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MYCOLOGICAL AND PATHOLOGICAL EXAMINATIONS OF CYTOSPORA CANKER OF SPRUCE

By

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ABSTRACT

MYCOLOGICAL AND PATHOLOGICAL EXAMINATIONS
OF CTYOSPORA CANKER OF SPRUCE

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Colorado blue spruce [Picea pungens] is a valuable landscape ornamental. Mature trees are often progressively disfigured by Cytospora canker. Cytospora canker is caused by Leucostoma kunzei [syn. Valsa kunzei]. The anamorphic form, Leucocytospora kunzei [syn. Cytospora kunzei] is most frequently observed. Several lines of research were undertaken to examine this fungal pathogen and disease. A collection of 487 isolates of L. kunzei were isolated from 196 cankers on 121 trees growing at several different sites. Leucocytospora kunzei was isolated from blue spruce as well as from five other coniferous host species.

Vegetative compatibility is an important biological feature of fungi. In L. kunzei, 44 vegetative compatibility groups (vc-groups), including eight multi-merge groups were identified. Vegetative compatibility groups did not segregate during conidiogenesis. The distribution patterns of vc-groups seemed to indicate a role for conidia in the dissemination of this pathogen. Isolates of L. kunzei from widely separated sites within Michigan, as well as between Michigan and Colorado isolates, were found which were in a common vc-group.

Some of the in vitro growth responses of L. kunzei were also

examined. The optimal water potential for mycelial growth was from -14 to -15 bars. The water potential of test media was adjusted by the addition of sucrose, mannitol, NaCl, KCl, or polyethylene glycol. NaCl and KCl proved to be unsuitable osmotica for use with L. kunzei. Leucocytospora kunzei grew well in vitro over a pH range of 5.0 - 7.0, optimal growth occurred at a pH of 6.2. Leucocytospora kunzei is well adapted to the internal environmental conditions of its spruce host.

In inoculation trials, using sixteen Cytospora s. lat. isolates, those isolates of L. kunzei from spruce and one from eastern white pine caused canker formation on wound inoculated branches of blue spruce. Cytospora s. lat. isolates from deciduous tree hosts did not incite canker formation on inoculated branches. Both excised and intact branches of mature trees were utilized. Excised branch inoculations reliably and accurately reflected responses in inoculated intact branches. Isolates of L. kunzei started from conidia were infective.

DEDICATION

I dedicate this dissertation to my wife and best friend, Linda Kay Leach. Her love, patience, understanding, and support have made this effort possible. I also want to dedicate it to our children, Anna Eru and Nathan Grey Leach-Proffer, who have added so much to our lives.

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SECTION 1

General Introduction

GENERAL INTRODUCTION

Colorado blue spruce (Picea pungens Engelm.) is a popular and widely planted ornamental conifer, prized for its color and dense pyramidal growth form (2). Although native to the Rocky Mountains in the western United States, blue spruce have been planted in the mid-western and northeastern part of the country as landscape trees (2). Blue spruce is unimportant as a timber species and is generally used for landscape specimens or as 'shelterbelts' or 'hedgerows' along property or lot lines. In the Midwest and Northeast, mature trees are often affected by Cytospora canker (7,14,19). It is of interest to note that reports of Cytospora canker on blue spruce in its native range are lacking.

The diffuse branch cankers associated with this disease cause the death of affected limbs (17). The needles on the affected limbs are first slightly off-colored and then progressively redden and eventually fall. Sometimes on blue spruce, the needles take on a purplish tint. Generally the lower branches are affected initially, often a single bottom limb will first be affected, which is often overlooked by the property owner. The disease, however, progressively affects limbs higher in the tree, spreading upward and laterally (3,5,7,17). Trunk cankers and tree mortality are rare in blue spruce (5,19). The aesthetic damage however, caused by the death of the branches, has a major impact on the value of this generally large and prominent

landscape tree.

Cytospora canker of blue spruce is caused by Leucostoma kunzei (Fr.) Munk ex Kern (13) [syn. Valsa kunzei] and is most frequently encountered in its anamorphic form, Leucocytospora kunzei (Sacc.) Urban [syn. Cytospora kunzei] (1,3,18,20). There are conflicting references in the literature dealing with this fungus. First, there are nomenclatural problems. For example, the combination Leucocytospora kunzei is not listed in the CMI Index of Fungi, although Urban (18) has been cited as making the combination. Author citations for Leucostoma kunzei are also not in total agreement (1,3,13,20). Secondly, Leucostoma and Leucocytospora have been considered to be subgroups within Valsa and Cytospora respectively, by some authors (19). The darkened conceptacle of compact tissues delimiting the perithecial and pycnidial stromata, however, puts this pathogen of spruce into the genus Leucostoma with its anamorphic form Leucocytospora (1,3,13,20). Leucostoma kunzei was used by Barr (1) to illustrate the genus. Pathologists should utilize this taxonomic classification.

In the United States, L. kunzei also affects other species of spruce (3,6,7,9,15,19) as well as some other species of conifers such as: eastern hemlock [Tsuga canadensis (L.) Carr.]; European larch [Larix decidua Mill.]; Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco]; eastern white pine [Pinus strobus L.]; and balsam fir [Abies balsamea (L.) Mill.] (3,8,13,19,21). Branch cankers are common in these other hosts, as in blue spruce. In addition, tree mortality has been reported on Douglas-fir (8,21) and Engelmann spruce [Picea pengelmannii Parry] (6).

Cytospora canker of blue spruce was first reported in 1933 by

Gilgut and Boyd (5). They described the symptoms of this desease as well as isolated the pathogen, Cytospora kunzei (identified by Wehmeyer). Gilgut in 1936 (4) fulfilled Koch's Postulates with the pathogen on Norway spruce [Picea abies (L.) Karst.] utilizing wound inoculations with spore suspensions (presumably conidial). The telemorph, Valsa kunzei, was associated with cankers on blue and Norway spruce by Marsden (15) in 1948 (again the fungus was identified by Wehmeyer). The work of Alma Waterman published in 1955 (19) remains as one of the foundation works on this disease and fungus. She compared fruiting structures and isolates in culture, of Valsa kunzei and the anamorphic Cytospora kunzei from various coniferous hosts. Although slight differences in perithecial stromata indicated three varieties within the species, pycnidial stromata were variable and not sufficiently different to indcate that more than one species was involved. She noted that the stromata development and marginal conceptacle were characteristic of Leucostoma and Leucocytospora as described by Von Höhnel, but still used Valsa and Cytospora for her classification scheme. In 1981, Kamiri and Laemmlen (12) reported on some of the epidemiological features of this fungus. They found conidia to be common and to be produced and viable essentially throughout the growing season, while ascospores were rare and seemed to be released only for a short time in the spring. They also studied some of the in vitro responses of the fungus in respect to temperature optima for mycelial growth and conidia germination.

Several workers have noted an association between drought stress and the severity of this disease on spruce and other hosts (6,9,19,21) in the field. Inoculation studies by Kamiri and Laemmlen in 1981 (11) and by Schoeneweiss in 1983 (16) verified these observations. Water

stress did increase the susceptibility of inoculated 5 year-old trees to canker formation by L. kunzei. Inoculation studies have also lead to some disagreement as to whether conidia are capable of causing canker initiation. While some authors found inoculations utilizing mycelium of isolates started from either conidia of ascospores to be infective (9,16,19), Kamiri and Laemmlen (11) indicated that only mycelium started from ascospores was capable of inciting canker formation on inoculated trees.

Attempts to control Cytospora canker of spruce using fungicides have been ineffective or inconclusive (10,19). The current control strategies are those recommended by Strong (17) in 1953 and include: removing infected branches and burning them, continuous monitoring for new sympotmatic branches, and fertilization and watering.

Leucocytospora kunzei has been isolated from non-symptomatic tissues (14). For that reason, the term 'affected tree' is used in preference to 'infected tree', to describe trees in which there is active canker expansion taking place. It appears that trees which show no symptoms of Cytospora canker are in fact already colonized to some extent by the fungus. Leucostoma kunzei may be part of the normal bark mycoflora, but can cause canker formation under certain predisposing stresses.

The purpose of this research was to broaden the available information dealing with the fungus Leucostoma kunzei and the disease Cytospora canker of blue spruce. Many of the areas investigated were done to supplement or answer questions posed by previous findings. Areas of investigation ranged from basic research in the in vitro growth responses of L. kunzei to more applied pathogenicity studies. In addition, a new avenue of investigation dealing with vegetative compatibility

in L. kunzei was undertaken which has provided both basic information, and has applications and implications for plant pathology. By increasing the amount of information available, this research should contribute to our efforts to manage this serious disease of an important landscape ornamental.

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SECTION 2

Vegetative compatibility groups in *Leucocytospora kunzei*

INTRODUCTION

Colorado blue spruce (Picea pungens Engelm.) is a popular and widely planted landscape ornamental in the northeastern and midwestern United States. Cytospora canker of blue spruce is an important and common disease of mature blue spruces in these areas (13,22). The diffuse branch cankers characteristic of this disease are caused by Leucostoma kunzei (Fr.) Munk ex Kern [syn. Valsa kunzei] which is most frequently seen in its anamorphic form, Leucocytospora kunzei (Sacc.) Urban [syn. Cytospora kunzei] (5,9,23). Leucocytospora kunzei also occurs on other species of spruce and various conifers (13,22). Although rarely fatal, the loss of branches due to this pathogen seriously detracts from the aesthetic value of this important landscape species.

Current control recommendations call for the removal of infected limbs as they occur. Being prized for their conical crown, which extends symmetrically down to ground level, the removal of branches also detracts from the overall appearance of these specimen trees. Attempts to use fungicides to control this pathogen have not been successful or were inconsistent in their action (18,22).

Promising results have been achieved in controlling the diffuse cankers caused by Cryphonectria parasitica (Murr.) Barr [syn. Endothia parasitica] on American chestnut using a biological control. 'Hypovirulent' isolates of C. parasitica exist which are less virulent than the normal isolates and trees can often survive and compartmentalize

infection. The 'hypovirulence' trait can be transmitted from a hypovirulent isolate of the fungus to a healthy virulent isolate *in vitro* or *in vivo* (3,4,14,15,19).

One potential benefit of developing a biological control for *L. kunzei* on blue spruce similar to the 'hypovirulence' system on American chestnut is that it may be able to prevent the aesthetic damage associated with both the disease and the current control strategy. If hypovirulent-like isolates of *L. kunzei* could be established on blue spruces then they may be able to convert any virulent isolates of *L. kunzei* present or introduced later, thus preventing limb losses. Because of their high value and use as ornamental trees, labor and monetary considerations would probably be tolerable.

Transmission of hypovirulence in *C. parasitica* requires an anastomosis between donor and recipient isolates and the transfer of a double stranded RNA 'virus-like particle'(3,4,14,15,19). Vegetative compatibility refers to the ability of two hyphae to make contact, fuse, and exchange nuclear and/or cytoplasmic materials (19). In *C. parasitica* there are many vegetative compatibility groups (vc-groups) (1,19,21). Vegetative compatibility, or rather incompatibility, has been shown to limit the effectiveness of using hypovirulence as a biological control, due to its regulation of the anastomosis process. Vegetative compatibility groups (vc-groups) have been documented in several fungi (1,6,8,10,11, 12,16,17,20) and may present a complicating factor in developing a biological control program.

The main purpose of this investigation was to examine vegetative compatibility in *L. kunzei*. A large collection of *L. kunzei* isolates was needed to accomplish this. Additionally during the culturing of

the isolates, visual screening for irregular isolates of unusual cultural morphology could be conducted. Hypovirulent isolates of C. parasitica have often had an altered cultural morphology (19).

MATERIALS AND METHODS

Isolate collection- Cankered branches of blue spruce and other conifers were removed from the trees. The trees were located in various Michigan cities and from one site near Colorado Springs, Colorado. Most of the trees were located in residential areas, while some were from cemetary plantings. Records were maintained of the species of tree, the location, and the proximity of other cankered trees. The collections were made from 1983 through 1985.

Isolation and culturing of L. kunzei- Cankered branches were brought into the laboratory and isolations were made either from infected host tissue or from conidial cirrhi. For tissue isolations, the cankered branch was surface disinfested by wiping with 95% ethanol and flaming. The bark was then aseptically removed. Pieces of the underlying discolored cambial/cortical tissues were removed and placed in petri plates containing Potato Dextrose Agar (PDA). When possible, isolations were made from tissues at the interface of healthy and diseased areas. For conidial isolations, cankered branch segments were soaked in a 5% sodium hypochlorite solution for 10 minutes, rinsed with distilled water, and placed in a moist chamber. Cirrhi of conidia could soon be seen on the surface of the branch and could be collected. Isolates were started from masses of conidia and/or from a single conidium. Single conidium isolates were obtained by a combination of a dilution series

of a spore suspension and streak plating the conidial suspension onto PDA. Isolate records were maintained which indicated the host of the isolate, the particular canker on the tree, and in some cases, the location along the cankered branch where the isolate was taken.

Cultures were transfered to a spruce decoction agar maintenance medium (SBA). SBA is prepared by soaking approximately 100 g of spruce bark shavings and twigs in 1000 ml of distilled water at 95°C for 45 minutes. The solution was filtered through four layers of cheesecloth and 20 g of Difco Bacto-Agar was added per liter of decoction fluid. This was then sterilized in an autoclave and later poured into 100X15 mm petri plates. The identity of the resulting cultures was verified through microscopic observation of the developing fruiting bodies and by cultural morphology on PDA, SBA, and 2% Malt Extract Agar (MEA). Colonies with unusual cultural morphology were looked for.

Coding system- A coding system was devised to keep track of the isolates. The code has four main parts. The key to the code is shown in Figure 1. The site codes are listed in Table 1.

VC-groups: isolate conditioning- Pilot experiments showed that for optimal results, the L. kunzei isolates to be paired needed to be preconditioned. Each isolate was transfered to, and grown on 2% Water Agar (WA) prior to the pairing step. Isolates were allowed to grow in the dark at 26°C. Isolates of L. kunzei grow slowly on WA but remain viable and conditioned as long as the WA medium is not allowed to dry out.

VC-groups: isolate pairing- The isolates were paired to determine vc-groups, adapting the systems used with other fungi (1). Plugs of each isolate to be paired were cut from the WA conditioning plates

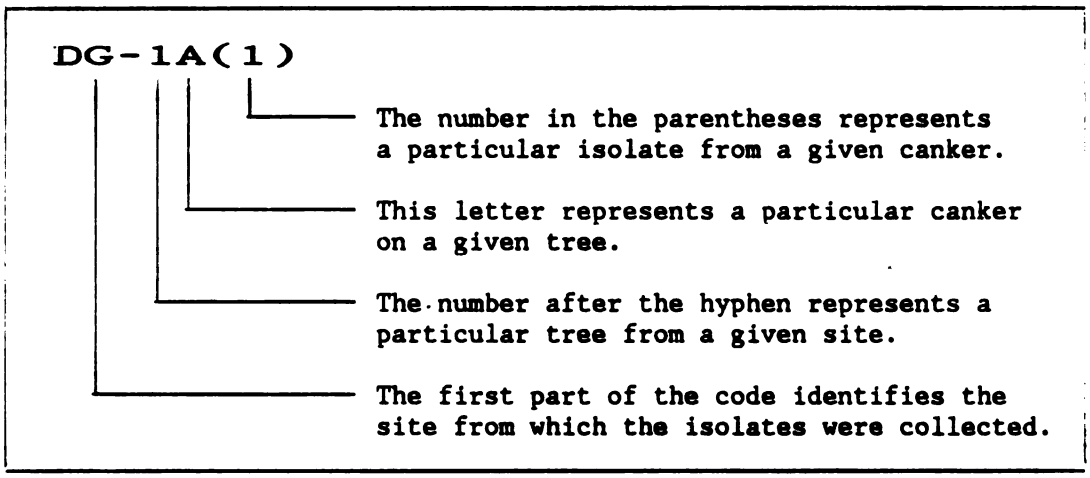

FIGURE 1. The coding system used to identify isolates of Leucocytospora kunzei.

TABLE 1

Collection sites from which isolates of *Leucocytospora kunzei* were obtained.

Site Code	Location
BF	Northwest corner of Beaumont and Forest Roads, Michigan State University, East Lansing, MI
COL	Colorado Springs, Colorado[1]
DG	Dow Gardens, Midland, MI
EL	East Lansing, MI
F	Frankenmuth, MI
FK	Franklin, MI
H	Haslett, MI
HL	Holt, MI
M	Midland, MI
MAS	Mason, MI
MSU	Michigan State University, East Lansing, MI
OK	Okemos, MI
R	Richville, MI
V	Vassar, MI
BC	Bay City, MI

[1] These isolates of *L. kunzei* came from a forest stand of Engelmann spruce growing at Kenosa Pass, Pike National Forest. These isolates are the only ones isolated from a natural planting of spruce.

(the plugs were 5 mm in diameter) and transfered to 100X15 mm petri plates containing 40-45 ml of PDA. Plugs were placed 1 cm from each other. Twenty one plugs could be placed on each pairing plate. A standardized grid pattern was used (Figure 2) to facilitate placement and record keeping. All isolates were paired to themselves (as controls) and to each of the other isolates. There were two replicates of each pairing plate. The pairing plates were sealed with Parafilm M®, kept in low light at 26°C, and examined daily.

VC-groups: evaluation- VC-groups were determined as previously described by Anagnostakis (1). Colonies of isolates within the same vc-group merge together. A black, barrage-like, reaction zone develops along the line of contact between two isolates contained in different vc-groups.

VC-groups: conidiogenesis- Vegetative compatibility among conidial isolates taken from cankers and from tissue isolates from the same canker were examined. In a separate experiment, 80 single conidium isolates obtained from a mass of conidia produced by a single pycnidium were cultured and paired for vc-grouping. The pycnidia were being produced on an excised branch inoculated with an isolate of L. kunzei [EL-16B(5)] and sporulation was induced as previously described.

RESULTS

Isolate collection- A total of 487 isolates of L. kunzei were isolated and cultured (Table 2). The isolates were collected from a total of 196 cankers from 121 trees. Most of the isolates were from blue spruce. Isolates of L. kunzei were also isolated from Engelmann, white, and Norway spruce; eastern white pine; eastern hemlock; and

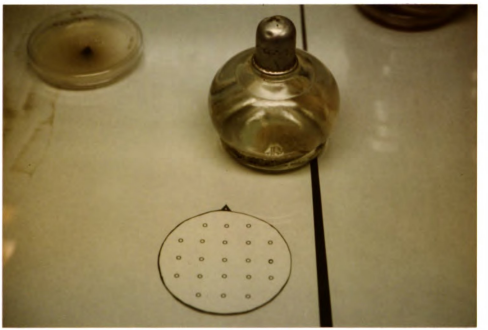

FIGURE 2. Vegetative compatibility pairing plates.

A standardized grid was used to maintain consistant distances between the isolate plugs. A total of 21 plugs could be used on one pairing plate.

TABLE 2

List of isolates of *Leucocytospora kunzei*: original host and source.

Isolate	Host Species	Source[a]
BC-1A(1)	*Picea pungens*	T
-1B(1)	"	T
-3A(1)	"	T
-3B(2)	"	T
BF-6A(1)	"	T
-6A(2)	"	T
-6A(3)	"	MC
-6B(1)	"	T
-6B(2)	"	T
-6B(3)	"	T
-6B(4)	"	T
-6C(1)	"	T
-6E(1)	"	T
BF-7A(1)	"	T
-7A(2)	"	MC
-7B(1)	"	T
-7C(1)	"	T
-7C(2)	"	T
-7C(5)	"	T
-7C(6)	"	T
-7C(7)	"	MC
-7D(1)	"	T
-7D(2)	"	T
-7D(3)	"	T
-7D(5)	"	MC
-7D(10)	"	SC
-7D(11)	"	SC
-7E(1)	"	T
-7E(2)	"	MC
-7E(10)	"	SC
-7F(1)	"	T
-7F(2)	"	T
-7F(3)	"	MC
-7G(1)	"	T
-7G(5)	"	T
-7G(6)	"	T

[a] Souces of isolate: T-infected tissue, MC-mass of conidia, SC-single condidium.

TABLE 2 (cont'd.)

Isolate	Host Species	Source[a]
BF-7H(1)	Picea pungens	T
-7H(2)	"	T
-7H(3)	"	MC
-7H(4)	"	MC
-7I(1)	"	T
-7I(2)	"	T
BF- WP	Pinus strobus	T
COL-1A(1)	Picea engelmannii	T
-1A(2)	"	T
-1A(3)	"	T
-1A(4)	"	T
-1A(5)	"	T
COL-3A(1)	"	T
-3A(2)	"	T
-3A(3)	"	T
-3A(4)	"	T
-3A(5)	"	T
COL-4A(1)	"	T
-4A(2)	"	T
-4A(3)	"	T
-4A(4)	"	T
COL-5A(1)	"	T
-5A(2)	"	T
-5A(3)	"	T
COL-6A(1)	"	T
-6A(2)	"	T
-6A(3)	"	T
-6A(4)	"	T
-6A(6)	"	T
-6A(7)	"	T
DG-1A(1)	Picea pungens	T
-1A(2)	"	T
-1A(3)	"	T
-1B(1)	"	T
-1B(2)	"	T
DG-2A(1)	"	T
-2A(2)	"	T
-2A(3)	"	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

TABLE 2 (cont'd.)

Isolate	Host Species	Source[a]
DG-3A(1)	Picea pungens	T
-3A(2)	"	T
-3A(3)	"	T
-3A(4)	"	T
DG-4A(1)	Picea glauca	T
-4A(2)	"	T
-4B(2)	"	T
-4D(1)	"	T
-4D(2)	"	T
EL-2B(3)	Picea pungens	T
-2B(4)	"	T
EL-3A(1)	"	T
-3A(2)	"	T
-3A(3)	"	T
-3A(4)	"	T
EL-8A(1)	Picea abies	T
-8A(2)	"	T
-8A(3)	"	T
EL-9A(1)	"	T
-9A(2)	"	T
-9B(1)	"	T
-9B(2)	"	T
-9B(3)	"	T
-9C(1)	"	T
EL-10A(1)	Picea pungens	T
-10B(1)	"	T
-10B(2)	"	T
EL-12A(1)	"	T
-12B(1)	"	T
-12B(2)	"	T
EL-13B(1)	Pseudotsuga menziesii	T
-13B(2)	"	T
EL-14A(1)	Picea pungens	T
-14A(2)	"	T
-14A(3)	"	T
-14A(4)	"	T
EL-15A(3)	"	T
-15A(4)	"	T
EL-16B(1)	"	T
-16B(2)	"	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

Table 2 (cont'd.)

Isolate	Host Species	Source[a]
EL-16B(3)	Picea pungens	T
-16B(4)	"	T
EL-17A(1)	"	T
-17A(2)	"	T
-17A(3)	"	T
-17B(1)	"	T
-17B(3)	"	T
-17B(4)	"	T
-17B(5)	"	T
-17B(6)	"	T
EL-21A(3)	Picea glauca	T
-21B(1)	"	T
-21B(2)	"	T
-21B(3)	"	T
EL-22A(2)	"	T
-22A(3)	"	T
EL-23A(1)	Picea pungens	T
-23B(1)	"	T
-23B(2)	"	T
EL-24A(2)	"	T
EL-25A(1)	"	T
-25A(2)	"	T
-25A(3)	"	T
EL-26B(1)	Picea glauca	T
-26B(2)	"	T
EL-27A(1)	"	T
-27A(2)	"	T
-27A(3)	"	T
-27B(1)	"	T
-27B(2)	"	T
-27C(1)	"	T
-27C(2)	"	T
-27D(1)	"	T
-27D(2)	"	T
EL-28A(1)	"	T
-28A(2)	"	T
EL-29A(1)	"	T
-29A(2)	"	T
-29B(1)	"	T
-29B(2)	"	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

TABLE 2 (cont'd.)

Isolate	Host Species	Source[a]
EL-29C(1)	Picea glauca	T
-29C(2)	"	T
EL-30A(1)	"	T
-30A(2)	"	T
-30A(3)	"	T
-30B(1)	"	T
-30B(2)	"	T
-30B(3)	"	T
-30B(4)	"	T
-30C(1)	"	T
-30C(2)	"	T
EL-31A(1)	Picea pungens	T
-31B(1)	"	T
-31B(2)	"	T
-31C(1)	"	T
-31C(2)	"	T
F-1A(1)	"	T
-1A(2)	"	T
F-2C(1)	"	T
-2C(2)	"	T
F-3A(3)	"	T
F-4A(1)	"	T
-4A(2)	"	T
-4B(1)	"	T
-4B(2)	"	T
F-5B(1)	"	T
-5B(2)	"	T
F-6A(1)	"	T
-6A(2)	"	T
F-7A(1)	"	T
-7A(3)	"	T
-7A(4)	"	T
F-8B(1)	"	T
-8B(2)	"	T
-8B(3)	"	T
F-9A(1)	"	T
-9A(2)	"	T
-9A(3)	"	T
F-10A(1)	Picea abies	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

TABLE 2 (cont'd.)

Isolate	Host Species	Source[a]
F-10A(2)	Picea abies	T
F-11A(1)	"	T
F-12A(1)	"	T
-12A(2)	"	T
-12A(3)	"	T
-12A(4)	"	T
FK-1A(1)	Picea pungens	T
-1A(2)	"	T
-1A(3)	"	T
-1A(4)	"	T
-1A(5)	"	T
-1B(1)	"	T
-1B(2)	"	T
-1B(3)	"	T
-1B(4)	"	T
-1C(2)	"	T
-1D(1)	"	T
-1D(2)	"	T
-1D(3)	"	T
FK-2B(1)	"	T
-2B(2)	"	T
H-1B(1)	"	T
H-2B(1)	Picea glauca	T
H-3A(3)	"	T
H-4A(2)	Picea pungens	T
H-5A(2)	"	T
-5B(2)	"	T
-5C(1)	"	T
-5C(2)	"	T
-5C(5)	"	T
-5D(1)	"	T
-5D(2)	"	T
-5D(3)	"	T
H-6A(1)	"	T
-6A(2)	"	T
H-7D(1)	"	T
-7D(2)	"	T
H-8A(1)	Picea glauca	T
-8B(1)	"	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

TABLE 2 (cont'd.)

Isolate	Host Species	Source[a]
H-8B(2)	Picea glauca	T
-8C(1)	"	T
-8C(2)	"	T
H-9A(1)	Picea pungens	T
-9A(2)	"	T
-9B(1)	"	T
-9B(2)	"	T
H-10A(1)	"	T
-10A(2)	"	T
-10B(1)	"	T
-10B(2)	"	T
-10C(1)	"	T
H-11A(1)	"	T
-11A(2)	"	T
-11B(1)	"	T
-11C(1)	"	T
-11C(2)	"	T
H-12A(1)	Picea glauca	T
HL-1B(1)	Picea pungens	T
-1B(2)	"	T
-1B(3)	"	T
-B(4)	"	T
HL-2C(1)	"	T
-2C(2)	"	T
-2C(3)	"	T
-2C(4)	"	T
HL-3A(1)	Picea abies	T
-3A(2)	"	T
-3A(3)	"	T
-3A(4)	"	T
-3A(6)	"	T
-3A(7)	"	T
HL-4A(2)	Picea pungens	T
-4B(1)	"	T
-4B(2)	"	T
-4B(3)	"	T
-4B(4)	"	T
-4C(1)	"	T
-4C(3)	"	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

TABLE 2 (cont'd.)

Isolate	Host Species	Source[a]
HL-4D(1)	Picea pungens	T
-4D(2)	"	T
-4D(3)	"	T
HL-5C(1)	"	T
-5C(2)	"	T
-5C(3)	"	T
-5D(1)	"	T
-5D(2)	"	T
-5D(3)	"	T
HL-6A(1)	"	T
-6A(2)	"	T
-6A(3)	"	T
-6A(4)	"	T
HL-7A(1)	"	T
-7A(2)	"	T
-7A(3)	"	T
-7A(4)	"	T
-7B(1)	"	T
-7B(2)	"	T
-7C(1)	"	T
-7C(2)	"	T
-7C(3)	"	T
-7C(4)	"	T
HL-8A(1)	Tsuga canadensis	T
-8B(1)	"	T
-8B(2)	"	T
-8B(3)	"	T
-8B(4)	"	T
-8B(5)	"	T
-8B(6)	"	T
M-1A(1)	Picea pungens	T
-1A(2)	"	T
-1A(3)	"	T
M-2A(1)	"	T
-2A(2)	"	T
M-3A(1)	Picea abies	T
-3A(2)	"	T
M-4B(1)	Picea pungens	T
-4B(2)	"	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

TABLE 2 (cont'd.)

Isolate	Host Species	Source[a]
M-5A(1)	Picea pungens	T
M-6B(2)	"	T
M-7A(3)	"	T
-7A(4)	"	T
-7B(1)	"	T
-7B(2)	"	T
-7B(3)	"	T
MAS-1B(3)	"	T
MAS-2A(1)	Picea abies	T
MAS-3A(5)	"	T
-3A(6)	"	T
-3A(7)	"	T
-3A(8)	"	T
MAS-4B(1)	Picea pungens	T
-4B(2)	"	T
-4B(3)	"	T
-4B(4)	"	T
-4B(5)	"	T
-4B(6)	"	T
-4D(1)	"	T
-4D(2)	"	T
-4D(3)	"	T
-4D(4)	"	T
-4D(5)	"	T
MAS-5A(1)	"	T
-5A(2)	"	T
-5B(1)	"	T
-5B(2)	"	T
-5B(3)	"	T
-5B(4)	"	T
-5B(5)	"	T
-5B(6)	"	T
-5C(1)	"	T
-5C(2)	"	T
-5C(3)	"	T
-5C(4)	"	T
MSU-1A(1)	"	T
MSU-2A(1)	"	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

TABLE 2 (cont'd.)

Isolate	Host Species	Source[a]
MSU-2A(2)	Picea pungens	T
-2A(3)	"	T
-2B(1)	"	T
-2B(2)	"	T
-2B(3)	"	T
MSU-3A(1)	"	T
MSU-4A(1)	"	T
MSU-5B(1)	Picea abies	T
-5B(2)	"	MC
-5B(3)	"	MC
-5B(4)	"	SC
-5B(5)	"	SC
-5B(6)	"	SC
-5B(7)	"	SC
-5B(8)	"	SC
-5B(9)	"	SC
-5B(10)	"	SC
-5B(11)	"	SC
-5B(12)	"	SC
OK-1A(1)	Picea pungens	T
-1A(2)	"	T
-1B(1)	"	T
-1B(2)	"	T
-1B(3)	"	T
OK-2A(1)	"	T
-2A(2)	"	T
-2C(1)	"	T
-2C(2)	"	T
OK-3C(1)	"	T
-3C(2)	"	T
-3C(3)	"	T
OK-4A(1)	"	T
-4A(2)	"	T
-4B(1)	"	T
-4B(2)	"	T
-4B(3)	"	T
-4B(4)	"	T
OK-5A(2)	"	T
OK-6C(1)	Picea glauca	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

TABLE 2 (cont'd.)

Isolate	Host Species	Source[a]
OK-7A(1)	Picea glauca	T
-7A(2)	"	T
OK-8C(1)	Picea pungens	T
-8C(2)	"	T
OK-9A(1)	Picea glauca	T
-9A(2)	"	T
-9A(3)	"	T
-9B(1)	"	T
-9B(2)	"	T
-9C(1)	"	T
-9C(2)	"	T
-9C(3)	"	T
OK-10A(1)	"	T
-10A(2)	"	T
-10B(1)	"	T
-10B(2)	"	T
-10B(3)	"	T
-10C(1)	"	T
-10C(2)	"	T
OK-11A(1)	Picea pungens	T
-11A(2)	"	T
-11B(1)	"	T
-11B(2)	"	T
OK-12A(1)	"	T
-12A(2)	"	T
-12B(1)	"	T
-12B(2)	"	T
-12B(3)	"	T
R-1A(1)	"	T
-1A(2)	"	T
-1B(1)	"	T
-1B(2)	"	T
-1B(3)	"	T
-1B(4)	"	T
R-2A(1)	"	T
-2A(2)	"	T
-2A(3)	"	T
-2A(4)	"	T
-2B(1)	"	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

TABLE 2 (cont'd.)

Isolate	Host Species	Source[a]
R-2B(2)	Picea pungens	T
-2B(3)	"	T
-2C(1)	"	T
-2C(2)	"	T
-2C(3)	"	T
V-1A(1)	"	T
-1A(2)	"	T
-1A(3)	"	T
-1D(1)	"	T
-1D(2)	"	T
V-2B(1)	"	T
-2B(2)	"	T
-2B(3)	"	T
-2C(1)	"	T
-2C(2)	"	T
-2E(1)	"	T
-2E(2)	"	T
-2E(3)	"	T
V-3A(1)	"	T
V-4D(1)	"	T
-4E(1)	"	T
-4E(2)	"	T
V-5B(1)	"	T
-5B(2)	"	T
-5B(3)	"	T
-5C(1)	"	T
-5C(2)	"	T
-5C(3)	"	T
-5C(4)	"	T
V-6B(2)	"	T
V-7A(1)	Picea glauca	T
-7A(2)	"	T
-7A(3)	"	T
V-8A(1)	Picea pungens	T
-8A(2)	"	T
-8A(3)	"	T
-8C(1)	"	T
-8C(2)	"	T
-8C(3)	"	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

TABLE 2 (cont'd.)

Isolate	Host Species	Source[a]
V-9A(1)	Picea pungens	T
-9A(2)	"	T
-9D(1)	"	T
-9D(2)	"	T
V-10D(1)	"	T
V-11A(2)	Picea abies	T
-11A(3)	"	T
V-12A(1)	Picea glauca	T
V-13A(1)	"	T
-13A(2)	"	T
-13A(3)	"	T
V-14A(1)	"	T
-14A(2)	"	T
-14A(3)	"	T
V-15A(1)	Picea pungens	T
-15A(2)	"	T
V-16B(1)	"	T
V-17A(1)	"	T

[a] Sources of isolate: T-infected tissue, MC-mass of conidia, SC-single conidium.

Douglas-fir.

Cultural morphology- The appearance of L. kunzei isolates in culture is affected by the type of medium and by lighting. On all of the media, the colonies were generally appressed, without any 'fluffy' aerial hyphae. Colonies generally started out buff colored and in the light progressively turned amber, and then darkened to a grey/brown. On any given medium the isolates all looked similar. However, there were a few exceptions. Several isolates remained white even under the lights. Isolates which remained white were: EL-10B(1), F-2C(1)(2), V-4D(1), V-10D(1)(2), and HL-7B(1)(2), 8 isolates in all. Isolate EL-21A(3) which was isolated from a white spruce grew slowly in culture.

Vegetative compatibility groups- The colonies of the L. kunzei isolates grew out from the WA plugs which were placed on the PDA pairing plates and made contact with the neighboring colonies (Figure 3.). After 7 to 10 days the type of response could be scored. Using the collection of 487 isolates at least 44 vc-groups were identified within L. kunzei. Of these 44 vc-groups, 36 single discrete groups were found (Table 3.) and 8 multi-merge groups were noted (Table 4.). Multi-merge groups showed compatible reactions to isolates contained in different vc-groups, which were themselves incompatible (Figure 4.).

VC-groups: conidiogenesis- In those cases where isolates of L. kunzei were started from tissue and from conidia produced on the same canker, the isolates were always in the same vc-group. Conidial isolates from a given pycnidium were also in the same vc-group upon testing. In the supporting experiment, 80 monoconidium isolates obtained from the cirrhi of a single pycnidium were paired and all were contained in the same vc-group as the original isolate producing the pycnidium.

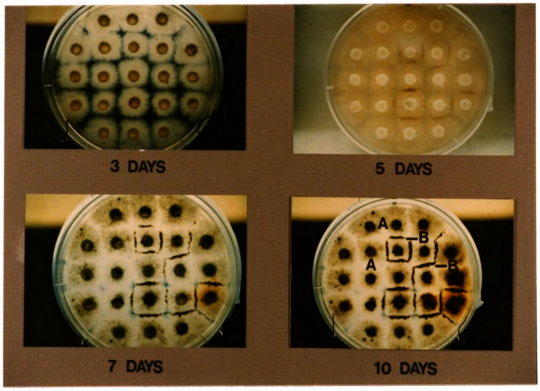

FIGURE 3. **Determination of vegetative compatibility groups.**

After the pairings are started the colonies expand and make contact. Compatible colonies merge along the line of contact (A). In the incompatible reaction, a black 'barrage-like' reaction line develops along the line of contact of the two incompatible colonies (B).

TABLE 3

Vegetative compatibility groups in *Leucocytospora kunzei*.

VC-GROUP	Isolates in the vc-group
1.	R-1A(1,2)
2.	R-1B(1,2,3,4)
3.	R-2A(1,2,3,4), R-2B(1,2,3), R-2C(1,2,3)
4.	EL-10B(1), F-2C(1,2), HL-7B(1,2)
5.	F-1A(1,2), F-4B(1,2), M-3A(1,2), H-9A(1,2), H-11A(1,2)
6.	F-3A(3), F-4A(1,2)
7.	F-5B(1,2), F-7A(1,3,4), FK-1A(1,2,3,4,5), FK-1B(1,2,3,4), HL-2C(1,2,3,4), HL-3A(1,2,3,4,6,7)
8.	F-6A(1,2), F-8B(1,2,3), H-1B(1,2), H-2B(1), H-3A(3)
9.	F-9A(1,2,3), F-12A(1,2,3,4), M-4B(1,2), M-5A(1), M-7A(3,4), M-6B(2), F-10A(1,2), F-11A(1)
10.	DG-1A(1,2,3), DG-1B(1,2), DG-2A(1,2,3)
11.	DG-3A(1,2,3,4), V-4E(1,2), V-7A(1,2,3), V-8A(1,2,3), EL-17B(1,3,4,5)
12.	DG-4A(1,2), DG-4B(2), V-11A(2,3), H-4A(2), H-12A(1), EL-25A(1,2,3)
13.	DG-4D(1,2), M-7B(1,2,3), MSU-5B(1,2,3,4,5,6,7,8,9, 10,11,12)
14.	M-1A(1,2,3), V-15(1,2), V-16B(1), V-17A(1)
15.	M-2A(1,2)
16.	MSU-2A(1,2,3)
17.	MSU-2B(1,2,3), FK-2B(1,2), EL-16B(1,2,3,4), EL-27C(1,2), EL-28A(1,2), EL-30A(1,2,3), EL-30B(1,2,3,4), EL-30C(1,2), EL-15A(3,4)

TABLE 3 (cont'd.)

VC-GROUP	Isolates in the vc-group
18.	BC-3A(1), BC-3B(2)
19.	EL-3A(1,2,3,4), EL-6A(1,2,3)
20.	BF-6A(1,2,3), BF-6B(1,2,3,4), BF-6C(1), BF-6E(1), BF-7A(1,2), BF-7B(1)
21.	BF-7C(1,2,5,6,7), BF-7D(1,2,3,4,5,10,11), BF-7E(1,2,10), BF-7F(1,2,3), BF-7G(1,5,6), BF-7H(1,2,3,4), BF-7I(1,2), EL-29A(1,2), EL-29B(1,2), EL-29C(1,2), EL-31A(1), EL-31B(1,2), EL-31C(1,2), EL-26B(1,2), EL-27A(1,2,3), EL-27B(1,2), EL-27D(1,2), EL-21A(3), EL-21B(1,2,3), EL-22A(2,3), OK-1A(1,2), OK-1B(1,2,3), OK-4A(1,2), OK-4B(1,2,3,4), OK-5A(2), OK-6C(1), OK-7A(1,2), OK-8C(1,2), OK-9A(1,2,3), OK-9B(1,2), OK-9C(1,2,3), OK-10A(1,2), OK-10B(1,2,3), OK-10C(1,2), OK-11A(1,2), OK-11B(1,2), OK-12A(1,2), OK-12B(1,2,3), BF- WP
22.	FK-1C(2), FK-1D(1,2,3)
23.	V-5B(1,2,3), V-5C(1,2,3,4), V-6B(2), V-13A(1,2,3), V-2C(1,2), V-2E(1,2,3), HL-5C(1,2,3), HL-5D(1,2,3), HL-6A(1,2,3,4), HL-7A(1,2,3,4), HL-7C(1,2,3,4), COL-1A(1,2,3,4,5)
24.	V-9A(1,2), V-9D(1,2), V-8C(1,2,3)
25.	V-4D(1), V-10D(1,2)
26.	V-3A(1), V-12A(1), V-14A(1,2,3)
27.	HL-8A(1), HL-8B(1,2,3,4,5,6)
28.	MAS-1B(3), MAS-2A(1), MAS-4B(1,2,3,4,5,6), MAS-4D (1,2,3,4,5), COL-4A(1,2,3,4)
29.	MAS-3A(5,6,7,8)
30.	H-5A(2), H-5B(2), H-5C(1,2,5), H-5D(1,2,3), H-6A(1,2), H-8C(1,2), H-10A(1,2), H-10B(1,2), H-10C(1), H-11C(2)
31.	H-9B(1,2), H-11B(1), H-11C(1)
32.	EL-13B(1,2)
33.	EL-23A(1), EL-23B(1,2)

TABLE 3 (cont'd)

VC-GROUP	Iaolates in the vc-group
34.	EL-24A(1,2)
35.	COL-3A(1,2,3,4,5)
36.	COL-5A(1,2,3), COL-6A(1,2,3,4,6,7)

TABLE 4

Multi-merge vegetative compatibility groups in *Leucocytospora kunzei*

Multi-merge group	Isolates in the group	Compatible vc-groups	Late incompatibility[a]
MM1	EL-2B(3,4), EL-9A(1,2), EL-9B(1,2,3), EL-9C(1), EL-12A(1), HL-1B(1,2,3,4), EL-12B(1,2), MSU-1A(1), HL-4A(2), HL-4D(1,2,3), HL-4C(1,3), MSU-3A(1), HL-4B(1,2,3,4), MSU-4A(1)	20,33	
MM2	BC-1A(1), BC-1B(1), V-1A(1,2,3), V-1D(1,2) V-2B(1,2,3)	1,19	
MM3	MAS-5A(1,2), MAS-5B(1,2,3, 4,5,6), MAS-5C(1,2,3,4), EL-14A(1,2,3,4)	2,10	
MM4	OK-2A(1,2), OK-2C(1,2)	30,MM6,MM7, MM8	31,MM5
MM5	OK-3C(1,2,3)	30,MM6,MM7, MM8	31,MM4
MM6	EL-8A(1,2,3), EL-10A(1), EL-10B(1,2)	30,MM4,MM5, MM7,MM8	31
MM7	EL-17A(1,2,3), EL-17B(6)	30,31,MM5 MM6,MM8	MM4
MM8	H-8A(1), H-8B(1,2)	30,31,MM4, MM5,MM6,MM7	

[a] Late incompatible reactions occurred between some of the multi-merge groups. In this case the black reaction line did not appear until much later than normal. Most reactions were very visible at 7 days, late incompatibility showed up at 14-20 days.

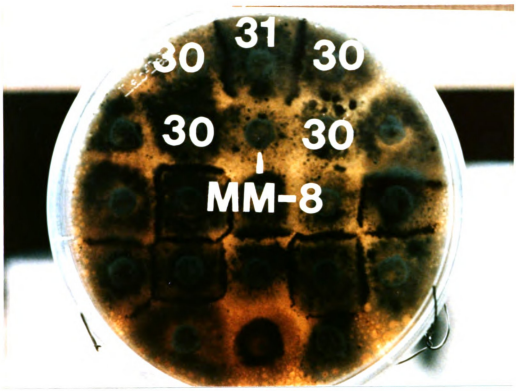

FIGURE 4. The multi-merge response.

The isolate in vc-group MM-8 is compatible with isolates in vc-groups 30 and 31. VC-groups 30 and 31 however, are incompatible.

Vegetative compatibility groups: distribution patterns

Within a canker- Within a single canker, generally all of the individual isolates were contained in a single vc-group. Upon examining 196 individual cankers, in only two cases were isolates obtained from a single canker which were in different vc-groups.

Within a tree- Generally, within a single tree when more than one canker was collected, the isolates obtained were in the same vc-group. It was not unusual however, for isolates to be contained in two vc-groups. One tree, BF-7, was sampled extensively. Of the nine cankers examined, seven yielded isolates in a single vc-group while the other two were in a vc-group which was the same as a neighboring tree.

Within neighboring trees- Several study sites included more than one tree in the planting in close proximity, Generally trees located near each other yielded isolates in the same vc-group of groups. For example, six trees sampled in a yard in East Lansing [EL-26 through EL-31] yielded isolates in only two vc-groups (vc-groups 17 and 21).

Within distant sites- Some vc-groups contained isolates which were collected from different, and in some cases widely separated sites within Michigan, and even included some Colorado isolates (Figure 5).

Among different tree species- Some of the vc-groups contained isolates which originally came from different tree species (Table 5). The L. kunzei isolates from eastern hemlock and Douglas-fir however, were not in the same vc-groups as any of the spruce isolates.

Among the white isolates- The white isolates formed two discrete vc-groups (vc-groups 4 and 25) and were not compatible with any of the normally pigmented isolates.

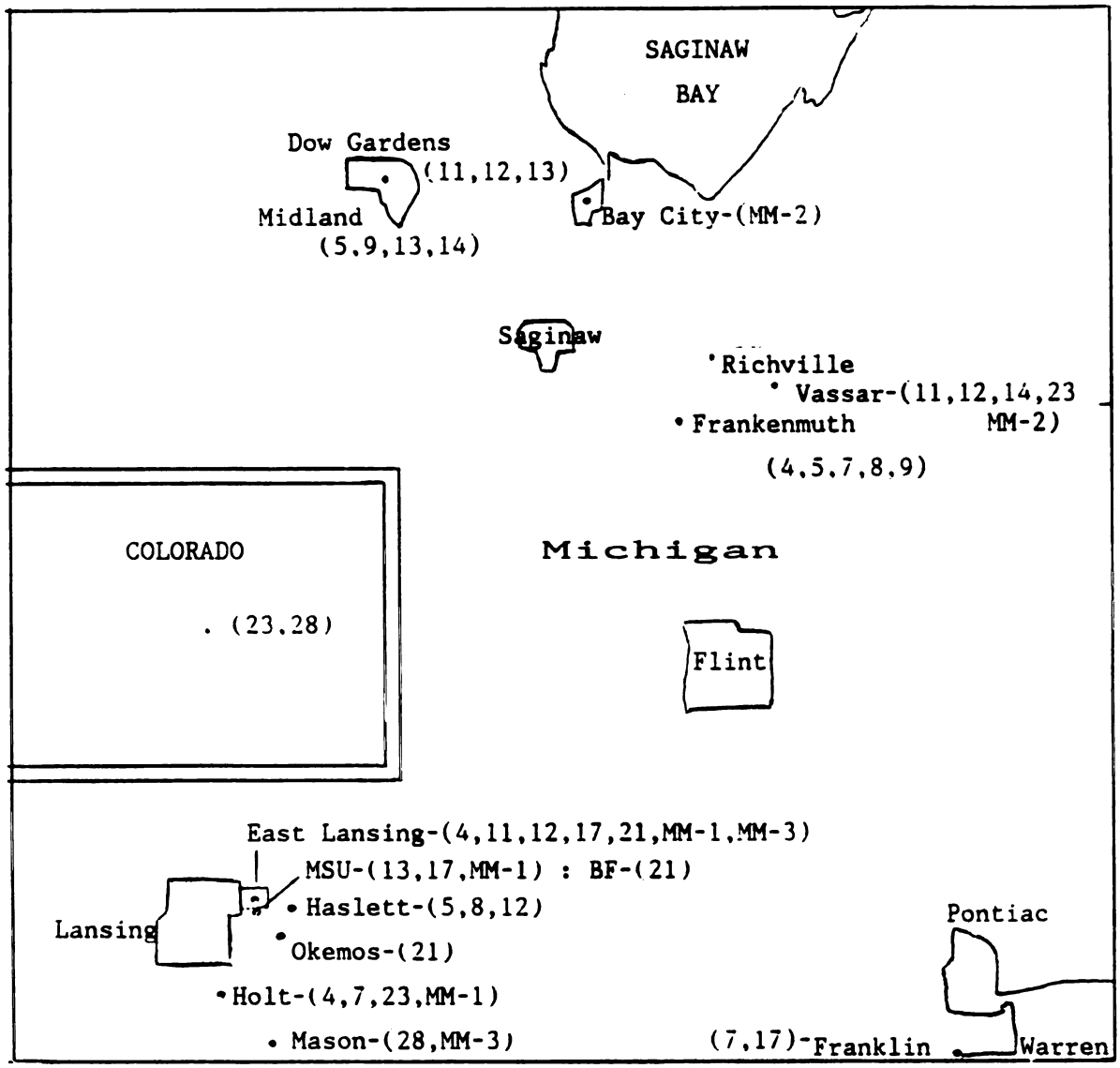

FIGURE 5. Vegetative compatibility groups found at more than one site.

VC-groups which contained isolates from different sites are listed by the sites from which they were isolated.

TABLE 5

Vegetative compatibility groups isolated from more than one host species.

vc-group	Hosts isolated from
5.	Picea pungens, P. abies
7.	P. pungens, P. abies
8.	P. pungens, P. glauca
9.	P. pungens, P. abies
11.	P. pungens, P. glauca
12.	P. pungens, P. abies, P. glauca
13.	P. pungens, P. abies, P. glauca
17.	P. pungens, P. glauca
21.	P. pungens, P. glauca, Pinus strobus
23.	P. pungens, P. glauca, P. engelmannii
26.	P. pungens, P. glauca
28.	P. pungens, P. abies, P. engelmannii
30.	P. pungens, P. glauca
MM1	P. pungens, P. abies
MM6	P. pungens, P. abies

DISCUSSION

Vegetative compatibility in fungi, as stated by Kulman, "refers to the characteristic enabling two fungi to make contact, fuse, and exchange cytoplasm or nuclear material." (19). Vegetative compatibility therefore is an important biological feature of fungi in that it governs heteroplasmon and heterokaryon formation respectively (7,8,19). Isolates which are vegetatively compatible make up a vc-group. Based on this survey of 483 isolates there appears to be at least 44 vc-groups in Leucocytospora kunzei. Large numbers of vc-groups have also been demonstrated in other ascomycetes: Cryphonectria parasitica (1,19,21), Podospora anserina (20), Neurospora crassa (10,12), and several Aspergillus species (6,8,11,16,17).

Vegetative compatibility has been found to be homogenic in all examined cases (1,2). That is, in order for two isolates to be vegetatively compatible they must have common alleles at one or more loci. Based on the number of vc-groups obtained for L. kunzei, there appears to be at least six loci involved in regulating vegetative compatibility in this fungus. This was determined according to the method of Anagnostakis (2), where the number of vc-groups is used to indicate the number of loci involved (where N equals the number of loci, the number of vc-groups = 2^N). This leaves the potential for at least 64 vc-groups to occur in L. kunzei. Evidence supports the idea that homologous alleles are not required at all of the compatibility loci for a compatible

reaction to occur. Alleles at some loci appear to have a quantitative effect while alleles at other loci produce a qualitative effect (3,8,). In L. kunzei the multi-merge groups demonstrate this feature in that they are compatible with more than one vc-group. This has been taken as an indication of close genetic relationships between the vc-groups, with homogenic alleles at many but not all alleles (2). This would indicate that isolates in vc-group 20, vc-group 33, and vc-group MM-1 are closely related to each other (Table 4.).

Vegetative compatibility governs hyphal anastomosis and the exchange of cytoplasmic or nuclear materials. The main biological impact of this is that it functions as a cellular defense mechanism against genetic infection (7,8). Genetic infection would include such agents as viruses, 'virus-like' particles, and/or defective mutant derivatives of essential cytoplamic elements (7). It would also include other nuclei. Hartl (12) suggests that vegetative compatibility, rather, vegetative incompatibility, may function to protect an established mycelium adapted to its environment from exploitive nuclei less well adapted to the environmental conditions.

Unfortunately vegetative compatibility works against using biological control agents such as viruses or virus-like agents, such as the dsRNA associated with hypovirulence in C. parasitica. Information gained here on L. kunzei with regards to vegetative compatibility will be useful in approaching future efforts in the biological control of this fungal pathogen. Fortunately multi-merge groups do occur which may serve as future 'delivery agents' for virus or virus-like biological control agents. Encouragingly, it has been noted in C. parasitica that there is sometimes conversion between some hypovirulent and virulent

isolates which are not vegetatively compatible (3,15). This occurs presumably due to the fact that the several loci involved in determination of vegetative compatibility effect different steps in the anastomosis and exchange process. In some cases enough steps may occur to allow the transfer of an infective virus-like agent, such as the ds-RNA particle in C. parasitica (19). The set of multi-merge isolates which showed some late incompatibility responses may be explained along these lines (Table 4.).

Examining the distribution of vc-groups in L. kunzei is not only important in a biological sense but has implications for pathological studies. Several factors seem to indicate that conidia do serve a role in the dissemination of this pathogen. It was shown that conidal isolates of L. kunzei were always in the same vc-group as their original isolate. Vegetative compatibility groups do not appear to segregate during conidiogenesis. This has also been noted with C. parasitica. No perithecia of L. kunzei were found during the 3 years of this research, and are rare on blue spruce. It has not been shown if vc-groups segegrate during ascosporogenesis in L. kunzei as they do in C.parasitica.

There was a tendency, at several levels, for a single or few vc-groups to predominate. Cankered branches within a single tree or in neighboring trees often yielded isolates within the same vc-group or groups. Generally within a single cankered branch, the isolates found were in the same vc-group. Trees located further apart showed more variety with regard to vc-groups. However, vc-group 21 didoccur widely in those sites centered around East Lansing (Table 3., Figure 5.). Short range spread via conidia could explain the predominance within cankers, tree, and neighboring trees of a single or few vc-groups. Long distance

spread may be due to ascospores which could account for some of the variety in vc-groups encountered. The tendency for a single or few vc-groups to occur on a tree is similar to the situation with chestnut blight in Europe. On chestnuts in Europe, a single or a few vc-group(s) occur in a tree or in neighboring trees. In the United States there are commony many vc-groups of C. parasitica within in a tree or single canker. In the United States ascospores have been found to disseminate this fungus but in Europe ascospores are rare and conidia serve as the primary means of dissemination (19). Also of pathological interest, several vc-groups were represented on more than one species of spruce. This in combination with the results of inoculation trials indicate that that most if not all species of spruce may be serving as reservoirs of inoculum capable of infecting other spruces. Here again vegetative compatibility has applications for pathological studies.

Another use of vegetative compatibility is as a marker for inoculation studies. By surveying the vc-groups present before inoculation trials it is then possible to use isolates known to be in different vc-groups for inoculation studies. Using vc-group analysis one can accurately verify the identity of the isolates recovered from any inoculations done on the tree. In this way vegetative compatibility can serve as a non-mutational marker for epidemiological studies. Using vc-groups as markers would also presumably avoid and unintentional selective pressures which might occur when using mutational markers.

No apparent 'hypovirulent' isolates of L. kunzei were found in this survey. The slow growing isolate, EL-21A(3), was used in inoculation experiments (isolate WS) and proved to be virulent. The pathogenicity of the white isolates has not been fully determined but it

appears that they are also capable of canker formation, at least in a few inoculated excised branches of blue spruce.

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SECTION 3

Wound inoculations of excised and intact branches of blue spruce with *Leucocytospora kunzei* and other *Cytospora s.lat.* isolates

INTRODUCTION

Blue spruce (Picea pungens Engelm.) is widely planted in the mid-western and northeastern United States as a landscape ornamental. The unique coloration and dense pyramidal crown of this tree make it a popular specimen tree (2). Blue spruce however, is often progressively disfigured by Cytospora canker (5,9,15,16). Cytospora canker of blue spruce is characterized by the occurrence of diffuse cankers on the limbs of mature trees (5,7,13,15,16). Cankered branches are commonly covered with resin exudations. Trunk cankers and tree mortality are not common in blue spruce (9.16) but the loss of branches associated with this disease destroys the symmetry and aesthetic value of these large and often strategically located landscape trees.

Cytospora canker of spruce is caused by Leucostoma kunzei (Fr.) Munk ex Kern, which is seen almost exclusively in its anamorphic form, Leucocytospora kunzei (Sacc.) Urban (1,3,17). This pathogen is still commonly refered to as Cytospora kunzei in the pathology literature (telemorph- Valsa kunzei) (5,7,8,9,11,12,15,16). The genera Valsa and Leucostoma and their associated anamorphic forms, Cytospora and Leucocytospora, are closely related. Leucostoma and Leucocytospora are considered by some to be subgroups within Valsa and Cytospora (1,14). For the purpose of clarity I will use Cytospora in the broader sense, Cytospora s. lat., which would include fungi in the genus Leucocytospora including L. kunzei.

Virtually every mature blue spruce in Michigan is affected by this

pathogen at some point in maturity. Blue spruce however, exists as a non-continuous population in the region, restricted primarily to widely separated sites as ornamental plantings. The spores of L. kunzei and Cytospora s. lat. are disseminated primarily via rainsplash (6,9,10). This combination of factors would seemingly make it unlikely that so many trees would be affected. This suggests the possibility that other tree species may be serving as sources of inoculum capable of infecting blue spruce.

Essentially every woody plant species examined has been found to be colonized by some species within Cytospora s. lat. (4,5). The various Cytospora spp. fungi have generally been treated as distinct species in the past, with 400 species having been described (14), many on the basis of their host association. There have been few pathogenicity or host range studies done with these fungi. The variability in Cytospora s. lat. conidiomata is high even on a single host species (14). The possibility exists that we may be dealing with a single or a few Cytospora s. lat. species which have a wide host range but have been treated as distinct species on the basis of their host association. Leucocytospora kunzei may owe its characteristic conidiomata type only to the particular nature of the bark of its spruce hosts, and Cytospora s. lat. isolates from other hosts, upon infecting spruce, may look similar.

The purpose of this research was primarily to get some indication as to whether other hosts of Cytospora s. lat. are serving as reservoirs of inoculum capable of infecting blue spruce. That is, are Cytospora s. lat. isolates from non-spruce hosts capable of infecting blue spruce.

The observed tendency of Cytospora canker of blue spruce to occur predominantly on mature trees (16) poses methodological problems when studying this disease. In Michigan, and other areas in the Midwest and Northeast, most mature blue spruces are located in highly visible and aesthetically important sites. This factor tends to restrict the amount of available material for experimentation. Past inoculation studies have utilized young nursery sized trees for experimentation (8,12,16), yet young trees are rarely affected by this disease. For that reason the applicability of using nursery sized trees for inoculation studies has been questioned (16). Because of the aforementioned criticism, these inoculations were done on mature blue spruces. In an effort to increase the amount of host material available for experimentation, a secondary goal of this research was to examine the efficacy of inoculating excised branch segments from mature trees to study the pathogenicity of isolates of Cytospora s. lat. on blue spruce.

MATERIALS AND METHODS

Isolates- Sixteen Cytospora s. lat. isolates were used in these inoculation experiments. Four of the isolates were from spruce hosts and twelve came from other tree species (Table 1). All of the isolates were originally isolated from infected cambial/cortical tissues except the isolate from Norway Spruce which was started from a mass of conidia. The isolates were grown on 2% Malt Extract Agar (2% MEA = 20 g Difco Malt Extract and 20 g Difco Bacto-Agar per liter of distilled water) at 26°C prior to inoculation.

Excised branch inoculations- Live healthy branches from mature

Table 1.

Origin and Identification of the 16 *Cytospora* spp. Isolates used to Inoculate Blue Spruce

Isolate Code	Original Host		Identification
	Spruce		
CBS	Colorado blue spruce	*Picea pungens*	*Leucocytospora kunzei*
CS	Colorado spruce	*P. pungens*	*L. kunzei*
NS	Norway spruce	*P. abies*	*L. kunzei*
WS	white spruce	*P. glauca*	*L. kunzei*
	Other Conifers		
DF	Douglas-fir	*Pseudotsuga menziesii*	*L. kunzei*
HM	eastern hemlock	*Tsuga canadensis*	*L. kunzei*
WP	eastern white pine	*Pinus strobus*	*L. kunzei*
	Deciduous Trees		
A	Alder	*Alnus* spp.	*Cytospora ambiens*
CM	Apple	*Malus domestica*	*C. ambiens*
BTA	Aspen, big tooth	*Populus grandidentata*	*C. chrysosperma*
HP	Aspen, 'hybrid'	*Populus* spp.	*C. chrysosperma*
OK	English oak	*Quercus robur*	*C. ambiens*

Table 1 (cont'd.)

Isolate Code	Original Host		Identification
M	Norway maple	*Acer platanoides*	*Cytospora annulata*
P	Peach	*Prunus persica*	*Leucocytospora leucostoma*
B	red copper beech	*Fagus sylvatica* c.v.	*C. ambiens*
RH	staghorn sumac	*Rhus typhina*	*C. ambiens*

blue spruce trees were collected and brought into the laboratory. Branches were collected in both the summer and winter months. Branches at least 2 cm in diameter were cut into sections 30-40 cm long and all of the smaller branchlets and needles were removed. The cut ends and branch stubs were sealed with molten paraffin wax to prevent drying. The site on the branch to be inoculated was surface disinfested by dabbing with 95% ethanol and flaming. Flap wounds 7 X 7 mm, were cut in the bark and small slabs of mycelium with the subtending agar medium were placed under the flaps. Sterile MEA was placed into the wounds for the control treatment. The flaps were closed and wrapped with Parafilm M®. The inoculated twigs were placed into loosely closed plastic bags to incubate for 7-10 days. A total of 403 inoculations were made on excised branches.

Intact branch inoculations- Live, intact branches of mature blue spruces were inoculated in the summer of 1984 and 1985. The wound-flap inoculations were done as described previously, but the surface disinfesting step was omitted. Inoculations were made along the branches between the points were the secondary or tertiary branchlets were growing. The small branchlets were not removed. The inoculations were wrapped with brightly colored tape to facilitate locating the inoculation sites later. The inoculations were examined after 3 weeks or more. A total of 289 inoculations were done on intact branches in the trees.

Evaluation- The wound inoculations were evaluated by stripping the bark off the branches and measuring canker development as indicated by a brown discoloration of the cambial/cortical tissues. Cankers were measured along the axis of the branches and at the widest point longitudinally.

RESULTS

The response of blue spruce to wound inoculations with Cytospora s. lat. was dependent on the particular isolate tested. On excised branches some isolates caused canker initiation, as indicated by the occurrence of expanding areas of discoloration in the cambial/cortical tissues. Other isolates and the sterile medium control did not cause canker initiation. However, the actual wound flaps were commonly discolored to some extent (Figure 1). Resin was not generally exuded at the inoculation site. Similar results occured on inoculated intact branches, with some isolates causing canker formation. Heavy resin exudations were noted at the inoculation sites where canker initiation was successful. Control inoculations or isolates incapable of initiating canker formation did not elicit the heavy resin exudation.

Based on their ability to incite canker formation on inoculated branches, the 16 Cytospora s. lat. isolates varied in their pathogenicity to blue spruce. Canker initiation and expansion was seen on both excised and intact branches when inoculated with the isolates taken from spruce or eastern white pine (Table 2). For each of the isolates tested, the percent canker initiation (Number of developing cankers/number of inoculations) was similar on both excised and intact branches.

Differences in the virulence of the four L. kunzei isolates from spruce were detected. Inoculations of excised and intact branches from an individual tree by these isolates resulted in cankers of various sizes. Measurements of the developing cankers indicated that the isolate

FIGURE 1. Canker initiation on an excised branch of blue spruce inoculated with Cytospora s. lat. isolates.

Isolate WS which was originally isolated from a spruce caused canker initiation on this inoculated excised branch segment. The other isolates failed to incite canker formation. This branch was evaluated 10 days post inoculation.

TABLE 2

The percent canker initiation on inoculated branches of blue spruce caused by isolates of Cytospora s. lat.

Original Host of the Isolate	% Canker Initiation Excised Branches	% Canker Initiation Intact Branches
Spruce		
Colorado blue	80.0	77.8
Colorado	85.0	86.7
Norway	80.0	93.3
white	96.3	90.1
Other Conifers		
Douglas-fir	0.0	0.0
eastern hemlock	0.0	0.0
eastern white pine	95.0	88.2
Deciduous Trees		
Alder	0.0	0.0
Apple	0.0	0.0
Aspen, big tooth	0.0	0.0
Aspen, 'hybrid'	0.0	0.0
English oak	0.0	0.0
Norway maple	0.0	0.0
Peach	10.0	0.0
Red copper beech	0.0	0.0
Staghorn sumac	0.0	0.0
Control	0.0	0.0

from white spruce was the most aggressive on that inoculated host tree (Table 3). However, in different inoculations using branches from other trees, no differences in virulence were indicated among the four L. kunzei isolates from spruce (Table 4).

Host effects on canker initiation and expansion were indicated in these experiments. Inoculations of excised branches from two different blue spruce trees growing at the same site resulted in differences in both the percent canker initiation and in canker expansion (Table 4). Canker initiation and expansion was greater on a tree which was predisposed to Cytospora canker, as indicated by the presence of naturally occurring cankers, than on a previously unaffected tree.

The percent canker initiation on excised branches inoculated with the four L. kunzei isolates from spruce was similar regardless of whether the branches were collected in the summer or the winter (Table 5). The increased efficiency seen across Table 5 is probably due to the refinement of the experimental technique.

DISCUSSION

Wound inoculations of both excised and intact branches of blue spruce indicate that most non-spruce hosts of Cytospora s. lat. are not serving as reservoirs of inoculum capable of infecting blue spruce. None of the Cytospora s. lat. isolates from deciduous trees were capable of inciting canker formation in inoculated blue spruce trees. Leucocytospora kunzei isolates from other conifers varied in their pathogenicity to blue spruce. Isolates of L. kunzei from Douglas-fir and eastern hemlock were not capable of inciting canker formation on blue spruce in this study.

TABLE 3

Comparison of canker lengths on excised and intact branches of blue spruce inoculated with four isolates of Leucocytospora kunzei.

Origin of Isolate	Excised Branches[1] 10 days (in mm)	Intact Branches[1] 22 days (in mm)
Colorado blue spruce	35.8 ± 20.9	26.4 ± 12.6
Colorado spruce	27.5 ± 10.1	56.0 ± 32.9
Norway spruce	62.3 ± 10.5	56.8 ± 24.4
White spruce	74.9 ±21.2	101.0 ± 22.7

[1]The excised branches came from the same tree upon which the intact branches were found, tree BF-8.

TABLE 4

Host effects on excised branch inoculations with four Leucocytospora kunzei isolates.

	Affected trees[1]		Unaffected trees[2]	
Isolate	% Canker Initiation	Size (mm)	% Canker Initiation	Size (mm)
CBS	88.9	60.4 ± 20.0	66.7	30.7 ± 11.8
CS	77.8	58.6 ± 15.5	33.3	39.0 ± 10.1
NS	90.0	55.6 ± 20.9	44.4	24.3 ± 13.4
WS	88.9	56.6 ± 17.0	33.3	26.7 ± 18.6

[1]This tree did have naturally occuring cankers at the onset of the study.

[2]This tree did not have naturally occuring cankers at the onset of the study.

TABLE 5

Percent canker initiation on excised branches of blue spruce inoculated with *Leucocytospora kunzei* isolates: Branches collected during different seasons.

L. kunzei Isolate	Season during which branches were collected		
	Summer 1984	Winter 1985	Summer 1985
CBS	77.8	77.8	100.0
CS	75.0	88.9	100.0
NS	62.5	90.0	100.0
WS	100.0	88.9	100.0

This agrees with the earlier findings of Waterman (16). However, in contrast to her findings, in this study the L. kunzei isolate from eastern white pine did prove to be pathogenic to blue spruce. This different response could be due to particular isolate differences or could be due to methodology. Waterman (16) utilized nursery trees for her inoculations and she also raised the question of the applicability of using nursery trees for inoculation. Using mature blue spruces already predisposed to Cytospora canker, as indicated by the natural occurrence of the disease, is important for studying this disease. Since mature trees are most commonly affected by this disease (7) it seems reasonable that they should be utilized when possible for assessing the pathogenicity or virulence of Cytospora s. lat. isolates on blue spruce.

No host specificity was detected among the isolates of L. kunzei from the various spruce hosts. Isolates of L. kunzei from white and Norway spruce were capable of inciting cankers of blue spruce upon inoculation. Further evidence in support of this was noted in vegetative compatibility studies with L. kunzei. Isolates of L. kunzei taken from neighboring white and blue spruces were found to be contained in the same vegetative compatibility group at one site.

These results have implications for developing a control strategy for Cytospora canker of blue spruce. On the one hand it simplifies the situation in that we probably do not have to consider deciduous trees as potential inoculum sources capable of infecting blue spruce. On the other hand, spruces of various species and potentially other conifers, as seen with eastern white pine, must be considered when developing control strategies for this disease. Even including

eastern white pine and all of the spruces as potential hosts and inoculum sources of L. kunzei, the ubiquitous occurrence of this disease throughout Michigan is surprising. Two possible explanations come to mind. In one case, perhaps there is an alternate method of long range dissemination. Windborne ascospores have not been reliably documented. Birds could easily be contaminated with the spores of L. kunzei. English sparrows are often seen perching in these trees. The pycnidia exude masses of conidia, which when wet, form slimy, spreading droplets along the surface of cankered limbs. Alternatively, the actual colonization of blue spruce may occur at an earlier time in the tree's history. Leucocytospora kunzei has been isolated from 'healthy' uncankered tissues. Perhaps infection occurs while the trees are young but cankers do not occur until some predisposing change occurs in the mature trees. If this is the situation, then control strategies will need to protect young trees in spite of the fact that cankers do not generally form on them.

Using excised branch inoculations appears to be a reliable and suitable method for catagorizing the pathogenicity and/or virulence of Cytospora s. lat. isolates on blue spruce. The response of both excised and intact branches of blue spruce to the wound inoculations with the various Cytospora s. lat. isolates was similar. No isolate which caused cankers in inoculated trees failed to cause canker initiation on excised branches. Inoculated excised branches also accurately reflected host effects noted in the field. In this examination, branches taken from a blue spruce predisposed to Cytospora canker, as indicated by the occurrence of the disease naturally in the tree, proved to be more susceptible to canker initiation and expansion than excised branches from a tree at the same site which did not have Cytospora canker prior

to inoculation.

If any differences in the response of inoculated excised and intact branches can be seen, it is that excised branches are more susceptible to canker formation than branches in the trees. This fact is reflected in two areas. In the first, canker expansion is faster in excised branches inoculated with L. kunzei than branches left intact on the tree. Secondly, in the only inconsistent response noted in these experiments, the L. leucostoma isolate from peach did incite canker formation on excised branches yet failed to incite cankers on intact branches (Table 2). This increased sensitivity in excised branches makes them even more suitable for screening Cytospora s. lat. isolates for being potentially pathogenic to blue spruce.

Using inoculated excised branches as a method to study Cytospora canker of spruce offers many benefits. It is a fast (7-10 days), sensitive, and reliable indicator of in vivo responses of blue spruce to inoculation. It utilizes mature tissues which are normally affected by the disease, and one can select branches from trees already predisposed to canker formation. Using these normally affected tissues avoids some of the complicating factors encountered by earlier workers who utilized nursery stock and found that they had to severely drought stress nursery trees in order for any canker formation to occur. This method also increases the amount of material at ones disposal for experimentation. A branch or branches may be collected from a tree and brought into the laboratory, avoiding the aesthetic damage field inoculations cause on these often key landscape trees. Convenience is also a factor in being able to do these tests in the laboratory. One potential application for this technique could be to test for

genetic resistance in blue spruce to L. kunzei. Branches could be tested from mature, seed producing trees without introducing or increasing the incidence of the disease in your seed producing stock.

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SECTION 4

Wound inoculations of excised and intact branches of blue spruce with conidial isolates of *Leucocytospora kunzei*

INTRODUCTION

Cytospora Canker of blue spruce (Picea pungens Engelm.) is caused by Leucostoma kunzei (Fr.) Munk ex Kern [syn. Valsa kunzei], which is most frequently seen in its anamorphic form, Leucocytospora kunzei (Sacc.) Urban [syn. Cytospora kunzei] (1,2,7). Some workers have indicated that mycelial inoculum from isolates of L. kunzei originating from conidia failed to cause cankers in wound inoculated trees while ascosporic isolates were infective (4). Other workers found conidial isolates to be capable of canker initiation in inoculated trees (3,6,7) . The purpose of these experiments was to re-examine the pathogenicity of conidial isolates of L. kunzei on inoculated intact and excised branches of mature blue spruce trees.

MATERIALS AND METHODS

Isolates- The isolates of L. kunzei used in these inoculation trials were isolated from either the infected tissues of a cankered spruce branch or from conidia produced on the same branch. Conidia were collected by first soaking the infected branch for 10 minutes in a 5% sodium hypochlorite solution and then rinsing them with distilled water. The cleaned branch was then placed into a moist chamber for 2 hours. Masses of conidia could be soon seen on the surface of the branches (Figure 1.) Conidial isolates were started from either masses of conidia or a single conidium. Isolations were made on Potato

FIGURE 1. Induced sporulation of Leucocytospora kunzei on spruce.

Masses of conidia produced by Leucocytospora kunzei on cankered branches of spruce placed in a moist chamber.

Dextrose Agar (PDA). After collecting the spores, the fruiting structures were hand sectioned and examined under the compound microscope to verify that the spores were indeed conidia. Single conidium isolates were obtained by placing a mass of conidia in sterile distilled water, shaking vigorously, followed by serial dilutions until discrete single conidia could be observed in a drop of the conidial suspension. The conidial suspension was then streak-plated onto PDA. Prior to inoculations all isolates were transfered and grown on 2% Malt Extract Agar (20 g Difco Malt Extract and 20 g Difco Bacto-Agar per liter of distilled H_2O) for 5-7 days at 26°C.

Inoculations- The wound inoculations on excised and intact branches from mature blue spruces were done as in previous experiments. Slabs of mycelium from the various isolates, with the subtending agar medium were used as inoculum with sterile medium inoculations for controls.

Experiment No. 1 - In experiment No. 1, the isolates of L. kunzei were isolated from a cankered branch of a mature blue spruce tree (BF-7) located at the Beaumont and Forest Road site described in previous sections. Inoculations were made on excised branches taken from the same tree at a later time. Four inoculation treatments were used (Table 1).

Experiment No. 2 - In experiment No. 2, the isolates of L. kunzei were isolated from the cankered branch of a Norway spruce (P. abies (L.) Karst.). These isolates came from the same cankered branch as the Norway spruce isolate used in the previous inoculation studies (NS). Inoculations were made on excised branches of blue spruce (from BF-6 which had been naturally infected prior to the onset of this experiment) predisposed to Cytospora canker. Inoculations on intact branches however, due to the limited availability of hosts, were made on two blue spruce trees

at the same site which had no prior history of Cytospora canker (BF-2 and BF-3). Several isolates were utilized (Table 2).

Evaluation- The wound inoculations were evaluated as previously described. Some of the excised branches in experiment No. 1 were examined at 6 days after inoculation and others at 10 days. The results were combined. The excised branches in experiment No. 2 were examined 10 days after inoculation. The intact branches were inoculated on 13 September, 1984 and were examined on 7 November, 1984.

RESULTS

The results of these experiments indicate that mycelial inoculum of L. kunzei isolates originating from infected tissues, masses of conidia, or from a single conidium are capable of inciting canker formation on inoculated blue spruce branches (Table 1,2). Additionally, in experiment No. 2, differences in the virulence among the conidial isolates were indicated (Table 3). Although canker initiation was seen on inoculated intact branches in experiment No. 2, the cankers were very small. In most cases the expansion was limited to only a few millimeters beyond the inoculation flap. Control inoculations failed to cause any expanding discoloration. Due to the limited expansion, no virulence differences were detected among the isolates on intact branch inoculations.

DISCUSSION

In this study, canker initiation occurred on both intact and excised branches of blue spruce wound-inoculated with mycelial fragments of

TABLE 1

Inoculations of excised and intact branches of blue spruce with isolates of Leucocytospora kunzei started from infected tissues or conidia. Experiment No. 1

Treatment or Isolate	Origin of Culture	Number of Inoculations	% Canker initiation[1] at ten days post inoculation
BF-7D(1)	infected tissue	11	72.7
BF-7D(m)	mass of conidia	11	100.0
BF-7D(11)	single conidium	11	81.8
Control	-	7	0.0

[1] % Canker Initiation = No. of inoculations with expanding cankers/No. of total inoculations.

TABLE 2

Inoculations of excised and intact branches of blue spruce with isolates of Leucocytospora kunzei started from infected tissues or conidia.

Experiment No. 2

Treatment or Isolate	Origin of Culture	Excised branches % Canker Initiation[1,2]	Intact branches % Canker Initiation[1,3]
MSU-5B(1)	infected tissue	80.0	50.0
MSU-5B(4)	mass of conidia	80.0	50.0
MSU-5B(6)	single conidium	100.0	83.3
MSU-5B(8)	"	100.0	50.0
MSU-5B(9)	"	100.0	50.0
MSU-5B(10)	"	80.0	66.7
MSU-5B(12)	"	80.0	66.7
Control	-	0.0	0.0

[1] % Canker Initiation = No.of inoculations with expanding cankers/ No. of total inoculations

[2] A total of five inoculations were made with each isolate.

[3] A total of six inoculations were made with each isolate.

TABLE 3

Mean canker lengths on excised branches of blue spruce inoculated with tissue and conidial isolates of Leucocytospora kunzei. Experiment No. 2

Treatment or Isolate	Origin of Culture	Mean Canker Length	
MSU-5B(1)	infected tissue	37.0 ± 18.1	ab[1]
MSU-5B(4)	mass of conidia	42.0 ± 7.8	ab
MSU-5B(6)	single conidium	55.8 ± 11.4	a
MSU-5B(8)	"	49.2 ± 19.5	ab
MSU-5B(9)	"	32.2 ± 11.1	b
MSU-5B(10)	"	45.5 ± 12.2	ab
MSU-5B(12)	"	48.8 ± 28.1	ab

[1]Those means followed by the same letter do not differ significantly LSD, $P = 0.05$.

L. kunzei isolates derived from various sources. Cultures originating from infected host tissue, masses of conidia, or from a single conidium were infective. This agrees with the findings of Jorgensen and Cafley (3), Waterman (7), Schoeneweiss (6), and with other inoculation trials presented in this thesis. In earlier inoculation studies done at Michigan State University by Kamiri and Laemmlen (4), monoconidial cultures failed to produce cankers in inoculated 5 year-old trees while monoascospore cultures were infective. Whether this was due to specific isolate characteristics or due to experimental technique is debatable. In this dissertation, inoculations with L. kunzei isolates from eastern hemlock and Douglas-fir failed to incite canker formation, so there is precidence for avirulent isolates. Waterman (7) obtained similar results. Additionally, 5 year-old blue spruce trees are not normally affected by Cytospora canker (3,7) and their use in inoculation expteriments has been questioned (7).

Inoculating excised branches of blue spruce with L. kunzei isolates accurately approximates the response of inoculations on intact branches in the tree (from the previous section). In experiment No. 2, the single conidium isolate, MSU-5B(9), appeared to be less agressive than the other isolates. Variability in the virulence of other L. kunzei isolates on blue spruce was detected in the previous section. Variability has also been documented among isolates of Leucocytospora leucostoma inoculated onto their peach host (8).

The inoculations of intact branches in experiment No. 2, produced small yet discrete cankers. The trees which were inoculated (BF-2 and BF-3) were at the same site utilized for the other inoculation experiments reported on in this thesis. Neither of these trees had

naturally occurring Cytospora cankers at the onset of this study. As indicated by the previous inoculation study, branches from non-predisposed trees showed less canker initiation and expansion upon inoculation. This inoculation experiment also supports that finding. The nature of the response however has not been accounted for and host genetic factors or seasonal effects may be a complicating factor here. These inoculations were done in the fall and it has been reported that L. kunzei grows maximally near 27°C (5), a much warmer temperature than the autumn temperatures in Michigan.

Perithecia of L. kunzei were never found on blue spruce during the 3 years of this doctoral research. Other workers as well, have reported that perithecia are rare on blue spruce (5). Pycnidia on the other hand, are common and numerous (2,5,7). Conidia do appear to be infective, based on wound inoculations with conidial suspensions (3). Additional support for considering conidia to be important in the dissemination of this fungus can be found in the vegetative compatibility work done with L. kunzei on blue spruce presented elsewhere in this thesis. Conidia appear capable of playing an important role in the spread of this fungus, particularly within a single tree and for short distances (less than 50 meters).

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SECTION 5

The effect of water potential on the *in vitro* growth of *Leucocytospora kunzei*

INTRODUCTION

Cytospora canker of blue spruce is caused by Leucostoma kunzei (Fr.) Munk ex Kern [syn. Valsa kunzei]. The anamorphic form of the fungus, Leucocytospora kunzei (Sacc.) Urban [syn. Cytospora kunzei] is most commonly encountered in the field (1). In the United States and Canada this pathogen has been found to cause branch and occasionally stem cankers on a variety of coniferous hosts (24). Colorado blue spruce (Picea pungens Engelm.) is an important and valuable ornamental tree species. Mature trees, however, are often seriously disfigured due to the branch mortality caused by L. kunzei. In blue spruce, and with other hosts of L. kunzei, drought stress has been associated with the severity of this disease (11,13,25). In experimental inoculations of Colorado blue spruces, canker formation and fungal colonization was greater in water stressed trees (14,20).

The purpose of these experiments was twofold. The first objective was to compliment and expand on the available basic information on the factors affecting the in vitro growth of L. kunzei (15). The second objective was to provide evidence as to whether the increased severity and canker expansion seen in association with drought stress and low tree water potentials was due to some predisposing effect on the host or whether it could be directly accounted for by the growth response of L. kunzei to water potential (Ψ).

MATERIALS AND METHODS

The test isolate- A selected isolate of Leucocytospora kunzei [EL-2B(3)] from a cankered branch of a blue spruce growing in East Lansing, Michigan, was grown in petri plates containing 2% Malt Extract Agar (2% MEA = 20 g of Difco Malt Extract and 20 g of Difco Bacto-Agar per liter of distilled H_2O). After 5-7 days, 6 mm plugs were transfered from near the margins of the colony to the water potential test plates.

Ψ test plates- The water potential of the Ψ test plates was adjusted to a range of water potentials by the addition of various osmotica or a matricum to the base 2% MEA medium. Leucocytospora kunzei grows very uniformly on this medium and forms colonies which are uniform from the center of the colony to the margins. For these experiments the Ψ test media were prepared by adjusting the water potential of the distilled water fraction by the addition of either sucrose, mannitol, NaCl, or KCl as osmotica or a matricum, polyethylene glycol 8000 [Mol. wt. 6000-8000] (21). With the osmotically or matrically adjusted solutions, a 2% Malt Extract medium was made. A solid 2% MEA medium was prepared with all osmotica. The matricum, polyethylene glycol (PEG), prevents the solidification of an agar medium (18), so a liquid 2% Malt Extract broth culture was utilized.

Water potential determination- The water potentials of the test media were determined prior to inoculation using a dew point hygrometer, Wescor model HR-33(T) with C-52 sample chambers (Wescor Inc., Logan,

Utah, 84321). Measurements with the dew point hygrometer were made at room temperature using the 'dew point mode' as described in the HR-33(T) instruction manual. There were four replicates at each water potential treatment in all experiments. The total range of the water potentials of the Ψ test media was from -0.7 bars to -110.0 bars. The water potential of the base 2% MEA was -0.7 bars.

Water potential and growth- After transfer of the inoculum plugs to 100X15 mm petri plates containing 25 ml of Ψ test medium, the plates were sealed with Parafilm® and grown at 26°C in the dark. The liquid broth cultures consisted of 150 ml of medium in a 300 ml Erlenmeyer flask. These were inoculated, fitted with foam stoppers, and placed on a shaker at room temperature (23°C ± 2°C) under ambient laboratory lighting.

Evaluation- The growth of L. kunzei was evaluated by measuring increases in colony diameter daily on solid media and by colony weight in liquid media. Increases in colony diameter, over a 48 hour period when all the isolates were actively growing, were used for the computations. The total weight of the colonies in the liquid medium was recorded after 1 week. The results of each experiment were statistically analyzed using analysis of varience.

RESULTS

The in vitro growth of L. kunzei was affected by the water potential of the culture medium and was dependent on the type of osmoticum or matricum utilized. The growth curves (Figure 1) for L. kunzei in response to water potential were similar, thought not identical when the medium

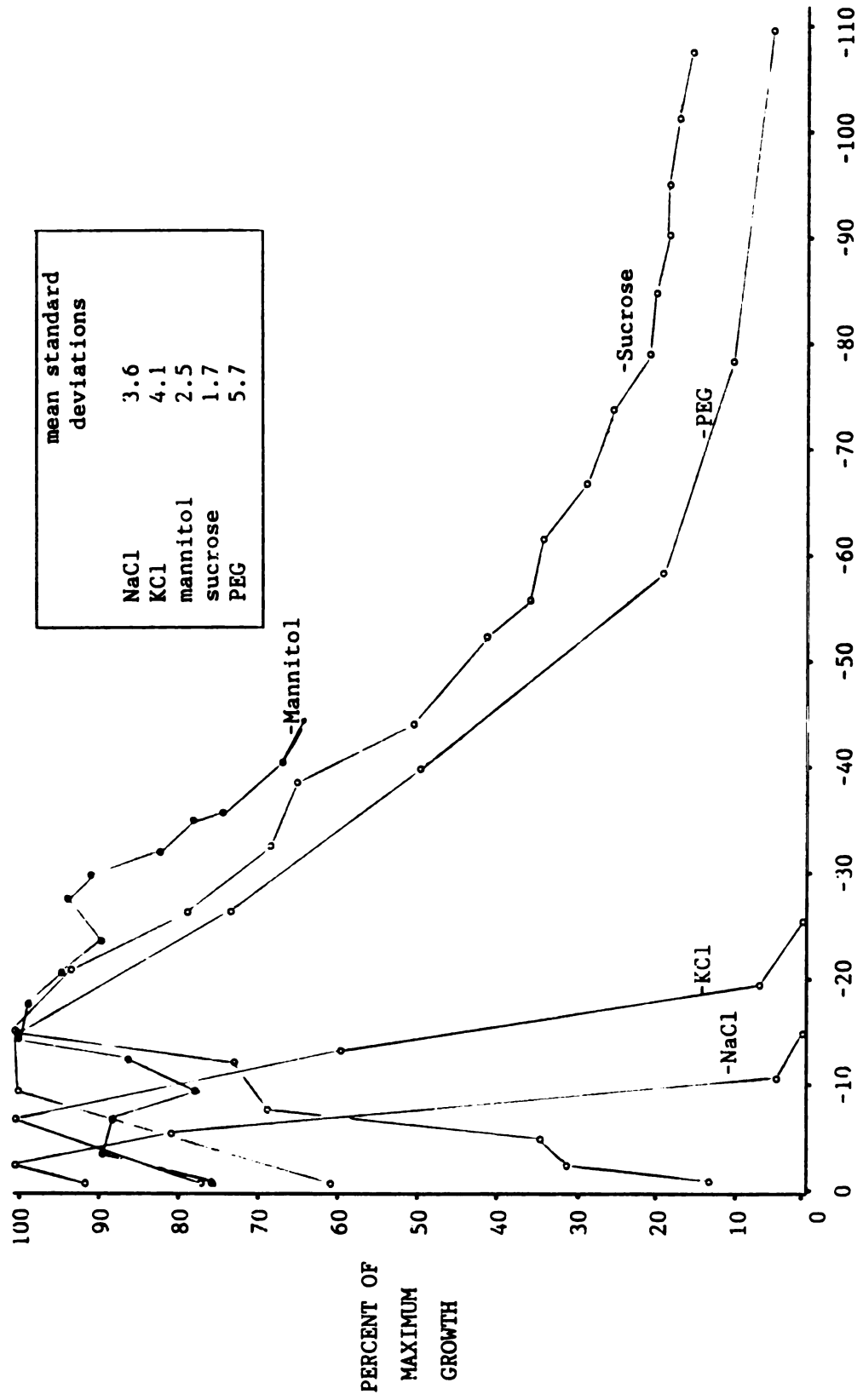

FIGURE 1. The effect of water potential on the in vitro growth of Leucocytospora kunzei.

was controlled osmotically with sucrose or mannitol and when controlled matrically with PEG 8000. Maximum growth occurred at a water potential of -14 to -15 bars, while growth was reduced to 50% of maximum at -40 to -50 bars. With the addition of sucrose and PEG 8000, growth was not inhibited at -100 bars. The growth response of L. kunzei to water potential changes was different where NaCl and KCl were utilized as osmotica. Maximum growth using NaCl and KCl as osmotica was at -2.7 and -6.7 bars respectively, while growth was inhibited at -14.8 and -26.5 bars respectively. The average maximum increase in colony diameter observed on solidified 2% MEA (-0.7 bars) was 13.9 mm/day. The average maximum increase in colony diameter observed for L. kunzei on Ψ adjusted media was 18.0 mm/day (Ψ = -14 to -15 bars).

DISCUSSION

Like all microorganisms, L. kunzei has certain optimal and minimal requirements for growth. One method commonly used to manipulate the water potential of artificial media is by the addition of various solutes to the media (4,6,7,9,18,23). If fungal growth responds similarly to water potential changes using different osmotica, then the complicating concern of specific solute effects can be discounted (9). High molecular weight polyethylene glycol has been utilized as an osmoticum but recently has been shown to affect water potential more as a matricum at high concentrations (21). Some fungi respond differently to water potential changes when it is controlled matrically rather than osmotically (7).

In these experiments, the growth of L. kunzei in response to water potential was similar where it was controlled osmotically with sucrose or mannitol and where it was controlled matrically with PEG. Growth was stimulated by slightly lowered water potentials, with maximum growth occurring at -14 to -15 bars. However, at higher water potentials (> -15 bars) the growth of L. kunzei was inhibited more in the liquid media (Figure 1). It is of interest to note that Hellkvist et al (10), while measuring the water potentials in Sitka spruce, noted solute potentials in spruce shoots ranging from -13 to -24 bars. This range of water potentials is near the growth optimum of L. kunzei. It appears that L. kunzei is well adapted to its conifer hosts.

While NaCl and KCl have been used as osmotica with other fungi (4,22,23) and have not been noted as being particlarly toxic to fungi (6), they do not appear to be suitable osmotica for use with L. kunzei. Fungal growth was inhibited at water potentials well above those noted with the other osmotica and matricum tested here. Growth was inhibited at water potential values higher than reported for many fungi (4,6,7, 22,23).

Several authors have noted the increased occurrence and severity of Cytospora canker on spruce and other hosts in relation to drought stress (11,13,14,25). Schoeneweiss tested that observation by inoculating Colorado blue spruces which were drought stressed and held at known water potential values (20). The role of the environment in predisposing plants to particular pathogens is well documented and accepted in plant pathology (5,19). Drought and the resulting water deficits it causes in plants has been shown to predispose a variety of woody and herbaceous

plants to disease. Several examples of drought stress and "bark moisture deficits" predisposing woody plants to canker-causing fungi can be found (2,3,8,14,16,20). A complicating factor however, which needs to be examined, is the possibility that the pathogens may have growth optima at lower water potentials which may account for the increased colonization and canker sizes on water stressed trees directly (20). Examples have been reported of plant pathogens which grow better at lower water potentials (7). In vitro growth studies address this issue.

With Cytospora canker of blue spruce, the evidence indicates that drought and low tree water potentials predispose spruce to canker formation and expansion by L. kunzei. In his experiments, Schoeneweiss found significantly greater canker expansion and fungal colonization on inoculated trees at -30 compared to -20 bars (20). In contrast, in these experiments the in vitro growth of L. kunzei was significantly lower at -30 versus -20 bars ($P = .01$). The increased growth of L. kunzei noted by Schoeneweiss cannot be accounted for simply by the growth response of L. kunzei to water potential changes. Whether the nature of the predisposition to canker formation in drought stressed trees is related to changes in sugar-carbohydrate levels in the inner bark as noted by Hodges (12) in drought stressed loblolly pines, or is due to some other factors, has yet to be addressed.

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SECTION 6

In vivo water potentials in blue spruce trees with and without a prior history of Cytospora canker

INTRODUCTION

Colorado blue spruce (Picea pungens Engelm.) is a popular and widely planted landscape ornamental in the midwestern and northeastern United States. The aesthetic value of these trees however, is often markedly reduced due to Cytospora canker. Cytospora canker of blue spruce is caused by Leucostoma kunzei (Fr.) Munk ex Kern [syn. Valsa kunzei] which is most frequently seen in its anamorphic form, Leucocytospora kunzei (Sacc.) Urban [syn. Cytospora kunzei] (1). The diffuse branch cankers caused by this pathogen characteristically kill the lower branches of mature trees first and then proceed to affect branches. higher in the tree. Trunk cankers in blue spruce are uncommon (9). In Michigan and other areas, canker development is rare in seedlings and juvenile tress less than 20 years old (6). At a given time, mature trees at the same site are generally not affected to the same degree.

Several workers have indicated a relationship between drought stress and the occurrence and severity of Cytospora canker on spruce and other coniferous hosts (3,4,9). In other experiments the water potential of blue spruce trees inoculated with L. kunzei has been shown to affect canker development (5,7). The purpose of this experiment was to investigate whether there is any simple relationship between the water status of blue spruces in the field and the differential occurrence of Cytospora canker on those trees.

MATERIALS AND METHODS

Study sites- Two study sites were used in this experiment. Both sites were on the properties of Michigan State University in East Lansing, Michigan. These sites were selected because of the presence of mature blue spruces in which Cytospora canker was affecting some but not all of the trees. Site No. 1 was located north of Baker Woodlot and Site No. 2 was located at the northwest corner of Forest and Beaumont Roads. In addition, each site contained younger unaffected trees as well.

Sampling- At each site, three mature trees with and without Cytospora canker were sampled. (Note- Site No. 2 was also used for inoculation experiments and L. kunzei was introduced into one of the three unaffected trees on a few branches. This tree was free of Cytospora canker before inoculation and was placed into the unaffected catagory for this experiment.) A small secondary or tertiary branchlet was removed from two separate limbs found at approximately breast height on each tree. The branchlets were 20-30 cm in length and were taken from midway along the main branches, near the point where the first needle-bearing branchlets could be found along the limb. The branchlets were shaded at this point by the more distal portions of other surrounding branches which form the dense perimeter of blue spruce trees. All samples were taken from the south-west side of the trees for the sake of uniformity. Branchlets were collected similarly from juvenile trees at site No. 1, but from limbs

knee to waist high. At site No. 2, the immature trees consisted of naturally seeded, suppressed seedlings less than 33 cm in height. The upper 20-30 cm of these seedlings were collected. In addition at Site No. 2, branch terminals 20-30 cm in length were collected from all of the mature trees for comparison to the branchlets taken within the perimeter of the tree. Immediately upon removal from the trees, the branchlets were enclosed in tightly rolled plastic bags (2) and placed into a cardboard box which was kept in the shade. The branchlets from both sites were collected from 12 noon to 1:30 pm on 3 September, 1985. The weather was sunny with a stiff breeze and the temperature was 28°C.

Measurements of Water Potential- The collected branchlets were brought into the laboratory immediately after their collection. Terminal portions, 15 cm in length, were recut immediately before being placed into a pressure chamber. Using a pressure bomb, the water potential of the branches was measured as described previously (2,7,8).

RESULTS

The average measured water potential of the branchlets from within the crowns of mature trees affected with Cytospora canker and from the mature and immature trees without Cytospora canker was similar within each of the sites (Table 1, 2). The average water potential of all the branchlets from Site No. 1 was -15.1 bars. The average water potential of all the branchlets from Site No. 2 was -13.3 bars. The water potential of branch terminals at Site No. 2 was lower in comparison to the branchlets closer to the trunk (Table 3). No

TABLE 1

Water potential in blue spruce branches taken from trees with and without cankers caused by Leucostoma kunzei. Site No. 1, East Lansing, Michigan.

	Mature Trees[a,b]		Juvenile Trees[a,b]
Branchlet	No Canker	Previous Canker	No Canker
1	14.7	16.8	15.4
2	14.9	15.2	14.6
3	14.8	14.7	14.8
4	15.1	14.6	14.7
5	14.9	15.2	15.4
6	16.0	15.0	15.0
Mean	15.1 z	15.3 z	15.0 z

[a] Water potential figures are given in (- Bars) and were measured using the pressure bomb technique.

[b] Means followed by the same letter do not differ significantly (ANOVA, F test, LSD, P=.05).

TABLE 2

Water potential in blue spruce branches taken from trees with and without cankers caused by Leucostoma kunzei. Site No. 2, East Lansing, Michigan.

	Mature Trees[a,b,]		Juvenile Trees[a,b]
Branchlet	No Canker	Previous Canker	No Canker
1	12.0	13.0	12.2
2	12.2	12.7	12.4
3	13.8	15.6	15.0
4	14.1	14.9	14.9
5	13.5	11.0	13.5
6	13.3	11.1	13.8
Mean	13.2 z	13.1 z	13.6 z

[a] Water potential figures are given in (- bars) and were measured using the pressure bomb technique.

[b] Means followed by the same letter do not differ significantly (ANOVA, F test, LSD, P=.05).

TABLE 3

Horizontal gradient of water potential along the branches of blue spruce with and without cankers caused by Leucostoma kunzei.
Site No. 2, East Lansing, Michigan.

Canker Status	Branchlet[ab] from midway along limb	Branchlet[a] from terminal portion	Change[a]
none	12.1	13.0	1.1
none	13.9	14.8	0.9
none	13.4	14.4	1.0
present	12.9	14.0	1.1
present	15.3	16.1	0.8
present	11.1	13.0	1.9

[a] Water potential figures are given in (- bars) and were measured using the pressure bomb technique.

[b] The water potentials in this column are the average of two measured limbs.

significant differences in the measured water potentials was detected between the three categories of trees tested.

DISCUSSION

The water potentials obtained in this experiment are comparable to those measured in Sitka spruce [P. sitchensis (Bung.) Carr.] in mid-August by Hellkvist et al (2). The water potential gradient observed between branchlets collected along the middle portion of a major limb and from the terminal end of the limb was also noted in that paper. The lower average water potential of the trees at Site No. 1 as compared to those at Site No. 2, reflects the more exposed location of that site. The trees at Site No. 1. were more open to the effects of wind and sunshine while the trees at Site No. 2 were more sheltered from the wind and were shaded somewhat by neighboring deciduous trees.

In examining the relationship of tree water status to the occurrence of Cytospora canker, an effort was made to examine those tissues initially affected by this disease. Based on observations of over 100 spruces affected by Cytospora canker in the field, L. kunzei seems to invade the main branches of blue spruce midway back towards the trunk rather than moving in from the terminal branch tips. For that reason, water potential measurements were taken from branchlets located within the crown of the trees. Lower branches were tested also because of the observed tendency of L. kunzei to affect the lower branches of blue spruce first (6,9). The type and time of day at which samples were taken were chosen to maximize the water potential stresses experienced

by the trees as indicated by Hellkvist et al (2).

Drought stress has been implicated as being a factor in the occurrence and severity of Cytospora canker of spruce by several authors (3,4,5,7,9). Based on these results, however, the water status of a tree alone does not appear to account for the observed apparent differences in susceptibility to Cytospora canker among individual mature trees at a given site. Likewise, the 'field resistance' of young trees to canker formation by L. kunzei cannot be explained simply by differences in tree water status. Young trees in this study were under the same water stresses as neighboring mature trees with Cytospora canker.

At this point, it appears the role that tree water potential plays in the occurrence of Cytospora canker of blue spruce is complex. Tree water status does not appear to singly account for observed differences in the susceptibility of mature trees or immature trees to cankers caused by L. kunzei. Genetic and other physiological factors are probably involved in regulating the time of occurrence and the severity of Cytospora canker of blue spruce. It is possible that under conditions of severe drought stress, differences in tree water potentials could become evident which were not detected in this study. This possibility could be examined in future research by artificially inducing more severe drought stresses on these trees.

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SECTION 7

The effect of pH on the *in vitro* growth of *Leucocytospora kunzei*

INTRODUCTION

Cytospora canker of blue spruce is caused by the fungal pathogen Leucostoma kunzei (Fr.) Munk ex Kern [syn. Valsa kunzei]. This fungus is most frequently seen in its anamorphic form, Leucocytospora kunzei (Sacc.) Urban [syn. Cytospora kunzei] (1). Like all microorganisms, L. kunzei has certain optimal and minimal requirements for growth. In order to understand plant disease it is often of importance to gather basic biological information about the pathogen itself. The purpose of this experiment was to examine the effect of pH on the in vitro growth of L. kunzei.

MATERIALS AND METHODS

Isolates- Four isolates of L. kunzei were used in this experiment. Isolates V-5C(3) and OK-4B(4) were originally isolated from Colorado blue spruce [Picea pungens Engelm.] while isolates EL-30A(1) and EL-8A(2) were originally isolated from white spruce [P. glauca (Moench) Voss] and Norway spruce [P. abies (L.) Karst.], respectively. All of the isolates were from cankered trees growing in Michigan. The isolates were grown in 100X15 mm petri plates containing 2% Malt Extract Agar (2% MEA = 20 g of Difco Malt Extract and 20 g of Difco Bacto-Agar per liter of distilled H_2O). After 5 days, 6 mm plugs were transfered from near the margins of the fungal colonies to the pH test media. Leucocytospora kunzei grows very well on this medium (2% MEA) and forms

colonies which are uniform. The pH of standard 2% MEA is 5.5.

pH test media- For this experiment the pH test media were prepared by adding sterile solutions of lactic acid or KOH to autoclaved but still unsolidified 2% MEA. The pH value for each test medium was measured with a Beckman pH meter. The pH values for the 14 test media were: 3.0, 3.3, 3.6, 4.1, 4.5, 5.0, 5.5, 6.2, 6.5, 7.0, 7.8, 8.5, and 10.4. Mycelial plugs of the L. kunzei isolates were placed in 100X15 mm petri plates containing 25 ml of the solidified pH test media, parafilmed closed, and grown in the dark at 26°C. There were four replicates at each pH for each of the four isolates of L. kunzei tested.

Evaluation- The growth of L. kunzei in response to pH was evaluated by measuring daily increases in colony diameter. The time from the inoculation of the pH test plates to the final measurements was 6 days. The results were analyzed statistically by analysis of variance.

RESULTS

The pH of the culture medium affects the in vitro growth rate of L. kunzei. The optimal pH for the growth of L. kunzei under these conditions was 6.2 with all of the isolates except OK-4B(4), which had a growth optimum at pH 6.7. Good growth occured over a range of pH values from approximately 5.0-7.0 (Figure 1). The in vitro growth of L. kunzei was inhibited during the time course of the experiment on media adjusted to a pH value of 3.0 [3.3 for OK-4B(4)] and at pH values greater than or equal to 8.5. Weeks later however, viable though irregular colonies did develop on those media of extreme pH. The

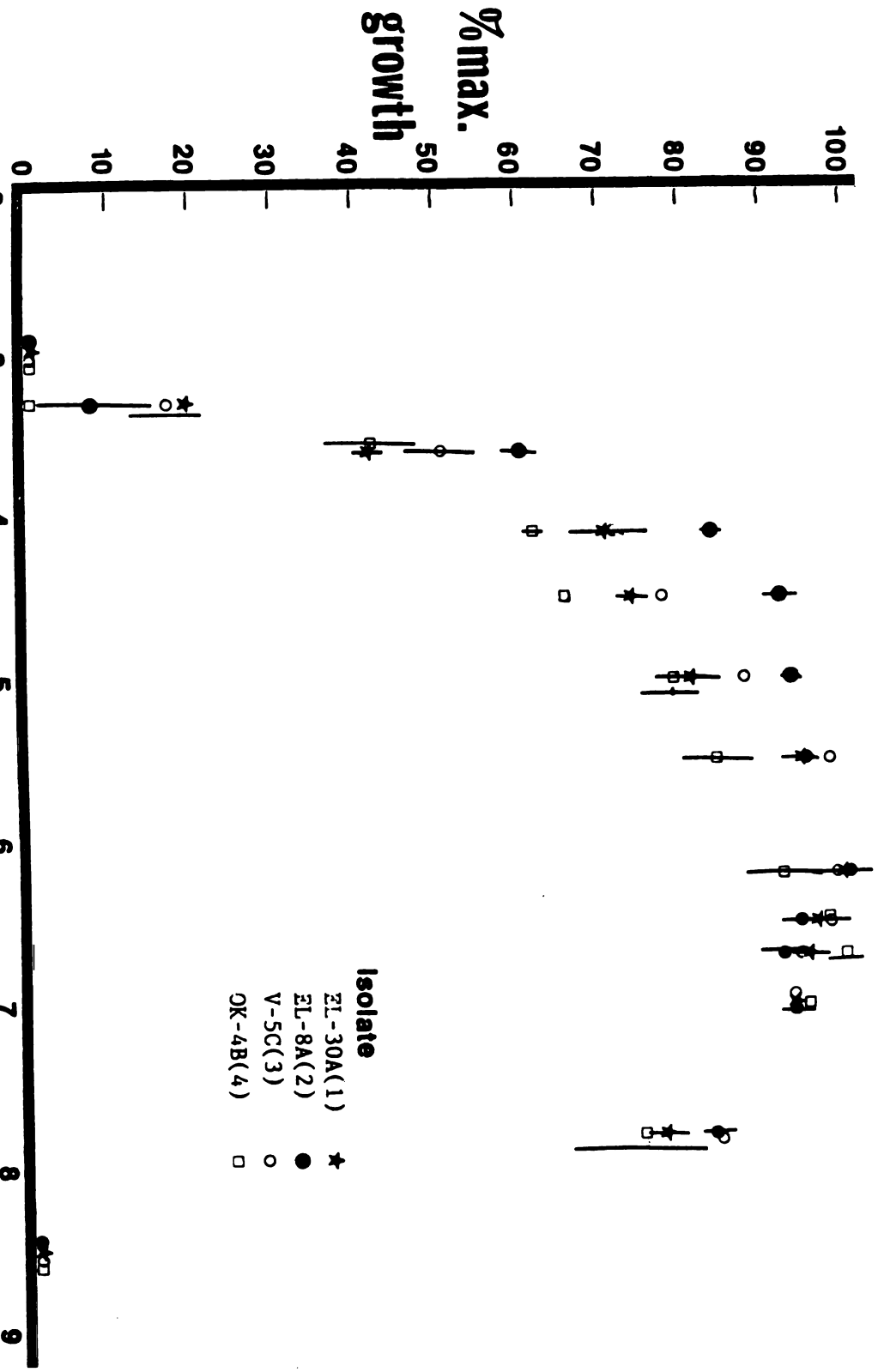

FIGURE 1. The effect of pH on the in vitro growth of Leucocytospora kunzei.

The average maximum increase in colony diameter per day for all of the isolates combined was 15.0 mm/day. There were significant differences in growth rates among the four isolates of L. kunzei tested under these environmental conditions (Figure 2).

DISCUSSION

Under given conditions a fungus will grow optimally over a range of pH values of the culture medium, and will fail to grow at high and low extremes (2). This experiment indicates that L. kunzei grows well on 2% MEA over a range of pH values from 5.0-7.0. Plant pathogenic fungi as a group tend to grow optimally on media adjusted to initial pH values of 5.0-6.5 and seem to form a 'biological type' among the fungi which prefer slightly acidic habitats (2). Two other fungi in the genus Leucostoma, L. cincta (Fr.) Von Höhnel and L. persoonii (Nit.) Togashi, which cause cankers on various stone fruits, prefer slightly more acidic conditions with reported optimal in vitro growth at pH values from 4.2-5.2 (3,4). Standard culture media such as Potato Dextrose Agar (PDA) and 2% MEA (pH 5.6 and 5.5 respectively) are suitable for isolating and culturing L. kunzei without making pH adjustments. These results cannot be regarded as absolute. The effects of pH on fungal growth are complex in nature and the mode of action leading to growth inhibition may not be the same at high and low hydrogen ion concentrations (2). Indeed, other environmental factors, such as temperature or the specific composition of the medium, can also affect the shape of the pH-growth response curve. For these reasons the results of this experiment cannot be taken as being the absolute effect of

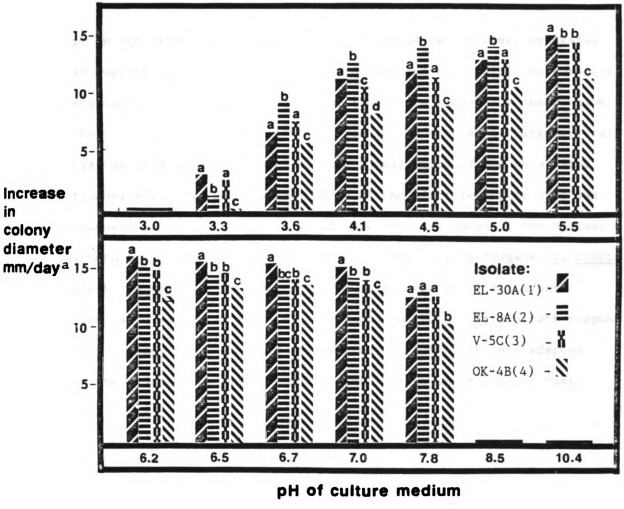

FIGURE 2. The response of four isolates of Leucocytospora kunzei to pH: Growth rates.

[a] Within each pH level, those values topped by the same letters are not significantly different (ANOVA, f-test, LSD, P=.05).

pH on the growth of L. kunzei. These results can however, be useful in suggesting a choice of a culture medium and for indicating possible ecological preferences. Along that line, in a supporting observation, the pH of cold water extracts of three pooled samples of bark/cortical tissues of blue spruce was 6.8. The actual value of the pH of live tissues would be lower, since the pH of the distilled water used was measured to be 7.4. This is within the observed range of the optimal growth of L. kunzei in vitro. The two stone fruit pathogens, L. cincta and L. persoonii, had lower pH optimums and the pH of peach bark/ cortical tissues was 4.4-5.6 (5). Based on the in vitro growth response of L. kunzei to pH and this observation, L. kunzei is well adapted to the hydrogen ion environment of the tissues in its spruce host.

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APPENDICES

APPENDIX A

Culturing Leucocytospora kunzei: Notes and observations.

1. The appearance of L. kunzei in culture is dependent on the type of growth medium and on lighting conditions. Lighting tends increase the amount of pigmentation of the colonies.

2. Colonies growing on Potato Dextrose Agar (PDA) under lights often 'staled-out' and ceased to grow before they filled the petri plate. The medium (PDA) was darkened. The pH of darkened PDA was higher than the pH of fresh PDA (7.1 versus 5.6 in one examination).

3. Based on cultural test, L. kunzei grows best on defined media utilizing maltose as a carbohydrate source.

4. Isolates of L. kunzei grown in axenic culture for two years were still pathogenic on inoculated blue spruce branches.

5. Twig or branch cultures are useful if pycnidia production is desired.

6. Leucocytospora kunzei was rarely recovered if the medium on which it was growing had dried down. "Potato chip cultures" are not viable.

7. Leucocytospora kunzei was best isolated from the discolored cambial/cortical tissues on branches which were not completely dead. If the discolored tissues were resin soaked, L. kunzei could still be recovered but it required more time. Avoid branches which are totally dead and lack needles.

APPENDIX B

Cytospora canker of spruce: Notes and observations.

1. Based on observations at homeowner properties, in many cases the occurrence and severity of Cytospora canker was greater on trees with root obstructions or soil related problems. Often trees near sidewalks and driveways would be first to be affected in a given yard or site.

2. Resinosis was not as pronounced on cankered branches of Norway and white spruce as compared to blue spruce.

3. Inoculations with L. kunzei isolates indicated that canker expansion was greatest in inoculated branches 1.5 to 3.0 cm in diameter. Inoculations on larger branches nearer to the trunk caused cankers which expanded but at a slower rate. Inoculations were best done on tissues at least two years old. Inoculations on new shoots generally failed to incite canker formation.

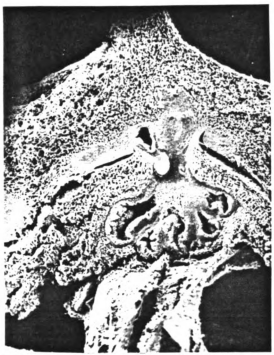
4. Cankered branches were placed on the ground in a brush pile. After one year, L. kunzei could be isolated from those branches which were not in contact with the soil. Few pycnidia however, were observed on the piled branches.

APPENDIX C

Other fungi frequently isolated from branches of blue spruce.

1. Aureobasidium spp.
2. Coniothyrium spp.
3. Phomopsis spp.
4. Epicoccum spp.

APPENDIX D



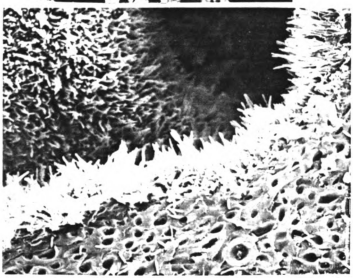
SCANNING ELECTRON MICROSCOPY

Leucocytospora kunzei

Free-hand sections of a pycnidium. Preliminary photographs.

l.s. through pycnidium.

30 X



Conidiophores lining the chambers.

1000 X

Conidiophores and Conidia.

15000 X