubers infected with *Rhizoctonia repens* Bern. Buxton and Perry (5) reported that the incidence of wilt in peas was decreased when plants were co-inoculated with *F. oxysporum* f. *pisi* and *F. solani*. The treatment of roots with *F. solani* was more effective when the inoculation was done two to four days prior to inoculation with *F. oxysporum* f. *pisi*. Perry (20) while discussing the mechanism underlying the reduction of pea wilt caused by *F. oxysporum* f. *pisi* by the use of *F. solani* suggested that as there occurred no competition between two fungi in the soil or in the rhizosphere of the plant, the reduction in wilt may be due to the toxic substances produced in the host plant in response to invasion by *F. solani*. He was, however, not able to demonstrate their presence convincingly. Smith (29) reported that the inoculation of tomato roots with spore suspensions of a species of *Micromonospora* (later identified as *Cephalosporium*) prior to their inoculation with *Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyder and Hansen inhibited the symptom development of *Fusarium* wilt in plants. Chisler *et al.* (6) suggested that the *Cephalosporium* may be acting internally in the plants exerting the inhibitory influence on the *Fusarium*. They also reported that increasing the time interval between inoculation of seedlings with the *Cephalosporium* sp. and inoculation with *F. oxysporum* f. *lycopersici* resulted in greater mitigation of symptoms. Long (15) reported the reduction of *Fusarium* wilt symptoms in cowpeas and Roy *et al.* (25) in cotton by the use of *Cephalosporium*. Chisler (7) reported that *Cephalosporium* did not inhibit or produce materials that inhibit the growth of *Fusarium*. The infection of plants with high levels of *Cephalosporium* caused rotting of the plants. Rodebaugh (24) proposed that *Cephalosporium* may be inhibiting *Fusarium* inside the tomato plant either indirectly by inhibiting pectin enzyme production which in turn resulted in the decrease of available carbohydrates necessary for its growth, or by some direct influence on it. Phillips (21), however, postulated that *Cephalosporium* treatment of tomato roots prior to *Fusarium* inoculation caused a physical block, which prevented spread of *Fusarium* through the host and thereby slowed wilt development.

MATERIALS AND METHODS

Verticillium albo-atrum Reinke and Berth. (hereafter called *Verticillium*) used in this research was originally isolated from cucumber. It was later found to be pathogenic to okra plants. *Cephalosporium* isolate No. 16 (hereafter called *Cephalosporium*) is a biotype from the original *Cephalosporium* isolated by Smith (29), and had been reported to reduce symptoms of *Fusarium* wilt of tomato, cowpea, and cotton (6, 15, 25). Both the fungi were maintained under mineral oil on potato-dextrose slants and stored at 5 C.

The okra variety, Dwarf Long Pod, was used throughout this study. Seeds were disinfested in 1:1 dilution of Clorox before planting in a steamed soil-sand mixture. Seven-day-old seedlings were used in all experiments.

Roots of the seedlings were inoculated using conidial suspensions of either *Verticillium* or *Cephalosporium*. Conidia used for inoculation were obtained from seven-day-old cultures on potato-dextrose broth. The flasks were incubated on a reciprocal shaker and the conidia were separated from the broth by centrifuging at 2,800 rpm for 15 min. The supernatant was discarded and an equal volume of water added to the sedimented conidia. This resulting conidial suspension was designated as normal concentration of the inoculum. Other concentrations of inoculum were prepared by appropriate dilutions of the normal concentration with water. Inoculation with *Cephalosporium* was accomplished by immersing the roots of the seedlings in the desired concentrations of inoculum for 72 hr. During the first 24 hr, the flasks containing seedlings and inoculum were kept on a wrist shaker, then removed for the balance of the inoculation period. Inoculation with *Verticillium* was accomplished by immersing the roots of okra seedlings for 15 min in conidial suspensions in flasks on a wrist shaker. Following this inoculation the seedlings were planted in steam sterilized soil.

Root-extracts were obtained by grinding roots with a mortar and pestle for 10 min. Seitz filtration (No. 3 filter) was used to sterilize the extracts. Stem exudates were collected in sterilized 3 mm squares of Whatman No. 1 filter paper.

Soils were infested with *Verticillium* and *Cephalosporium* by incorporating 1,000 ml of a seven-day-old broth culture of the respective fungus in 20 lb of double steam sterilized soil. The infested soil, before use, was incubated for 15 days under moist conditions.

For studying the effect of root exudates on growth of *Verticillium*, okra seedlings were raised under aseptic conditions on 2 percent agar in test tubes. Seedlings growing in this agar were inoculated by adding suspensions of *Cephalosporium* to the surface of the agar for 48 hr. Inoculated seedlings were then transferred to petri plates of potato-dextrose agar previously flooded with Verticillium conidia, and the effect of root exudates diffusing from the roots was observed on the growth of Verticillium.

For studying the effect of root extracts on the growth of Verticillium, the fungus was cultured in potato-dextrose broth containing root extracts from Cephalosporium infected or healthy roots. The root extract agar was composed of 5 g of dextrose, 15 g of agar, and extract from 200 g of roots/1,000 ml of water. At the end of 20 days, the fungus was harvested by filtering with Buchner's porcelain filter and Whatman filter paper No. 1. Weight of the fungus was determined after drying.

Vascular bundle discoloration was rated by the following index:

0 - no vascular bundle discoloration

1 - vascular bundle discoloration in roots only

2 - vascular bundle discoloration not above cotyledon node

3 - vascular bundle discoloration not above first leaf node

4 - vascular bundle discoloration lower than half way between the first leaf node and stem tip

5 - vascular bundle discoloration higher than half way between the first leaf node and stem tip

Wilting data are based on number of the plants showing typical symptoms like chlorosis, vein clearing, and loss of turgidity.

Further details of special techniques employed will be discussed along with relevant experiments.

In some of the experiments where experimental errors were not normally distributed with a common variance, the data before statistical analysis were subjected to logarithmic transformation as recommended by Steel and Torrie (30).

EXPERIMENTAL RESULTS

Effect of inoculating roots of okra with varying levels of Cephalosporium on the incidence of wilt

Preliminary investigations indicated that inoculation of okra roots with *Cephalosporium* prior to their inoculation with *Verticillium* sometimes increased the incidence of wilt instead of mitigating it. It was, therefore, considered necessary to study the effects of *Cephalosporium* alone on okra plants. The results from various experiments, the typical trend of which are summarized in Table 1, showed that *Cephalosporium* induced wilting in okra at high inoculum concentrations, while the plants inoculated with low concentrations remained apparently healthy. *Cephalosporium* was isolated from all treated plants. In apparently healthy plants the recovery was only from root zones while in wilted plants it was recovered from all portions of the stem. The invariable recovery of *Cephalosporium* from apparently healthy plants suggested the possibility of *Cephalosporium* inducing a hypersensitive reaction in plants at low concentrations of inoculum, in the same way as was reported by Muller (18), and Cruickshank *et al*. (8) in their studies on potatoes and peas respectively. It was, therefore, considered desirable to investigate this aspect thoroughly.

TABLE 1. Effect of inoculating okra with different levels of *Cephalosporium*

Cephalosporium Inoculum Level Spores/ml ($x 10^7$)	Average[a] Percentage Wilting after 4 Weeks	Average Vascular Bundle Discoloration
17.0	11.1	3.5
12.0	8.8	2.9
8.3	4.4	2.3
4.8	2.2	2.0
3.0	0	1.5
2.0	0	1.0

[a]Four replications.

Incidence of Verticillium wilt in Cephalosporium inoculated okra

Roots of seven-day-old seedlings were treated, prior to their inoculation with *Verticillium*, with six concentrations of *Cephalosporium* for 72 hr and then the plants were replanted in sterilized soil and the incidence of wilt recorded 4 wks later.

The incidence of *Verticillium* wilt in okra was significantly reduced by preinoculating the roots with low concentrations of *Cephalosporium* (Table 2, Fig. 1). *Cephalosporium* was recovered only from the root zones of healthy plants.

TABLE 2. Effect of inoculating roots of okra seedlings with different concentrations of Cephalosporium on the incidence of Verticillium wilt

Cephalosporium Inoculum Level: Spores/ml (x 10^7)	Average Percentage Wilting at the End of 4 Weeks[b]	Average Vascular Bundle Discoloration[b]
17.0	42.2	3.5
12.0	42.2	3.6
8.3	31.1	3.0
4.8	6.7*	1.5
3.0	8.8*	1.9
2.0	4.3*	1.0
Verticillium only[a]	48.8	3.5
Water only	0	0

[a]5.3 x 10^7 spores/ml.

[b]Four replications.

*Significant difference over control (Verticillium only) at 1% level.

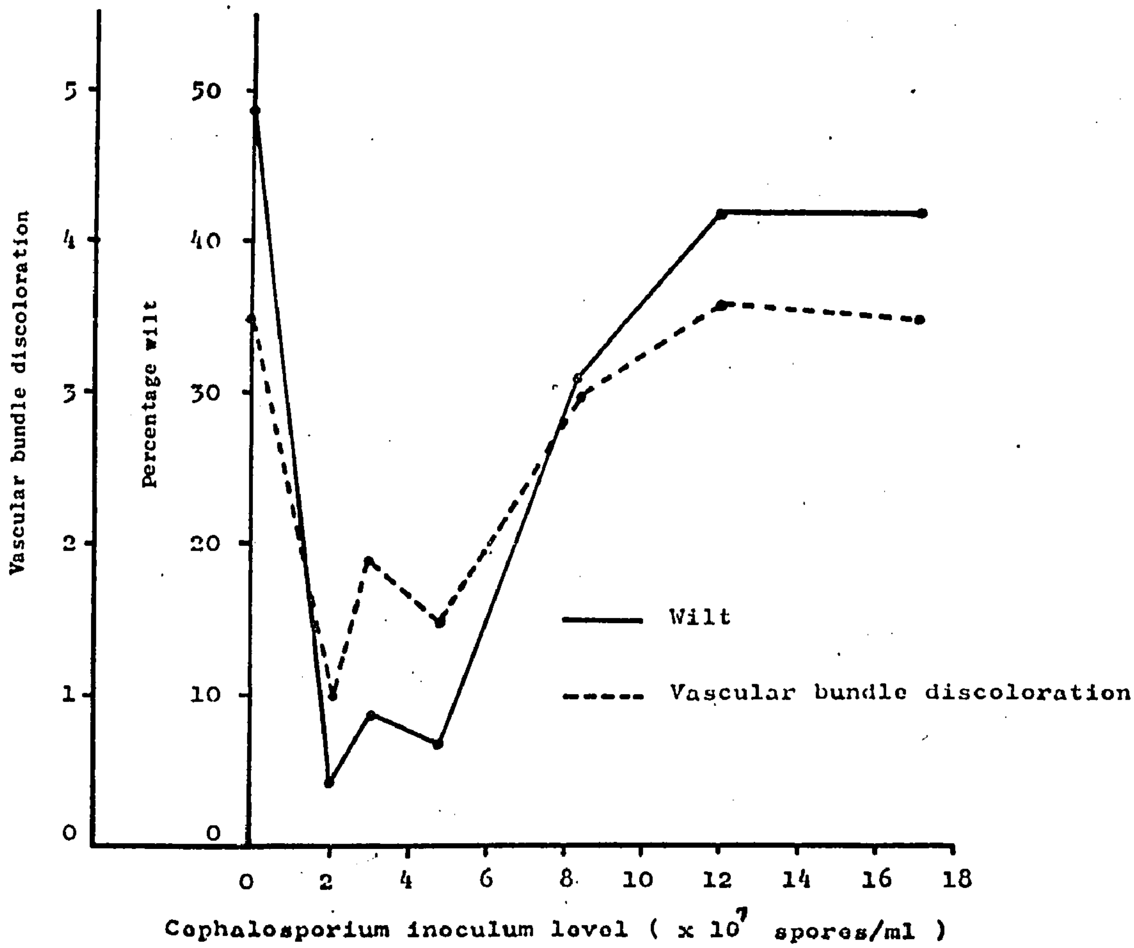


Fig.1.--Effect of Cephalosporium inoculation of okra roots on the incidence of Verticillium wilt and vascular bundle discoloration

Effect of Cephalosporisation of okra seeds on the incidence of Verticillium wilt

Clorox-treated seeds of okra were inoculated with various concentrations of Cephalosporium by dipping them in the inoculum for 24, 48, and 72 hr. The inoculated seeds, after shade drying, were planted in steamed sterilized soil. Seven-day-old seedlings were uprooted, inoculated with Verticillium and replanted.

The treatment of okra seeds with different levels of Cephalosporium for varying time durations did not affect the incidence of Verticillium wilt (Table 3); however, the inoculation of germinating seeds with low levels of Cephalosporium resulted in reducing the incidence of wilt significantly.

TABLE 3. Effect of inoculating seeds of okra with different concentrations of Cephalosporium for different times on the incidence of Verticillium wilt

Cephalosporium Inoculum Level	Average Percentage Wilt[a]		
	Inoculation Duration		
Spores/ml ($\times 10^7$)	24 hr	48 hr	72 hr
9.1	23.3	33.3	36.6
5.1	20.0	30.0	33.3
3.5	20.0	27.0	30.0
2.7	27.0	33.3	27.0
1.6	20.0	30.0	33.3
1.1	23.3	30.0	36.6
Verticillium only[b]	27.0	36.6	23.3
Water only	0	0	0

[a]Three replications.

[b]7.2×10^7 spores/ml.

The duration of inoculation time, however, did not exert any influence (Table 4, Fig. 2). Cephalosporium was invariably isolated from the root zones of apparently healthy plants. Seeds were inoculated at the time when the radical was emerging.

TABLE 4. Effect of inoculating germinating seeds of okra with different concentrations of Cephalosporium for various times on the incidence of Verticillium wilt

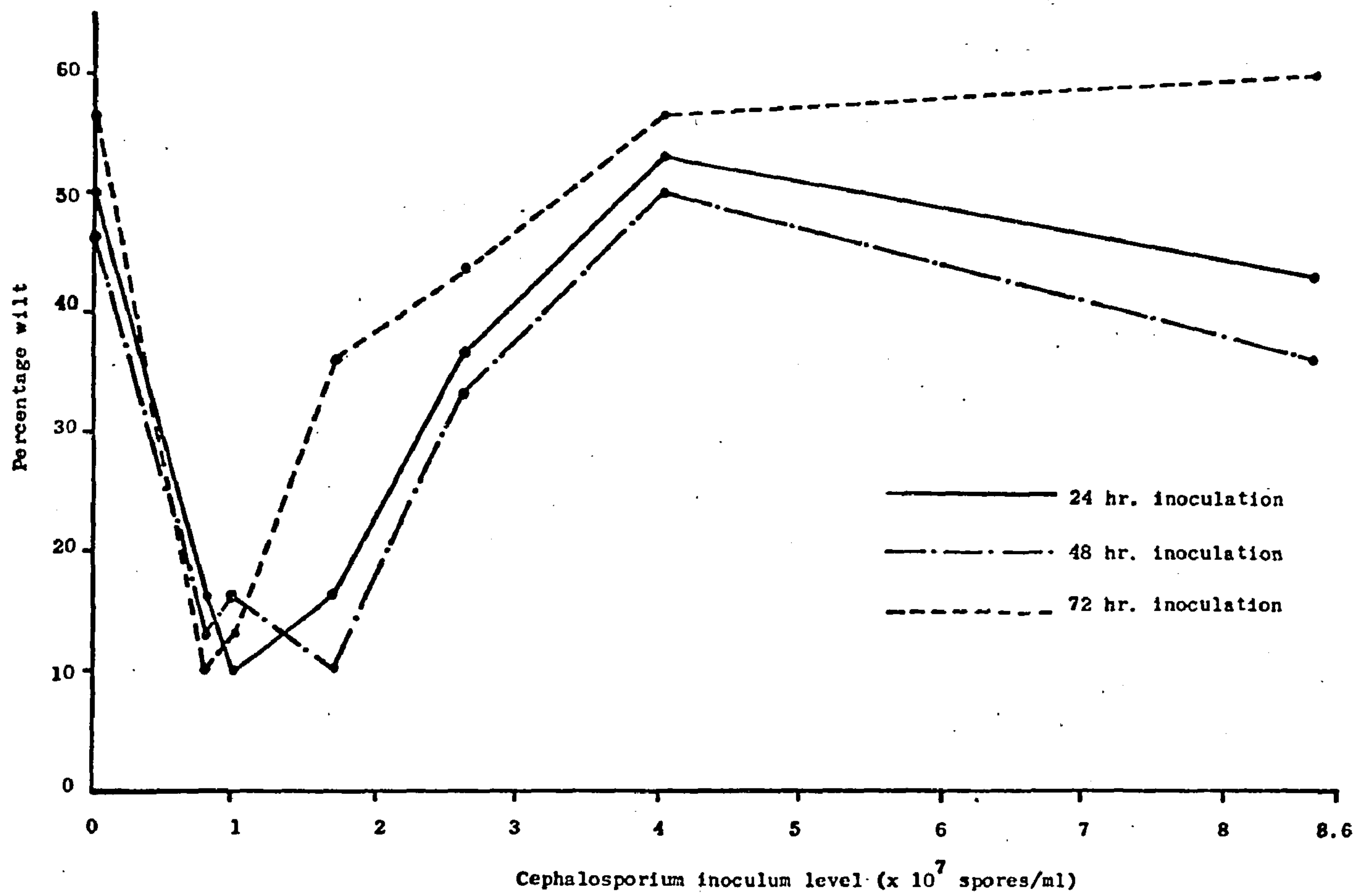
Cephalosporium Inoculum Level	Average Percentage Wilt[a]		
Spores/ml ($x 10^7$)	24 hr Inoculation	48 hr Inoculation	72 hr Inoculation
8.6	43.3	36.6	60.0
4.0	53.3	50.0	56.6
2.6	36.6	33.3	43.6
1.7	16.7*	10.0*	36.6
1.0	10.0*	16.7*	13.3*
0.8	16.7*	13.3*	10.0*
Verticillium only[b]	50.0	46.6	56.6
Water only	0	0	0

[a]Three replications.

[b]4.1×10^7 spores/ml.

*Significant difference over control (Verticillium only) at 1% level.

Fig. 2. Effect of Cephalosporium inoculation of germinating okra seeds on the incidence of Verticillium wilt



Effect of in-soil Cephalosporisation of okra roots on the incidence of Verticillium wilt

Clorox-treated okra seeds were sown in *Cephalosporium* infested soil and were allowed to grow after emergence for 12, 24, 36, 48, and 60 hr in the infested soil. The seedlings were then uprooted and inoculated with *Verticillium* (4.2×10^8 spores/ml) for 15 min and replanted.

The incidence of *Verticillium* wilt was significantly reduced in the case of those seedlings which grew in *Cephalosporium* infested soil for only 12 hr as compared to those which grew for longer periods (Table 5, Fig. 3). *Cephalosporium* was isolated from 87.5% of apparently healthy seedlings.

TABLE 5. Effect of in-soil inoculation of okra roots with *Cephalosporium* for various durations of time on the incidence of *Verticillium* wilt

Time the okra seedlings were in *Cephalosporium* infested soil prior to their *in vitro* inoculation with *Verticillium*[b]	Average[a] Percentage Wilt
12 hr	16.6*
24 hr	34.6
36 hr	54.6
48 hr	31.3
60 hr	37.5
Verticillium only[b]	39.6

[a]Six replications.

[b]4.2×10^8 spores/ml.

*Significant difference over control at 1% level.

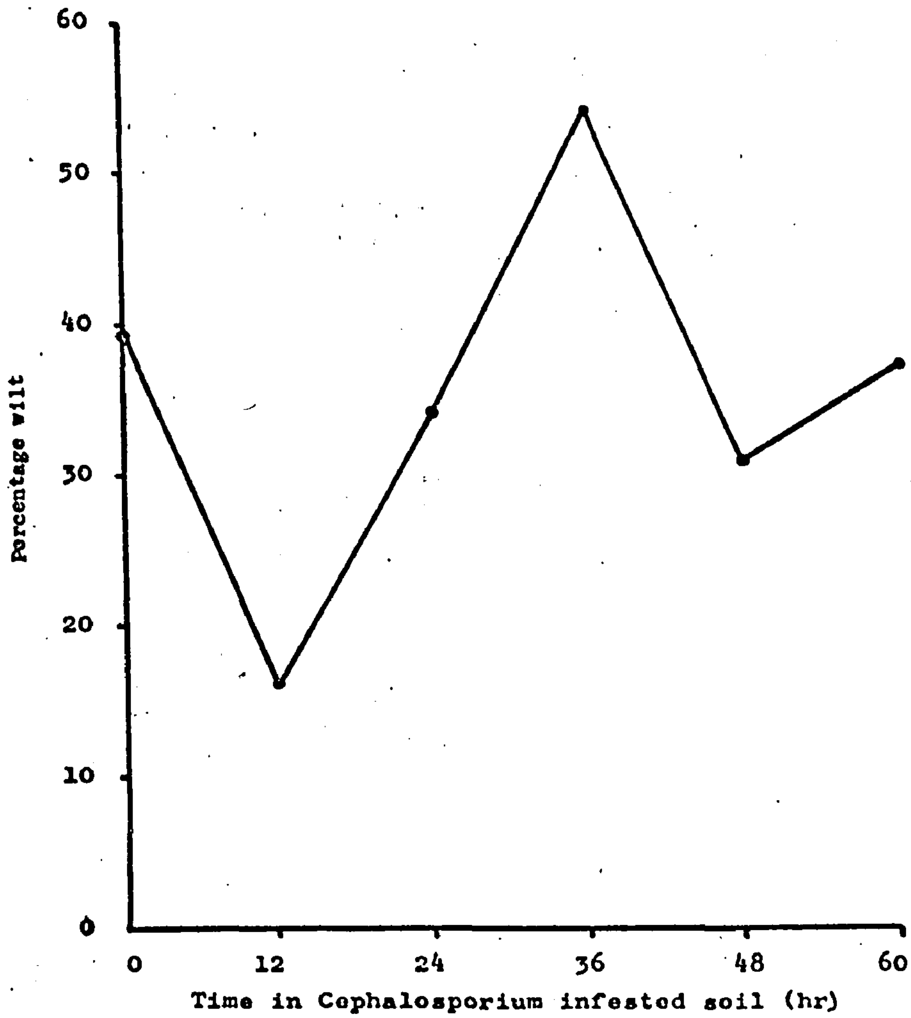


Fig.3.--Effect of growing okra in *Cephalosporium* infested soil for various time duration on the incidence of *Verticillium*-wilt

Effect of treating okra roots with Cephalosporium prior to their inoculation with Verticillium on the subsequent recovery of Verticillium

Roots of seven-day-old seedlings were inoculated with Cephalosporium for 72 hr and then transplanted in Verticillium infested soil. The seedlings were removed after 7 days and sections from each seedling were cultured for checking the presence of Verticillium.

The treatment of okra roots with Cephalosporium prior to their transfer to Verticillium infested soil reduced the recovery of Verticillium by 22.7 percent relative to the control (Table 6).

TABLE 6. Effect of pre-inoculation of okra roots with Cephalosporium on subsequent infection by Verticillium

Treatment	Percentage Recovery of Verticillium[a]	Percentage Decrease Over Control
Okra roots preinoculated with Cephalosporium[b]	54.5*	22.7
Okra roots not inoculated with Cephalosporium	70.5	

[a]On the basis of 200 sections from 40 plants.
[b]3.9×10^7 spores/ml.
*Significant difference over control at 5% level.

These preliminary experiments (Tables 1 to 6) indicate that incidence of Verticillium wilt of okra is reduced by Cephalosporium under certain conditions. The following were envisaged as possible mechanisms of wilt reduction by Cephalosporium: (1) Cephalosporium may be causing a physical block of xylem vessels and thus restricting the advance of Verticillium in the plants in the same way as has been reported in tomato wilt caused by Fusarium oxysporum f. lycopersici (21); (2) Cephalosporium may be antagonistic to the growth of Verticillium in plants as well as in soil; (3) plants may be responding to Cephalosporium infection by producing some anti-Verticillium principle. To determine a possible mode of action of Cephalosporium several investigations were initiated.

Microscopic examination of roots and stems of okra inoculated with low concentrations of Cephalosporium did not reveal any blockage of xylem vessels under the conditions of this study; however, light brown discoloration of xylem vessels was observed. From this it was concluded that the advance of Verticillium in okra plants was not restricted by Cephalosporium itself or through any kind of physical block induced by Cephalosporium, as had been found in a study on the mechanism of Fusarial wilt reduction in tomato by Cephalosporium (21). The above conclusion is based on the examination of more than 200 plants.

It was observed that Cephalosporium and Verticillium, while growing together on potato-dextrose agar or root-stem extract agar were not antagonistic to each other, and the relationship between them appeared to be like neutral symbionts (Table 7).

Culture filtrates obtained from seven-day-old cultures of Cephalosporium did not appear to affect the rate of growth and cultural characteristics of Verticillium (Table 8). After Seitz filtration, the filtrates were incorporated in dextrose agar at the rate of 500 ml in 1,000 ml of agar.

TABLE 7. Effect of *Cephalosporium* and *Verticillium* on their mutual growth *in vitro*

Treatment	Observations[a]
1. Spores of *Cephalosporium* and *Verticillium* seeded together on petri plates	Spores of both fungi germinated in an apparently normal manner. Petri plates were filled completely with mycelial growth after 36 hr of spore seeding
2. Petri plates first seeded with spores of *Cephalosporium* and after their germination, the plates were seeded with spores of *Verticillium*	Spores of *Verticillium* germinated normally. There was no apparent inhibitory effect of *Cephalosporium* on their germination or on their subsequent growth
3. Petri plates seeded with spores of *Cephalosporium* and mycelial bits of *Verticillium* at the same time	Germination of spores of *Cephalosporium* and growth of mycelium of *Verticillium* was normal. The mycelium of both fungi grew intermingling together. There was no apparent inhibition of any
4. Petri plates seeded with spores of *Cephalosporium* and after their germination, the plates were inoculated with mycelial bits of *Verticillium* at different places	Mycelium of the *Verticillium* was found growing normally without any apparent inhibitory effect of *Cephalosporium* on its growth
5. Petri plates were inoculated simultaneously with mycelial bits of *Cephalosporium* and *Verticillium*	No apparent inhibition in the growth of either *Cephalosporium* or *Verticillium* was noticed at the point of their contact. The mycelium of both intermixed and remained growing uninterrupted
6. Petri plates were inoculated first with a mixture of mycelium and spores of *Cephalosporium* and after 24 hr inoculated with a mixture of mycelium and spores of *Verticillium*	Spores and mycelium of the *Verticillium* germinated and grew normally. There was no apparent mutual inhibition of either fungus

[a]Five replications.

TABLE 8. Effect of filtrates from Cephalosporium broth cultures on the growth of Verticillium in vitro

Treatment	First Test: Average[a] Radial Growth at the End of 7 Days (mm)	Second Test: Average[a] Radial Growth at the End of 13 Days (mm)	Mycelium Growth Character-istics
I. Dextrose-culture filtrate agar			
(a) culture filtrate autoclaved	48.7	70.0	Appressed and compact
(b) culture filtrate not autoclaved	49.8	68.0	Appressed and compact
II. Dextrose agar	52.0	73.0	Appressed and compact

[a]Twelve replications.

Effect of root-extracts from Cephalosporium infected plants on the weight of Verticillium

In one of the preliminary experiments, it was observed that root extracts from Cephalosporium inoculated plants affected the cultural characteristics and growth of Verticillium, and that the inhibitory property of root extracts was destroyed by autoclaving. The effect of root extracts on the growth of Verticillium was further studied by culturing the fungus on potato-dextrose broth (PDB) having 25% of Seitz filtered root extracts. Dry weight of the mycelium was determined after 20 days.

The root extracts from Cephalosporium inoculated plants inhibited the growth of Verticillium to a significant extent. The inhibitory properties of root extracts were affected by the concentrations of Cephalosporium used for inoculating okra plants. There was a positive correlation between the concentration of the Cephalosporium inoculum used and the activity of the extracts (Table 9, Fig. 4). The experiment was repeated twice with the same trend of results.

TABLE 9. Effect of root extracts from Cephalosporium infected okra plants on the growth of Verticillium

PDB Plus Root Extracts from Okra Plants Inoculated with the Following Levels of Cephalosporium (Spores/ml x 10^7)	Average[a] Dry Weight in gm
8.8	0.0464*
5.0	0.0788*
3.4	0.1122 ⎤ *
2.0	0.1141 ⎦
1.1	0.1591 ⎤ *
0.6	0.1590 ⎦
PDB only	0.2153*
PDB plus root extracts from healthy plants	0.2341

[a]Eight replications.

*Significant difference over control at 1% level.

Germination of Verticillium spores was inhibited significantly by the root extracts from Cephalosporium inoculated plants. Germination was tested in root extracts diluted with an equal

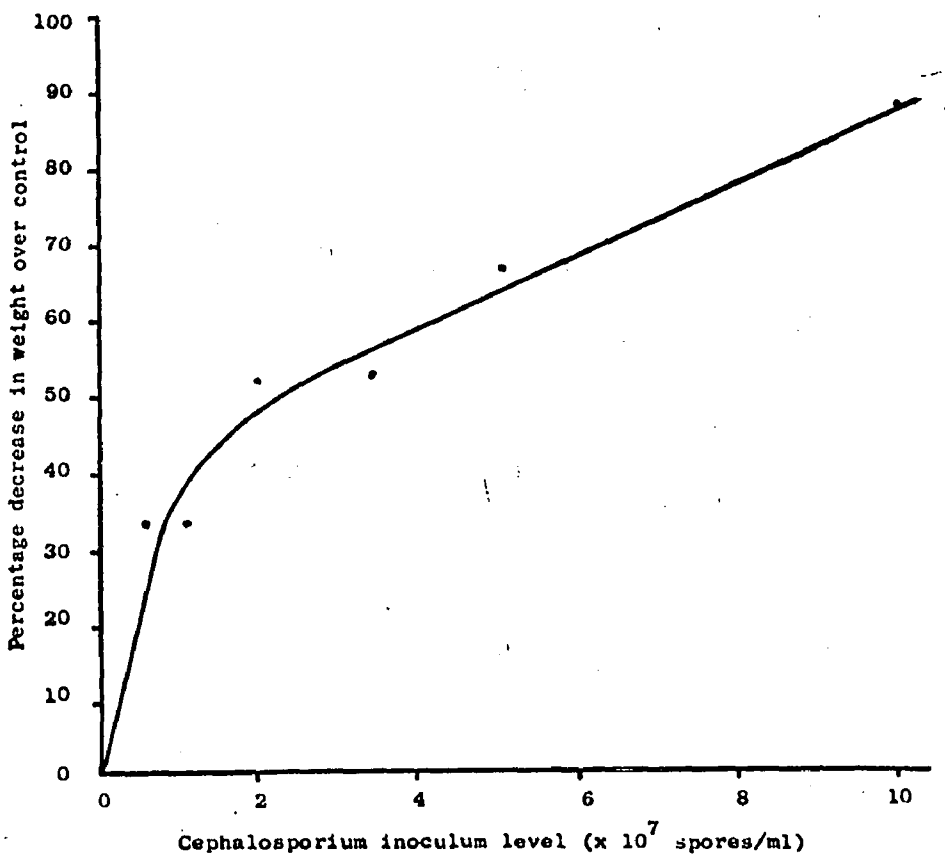


Fig. 4.--Effect of Cephalosporium inoculum levels on the inhibitory properties of okra roots extracts to Verticillium growth

volume of water. The inhibition of germination was from 58.2 to 72.2% (Table 10, Fig. 5).

TABLE 10. Effect of root-extracts from Cephalosporium inoculated okra plants on the germination of Verticillium spores

Extracts from Okra Plants, Previously Inoculated with the Following Levels of Cephalosporium (Spores/ml x 10^8)	Germination Range (Percent)	Average[a] Germination at the End of 72 hr (Percent)
4.0	20 - 40	27.8
2.4	29 - 38	31.8 *
1.1	27 - 40	33.8
0.8	39 - 45	41.8
0.5	39 - 42	40.4 *
0.2	39 - 42	40.6
Water only	77 - 89	84.6*
Extracts from healthy plants (control)	86 - 94	92.2

[a]Five replications.

*Significant difference over control at 5% level.

Root extracts were obtained from plants inoculated with different concentrations of Cephalosporium and the effect of these extracts on the germination of Verticillium conidia was studied to determine the lowest level of Cephalosporium inducing the inhibitory properties.

Root-extracts from the plants which were inoculated with 0.15 x 10^7 spores/ml or less did not seem to possess any inhibitory properties. Apparently the end point of the effective inoculum level was somewhere between 0.28 x 10^7 to 0.15 x 10^7 spores/ml (Table 11).

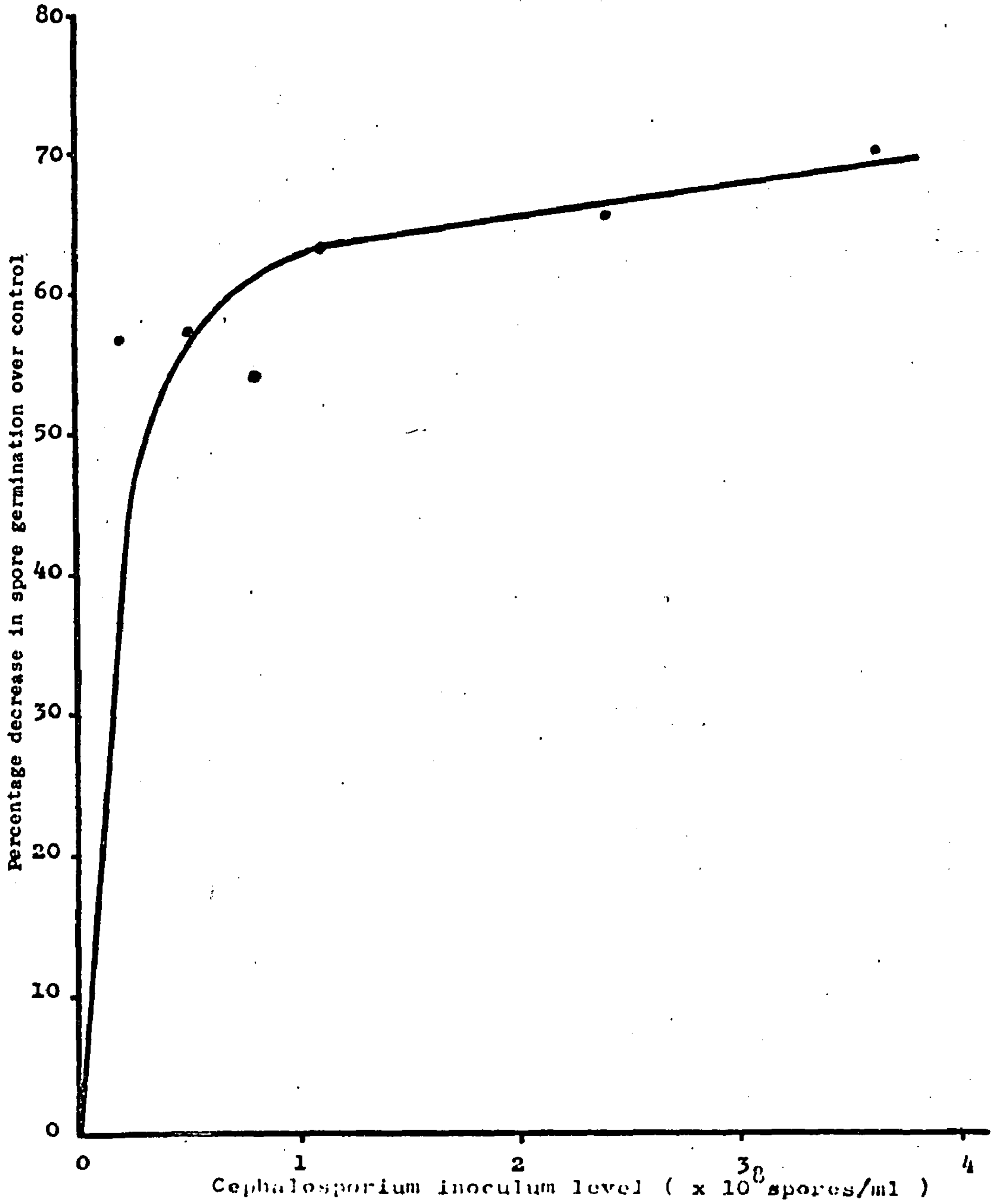


Fig.5.-- Effect of Cephalosporium inoculum level on the inhibitory properties of okra root extracts to Verticillium spore germination

TABLE 11. Effect of root-extracts from Cephalosporium inoculated okra plants on the germination of Verticillium spores

Root Extracts from Plants Inoculated with Following Levels of Cephalosporium (Spores/ml x 10^7)	Germination Range (Percent)	Average[a] Germination at the End of 72 hr (Percent)
2.04	39 - 53	42.2 *
0.91	39 - 54	46.0 *
0.59	58 - 69	61.8*
0.28	70 - 90	80.2*
0.15	86 - 95	89.0
0.08	85 - 96	92.5
0.03	85 - 95	90.5
Root-extracts from healthy plants	87 - 95	92.2

[a]Six replications.

*Significant difference over control at 1% level.

Effect of root-extracts from Verticillium inoculated plants on the germination of Verticillium spores

Roots of seven-day-old seedlings of okra from Clorox disinfested seeds were inoculated with Verticillium for 96 hr. Extracts from roots of these plants were sterilized by Seitz filtration and diluted with an equal volume of water before their effect on germination of Verticillium conidia was determined.

The germination of Verticillium spores was not inhibited by the root extracts from Verticillium inoculated plants (Table 12), as had been found in case of root extracts from Cephalosporium inoculated plants.

TABLE 12. Effect of root-extracts from Verticillium inoculated plants on the germination of Verticillium spores

Treatment	Germination Range (Percent)	Average[a] Germination at the End of 72 hr (Percent)
Extracts from Verticillium infected plants[c]	85 - 92	90.6[b]
Extracts from healthy plants	90 -100	94.8

[a]Ten replications.

[b]Difference is not significant over control.

[c]4.1×10^8 spores/ml.

Effect of spraying okra seedlings with spores of Cephalosporium on the incidence of Verticillium wilt

Seven-day-old seedlings from Clorox treated seeds planted in sterilized soil were sprayed with spores of Cephalosporium (9.6×10^6 spores/ml). The sprayed plants were transferred to 100% humidity chambers for 72 hr. The temperature in the chamber was maintained at 30 C. At the end of 72 hr in the humidity chamber, the roots of the plants were inoculated with Verticillium spores (1.5×10^7 spores/ml), and the incidence of wilt was determined three weeks later.

The spraying of okra plants with Cephalosporium spores prior to root inoculations with Verticillium did not reduce the incidence of wilt (Table 13), although Cephalosporium was found to be established in the leaves and stems of okra.

The results enumerated in the preceding pages indicated that invasion of okra roots by Cephalosporium prior to their

TABLE 13. Effect of spraying okra seedlings with spores of *Cephalosporium* on the incidence of *Verticillium* wilt

Plants sprayed with--	Replication	No. of Test Plants in Each Replication	Average Wilt (%)
Cephalosporium spores	1	30	100
	2	35	94.2
	3	25	88.0
	4	30	80.0
	5	31	74.4
		Average	87.3
Autoclaved water	1	25	80.0
	2	25	92.0
	3	30	86.6
	4	25	76.0
	5	30	76.6
		Average	82.2

invasion by *Verticillium*, perhaps induced the production of some toxic metabolite which had an inimical influence on *Verticillium* (both on the germination of spores and on the growth of mycelium). To gain some insight about the mode of action of the suspected toxic metabolite, the effect of stem exudates, stem extracts, and root exudates from *Cephalosporium* inoculated plants on the germination of *Verticillium* spores was studied.

Effect of stem-exudates and stem-extracts from Cephalosporium inoculated okra plants on the germination of Verticillium spores

Stem-exudates from okra previously inoculated with *Cephalosporium*, and from healthy plants were collected under aseptic conditions in 3 mm squares of Whatman No. 1 filter paper. The filter paper squares having stem exudates were transferred to plates of potato-dextrose agar flooded with *Verticillium* spores. There was no inhibition of spore germination under or around the filter paper squares. The spores germinated normally and mycelium ultimately covered the filter paper squares.

Stem-extracts from okra plants were obtained in the same way as described earlier for root-extracts. The extracts were sterilized by Seitz filtration. Squares of Whatman No. 1 filter paper soaked in extracts were placed on plates of potato-dextrose agar flooded with *Verticillium* spores. The stem-extracts like the stem exudates from *Cephalosporium* inoculated plants did not inhibit the germination of *Verticillium* spores (Table 14). From the preceding two experiments it was inferred that the postulated toxic metabolite was not translocated upward in the stem or if so then the quantity of the translocated metabolite was not enough to inhibit germination of *Verticillium* spores.

TABLE 14. Effect of stem-extracts from Cephalosporium inoculated plants on the germination of Verticillium spores in vitro

Extracts from Okra Plants Previously Inoculated with the Following Levels of Cephalosporium ($Spores/ml \times 10^8$)	Observations[a]
1.92	No inhibition of spore germination was noticed under or around the filter paper squares. The mycelium grew normally and ultimately covered the filter paper squares
1.08	
0.69	
0.40	
0.24	
0.09	
Stem-extracts from healthy plants	

[a]Seven replications.

Effect of root-exudates from Cephalosporium inoculated plants on the germination of Verticillium spores

Clorox disinfested seeds of okra were sown in 6" x 1" test tubes containing 2% agar under aseptic conditions. When the seedlings were two days old they were inoculated in situ for 48 hr with spore suspensions of Cephalosporium (4×10^6 spores/ml) and then were aseptically transferred to petri plates of potato-dextrose agar previously flooded with Verticillium spores. It was observed that there was no germination of Verticillium spores around the roots of 30% of the seedlings and later, marked inhibitory zones developed around them. The experiment was repeated six times with the same trend of results but the number of seedlings showing inhibitory zones never exceeded 30%. In one experiment, *Verticillium* spores were allowed to germinate before the *Cephalosporium* inoculated seedlings were transferred to the plates. In this case the further growth of germ tubes in the vicinity of the roots of about 38% of the seedlings stopped and inhibitory zones became apparent. The low percentage of inoculated okra seedlings showing inhibitory zones around their roots may be due to the failure of establishment of *Cephalosporium* in the majority of the cases. *Cephalosporium* was never isolated from the seedlings showing no inhibitory zones, while on the other hand *Cephalosporium* was always isolated from the seedlings with inhibitory zones around their roots. On the basis of these observations it was suspected that the inhibitory action of the postulated toxic metabolite induced by *Cephalosporium* was confined to the roots of okra plants.

DISCUSSION

Experimental results indicate that the treatment of okra roots with high concentrations of *Cephalosporium* invariably results in inducing wilt in plants while the treatment with low concentrations of inoculum has no apparent effects (Table 1). *Cephalosporium* seems to penetrate and become established in apparently healthy plants as it was consistently isolated from them. It also appears that the progress of *Cephalosporium* in okra plants inoculated with low concentrations is slow relative to the progression in those inoculated with high concentrations, because *Cephalosporium* was never isolated above hypocotyl level of the apparently healthy plants, whereas it was isolated right up to the tip from the wilted ones. Müller and Börger (19) have reported that when plants were inoculated with avirulent strains of pathogens a chemical principle was produced which afforded protection to them by inhibiting the growth of virulent pathogens. According to Müller (18), a definite concentration of the chemical principle was necessary for the inhibition of the pathogen but when its concentration crossed a certain limit it became toxic to the host plant itself. In the present study it is possible that the inoculation of okra plants with low concentrations of *Cephalosporium* may be inducing the production of a toxic metabolite in quantity which may not be enough to cause any apparent harm to the plants but may be adequate to inhibit the growth of *Verticillium* propagules. With the use of high concentrations of inoculum the production of the toxic metabolite may be in such quantity as to cause wilting in plants.

The experiments to study the effect of *Cephalosporium*-okra interaction on the incidence of *Verticillium* wilt indicate that the incidence of wilt is significantly reduced by pre-inoculating roots of okra with low concentrations of *Cephalosporium* (4.8×10^7 spores/ml and lower) (Table 2). Buxton and Perry (5) reported a lower incidence of wilt in peas by co-inoculating the plants with *Fusarium oxysporum* f. *lycopersici* and *F. solani*. Smith (29) has reported the control of wilt in tomato caused by *F. oxysporum* f. *lycopersici* by preinoculating the roots with a species of *Micromonospora*. Long (15) and Roy *et al.* (25) were able to reduce *Fusarium* wilt symptoms in cowpea and cotton respectively by the use of *Cephalosporium* sp.

The treatment of okra seeds with different concentrations of *Cephalosporium* for various durations of time is not effective in reducing the incidence of *Verticillium*. However, the treatment of germinating seeds with low concentrations of *Cephalosporium* prior to inoculation with *Verticillium* reduces the incidence of wilt significantly (Tables 3 and 4). The ineffectiveness of seed inoculation with *Cephalosporium* in controlling wilt may be due to the epigeal nature of cotyledons, so most of the inoculum may be carried above ground, thus reducing the amount in the vicinity of roots to below the effective range. This inference is based upon the observation that Cephalosporium was never isolated from the seedlings from Cephalosporium inoculated seeds while it was readily isolated from the seedlings growing from inoculated germinating seeds. The effectiveness of Cephalosporium treatment of germinating seeds in reducing the incidence of Verticillium wilt may be due to the exposure of emerging radicals to the controlled amount of Cephalosporium inoculum and thus the seedlings treated with low concentrations of inoculum show resistant reactions (Table 4 and Fig. 2).

The importance of low levels of Cephalosporium in inducing resistance in the plants is further confirmed by the varying degrees of wilt induced by altering the duration of root inoculation but keeping the inoculum level constant. The inoculation of roots with 1.7×10^7 spores/ml for 72 hr results in significantly higher incidence of wilt relative to those treated for 24 and 48 hr (Table 4, Fig. 2). It is presumed that with 72 hr inoculation period the roots contact more Cephalosporium relative to 24 and 48 hr inoculation periods. This assumption is further strengthened by the results obtained from in-soil inoculation of roots with Cephalosporium, where the incidence of wilt increases significantly as the duration of root inoculation increases from 12 hr to 60 hr (Table 5, Fig. 3).

The role of Cephalosporium in reducing the incidence of wilt in okra is further indicated with 22.7% less recovery of Verticillium from the seedlings inoculated with Cephalosporium prior to their inoculation with Verticillium (Table 6). It, therefore, seems reasonable to infer that preinoculation of okra roots with Cephalosporium somehow reduces the penetration and perhaps the subsequent development and progress of Verticillium in plants.

Experiments to study the nature of resistance induced by Cephalosporium inoculations of roots indicate that Cephalosporium neither causes nor induces any physical blockage of xylem vessels of inoculated okra plants. This observation suggests that the mechanism of Verticillium wilt reduction in okra by Cephalosporium is different from the mechanism of fusarial wilt reduction in tomatoes (21).

The non-occurrence of antagonism between Verticillium and Cephalosporium (Table 7), and the absence of any adverse influence of Cephalosporium metabolites on the growth of Verticillium (Table 8) indicates that Cephalosporium may be inducing some changes in the invaded host tissues which may ultimately inhibit the growth and development of Verticillium in plants. This inference is supported by the observance of inimical influence of root-extracts from Cephalosporium inoculated plants on the germination and subsequent development of Verticillium (Tables 9, 10, 11). As the root-extracts from Verticillium inoculated plants do not inhibit the germination of Verticillium spores (Table 12), it is presumed that the inhibitory properties of the extracts of Cephalosporium inoculated plants are the result of Cephalosporium-okra interaction and the place of effective interaction appears to be the roots of the plant. This inference is based on the observations that spraying of okra plants with Cephalosporium spores does not reduce the incidence of Verticillium wilt although Cephalosporium mycelium penetrates the leaf and stem tissues of the sprayed plants, and that root exudates from Cephalosporium inoculated plants inhibit the growth of Verticillium.

A collective look at all the observations and experimental data perhaps justifies the following conclusions on the mechanism of Verticillium wilt reduction in okra by Cephalosporium. The treatment of okra roots with low concentrations of Cephalosporium induces a hypersensitive reaction in plants which leads to the production of some anti-Verticillium metabolite in the infected tissues. This so-called toxic metabolite is partly exuded and partly retained in the roots where it inhibits the growth of Verticillium and hence lowers the incidence of wilt. When the roots of okra are inoculated with high concentrations of Cephalosporium, the production of anti-Verticillium principle crosses a certain optimum limit and becomes toxic to the plant tissues. At this stage, the relationship between Cephalosporium and Verticillium perhaps assumes a metabiotic nature and hence the increase in the incidence of wilt in plants inoculated with high concentrations of Cephalosporium.

The study of interaction between okra plants and Cephalosporium illustrates the importance of low levels of Cephalosporium in reducing the incidence of Verticillium wilt. In vitro studies on the effect of root extracts from Cephalosporium inoculated plants on spore germination and mycelial growth of Verticillium illustrates the effectiveness of higher concentrations of

Cephalosporium. This anomaly in results may be explained. It is presumed that the infection of okra with all concentrations of *Cephalosporium* results in the production of a toxic metabolite which is essentially anti-*Verticillium* in character but is toxic to the plants also at certain concentrations. When the plants are inoculated with high levels of *Cephalosporium*, the production of the toxic metabolite crosses the critical concentration necessary for *Verticillium* inhibition and attains the concentration which is toxic to the plant tissue. Under these conditions, it appears that anti-*Verticillium* properties of the toxic metabolite are masked, while in *in vitro* studies the living tissue is not involved and hence there is no masking of the anti-*Verticillium* character of the metabolite.

SUMMARY

Under the conditions of this research, inoculation of okra roots with low concentrations of Cephalosporium prior to their inoculation with Verticillium reduced significantly the incidence of Verticillium wilt.

The treatment of okra seeds with high as well as with low concentrations of Cephalosporium did not have any influence on the incidence of wilt; however, the inoculation of germinating seeds with low concentrations of Cephalosporium did reduce the incidence of wilt.

Preinoculation of okra roots with Cephalosporium retarded significantly the colonization of the plants by Verticillium. The reduction was about 23 percent.

Okra plants inoculated with Cephalosporium did not show any marked differences in their anatomy except light brown discoloration of xylem vessels.

Cephalosporium and Verticillium were not antagonistic to each other in culture. Metabolites of Cephalosporium did not affect the growth of Verticillium in culture.

Root extracts from Cephalosporium inoculated plants inhibited significantly the conidial germination and growth of Verticillium. The inhibitory properties of root extracts were destroyed by autoclaving.

Root extracts from *Verticillium* inoculated plants did not inhibit the growth of Verticillium in *in vitro* conditions.

Spraying of okra plants with *Cephalosporium* conidial suspensions prior to their inoculation with *Verticillium* did not have any effect on the incidence of *Verticillium* wilt.

Stem exudates and stem extracts from *Cephalosporium* inoculated plants did not inhibit the germination and growth of *Verticillium*, however, root exudates from *Cephalosporium* inoculated plants retarded the germination and growth of *Verticillium*.

On the basis of present study it is presumed that the nature of resistance induced by *Cephalosporium* in plants to *Verticillium* invasion is chemical. The inhibitory action of the chemical seems to be local instead of systemic.

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