QUANTITATIVE INVESTIGATIONS OF INFECTION AND COLONIZATION OF PEANUT ROOTS BY *Cylindrocladium crotalariae*

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Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Plant Pathology

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May, 1986

Blacksburg, Virginia

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(ABSTRACT)

Cylindrocladium black rot (CBR) is a destructive root disease of peanut caused by *Cylindrocladium crotalariae*. New and quantitative approaches were developed to estimate the inoculum potential for this soil-borne pathogen under soil-temperature tank conditions favorable for CBR development. Using cultural plating methods, numerous *C. crotalariae* infections (1 to > 1,000 per plant) were observed on asymptomatic root systems of peanut plants, susceptible and resistant to CBR, grown in naturally infested soils at 25 C. Regression-line slope values of $\log_{10}$-$\log_{10}$ plots for microsclerotial inoculum density versus the number of observed root infections per plant and per unit root length [0.98 (R^2=0.94) and 0.99 (R^2 = 0.94), respectively] indicated direct proportionality between the respective variables. Efficiency of inoculum for observed infection (percent of germinating microsclerotia that infect roots) estimates were high (near 100%), while efficiency of observed infection for necrosis (percent of infections that develop into necroses) estimates were low (0.27 to 0.28%). Observed infection rates, $I^o{}_r$ (the number of observed infections per m root per day per microsclerotium per g of

soil) were significantly lower (P = 0.001) for CBR-resistant 'Spancross' than for CBR-susceptible 'Florigiant'. The majority of observed infections did not appear to be restricted to outer cortical root tissues. A portion of observed infections near root surfaces resulted in subsequent colonization of the inner cortex and stele of plants for each cultivar. A significantly greater (P = 0.05) number of segments from steles of CBR-susceptible Florigiant taproots were colonized by C. crotalariae than those from CBR-resistant Spancross. Using naturally infested peanut field soils, a dose of 7.4 μg NaN_3 (sodium azide)/g soil was effective in reducing initial micro-sclerotial populations by 50%, based upon a highly significant dosage-response curve (R^2 = 0.96, P = 0.0001). CBR development was reduced significantly (P = 0.05) for Florigiant plants grown in C. crotalariae-infested soils treated with 7.5 μg NaN_3/g soil or higher, compared to plants grown in untreated soils. Sublethal doses of NaN_3 combined with biological control agents may be useful in the development of integrated CBR control measures.

DEDICATION

This dissertation is dedicated to my parents,

Tsuyako Kobayashi and Tommy Toshio

who have always valued formal education.

Their love, patience, guidance and understanding have been constant inspirations to me.

****

Happy 43rd anniversary, Mom and Dad!

ACKNOWLEDGEMENTS

The author expresses sincere gratitude and appreciation to many educators, colleagues and friends who have been instrumental in her professional development.

I thank Dr. Gary J. Griffin, my major advisor, mentor and immediate supervisor for his constant professional advice and criticisms, unwavering encouragement and moral support.

I sincerely appreciate and thank members of my advisory committee, Dr. Maynard G. Hale, Dr. N. L. Powell, Dr. R. J. Stipes and Dr. D. M. Porter for their time, patience, advice, and moral support. I also thank Dr. K. H. Garren and Dr. W. H. Wills for serving on my committee when needed.

Dr. L. I. Miller, another mentor (or tormentor), especially deserves a note of appreciation for his advice, kindness and unusual sense of humor.

It is unfortunate that physical space does not permit the acknowledgement of the individual names of all the faculty, technical and secretarial staffs and graduate students (past and present) in the Department of Plant Pathology, Physiology, and Weed Science, elsewhere in the University, or community. Your friendship, advice, comradeship and moral support will be long remembered. Thanks for the memories!

For financial support, I acknowledge the Commonwealth of Virginia and PPG Industries, Pittsburgh, Pennsylvania.

Finally, for their encouragement and moral support, my parents and my sister, Karen.

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INTRODUCTION and OBJECTIVES

Species of Cylindrocladium are prominent pathogens of many hosts in temperate, subtropical and tropical areas of the world. Root and crown rots, leaf spots or needle blights and stem cankers have been reported on many field and vegetable crops, ornamentals and forest nursery or large trees. Cylindrocladium crotalariae, one of the major pathogenic species of the genus, causes a root rot of a number of hosts, including blueberry (5), azalea (5) and peanut (1).

In 1965, Cylindrocladium black rot (CBR) on peanut was first observed in Georgia (1, 8). CBR was reported in Virginia and North Carolina in 1970. The disease has been associated with severe pod losses wherever the disease occurs (2, 3). Symptoms include chlorosis, necrosis and wilting. Wilting is caused by extensive root rot. Occasionally, an entire field of peanuts may be destroyed.

Microsclerotia are the primary survival propagules of C. crotalariae in soil. Microsclerotia are also thought to serve as the primary infective propagule of the fungus. They may be found within the cortex of colonized peanut roots. Upon decomposition of the root, microsclerotia are released into the soil. Even though microsclerotial densities may be reduced significantly following periods of extreme cold or drought (4), they manage to survive in the soil and are still capable of causing disease in later years.

A basic and comprehensive knowledge of the ecological interactions between the host and pathogen is prerequisite to recommendation of effective control strategies. Precise quantitative information regarding inoculum potential relationships of C. crotalariae, and the route of infection and colonization by C. crotalariae are especially important. Such knowledge regarding inoculum potential relationships may also be useful in the development of predictive analyses of CBR. Efforts to control Cylindrocladium black rot of peanut with the use of resistant cultivars in concert with soil fumigants have been tested (6, 7). Management of this disease also may be fostered through the integration of sublethal fumigant doses (to the pathogen) with application of other biological control agents.

The major objectives of this study were: (i) to determine infection rates and microsclerotial density-infection relationships of C. crotalariae on peanut roots in naturally infested soil, (ii) to determine the extent of infection and colonization by C. crotalariae of CBR-susceptible and CBR-resistant peanut roots, and (iii) to determine dose-response relationships of C. crotalariae to sodium azide in naturally infested soil, and to determine if sublethal doses of sodium azide in combination with microbial antagonists control CBR.

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CHAPTER I

LITERATURE REVIEW

BIOLOGY OF Cylindrocladium crotalariae and DISEASE CYCLE

Cylindrocladium crotalariae Loos (Bell & Sobers [=Calonectria crotalariae Loos Bell & Sobers)], is a soilborne pathogen which is classified botanically as a member of the imperfect fungi, the Deuteromycetes. The fungus causes Cylindrocladium black root rot (CBR) of peanut, a destructive root and pod rot of susceptible varieties of peanut, Arachis hypogaea (17). Its teliomorph, Calonectria crotalariae, is an ascomycete, which forms perithecia on colonized roots, crowns and pods, under conducive conditions. The fungus reproduces as uni- to multiseptate conidia, ascospores and microsclerotia. Microsclerotia are aggregates of thick-walled, dark-pigmented cells, usually 25- to 103-μm in width from naturally infested peanut fields (71, 129) and are found in the rhizospheres and tissues of colonized peanut roots. Krigsvold et al (99) found that root exudates influence microsclerotial germination in peanut rhizospheres, thus, the primary infection court for C. crotalariae is thought to be near the region of root tips. Following germination, penetration of root surface tissues may occur after 12-24 hrs, and subsequent penetration to the root stele may occur within 48 hrs (90). After a growing season, microsclerotia may be released from

decomposing, diseased roots and spread from field to field by combines (137) or other farm machinery and by irrigation water flow (98). Although microsclerotia may survive in the soil for many years, microsclerotial populations may be reduced by prolonged periods of freezing temperatures (71, 125, 133) as well as drought (71). Soybeans and black walnut are are the only other known economic hosts for this pathogen in Virginia. Although C. crotalariae has been isolated from freshly harvested peanut seeds, the fungus is not considered a seed-borne inhabitant.

Perithecia formed during moist periods, exude ascospores (137) which may spread within the field by rainwater splash. The importance of ascospores is believed to be limited to short-distance spread. Total populations (conidia plus ascospores plus microsclerotia) of C. crotalariae have not differed significantly from microsclerotial populations within a growing season (Griffin and Tomimatsu, unpublished data).

Although no races of this pathogen have been reported, the genetics of virulence of C. crotalariae to Arachis hypogaea appear to be complex (22, 25). Virulence variability has been suggested to be due to the predominantly binucleate mycelia (some were observed to be either uninucleate or trinucleate) of C. crotalariae (91).

SYMPTOMATOLOGY and INFLUENCE of ENVIRONMENTAL FACTORS on CBR SEVERITY

Above-ground symptoms of the disease include chlorosis and

wilting of the foliage commonly observed in late July to August. Roots with characteristic black lesions and cortical decay also have been observed early in the season. Peanut plants without shoot symptoms but with severly rotted taproots have been noted (Griffin and Tomimatsu, unpublished), due to the production of adventitous roots (78).

CBR development in Virginia and North Carolina is strongly favored by heavy rainfall in June and/or July, followed by extensive dry periods in August and September (126, 127). High rainfall early in the growing season, sufficient to provide soil moisture near field capacity, is presumably necessary for extensive root infection and severe root rot. Expression of aboveground symptoms may be further intensified by subsequent periods of moisture stress on the plant.

Root rot severity has been observed to be influenced by abiotic components such as soil particle size (22, 24), soil type (13), nutrients (121), temperature and moisture (71, 126, 128). Also, biotic components such as plant parasitic nematodes, particularly _Meloidogyne_ spp. and _Macroposthonia_ spp. (45, 46), variation in pathogen virulence (135) and _C_. _crotalariae_ microsclerotial densities influenced disease severity. Barron and Phipps (13) observed an increase in root rot severity when recommended rates of the herbicides, dinitramine and dinoseb, were applied to field microplots. The impact of dinitramine treatments on the root rot severity appeared to be dependent on soil type, microsclerotial

density and herbicide rate.

CBR MANAGEMENT STRATEGIES

Control of plant diseases should place major emphasis on 1) the use of procedures that eliminate pathogens in soil or effectively suppress or avoid their pathogenic capacity, 2) use of plant propagules free of the pathogen, and 3) use of resistant cultivars when available (37). All of these principles have been applied toward the development of practical management of CBR, either singly or in combination.

Resistance to CBR.

Resistance to CBR can be evaluated from two practical aspects (120): differences in disease incidence and severity (i.e. pathogen growth) and differences in microsclerotial production. Based on disease incidence or severity, several evaluations of CBR resistance have been conducted in naturally infested fields (33, 34, 56, 66, 67, 120, 125, 137, 165), in microplots (120) and in greenhouse studies (76, 77, 78, 120, 125, 138).

Sources of CBR resistance originated from three Virginia types, one Valencia-type and several Spanish-type varieties of peanuts based on disease severity (20, 33). Selections were made for those peanut genotypes which exhibited reduced root rot severity to CBR and those which had agronomic traits desirable for peanut production.

Hadley et al (77) examined crosses of NC3033 (CBR-resistant),

Argentine (CBR-resistant), NC 2 (CBR-susceptible) and Florigiant (CBR-susceptible) and showed that resistant selections could be made from the progeny of 2 CBR-resistant cultivars and NC2. Florigiant produced susceptible progeny in all crosses. Based on these studies under optimum greenhouse conditions, they attributed resistance to additive genetic effects, and suggested that early generation selection for CBR resistance would be effective. Coffelt (33) in field trials, also found appreciable heritable CBR-resistance in the Spanish cultivars, and this resistance was observed to be governed solely by nuclear genes. The Spanish cultivar Spancross exhibited root rot resistance similar to that of one of its parents, i.e., Argentine.

Although reduced microsclerotial populations following growth of CBR-resistant cultivars have been examined in a few reports (67, 153), the microsclerotial densities in roots of CBR-susceptible and CBR-resistant cultivars generally increased with the root-rot ratings (120). Peanut genotypes presently are selected on the basis of several agronomic traits, e.g. large fruit and adequate yield. A negative correlation was found between resistance to CBR and with either large pod size or with adequate pod yield (67).

In a screening test of 11 Va-type peanut cultivars and cultivar Spancross (CBR-resistant), significant differences (P=0.05) in the percentage of dead plants due to CBR were observed for the Va-type cultivars in fields which had a 15% or less disease incidence (56).

One of four fields, however, had much higher disease incidences (i.e., > 27%) in which significant differences could not be detected among the 11 Va-type cultivars. Cultivar Spancross had appreciably fewer dead plants than any of the Va-type cultivars in all four fields.

Variation in disease incidence among Virginia-type peanut cultivars has been attributed to interactions among different fields and genotypes (18, 56, 125, 165). A clustered spatial distribution of the microsclerotia of C. crotalariae in naturally infested field soils (80, 154) was considered to contribute to the variation of CBR resistance in field trials among CBR-susceptible Florigiant and moderately resistant NC8C and 2 other breeding lines (120).

Soil Fumigation Studies

Treatment of soils with fumigants such as sodium or potassium azide (77) or methyl isothiocyanate (88) previously have been regarded as economically unfeasible for treatment of large infested areas but may be useful for small areas. In earlier studies, rates of preplant sodium azide (NaN_3) applications as low as 9 - 18 kg NaN_3/ha produced the highest peanut yields and lowest CBR disease severity ratings of any of the other chemical treatments, including pentachloronitrobenzene (PCNB), benomyl (93) and other nematicides (132). Rodriguez-Kabana and Backman (132) observed that a single post-emergence application of 12 lb NaN_3/A (13.5 kg NaN_3/ha)

or, in combination with a pre-plant treatment of less than 36 lb NaN_3/A (40.4 kg NaN_3/ha), had the greatest potential to reduce CBR incidence. Substantial increases in yield were especially noted when preplant applications of 22-45 kg NaN_3/ha were followed by over-treatment 30-40 days later of 5-8 kg NaN_3/ha (119). Hanounik and Osborne (unpublished) observed a significant decrease in CBR incidence on susceptible peanut cultivars following NaN_3 applications under greenhouse conditions. They attributed the disease incidence to a reduction in microsclerotial levels of <u>C</u>. <u>crotalariae</u>. The potential use of NaN_3 as a control agent for CBR has been supported by studies which reported significant reductions in CBR incidence on peanuts (132, 137, 164), as well as decreased microsclerotial populations (Hanounik and Osborne, unpublished data) following soil fumigation. More recently, significant reductions in the percent of CBR-symptomatic plants (based on shoot symptoms) have been observed in fields fumigated with Busan® or Vapam® (124). Decreased microsclerotial populations also were observed in soils fumigated with Busan® 1020 (@ 10 & 20 gal/A) or Vapam® (20 gal/A) and planted to NC8C (moderately CBR-resistant cultivar); whereas in fumigated soils planted to Florigiant, microsclerotial populations increased (124).

<u>Integrated Control Measures for CBR and Other Diseases.</u>

Suppression of disease via the use of biological agents, <u>in toto</u> or in part offers a powerful means to increase yield by

inhibition or destruction of pathogen inoculum, protect plants against infection, or increase the ability of plants to resist pathogens. Studies which employ soil fumigation techniques integrated with alternative disease-preventive methods may reveal the greater potential yield, in spite of abiotic stresses, if pathogens favored by the stresses can be controlled.

Sublethal concentrations of soil fumigants have been applied to soils to induce biological control of a pathogen by encouraging high populations of antagonistic microflora (37). A classic example of this type of integrated control involved the management of _Armillaria mellea_ in orchards using the combination of carbon disulfide (CS_2) and _Trichoderma viride_. Effectiveness apparently resulted from CS_2 killing the exposed mycelia and rhizomorphs, and weakened the remaining propagules in some undetermined manner, which enabled _T. viride_ to kill them. Bliss (26), concluded that CS_2 would kill _Armillaria_ at treatments higher than the tested dosage of 453.6 kg a. i./ha. However, _Trichoderma_ was the effective agent at lower concentrations of CS_2 using 338.5 kg a.i. per ha. _Armillaria mellea_ survived at least six years in citrus roots in non-treated soil that contained _Trichoderma_. Carbon disulfide treatment resulted in kill of the pathogen in roots only when _T. viride_ was present and was ineffective in sterilized soil (26).

Elad et al (50) reported the efficacy of methyl bromide fumigation combined with application of _Trichoderma harzianum_

into strawberry nursery beds and fruiting fields to protect plants from black root rot caused by Rhizoctonia solani. This protection was extended further when the plants were transferred to commercial fields. Soil fumigation disinfested the nursery beds very efficiently; however, some propagules of the pathogen apparently remained in the treated nursery soils which were later shown pathogenic on bean seedlings, in separate greenhouse experiments. Fumigation with methyl bromide (500 kg a.i./ha, 98% MeBr) favored the pathogen's spread, probably due to killing of the antagonists present in nonfumigated soil. Treatment with the Trichoderma preparation (wheat bran preparation, 40-50 g dry wt/m^2) slowed the spread of R.solani from the infested plants in the nursery beds to areas free of plants or stolons (40% reduction as compared to the control), when this treatment was spread over the row, as opposed to treatment at *the transplanting site.*

Sodium azide has been used conventionally as a broad-spectrum, pre-plant fumigant to control weeds, nematodes, insects and other plant pests in soil. Trichoderma spp. and other genera of the Monialies were found to be tolerant of annual applications of 134.5 kg NaN_3/ha over a 3-yr period (94). Populations of Fusarium spp., Pythium spp. and Trichoderma spp. decreased initially by 1% following NaN_3 application, but increased toward the end of the season. Species of Trichoderma have the ability to rapidly re-colonize fumigated soils and establish high populations (37, 86).

Also, some species of Trichoderma exhibit a wide diversity of microbial antagonisms (i.e., antibiosis, hyerparasitism and hyphal interaction) (40, 41, 42).

Azides, in general, are thought to be respiratory inhibitors. In vitro studies have yielded results which show the inhibitory effects of azides on numerous microbial transformations (93). Critical concentrations of NaN_3 have been shown to inhibit respiration only slightly but carbon assimilation and synthesis for Escherichia coli were inhibited completely (32). Spiegelman (147) observed that certain microorganisms were unable to use the energy of anerobic glycolysis in forming adaptive enzymes in the presence of azide. Sodium azide (86) and sodium azide + NH_3 (114) have been shown to inhibit soil nitrification. Hughes and Welch (86) emphasized that acidity promotes hydrolysis of KN_3 in aqueous solution with formation of volatile hydrazoic acid: $KN_3 + H_2O \longrightarrow KOH + HN_3$. Parochetti and Warren (118) provided conclusive evidence to indicate that KN_3 is rapidly decomposed in acidic soils, to the more biologically active form, HN_3. Hydrazoic acid is then rapidly converted to N_2O, N_2 and NO (21, 156).

Selectivity of soil fumigation results from a combination of differential tolerances and from escape by microorganisms (37, 96). Soil fumigants exist as gradients in the soil distal from injection sites, thus enabling cells and/or microorganisms to survive where the dosages are sublethal. Recolonization of a

fumigated soil following selective kill is accomplished primarily by those survivors having the shortest response time and the fastest growth rate. Of the microbial population, species of Trichoderma and Mucor are usually the first fungi to recolonize fumigated soil, whereas the Pseudomonas spp. are usually the first bacteria. Low microbial populations remain following soil fumigation, however. Root pathogens, e.g., Pythium spp. may also rapidly colonize the soil. Cook and Baker (37) advocate the use of a proper delay between soil fumigation and the sowing of the crop which may provide a greater advantage for nonpathogens to increase their soil populations. Management of the disease may be extended by supplementing fumigated soils with the addition of appropriate antagonists at the appropriate time.

INOCULUM POTENTIAL

The capability of a plant pathogen to infect and colonize susceptible plant tissues is especially important in studying the disease epidemic in order to initiate appropriate control and/or disease management procedures. Concepts of these ecological interactions between soil-borne pathogens and infection courts of their hosts underlying disease development have progressed over a period of about 30 years. Inoculum potential has been the preferred term to encompass these relationships, although the plethora of reviews (6, 8, 43, 44, 58, 61, 85, 109, 163), and journal articles citing appropriate and improved methodology have provided plant

pathologists continued refinement which has resulted in disagreement and has led to divergence of the concept. Additionally, alternative approaches, e.g. infection rates (157, 158), and concepts also have aided our comprehension regarding a pathogen's relative vigor to initiate infection and the disease process.

Apart from the genetic capacities of a given population of a pathogenic species (i.e., physiological races), there are obvious ecological considerations and morphological characteristics which influence inoculum potential. Inoculum may be organized into complex units (which affect size), such as in a cluster of cells. The inner cells may be protected against toxic materials and these adjacent cells may also contribute toward development of a single germ tube. When such a multicellular unit of inoculum produces several germ tubes, these together should produce more of an enzyme needed for invasion of the host than a single cell (assuming enzymatic degradation participation in pathogenesis). Size (23, 82) and nutritional vigor (123) of the propagules have been found to influence disease severity and thus, inoculum potential. For example, CBR severity at the same inoculum density, was more severe for large microsclerotia (i.e., microsclerotia collected on a 150-μm pore size sieve) than with smaller microsclerotia [those collected on sieve sizes between 38-μm and 106-μm pore size (23).

Historical Development of Concept.

Between 1932 and 1960, the term inoculum potential was treated

comparable to inoculum density. For example, Lin (105) used inoculum potential for the number of spores in pycnidia on leaf surfaces of Septoria apii. Horsfall, first in 1932 (83) and subsequently in 1945, equated "inoculum potential" with "spore load", referring to Heald's demonstration of the relationship between spore load and the development of bunt, caused by Tilletia caries and T. foetida in wheat. He (84) referred later to the application of the term by Zentmeyer and his associates as "the equilibrium between number of hosts, number of spores, randomness of host distribution and weather factors."

Effects of the environment on inoculum potential were considered by Garrett (57) who expressed the concept in relation to energy. Specifically, inoculum potential is the "...energy of growth of a parasite available for infection of a host at the surface of the host organ to be infected." Inoculum potential may be increased in either or both of two ways:

a) by increase in the number of infecting units, or propagules of the fungus per unit of root surface; or,

b) by increasing the nutritional status of the propagules.

The term "energy of growth" implied that infective hyphae may grow from the inoculum, and it may further be capable of producing hyphae at a certain density per unit area of host surface having a certain vigor, as determined by the nutrient status of the hyphal tips. It should be expected, that carbon reserves in the

germinating spore would limit the energy of growth and the infectivity of the germ tube more commonly than does any other nutrient, as carbon reserves would be used by the respiration of the dormant spore, whereas nitrogen and other mineral nutrients would not. Further, the energy of growth from the inoculum is conditioned also by a third factor, the collective effect of environmental conditions. Since environmental conditions vary from optimal to completely inhibitory, they determine the actual or realized energy of growth ranging from a possible maximum down to zero. Endogenous nutrients of inoculum may be augmented by exogenous nutrients from the environment, most commonly by exudation of nutrients, principally amino acids and sugars, from the host surface. Snyder (145) noted that carbon and nitrogen-compounds essential to the pathogen may be stored in the resting propagule or substrate in which it is lodged; inorganic N (primarily) may be provided in the soil solution and undecomposed crop refuse in the soil may provide additional sources of carbon. Generally the action of exudates on fungal germination is not specific (107, 140, 142, 143, 156); as both hosts and non-hosts may supply the stimulus and germination of resting structures (e.g. 69, 99) in the rhizosphere.

However, there have been a few reports (notably, 99, 141) where the action of root exudates of susceptible and resistant species of a host have fostered differential germination of a pathogen *in situ*. Specific examples will be discussed further in another section.

Other studies have also supported the general hypothesis of increased propagule germination of the pathogen in rhizospheres of susceptible cultivars in vitro (28, 155) and some (4, 5, 106, 110, 111) have noted higher numbers of bacterial populations or fungal populations (149), or in rhizospheres of susceptible host cultivars than their respective resistant host cultivars. Zilberstein et al (167) found that germination on agar and pathogenicity of Verticillium dahliae microsclerotia were affected by host origin and growth medium.

Criticism of this rather vague concept of inoculum potential has evolved primarily with the difficulty of defining or quantifying energy of growth. Garrett (59) noted that the most helpful data in determining energy of growth by an inoculum stemmed from experiments on infectivity of fungal spores. However, even with the most sophisticated techniques, the "infectivity" of the inoculum varies with the experimenter, the methodology and the organism(s) involved. This has been demonstrated amply in many reports.

Dimond and Horsfall (43) further equated inoculum potential with disease: "...the result of the action of the environment, the vigor of the pathogen to establish an infection, the susceptibility of the host and the amount of inoculum present." They thought it convenient to express inoculum potential as a form of potential energy in a physical sense. Since all forms of energy contain an intensity factor and a capacity factor, the 'energy' required to establish an infection would be based on the

resultant action of intensity and capacity factors. The amount of inoculum present was considered the intensity factor, whereas, environmental factors such as temperature, moisture and host susceptibility contributed to the capacity factors of inoculum potential. Wilhelm (161, 162) used the term infection index to relate to Horsfall's (83, 84) concept of inoculum potential; the infection index was the percentage of the plant population that became infected in infested soil, e.g., the disease incidence. Wilhelm (162) further argued that potential, i.e. the capacity to do work is a somewhat nebulous concept, and proposed that inoculum potential be equal to the amount of viable inoculum. Other investigators have also equated the amount of viable inoculum to inoculum potential (81, 102, 139).

The sum of all intensity and capacity factors sensu Dimond and Horsfall (43) which influence the inoculum would be the inoculum potential sensu Garrett (57). This value combined with the set of factors (environment and host) underlying resistance to disease during its life cycle should give a predictive index of disease severity (6).

Mitchell (109) proposed the term, absolute inoculum potential and defined it as a "measure of the maximum capacity of a pathogen *population to infect a population of fully susceptible host plants* under conditions optimum for infection." This concept is not too different from that of Garrett's definition of inoculum potential.

By this he meant that one had to measure the maximum possible capacity for infection by a population of the pathogen; and therefore he felt one should be concerned only with the ability to cause infection and not "confuse the issue by involving factors which only affect subsequent disease development." Short-time exposure of susceptible host tissue under optimum conditions for infection and subsequent plating or direct examination of such tissue may help determine frequency of infection. Stanghellini et al (148) estimated the AIP of *Pythium aphanidermatum* in the root-soil interface by the use of potato tuber tissue (a natural host), under optimal soil moisture and temperature conditions. Their results indicated that the oospore inoculum density per unit of infested area of the root-soil interface was not the factor restricting the number of lesions on the sugar beet tap root, but environmental factors (i.e. sub-optimal soil moisture and/or temperatures), limited expression of the AIP of *P. aphanidermatum*. They further pointed out that the infection capacity of a propagule at the time infection occurs is a function of the energy available to or possessed by the propagule approached by the root or other plant part. Thus, in many cases, the energy available to propagules in the "pre-colonization" phase results in a substantial increase in the biomass component of the inoculum potential. As indicated earlier, this approach is not too different from that of Garrett (57).

<u>Methodology</u>

The qualitative aspects of Garrett's concept (57), and the sentiments expressed by Baker (6), Dimond and Horsfall (44) and Wilhelm (163) illustrated the necessity to further develop the quantitative aspects of inoculum potential. Previous methods, such as employment of hosts as indicators, correlations with activity or inoculum density and use of fundamental units, (e.g. CO_2 evolution and calorimetry) have been used as evaluations of the inoculum potential of a specific pathogen under defined conditions. An adequate summary of these techniques and their associative pitfalls were reviewed by Baker (6).

The development of selective media and special isolation techniques has enabled plant pathologists to precisely quantify pathogen populations in the soil. Efforts relating population density to the propagule's size of the pathogen (30), infection potential (30), disease severity (22, 23, 77), disease incidence (transformed to correct for random multiple infections by the Gregory multiple infection correction [Gregory (68)] (3, 10, 75, 160) and slope values of disease progress curves (131) have been reported as estimators of inoculum potential. The multiple infection correction was first reported by Gregory (68) who used the factor to estimate infections of the leaf pathogens <u>Phytophthora infestans</u> and <u>Gymnosporangium juniperi-virginianae</u> and <u>G</u>. <u>clavariaeforme</u>. Other

approaches have utilized competitive saprophytic ability, (59), a biological resistance value of the soil (27), and infection rates (157, 158), as relative estimates of inoculum potential for a given pathogen.

The inoculum potential of a pathogen could be understood further with the quantitative aids provided by: (a) equations estimating rhizosphere width, (b) disease progress curves or infection rates, and (c) elucidation of mathematical relationships between inoculum density and disease incidence. The models and analyses, which emphasize the density component, have been suggested by Baker et al (10) and Baker (7) for treatment of inoculum potential.

In a simple formula, Baker (7) equated disease to the inoculum potential (of the pathogen, i.e., its population density having a certain amount of vigor and virulence), modified by the capacity or environmental factors (43), multiplied by the disease potential. Disease potential (65) was defined as the susceptibility of the host over the period of its life cycle as influenced by environmental effects. Baker's formula (7) is consistent with the concept of inoculum potential of Garrett (57).

The models of Baker were established on the principles of physical and surface chemistry where the inoculum lies in a three-dimensional tetrahedron with the soil particles; the number of ensuing infections were assumed to be directly proportional to the spores at

the root surface. However, Vanderplank (158) stated that even after the most thorough mixing, spores would not be distributed more than at random. As the number of infections are assumed to be directly proportional to the number of spores at the root surface, the infections were never restricted by the number of susceptible sites and the possibility that many spores may have competed for the same sites of infection was ignored by Baker et al (10). However, competition for the same infection sites would occur theoretically, only at high inoculum densities. In these models, increases in the inoculum density of soilborne fungal plant pathogens result in rapidly increasing disease incidence until a certain level of inoculum is reached, beyond which the rate of increase levels off.

Slope values of inoculum density-disease incidence [transformed by $\log_e (1/1-y)$; y represents the percent disease incidence, (68)] curves provided quantitative estimates to detect the influence of various biological, physical and chemical environmental factors on the curve. $\log_{10}$-$\log_{10}$ transformations of ID-DI relationships for soil-borne pathogens were used to distinguish influences of the rhizoplane from the rhizosphere on the establishment of a successful infection (10). The $\log_{10}$ transformation (10) was used for uniformly distributed propagules in the soil (according to Baker et al) (10), and straightened the normally expected curvilinear relationship between these two variables.

The slope value (b of a first-order linear equation) of a

log_{10}-log_{10} transformed relationship was expected to be 1.0, if successful infections could be established from a certain distance in a volume of a rhizosphere and 0.67, if they operated <u>only</u> on the rhizoplane for inoculum distributed about a fixed infection court. If the system involved a moving infection court, e.g. a moving root tip, the slope of the transformed inoculum density-disease incidence curve was expected to be 0.67. The position of the inoculum density-disease incidence curves indicated the <u>efficiency</u> of propagules to incite disease symptoms; i.e., the farther to the right the curve is, the more propagules required to produce an infection. For <u>C</u>. <u>crotalariae</u>, several investigators have reported slope values of 0.67 (77, 80, 126, 154), which suggested a rhizoplane influence according to the Baker models (10). In greenhouse studies, Hanounik et al (77) observed differences in positions and slopes of log-log and log-probit transformations of inoculum density-disease <u>severity</u> curves of <u>C</u>. <u>crotalariae</u> on the CBR-susceptible 'Florigiant' and CBR-resistant 'Spancross' in NaN_3-treated and untreated soils. The pattern of increase in disease severity was influenced significantly by soil chemical treatment and host genotype. Differences in positions and slopes of the inoculum density-disease curves were attributed to differences in host defense mechanisms and to soil chemical treatments employed. Disease indices, as well as disease transformation values based on the severity index, were lower for 'Spancross' than for 'Florigiant' in both untreated soil-less mixes

for all inoculum densities that were examined.

In field studies, it may not be possible to distinguish between the rhizoplane and rhizosphere effects from slopes of $\log_{10}$ ID vs $\log_{10}$ DI for host-pathogen interactions. In the work of Griffin and Tomimatsu (70), slopes of ID-DI curves were modified by the use of a multiple infection transformation (159) for C. crotalariae inoculum, which was known to be clumped. This correction did not alter significantly the estimated slope value based on the Poisson distribution, but in neither case (i.e., between the transformation based on the Poisson nor the negative binomial) was the estimated value significantly different from 1.0 or 0.67.

The use of the above mathematical models to describe inoculum density-disease incidence (ID-DI) relationships has met with some criticism (53, 62, 72, 103, 158), and alternative approaches have been suggested (53, 62, 64, 72). Grogan and his colleagues (72) *argued that the number of randomly distributed propagules within a* finite layer of soil adjacent to the root surface, and not the distance between propagules, as indicated by Baker (10), determined the number of infections that could occur. Thus Grogan et al (72), as well as Vanderplank (158) treated the rhizoplane as a very small volume adjacent to the root surface. Their treatment is similar to that concept of the rhizoplane as originally defined by Clark (31). Gilligan (62) reasoned that the number of propagules at

the rhizoplane should include all the propagules that were originally within the volume occupied by the host root. Whereas Baker's models deal with a surface phenomenon for the rhizoplane effect, the alternative models of Vanderplank (157), Grogan et al (73) and Gilligan (64) treat the rhizoplane as a volume phenomenon.

Measurement of rhizosphere width.

The distance to which exudates diffuse into the soil represents the upper limit or the distance to which propagules can respond to roots for the triggering of propagule germination (= the distance of the rhizosphere effect). For example, root exudates stimulated propagule germination of microsclerotia of *Verticillium dahliae* (54) and of *C. crotalariae*, *in vitro* (99). The distance of the rhizosphere effect may determine the efficiency of a pathogen to establish a successful (7) or successful, progressive infection (57, 60). Such infections eventually would lead to necrosis. The establishment of a successful infection may require more than a population of a virulent pathogen at an infection court. The threshold value for the energy of growth from the inoculum below which infection has failed to occur may be exceeded by that of a single, typical fungal hypha of the particular species. In earlier literature, it was surmised that the rhizosphere width was between 1 and 2 mm based on the criterion of increases in the population densities of bacteria and fungi to indicate the extent of influence of root exudates in soil (134). Using a root slide technique, Griffin (69) found by direct micro-

scopic observation that chlamydospore germination of F. oxysporum, a common peanut root colonizer, was nearly 100% at the root surface and decreased gradually to zero at 1.73 mm from the root surface. Thus, propagule germination was influenced predominantly by the rhizoplane (sensu Clark), but not limited to it. Newman and Watson (112) considered the quantity of exudates and their distribution over time, based on diffusion rates and absorption by organisms. Under ideal conditions, results of their model suggested that the rhizosphere effect would extend approximately 1 to 2 mm at the initiation of exudation before significant microbial activity occurred; however, once microbial growth was initiated, the rhizosphere lessened to a distance of considerably less than 1 mm. Based on the data of Evans et al (51, 52), the rhizosphere width was calculated to approximately 100 μm for Verticillium dahliae. For a few fungi, large distances have been reported.

A theoretical estimate of the width of the rhizosphere was found to be 0.64 mm in accordance with Ferriss' model (53), which assumed 100% propagule germination and infection within the calculated rhizosphere width when competence equals 1.0. Competence sensu Ferriss (53), was based on "competence distance" (72) which referred to the "maximum distance a propagule can lie from a root surface and still have a chance of causing infection." A number of factors, e.g. host resistance, environmental effects and a non-random distribution of inoculum would reduce the value of competence below one,

and thus cause an underestimation in the width of the rhizosphere (53). This was exactly the case when Drury et al (49) calculated a theoretical rhizosphere width based upon the direct microscopic observations by Griffin (69) of Fusarium oxysporum chlamydospore germination in peanut rhizospheres. Gilligan (63) reported a maximum zone of potential infection to be 10 mm for Gaeumannomyces graminis var. tritici. Sclerotia of Sclerotium cepivorum, responded to Allium sp. roots at distances of over 1 cm (35).

In preference to the term rhizosphere, Gilligan (64) recently proposed the term pathozone, which refers to the region of soil surrounding a host unit within which the center of a propagule must lie for infection of the host unit to be possible. The term is widely applicable to infection courts other than the root, e.g., seed or hypocotyl, but does not distinguish the stimulating effects of the host organs, e.g. root exudates, on propagule germination and infection from other stimulators, endogenous or exogenous.

Inoculum efficiency.

Measurement of the width of the rhizosphere or pathozone is important but the concept did not make allowance for efficiency of propagules to cause infection (8, 9, 49), a necessary component of inoculum potential. Furthermore, as noted by Drury et al (49), competency refers more to "what inoculum should be able to do rather than what it has done."

Preliminary calculations of efficiency of propagules of

R. solani was found to be below 10% (19). Gilligan (62) arbitrarily assigned a value of 50% for the efficiency of propagules to induce infections. This would seem to imply that only those propagules which germinate in the rhizosphere have an opportunity to cause infection. Grogan et al (72) proposed the use of competence volume, which stated that only those propagules that are biologically competent and located in the "competence volume" of soil near the root surface have any chance of causing infections.

Efficiency of propagule germination and infection is a more preferable term to inoculum potential since all propagules near an infection court may not participate ultimately in the infection of the host (9). "Efficiency is measured after the fact", and is not an indication of theoretical expectations (49). Reported calculations based on this estimate vary, depending on the pathogen and host involved, the methods and the materials of each investigation.

Black and Beute (22) used inoculum efficiency to indicate the relationship between root rot severity and inoculum densities. They used efficiency as the capacity to produce a given severity of root rot with a minimum number of C. crotalariae microsclerotia. Furthermore, "inoculum efficiency" was used (preferable to efficiency for inoculum to cause infection), because they felt that the severity of root rot reflected various probabilities (among treatments) of obtaining one lesion for each

microsclerotium and also for various lesion expansion rates.

Infection rates.

"Inoculum potential cannot be quantified" (158). All factors of host, pathogen and environment interact and are pooled in the concept of disease. Vanderplank (158) claimed that such investigations were blind alleys, even with the concept of ED_{50} (effective inoculum density to affect 50% of the plant population); based upon the concept that inoculum potential determines disease and disease measures inoculum potential. Vanderplank (158) proposed the use of infection rates, as providing adequate estimates of "inoculum potential" and thereby, adequate 'concepts' of the epidemiology of the pathogen. However, the approaches used by Vanderplank do not consider the host nor capacity factors. Huisman (87) has stressed the importance of host-dependent infection rates for soil-borne pathogens.

Vanderplank (158) stated that a single number denoting an infection rate quantified the entire interaction and pooling of factors. However, infection rates also have their drawbacks. There have been many reports which consider infection rates for foliar pathogens, however such is not the case for root-infecting fungi. Vanderplank (158, 159) was among the first to calculate infection rates for soil-borne pathogens, the number of infections for each case was represented by an estimate to account for monocyclic [simple-interest disease (sensu Vanderplank, 158)].

The use of disease progress curves to deduce or prove infection mechanisms has not been properly supported with experimental observations (87, 122). Rates express the response or behavior of the system to external or internal factors in relation to the previous state. They primarily change disease intensity, but not necessarily the underlying pattern. Thus rates are variable (95).

Disease progress curves which represent the transformed number of infections based upon the disease incidence or disease severity ratings contribute to the second misuse of the models of Vanderplank (158). The confusion appears to result from the use of pathogen growth rates in tissue with the infection rates. Although growth of the pathogen can affect the number of new infection sites indirectly through effects on inoculum production, the growth of the pathogen in the host was irrelevant, as Vanderplank's models employ the number of infection sites or infection loci (87). This has been observed for other soil-borne pathogens (29, 158).

The assumption that lack of pathogen multiplication (i.e. new infections do not contribute to further infections) may be an insufficient criterion for the use of the simple-interest model [for any particular disease] without other applicable criteria (87). Use of the simple-interest equation requires that there be a constant arrival rate of pathogens per unit of host tissue for the entire time period over which infections are measured. Huisman (87) noted further, that in a simple-interest disease system, parent

infections yielded a constant quantity of new infections per unit of time ("i.e., through a constant rate of inoculum production dispersal and infectivity"); and that these conditions were not met for most of the diseases induced by soil-borne pathogens which have been cited previously (11,158, 166).

Part of the problem in evaluating inoculum potential has resulted from estimating the number of infections which can incite disease recognizable as macroscopic symptoms. The Gregory multiple infection (68) transformation is valid only when the organism or event (in this case, disease incidence) is distributed at random. It is useful when plants, infected early, do not contribute to subsequent infections, or when the source of inoculum is constant. The quantity in the denominator, $(1-y)$, is a correction factor to allow for a decreasing proportion of healthy tissue left for infection. Another problem has been the estimation of inoculum potential, "after the fact", i.e. attempting to quantify the pathogenic vigor of inoculum after the development of symptoms, instead of prior to disease onset.

The above approaches and methods have been used in attempts to denote differences in pathogenic vigor of inoculum or differences in resistance of host cultivars for many host-pathogen interactions. For root diseases, however, information regarding inoculum potential estimates and infection rates is sparse for quantitative analysis to attempt to distinguish between resistant and susceptible

cultivars.

V. INFECTION DEVELOPMENT AND ROOT COLONIZATION

Generally, when the pathogen has a high inoculum potential it is highly virulent, or it has access to a substantial nutrient source, or both. Subsequently, there is a high rate of invasion of host tissues and host responses are limited or absent. Physiological characters of the host presumably determine the range of inoculum potentials against which it can make a positive response to incipient infection (14). However, other factors may also influence the host's response to the pathogen.

Infection establishment and root colonization.

Establishment of a successful infection on susceptible root tissues by *C. crotalariae* could be attributed to inadequate defense mechanisms or to a breakdown in host defense mechanisms (78, 79, 90) once the mycelium penetrated the root surface cortical cells, in addition to the "genetic potential (virulence) of the pathogen." Harris and Beute (79) found no differences in the basic formation of original or additional taproot periderms or in the suberization of periderm in susceptible (cvs. Florigiant and NC2) or resistant peanut lines, (cvs. Argentine and NC 3033). They observed extensive periderm more frequently in resistant lines, however. The resistant cultivar NC 3033 remained highly resistant with four microsclerotia per mm^2 of taproot, whereas susceptible Florigiant was moderately to severely diseased with one microsclerotium per mm^2 of taproot (78).

Some workers obtained isolates of C. crotalariae from lesions on roots of CBR-resistant plants. These isolates of C. crotalariae were observed to be more adapted to resistant NC 3033 than others (22, 74). Isolates from roots of resistant NC 3033 seedlings and from microsclerotia in field soil following harvest of NC 3033 had high general virulence (22, 23), i.e., virulence effective against all peanut genotypes (75).

Information regarding the extent of colonization of host tissues for other soil-borne pathogens has been reported in studies conducted with V. dahliae (2, 54, 104), Fusarium spp. (1) and G. graminis var. tritici (146). Such knowledge in combination with histopathological and histochemical results would provide further insight into CBR pathogenesis. When exposed to root exudates of resistant or susceptible hosts, dormant propagules of Verticillium and Fusarium generally germinate and colonize the surface of roots of a wide variety of plants, including both host and non-host species (51, 54, 93, 100, 101, 141). Krigsvold et al (99), reported low levels of germination of C. crotalariae microsclerotia in 1-mm wide rhizospheres of CBR-susceptible and CBR-resistant cultivars of peanut, 39.8 and 33.9% germination, respectively. Also, Schippers & Voetberg (141) noted differential germination of F. oxysporum f. sp. pisi on resistant and susceptible pea cultivars. Verticillium dahliae grew more extensively and formed more

microsclerotia along the root surface of immune wheat than over roots of susceptible pea (104). Pathogenic and nonpathogenic strains of Verticillium spp. and Fusarium spp. penetrated through young root tissues and into the xylem in some plants (15, 36, 115), without apparent harm to the plant (36), but wounds may considerably increase the amount of infection, especially by Fusarium. Immune reactions of non-host corn and soybeans were associated with levels of resistance against direct penetration by F. oxysporum f. sp. melonis (12) as well as for weed non-hosts, Oryzopsis, Digitaria, Amaranthus, and Malvua against F. oxysporum f. sp. lycopersici (92). Nonpathogens related to pathogens commonly occupy the same ecological niche; thus, nonpathogenic members of F. oxysporum occur with pathogens in plant roots (37).

Investigations regarding the mechanism of root colonization by fungi and bacteria have been renewed, especially with the increased emphasis on the control or management of plant diseases with biological agents (37). The majority of these reports have considered infection and colonization of susceptible and resistant cultivars of select plant species under carefully controlled conditions and the use of virulent isolates of the respective pathogens. Few reports have used several isolates of the pathogen (146) and none, at the present time, have used naturally infested soils. Earlier investigations (47, 48, 115) considered the role of fungal

colonization in saprophytic decay of other plant species, e.g. ryegrass (160) and bean.

Waid (160), examined the microflora involved in decomposition of ryegrass roots, by microscopic observation and by plating out intact roots, or dissected fragments of outer cortex or of inner cortex plus the stele. No fungi were observed either penetrating the endodermis or within steles of "white", healthy roots possessing a cortex.

Dix (47) observed Cylindrocladium spp. as a minor root-invading fungus of Phaseolus vulgaris; the frequency of isolation of Cylindrocladium spp. increased initially with progression of decomposition, but subsequently declined as the root matured. The fungus may have played a part in the early stages of decay, but proved to be a poor competitor with Fusarium. Fusarium oxysporum was observed as an early secondary colonizer (but not a pioneer colonist) of the bean root surface and as an invader of root tissues (48).

Reportedly, F. oxysporum has been associated as part of the root-surface microflora of a number of plants, including leek (116), blue lupine (113), rye grass and cocksfoot (160), bean (47, 152), barley and cabbage (117), and white clover (144). However, only a few investigators have examined the extent of their colonization into the subepidermal layers of the root. Fusarium oxysporum was isolated from the inner cortical tissue of asymptomatic roots of dwarf bean (115), white clover (144), ryegrass (160), as well as from feeder roots of various species of the Gramineae treated with 0.1% $HgCl_2$ for

30 sec (referred to as inner root tissues (97). Disagreement regarding the role of the fusaria has been reported previously (152). Some reports considered that nonpathogenic strains absorbed organic substances excreted from root cells, and had little or no effect on root physiology. Others regarded the colonization of roots as representing an association which must affect the metabolism of root cells and absorption of nutrients by the roots. Fusarium oxysporum has also been suggested as an important agent in root decomposition (152).

In Georgia, F. oxysporum, F. solani, F. roseum or F. moniliforme have been recovered from the subterranean portions of the plant, where they apparently flourished without causing disease symptoms (89). In some instances, however, pathogenesis is somewhat obvious as chlorosis of leaflets, and slight wilting followed by plant death (89, 108). The fungus usually attacks the taproot just below the soil surface and causes formation of elongated, sunken, brown lesions. Injury by Fusarium spp. is usually minimal, even though primary and secondary rootlets have been attacked (89). These fungi appear to be pathogenic only under rarely occurring circumstances (89), since peanut plants were attacked by F. oxysporum and F. roseum (16) under gnotobiotic conditions favorable for growth of the fungi. The exudates from rapidly expanding tissue have been suggested to be partly responsible for invasion of tissue by Fusarium spp (130). Indeed, germination of

chlamydospores of *F. oxysporum* was observed to be *predominantly* a rhizoplane influence (69), but not exclusively.

Verticillium spp. (55, 102, 150, 151) and *Fusarium* spp. (1) were observed to penetrate resistant and susceptible cultivars of their respective hosts to about the same degree. Garber and Houston (55) were unable to relate the quantity of vessel elements invaded at primary invasion sites to differences in levels of *Verticillium* wilt tolerance between two varieties of cotton. However, more extensive and intensive fungus development in the xylem of the less-tolerant variety was observed in tissue sections removed from the older part of the root. Similarly, Lacy and Horner (102, 103) reported no differences in the number of invasion sites by *V. dahliae* on root surfaces of both wilt-resistant and wilt-susceptible cultivars of mint. More extensive systemic invasions of wilt-susceptible roots were noted, however.

Restriction of root colonization resulting from host reaction at the endodermis has been observed by some workers (e.g. 151) and may be important in the resistance of resistant cultivars of pea to *F. oxysporum* f. sp. *pisi* and resistant cultivars of tomato to *F. oxysporum* f. sp. *lycopersici* and *V. albo-atrum*. There may be other factors indirectly responsible for establishing (a) defense mechanism(s) in the host plant. For example, induced resistance in wheat was triggered by *Phialophora radicicola* var. *graminicola* and thus prevented *G. graminis* var. *tritici* from spreading

longitudinally in the stele. Based upon histological observations, using phloroglucinol-HCl or aniline sulfate for lignin, or Sudan Black B for suberin, *P. radicicola* apparently elicited considerable lignification and suberization of the inner tangential wall of the endodermis (146), since no root necrosis nor colonization was observed (38, 39).

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CHAPTER II

INOCULUM POTENTIAL OF *CYLINDROCLADIUM CROTALARIAE*: INFECTION RATES AND MICROSCLEROTIAL DENSITY-ROOT INFECTION RELATIONSHIPS ON PEANUT

INTRODUCTION

Cylindrocladium black rot (CBR) of peanut (*Arachis hypogaea* L.), caused by *Cylindrocladium crotalariae* (Loos) Bell & Sobers, is a destructive root rot that in some years can kill nearly all peanut plants in infested areas of a field (22). Some progress has been made on the study of inoculum potential relationships of this pathogen, especially in regard to inoculum density-disease incidence relationships (13, 21, 24) and in regard to the influence of physical environmental factors on microsclerotium germinability (11, 23). Nothing is known, however, about the quantitative relationship between microsclerotium germination in the rhizosphere and observed root infections. As is indicated in a review by Baker (2), a similar situation exists for other root-infecting fungi. Also, except for analyses of Vanderplank (26, 27) and the work of Kannwischer and Mitchell (16), with black shank of tobacco, little is known about infection rates of soilborne pathogens.

Krigsvold et al (17, 18) demonstrated that a high percentage (39.8 %) of the microsclerotia of *C. crotalariae* germinated in a 1-mm-wide volume of rhizosphere soil collected from defined regions

of peanut root tips. If inoculum efficiency (percent of germinating propagules that infect roots) is high, this germination in the rhizosphere could lead to development of many root infections on each peanut plant at microsclerotial inoculum densities that occur in nature. Infection rates (infections per plant per unit time) and overall slope values of arithmetic inoculum density-infection plots would be high also. All or a portion of the infections could lead to development of lesions and CBR symptoms. If inoculum efficiency is low, due to low endogenous reserves, inadequate exogenous nutrients (exudates), or antagonism in the peanut rhizosphere or host-defense mechanisms, few or no infections would occur on an individual peanut plant. As pointed out by Baker (2), little is presently known about inoculum efficiency and, to our knowledge, no estimates based on propagule germination data have been made for root-infecting fungi.

This paper reports the occurrence of numerous observed infections of _C_. _crotalariae_ on asymptomatic root systems of field-grown and greenhouse-grown peanut plants (determined by plating methods), infection rates for observed root infections, and the relationship between microsclerotial inoculum density and the number of observed infections. In addition, estimates are made for efficiency of inoculum for observed infection, and efficiency of observed infection for necrosis (percent of infections that develop into necroses). Portions of the information here have been presented in a preliminary report (25).

MATERIALS AND METHODS

Field study. In September 1979, in Southampton County, VA, a *plot measuring 4.6 m long by 3.9 m (four rows) wide was established* in an area of a peanut field in which the plants were showing CBR symptoms. The field was known to be infested with C. crotalariae since 1974. All plants in the plot were excavated, the roots were washed, and the plants were rated for shoot symptoms, root rot, and discoloration. A composite soil sample was made from soil cores (2 cm diameter X 20-25 cm deep) taken every 30.5 cm, in each of the three interrow areas. After transport to the laboratory and thorough mixing, the soil sample was assayed for microsclerotia of C. crotalariae by the method of Griffin (8). Asymptomatic root systems of 10 plants were washed for an additional 25 min in running tap water and the entire root systems were cut into portions as extensive (long) as possible for plating on 9-cm-diameter petri plates containing sucrose-QT medium (8). For each plant the total length of plated roots (fine, lateral, and taproots) was measured with a ruler. Colonies of C. crotalariae growing from roots were counted after 5-7 days of incubation at 25 C. Extreme care was taken to prevent duplicating the recording of colonies that originated from both ends of a cut root or that were closely associated but were not clearly discrete units. The number of discrete colonies of C. crotalariae growing from asymptomatic roots was used as a measure

of the number of observed apparent infections (hereafter referred to as observed infections).

Infection rate and relationship between microsclerotial inoculum density and the number of observed infections. Generally, in the greenhouse studies, C. crotalariae-free peanut field soil (sandy loam, pH = 5.2) was mixed with naturally infested soil containing high populations of microsclerotia of C. crotalariae to obtain the desired inoculum densities. Initial microsclerotial populations were determined by the method of Griffin (8). Soil with high microsclerotial populations was obtained from the field by excavating the root zones of plants with symptomatic shoots, removing intact roots, and mixing the soil thoroughly before using it as a source of inoculum. Thiram-treated peanut seeds of CBR-susceptible cultivars (Florigiant or VA-72-R) were planted four per 1-L plastic container (11-cm diameter). These pots were placed in a temperature tank at 25 C on a floating apparatus with a manifold underneath to allow for water drainage from the pots. Plants were watered daily or as needed to maintain the moisture level near field capacity. Following the plant-growth period, plants were removed gently and washed free of remaining soil in the same manner as used in field studies. Shoot symptoms, as well as percentage root necrosis and discoloration, were noted for each plant. In the infection-rate study, observed infections per plant were determined on 10, 10, and four asymptomatic root symptoms for the 21-, 42-, and 63-day periods, respectively.

For each period, plants were removed from pots until 10 asymptomatic root systems were obtained. Estimated infections of Vanderplank (26, 27), determined from $\log_e (1/1-y)$ in which *y* is root rot incidence, were based on 14, 29, and 58 plants for the same periods. In the inoculum-density experiment, entire root systems of three plants with asymptomatic roots were plated for each of the microsclerotial densities after 21 days. Observed infections were determined from plated entire asymptomatic root systems, as described previously. Root lengths (fine, lateral, and taproots) were measured for each plant with a ruler.

Lesion development by C. crotalariae isolates from observed infections. Isolates of *C. crotalariae* obtained from asymptomatic *roots and necrotic portions of roots were grown on plates of Hunter's* medium (15) in agar for at least 6 wk at 25 C. Strips of agar medium (1 x 3 cm), containing mature microsclerotia, were washed for 8 hr in running tap water to remove exogenous nutrients and enzymes before they were macerated in a blender and wet-sieved on a 25-μm sieve for 10 min. Microsclerotia were suspended in enough distilled water to give a concentration of 150 microsclerotia per 0.5 ml for each isolate. A 0.5-ml aliquot was pipetted onto and spread over washed 0.5 x 1.0-cm strips of water agar. The strips of microsclerotial inoculum were placed on a glass plate (root-slide) designed by Krigsvold (17). The agar strip was covered with a plastic screen and held in place with rubber bands. A glass rod, bent to form a "V",

was used for guiding the peanut taprooot toward the agar strip of microsclerotia. The root-slide was placed at an acute angle in a 1-L plastic pot, having a window on one side (hereafter called a windowed pot) to monitor the growth of the taproot, root necrosis and root discoloration. Unsterile peanut-field soil free of C. crotalariae was placed on top of the root-slide in the windowed pot. A pre-germinated peanut seed (radicle length, 1.5 cm) was placed in each pot. Plants were subirrigated twice daily and incubated at 25 C in a growth chamber with a 14-hr photoperiod at 4,500 lux. After 2-3 wk of incubation, depending on taproot growth rate, each plant was carefully removed, washed free of soil, and lengths of each necrotic and discolored area of the taproot were measured. Symptomatic tissue was plated on sucrose-QT medium (8) for isolation of the pathogen.

RESULTS

Infection of field-grown peanut plants by C. crotalariae. Of the 80 15-wk-old peanut plants excavated from the field plot in September 1979, 41% had root necroses, while only 25% of the plants had CBR shoot symptoms (chlorosis, wilting, or necrosis). Surface discoloration of fine roots, commonly observed in field- or green-house-grown peanut plants in the absence of C. crotalariae, was not considered a CBR symptom. Six of 10 asymptomatic root systems assayed were colonized by C. crotalariae and had a mean of 36.3 observed root infections per infected plant (range, 1 to 114); observed infections were found on fine roots as well as on the taproots and main lateral roots (Table 1). Four of the 10 asymptomatic root systems were not colonized by C. crotalariae. The inoculum density of the composite sample of soil cores from the plot was 0.2 microsclerotia per gram of soil.

Infection rate and disease progress. The disease-progress curves (Fig. 1) showed an increase in the number of observed infections and estimated infections ($\log_e [1/1-y]$, in which y is equal to the proportion of peanut plants with rot) over time. Root length increase between 21 and 63 days is indicated also in Fig. 1. The average number of observed infections per plant were 161 at 21 days, 470 at 42 days, and 1,080 at 63 days. The number of estimated infections per plant were 0.33 at 21 days, 1.05 at 42 days, and 2.66 at 63 days. The incidence of root rot (not shown in Fig. 1) was

Table 1. Number of observed infections caused by Cylindrocladium crotalariae on asymptomatic taproots, lateral and fine roots of 10 field-grown cultivar Florigiant peanut plants after 15 wk

Plant Code	Root length (m)	Observed infections: Per plant	Per meter of root: Fine	Lateral + taproots	All roots
A	4.4	52	5.0	24.0	11.8
B	8.2	11	0.5	2.6	1.3
C	2.7	0	0.0	0.0	0.0
D	4.4	1	0.0	0.4	0.2
E	5.9	114	14.5	25.0	19.3
F	7.8	0	0.0	0.0	0.0
G	4.3	0	0.0	0.0	0.0
H	4.1	27	1.2	1.4	6.6
I	5.4	0	0.0	0.0	0.0
J	4.1	13	4.3	1.3	3.2

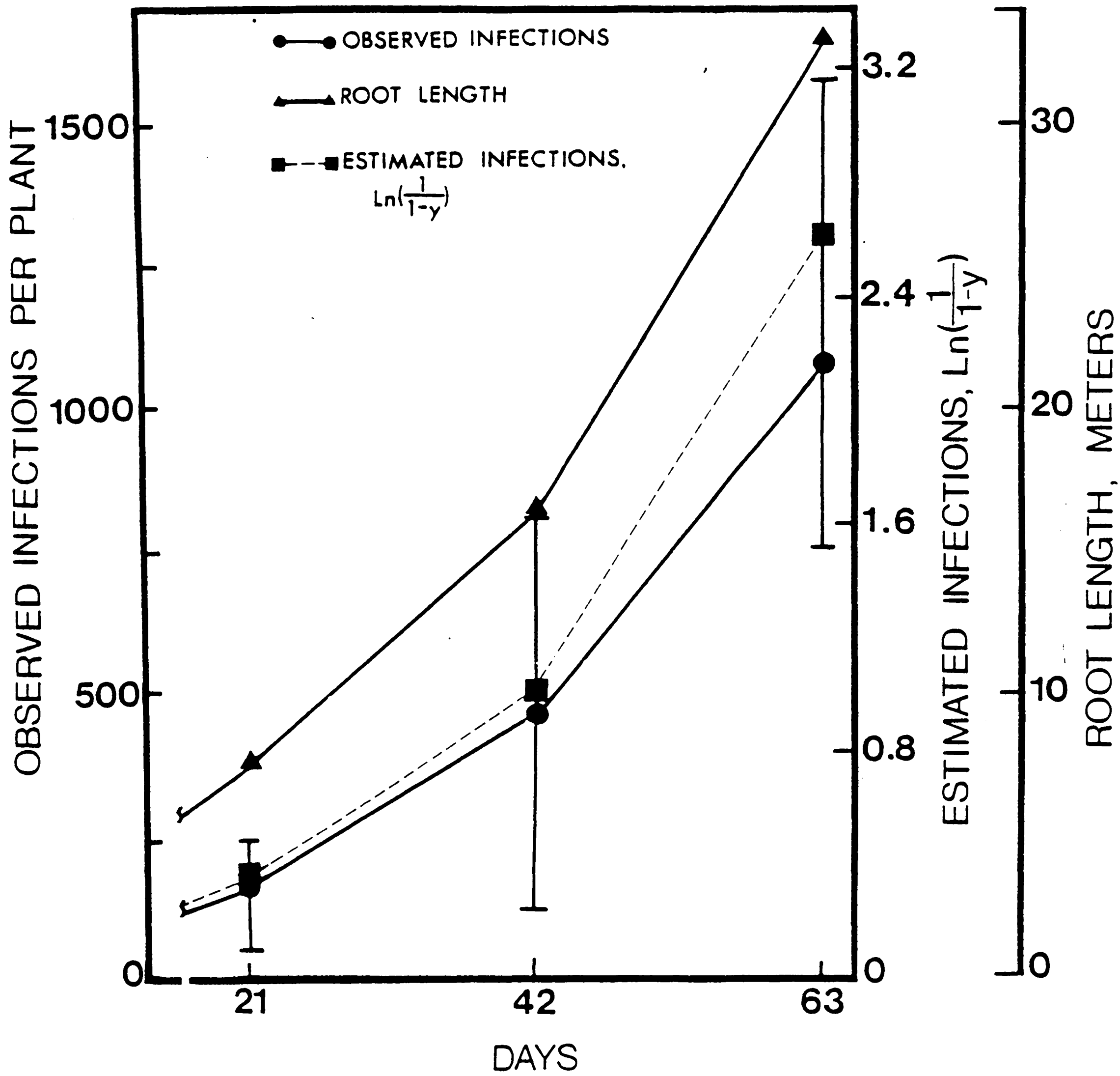

Fig. 1. Number of observed root infections caused by Cylindrocladium crotalariae per cultivar Florigiant peanut plant, estimated root infections per plant, and root length per plant after 21, 42 and 63 days at 25 C.

28.3% at 21 days and 93.0% at 63 days. Based on interpolation of th infection-rate curves, each plant had about 300 observed root infections when 50% of the plants had root necroses (equals estimated infection value of 0.693). The infection rate for observed infections on a unit-root-length basis, I^{o}_{r}, increased slightly for each of the three 21-day periods. Calculated values of 0.120, 0.162, and 0.199 observed infections per meter of root per day per microsclerotium per gram of soil were obtained for the first, second, and third 21-day periods, respectively. On a per-plant basis, the two infection rates, R_o, for observed infections, and R_e, for estimated infections (26, 27), increased greatly over the three 21-day periods. Values for R_o were 0.84, 1.62, and 3.19 observed infections per plant per day per microsclerotium per gram of soil for the first, second, and third 21-day periods, respectively. Values for R_e (based on $\log_e [1/1-y]$) in which y is the proportion of the plant population with root necrosis) were 0.0017, 0.0038, and 0.0084 estimated infections per plant per day per microsclerotium per gram of soil, for the first, second, and third 21-day periods, respectively. In these and other greenhouse tests, observed infections were distributed over all portions of the root system and many were located near the root tips. No perithecia of *Calonectria crotalariae* (Loos) Bell & Sobers were visible on any plants in the course of the experiments.

Surface-sterilization tests. To determine if observed infections were restricted to the outer cortical tissues of peanut

roots (peanut root has no epidermis), washed roots were treated with two concentrations of NaClO. Generally, there was a reduction in the number of observed infections per meter of root length for roots treated with the two concentrations of NaClO (0.1 and 0.25%), compared to the washed controls (Table 2). Overall, treatment of five entire root systems with 0.1% NaClO gave 24.3% reduction of observed infections, compared to the washed controls, and treatment with 0.25% NaClO resulted in a 43.7% reduction. Despite this reduction in observed infections per meter of root for NaClO-treated root systems, the average number of observed infections per meter of root was not significantly different from that of the washed controls (P=0.05). Thus, most observed (apparent) infections were probably not limited to surface cortical cells.

<u>Inoculum density experiments</u>. One or more observed root infections were found on all except one of the plants in the inoculum density experiment after 21 days. For the arithmetic plot, first-order regression equation analyses indicated the average number of observed infections per plant increased in direct proportion to the microsclerotial density (Fig. 2). The slope value of this straight line is 21.4 with an R^2 value of 0.95. A similar relationship was found also in an arithmetic plot (Fig. 3) of observed infections per meter of root vs the inoculum density (slope = 2.3, R^2 = 0.95). As there appeared to be some apparent curvature in the data of both plots, second-order regression (quadratic) equations also were

Table 2. Effects of two concentrations of NaClO on the number of observed <u>Cylindrocladium crotalariae</u> infections on asymptomatic root systems of peanut

	Observed infections per meter of root[a]			
Root type	Washed[b]	0.1% NaClO[c]	Washed[b]	0.25% NaClO[c]
Fine	11.6	8.0	23.1	13.1
Lateral	31.2	22.0	53.1	32.2
Taproots	21.0	24.0	50.3	20.3
All roots	23.0	17.4[d](-24.3%)	37.8	21.3[d](-43.7%)

[a]Five root systems were used for each treatment.

[b]Washed in running tap water for 25 min.

[c]Washed in running tap water for 25 min plus treatment with NaClO for 30 sec.

[d]Data are not significantly different from washed root systems, according to the F-test (P=0.05). The percentage reduction in number of observed infections per meter of root due to treatment with NaClO is indicated in parentheses.

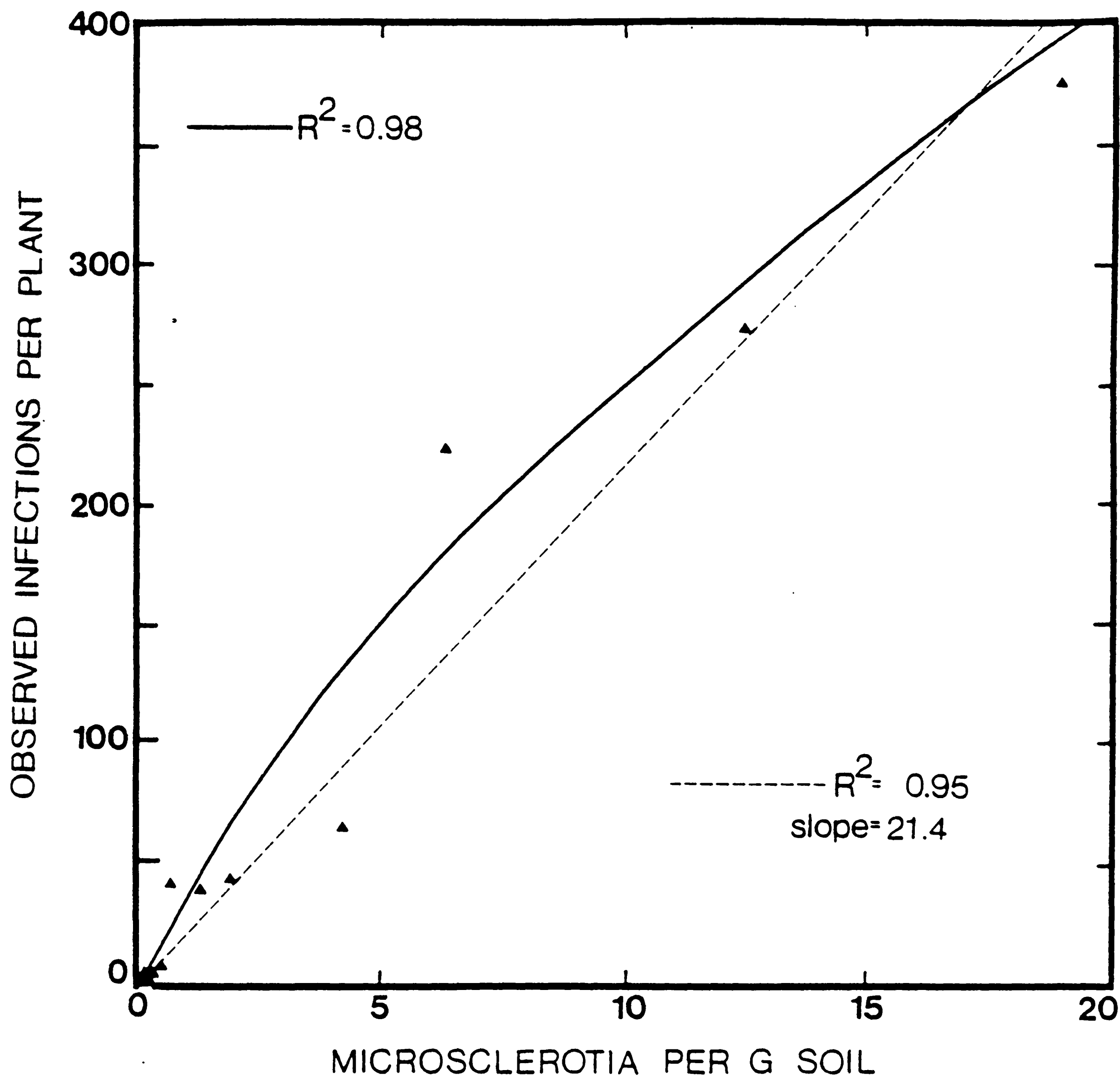

Fig. 2. Arithmetic plot of first-order and second-order linear regression curves for the relationship between numbers of observed infections caused by *Cylindrocladium crotalariae* per cultivar Florigiant peanut plant and numbers of microsclerotia per gram of soil.

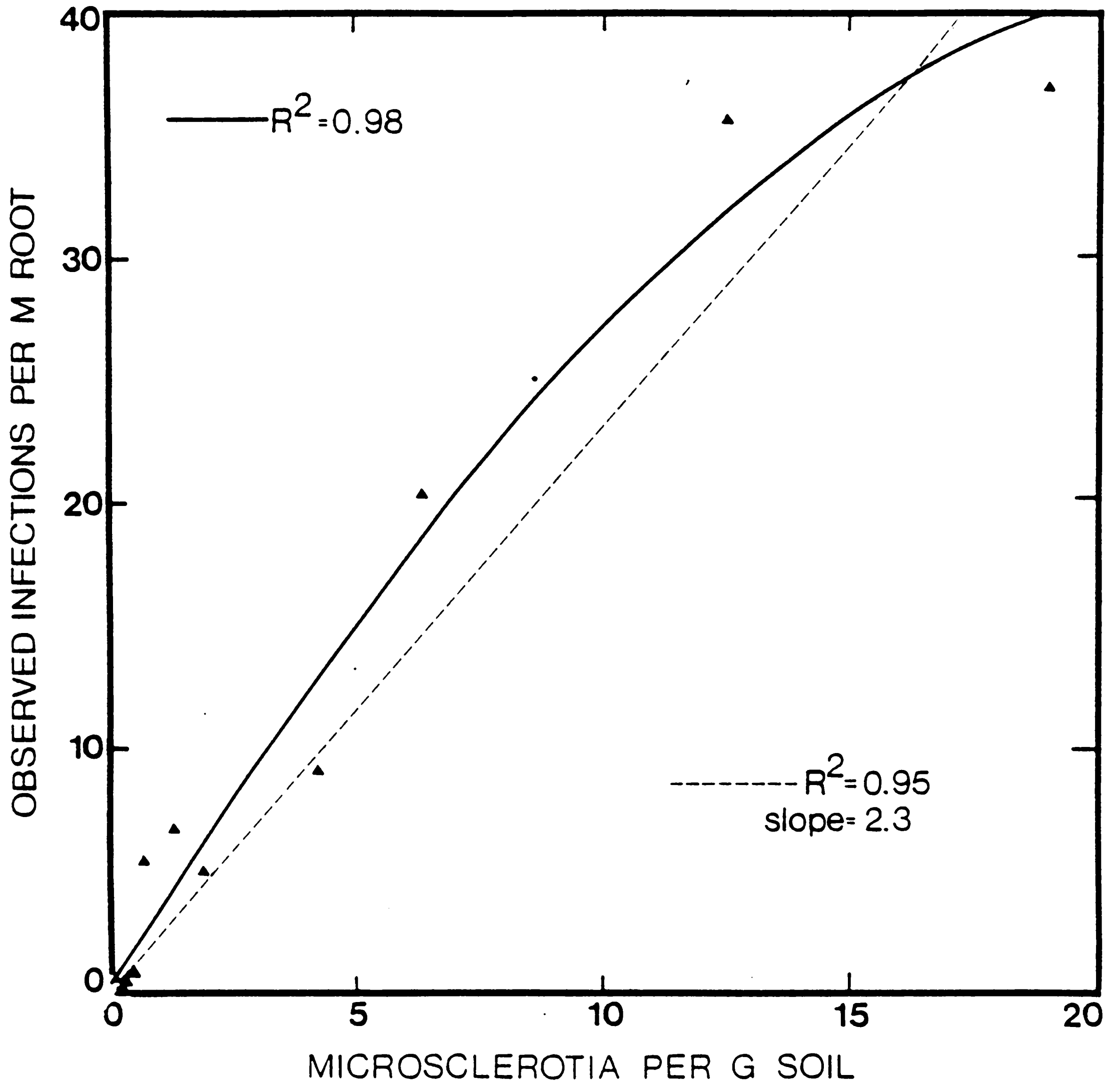

Fig. 3. Arithmetic plot of first-order and second-order linear regression curves for the relationship between numbers of observed infections caused by _Cylindrocladium crotalariae_ per meter of root and microsclerotia per gram of soil.

examined (3). For the second-order regression equations, there was more curvature in the regression line in the upper region of the curve for the infections-per-meter-of root plot than in the infections-per-plant plot. Table 3 shows the results of statistical analyses designed to test the fitness of the first-order and second-order regression equations. The robust form (14) of both equations deemphasized the five highest inoculum density data points (upper region of the curve) most removed from the regression lines, as these points strongly influenced regression analysis. Based upon parameter estimates and on error and R^2 values, both the robust form and the least-square form of the second-order regression equations gave a better fit than the first-order equation. However, when the highest inoculum density data point was omitted from the data set, the coefficient for the X^2 term in the second-order regression equation was not significant ($P= 0.05$). Thus, the first-order regression equation appeared to be the best fit. At 3 wk, approximately the same number of observed infections per plant (195) are predicted by the first-order equation for 9.1 microsclerotia per gram of soil, as was found in the time-course experiment that utilized this inoculum density (Fig. 1).

Log_{10}-log_{10} plots of microsclerotial inoculum density vs observed infections per plant and per 10 m of root are shown in Figs. 4 and 5, respectively. Both plots had slope values near 1.0, again indicating that the number of observed infections increased in

Table 3. Statistics of first- and second-order regression analyses of the relationship between the number of *Cylindrocladium crotalariae* observed infections per meter of root and microsclerotial density

Regression equation		Sum of absolute error	R^2	Variable	Estimate
$y = bX + E$	Least-square form	28.96	0.95	X	2.30
	Robust form	28.91	0.98	X	2.26
	Least-square form[a]	13.90	0.98	X	2.89
$y = b_1X + b_2X^2 + E$	Least-square form	18.85	0.98	X	3.72
				X^2	-0.09
	Robust form	17.97		X	3.87
				X^2	-0.10
	Least-square form[a]	13.70	0.98	X	2.99
				X^2	-0.009[b]

[a]Includes all data points except highest inoculum density.

[b]Parameter estimate is not significant (P = 0.05).

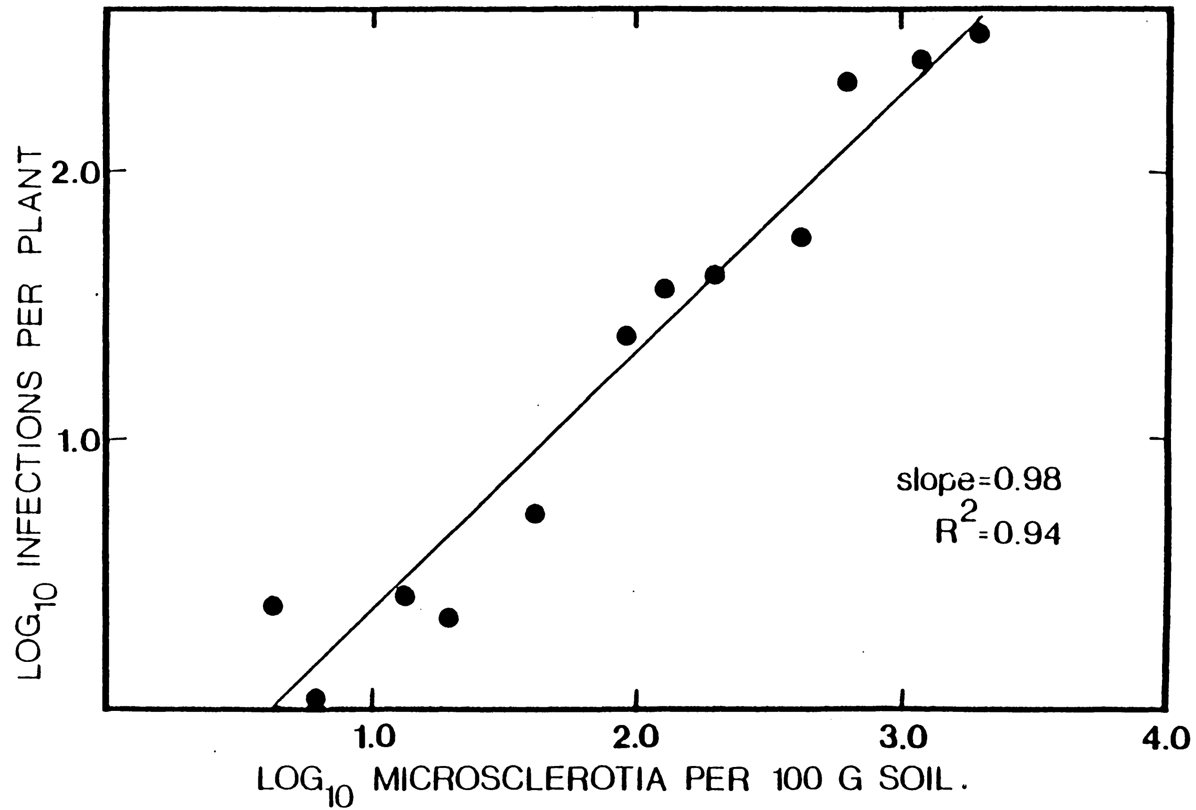

Fig. 4. Relationship between $\log_{10}$ numbers of observed infections caused by _Cylindrocladium crotalariae_ per cultivar Florigiant peanut plant and $\log_{10}$ of the number of microsclerotia per 100 g of soil. Values are the means of three replicates.

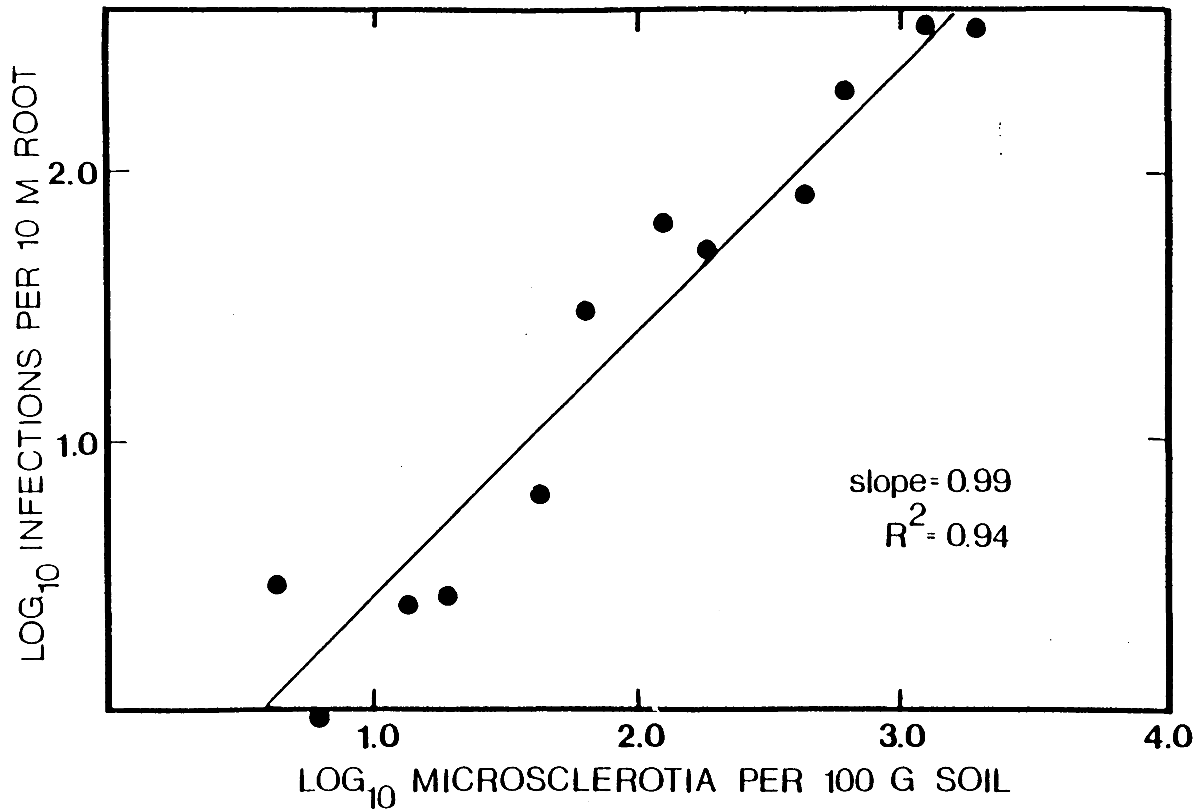

Fig. 5. Relationship between $\log_{10}$ numbers of observed infections caused by *Cylindrocladium crotalariae* per 10 m of root and $\log_{10}$ of the microsclerotia per 100 g of soil. Values are the means of three replicates.

direct proportion to inoculum density. An R^2 value of 0.94 was obtained for each of the data sets of Figs. 4 and 5. Based on the models of Baker, et al (1,4), a rhizosphere host-pathogen interaction is indicated by the slope values obtained.

Lesion development on taproots. The results in Table 4 show the average lengths and average rates of lesion development for four isolates of *C. crotalariae*, each obtained from observed infections or from lesions. Lesion isolates produced higher lesion growth rates and larger lesions than observed-infection isolates, but the differences in both cases were not significantly different (P=0.05).

Efficiency of inoculum and efficiency of infection. The estimate of inoculum efficiency for observed infection of temperature-tank-grown (25 C) VA-72-R peanut plants by germinating microsclerotia of *C. crotalariae* after 3 wk was high (about 100%). This estimate was based on the data that Krigsvold (17) obtained for percent microsclerotium germination (39.8%) in a 1-mm-wide zone of rhizosphere soil collected from defined regions of VA-72-R peanut root tips at 25 C, and on data obtained in the present study, which included root length per VA-72-R plant, and the number of observed root infections per VA-72-R plant. The infection rate, I^{o}_{r}, for VA-72-R plants (0.091 to 0.108 observed infections per meter of root per day per microsclerotium per gram of soil) was somewhat lower than that obtained for Florigiant plants. The volume of rhizosphere soil that root tips pass through, as they grow, was calculated from

Table 4. Lengths and rates of necroses on peanut taproots caused by lesion and observed-root-infection isolates of _Cylindrocladium crotalariae_ at 25 C

Isolate source	Isolate number	Length of necrosis[a] (mm)	Overall mean[b] (mm)	Rate of lesion development[a] (mm per day)	Overall mean[b] (mm per day)
Observed root infections	N3	4.3		0.2	
	N4	31.4		1.2	
	N10	20.0		0.8	
	N13	45.3	25.3 A	1.8	1.0 A
Root lesions	R13	31.4		1.2	
	R40	47.0		2.3	
	R39	31.6		1.2	
	R44	36.2	36.6 A	1.3	1.5 A

[a]Based on three replicates for each isolate on cultivar Florigiant peanut plants.

[b]Means followed by the same letter are not significantly different (P = 0.05).

the mean plant root length, the radius of the root tip plus rhizosphere soil (1.13 mm) and the radius of the root tips alone (0.13 mm). The weight of rhizosphere soil was calculated from the bulk density (1.4 g/cm^3) of the soil and the volume of the rhizosphere soil. The number of microsclerotia germinating per plant was calculated from the percent germination data of Krigsvold (17), the inoculum density (9.1 microsclerotia per gram of soil), and the weight of rhizosphere soil per plant. Efficiency of inoculum was calculated as follows:

Efficiency of inoculum for observed infection =

$$(O_{ti}/G_{ti}) \times 100 ,$$

in which O_{ti} represents the number of observed infections per plant at time, t_i; and G_{ti} represents the estimated number of microsclero tia germinating in a 1-mm zone of rhizosphere soil per plant at time, t_i. The time between microsclerotium germination and infection (infection time) is estimated to be short and should not critically influence these calculations. That the inoculum efficiency estimate was greater than 100% may be due, in part, to a low estimate for percentage microsclerotium germination; high propagule densities are required for these tests and this can lower percent germination in soil (9). Also, as developed later, infection courts other than the root tip may have played a role in root colonization.

Since the increases in the two infection rates, R_o and R_e, were somewhat similar, and since $\log_e (1/1-y)$ may be a good estimate of

those observed infections that develop into necroses, it may be reasonable to develop the following estimate for efficiency of observed infection for necrosis:

Efficiency of observed infection for necrosis =

$$[\log_e(1/1-y)_{t_2}]/O_{t_1} \times 100 ,$$

in which the numerator represents the estimated number of infections per plant at a comparable time, t_2; and the denominator represents the number of observed infections per plant at a given time, t_1, where $t_2 - t_1$ is equal to the incubation period minus the germination period and infection time estimate (=incubation period - [germination period + infection time]). The relationship, $\log_e (1/1-y)$, estimated infections per plant, was used to calculate this efficiency estimate since discrete lesions due to *C. crotalariae* on peanut roots could not be counted reliably in any of the tests conducted in this investigation. Based on the lesion growth-rate experiment and the data of Krigsvold (17), $t_2 - t_1$ was estimated to be 5 days.
Infection efficiency estimates for the experiment reported in Fig. 1 ranged from 0.27 to 0.28% for the three 21-day periods. An efficiency of inoculum for necrosis estimate for VA-72-R plants after 3 wk, calculated with $\log_e (1/1-y)$, was 0.85%.

DISCUSSION

Multiple observed infections of _C. crotalariae_ were found over all areas of asymptomatic fine, taproots, and lateral roots of field-and greenhouse-grown peanut plants. Many infections were located near the root tips which, according to the observations of Krigsvold (17), are probably the primary infection courts. Thus, as the root tip grows, the infection site becomes further removed from the root tip. Based on the root surface-sterilization results, most observed root infections probably were not limited to surface cortical cells.

Previous fungal root-colonization studies used root segments 2 cm (5, 6, 19) or smaller (19, 20). For plant pathogens, overestimating the number of observed infections at high inoculum densities could cause the regression line to curve upward in the upper region of arithmetic inoculum-density plots. This was not observed here for second-order regression curves (Figs. 2 and 3).

The number of observed infections per asymptomatic peanut root system was generally more variable for field-grown plants than for greenhouse-grown plants. That some plants from the field plot were not colonized by _C. crotalariae_ may have been due to the clumped or nonrandom _C. crotalariae_ microsclerotial pattern found previously in this peanut field (24). For the greenhouse tests, microsclerotia-infested soil was thoroughly mixed previous to all experiments, which probably precluded clumping of inoculum.

The disease-progress study indicated that the infection rate, I^o_r, for observed infections increased over the three 21-day periods of the experiment. R_e and R_o increased greatly over the same period. In contrast to I^o_r, Vanderplank's (26) infection rate ($R \sim R_e$) does not allow for increased rates of host root length growth in simple-interest disease. The value of R_{ot}, where R_o is modified from Vanderplank's equation by using the number of observed infections per plant; instead of $\log_e (1/1-y)$, is equivalent to $I_{rt}{}^o l_t$. where l_t is the change in length of root per plant after any time, t. To our knowledge, I^o_r is the first measurement of an infection rate for a root-infecting fungus that is based on observed root infections and is expressed on a unit-root-length and unit-inoculum basis. In inoculum-potential studies, I^o_r should be more closely related to the effect of capacity factors on inoculum than R_o or R_e, since I^o_r takes account of host growth rate. As indicated, I^o_r increased over the period of the experiment, which suggests that root exudation or the number of infection sites per unit root length increased with time. This may result from root diameter growth and associated exudation (10) or from emergence of lateral and fine roots through the root cortex; there is more root branching as peanut root systems get older and these root emergence points may be sites of root exudation (12) and infection.

In absolute terms, the multiple-infection correction (1, 26, 27) greatly underestimated the number of observed root infections per

plant, but appeared to be roughly proportional to it. Estimated infections never exceeded a mean of three per plant, whereas observed root infections reached over 400 times this value. This appeared to be so because most observed root infections did not progress to necroses within a short period of time; i.e., incubation period minus the time required for microsclerotium germination and host infection. That most observed infections did not cause necrosis may have been due to low inoculum potential of individual microsclerotia (vs many microsclerotia on water agar strips in the lesion tests), differences in host resistance or disease proneness among plants (Florigiant is a multiline cultivar), and/or differences in pathogenicity among _C. crotalariae_ isolates. The lack of necrosis resulting from observed infection is reflected in the low estimate of efficiency of infection for necrosis (avg, 0.28%), or efficiency of _inoculum_ for necrosis, and this appeared to be much more limiting to disease development than was inoculum efficiency for observed infection, for which a high estimate was obtained. The latter is in agreement with the lack of appreciable _C. crotalariae_ germ tube lysis in the peanut rhizosphere observed by Krigsvold (17). Use of estimated infections, from the proportion of necrotic roots, to calculate efficiency of infection is presented here as a preliminary approach to estimating efficiency of infection for necrosis, and may result in underestimation. As indicated, the opposite occurred for the efficiency of inoculum for observed infection estimates.

To our knowledge no previous attempt has been made to calculate efficiency of infection or efficiency of inoculum (based on rhizosphere germination data) for root-infecting fungi.

The number of observed infections per plant, as well as observed infections per unit root length, increased in direct proportion to the inoculum density in the arithmetic plot. Although data points may be interprcted as fitting a curve with a decreasing slope, especially in Fig. 3, the $\log_{10}$-$\log_{10}$ plots of the same variables (Figs. 4 and 5) predict a straight-line relationship (3), since the slopes for each of the logarithmic plots were approximately 1.0. Furthermore, the results in Table 3 show the linear relationship to be the best fit, assuming that little confidence is placed in the highest inoculum density. The latter may have resulted in competition for, or overlapping of, infection sites. That the slope of the curve did not increase suggests that observed infection development from root-root contacts was probably not a factor at high inoculum densities. The inoculum density values required for 50% infection (ED_{50}) at 21 days were below 0.04 microsclerotia per gram of soil, since all plants examined at this inoculum density had observed infections.

The slope values of the two $\log_{10}$-$\log_{10}$ plots are close to 1.0 which suggests that the inoculum-host interaction follows a rhizosphere influence, as predicted by Baker et al (1, 4). Other investigators (13, 21, 24) using $\log_{10}$ microsclerotia per unit of soil

observed slope values closer to 0.67. Although two studies were field studies, where experimental conditions were not optimal, the 0.67 slope suggests a rhizoplane or root-contact influence (1, 4). That Krigsvold (17) observed greater than 33% microsclerotium germination in the inner 1-mm layer of rhizosphere soil of both CBR-susceptible cultivar VA-72-R and CBR-resistant cultivar Argentine peanut root tips suggests that microsclerotium germination is restricted to near the root surface, but is not limited to contact of microsclerotia with the root surface. Further research is required to clarify why slopes of 0.67 or lower have been obtained in other studies. Possibly, only large microsclerotia or several microsclerotia in an organic matter particle that are in contact with the peanut root surface (as in the water-agar-strip tests), where exogenous nutrients (exudates) are high and the inoculum potential would be greatest, give rise to what Garrett (7) and Baker (1) consider to be successful progressive infections and successful infections, respectively. These result in symptoms and may be estimated by $\log_e (1/1-y)$. Underestimation of infections by $\log_e (1/1-y)$ does not appear to be a critical factor (1, 2, 26, 27), as disease incidences were low in previous studies. In a field study, a clumped inoculum pattern in the field appeared to contribute to lowering $\log_{10}$-$\log_{10}$ slope values for this pathogen-host combination (24). Capacity factor differences may be important also (2).

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CHAPTER III

INFECTION RATES AND EXTENT OF INFECTION AND COLONIZATION BY CYLINDROCLADIUM CROTALARIAE ON ROOTS OF CBR-SUSCEPTIBLE AND CBR-RESISTANT CULTIVARS OF PEANUT

INTRODUCTION

Cylindrocladium crotalariae Loos (Bell and Sobers) [=teliomorph, Calonectria crotalariae Loos (Bell and Sobers)] is a soil-borne fungus which causes Cylindrocladium black root rot (CBR), a destructive root disease on peanuts in the Virginia-North Carolina peanut-growing region. In some years under conducive conditions, CBR may destroy nearly all plants in an infested field.

Cultivars resistant to CBR have been developed and the factors responsible for resistance originated from Spanish-type varieties (3). However, root rot incidence and CBR-disease severity have been reported to vary among locations and within cultivars. Differences in microsclerotial germination and subsequent infection and colonization of host tissues may contribute to the expression of root necrosis by the host in response to C. crotalariae. Other biotic factors, e.g. colonization of root tissues by other organisms, may also affect host response and alter symptom expression.

Krigsvold, et al (11) found that a higher percentage of microsclerotia (the primary survival propagule of C. crotalariae)

germinated in response to exudates produced by root tips of CBR-susceptible cultivar VA-72-R than in response to exudates of CBR-resistant cultivar Argentine, in 1-mm wide rhizospheres of each cultivar.

In the preceding chapter, observed root infections of C. crotalariae [i.e., discrete colonies of the fungus growing from asymptomatic peanut roots plated on sucrose-QT medium, selective for C. crotalariae (5)] were reported on CBR-susceptible cultivar Florigiant, and there was a direct relationship between the initial microsclerotial density and observed infections per m of root. Furthermore, only a few of these observed infections led to necrosis. At present, no information regarding observed infection rates for CBR-resistant cultivars has been reported.

Harris and Beute (8) found no qualitative differences in the formation of periderms in peanut taproots of CBR-susceptible or CBR-resistant cultivars when challenged with microsclerotial inoculum from a virulent isolate of C. crotalariae. However, fewer wound periderms were observed in cultivars NC3033 and Argentine (CBR-resistant), than in cultivar NC2. Hadley, et al (6) previously had rated NC2 as CBR-susceptible, due to its high root rot severity index, even though results from heritability analyses indicated NC2 as a suitable source of CBR resistance.

The major objectives of this study were to examine observed infections on asymptomatic roots of a CBR-susceptible cultivar and a

CBR-resistant cultivar, to determine the observed infection rates, of _C_. _crotalariae_ for each cultivar and to assess the extent or depth of colonization by _C_. _crotalariae_ in asymptomatic root tissues, for each cultivar. Results on the extent of infection and colonization of peanut roots by _Fusarium_ spp. are also reported.

MATERIALS AND METHODS

Plant and soil material. Cultivar Florigiant, and the CBR-resistant cultivar, 'Spancross' were used for all experiments. C. crotalariae-free peanut field soil (sandy loam, pH = 5.2) was mixed with naturally infested field soil containing high populations of microsclerotia of C. crotalariae to obtain the desired inoculum densities. Soil with high microsclerotial populations was obtained from a field with a history of CBR, in Southampton Co. Va, by excavating the root zones of plants with symptomatic shoots, removing intact roots, and mixing the soil thoroughly before using it as a source of inoculum. This naturally infested soil was stored at 25 C, until needed, in plastic bags with three pinholes for gas exchange.

Infection rate studies. Seeds were pre-germinated in moist paper towels (3 days for Spancross and 4 days for Florigiant) before planting them, 4 seeds per pot in 1-l plastic containers (11-cm diameter) in C. crotalariae-infested soils. Soils were thoroughly mixed for at least 15 min in a cement mixer and initial inoculum levels of soils were determined before each experiment by wet-sieving and dilution-plating onto plates containing sucrose-QT medium (5). The pots were placed in temperature tanks at 25 C on floating apparatuses with manifolds underneath to allow for water drainage from the pots. Plants were watered daily or as needed to maintain the moisture level near field capacity or -0.33 bars.

Following 3 wk, plants were removed gently, shaken to loosen surrounding soil, and roots were washed free of remaining soil. Using a cheesecloth-beaker assembly, entire asymptomatic root systems of plants were washed for an additional 25 min in running tap water. The entire root systems were cut into portions as long as possible for plating on 9-cm diameter petri plates containing sucrose-QT medium. The total length of plated roots (fine, lateral and taproot) was measured with a ruler for each plant. Colonies of C. crotalariae growing from roots were counted after 5-7 days incubation at 25 C. Extreme care was taken to prevent duplicating the recording of colonies that originated from both ends of a cut root or that were closely associated but were not clearly discrete units. The number of discrete colonies of C. crotalariae growing from asymptomatic roots was used as a measure of the number of observed infections.

Root dissection studies. Seeds were pre-germinated in the manner as described previously for the infection rate studies. For these experiments, six Florigiant seeds per pot and seven Spancross seeds per pot were planted to obtain large populations of taproots for each cultivar. Initial microsclerotial density levels were determined before planting and placing the pots in soil temperature tanks as described above. After 3 wk, plants of each cultivar were removed and washed in the same manner as for the infection-rate tests.

Lateral and fine roots were removed from taproots that were free

of visible root necrosis. The tap root surface was scrubbed gently and briefly with a soft-bristle toothbrush to remove the outermost cortical cell layer(s) before cutting into 1-cm segments. The "inner" cortex (hereafter referred to as cortex or cortical) was stripped from each segment with a sharp razor blade before plating the cortical tissue segments on sucrose-QT selective medium (5). The 1-cm stelar segments, with few or no cortical tissue remnants, were shaken in 100-ml of sterile distilled water for at least 1 min before transferring to plates containing sucrose-QT medium. After 5-7 days at room temperature (25-28 C) the number of segments of cortex and stele tissues with C. crotalariae and Fusarium spp. were determined for each segment of each cultivar.

RESULTS

Observed infection rates and observed infection density of *C. crotalariae* on CBR-susceptible and CBR-resistant peanut cultivars. The infection rates, $I^o{}_r$ [number of observed infections per meter root per day per microsclerotium per g soil (Chapter II)] were higher for Florigiant than for Spancross, in all trials (Table 5). Although the average infection rate for 46 Florigiant plants was 28.9% greater than the average of 26 Spancross peanut plants, the difference was not significant at P=0.05 when the eight total trials were considered. However, when one trial, with anomalous results, was omitted from the statistical analysis, a highly significant difference of 62.5% (P = 0.001) was obtained between the average infection rates for the two cultivars.

In a similar experiment, the average infection rate, $I^o{}_r$, for ten 'Argentine' (CBR-resistant) peanut plants was significantly lower (P=0.001) ($I^o{}_r$ = .026 infections per m root per day per microsclerotium per g of soil) than the average infection rate obtained for ten Florigiant peanut plants ($I^o{}_r$ = 0.072 infections per m root per day per microsclerotium per g soil per day) after 3 wk. This infection rate for Florigiant was somewhat greater than reported in Table 5. Observed infections of *C. crotalariae* were found over all portions of the root systems (fine, lateral and taproots) of the CBR-susceptible cultivar (Florigiant) and the

Table 5. Observed infection rates of Cylindrocladium crotalariae on asymptomatic roots and associated probability levels of significance on two cultivars of 3-wk-old peanut plants susceptible or resistant to Cylindrocladium black rot (CBR) in separate trials in greenhouse soil temperature tanks at 25 C

Florigiant (CBR-susceptible)			Spancross (CBR-resistant)			
I_r^o [a]	No. of Plants [b]	m of root tissue [c]	I_r^o [a]	No. of Plants [b]	m of root tissue [c]	P-values [d]
0.049	46 (5)	430.4	0.038	26 (3)	143.0	0.12
0.052	36 (4)	344.9	0.032	16 (2)	84.1	0.001

[a] Infection rate, I_r^o = number of observed infections per m root per day per microsclerotium per gram of soil.

[b] Numbers in parentheses indicate the number of trials. The initial microsclerotial densities were 17.4, 12.6, 16.4, 9.4 and 10.8 microsclerotia per gram soil for 5 trials of Florigiant, and were 9.4, 9.4 and 10.8 microsclerotia per gram soil for 3 trials of Spancross. The initial microsclerotial densities were 17.4, 12.6, 16.4 and 10.8 microsclerotia per gram soil for 4 trials of Florigiant, and were 9.4 and 10.8 microsclerotia per gram of soil for 2 trials of Spancross.

[c] Total length of root tissue plated on sucrose-QT medium for the indicated number of plants.

[d] Levels of significance between infection rates were determined according to Student's t-test.

CBR-resistant cultivar (Spancross) (Appendix A), following 3 wk of incubation in C. crotalariae-infested soil.

The average number of infections per m root, the average number of infections per plant and the average root length per plant were significantly higher for five trials with the CBR-susceptible cultivar, Florigiant, than for three trials of the CBR-resistant cultivar, Spancross (P=0.001) (Appendix A) (hereafter, five-of-five trials of Florigiant and three-of three trials of Spancross will be referred to as "eight trials").

Extent of colonization by C. crotalariae and Fusarium spp. of asymptomatic taproots of 3-wk-old CBR-resistant and CBR-susceptible cultivars of peanut. Cylindrocladium crotalariae generally was recovered from a greater number of dissected cortical and stelar tissue segments of asymptomatic taproots of CBR-susceptible Florigiant than of CBR-resistant Spancross (Table 6). A significantly higher percentage of the stelar segments were colonized by C. crotalariae for Florigiant than for Spancross (P = 0.05). However, the differences in the percentage of cortical tissues colonized by C. crotalariae were not significant (P=0.05) between the two cultivars.

There were significantly higher percentages of both cortical and stelar tissue segments colonized by Fusarium spp. from taproots of the CBR-resistant cultivar, Spancross, than for the CBR-susceptible cultivar, Florigiant (P = 0.05). Representative isolates of Fusarium spp. were transferred to blocks of potato-dextrose agar

Table 6. Numbers and percentages of 1-cm segments yielding *Cylindrocladium crotalariae* and *Fusarium* spp. from dissected cortex and stele tissues of 3-wk-old asymptomatic taproots from CBR-resistant and CBR-susceptible peanuts grown in greenhouse temperature tanks at 25 C [a]

Parameter	Peanut Cultivar Susceptible (Florigiant) [b]	Resistant (Spancross)
Number of 1-cm segments, dissected and plated	1198	1063
% cortex segments colonized by *C. crotalariae*	13.4	12.1
% stele segments colonized by *C. crotalariae*	6.2 *	3.8
% cortex segments colonized by *Fusarium* spp.	89.7 *	92.4
% stele segments colonized by *Fusarium* spp.	66.4 *	79.5

[a]Average microsclerotial density (for five trials): 30.2 microsclerotia/g soi

[b]Means compared horizontally, followed by an asterisk (*) are significantly different at P = 0.05, according to Student's t-test.

(Difco Labs, Philadelphia PA), allowed to grow on water agar and identified as Fusarium oxysporum, based on microconidiophore morphology.

Figure 6 shows a schematic representation of the extent of infection and colonization of CBR-susceptible Florigiant and CBR-resistant Spancross root tissues based upon the results obtained in this study. The average number of infections per m root, on a linear scale, adjusted to an equivalent inoculum density (~10 microsclerotia per g soil), is represented by the relative lengths on the outer cortex surfaces (A) for each cultivar. The width of the shaded regions of the inner cortex (B) and stele (C) were based upon the percent of cortical or stelar segments from taproots of the respective cultivars colonized by C. crotalariae, adjusted to an equivalent inoculum density, 10 microsclerotia per g soil.

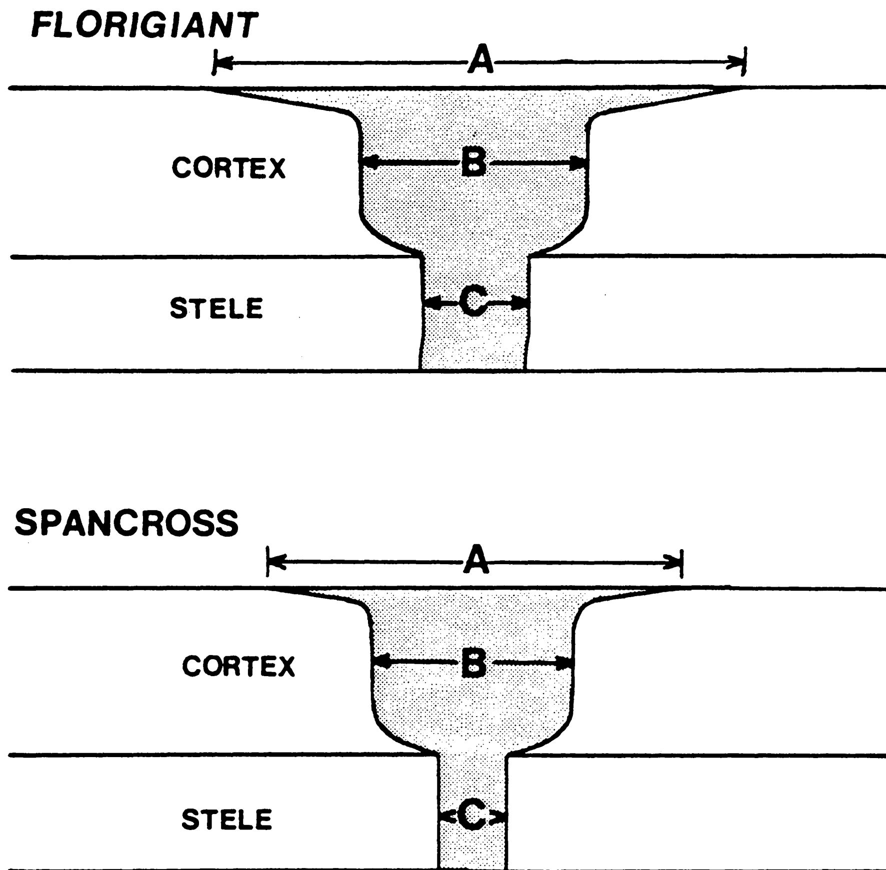

Fig. 6. Schematic representation of the extent of infection and colonization of CBR-susceptible Florigiant and CBR-resistant Spancross asymptomatic peanut taproot tissues by C. crotalariae following 3 wk growth in naturally infested peanut field soil in greenhouse temperature tanks at 25 C. Diagram is based upon the results for the number of observed infections per m root tissue for 46 Florigiant and 26 Spancross plants. On a linear scale, the shaded areas represent the number of infections per m root tissue (adjusted to an equivalent inoculum density of 10 microsclerotia per g soil), on the (A) outer cortex surface, in the (B) inner cortex, and in the (C) stele. For diagrammatical purposes, the widths of the cortex and the stele for each respective cultivar have been drawn equal. The bar in the lower left hand portion of the figure represents 1 infection or colonization per m root tissue.

DISCUSSION

Generally, the infection rates, $I^o r$, the number of observed infections per m root and the number of observed infections per plant for the CBR-susceptible cultivar Florigiant were higher than those observed for the CBR-resistant cultivars, Spancross and Argentine. These results are in agreement with observations by Krigsvold, et al (11), who reported a significantly higher percentage of microsclerotial germination in 1-mm wide rhizospheres of CBR-susceptible VA-72-R, in contrast to CBR-resistant Argentine.

Anomalous results obtained in one trial using plants of both Florigiant and Spancross influenced the overall infection rates for each of the two cultivars (Table 5). The results of these eight trials, conducted at slightly different inoculum densities, were combined since the number of _C. crotalariae_ infections per m root were found to be directly proportional to the initial microsclerotial density (Chapter II). The change in the variance-to-mean ratios, V/m (Appendix B), for the average infection rates of the resistant cultivar, Spancross, may account for the loss of significant differences between the two cultivars, when the anomalous trial is included in the analysis. Including the anomalous data increased the V/m only 30.8% for the susceptible cultivar Florigiant, but increased the V/m by 61.9% for the resistant cultivar Spancross (represented by 10 plants). Also, there is a smaller percent difference in the

average infection rates, $I^{o}{}_{r}$, between the two cultivars (28.9%), relative to the resistant cultivar, when the eight trials were considered, as compared to a difference of 62.5%, when the anomalous trials were deleted from the analysis (i.e., six trials).

The outer cortex surfaces of the segments from the cultivars used in the root dissection study were not plated and the number of surface root infections were not determined. For these experiments, only tissues from the inner cortex and stele were plated, due to the tedious nature of precisely removing a well-defined area of the outer cortical surfaces from root segments of either cultivar. It also was necessary to use high populations of plants since only the taproots could be dissected precisely.

The percent of cortical tissue segments yielding C. crotalariae was slightly higher for Florigiant than for Spancross (Table 6 and Fig. 6). Examination of Fig. 6 suggests that not all of the infections observed on outer cortex surfaces of both cultivars result in subsequent colonization of the inner cortex. Similarly, for both cultivars, not all colonizations of the cortex result in subsequent colonization of the stele.

There was a significantly higher percentage of stelar tissues colonized by C. crotalariae for Florigiant than for Spancross (Table 6 and as shown by the larger shaded region of the stele tissues of Florigiant relative to Spancross in Fig. 6). This reduced colonization of the stele in Spancross may be due to less breachment

of wound periderms of the inner cortex noted in other CBR-resistant lines (8), or other resistance mechanisms operating in the inner cortex or at or near the endodermis in Spancross. These mechanisms are perhaps absent or less-effective (17) in Florigiant (8).

Greater susceptibility of a genotype to C. crotalariae may be due to a smaller amount of root tissue to infect and colonize in Florigiant than Spancross. However, measurements of the combined widths of several cross sections of peanut taproots (of both cultivars) suggested other factors to be responsible for susceptibility, since the combined cortical widths of two different sections of Spancross taproots were slightly lower than those observed for Florigiant taproots (Appendix C). Thus, the fungus had a greater width of tissue to colonize in Florigiant than Spancross. In contrast, there was a significantly wider stele (36.1%) in Florigiant than in Spancross (P=0.05). It should also be noted that the genetic background is vastly different for the two cultivars used in this study. Florigiant is a multiline of seven sibling lines of Arachis hypogaea (15) and Spancross is a hybrid of A. hypogaea and A. monticola (13).

Further research is necessary to establish the relative importance of lateral root emergence points in the pathogenesis of peanut roots by C. crotalariae. In this study, significantly fewer colonizations were observed in the stele of Spancross taproots than in Florigiant, which may have been due to the production of fewer

lateral root emergence points (as suggested by a smaller circumference of the pericycle) near germinating microsclerotia, or resistance barriers (or other resistance mechanisms) in the inner cortex or at the endodermis. Root emergence points have been reported previously as sites of root exudation (7). As indicated, Krigsvold, et al (11) noted that these exudates from cultivars susceptible and resistant to CBR fostered microsclerotial germination in the rhizosphere. Harris and Beute (8) also thought these root emergence points were important in the root infection of taproots from CBR-susceptible and CBR-resistant cultivars by _C. crotalariae_.

Necrosis in stelar tissues of peanut hypocotyls was reported by Johnson and Beute, (9) and in taproots (8) several cells in advance of hyphae, whereas, Milholland (12) reported invasion of the stele of symptomatic roots of highbush blueberry by _C. crotalariae_. Hyphae of _C. crotalariae_ were observed in the stele of taproots only in the presence of a severe lesion (8). In this study, _C. crotalariae_ was observed in dissected stelar tissues from asymptomatic taproots of both Florigiant and Spancross. Thus, following microsclerotial germination at the root tips (11), _C. crotalariae_ probably infects and colonizes root tissue from the surface of the outer cortex through the inner cortical layers toward the vascular tissues, previous to root tissue necrosis. Longitudinal root colonization, not determined in this study, may also occur.

While formation of resistance barriers may aid in arresting infection and/or inhibiting colonization of tissues by C. crotalariae, other factors should be investigated. In this study, strains of F. oxysporum (as some representative isolates were identified), were observed in significantly higher numbers on asymptomatic root segments of cortical and stelar tissues of Spancross than of Florigiant. The differences were slight in the cortex, but appreciable in the stele. Fusarium oxysporum is a common colonist of root tissues (4, 14), and in some studies, has also been found in the vascular tissues (10, 15, 16, Table 6) without any apparent harm to the plant (2, 4, 14, 15). However, Bell (1) demonstrated root and shoot necrosis of peanut following inoculation of germinated peanut seed (previously sterilized) with F. oxysporum and F. roseum in a gnotobiotic environment.

Further studies are needed to determine the role(s), if any, of the Fusarium spp. observed in or near the vascular tissues of the taproots of CBR-resistant cultivars. Possibly, the organisms contribute, in some way, toward reducing the extent of stelar colonization by C. crotalariae in the resistant cultivar. In another study (Chapter IV), a significant reduction (P=0.05) in the average root rot severity index was obtained under greenhouse conditions for 9-wk-old Florigiant peanuts grown in C. crotalariae-infested peanut field soil amended with laboratory-grown conidia of F. oxysporum and Aspergillus niger.

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CHAPTER IV

MICROSCLEROTIAL RECOVERY OF CYLINDROCLADIUM CROTALARIAE AND DEVELOPMENT OF CYLINDROCLADIUM BLACK ROT ON PEANUTS FOLLOWING TREATMENT OF NATURALLY INFESTED PEANUT FIELD SOILS WITH SODIUM AZIDE AND MICROBIAL ANTAGONISTS

INTRODUCTION

Potassium and sodium azide are inhibitors of the cytochrome oxidase system in respiratory metabolism largely due to the liberation of hydrazoic acid (6, 18).

In field studies, sodium azide (NaN_3) has been shown to reduce *Cylindrocladium black root rot (CBR) on peanut (15, 16, 20) caused by* the fungus Cylindrocladium crotalariae (Loos) Bell & Sobers (1). The disease was first observed in the Virginia-North Carolina regions in 1970 (4), and by 1976, it was found in all peanut-producing counties of Virginia and North Carolina (14).

For control of CBR, rates of 10-40 lb NaN_3/A., (11.2-44.8 kg NaN_3/ha) for a 15-cm depth were effective when the chemical (in the granular form) was disk-incorporated into soils (16). Post-emergence applications (either singly or in combination with pre-plant applications) of lower rates of 12 lbs NaN_3/A (13.5 kg NaN_3/ha) (15), and 4-7 lbs NaN_3/A (4.5-7.8 kg NaN_3/ha) reduced disease incidence. Only partial control of CBR was obtained with this biocide in Virginia (6). Not all propagules (primarily microsclerotia) of C. crotalariae were killed by NaN_3, however.

Although NaN_3 has been effective in the reduction of CBR development on field-grown peanuts, fumigation of large infested areas has been regarded as economically unfeasible. Furthermore, Cook and Baker (3) advocated the use of soil fumigation to provide a standard to measure the progress that has been accomplished with experimental biological control methods.

The use of sublethal doses of carbon disulfide to control Armillaria mellea is a classical study which integrated chemical control against Armillaria root rot of citrus and peach roots with biological control of additions of Trichoderma spp. inoculum to treated trees (2).

Horsfall (7) stated that one of the least investigated areas in plant disease control is the relation between inoculum potential and the amount of fungicide required for effective control of the pathogen.

Presently, there are no reports regarding the response of C. crotalariae microsclerotial populations to different levels of NaN_3 treatment. Portions of the information presented in this study may offer insight for further investigations concerning the economical application of sublethal dosages of sodium azide for control of soil-borne pathogens. The objectives of this study were to determine microsclerotial population densities following the application of different levels of NaN_3 to naturally infested peanut field soils and to examine the extent of root rot severity on greenhouse-grown peanut

plants following growth in NaN_3-treated soils. Results from tests which integrated sublethal doses of NaN_3 to C. crotalariae with selected microbial antagonists are réported also.

MATERIALS AND METHODS

Preparation of C. crotalariae infested soil. Naturally infested soil (loamy sand, pH=5.0, with 0.5 % organic matter) was collected from a peanut field with a history of CBR. The water content at -0.33 bars (field capacity) was 5.3 percent. Further characteristics of the soil from this field are listed in Appendix D. To increase microsclerotial populations, necrotic roots of naturally infected peanut plants were macerated in a blendor and sieved through nested coarse- and fine-mesh sieves. The microsclerotia embedded in the tissues were recovered on the fine-mesh sieve (500-μm pore size) and added to the soils collected from the rhizospheres of CBR-diseased plants. The range of the final microsclerotial densities of three (similarly) infested soils was 480-760 microsclerotia/g soil. These soils were kept moist (~8 % moisture) in plastic bags with pinholes for gas exchange at 25 C until needed.

Fumigation of naturally infested soil with sublethal doses of sodium azide. Five doses of sodium azide (NaN_3) (8 % a. i. SMITE Pittsburgh Plate Glass Industries, Pittsburgh, PA) were selected for examination of their efficacy in reducing microsclerotial populations of naturally infested peanut field soil, based on preliminary fumigation studies with artificially-infested soils.

Four hundred grams of one of the infested soils (at 9 % soil moisture), were treated with 15 μg NaN_3 per g soil and hand-mixed for 15 min before preparing serial logarithmic dilutions with untreated

C. crotalariae-infested soil for the remaining five treatments (0.5, 1.0, 1.5, 5.0 and 10.0 µg NaN_3/g dry soil). All NaN_3 treatments were calculated on a per gram dry weight soil basis. Each of four 40-g replicates per dosage was hand-mixed an additional 2 min before placing the aliquot in 50-ml beakers and covering with aluminum foil with 3 small pinholes to allow for gas exchange. The beakers were placed in an incubator at 25 C for 4 days before assaying microsclerotial populations by soil-dilution plating on sucrose-QT selective medium (5). Colonies of C. crotalariae were counted from each of 10 plates, for each replicate of each treatment following 5-7 days incubation at 25 C.

Preparation of bacterial and fungal antagonists for greenhouse studies. Cultures of Pseudomonas cepacia (Burkholder) were maintained in tubes of sterile distilled water (6 C) until needed. The original isolates were obtained from Dr. William Ayers, USDA-ARS, Beltsville, MD. Agar cultures of Fusarium oxysporum Schlecht. and Aspergillus niger van Tiegh. were maintained on slants of potato dextrose agar (PDA) until needed. Isolates of both fungi were obtained from roots of peanut plants or from peanut field soils. Fusarium oxysporum isolates were identified on the basis of microconidiophore morphology following fungal growth onto plates of water agar from blocks of potato dextrose agar (PDA). Cultures of Trichoderma harzianum Rifai were maintained on slants of PDA until needed. The isolates of T. harzianum were obtained from

Dr. R. Baker, Colorado State University, Fort Collins, CO, and from Dr. H. D. Wells, USDA-ARS, Tifton, GA.

Slant cultures (~24-hr old) of each of 2 isolates of P. cepacia grown on PDA were harvested and washed (to remove exogenous nutrients) by pipetting 10 ml of sterile distilled water and gently scraping the cells from the agar slants with a rubber policeman. After harvesting each slant twice in this manner, the suspensions were centrifuged and washed twice with sterile distilled water before initial density levels were determined with a bacterial cell counter. Seven-day-old slants of F. oxysporum were harvested in the same manner. Agar plates (~12-day-old) of A. niger and of T. harzianum were harvested in the same manner, except following washing, the respective suspension was filtered first through several layers of cheesecloth to remove mycelial fragments before centrifugation. One-tenth ml of a 0.25 % Tween-20 [polyoxyethylene (20) sorbitan monolaurate, Atlas Chemical Industries, Inc., Wilmington, Delaware] was added to the suspension before centrifugation. The fungal pellets were washed, resuspended twice with sterile distilled water, and centrifuged before the initial densities were determined with a hemacytometer.

Interactions of sublethal treatments of sodium azide, C. crotalariae and antagonistic microflora. Several studies were conducted in the greenhouse to determine root rot severity following amendments of naturally infested peanut field soil with presumable

antagonistic microflora and/or sublethal treatments of NaN_3 to C. crotalariae. In addition, the percent of root segments colonized by C. crotalariae following growth in these different treatments was examined in selected studies. Unless otherwise stated, all studies were conducted in soil temperature tanks adjusted to 25-26 C, which has been reported as the optimal temperature regime for CBR development (13). Microsclerotial levels were adjusted to an initial density of ~10 microsclerotia/g soil, and were determined by soil-dilution plating on sucrose-QT selective medium (5). Following determination of sublethal dosages of NaN_3 to C. crotalariae (from the procedure above), C. crotalariae-free and C. crotalariae-infested soils were treated with the appropriate levels of NaN_3 and mixed for 15 min in a cement mixer. These soils were placed in large plastic bags with pinholes for gas exchange and were incubated at temperatures of 18 to 32 C to complete the fumigation. The temperature variation was caused by the necessity of incubating the large volumes of soil in the greenhouse.

Four days after the NaN_3 treatment (or fumigation), suspensions of the antagonist(s) were mixed into the soils in a cement mixer for 20 min. Ten days following NaN_3 treatment, the treated soils were placed in pots and four peanut seeds, cv. Florigiant, were planted in 1-L pots (11-cm diameter). The pots were placed in a temperature tank at 25 C on a floating apparatus with a manifold underneath to allow for water drainage from the pots. Plants were watered daily or

as needed to maintain the moisture level sufficient for plant growth. Each treatment in each of the experiments had at least 13 pots. Due to inadequate space in the soil temperature tanks, only selected treatments were investigated. After 8 to 9 wk of incubation, plants were removed gently, washed free of remaining soil and the root systems were evaluated on a scale of 0 to 5 (0 = no visible root necrosis, 1 = 1 to 10% root necrosis, 2 = 11 to 25% root necrosis, 3 = 26 to 50% root necrosis, 4 = 51 to 75% root necrosis, and 5 = > 75% root necrosis) for root rot severity. Several tissue biopsies were performed on symptomatic roots and plated on sucrose-QT selective medium (5) to confirm the presence of *C. crotalariae*. Mean comparisons for treatments in which *C. crotalariae*-infested soils were used, were analyzed using horizontal and vertical single degree-of-freedom planned F-comparisons for most of the experiments (17). Each experiment included the appropriate controls using *C. crotalariae*-free soils, however these were not included in the data analysis, except where noted.

For selected colonization studies, root systems of several plants from each treatment were washed for 25 min in running tap water to remove surface contaminants. Twenty 2-cm segments were excised from asymptomatic root tissues from each of the washed root systems. The segments were plated on selective sucrose-QT medium (5) and after 5-7 days incubation at 25 C, the percent of root segments colonized by *C. crotalariae* were determined for each treatment.

RESULTS

Recovery of C. crotalariae microsclerotia following fumigation of naturally infested soil with NaN_3. Populations of C. crotalariae were recovered from all untreated and NaN_3-treated, C. crotalariae-infested soils (or infested soils) (Fig. 1). Increasing doses of NaN_3 from 2.5 µg a.i. NaN_3 and higher, reduced the microsclerotial population density of C. crotalariae. The regression equation for the arithmetic relationship between the average population density of microsclerotia recovered from each of four treatments and the NaN_3 concentration was Y=-25.2 + 370.7, (R^2=0.96, P = 0.0001). The greatest decrease in percent microsclerotial recovery (~93%) was observed in C. crotalariae-infested soils treated with 10.0 µg NaN_3 per g soil.

Based on this dosage-response curve, the effective dose of NaN_3 at which 5% of the initial microsclerotial population was recovered (ED95) was calculated as 14.0 µg NaN_3/g soil. Correspondingly, the NaN_3 concentration at which only 50% of the initial population (ED50) was recovered was 7.4 µg NaN_3/g soil.

Cylindrocladium black rot development on peanut roots following growth in naturally infested peanut field soils fumigated with NaN_3. Root rot development at 25 C was observed on all plants from fumigated, and non-fumigated C. crotalariae-infested soils (Table 7). Plants grown in non-fumigated, C. crotalariae-infested soils had the greatest root rot severity rating. A significant reduction of root

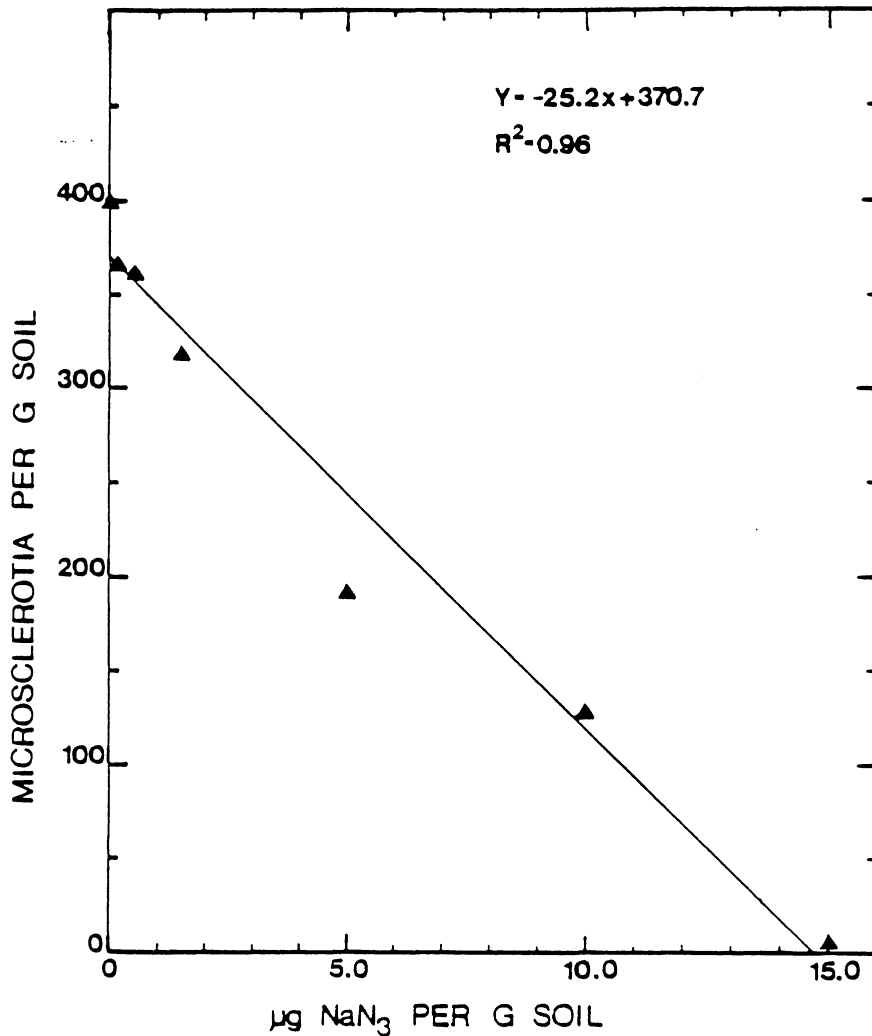

Fig. 7. Dosage-response relationship of the recovery of *Cylindrocladium crotalariae* microsclerotia from naturally-infested peanut field soils after four days of fumigation with NaN_3 at 25 C ($P = 0.0001$). Values are the averages of four replicates for each NaN_3 concentration.

Table 7. Root rot development and percent colonization of asymptomatic and symptomatic peanut roots by *Cylindrocladium crotalariae* on 8-wk-old Florigiant plants grown in *C. crotalariae*-infested soils at 25 C following fumigation with different levels of sodium azide

Dosage, µg NaN_3/g *C. crotalariae*-infested soil[a]	Number of Plants	Root Rot Severity Index[b]	Percent of root segments yielding *C. crotalariae*	
			Asymptomatic[d]	Symptomatic
0	44	2.5 A[c]	5.0	92.0[e]
2.5	45	2.3 A	1.5	93.0[e]
7.5	30	1.7 B	0.0	0.0
10.0	34	1.3 C	0.0	0.0
0.0, *C. crotalariae*-free	41	1.5 BC	0.0	0.0

[a] Unless stated otherwise, initial density of *C. crotalariae*-infested soil was 9.8 ms/g soil.

[b] Root rot severity index: 0 = no visible root necrosis or some brown discoloration, 1 = 1 to 10% root necrosis and/or minimal black necrotic lesions on tips of fine roots, 2 = 11 to 25% root necrosis, 3 = 26 to 50% root necrosis, 4 = 51 to 75% root necrosis and 5 = >75% root necrosis.

[c] Means followed by the same letter are not significantly different at P = 0.05, according to the Duncan's Multiple Range Test.

[d] Based on 20 2-cm segments of asymptomatic root tissues of 5 plants.

[e] Based on 200 tissue biopsies of necrotic tissues.

rot severity (P=0.05) was observed for plants grown in C. crotalariae-infested soils treated with 7.5 μg or 10.0 μg NaN_3 per g soil versus no fumigation. Thus, rates of SMITE less than 7.5 μg a.i. NaN_3 per g soil were used in successive experiments.

Cylindrocladium crotalariae was recovered from asymptomatic and symptomatic root tissues of Florigiant plants grown in non-fumigated, C. crotalariae-infested soils, as well as C. crotalariae-infested soils treated with 2.5 μg NaN_3 per g soil (Table 7). There was a greater percent of asymptomatic root segments yielding C. crotalariae from plants grown in nonfumigated C. crotalariae-infested soils than from those recovered from fumigated, C. crotalariae-infested soils. Cylindrocladium crotalariae was not recovered from symptomatic tissues of plants grown in C. crotalariae-infested soils treated with 7.5 or 10.0 μg NaN_3 per g soil. Due to the small sample size and low, proximal percentages of asymptomatic root segments colonized by C. crotalariae, proper statistical analyses could not be employed.

Root systems of peanut plants grown under field or greenhouse conditions have a surface brown discoloration, usually not attributable to necrosis caused by C. crotalariae.

No trends were observed between the average fresh weight of root tissue nor the average stem height and the different NaN_3 treatments (Appendix E). However, a lower average fresh root weight was noted for plants recovered from infested soils

treated with 7.5 μg a. i. NaN_3 per g soil than for plants grown in other soil treatments. The average stem height for plants grown in untreated and C. crotalariae-free soils was higher than those observed for plants recovered from other soil treatments.

Cylindrocladium black rot development on peanut roots following growth in naturally-infested peanut field soils treated with NaN_3 amended with antagonists. Table 8 shows the average root rot indices for peanuts grown in fumigated and non-fumigated C. crotalariae-infested soils with or without amendments of P. cepacia. By horizontal comparisons, P. cepacia significantly reduced (P = 0.05) root rot in fumigated soil but not in non-fumigated soil. The reverse was found in soils without the P. cepacia amendments. The average root rot severity indices were not appreciably different for plants grown in the other three soil treatments. The lowest root rot severity indices, 0.37 and 0.35, (not shown in Table 8) were obtained for plants grown in C. crotalariae-free soils without NaN_3 and for those grown in C. crotalariae-free soils amended with P. cepacia. Colonies of C. crotalariae were recovered from asymptomatic root tissue segments of plants grown in all treatments which used C. crotalariae-infested soil. There was a higher percentage of asymptomatic root segments colonized by C. crotalariae from plants grown in fumigated, C. crotalariae-infested soils with the bacterial amendment than for the other treatments.

Table 8. Root rot development and percent colonization of asymptomatic root segments by *Cylindrocladium crotalariae* from 8-wk-old Florigiant peanuts grown in *C. crotalariae*-infested soils at 25 C following treatments of NaN_3 and/or amendments of *Pseudomonas cepacia* (PC)

Dosage, µg NaN_3/g *C. crotalariae*-infested soil[a]	Average Root Rot Rating[b]		% root segments colonized by *C. crotalariae*	
	+PC[c]	-PC	+PC	-PC
2.5 *[d]	2.4[e] (38)[f]	3.2[e] (35)	4.0	3.0
0.0 *[d]	2.6 (37)	2.7 (39)	2.0	3.0

[a]Initial density of *C. crotalariae*: 9.6 microsclerotia per g soil.

[b]Root rot rating scale: 0 = no visible root necrosis, 1 = 1 to 10% root necrosis, 2 = 11 to 25% root necrosis, 3 = 26 to 50% root necrosis, 4 = 51 to 75% root necrosis, and 5 = > 75% root necrosis.

[c]+PC: Where noted, soils were amended with 1.0×10^6 cells/g soil

[d]Values followed by an asterisk (*) are significantly different at P = 0.05 by vertical comparison for the NaN_3 dosage levels.

[e]Values followed by an "e" are significantly different at P = 0.05 by horizontal comparison for the bacterial amendment.

[f]Numbers in parentheses represent number of plants considered in root rot evaluations.

Cylindrocladium crotalariae was also recovered from several biopsies of necrotic tissues from symptomatic roots, of all treatments using C. crotalariae-infested soils.

In a subsequent experiment, significantly greater (P=0.05) root rot severity ratings were observed for plants grown in infested soils treated with 0, 2.5, and 5.0 μg NaN_3/g soil in the presence of P. cepacia than those grown in infested soils without P. cepacia treated with the same levels of NaN_3 (Appendix F).

Cylindrocladium crotalariae was recovered from plated asymptomatic root segments of all treatments which utilized C. crotalariae-infested soil. An appreciably higher percentage of asymptomatic root segments yielded C. crotalariae from plants grown in C. crotalariae-infested soils treated with 2.5 μg NaN_3 per g soil than from plants grown in infested soils without NaN_3 and P. cepacia.

Significantly lower root rot severity indices were observed on plants grown in C. crotalariae-infested soils amended with a mixture of A. niger and F. oxysporum, with or without NaN_3 fumigation, than for those grown in C. crotalariae-infested soils without the fungal amendment (Table 9). However, no significant differences were observed in the root rot severity ratings between the two levels of NaN_3 in the presence or absence of the fungal amendment.

In a subsequent greenhouse experiment, using an amendment of several root and soil isolates of Fusarium oxysporum (added as

Table 9. Root rot development on 9-wk-old Florigiant plants grown in C. crotalariae-infested soils between temperatures of 18 to 32 C following chemical treatments of sodium azide and/or a mixture of Aspergillus niger and Fusarium oxysporum.

NaN_3 dosage, μg NaN_3/g C. crotalariae-infested soil[a]	Average Root Disease Severity Index[b]	
	+ Fungal Amendment[c]	- Fungal Amendment[c]
5.0	2.9 *[d] (15)	3.2 (20)
0.0	2.9 * (15)	4.1 (19)

[a]Initial density of C. crotalariae: 11.9 microsclerotia per g soil.

[b]Root rot rating scale: 0 = no visible root necrosis, 1 = 1 to 10% root necrosis, 2 = 11 to 25% root necrosis, 3 = 26 to 50% root necrosis, 4 = 51 to 75% root necrosis, and 5 = >75% root necrosis.

[c]Fungal amendment: Mixture of 5 x 10^4 F. oxysporum conidia per g soil and 1.5 x 10^5 A. niger conidia per g soil.

[d]Values followed by an asterisk (*), are significantly different (P = 0.0[illegible] by horizontal comparison within each level of NaN_3.

1.4 X 10^5 spores/g soil), general seed decay and hypocotyl rot was observed on Florigiant plants grown in C. crotalariae-infested soils, with or without NaN_3 fumigation (at 5.0 µg NaN_3/g soil).

Appendix G shows the average root rot ratings for plants grown in C. crotalariae-infested soils treated with 0, 2.5 and 5.0 µg NaN_3/g soil with or without T. harzianum amendment. Plants with the lowest root rot ratings were obtained from non-fumigated, C. crotalariae-infested soil either with or without the addition of T. harzianum. Highest root rot ratings were observed on plants from C. crotalariae-infested soils treated with 2.5 µg NaN_3/g soil and amended with T. harzianum. The differences in root rot severity indices among the treatments were not statistically significant ($P = 0.05$).

Results of a test which utilized a mixture of three different isolates of T. harzianum are presented in Appendix H. The average root rot index for plants grown in NaN_3-treated, C. crotalariae-infested soil with the fungal amendment was slightly lower than for those plants grown in similarly C. crotalariae-infested soils treated with NaN_3 and amended with T. harzianum. However, the highest root rot severity index was obtained for plants grown in non-fumigated, T. harzianum-amended, C. crotalariae-infested soils. The differences in the root rot severity indices among the treatments were not statistically significant ($P = 0.05$).

DISCUSSION

Initial microsclerotial densities of germinable C. crotalariae decreased linearly ($P = 0.0001$) in response to increasing doses of NaN_3 of naturally infested peanut field soils, after four days of fumigation. The dosage level of 7.4 μg NaN_3/g soil was found to reduce initial microsclerotial populations by 50 percent. Correspondingly, significant reductions ($P = 0.05$) in root rot severity indices were observed on Florigiant plants grown in C. crotalariae-infested soils treated with 7.5 μg NaN_3/g soil or higher, as compared to those plants grown in infested soils treated with lower levels of NaN_3. Many microsclerotia were recovered from 5.1-cm thick clods of high-clay, C. crotalariae-infested soil, treated with 50 μg NaN_3/g soil after 4 days, as well as from surfaces of high-clay, C. crotalariae-infested soils which had been treated with a surface application of 10 μg NaN_3/g soil (18).
Further research is necessary to clarify the possible fungistatic or fungicidal activities of NaN_3 to C. crotalariae. Fungistatic activity of Sclerotium rolfsii was demonstrated for low levels of NaN_3 (< 5 μg NaN_3/g soil), whereas, fungicidal activity was demonstrated by higher doses of 10 μg NaN_3/g soil, based on the inability to recover sclerotia after 7 days from treated soils (19).

The results of these studies may be valuable toward the development of CBR control methods which integrate sublethal doses of NaN_3 with biological control agents, or with other disease control

methods. Promising results for reducing CBR severity were obtained using sublethal levels of NaN_3 (at 5.0 μg NaN_3/g soil) in combination with Pseudomonas cepacia or a mixture of F. oxysporum and A. niger. However, these results were not confirmed in subsequent trials. In two separate greenhouse trials, isolates of T. harzianum were tested also, with conflicting results.

Phipps (12, 13) noted decreased microsclerotial populations, as well as the percent of CBR-symptomatic plants (based on shoot symptoms) in soils fumigated with Busan® or Vapam® and planted to a CBR-resistant cultivar. However, increased microsclerotial populations were recovered from fumigated, infested field soils planted to a CBR-susceptible variety.

For a successful integrated chemical-biological control approach to be operable in fumigated soils, the controlling organism must be more tolerant of the fumigant than the pathogen, or have the ability to recover more rapidly from its effects (10). The classical study of this type of integrated control was reported for control of Armillaria mellea by Trichoderma spp. in soils fumigated with carbon disulfide (2). Further experimentation is required to clarify the inconsistent results obtained from greenhouse studies which integrated use of chemical control with biological control agents. The selectivity of soil fumigation has been reported to affect the differential tolerances and escape by microorganisms (3). Perhaps the competitors used in this study were unable to survive the trace

residual fumigation, or they could not become a major component of the soil microflora to reduce root rot severity. Although nearly 100 % viability of log-phase or active cultures have been obtained for other bacterial species (Tomimatsu, unpublished) as well as other fungal species (G. J. Griffin, personal communication), the antagonists to C. crotalariae may have been in a more fragile state and thus, more susceptible to stress factors imposed by fumigated soils. Others (7, 8) have reported survival and/or re-population of soils by Trichoderma spp. and other competitive microorganisms within 5 weeks, following treatment with greater levels of NaN_3 than those used in this study. A reduction in fungal populations was observed after 2 weeks, however.

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CHAPTER V

SUMMARY

Cylindrocladium crotalariae is a soil-borne pathogen which causes Cylindrocladium black rot (CBR), a destructive root disease of peanuts in the Virginia-North Carolina regions. Approaches for effective CBR management have been somewhat elusive, due in part to the reported influence of environmental factors on disease severity (Chapter I). Comprehensive information regarding the inoculum potential relationships of and the route of infection and colonization by C. crotalariae on peanut roots may prove useful for the development of effective control strategies.

Evaluations of inoculum potential for a soil-borne pathogen have relied upon mathematical relationships between the inoculum density and disease incidence (1), or measurements of infection rates (10, 11). Both approaches previously utilized the number of infections estimated from transformations of disease incidence. In the present study, cultural techniques were used to enumerate observed (apparent) infections on asymptomatic peanut roots, following germination of C. crotalariae microsclerotia near the infection courts.

Numerous infections of C. crotalariae were observed on taproots, fine and lateral roots from two cultivars, Florigiant (CBR-susceptible) and Spancross (CBR-resistant), which were grown in naturally infested peanut field soils under greenhouse temperature

tank conditions (25 C), conducive for CBR development. Slope values near 1.0 of $\log_{10}$-$\log_{10}$ plots for microsclerotium density versus the number of observed infections (on a per plant or a per m root length basis for Florigiant) indicated direct proportionality between these two variables. Estimates of the efficiency of inoculum (percent of germinating microsclerotia that infect roots) and efficiency of infection (percent of infections that develop into necroses), were calculated for CBR-susceptible cultivars. A high estimate for the efficiency of inoculum for observed infection (near 100%) was found for CBR-susceptible 'VA-72-R' (Chapter II). This high estimate for efficiency of inoculum is in agreement with the lack of appreciable C. crotalariae germ tube lysis in the peanut rhizosphere observed by Krigsvold et al (7). In contrast, a low estimate for the efficiency of infection for necrosis was calculated for Florigiant (~0.28 %). Thus, the infection efficiency for necrosis appeared to be more limiting to CBR development than the efficiency of inoculum for infection.

A new and improved infection rate for soil-borne pathogens, I^{o}_{r} (the number of observed infections per m root per day per microsclerotium per gram of soil), was calculated also (Chapter II). Previous estimations of infection rates (10, 11) had ignored the rate of host growth as a component of simple-interest disease. Significantly lower (P=0.001) values of I^{o}_{r} were calculated for 3-wk-old Spancross plants than for Florigiant. The results from the infection rate

studies are in agreement with Krigsvold et al (7) who observed significantly lower (P < 0.001) germination of C. crotalariae microsclerotia in rhizospheres of CBR-resistant 'Argentine' than for CBR-susceptible 'VA-72-R'.

Based upon surface-sterilization studies (Chapter II), the majority of observed infections were not restricted to root surface cortex tissues. A portion of the infections on the cortex surface resulted in colonization of the inner cortex, and in subsequent colonization of the stele for both Florigiant or Spancross (Chapter III).

It is probable that C. crotalariae infects and colonizes root tissue from the surface of the outer cortex, through the inner cortical layers and inward to the vascular tissues (Chapter III, 4, 6), following microsclerotial germination at peanut root tips (7), or other infection courts (Chapters II and III). Lateral colonization by C. crotalariae along the surfaces of the cortex tissue was not examined in these studies. This route of infection and colonization and the low estimates of efficiency of infection for necrosis from field (3) and greenhouse studies (Chapter II) suggest that CBR may be a debilitative, slow cortical root disease as originally proposed by Jackson and Bell (5).

As noted in Chapter II, differences in capacity factors (1) may be important in the colonization or the establishment of a successful, progressive infection [sensu Garrett, (2)] and subsequent

development of CBR symptoms on roots. Fungal colonists of root tissues may affect CBR pathogenesis, and subsequent disease severity. Greater numbers of cortex and stele segments of CBR-resistant Spancross were colonized by Fusarium spp. than those segments from CBR-susceptible Florigiant (Chapter III). Further research is necessary, however, to clarify the role(s) of these colonists at the cortex surface or within root tissues. Indeed, they may serve as antagonists to C. crotalariae.

The soil biocide, sodium azide (NaN_3), effectively reduced the initial microsclerotial density in soil (25 C) and the percent of root tissue infected and colonized by C. crotalariae, under greenhouse conditions, at rates of NaN_3 much lower than those used in previous studies for control of CBR in the field. Sodium azide, at doses sublethal to C. crotalariae, combined with microbial antagonists may be useful in the development of integrated CBR control strategies. However, inconsistent results regarding CBR development on Florigiant plants were obtained for several studies (Chapter IV) which utilized C. crotalariae-infested soils treated with sublethal doses of NaN_3 integrated with microbial antagonists. Possibly, these inconsistencies may have been due, in part, to the trace residual biocidal activity of NaN_3 to the antagonists, even though these dosages were sublethal to C. crotalariae. Phipps (8, 9) reported good control of CBR in field studies, by the

integration of planting the moderately CBR-resistant 'NC8C' with soils fumigated with sodium N-methyldithiocarbamate.

In summary, the techniques and approaches used in these studies have provided an improved understanding of the inoculum potential relationships of C. crotalariae infections on peanut roots. Knowledge of inoculum potential relationships may prove useful for the development of predictive analyses of CBR in the field. Knowledge of the route and degrees of infection and colonization of peanut roots from CBR-susceptible and CBR-resistant cultivars by C. crotalariae, following microsclerotial germination near infection courts, may provide information regarding the events involved in C. crotalariae pathogenesis and CBR resistance. Results from these studies, combined with the knowledge of effective approaches which reduce the inoculum potential of C. crotalariae, may be instrumental in the development of CBR control strategies.

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Appendix A. Average number of *Cylindrocladium crotalariae* infections per m root, average number of infections per plant and average root lengths of two cultivars of peanut, grown for 3 weeks in *C. crotalariae*-infested soil in greenhouse temperature tanks at 25 C

Cultivar	No. of Plants[a]	Average no. infections per m root	Average no. infections per plant	Average root length, m
Susceptible[b]	46	13.8 *[c] (2.8-38.7)[d]	129.5 * (23-354)	9.4 * (8.1-10.8)
Resistant[e]	26	7.8 (2.9-14.7)	41.5 (19-113)	5.7 (4.1-7.2)

[a] Combination of five of five trials for the susceptible cultivar and three of three trials for the resistant cultivar. The initial microsclerotial densities were 17.4, 12.6, 16.4, 9.4 and 10.8 microsclerotia per g soil for Florigiant, and were 9.4, 9.4 and 10.8 microsclerotia per g soil for Spancross.

[b] Cultivar Florigiant was used in these trials as the susceptible cultivar to Cylindrocladium black rot.

[c] Means followed by an asterisk (*) are not significantly different at P = 0.05 between cultivars, according to the Student's t-test.

[d] Numbers in parentheses represent range of observed values.

[e] Cultivar Spancross was used in these trials as the resistant variety to Cylindrocladium black rot.

Appendix B. Means, variance-to-mean ratios and percent differences of values for infection rates, I^{o}_{r}, and for infections per m root for eight combined trials and for six combined trials of CBR-susceptible cultivar Florigiant and CBR-resistant cultivar Spancross

	Eight trials		Six trials	
Variable	Susceptible	Resistant	Susceptible	Resistant
Number of Plants	46	26	36	16
Average micro-sclerotial density, (ms/g of soil)	13.3	9.9	14.3	10.1
Infection rate, I^{o}_{r}[a]				
Mean infection rate,	0.049	0.038	0.052	0.032
% difference between cultivars[b]	28.9		62.5	
V/m ratio[c]	0.0208	0.0068	0.0159	0.0042
% change[d]			30.8	61.9
Infections per m root				
Mean infections per m root	13.8	7.8	16.0	6.7
% difference between cultivars[b]	76.9		> 100	
V/m ratio[c]	5.89	1.39	4.92	0.88
% change[d]			19.7	60.0

[a] I^{o}_{r} = number of C. crotalariae infections per m root per day per microsclerotium per g soil.

[b] Percent difference between cultivars, relative to the resistant cultivar.

[c] V/m ratio = the ratio between the variance and the mean of the respective variable, I^{o}_{r} or the number of infections per m root.

[d] Percent change between the analyses for eight combined trials and for six combined trials, relative to the six combined trials for each respective cultivar.

Appendix C. Widths of cortex and stele tissues from cross-sections of two different taproot regions from CBR-resistant and CBR-susceptible peanut cultivars following 3 weeks growth at 25 C in _C. crotalariae_-infested peanut field soil

Distance from transition region[a], mm	Average tissue widths, μm for each cultivar[b]			
	Cortex		Stele	
	Susceptible	Resistant	Susceptible	Resistant
10	983.5 *[c]	749.1	1,934.7 *	1,039.8
60	412.9 *	612.3	759.5	683.3
Combined	1,396.4	1,361.4	2,694.2 *	1,723.1

[a] Transition region: junction of the hypocotyl tissue with root tissue.

[b] The CBR-susceptible cultivar used in these trials was 'Florigiant', and the CBR-resistant cultivar was 'Spancross'. The initial microsclerotial density was 9.4 microsclerotia per gram of soil.

[c] Means followed by an asterisk (*) are significantly different ($P = 0.05$), between cultivars for each tissue type according to Student's t-test.

Appendix D. Physical and chemical characteristics of a peanut field soil from southcentral Virginia used in all greenhouse studies

		Drake soil
	pH	5.5
	CaO[a]	601
	MgO[a]	90
	P_2O_5[a]	280+
	K_2O[a]	118
	NO_3-N[b]	19.5
	NH_4-N[b]	2.21
	% Organic matter	0.5
% Water content at:	- 0.10 bars	9.81
	- 0.33 bars	5.04
	-15.20 bars	1.39
% Sand:	Very coarse	0.10
	Coarse	0.70
	Medium	5.74
	Fine	50.15
	Very fine	24.27
	Total sand	80.97
	% Silt	14.80
	% Clay	4.23
Textural class		Loamy sand

[a]Expressed as kg/ha.

[b]Expressed as µg/g.

Appendix E. Average fresh weights of root tissues and average fresh heights of stem tissues of 8-wk-old Florigiant peanut plants grown in uninfested and *Cylindrocladium crotalariae*-infested peanut field soils following fumigation with different levels of sodium azide

NaN_3 treatment, µg NaN_3/g infested soil[a]	Number of Plants	Average Fresh Weight of Root Tissue, g[c]	Average Fresh height of Stem tissue, cm[b c]
0	44	0.72	19.8
2.5	45	0.72	16.6
7.5	30	0.69	17.0
10.0	34	0.76	18.5
C. crotalariae-free, 0	41	0.75	21.9

[a]Unless stated otherwise, initial density of *C. crotalariae*-infested soil was 9.8 microsclerotia per g soil.

[b]Measured from cotyledons to the top of the plant.

[c]No significant differences were detected at P = 0.05 level, according to Duncan's Multiple Range Test.

Appendix F. Root rot development and percent colonization of asymptomatic root segments by Cylindrocladium crotalariae from 8-wk-old Florigiant peanuts grown in C. crotalariae-infested soils following treatments of NaN_3 and/or amendments of Pseudomonas cepacia at 25 C.

Dosage, µg NaN_3/g C. crotalariae-infested soil[a]	Average Root Rot Rating[b]		% root segments colonized by C. crotalariae[c]	
	+PC	-PC	+PC	-PC
5.0	2.8*[d] (37)[e]	2.4 (38)	3.0	2.5
2.5	3.1* (35)	2.6 (28)	13.0	55.0
0.0	2.5* (33)	2.0 (30)	10.0	28.0

[a]Initial microsclerotial density of C. crotalariae: 9.7 microsclerotia per g soil. Where noted, soils were amended with 1.1×10^6 P. cepacia cells/g soil.

[b]Root rot rating scale: 0 = no visible root necrosis, 1 = 1 to 10% root necrosis, 2 = 11 to 25% root necrosis, 3 = 26 to 50% root necrosis, 4 = 51 to 75% root necrosis, and 5 = >75% root necrosis.

[c]Based on 10 2-cm segments of asymptomatic root tissues of each of 20 plants per treatment.

[d]Values followed by an asterisk (*) were significantly different (P = 0.05) in the presence of P. cepacia, using horizontal comparisons, within each sodium azide level.

Appendix G. Root rot development of 8-wk-old Florigiant plants grown in C. crotalariae-infested soils a5 25 C following soil treatments of sodium azide and/or amendments of Trichoderma harzianum

NaN_3 Dosage, µg NaN_3/g of C. crotalariae-infested soil[a]	Average Root Rot Severity Index[b]	
	+T. harzianum[c]	-T. harzianum
5.0	2.6 (48)[e]	d
2.5	2.9 (51)	d
0.0	2.4 (55)	2.5 (55)

[a]Initial C. crotalariae density: 9.4 microsclerotia per g soil

[b]Root rot rating scale: 0 = no visible root necrosis, 1 = 1 to 10% root necrosis, 2 = 11 to 25% root necrosis, 3 = 26 to 50% root necrosis, 4 = 51 to 75% root necrosis, and 5 = > 75% root necrosis.

[c]Soils amended with 4.8×10^4 T. harzianum conidia per g soil.

[d]No plants were grown for these treatments.

[e]Numbers in parentheses indicate the number of plants for each treatment. No significant differences were observed for root rot severity indices using single degree-of-freedom planned F-comparisons.

Appendix H. Root rot development of 20-wk-old Florigiant peanut plants grown in *C. crotalariae*-infested peanut field soil at 25 C following chemical treatments of sodium azide and/or fungal amendments of a mixture of *Trichoderma harzianum* isolates

NaN_3 Dosage, μg NaN_3/g of *C. crotalariae*-infested soil[a]	Average Root Rot Severity Index[b]	
	+*T. harzianum*[c]	-*T. harzianum*
5.0	3.4 (24)[d]	3.0 (27)
0.0	3.6 (26)	3.5 (29)

[a]Initial *C. crotalariae* density: 10.1 microsclerotia per g soil

[b]Root rot rating scale: 0 = no visible root necrosis, 1 = 1 to 10% root necrosis, 2 = 11 to 25% root necrosis, 3 = 26 to 50% root necrosis, 4 = 51 to 75% root necrosis, and 5 = > 75% root necrosis

[c]Where noted, soils were amended with 1.5×10^6 *T. harzianum* conidia per g soil

[d]Numbers in parentheses indicate the number of plants for each treatment. No significant differences were observed for root rot severity indices using single degree-of-freedom planned F-comparisons.

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