A COMPARATIVE STUDY OF VIRULENT AND AVIRULENT ISOLATES OF *CHALARA ELEGANS* ON ROOTS AND SHOOTS OF BEAN (*PHASEOLUS VULGARIS*)

by

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in the Department
of
Biological Sciences

SIMON FRASER UNIVERSITY
December 1997

APPROVAL

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Degree: MASTER OF SCIENCE

Title of Thesis:

A comparative study of virulent and avirulent isolates of *Chalara elegans* on roots and shoots of bean (*Phaseolus vulgaris*).

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A COMPARATIVE STUDY OF VIRULENT AND AVIRULENT ISOLATES OF CHALARA ELEGANS ON ROOTS AND SHOOTS OF BEAN (PHASEOLUS VULGARIS)

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ABSTRACT

Chalara elegans Nag Raj and Kendrick, also known by the name *Thielaviopsis basicola* (Berk. and Br.) Ferr., causes black root rot disease on many plants including bean (*Phaseolus vulgaris*). This thesis describes the infection process and host response using virulent (BK28) and avirulent (BK28R) isolates of *C. elegans* on root and shoot tissues of two cultivars of bean. A study of the relationship of inoculum level to lesion occurrence showed that 10 - 36 phialospores were required to initiate infection at point inoculation sites. Lesions appeared on both hypocotyls and primary roots inoculated with BK28 at 3 - 4 days after inoculation, and continued to enlarge after their initial appearance. The BK28 isolate penetrated directly, and infection hyphae were observed in epidermal cells of bean roots by 30 to 36 hours after inoculation and by 36 to 42 hours in hypocotyl tissues. Isolate BK28 produced secondary phialospores; germination and penetration by secondary phialospores were observed only on roots. The avirulent isolate BK28R rarely penetrated either hypocotyl or root tissues. Where penetration occurred, infection hyphae of BK28R were observed only in epidermal cells of hypocotyl tissues, and in both epidermal and cortical cells of roots. Sporulation by BK28R was not observed on either root or hypocotyl tissues, whereas BK28 sporulated at infection sites on both roots and hypocotyls. Appressorium-like enlargements were produced infrequently by the two isolates. The frequency of infection was not significantly different between bean cultivars, on either roots or hypocotyls. A greater proportion of

phialospores germinated on root compared to hypocotyl tissues of both cultivars. Isolate BK28 penetrated at a significantly higher frequency than BK28R, on both root and hypocotyl tissues. Both isolates caused browning of colonized cells at infection sites. BK28 on root and hypocotyl tissues, and BK28R on root tissues, appeared to be able to grow from necrotic cells into adjacent, apparently viable cells. Suberin was detected in the epidermis of hypocotyls but not in the root epidermis; lignin was not observed in epidermis of either hypocotyls or roots.

Dedication

To my wife Trini, Papa, Mama and Bapak
for their prayers

Acknowledgments

I would like to thank Dr. J. E. Rahe, my senior supervisor, for his guidance, ideas and support throughout my study. I would like also to extend my appreciation to Dr. M. M. Moore for her ideas and encouragement of my research and for reviewing and providing suggestions on the manuscript.

I would like to thank Dr. Z. K. Punja for providing the isolates of *Chalara elegans*. I am indebted to Dr. V. Bourne and Mr. M. Weiss for their help with the scanning electron microscopy, and to the Pacific Agri-Food Research Center (Summerland) for allowing me to use their scanning electron microscope.

I would like to thank Mr. Robert Balsaw for his help with the statistical analysis. Also, special thanks are due to all in the lab as well as my Indonesian friends at Simon Fraser University and at The University of British Columbia for their assistance and friendship.

Finally, I would like to acknowledge the Asian Development Bank (ADB) for funding my study through the Six Universities Development and Rehabilitation (SUDR) project, and the World University Service of Canada (WUSC) for its assistance, and particularly the Indonesian Programme staff at WUSC Office in Ottawa.

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GENERAL INTRODUCTION

Although plants are continuously in contact with a variety of potential pathogenic microorganisms, successful infections are rare (Davis, 1992), due to the specificity of relationships between host and pathogens (Graniti, 1976). According to Brian (1976), whether a microorganism becomes pathogenic or not is determined early in the process of infection. A number of stages occur during plant-parasite interactions. These stages can be "switching points", where an incorrect response by the pathogen can result in resistance. To successfully infect a pathogen must penetrate through the plant surface, an outer structural defense, using digesting enzymes and mechanical forces, and then overcome active plant defenses (Hadwiger, 1984).

1.1. Events of Fungal Pathogenesis

1.1.1. On the plant surface

1.1.1.1 Attachment

Following the dispersal of spores, they must arrive on a substrate to germinate and to produce a new mycelium. For saprotrophs, this process is not a crucial event if enough moisture and abundance of dead organic material are available. However, it is often necessary for spores to attach to a substrate prior to germination (Wessels, 1997). To attach to a surface, it is generally thought that a specific interaction occurs between the spores and the host surface (Clement *et al.*, 1994). The surface of plants can be extremely

hydrophobic, depending on its chemical composition and topography (Holloway, 1971). Active adhesion of spores can play a role. For example, upon hydration, spores of the rice blast fungus, *Magnaporte grisea*, produce a material from the site of future germ-tube formation that can tightly adhere to hydrophobic surfaces (Hamer et al, 1988). The adhesive materials released by fungi are called hydrophobins and are hydrophobic proteins containing eight cysteine residues (Wessels, 1996).

The germ tube or the appressorium (a swelling on germ tube or hypha) must adhere tightly to the hydrophobic surface to counteract the force generated by the infection peg, which arises therefrom and penetrates the epidermis (Wessels, 1997). According to Nicholson and Kunoh, (1995), germlings of most pathogenic fungal species adhere best to hydrophobic surfaces, some to hydrophilic surfaces, and only a few species show adherence to both kinds of surfaces.

1.1.1.2. Germination

Spore germination is the first and most crucial event in the life cycle of most fungi. This stage involves a transition from dormancy to active growth and is accompanied by profound structural and biochemical changes. The mobilization of stored carbon sources like lipids, carbohydrates, or proteins is included in the process (Kolattukudy and Koller, 1983). The requirement for additional exogenous nutrients is important in the germination process for most fungi. Shepherd (1994) stated that exogenous materials, primarily amino acids and sugars, present in plant exudates, and the amount and constitution of the exudates depend on the plant species, variety, the age and the part of plant (Parry, 1990). In general, different pathogens respond differently to

these resources; for example, *Macrophomina phaseolina*, *Phytophthora* spp. and *Verticillium dahliae* respond to amino acids, but not to sugars, while propagules of *Fusarium* spp. and *Pythium* spp. respond to amino acids and sugars (Shepherd, 1994; Nelson, 1990).

In addition, factors that are present in the rhizosphere affect the germination and development of pathogenic fungi. For instance, the germination of chlamydospores of *Phytophthora cinnamomi* in soil is enhanced by the addition of antibiotics (vancomycin and nystatin) (Mircetich and Zentmyer, 1970). This suggests that fungi that are able to tolerate toxic compounds are most likely to outgrow others.

Germination is necessarily accompanied by the biosynthesis of nucleic acids, proteins, membrane system and the cell wall, and if fungal spores germinate in an unfavorable environment, they are unlikely to survive. Therefore, it is crucial for spores to remain dormant until a host plant is available (Kolattukudy and Koller, 1983).

1.1.2. Inside the host tissues

When spores have successfully dispersed and grown on the surface of a host, entry or penetration into the viable tissues becomes the next stage for continuing the life cycle. The initial site of contact between a pathogen and the surface of the host is described as the infection court (Dickinson and Lucas, 1982).

1.1.2.1. Entry via natural openings and wounds

Many natural openings that are relatively unprotected can facilitate entry of fungal pathogens. Stomata, lenticels, leaf traces, and cracks where secondary roots emerge

through the cortex are available for penetration. Among these natural openings, stomata are the main portal of entrance for many foliar pathogens.

According to Tainter and Baker (1996), fungi enter stomata in two ways: (1) growth of a germ tube or vegetative hypha between the guard cells and into the substomatal chamber without forming an appressorium, and (2) formation of an appressorium over a stoma and production of a penetration peg that enters between the guard cells. Orientation of the hypha across the long axis of the epidermal cells provides a better chance to come across a stoma.

Many pathogens can only gain entry to their hosts through wounds. Damage may result from surface breakage, environmental influences, animal, and insect activities, or by natural processes of plant growth, for instance, leaf scars or sites of lateral root emergence (Isaac, 1992). Wounds created as a result of grafting, pruning, farming and harvesting methods may also lead to plant infections.

1.1.2.2. Penetration of intact tissue surfaces

To penetrate directly, fungi must break through an external layer of wax, cutin, pectin and a network of cellulose fibrils to reach the living protoplasm. Fungi have evolved diverse invasion strategies to overcome these barriers. For instance, direct penetration of hosts by fungi is associated with the development of hyphal modifications known collectively as infection structures (Dickinson and Lucas, 1982). The morphogenetic events leading to formation of the infection structure often depend on specific signals provided by the plant surface (Mendgen *at al.*, 1996).

1.1.2.3. Colonization

After pathogens have obtained entry into hosts, the next stage is to advance into the plant tissues and to establish a parasitic relationship. Pathogens have unique mechanisms which enable them to colonize the host; for example, the objective of a biotroph or an obligate pathogen is to obtain nutrients from the host without killing it. Therefore, colonization by biotrophs must cause minimal disruption to the host plants. On the other hand, colonization by necrotroph pathogens may not be affected by host tissue disturbance (Parry, 1990; Isaac, 1992).

According to Wheeler (1975) and Agrios (1993) non-obligate pathogens establish colonization by employing enzymes, growth regulators and toxins. However, this is not the case for obligate parasites because they do not grow on the dead cells (Oku, 1994). Isaac (1992) stated that the outcome of pathogen invasion is the result of interactions that are dependent on the properties of both pathogens and hosts and are mediated by a range of factors. Host resistance can influence the spread of the pathogen, and lead to minimal disease outcome. Also, the characteristics of the growth habit and life cycle of individual pathogens can influence the invasion of their hosts. For example, powdery mildew pathogens (*Erysiphe pisi* and *E. graminis*) grow only on the outer surface of plants and only penetrate epidermis cells.

1.2. Mechanisms of Host Defense to Pathogens

During the co-existence between plants and fungi, a highly complex and intricate relationship has evolved. In general, plants tend to reject fungi. Pathogenic fungi have

evolved unique systems to attack plants; however, plants in turn have evolved the ability to defend themselves. Therefore, the vast majority of fungi are saprophytes and no more than 10 % of the ~ 100,000 known fungal species are able to colonize plants, and not all of them are capable of causing diseases (Knogge, 1996; Misaghi, 1982). According to Oku (1994) the resistance of plants against microbial attack seems to be the rejection reaction. Hammond-Kosack and Jones (1996) proposed that reasons for pathogen failure are (1) plants are unable to support the nutrient requirements of a potential pathogen, (2) plants have preformed structural barriers or toxic compounds that restrict successful infection to only certain pathogen species, and (3) defense mechanisms are triggered by plants upon recognition of contact by a fungus.

1.2.1. Nutrients as factors affecting pathogenesis

1.2.1.1. Prepenetration and penetration phases

Host nutrients play an important role in the initial contact with pathogens (Hancock and Huisman, 1981). Exudation of carbon assimilates onto plant surfaces is considered as a major factor in determining the outcome of infection of many soil- and air-borne pathogens (Blakeman, 1971; Schroth and Hildebrand, 1964). There is circumstantial evidence that nutrients on plant surfaces are exudated largely from the apoplast (Hancock and Huisman, 1981). As an example, germ tubes and appressoria formed by *Botrytis squamosa* and *Colletotrichum lindemuthianum* orient toward the anticlinal cell walls of leaves (Clark and Lorbeer, 1976; Mercer *et al.*, 1971).

Host exudates that contain nutrients such as sugars or amino acids are important not only to stimulate germination but also to set the stage for subsequent infection

processes. Several studies indicate that the composition of substances in exudates controls morphogenic and physiological activities during penetration. For instance, infection cushions are formed by many root-infecting fungi in response to exudates from non-cutinized surfaces of the hypocotyl and root (Tainter and Baker, 1996).

1.2.1.2. Post penetration phase

After infection has been established, plant pathogens grow and multiply on and in host plants by consuming components of host plant nutrients. The most significant movement of nutrients from host to pathogen occurs during the post penetration phase, and the demand for host nutrients is particularly great during reproduction. Parasites may absorb nutrients directly from the apoplast region or stimulate the diversion of host nutrients from a distance to sites of infection. When the nutrients are suitable, the pathogen may develop and the disease outbreak may be severe. On the other hand, unsuitable nutrients may cause the resistance of plants to pathogens. The ability of plant pathogens to absorb photoassimilates and other nutrients from their hosts determines the success of infection (Hancock and Huisman, 1981; Oku, 1994; Spencer-Phillips, 1997).

1.2.2. Preformed structural and chemical host resistance

Preformed resistance to plant pathogens occur normally in a healthy plant and are not produced as a result of stimulus from invading pathogens. The mechanisms of preformed or passive resistance can be structural or chemical. The initial contact between fungi and plants most usually occurs at the cuticle, which overlies the epidermal cell walls, and it may function as a barrier (Isaac, 1992). The cuticle is composed of an insoluble polymer cutin, embedded in a complex mixture of hydrophobic materials

collectively called wax (Kolattukudy and Koller, 1983). For pathogenic fungi that enter the plant by direct penetration, the physical properties of the plant surface such as the hardness and thickness of cuticle affect penetration (Oku, 1994). The cuticle provides a significant barrier to penetration by *Botrytis cinerea* (Elad and Evensen, 1995).

Also, the intact plant cell wall is an effective preformed structural barrier against pathogen attack. With very small pores, cell walls block access by pathogens to the protoplast. Penetration therefore requires enzymes or mechanical forces for breaking the walls (Brett and Waldron, 1990). Plants whose cell walls are resistant to enzymatic degradation may minimize or prevent infection, as for *B. cinerea* (Elad and Evenson, 1995).

Plants may contain substances that inhibit some pathogens. The distribution of preformed inhibitors within plants is often tissue specific and there is a tendency for these compounds to be concentrated in the outer cell layers of plant organs. Catechol and protocatechuic acid (which are found in onion scales), saponins (which are found in many plants, including tomato), cyanogenic glucosides (which are found in many plants, including lima bean) are some examples of preformed antimicrobial compounds that act as components of plant defense systems (Osbourn, 1996; Strange, 1993).

1.2.3. Induced resistance.

Induced resistances or active resistance mechanisms operate specially in response to pathogen invasion and are most commonly associated with major gene resistance. This resistances shows high protection to specific pathogens by specific hosts with associated changes in gene expression (Ryals *et al.*, 1996). The genes encoding the

induced defenses have been cloned and study extensively (Uknes *et al.*, 1992). The induced resistance involves structural and chemical responses. Some of these defenses may involve the production of suberin, lignin and a hypersensitive reaction that may slow the spread of the pathogen in tissues.

1.2.3.1. Suberin formation

Compared to lignification, suberization has been less studied and most interest has focused on suberization due to wounding (Aist, 1983). Little is known about the chemical composition of suberized layers. Kolattukudy and Koller (1983) have proposed a model for the structure and formation of suberin. Suberin is composed primarily of long chain (C-20 to C-30) fatty acids, alcohols and hydroxy fatty acids or dicarboxylic acids deposited between the plasma membrane and cell walls, and the deposition of suberin typically occurs in layers with waxes. Aromatic components are proposed as precursors of suberin (Kolattukudy, 1984). Suberin is a constituent of healthy plant tissue, but its synthesis may be enhanced by challenge with microorganisms (Strange, 1993).

According to Kolattukudy (1984), suberin functions (1) as a barrier to moisture diffusion, (2) as a barrier to solute movement, (3) as a stress response, and (4) as a barrier to microbial attack. Resistance to diffusion of water is correlated with deposition of an aromatic matrix of suberin and waxes. Especially in the roots, suberin forms a barrier to solute movement in the plant. Suberin in the endodermis may keep the apoplastic transport inside the vascular system (Peterson *et al.*, 1981; Robards *et al.*, 1979).

When a plant is under stress by wounding or nutrient deficiency, it increases the deposition of suberin (Kolattukudy and Dean, 1974; Pozuelo *et al.*, 1984). Cell walls that contain suberin show a significant barrier to penetration by pathogenic organisms, and there are relatively few pathogens that can invade tissues protected by suberized cell layers (Kolattukudy and Koller, 1983). Biggs (1989) showed that the resistance of peach to the canker-inducing fungus *Leucostoma persoonii* correlates with suberin accumulation.

1.2.3.2. Lignification

Lignification is the covalent binding and polymerization of phenylpropanoids, predominantly sinapyl, coniferyl and p-coumaryl alcohols, occurring in the cell wall or related matrices. Other phenylpropanoids, like p-coumaric, ferulic and p-hydroxybenzoic acids, are usually esterified to the polymer (Aist, 1983). These are polymerized in a free-radical process involving H_2O_2 and peroxidase to form a complex structure (Strange, 1993).

Two kinds of lignin exist in the plant host, healthy lignin with empirical formula $C_9H_{10.30}O_{2.20}(OCH_3)_{1.16}$ and disease lignin with empirical formula $C_9H_{8.33}O_{2.80}(OCH_3)_{0.75}$ Healthy lignin is composed of syringylpropane units and disease lignin primarily consists of guaiacylpropane units. Wound lignin is the same or similar to disease lignin (Asada *et al.*, 1979). Lignin is formed naturally or in response to pathogens and it is derived via the shikimic acid pathway with phenylalanine and tyrosine as intermediates (Bird, 1988).

The roles of lignin in resistance are increasing the mechanical force required for penetration, increasing the resistance of cell walls to enzyme degradation and creating an

impermeable barrier to the flow of nutrients and toxins. Also, lignin precursors and the free radicals could be directly toxic to the invading microorganisms and may lignify the fungal hyphae (Ride, 1978).

1.2.3.3. Hypersensitive reaction

Many studies regarding the hypersensitive response have been done not only because it is a visible example of the dynamic role of the host in the early stage of pathogen attack, but also because it confers a high degree of resistance to pathogen invasion (Dickinson and Lucas, 1982). The hypersensitive response is a rapid collapse of tissue that is genetically programmed in the plant as output of new host transcription and translation (Dixon *et al.*, 1994; Dangl *et al.*, 1996). The hypersensitive response is a very common reaction in plants challenged with avirulent pathogens (Strange, 1993). A variety of defense reactions such as phytoalexin accumulation, lignification, production of hydrolytic enzymes such as chitinases and glucanases are associated with the hypersensitive response. However, it is still unclear whether the hypersensitive response causes disease resistance by blocking nutrient access to pathogens or by releasing anti-microbial compounds from dying host cells (Dangl *et al.*, 1996).

1.3. Bean (*Phaseolus vulgaris*) and *Chalara elegans* as a Model for Study of Plant Pathogen Interaction

1.3.1. *Phaseolus vulgaris*

P. vulgaris is an important edible legume that is consumed in many different forms. Consumption of dry bean is most frequent but other forms also have importance. In Latin America and Africa, a green phase of seeds can be harvested and consumed fresh. In some parts of the world, mostly Africa, bean leaves are consumed as a vegetable. Also, the immature green pods while still fiberless, called snap bean, French bean, or green bean, are consumed by people in North America, Latin America, Europe, Africa and Asia. Dry beans mainly supply protein and calories, while snap beans supply low protein and calories with more vitamins and minerals. Among vegetable bean products, snap or green beans are the most significant form used, due to their wide geographical distribution, relatively large production, potential as an income source, and their role as a source of vitamins and minerals (Silbernagel *et al.*, 1991).

1.3.1.1. Morphological aspects

The main stem of the bean plant derives from the axis of the seed embryo. It forms a succession of nodes and is easily distinguished from the lateral axes, because of its large diameter and direct link with the root below. In addition, the presence of the cotyledons distinguishes the stem from lateral axes (Debouck, 1991).

On mature plants, the main stem starts at the insertion of the root system, and epicotyl and hypocotyl are still visible. The lateral axes are inserted at nodes, in the axils of the leaves. The appearance of flowers depends on the kind of cultivar; the first flower-

producing buds occur at rank 3 on axillary buds (Debouck, 1991). A sketch of the bean plant is shown in Fig. 1.

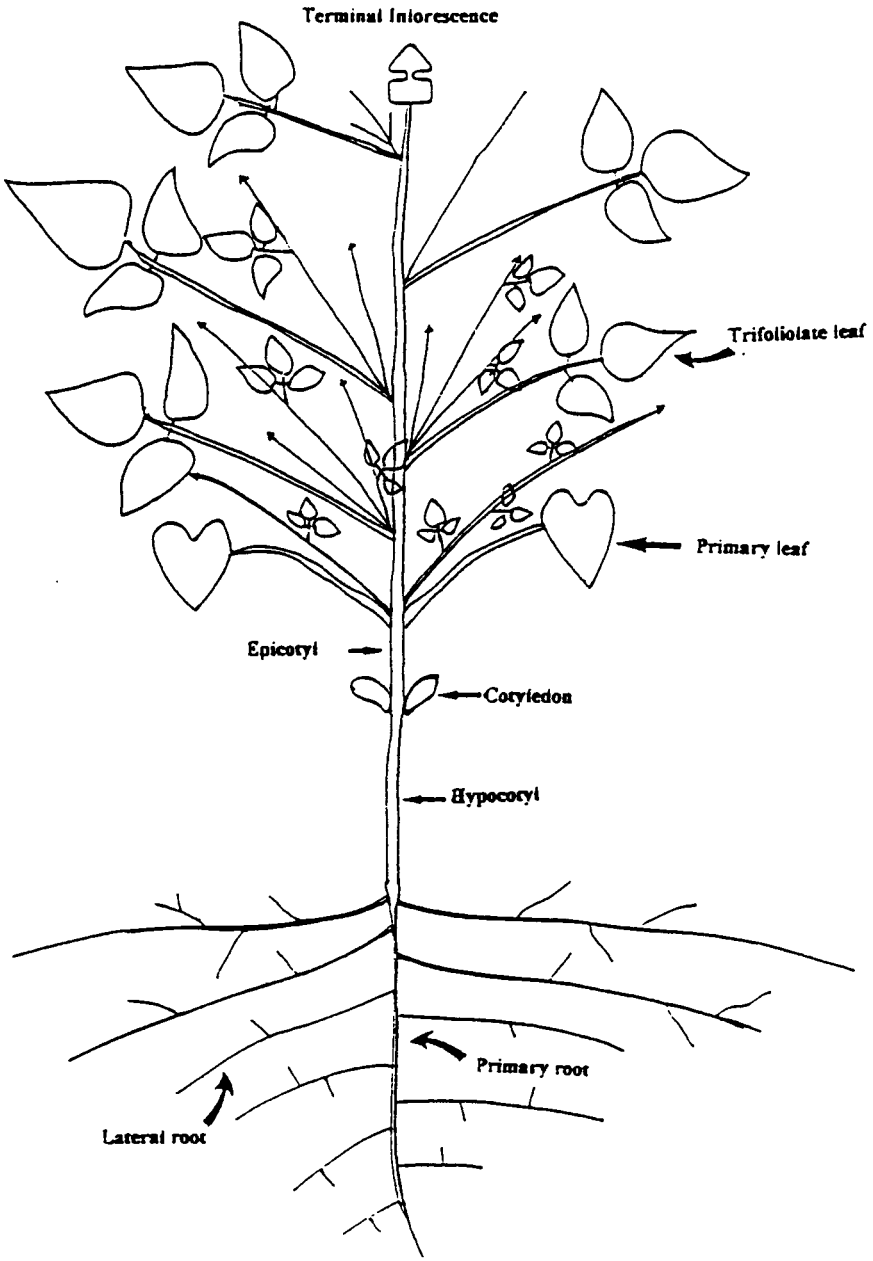


Fig. 1. A sketch of determinate type of bean plant (*Phaseolus vulgaris*) (Debouck, 1991 with modification).

1.3.1.2. Bean cultivars

Bean cultivars can be classified according to many criteria (Voysest and Dessert, 1991). Classification can be based on the stage of plant maturity when beans are consumed (Purseglove, 1968), or on seed characteristics such as the color, brilliance, shape and size of the seeds. Classification by growth habit is also used to describe bean cultivars. Determinate and indeterminate are two types of growth habits. Determinate cultivar develop a terminal inflorescence, whereas indeterminate cultivars do not, and thus continue to grow indefinitely (Voysest and Dessert, 1991).

1.3.2. *Chalara elegans*

1.3.2.1. Classification and identification

Chalara elegans Nag Raj & Kendrick [(*Thielaviopsis basicola* (Berk. & Br.) Ferr.)], according to Nag Raj and Kendrick (1975), belongs to

Kingdom : Fungi

Division : Eumycota

Subdivision : Deuteromycotina (Fungi Imperfecti)

Class : Moniliales

Genus : *Chalara*

Species : *Chalara elegans*

C. elegans produces two kinds of spores, phialospores and chlamydospores. The two kinds of spores can be observed on many agar media. The name *C. elegans* that is used by Nag Raj and Kendrick (1975) is based on the phialidic spore of the fungus. Phialospores are produced in 24 hr on Difco® Potato Dextrose Agar (PDA) or on 5 - 15

% carrot juice agar. Phialospores vary in size, 8 - 17 µm x 3 - 5 µm, with slightly rounded ends (Shew and Meyer, 1992). The production of chlamydospores occurs within 4 days at room temperature (22 - 28 ^{0} C) in the dark. Chlamydospores are produced as a linear chain of 5 - 7 cells that separate at maturity, such that the individual cells are able to germinate. The size range of individual chains is 6.5 - 14 x 9 - 13 µm (Nag Raj and Kendrick, 1975; Shew and Meyer, 1992).

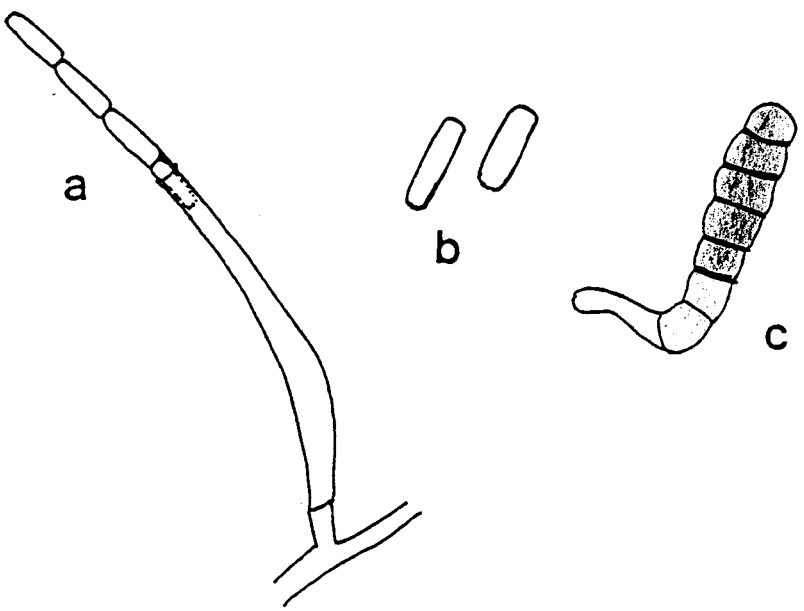


Fig. 2. Conidia of *Chalara elegans*. Phialospores (endoconidia) emerging from conidiophore (a), ungerminated phialospores (b) and chain of chlamydospores (c). The two types of conidia are produced in the same medium.

1.3.2.2. Host range and distribution

C. elegans is a soil-borne pathogen with saprophytic ability that infects a wide range of cultivated and uncultivated hosts (Yarwood, 1981; Gayed, 1972). The fungus has been found in at least 46 countries (Shew and Meyer, 1992). Important agricultural hosts are cotton, bean, carrots, pansies, peanuts and tobacco (Hood and Shew, 1996a). On cotton, *C. elegans* was very damaging in the early stage of seedling development (Johnson and Doyle, 1986). On bean, *C. elegans* can be a potentially serious and destructive pathogen, when a favorable environmental condition is available, such as temperature between 25 - 28 ^{0} C and high soil moisture (Christou, 1962). For example, in southwestern Ontario, *C. elegans* is an important pathogen on tobacco and poses a threat to new crops of legumes, including beans (Reddy and Patrick, 1989).

1.3.3.3. Inoculum production and pathogenicity test

Several selective media that have been described for isolation of *C. elegans*, including rose bengal agars (RB-0, RB-M1, and RB-M2) (Tsao, 1964; Tsao and Canetta, 1964), V8 juice dextrose-yeast agar (VDYA) (Papavizas and Davey, 1961). VDYA-pentachloronitrobenzene (VDYA-PCNB) (Papavizas, 1964), *T. basicola* medium-carrot (TBM-C) and *T. basicola*-V8 (TBM-V8) (Maduewesi *et al.*, 1976), *T. Basicola*-carrot-etridiazolnystatin (TB-CEN) (Specht and Griffin, 1985), TB-CEN-pentachloronitrobenzene (TB-CENP) medium (Holtz and Weinhold, 1994), and semiselective medium (TB-2RBA) (Chittaranjan and Punja, 1994). All of these media have been used to detect and estimate the inoculum density of *C. elegans* in soils. For producing a large amount of inoculum, *C. elegans* can be cultured on V8 juice agar,

carrot juice agar or potato dextrose agar. Phialospores are produced abundantly on V8 juice agar (Shew and Meyer, 1992; Chittaranjan and Punja, 1994).

Pathogenicity tests and cultivar evaluations are done mostly with phialospores of *C. elegans*, since the phialospores are produced in abundance, easily recovered from agar media and they germinate quickly, 7 - 10 hr after inoculation on tobacco root (Tahiri-Alaoui *et al.*, 1993), and invade tissues by 24 - 30 hr after inoculation (Chittaranjan and Punja, 1994).

1.3.3. Advantages of the model

Bean and *C. elegans* are a good model for study of host pathogen interactions because (1) beans can be easily grown in large numbers under controlled conditions, are grown worldwide, have tissues that are easily observed under light microscope, and respond to pathogens with elucidated defense mechanisms, such as hypersensitive responses, phytoalexin production and other responses (Pierre and Wilkinson, 1969; Pierre, 1971; Rahe, 1973), and (2) *C. elegans* is easily cultured and attacks both root and hypocotyl tissues of bean. Therefore, comparative studies of host-pathogen interactions can be conducted on both root and shoot.

1.4. Objectives of the Research

The objectives of the research were (1) to compare the histology of infection by a virulent and an avirulent isolate of *C. elegans* on beans, (2) to compare the infection process and host responses on root and shoot tissues, and (3) to discover the histological basis for inability of the avirulent isolate to infect beans.

The isolates of *C. elegans* that were used differed in regard to dsRNA. Isolate BK28 was isolated from cotton and contained dsRNA; isolate BK28R was obtained as a sector from a colony of BK28 and contained no dsRNA (Bottacin *et al.*, 1994).

Spore germination, appressorium formation, penetration and colonization by the virulent and avirulent isolates of *C. elegans* were examined by microscopy, and early formation of germ tubes of the two isolates was examined under scanning electron microscope. Response of beans in terms of cell browning and hypersensitive reaction was examined, as well as the production of suberin and lignin. Two cultivars of bean, Tendergreen and Kentucky Wonder were chosen on the basis of a preliminary experiment in which Tendergreen appeared the less susceptible than Kentucky Wonder. Primary roots and hypocotyl of seedlings grown under soilless laboratory condition were used in this experiments.

Chapter II

EFFECT OF INOCULUM LEVELS OF *CHALARA ELEGANS* ON THE OCCURRENCE OF SYMPTOMS AT POINT INOCULATION SITES ON CULTIVARS OF BEAN (*PHASEOLUS VULGARIS*)

2.1. Introduction

Chalara elegans causes extensive root rot and suppression of yield on burley tobacco (Shew and Shoemaker, 1989). Resistant cultivars are the primary method for controlling the disease (Wilkinson, 1991), because fumigation had little effect on the pathogen or subsequent incidence of the disease (Shew and Shoemaker, 1993). Reddy and Patrick (1989) stated that *C. elegans* was a serious potential threat to some of the new crops being proposed as alternatives to tobacco in southern Ontario, including bean (*Phaseolus vulgaris*). *C. elegans* produces hyaline, thin-walled phialospores (endoconidia) and large, pigmented, thick-walled chlamydospores which serve as propagules for long term survival in the soil (Tsao and Bricker, 1966). Phialospores of *C. elegans* are used as inoculum in pathogenicity studies and cultivar evaluations. They are produced abundantly in axenic culture, and are easily recovered from culture media (Chittaranjan and Punja, 1994). They germinate rapidly and are able to create infections as soon as 12 hours after inoculation on cotton (Mauk and Hine, 1988), and within 24 hours on wounded carrot tissues (Punja, *et al.*, 1992).

Study of the resistance of plant hosts and the potential of pathogens to initiate infection often requires information about the relationship of inoculum concentration to disease incidence. Such information can also be used to develop screening tests for

detecting resistance in progenies from plant breeding programs. The objective of this study was to determine the effect of phialospore concentration on the frequency of infection on root and shoot tissues of two cultivars of bean (*P. vulgaris*) which had shown different levels of susceptibility in a preliminary experiment, and to compare these relationships on light- and dark-grown plants. Potential applications of this information include quantification of possible differences in host susceptibility related to cultivar, tissue or environment, and to serve as a basis for selecting phialospore concentrations to be used in subsequent research.

2.2. Materials and Methods

The two cultivars of beans used in this study, Tendergreen (TG) and Kentucky Wonder (KW), were obtained from Willhite Seed Co., Poolville, Texas. In a preliminary study, TG appeared to be less susceptible than KW to *C. elegans*. Isolate BK28 of *C. elegans* was grown on V8 juice agar and stored at 10^0 C. Originally isolated from cotton (Bottacin *et al.*, 1994), this strain was kindly provided by Z.K. Punja (Simon Fraser University).

Seeds were surface sterilized in 1 % solution of sodium hypochlorite for 15 minutes, rinsed with deionized water and placed between two sheets of moistened seed germination paper (Anchor Paper, St. Paul. MN) laying on top of a sheet of waxed paper. The papers and seeds were then rolled into a cylinder ('rag doll'), which was placed upright in an incubator at 28^0 C. After 5 days, germinating seeds of uniform size were transferred to clean rag dolls, and placed in a growth room with 12 hours of light (4 GE

Power Groove Cool White bulbs) and temperature 23 - 26^0 C. The seedlings were kept moist by addition of water, which ascended the seed germination paper from the bottom by capillary action.

To prepare inoculum, sterile water was added to 2-week old fungal cultures of isolate BK28 (virulent isolate) growing on V8 juice agar. The culture surfaces were rubbed gently with a glass rod to suspend the phialospores, and the suspension was filtered through glasswool to separate the phialospores from mycelium and chlamydospores.

Spore numbers in suspensions of phialospores in sterile water were estimated using a haemocytometer and the suspensions were then diluted to the required concentrations. Suspensions containing 1 x 10^6, 2 x 10^5, 4 x 10^4 and 8 x 10^3 phialospores/ml and sterile water (control) were applied as 5 µl droplets, using a micropipet, onto the surface of primary roots and hypocotyls of 1 week old bean seedlings. Each individual plant received all of the spore concentrations and water control as droplets spaced 2 cm apart in random order on hypocotyls, and 1 - 1.5 cm apart on primary roots. The experiment utilized three replications, each containing 10 individual seedlings receiving all treatments randomly.

After inoculation, the seedlings were kept uncovered on the moist rag doll laying on the tray (Fig. 3) in a dark moist chamber for about 12 h, then transferred to a lab bench until the inoculum droplets had almost dried and then rolled again into rag dolls that were placed in either a dark room (dark-grown plants) or a growth room (light-grown plants), both at room temperature 23 - 26^0 C. The rag dolls were kept moist with

deionized water as required. Observations were done at day 5, 10 and 15 after inoculation by scoring each inoculation site as + (positive) or - (negative) with regard to visible symptoms. The data percentage of symptomatic sites were transformed to $\sin^{-1}\sqrt{y}$ for analysis of variance using generalized linear model procedure on SAS package (SAS Institute Inc., 1996) with factorial analysis. For simple linear regression analysis, concentrations were log transformed. Data for the sites that had received water droplets as control were excluded from the analyses since no symptoms were observed at any of these sites.

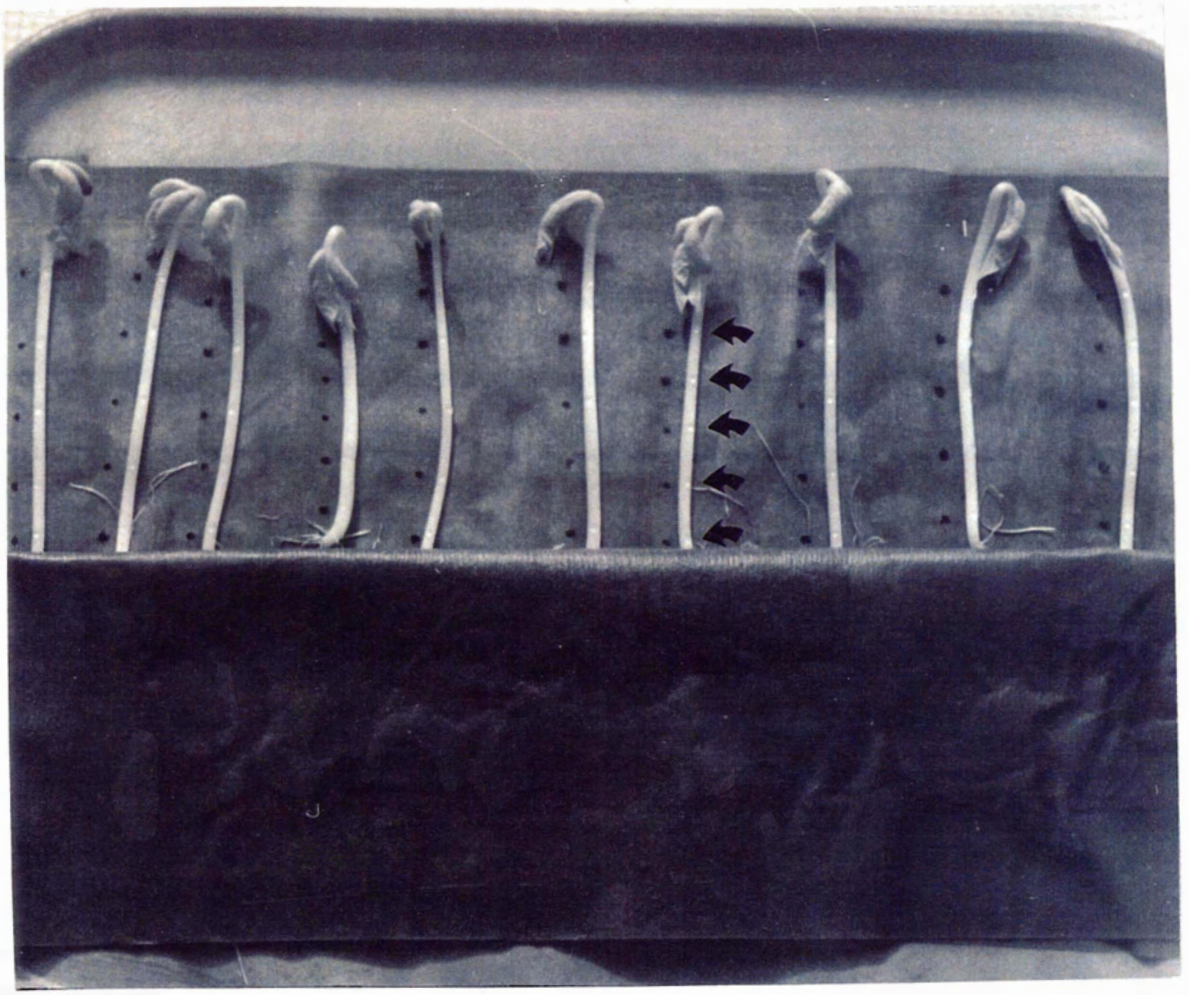


Fig. 3. Experimental setup used for point inoculation. Each bean seedling received all concentrations of phialospores in random order as 5 μL droplets applied at locations indicated by arrows. Roots (not shown) were treated similarly.

2.3. Results

Necrotic lesions first began to appear at point inoculation sites on hypocotyls and roots by 3 to 4 days after inoculation. The lesions were initially brown and become progressively darker due in part to pigmented reaction products in host tissues. Lesions on hypocotyls increased in size for a few days after their initial appearance, and ranged from 3.4 - 15.6 mm in length and 0.2 - 2.1 mm in width by day 10 after inoculation.

The percentage of symptomatic inoculated sites on hypocotyls was significantly affected by the number of phialospores in the inoculum drop ($P = 0.0001$), and by cultivar ($P = 0.03$). Similar relationships occurred at point inoculation sites on roots. Overall, light-grown and dark-grown plants did not differ in susceptibility, but a significant interaction between cultivar and light treatment was observed ($P = 0.04$). Lesions on the hypocotyls of light-grown plants were more sharply demarcated from adjacent healthy tissue than on dark-grown plants (Fig. 4); lesions on the two cultivars are shown in Fig. 5.

Table 1 shows the mean percentage of point inoculation sites with symptoms at 5, 10 and 15 days after inoculation for both cultivars, in relation to the numbers of spores applied. The difference in susceptibility of the two cultivars is documented by the data presented in Table 2. The number of symptomatic inoculation sites was 12% - 15 %

greater on hypocotyls of Kentucky Wonder than on Tendergreen. Mean percentages (both cultivars) of symptomatic inoculation sites on root and hyopocotyl tissues are presented in Table 3. These data show that the number of symptomatic sites was significantly greater on roots than on hypocotyls at 5000 and 1000 phialospores per inoculation site.

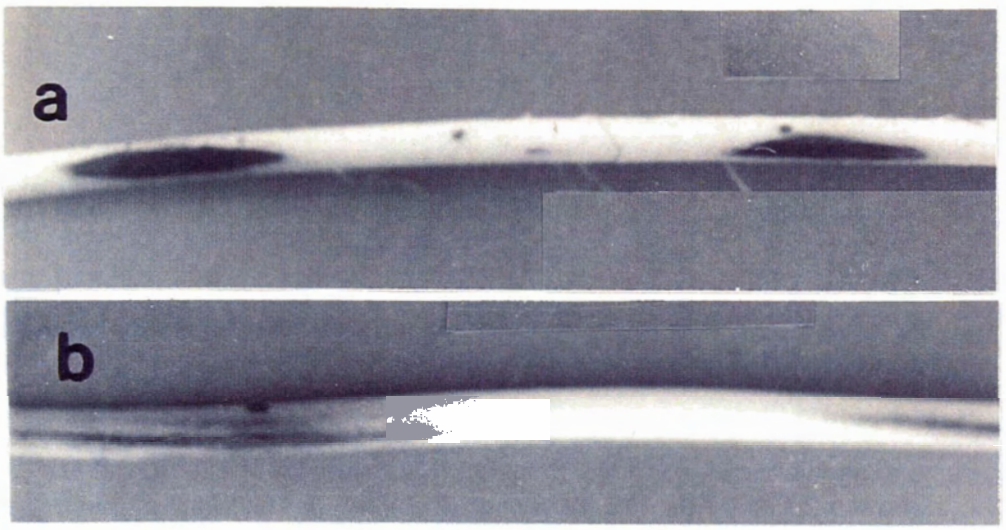


Fig. 4. Typical appearance of lesions on hypocotyls (cv. TG) of light-grown (a) and dark-grown (b) bean seedlings at day 15 after point inoculation with droplets that contained different numbers of phialospores of *Chalara elegans*.

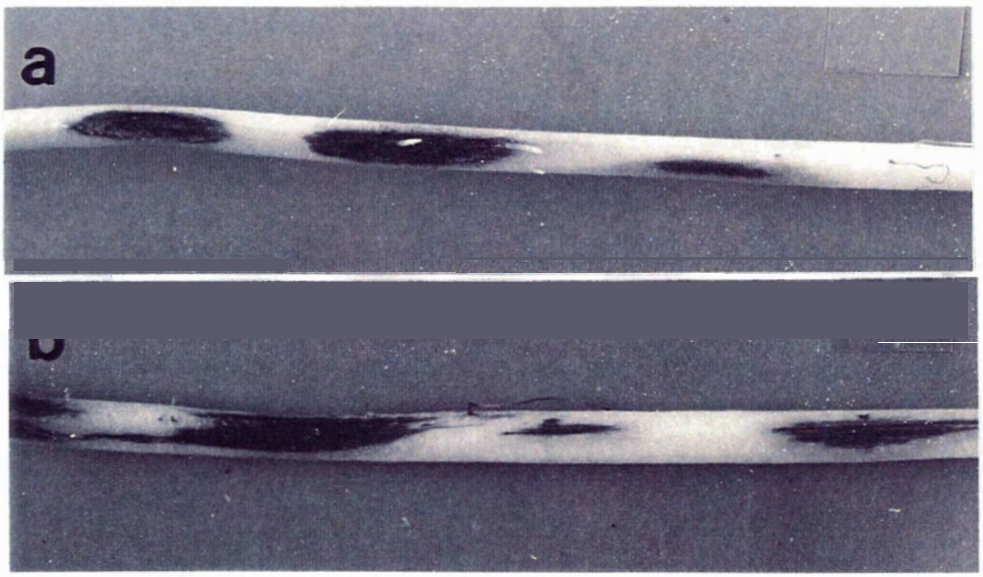


Fig. 5. Lesions on etiolated hypocotyls of cv TG (a) and KW (b) of light-grown bean seedlings at 15 days after point inoculation with droplets containing different number of phialospores of *Chalara elegans*.

Table 1. Relationship between the number of phialospores of *Chalara elegans* and the occurrence of lesion (%) [1] at point inoculation sites on hypocotyls of *Phaseolus vulgaris*

Day post inoculation	Number of phialospores / site			
	5×10^3	1×10^3	2×10^2	4×10
5	55.00 a	35.83 ab	28.33 b	8.33 c
10	65.00 a	43.33 b	33.33 b	9.17 c
15	70.00 a	46.67 b	33.33 b	9.17 c

Value in a row, followed by the same letter/s are not significantly different from each other ($P \leq 0.05$) according to Bonferroni's test.

[1] Means are average of non-transformed percentages of symptomatic sites of three replications, each replication consists of 10 observations; pooled data for cv TG and KW in light and dark-grown (n = 12)

Table 2. Percentage [1] of symptomatic point inoculation sites on hypocotyls of two cultivars of *Phaseolus vulgaris* at 5, 10 and 15 days after inoculation with *Chalara elegans*, isolate BK28.

Day post Inoculation	Cultivar	
	Tendergreen	Kentucky Wonder
5	24.58 a	39.16 b
10	30.83 a	44.58 b
15	33.75 a	45.83 b

Value in a row followed by the same letters are not significantly different ($P \leq 0.05$) according to Bonferroni's test.

[1] Means are average non-transformed percentages of symptomatic point inoculation sites of three replications, each replication consists of 10 observation; pooled data of light- and dark-grown beans, at all concentrations of inoculum (water controls excluded) (n=24)

Table 3. Percentage [1] of symptomatic point inoculation sites on roots and hypocotyls of *Phaseolus vulgaris* inoculated with *Chalara elegans*, isolate BK28

Number of Phialospores per Sites	Bean Part	
	Primary Root	Hypocotyl
4×10^1	5.00 a	10.00 a
2×10^2	36.67 a	40.00 a
1×10^3	71.67 a	46.67 b
5×10^3	90.00 a	68.33 b

Value in a row followed by the same letter is not significantly different ($P \leq 0.05$) according to Bonferroni's test.

[1] Means are averages of three replications (each replication consists of 10 observations) of non-transformed percentages of symptomatic point inoculation sites for light grown of cv. TG and KW at 10 days after point inoculation. (n = 6)

Fig. 6 shows that symptomatic inoculation sites on hypocotyls of dark- and light-grown plants of both cultivars were positively correlated with number of phialospores in the drops; similar results were found on roots (Fig. 7). Based on these data, the extrapolated minimum numbers of phialospores required to initiate infection on either hypocotyl or root tissues ranged between 10 and 36 phialospores per site.

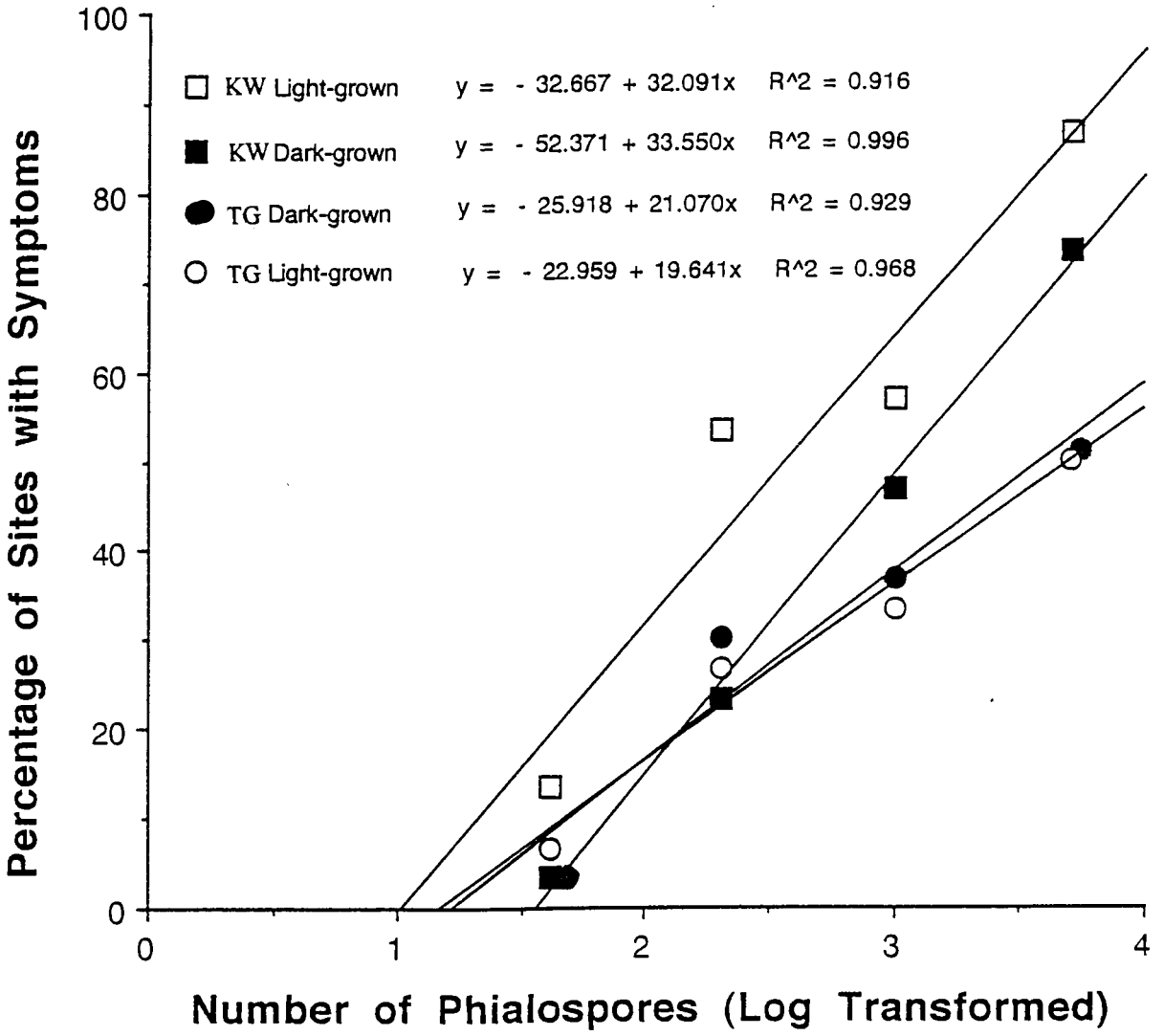


Fig. 6. Relation between number of phialospores of *Chalara elegans* and symptomatic point inoculation sites on hypocotyls of dark-grown and light-grown bean seedlings (*Phaseolus vulgaris*) at 10 days after inoculation.

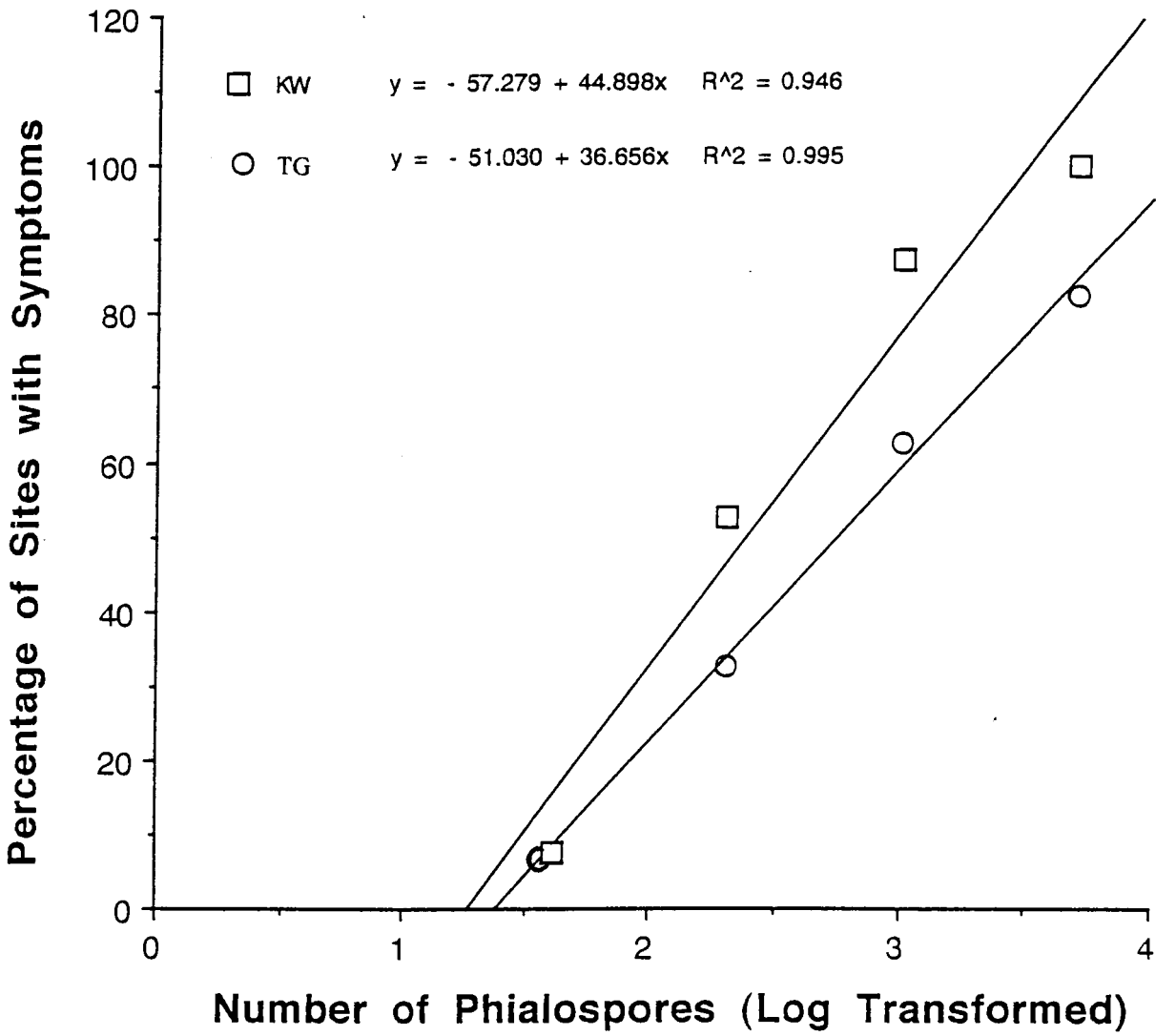


Fig. 7. Relation between number of phialospores of *Chalara elegans* and symptomatic point inoculation sites on roots of light-grown bean seedlings (*Phaseolus vulgaris*) at 10 days after inoculation.

2.4. Discussion

Although *C. elegans* is a primary incitant of disease on tobacco, it is a component of root rot disease complexes on many plants including bean. Host resistance is the most economical and potentially the most effective tool for management of root diseases and soilborne pathogens (Shew and Shew, 1994). Breeding for resistance is facilitated by inoculation procedures that permit detection of significant resistance. Accurate and rapid assay methods are needed to screen backcross progenies (Tu, 1992). Spraying, dipping and brushing of inoculum onto test plants are three inoculation methods that have been used in resistance breeding (Kruger *et al.*, 1977; Tu and Ayslesworth, 1980). However, spraying of inoculum tended to produce variable results in which many susceptible plants escaped infection, the dipping method appeared to be too severe for young germinating seedlings, and brush application was laborious and inefficient (Tu, 1992). These problems are mainly related to evaluations in green house, screen house and growth chambers (Beebe and Corrales, 1991), which are often used in the preliminary screening of large numbers of cultivars or seedling progenies before conducting field tests.

In the present study, we found that application of droplets of inoculum containing phialospores of *C. elegans* at point inoculation sites produced a strong correlation between inoculum concentration and symptom development. Although laborious, the method provided a sensitive means for detection of small but significant differences in susceptibility. Such differences were shown to occur both between the two bean cultivars and between the root and hypocotyl tissues.

On tobacco, there are three types of resistance to black root rot caused by *C. elegans* (Clayton, 1969). The first type, immunity, is controlled by a single dominant gene. The second type, which is expressed as a high level of resistance, is controlled by a combination of dominant and recessive genes, and the third type, which confers a moderate level of resistance, is controlled by a group of recessive genes. No published information was found regarding resistance of bean to *C. elegans*. Throughout the preliminary experimentation, *C. elegans* (isolate BK28) was a pathogen to all bean cultivars tested (data not shown). Factors that underlie differences in the susceptibility of bean cultivars may exist before the infection process is initiated or be induced during the early stage of host-pathogen interactions. Bateman and Lumsden (1965) showed that one of the factors affecting the susceptibility of bean seedling hypocotyls to *Rhizoctonia solani* was the calcium content and mobilization to sites of infection.

The results indicated that roots were more suceptible than hypocotyls at the high numbers of phialospores. Stockwell and Hanchey (1984) found that root exudates affected the number of infection cushions of *R. solani* on bean. Living plants release diverse organic subtances from their root system into the rhizosphere via active excretion, leakage, abrasion and wounding, and by cell death, mechanisms referred to collectively as root exudation (Shepherd, 1994).

Apparently, the percentage of lesion was higher on light-grown cv. Kentucky Wonder. Light provides energy for photosynthesis as well as environmental signals that affect plant development (Thompson and White, 1991). Light also affects the production of enzymes and growth regulators (Hart, 1988). For example, phenylalanine ammonia-

lyase (PAL) increases in response to UV light (Thompson and White, 1991; Kuhn *et al.*, 1984; Chappel and Hahlbrock, 1984). Increases in PAL activity often occur during induced defense response in plants (Kombrink and Somssich, 1995; Ouchi, 1983). In contrast, ethylene production often is reduced by light (Hart, 1988), particularly in the plumular hook portion of pea (Goeschl *et al.*, 1967) and in the bean hypocotyl hook (Kang and Ray, 1969). Ethylene may function in the expression of host resistance (Sticher, *et al.*, 1997). The temperature at the surface of the seedlings with rag dolls that were exposed to light may have been higher than that for dark-grown seedlings. According to Lloyd and Lockwood (1962) root rot caused by *C. elegans* on peas was most severe at soil at 28 ^{0} C, when plants were under stress.

To gain entrance into plant tissues, fungi generally secrete hydrolytic enzymes such as cutinase, cellulases, pectinases, and proteases (Knogge, 1996). Since 10 or more phialospores at point inoculation sites appeared to be required to initiate infection, it might be inferred that a critical concentration of some extracellular product associated with spore germination is required for infection of hypocotyl and root tissues by *C. elegans*.

Chapter III

COMPARATIVE HISTOPATHOLOGY OF VIRULENT AND AVIRULENT ISOLATES OF *CHALARA ELEGANS* ON ROOT AND SHOOT OF BEAN (*PHASEOLUS VULGARIS*)

3.1. Introduction

Some studies of the pathogenicity and histopathology of *Chalara elegans* have been reported for tobacco, cotton and carrot (Allison, 1938; Stover, 1950 Mauk and Hine, 1988; and Punja, *et al.*, 1992) and beans (Christou, 1962; Pierre and Wilkinson, 1969). In spite of the wide host range of *C. elegans*, some variability in susceptibility of bean to two isolates of the fungus was found in preliminary experiments. In addition, that research also revealed a substantial difference in susceptibility between root and shoot tissues of bean cultivars. There is little information in the published literature about differences in susceptibility of bean cultivars and the basis for differences in the virulence of different isolates of *C. elegans*. Although *C. elegans* is pathogenic on both root and shoot tissues of bean, information about its comparative histopathology on these tissues has not yet been reported. We describe here a comparative analysis of the infection process by virulent and avirulent isolates of *C. elegans* on root and shoot tissues of *P. vulgaris*. The objectives of this study were to (1) determine the basis for differences in the virulence of *C. elegans* isolates, (2) to compare infection process on root and shoot tissues, and (3) to identify histological components of host response to infection.

3.2. Materials and Methods

Two cultivars of beans, Tendergreen (TG) and Kentucky Wonder (KW), and BK28R (avirulent) and the BK28 (virulent) isolates of *C. elegans* were used in this study. Isolate BK28 was originally obtained from cotton, and isolate BK28R originated as a sector in an axenic colony of BK28 (Bottacin *et al.*, 1994). These isolates were kindly provided by Z.K. Punja (Simon Fraser University, Burnaby, Canada).

Seeds were surface sterilized in a 1 % solution of sodium hypochlorite for 15 minutes, rinsed with deionized water and placed between two sheets of moistened seed germination paper (Anchor Paper, St. Paul. MN) laying on top of a sheet of waxed paper. The paper and seeds were rolled into a cylinder ('rag doll'), and placed upright in an incubator at 28^0 C. After 5 days, germinating seeds of uniform size were transferred to clean rag dolls, and placed in a growth room with 12 hours of light and temperature 23 - 26^0C.

To prepare inoculum, sterile water was added to 2-week old fungal cultures growing on V8 juice agar. The culture surfaces were rubbed gently with a glass rod to suspend the phialospores, and the suspension was filtered through glasswool to separate the phialospores from mycelium and chlamydospores. Inoculation was done by spraying a suspension containing 1 x 10^6 phialospores / ml onto the surface of roots and hypocotyls of 7-day old seedlings. After inoculation, the seedlings were kept uncovered in a dark moist chamber for about 6 hours, then rolled again into rag dolls and returned to the growth room. The rag dolls were kept moist with deionized water as required. The experiment was done in completely randomized design with five replications.

Observations were done at 6, 18, 30, 42, 60, 78, 96 and 144 hours after inoculation. The observation at 96 and 144 hours after inoculation were to document the symptom phenotype caused by *C. elegans.*

Whole hypocotyls and roots of seedlings inoculated by isolate BK28R (avirulent) and BK28 (virulent) were collected at 6, 18, 30, 42, 60, and 78 hours after inoculation, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.0 for approximately 12 hours, rinsed with 25% ethanol, placed in test tubes containing 50% ethanol and then kept at 10^{0} C prior to examination. Strips of epidermis from hypocotyls and small pieces of root were stained with Toluidine Blue O and examined at 400 X magnification to assess the number of germinating spores, appressorium formation, penetration and the appearance of colonized cells for each tissue and time of sampling. Data were taken from three randomly selected fields of view on each of three epidermal strips from hypocotyls and pieces of primary root from each of five replicate plants.

Seedlings were observed under the stereomicroscope at 96 and 144 hours after inoculation and the length of lesions at separate locations near the bottom, middle and top of both hypocotyls and roots were recorded. Measurements were done using a calibrated micrometer.

Data for the number of infection sites / cm^{2} of beans inoculated with BK28R and BK28 isolates, and the length of lesions on hypocotyls and roots inoculated with the BK28 isolate were analyzed with generalized linear model (GLM) with factorial analysis on SAS package release 6.11. Average proportions of phialospore germination on shoots

and roots of each bean cultivar were analyzed by GENMOD procedure with logistic link on SAS release 6.11 software (SAS Institute Inc., 1996).

Separate experiments were done to study the qualitative infection process of *C. elegans*. Samples of hypocotyls and primary root inoculated as described above were collected at 6, 12, 36, 42, 66, 78, 104 hours and 10 days after inoculation. Free hand sections were observed under a microscope and some tissues were embedded in JB-4® embedding medium (J.B.EM Services Inc., Dorval, Quebec). For embedding, bean hypocotyls and roots were cut into 0.5 cm lengths, fixed as already described, and dehydrated through a series of ethanol (30, 50, 75, 90 and 100 %). To prepare the JB-4® embedding medium, 0.90 grams of dry catalyst C (benzoyl peroxide) was added to 100 ml of JB-4 solution A (2-butoxyethanol) and mixed until dissolved. 1 ml of JB-4 solution B (N, N-dimethylaniline) was added to 25 ml of solution A containing the catalyst. This solution was placed in an ice bath to prevent premature polymerization. Tissues were put into BEEM capsules containing the JB-4® embedding medium and incubated under anaerobic conditions for 90 minutes at room temperature to allow polymerization to occur (J.B. EM Services Inc., 1988). Blocks containing the embedded tissues were cut with a dry glass knife and collected with a forceps. Toluidine Blue O was used for staining prior to observation with a compound microscope.

Scanning electron microscopy was used to study qualitative aspects of early stages of spore germination and formation of specific infection structures. Point inoculation, inoculated with 5 µL per droplets containing 1 x 10^3 phialospores per droplet, were sampled at 7, 15 and 36 hours after inoculation. Segments of tissues approximately 0.5

cm in length were cut and placed into 0.1 M sodium cacodylate buffer. The segments were then fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.0 for approximately 12 hours, rinsed with 0.1 M sodium cacodylate buffer three times, post-fixed for 1 hour in 2 % osmium tetroxide-0.1 M sodium cacodylate. After post-fixing, segments were washed three times in the same buffer. They were then dehydrated through a series of ethanol (30, 50, 70, 90, 100 %), followed with absolute ethanol and amyl acetate (3 : 1, 1 : 1, 1 : 3, and three times in 100 % amyl acetate) (Tahiri-Alaoui *et al.*, 1993). Critical point drying was done with liquid CO_2 as the transitional fluid. Dried segments were mounted on aluminum stubs, and coated with a thin layer (approximately 20 to 30 nm) of gold and stored in containers (Bozzola and Russell, 1992). Specimens were observed on a scanning electron microscope Hitachi Model S-2500; images were captured and stored on zip disks.

3. 3. Results

3.3.1. Macroscopic aspects of symptom development

Lesions first appeared on both hypocotyls and primary roots that had been inoculated with the virulent isolate at 3 - 4 days post inoculation. Lesions continued to enlarge for an additional 3 - 4 days after their initial appearance (Table 4). The lesions were initially brown and become progressively darker with time, starting from the centers. The dark color was partly due to pigmented host reaction products that accumulated in the affected cells, and partly due to the production of dark brown-black chlamydospores on the surface of the lesions. Factorial analysis of the data for 96 and

144 hours after inoculation showed that lesion length on roots and hypocotyls differed significantly (P = 0.0001) (Table 4). Lesion length on hypocotyls of the two cultivars also differed significantly (P = 0.04), but not on roots (P = 0.9) (Table 5).

Tiny brown lesions occurred infrequently on hypocotyls inoculated with the avirulent isolate; these appeared at 4 days post inoculation on KW and at 6 days post inoculation on TG. No lesions occurred on primary roots of either cultivars following inoculation with the avirulent isolate. Symptoms on bean roots and hypocotyls inoculated with virulent and avirulent isolates of *C. elegans* at 6 days post inoculation are shown in Fig. 8.

Table 4. Development of lesions on roots and hypocotyls of bean, *Phaseolus vulgaris*, inoculated with the virulent isolate (BK28) of *Chalara elegans*.

Hours after inoculation	Mean length of lesions[1] (mm)	
	Hypocotyl	Primary Root
96	1.88 a	0.27 a
144	3.92 b	0.41 b

Values in a column followed by the same letter do not differ significantly (P<0.05) according to Bonferroni's test

[1] Mean of lenght of cv. TG and KW (n = 10).

Table 5. Mean length of lesions (mm)[1] on roots and hypocotyls of two cultivars of bean, *Phaseolus vulgaris*, inoculated with a virulent isolate (BK28) of *Chalara elegans*

Cultivar	Bean Part	
	Hypocotyl	Primary Root
TG	2.57 a	0.34 a
KW	3.23 b	0.34 a

Values in a column followed by the same letter do not differ significantly (P<0.05) according to Bonferroni's test.

[1] Mean of lengths at 96 and 144 hours post inoculation (n = 10).

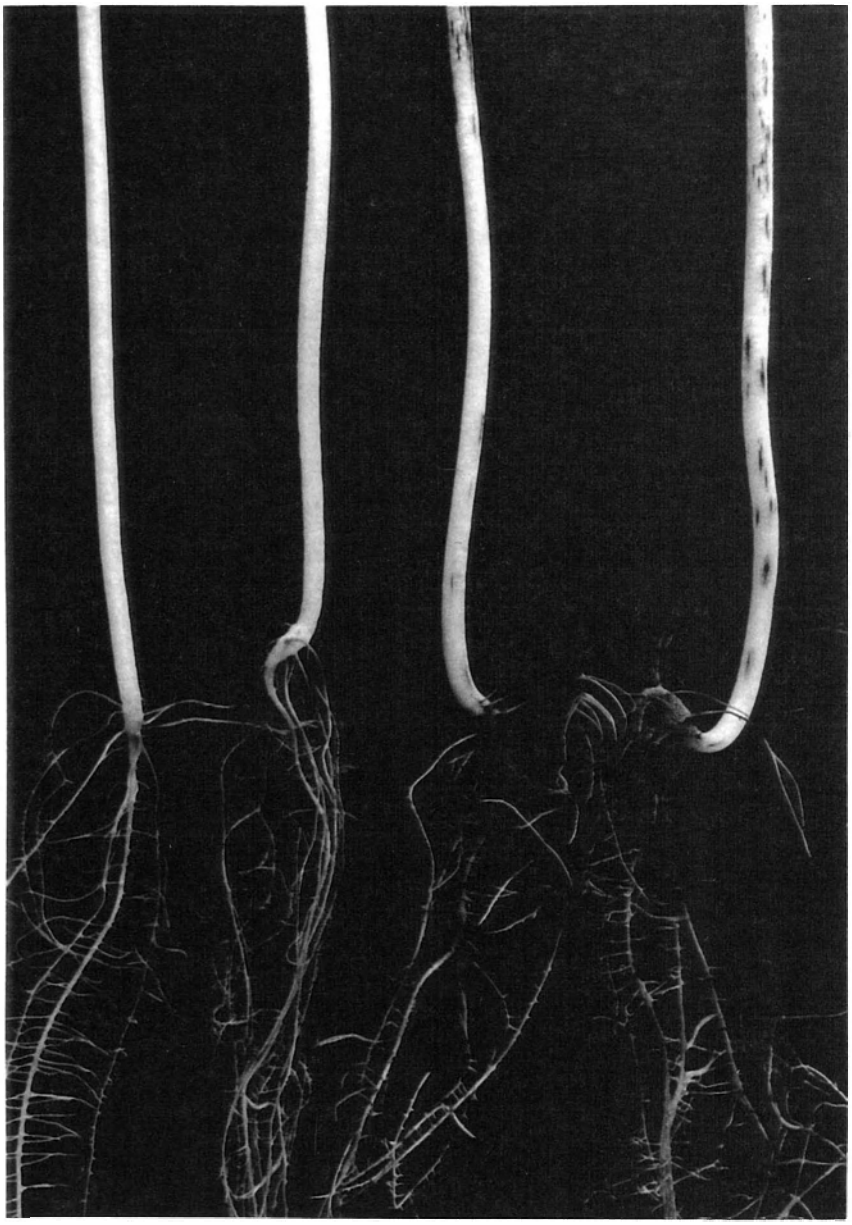


Fig. 8. Appearance of symptoms at 6 days after inoculation of two cultivars of bean, *Phaseolus vulgaris* with virulent (BK28) and avirulent (BK28R) isolates of *Chalara elegans*. From left to right are TG/BK28R, KW/BK28R, TG/BK28, KW/BK28.

3.3.2. Microscopic aspects of infection development

The microscopic aspects of infection by isolates BK28R and BK28 were similar in both cultivars of bean. Therefore, the descriptions refer to both bean cultivars. In addition, the quantitative kinetics or time course of infection for the two isolates were enumerated from the two experiments.

Virulent isolate (BK28) of *Chalara elegans*

Spores, size 7.50 to 18.75 x 2.50 to 3.75 µm, attached to the bean surface mostly at epidermal cell junctions, began germination prior to 6 hours after inoculation, typically with a single germ tube. Germination with two germ tubes was observed in 3 out of 3403 germinated spores . Germ tubes were 1.25 to 1.50 µm in width near to the spore and 1.25 to 3.25 µm wide near to the tip. Phialospores of the BK28 isolate germinated faster on roots than hypocotyls (Fig 9A, 9B). Inoculum of BK28 isolate produced secondary phialospores, which formed on germinating germ tubes. They were distinguished from primary phialospores by their thinner walls, smaller size and pyriform shape (Punja, 1992). Often, the secondary phialospores germinated and appeared to penetrate on root (Fig. 10C). This is the first time germination and penetration by secondary phialospores has been reported.

Successful germ tubes were typically short and terminalized with slight tip swellings or infrequent appressorium-like enlargements (Fig 10A) and penetrated directly at epidermal cell junctions on hypocotyls. On primary roots, germ tubes appeared to enter through natural openings (Fig. 10B) as well as penetrated directly. Germ tubes that failed to penetrate appeared to continue growing on the tissue surfaces.

Primary hyphae inside host cells were first observed between 30 to 36 hours after inoculation on primary roots, and between 36 to 42 hours after inoculation on hypocotyls. After entering the cell lumen, the infection hypha formed septa and branched repeatedly until the cell lumen was crowded with hyphae. The fungus then invaded adjacent cells by producing long or short fine hyphae (Fig. 11A), or by coarser septate hyphae that appeared either long and unconstricted or as a chain of bulbous cells (constricted hyphae) (Fig. 11B, 11C). The type of hyphae produced appeared to depend on the type and size of the colonized host cells. Mostly long unconstricted, sparsely branch hyphae were produced in the epidermis, and long unconstricted as well as constricted hyphae formed in the cortical tissues. During later stages of host colonization, unconstricted hyphae emerged from the colonized tissues and grew transversally on the surface and produced longitudinal branches. By 78 hours after inoculation, hyphae on the epidermal surface produced new phialospores and by 96 hours after inoculation, chlamydospores were present. At 104 hours after inoculation, the fungus had advanced to the endodermis of either root or hypocotyl tissues. At 10 days after inoculation macroconidia and endoconidia were observed inside the vascular tissue of hypocotyls. Sporulation was not observed inside the vascular system of primary roots.

Avirulent isolate (BK28R) of *C. elegans*

Phialospores of isolate BK28R, size 8.75 to 21.75 x 3.75 to 6.25 μm, germinated and produced germ tubes that grew on the epidermal surfaces of roots and hypocotyls, but mostly failed to penetrate (Fig. 9C and 9D). The width of germ tubes near the spore was 1.25 to 1.88 μm and near the tip was 0.94 to 2.5 μm. Long germ tubes attached and

appeared to grow in contact with the host surface topography. Some infection hyphae penetrated and entered the epidermal cells by 30 - 36 hours after inoculation on roots, and 42 - 60 hours after inoculation on hypocotyls. Infection hyphae on the epidermis of hypocotyls or root were hyaline, constricted and appeared relatively bigger than infection hyphae of the virulent isolate (Fig. 12A). Infection hyphae of BK28R penetrated only the epidermal cells of hypocotyls, while on the roots they sometimes advanced into cortical tissue (Fig. 12B). Constricted and unconstricted hyphae were formed by the BK28R isolate in root tissue. However, secondary phialospores and sporulation by this isolate were not observed.

Appressorium formation

There is controversy in the literature as to whether *C. elegans* forms appressoria upon germination. In my experiments, germinated phialospores infrequently produced appressorium-like enlargements at the tips of germ tubes. The width at the tips of germ tubes lacking such enlargements ranged from 1.25 µm to 2.40 µm. On roots, 1 - 6 % and 2 - 6 % of germinating phialospores of virulent and avirulent isolates, respectively, produced terminal swellings > 2.40 µm in width, while on hypocotyls the frequencies of appressorium-like swellings were 0 - 2 % and 0 % for the virulent and avirulent isolates, respectively. The lower percentages of each range were observed on cv. TG and the higher percentages on cv. KW, and represent averages of data from 6 to 60 hours after inoculation.

Fig. 9. Scanning electron microscope image of germination of BK28 (virulent isolate) and BK28R (avirulent) isolates of *Chalara elegans* on cv. KW at 36 hours after inoculation. A, Germination of phialospores of BK28 on hypocotyl. B, Phialospores of BK28 on root. C, Phialospores of BK28R on hypocotyl. D, Phialospore of BK28R on root (arrow heads).

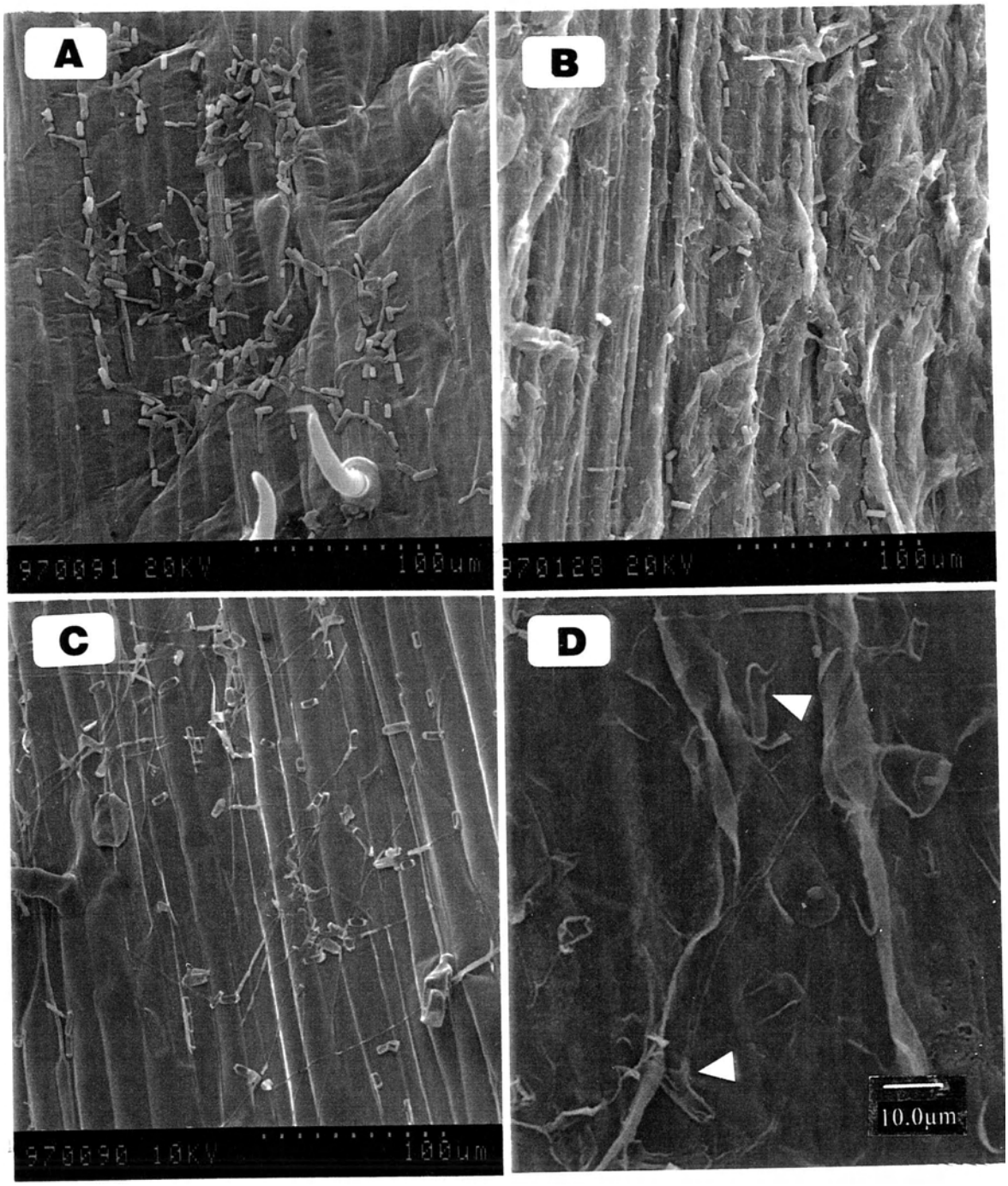

A
970091 20KV
100um
B
970128 20KV
100um
C
970090 10KV
100um
D
10.0µm

Fig. 10. Germination of phialospores of BK28 isolate of *Chalara elegans* on cv. KW; on hypocotyl (A), on root (B); a secondary phialospore germinated on root (C). ph=phialospore; gt=germ tube; rh=root hair; sp=secondary phialospores

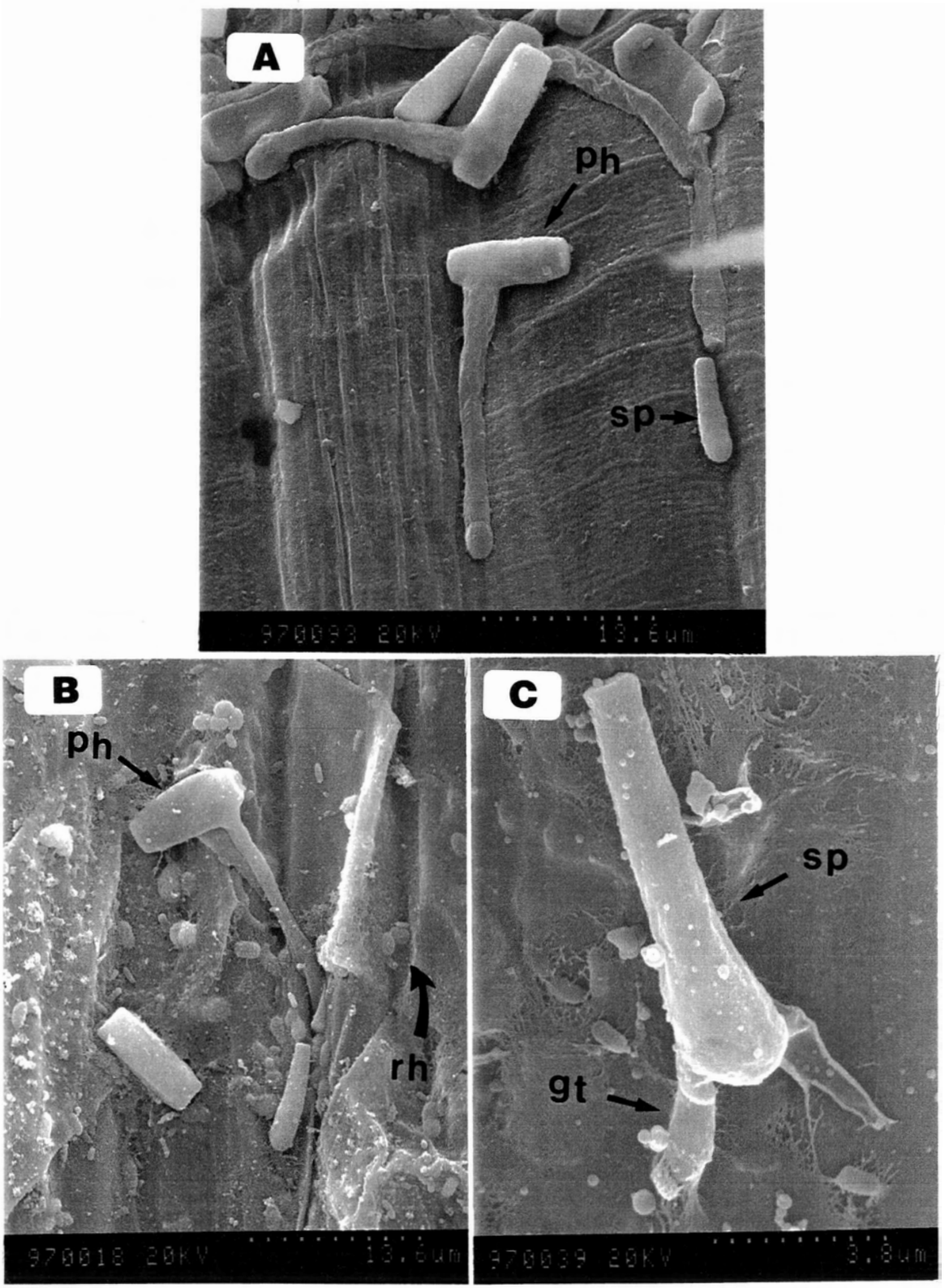

A
ph
sp
B
ph
rh
C
sp
gt

Fig. 11. Colonization of bean tissues by BK28 (virulent isolate) on primary root of cv. KW at 1000 X magnification. A, Long (large arrow) and short (small arrow) fine hyphae emerging from cell, B, Long unconstricted hyphae (arrow) emerging from a cell that is full of hyphae; C, Short septated and constricted (small arrow) and long septated and unconstricted (large arrow) hyphae in cortical cells.

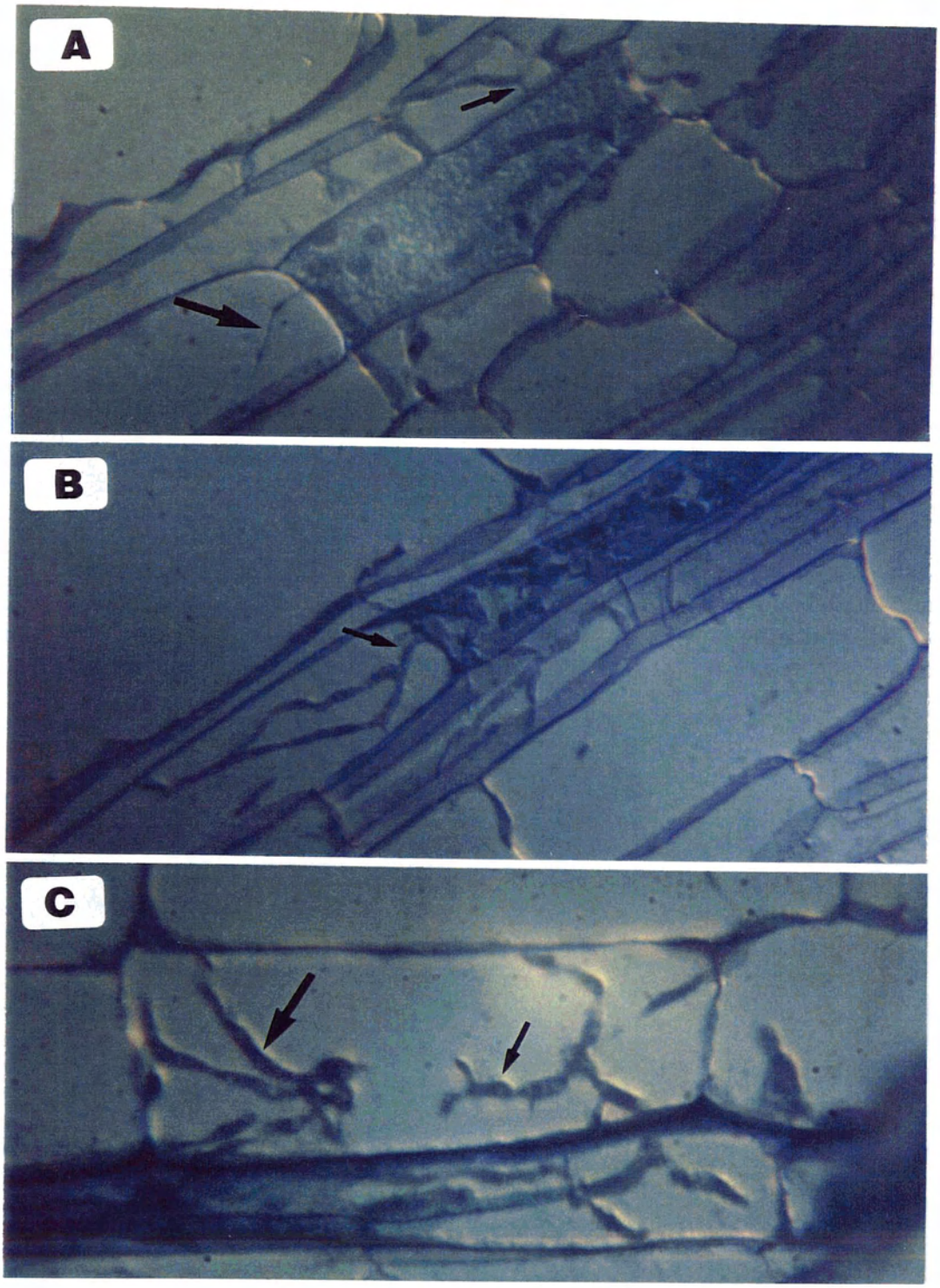
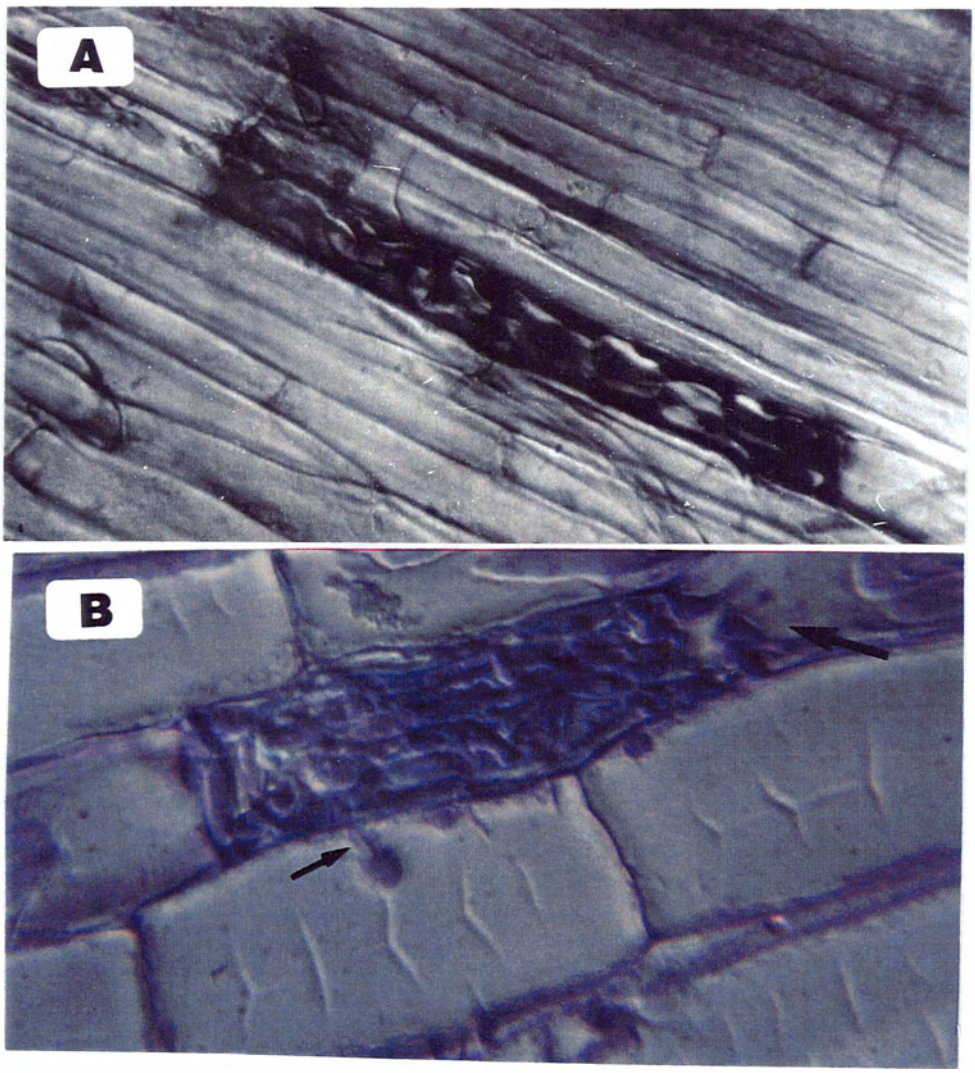

A
B
C

Fig. 12. Colonization by BK28R (avirulent) isolate of *Chalara elegans* on primary root of cv. KW at 1000 X magnification. Hyaline constricted hypha appeared unable to escape from hypersensitive epidermal cell (A), avirulent isolate was able to escape by long thin hypha (small arrow) to adjacent cell, or appeared due to cell wall breakage (large arrow) (B).


A
B

3. 3. 2. 1. Quantitative study of phialospore germination

Analysis of the data for spore germination using generalized linear model with logistic link (Genmod on SAS package, type 3 analysis) showed that germination of phialospores was significantly higher on roots than on hypocotyls (P = 0.0001). Time, i.e., hours after inoculation, was significant (P = 0.0001), whereas between isolates (P = 0.12) and cultivars (P = 0.33) were not. Significant interactions included time x isolate (P = 0.0001), time x cultivar (P = 0.017), time x tissues (root, hypocotyl) (P = 0.044) and tissues x cultivars (P = 0.015). Percentages of spore germination by isolates BK28R and BK28 of *C. elegans* on roots and hypocotyls on the two cultivars of bean as well as the predicted germination generated by Genmod procedure of SAS with logistic link are shown in Fig. 13.

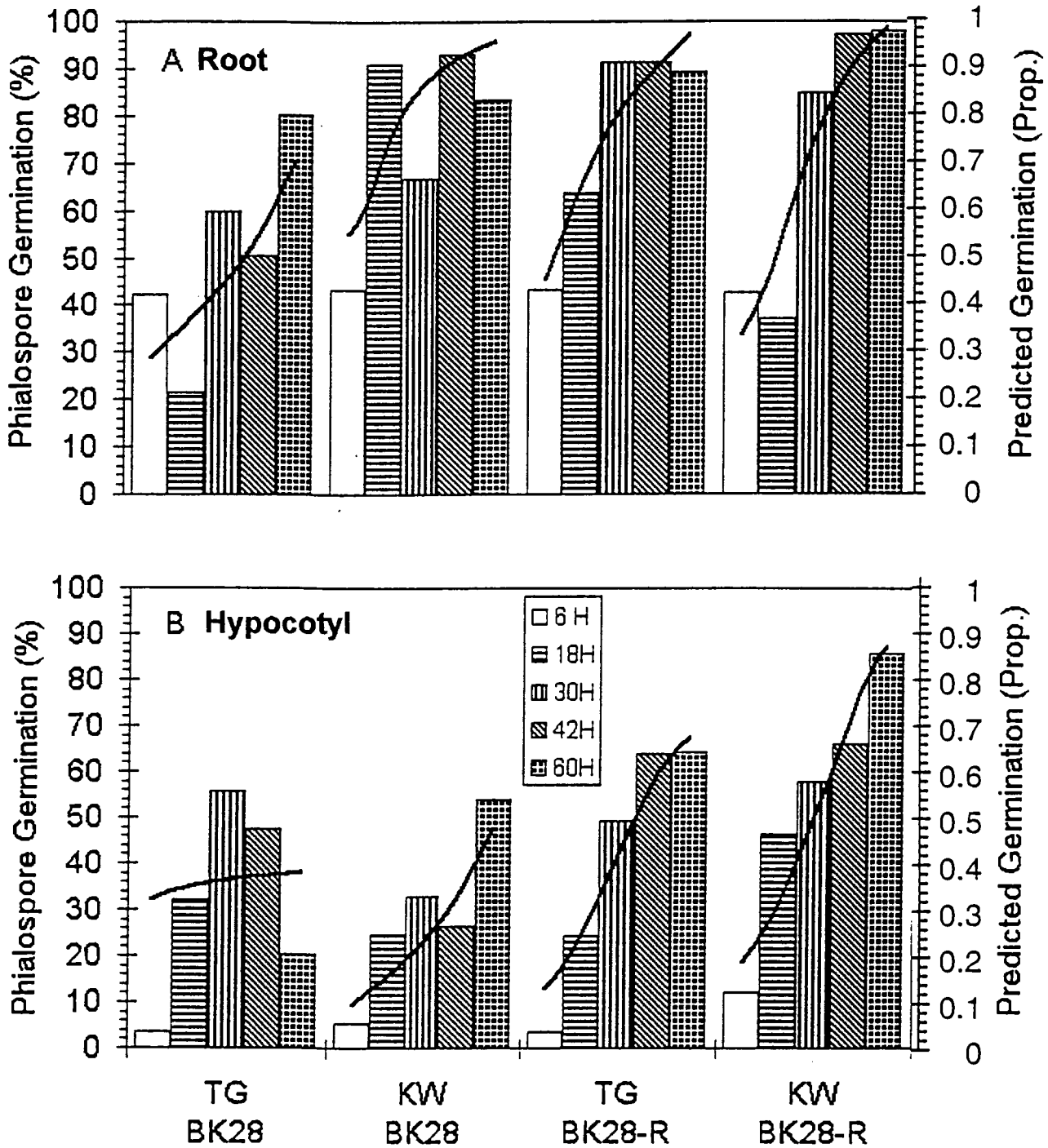


Fig. 13. Germination of phialospores of BK28 (virulent) and BK28R (avirulent) isolates of *Chalara elegans* on root (A) and hypocotyl (B) at 6, 18, 30, 42 and 60 hours after inoculation. Histograms are the percentage averages of observed values, and solid curves are predicted proportion values using Genmod procedure with logistic link on SAS release 6.11.

3. 3. 2. 2. Site colonization

When an infection hypha penetrated an epidermal cell, the cells sometimes but not always became brown and hypersensitive in appearance. Hyphae of the virulent isolate (BK28) on primary roots and hypocotyls grew beyond the browning or hypersensitive reacting cells. On the other hand, hyphae of the avirulent isolate (BK28R) were restricted to epidermal cells of hypocotyls and to cortical root cells. Until 78 hours after inoculation, no significant differences were found between the cultivars in the numbers of infection sites on root ($P = 0.69$), and on hypocotyl ($P = 0.22$) tissues. Infection frequencies for BK28 and BK28R were significantly different on root ($P = 0.06$), as well as on hypocotyl ($P = 0.02$). Hours after inoculation was significant on roots ($P = 0.0001$) and was not significant on hypocotyls ($P = 0.21$). Numbers of infection sites on root and hypocotyl by BK28R and BK28 isolates are shown on Table 6, and numbers of infection sites at different times after inoculation are presented in Table 7. The number of colonized cells at infection sites on hypocotyls increased only slightly between 42 and 78 hours after inoculation, whereas hyphae at infection sites in roots spread more rapidly during this period (Fig. 14). Cells colonized by either isolate were usually necrotic. Hyphae of the virulent isolate BK28 appeared able to grow out of necrotic cells in both root and hypocotyl tissues; hyphae of the avirulent BK28R apparently growing from necrotic cells were seen occasionally in roots, but not in hypocotyls (Fig. 15).

Table 6. Number of infection sites per cm^2 on bean roots and hypocotyls inoculated with virulent isolate (BK28) and avirulent isolates (BK28R) of *Chalara elegans*.

Isolate	Mean # of Colonized Site / cm^2 [1]	
	Primary Root	Hypocotyl
BK28R	5.18 a	0.18 a
BK28	12.29 b	1.63 b

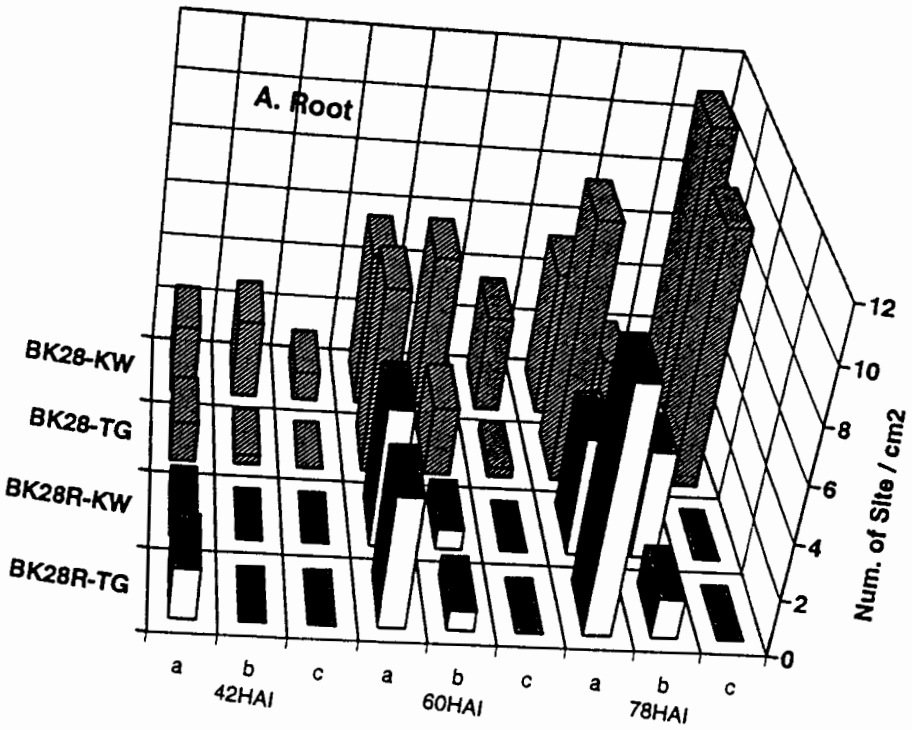
Values in column, followed by the same letter are not significantly different from each other ($P \leq 0.05$) according to Bonferroni's test.
[1] Means of infection sites on cv. TG and KW at 42, 60 and 78 hours after inoculation (n = 30).

Table 7. Number of infection sites per cm^2 at 42, 60 and 78 hours after inoculation with virulent (BK28) and avirulent (BK28R) isolates of *Chalara elegans* on roots and hypocotyls.

Hours Post Inoculation	Mean # of Colonized Sites / cm^2 [1]	
	Primary Root	Hypocotyl
42	2.88 a	0.18 a
60	8.65 ab	1.05 a
78	14.68 b	1.49 a

Values in column, followed by the same letter are not significantly different from each other ($P<0.05$) according to Bonferroni's test.
[1] Means of pooled data for isolate BK28 and BK28R on cv. TG and KW (n = 10).

Fig. 14. Apparent size of infection sites on root and hypocotyl tissues of two cultivars (KW, TG) of bean *Phaseolus vulgaris* at 42, 60 and 72 hours after inoculation with virulent (BK28) and avirulent (BK28R) isolates of *Chalara elegans.* a. 1 - 5 cells colonized, b. 6 - 10 cells colonized, and c. More than 10 cells colonized by the pathogen.


A. Root
BK28-KW
BK28-TG
BK28R-KW
BK28R-TG
a b c a b c a b c
42HAI
60HAI
78HAI
0
2
4
6
8
10
12
Num. of Site / cm2

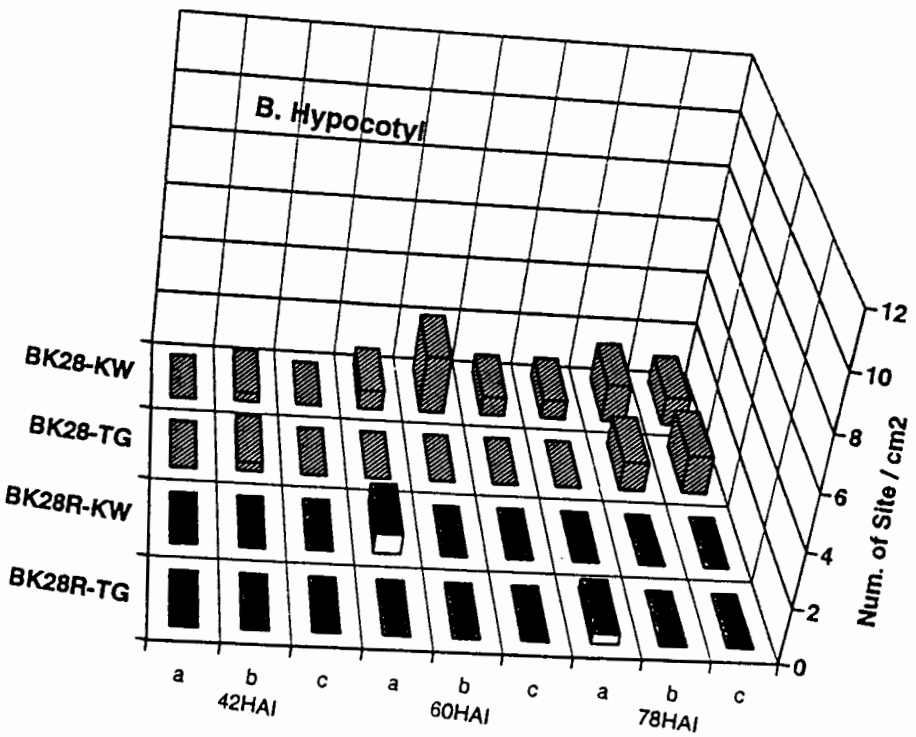
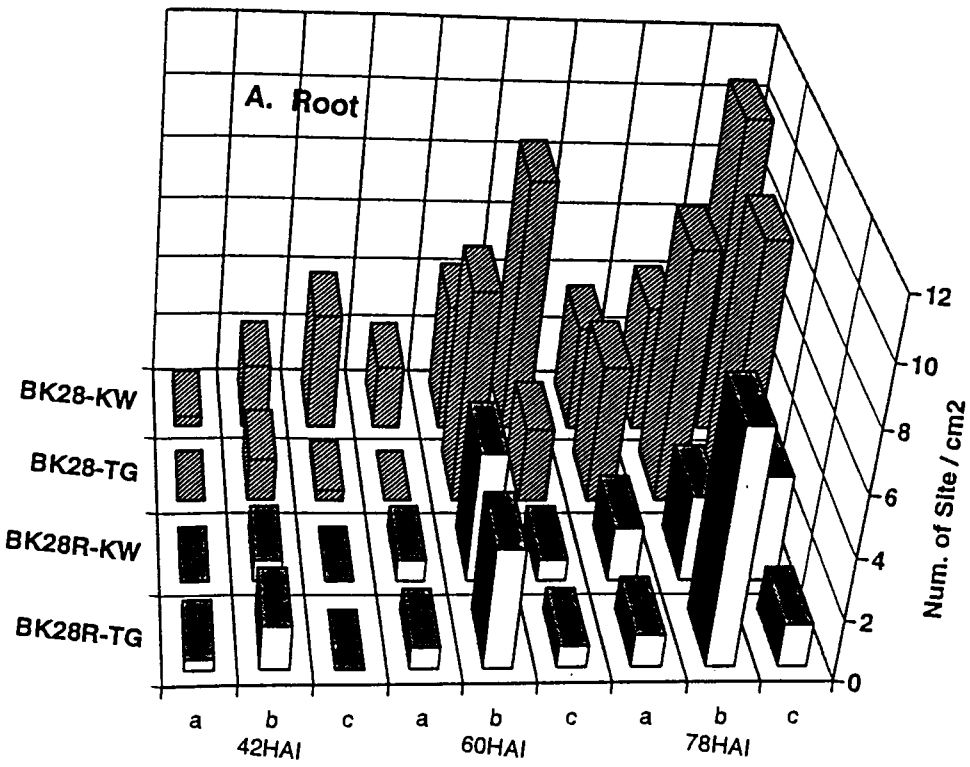
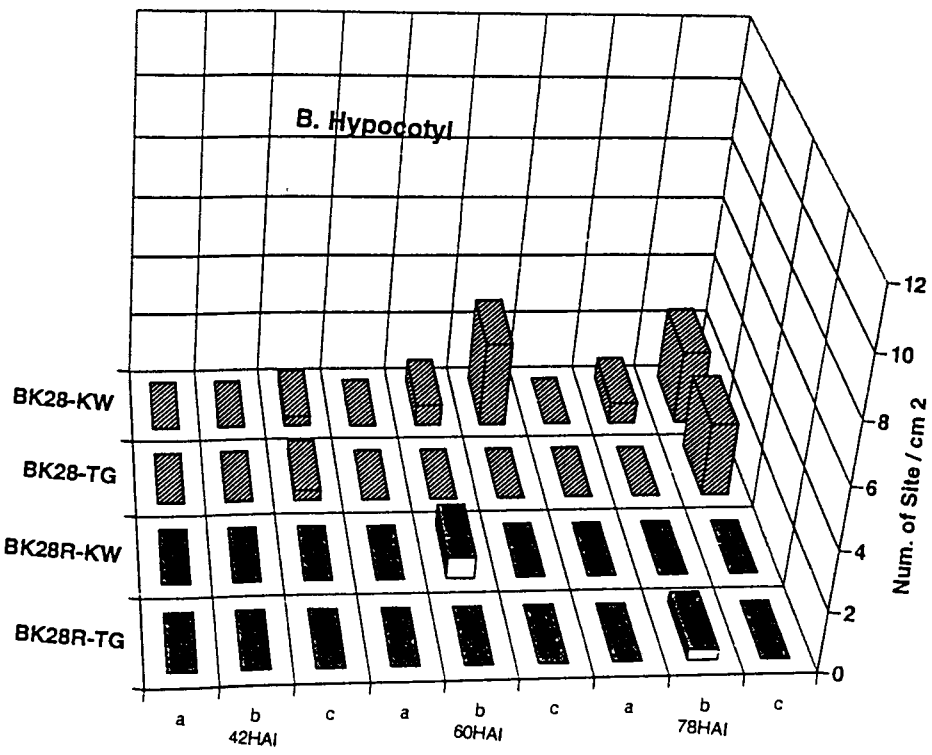

B. Hypocotyl
BK28-KW
BK28-TG
BK28R-KW
BK28R-TG
a b c a b c a b c
42HAI
60HAI
78HAI
0
2
4
6
8
10
12
Num. of Site / cm2

Fig.15. Appearance of infection sites on root and hypocotyl tissues of two cultivars (KW, TG) of bean, *Phaseolus vulgaris,* at 42, 60 and 78 hours after inoculation with virulent (BK28) and avirulent (BK28R) isolates of *Chalara elegans.* a. Colonized cells appear viable, b. All colonized host cells appear necrotic, and c. Hyphae appear to be growing out of necrotic cells into adjacent viable cells.


A. Root
BK28-KW
BK28-TG
BK28R-KW
BK28R-TG
Num. of Site / cm2
0
2
4
6
8
10
12
a b c a b c a b c
42HAI
60HAI
78HAI


B. Hypocotyl
BK28-KW
BK28-TG
BK28R-KW
BK28R-TG
Num. of Site / cm 2
0
2
4
6
8
10
12
a b c a b c a b c
42HAI
60HAI
78HAI

3. 4. Discussion

C. elegans is a hemibiotrophic pathogen (Hood and Shew, 1996b). It infects living tissues in the same way as biotrophs, but as with necrotrophs, can develop and sporulate on the dead tissues (Luttrell, 1974). *C. elegans* can infect bean roots, as well as the shoots (hypocotyl), a capability shared by relatively few fungi. The root and shoot are initiated as a single continuous structure during the development of the embryo, and develop into two structures with different functions (Raven, *et al.*, 1987). Thornley (1972) proposed a model in which growth is dependent on the supply of carbon from the shoot by phloem transport, and nitrogen from the root by xylem transport.

The results of these experiments showed that the development of *C. elegans* was affected by the type of tissues of bean. More lesions occurred on roots, and larger lesions developed on hypocotyls than on roots. *C. elegans* is a soil-associated pathogen and it has developed an invasion strategy that targets the root. According to Mendgen *et al.* (1996), the formation of infection structures often depends on signals provided by the plant surface as a prerequisite for a specific mode of invasion. The most significant movement of nutrients between host and pathogens occurs during colonization of the host tissues. Some parasites may absorb nutrients directly from the apoplast region, the area outside the cell or associated cell wall regions of a plant with passive movement of water and solutes, while others may stimulate the diversion of host nutrients to sites of infection (Hancock and Huisman, 1981).

When phialospores of *C. elegans* land on bean tissues, the first factor that may be critical for germination is the availability of nutrients to the pathogen. Thus, from this study, it would appear that roots of bean may provide essential nutrients for germination of phialospores of *C. elegans* via leakage from natural openings that occur on the root. It has been shown that propagules of *C. elegans* germinated in response to nutrient addition and were stimulated by exogenous nutrients and plant root exudates (Linderman and Toussoun, 1968). Exudation is considered to be important in parasitism for stimulating germination (Hancock and Huisman, 1981). Other soil factors can also affect fungal germination; for example, Meyer *et al.* (1994) showed that suppressed the germination of phialospores of *C. elegans*. However, since the beans used in my experiments were grown in the absence of soil, soil factors did not affect the phialospore germination that I observed.

In order to colonize a host, fungal pathogens must penetrate the cell wall to overcome the first line of plant defense. Both physical and chemical means of penetration have bean described (Isaac, 1992). The abilities to grow and reproduce in colonized tissues are also important factors that determine the success of plant pathogens. The virulent isolate of *C. elegans* possessed these capabilities, while the avirulent isolate had a reduced capacity to penetrate. Phialospores of both isolates germinated on roots and shoots. Germ tubes of the virulent isolate generally penetrated directly while those of the avirulent isolate generally grew on the surface and failed to penetrate.

As for other plants, beans have evolved to co-exist with pathogens. Resistance is the rule and infection is a rare exception (Bell, 1980; Lamb, 1994). Bean cuticle, cell

walls and nutrients appear to play a passive but important role in the defense against the avirulent isolate. However, the virulent isolate was apparently not affected by those defenses. The cuticles consist of hydroxy fatty acids esterified with either phenolics (cutin) or primary alcohols (waxes). The cell wall consists of polymers of carbohydrates (cellulose, hemicellulose, pectin), amino acids (hydroxyproline-rich glycoprotein, glycine-rich protein, enzymes) and phenolic compounds (lignin, phenolic esters) (Kombrink and Somssich, 1995). That the cuticle and epidermis cell wall may prevent the development of *C. elegans* was indicated by the lower number of colonized cells on hypocotyls compared to roots. Histochemical analysis showed that cuticle and epidermis of hypocotyl were Sudan positive, indicating the presence of suberin or wax, whereas the root epidermis was Sudan negative. My research supports that the hypothesis that a major difference between the BK28 and BK28R isolate are that the latter isolate lacks some of the abilities needed to overcome the barriers represented by the host epidermal surface.

Chapter IV

HISTOCHEMICAL ANALYSIS OF SUBERIN AND LIGNIN IN ROOT AND SHOOT TISSUES OF BEAN (*PHASEOLUS VULGARIS*) INOCULATED WITH VIRULENT AND AVIRULENT ISOLATES OF *CHALARA ELEGANS*

4. 1. Introduction

Plants co-exist with microorganisms in nature but infectious disease is rare. Plant-associated pathogen failure may result from (1) inability of plants to support the niche requirements of potential pathogens, (2) structural barriers or toxic compounds that prevent infections (Hammond-Kosack and Jones, 1996), or (3) initiation of specific response mechanisms that confer resistance when challenged by pathogens (Dixon *et al.*, 1994). Resistance responses include diverse mechanisms such as secretion of fungal cell wall degrading enzymes and other pathogenesis-related proteins, phytoalexins (Hammond-Kosack and Jones, 1996), as well as suberin (Kolattukudy and Koller, 1983) and lignin production (Asada *et al.*, 1979). The objective of this study was to determine the occurrence of suberin and lignin in the tissues of root and hypocotyl of bean during early stages of interaction with *Chalara elegans*, the causal agent of black root rot disease.

4. 2. Materials and Methods

Seed of beans cv. Tendergreen (TG) and Kentucky Wonder (KW) were surface-sterilized with 1 % sodium hypochlorite for 15 minutes and allowed to germinate

between two sheets of moistened seed germination paper (Anchor Paper, St. Paul, MN) laying on top of a sheet of waxed paper. The three papers containing the seeds were rolled into cylinder (rag dol), and placed upright in an incubator at 28 ^{0} C. After 5 days, seedlings of uniform size were transferred to clean rag dolls, and placed in a growth room with 12 hours of light and temperature 23 - 26 ^{0} C. Inoculum was prepared as described in Chapter II.

Roots and hypocotyls of cvs. TG and KW were inoculated with BK28 (virulent) and BK28R (avirulent) isolates of *C. elegans*. Suspension of phialospores containing 2 x 10 ^{5} cells per ml were applied as 5 µL droplets at point inoculation sites using a micropipet. Droplets of sterile water were used as control. Each treatment (inoculum, cultivars and hours after inoculation) was examined on three replicate seedlings, each inoculated at two sites. Treatments on roots and hypocotyls were done on separate seedlings. Inoculation sites were identified by marking on the germination paper next to the inoculation sites. Segments of 0.5 cm length, containing an inoculation site in the middle, were collected at 7, 15 and 36 hours after inoculation. The segments were fixed in 2.5 % glutaraldehyde in 0.1 sodium cacodylate buffer at pH 7.0 for 12 hours, rinsed with 25 % ethanol, and stored in 50 % ethanol before observation. Under a stereozoom microscope, three transverse sections were cut free-hand as thinly as possible with a razor blade from the middle area of each of the fixed segments.

To detect suberin, the sections were immersed in a filtered, saturated solution of mixed Sudan III and IV (Jensen, 1962), rinsed in 70 % ethanol and immersed in 70 %

glycerol before being placed onto glass slides for observation. The stained sections were examined using a Meji stereozoom microscope with a Lumina Model F0.150 light source. A red color was interpreted as positive for suberin.

A histochemical test for lignin was done using the Weisner phloroglucinol-HCl test (Jensen, 1962). Transverse sections were flooded with a filtered saturated aqueous solution of phloroglucinol in 20% HCl for 5 minutes, and placed on glass slides containing several drops of phloroglucinol in 20% HCl to prevent drying while observing under dissecting microscope. Red-violet or pink to bright red color was interpreted as lignin (Jensen, 1962).

Color responses in the stained sections were rated as follows:

0 = Negative color reaction of suberin or lignin,

1 = Positive color reaction with discontinuous staining in the responding tissue regions,

2 = Positive color reaction with continuous staining in the responding tissue regions.

Indices for suberin and lignin were generated by the formula:

$$\text{suberin or lignin index} = \frac{\text{sum of all numerical ratings}}{\text{total number of observations x maximum rating (2)}}$$

4. 3. Results

A red color indicative of suberin appeared in root endodermis in sections treated with a combination of Sudan III and IV (Fig. 16). Both bean cultivars, inoculated with BK28 (virulent) and BK28R (avirulent) isolates of *C. elegans*, and uninoculated control

seedlings yielded similar results. Fig. 17 shows that suberin index increased over time, except in the case of cv. TG inoculated with water.

In cross sections of hypocotyls, staining occurred in the epidermis, in the vascular cambium and endodermis, and at the base of trichomes. The index of suberin in epidermis and vascular cambium was the highest in sections of bean inoculated with virulent isolates (Fig. 18 and 19).

The phloroglucinol-HCl lignin test gave a positive reaction in the pericycle, phloem and xylem of root sections (Fig. 20 and 21). Xylem of roots gave a red bright color that persisted longer than the pink color produced in phloem and pericycle cells. Xylem had the highest index of suberin and it was followed by phloem and pericycle, respectively.

On hypocotyls, xylem and vascular cambium produced a bright red color and vascular cambium reacted with a pink color (Fig. 22). Similar lignin indices were obtained for all treatments (Fig. 23).

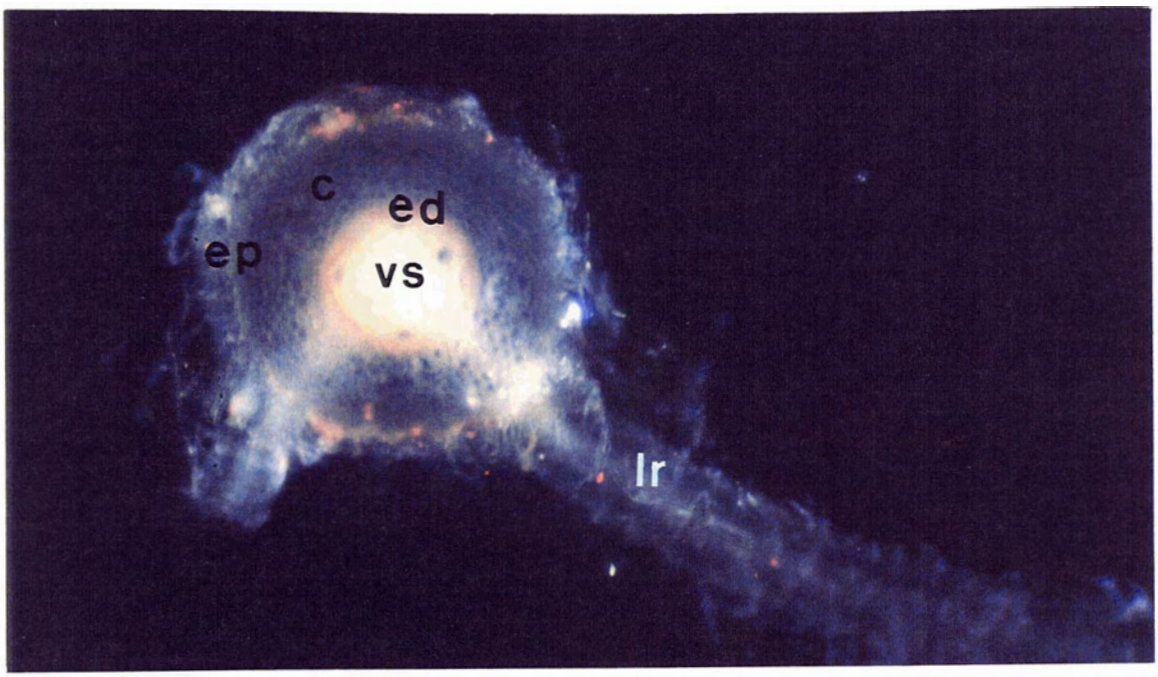


Fig. 16. Cross section of primary bean root of cv. TG treated with Sudan III and IV for suberin. Bean root were inoculated with isolate BK28 of *Chalara elegans* and sampled at 36 hours after inoculation.
lr=lateral root, ep=epidermis, c=cortex, ed=endodermis, vs=vascular system

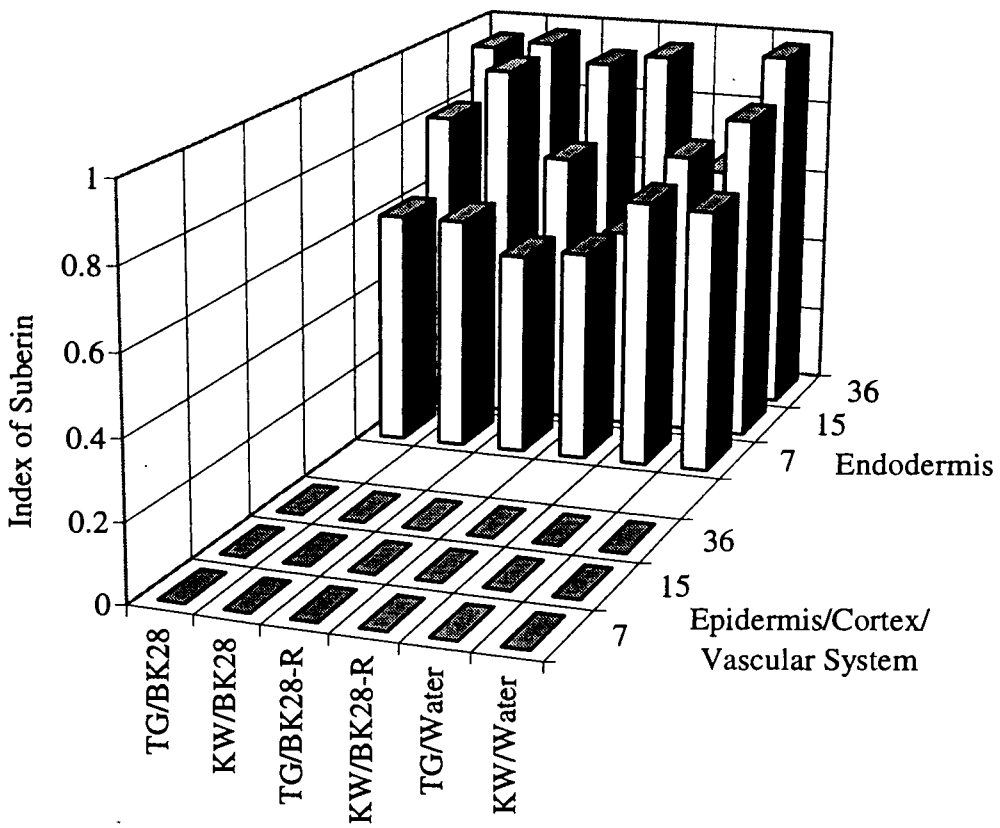


Fig 17. Index of suberin in primary root tissues of bean (*Phaseolus vulgaris*). 7, 15, and 36 are the hours after inoculation.

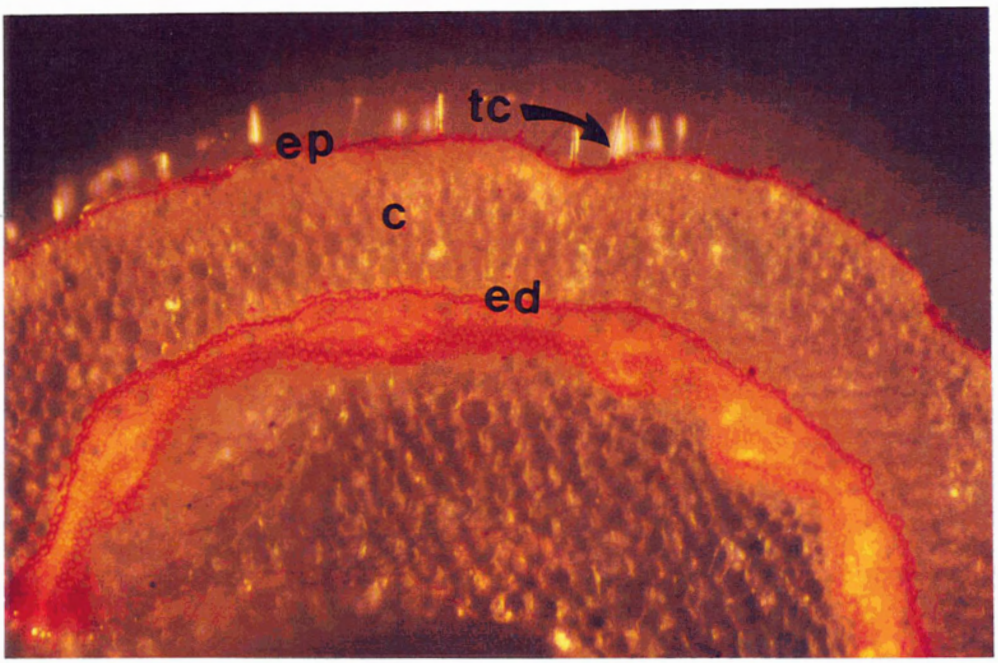


Fig. 18. Cross section of bean hypocotyl of cv. TG treated with Sudan III and IV for suberin. Hypocotyls inoculated with isolate BK28 of *Chalara elegans* and sampled at 36 hours after inoculation.
tc=trichome, ep=epidermis, c=cortex, ed=endodermis

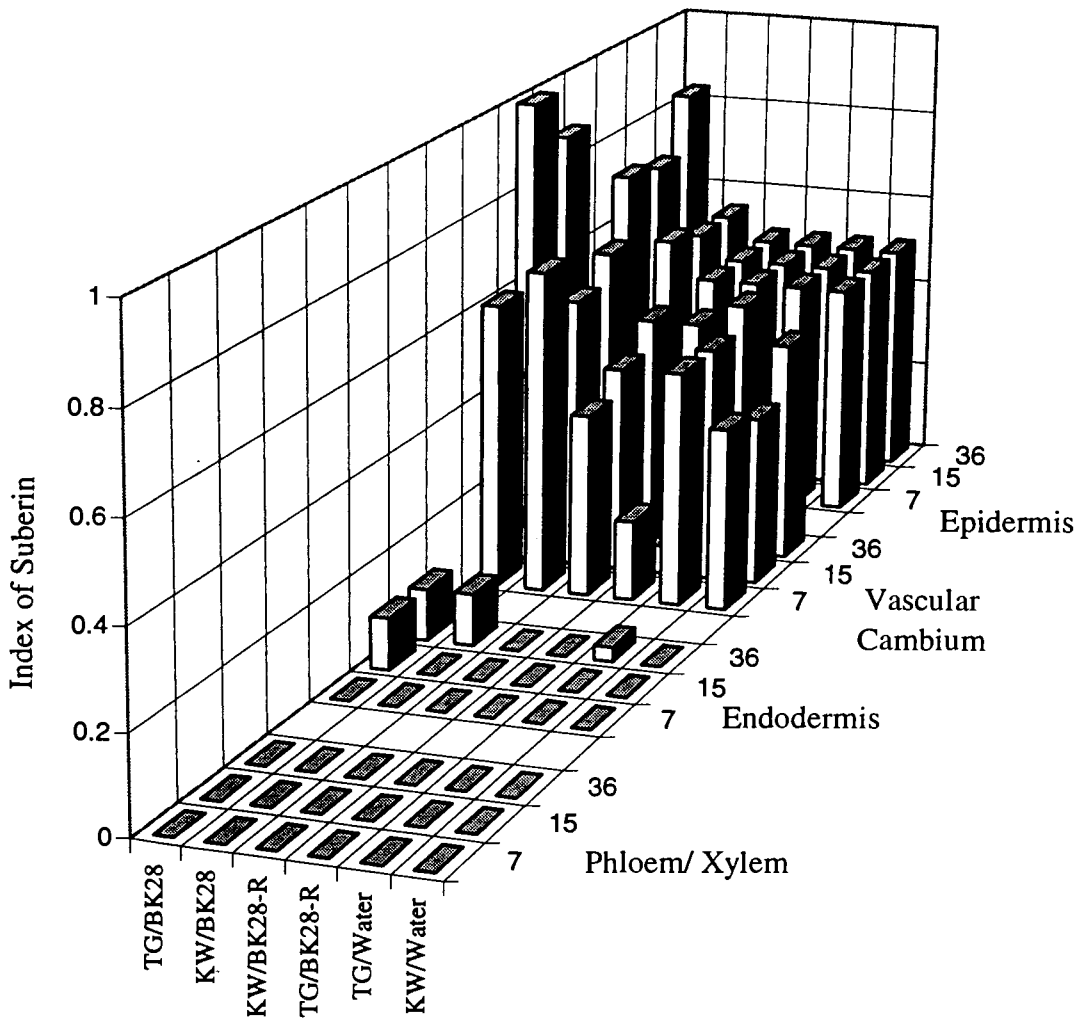


Fig. 19. Index of suberin in hypocotyl tissues of bean (*Phaseolus vulgaris*). 7, 15, and 36 are the hours after inoculation.

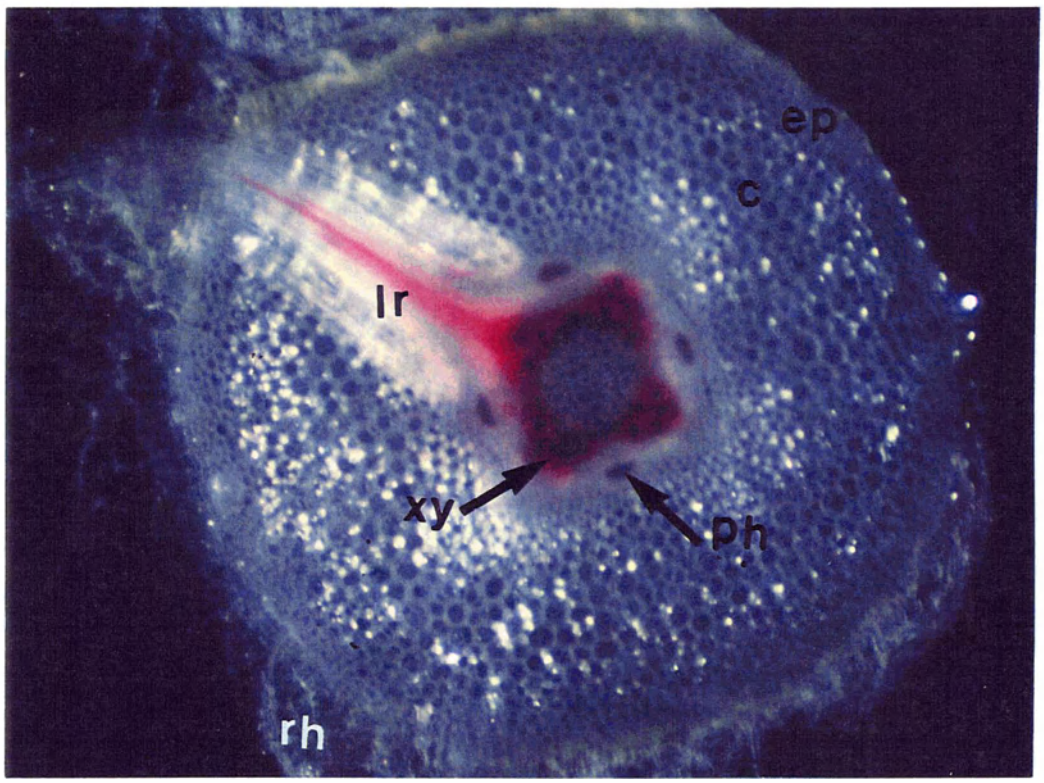


Fig. 20. Phloroglucinol-HCl test for lignin in primary root of cv. TG inoculated with isolates BK28 of *Chalara elegans* at 36 hours after inoculation.
rh=root hair, lr=lateral root, ep=epidermis, c=cortex, xy=xylem, ph=phloem

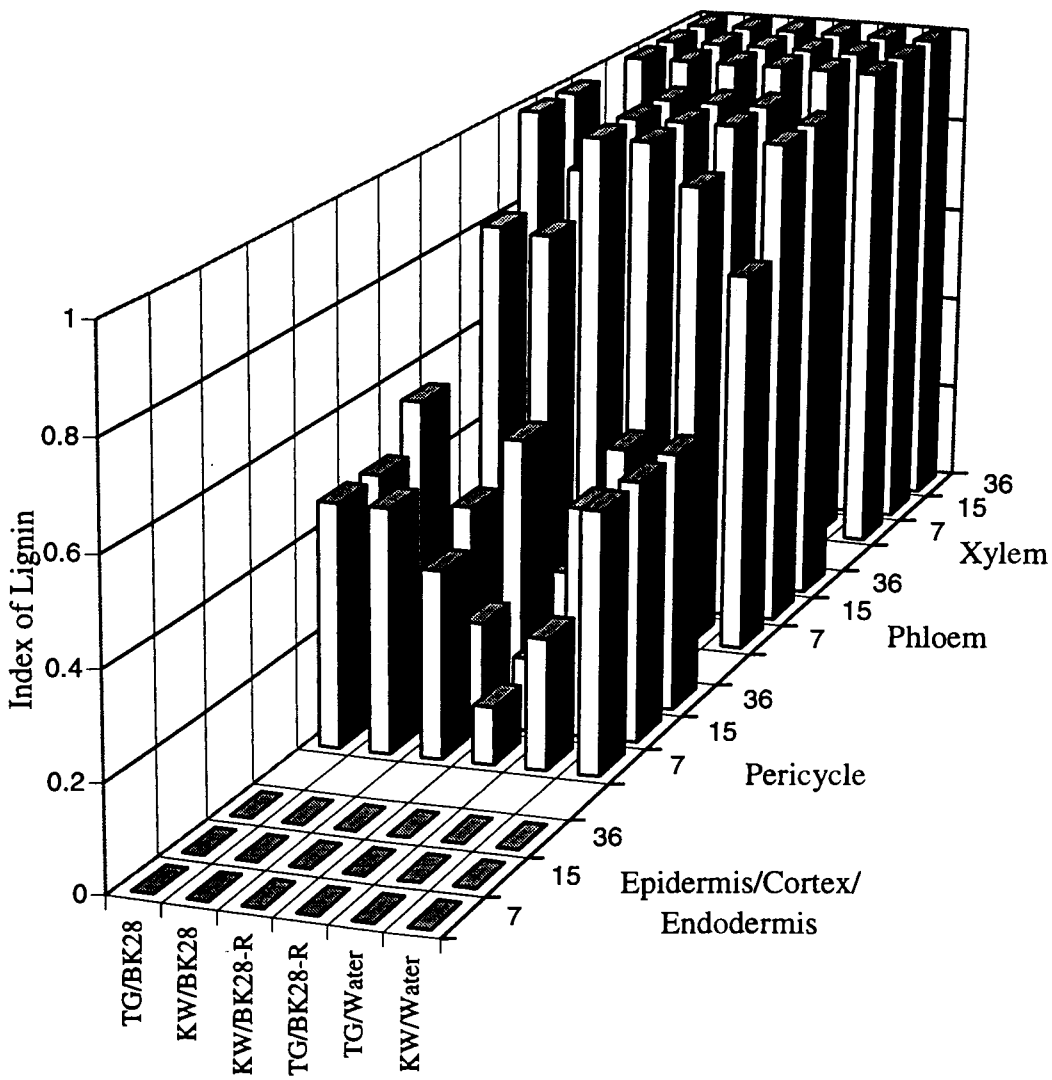


Fig. 21. Index of lignin in primary root tissues of bean (*Phaseolus vulgaris*). 7, 15, and 36 are the hours after inoculation.

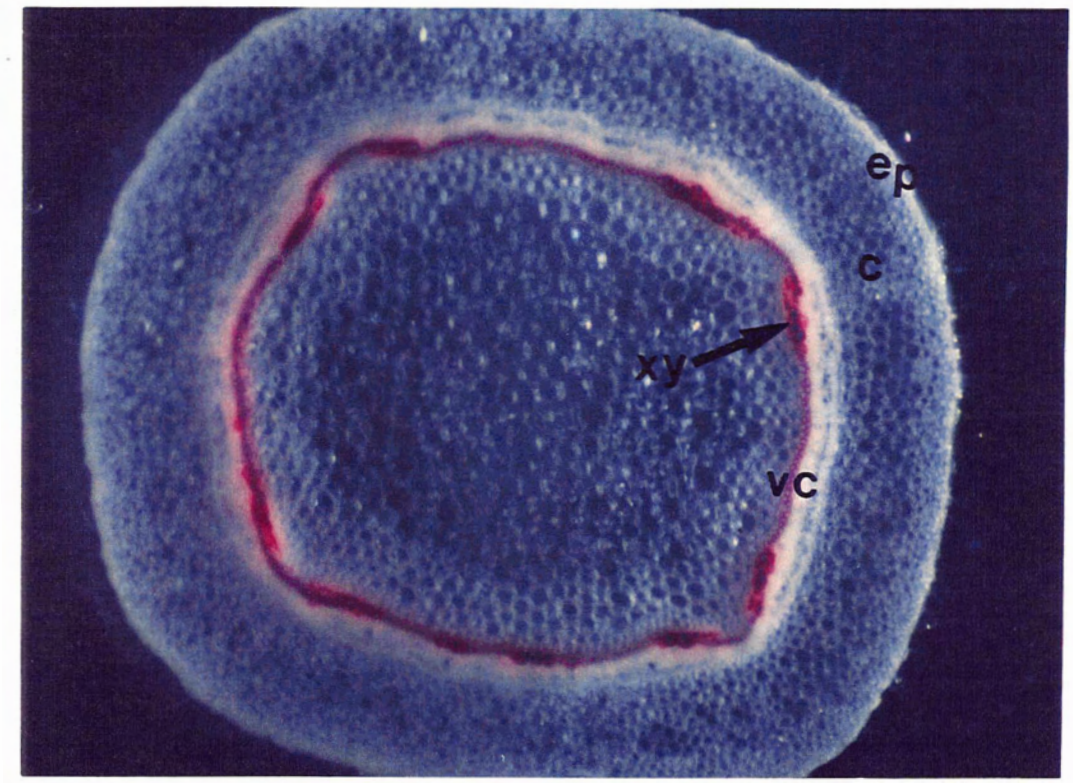


Fig. 22. Phloroglucinol-HCl test for lignin in hypocotyl of cv. TG inoculated with isolates BK28 of *Chalara elegans* and sampled at 36 hours after inoculation. ep=epidermis, c=cortex, xy=xylem, vc=vascular cambium

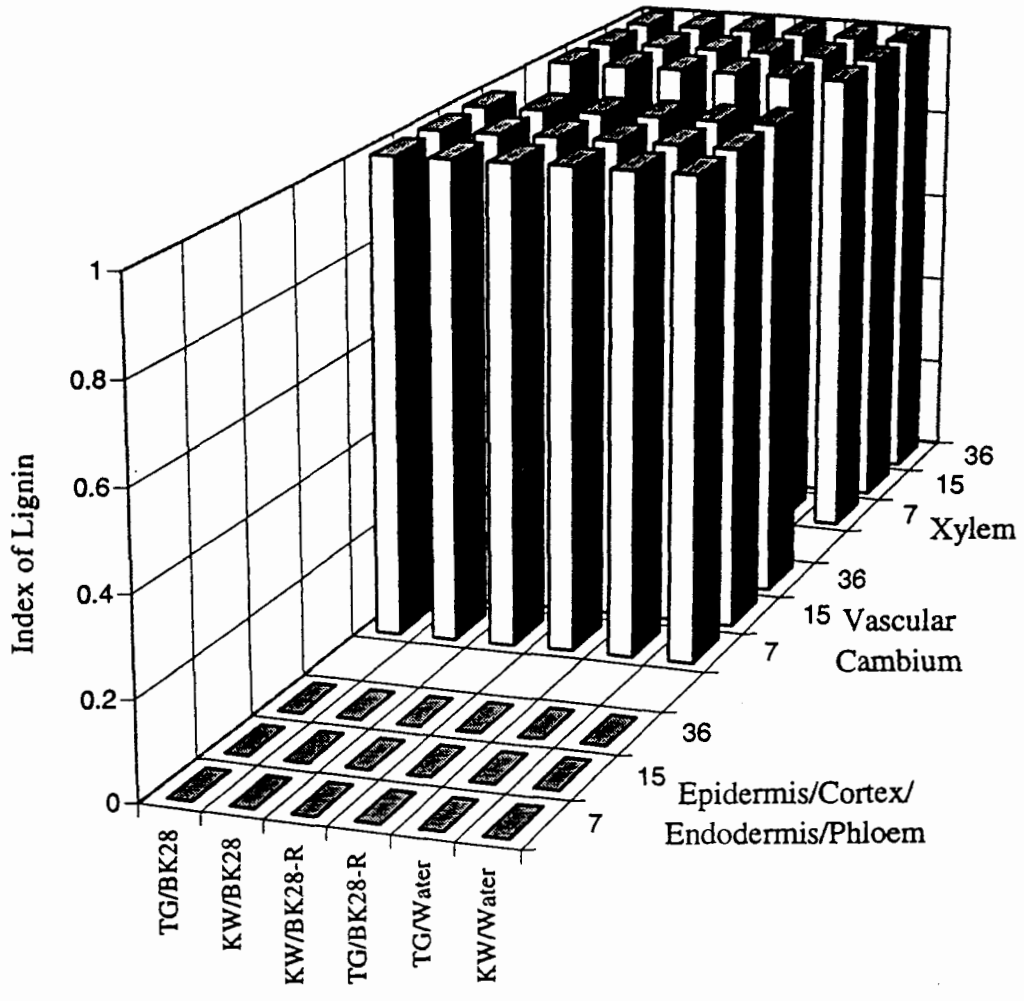


Fig. 23. Index of lignin in hypocotyl tissues of bean (*Phaseolus vulgaris*). 7, 15, and 36 are the hours after inoculation.

4. 4. Discussion

The combination of Sudan III and IV as a method to detect the presence of suberin in plant tissues gives similar results to those of other methods such as Sudan Black B, Nile Blue, residual UV fluorescence, sulfuric acid resistance and phloroglucinol quenching plus ultraviolet excitation (Brammall and Higgins, 1988; Rittinger *et al.*, 1987). The phloroglucinol-HCl test for lignin is considered to be the most sensitive among the methods for histochemical detection of lignin despite questions regarding the specificity of the test (Sherwood and Vance, 1976). For example, phloroglucinol-HCl was more sensitive than the Maule procedure or chlorine-sulfite tests (Brammall and Higgins, 1988; Rittinger *et al.,* 1987) for detection of lignin.

The suberin and lignin tests were performed with the assumption that phialospores of *C. elegans* were on the tissue surface and developing as described in Chapter III. Direct evidence for the presence of infection sites was not observed in the stained free-hand sections. Roots and hypocotyls of bean reacted differently to suberin and lignin tests. On root, suberin was found in the endodermis; while lignin was located in xylem, phloem and pericycle. In contrast, in hypocotyl, suberin was detected in epidermis, vascular cambium and sometimes in endodermis; whereas lignin was recorded in xylem and vascular cambium.

Lignin and suberin that found on endodermis and vascular system may not be involved in the early defense to the pathogen but they act as barrier to apoplastic transport system of the plant. This is because those tissues are not directly in contact with the

pathogen (Robards *et al.*, 1979), and Peterson *et al.*, (1981) suggested that suberin in the endodermis act as barrier to apoplastic transport

Lignin was not detected in the epidermis of hypocotyls and roots and is contradictive to the assumption that lignification occurs first and is followed by suberization (Kolattukudy, 1984; Rittinger *et al.*, 1987). However, it can be explained by the fact that lignin contains 3 major monomers, namely *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) (Boudet *et al.* 1995), and the phloroglucinol-HCL test only determine the guaiacyl lignin (Lewis and Yamamoto, 1990). First, there is no lignin production in bean epidermis in response to *C. elegans*. The second there is other type of lignins that could not be detected by the phloroglucinol-HCL test. The third is lignin occurs in the late interaction. Zeier and Schreiber (1997) found that structure of lignin and suberin on epidermal cells of root of *Clivia miniata* were different from those in endodermis and xylem vessels. Stockwell and Hanchey (1987) found on bean that plorogluconol-HCL test reacted positively with lesions and lesion border caused by *Rhizoctonia solani*.

According to Kolattukudy (1984), suberization of cell wall provides an effective barrier to pathogen penetration. Therefore, suberin that appeared on epidermis of hypocotyl may play a role in resistance to *C. elegans*. Results of my research indicating that suberin in epidermal cell of hypocotyls may hinder the fungal penetration was supported by the finding of lower numbers of successful infection sites on hypocotyls than root.

Chapter V

CONCLUSIONS AND FUTURE RESEARCH

Plants coexist in nature with an enormous number of potential pathogens and most plants remain healthy in that co-existence. Resistance is the rule and susceptibility is the exception (Rodriguez and Redman, 1997). Also, among the pathogenic fungi exist different levels of virulence. Questions such as what mechanisms distinguish a saprophytic fungus from a plant pathogen, and what mechanisms make one species more virulent than another have been a focus of recent research (Knogge, 1996). Another interesting question is why most pathogenic fungi only infect particular parts of their hosts. Considering the presently available understanding of these questions, this thesis aimed to unravel mechanisms that distinguish an avirulent isolate from a virulent isolate of *C. elegans* with regard to infection on root and shoot tissues of bean (*P. vulgaris*).

5.1. Relationship of Number of Phialospores to Lesion Occurrence

It was deemed important to study the effects of phialospore concentration on infection, because there is no available published information regarding this relationship for *C. elegans* on bean.

The study showed that 10 - 36 phialospores of *C. elegans* were required to initiate infections on both primary roots and hypocotyls on bean. Beyond this minimum, the number of phialospores was positively correlated with the number of lesions. Why

should more than one spore be required ? Possibly, hydrolytic enzymes may be produced by phialospores on the host surface, and a critical amount may be required for penetration.

A reproducible method of inoculation is important. The information that was obtained provided a basis for the inoculation procedures used in the research. Point inoculation sites used in the experimentats permitted detection of small differences in susceptibility between bean cultivars. This method might have application as an inoculation procedure for resistance screening of progenies in bean breeding programs.

5.2. Comparative Study of Virulent and Avirulent Isolates of *Chalara elegans* on Root and Shoot Tissues of Bean.

The present study indicated that germination of phialospores was higher on roots than on hypocotyls; the avirulent isolate did not differ from the virulent isolate in regard to the proportion of phialospores that germinated. Exudation from root tissues may have caused the higher germination on roots. Exudation plays an important role in stimulating spore germination (Hancock and Huisman, 1981). Additionally, the number of infection sites was higher on roots than on hypocotyls.

The ability to form penetration hyphae on bean tissues distinguished the avirulent from the virulent isolate of *C. elegans* on bean. The virulent isolate actively established a colonizing phase by penetrating epidermal surfaces of both root and hypocotyl tissues. In contrast, the avirulent isolate generally failed to penetrate, and in those instances where

entry was detected, the hyphae were restricted in the epidermis of hypocotyls and cortical cells of roots. Sporulation was not observed by the avirulent isolate on beans.

5.3. Suberin and Lignin in Roots and Shoots Inoculated with Virulent and Avirulent Isolates of *Chalara elegans*.

Suberin and lignin have been proposed as inducible host defense mechanisms (Kolattukuday, 1984; Bird, 1988; Ride, 1978). However, the early stage of infection by *C. elegans* on bean roots and shoots gave no indication of inducible lignin. However, because of the many forms of lignin and the specificity of particular histochemical tests, it is possible that an undetected lignification response may have occurred. Shoot epidermis stained positively for suberin; while epidermis of primary roots gave negative suberin test. The absence of suberin in epidermis of roots relates to one of physiological functions of the root, namely absorption. On the other hand, suberin in the epidermis of hypocotyls may have contributed to the lower number of lesions on hypocotyls caused by *C. elegans*.

5. 4. Future Directions

The results showed that cessation of germ tube elongation and formation of penetration hyphae were prerequisites for the pathogenesis of *C. elegans* on bean. We found that germ tubes of isolate BK28R generally kept growing and rarely penetrated. Additionally, where penetration did occur, colonization by the avirulent isolate was restricted to epidermal cells of hypocotyls and to several layers of the root cortex. Pre-

infection phases of host pathogen interaction such as attachment are current foci of research. It will be important to determine if there is a relation between attachment of the phialospores and the ability of *C. elegans* to form infection hyphae. Attachment is an important factor in fungal infection and is required in disease development (Kwon and Epstein, 1993). Attachment by ungerminated conidia and conidial germlings of *Colletotrichum graminicola* occurs on corn leaves (Mercure *et al.*, 1994).

The virulent isolate (BK28) used on this study was an isolate from cotton and contained dsRNA; the avirulent isolate (BK28R) contained no dsRNA. The presence of dsRNA has been reported for a large number of pathogens such as *Rhizoctonia solani* (Castanho *et al.*, 1978), *Pyricularia oryzae* (Hunst *et al.*, 1986), *Cryphonectria parasitica* and *Ustilago maydis* (Wood and Bozarth, 1973) and *C. elegans* (Bottacin *et al.*, 1994). Bottacin *et al.*, (1994) found that 36 of 43 strains of *C. elegans* contained dsRNA. According to Nuss and Koltin, (1990) the presence of dsRNA reduces the virulence of pathogens; however, recent information indicates that dsRNA may be associated with enhanced virulence. Isolates of *C. elegans* with reduced amounts of dsRNA that were derived from dsRNA-containing wild types were generally less virulent than the corresponding wild types (Punja, 1995). Although the relation of dsRNA to virulence is not known (Nuss and Koltin, 1990), Marcus *et al.*, (1986) showed that a virulent isolate of *R.* solani differed from a less virulent isolate containing no dsRNA in production of pectolytic enzymes; the less virulent isolate of *R. solani* produced no endopectinolyase. In my study on beans, an isolate of *C. elegans* containing no dsRNA (BK28R) generally failed to penetrate actively the intact host tissues and was unable to

colonize and sporulate on this host. The molecular basis for this phenomenon is not known. Therefore, further study is needed to know the role of dsRNA in *C. elegans*.

Lignin is derived from different phenylpropyl alcohols, namely coniferyl, sinapyl and *p*-hydroxyphenylpropyl alcolyls (Boudet *et al.*, 1995). The phloroglucinol-HCL test reportedly detects only the guaicyl structure which is derived from coniferyl alcohol (Lewis and Yamammoto, 1990). It is possible that induced lignin occurred in bean but lacked the particular structure needed to react to phloroglucinol-HCL. Therefore, experiments designed to determine the presence of other forms of lignin are needed.

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