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THE UNIVERSITY OF ALBERTA

HOST-PARASITE INTERACTIONS BETWEEN *Endocronartium harknessii* AND *Pinus contorta*

BY

ERIC ANDREW ALLEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN

PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

FOREST PATHOLOGY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

SPRING 1989

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Host-parasite interactions between Endocronartium harknessii and Pinus contorta.

Dr. P.V. Blenis

Dr. Y. Hiratsuka

THE UNIVERSITY OF ALBERTA

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Canada

Date: Dec 16, 1988

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Host-parasite interactions between Endocronartium harknessii and Pinus contorta submitted by Eric Andrew Allen in partial fulfillment for the degree of Doctor of Philosophy in Forest Pathology.

.....
Supervisor, Dr. P.V. Blenis

.....
Dr. Y. Hiratsuka

.....
Dr. J.P. Tewari

.....
Dr. B. Dancik

.....
Dr. D.D. Cass

.....
Dr. E.M. Hansen

Date Dec. 12, 1988

ABSTRACT

Three aspects of the Endocronartium harknessii / Pinus contorta pathosystem were examined: symptom development in infected pine seedlings, resistance reactions, and the axenic culture of E. harknessii from infected pine tissue.

The development of external symptoms of infection was observed for a period of seven weeks following inoculation. Symptom expression was quite variable, but was shown to be linked to physiological and anatomical events occurring in underlying tissues.

Resistance to E. harknessii was observed in 3, 10, 20, and 33-month old lodgepole pine seedlings. Three sites of resistance were postulated, the epidermis, cortex, and cambium. In cases of epidermal resistance, penetration of the epidermis occurred, but infection was prevented by an apparent hypersensitive response. Cortical resistance occurred where infected cells in the cortex were successfully isolated by necrophylactic periderm and the infected tissue was exfoliated with the rhytidome. In cambial resistance, the fungus progressed to the vascular cambium where infected cells and cambial initials were inactivated. This resulted in abnormal secondary xylem development, characterized by a zone of pathological tissue extending from the pith to the epidermis. In a number of cases, cambial function was restored and infected lesions were overgrown. Live mycelium often survived within cortical lesions and in some cases escaped, reinvading healthy cortical cells. Such infections were usually blocked, but occasionally the fungus progressed to reinfect the cambium. These latent-type

infections resulted in the initiation of gall formation up to one year after initial resistance to infection occurred.

Axenic cultures of E. harknessii were established from infected pine tissue. Contaminant-free explants of gall tissue were incubated on a defined growth medium establishing dual-cultures of pine and fungal cells. When fungal growth was well-established (on about 2% of the explants) fungal cells were separated from the host tissue and grown axenically. Subcultured axenic colonies were maintained for more than one year. Colonies were orange-colored, and consisted of hyaline, septate hyphae, arising from a mass of vesicular cells. The vesicular cells varied in size and contained yellow-orange lipid bodies. The nuclear condition of axenically-grown E. harknessii cells was both monokaryotic and dikaryotic; occasionally multinucleate or akaryotic.

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CHAPTER I

INTRODUCTION

Endocronartium harknessii (J.P. Moore) Y. Hiratsuka is the causal agent of western gall rust, a fungal disease of hard pines. It is widely distributed throughout North America and can cause severe damage in infected stands (Hiratsuka and Powell, 1976). Major outbreaks are rare and the impact of the disease is not high in natural forests. However, as intensive management techniques play a more prominent role in forest practice, the importance of western gall rust will likely increase. Changes in microclimatic conditions resulting from spacing and thinning operations may affect patterns of spore release and infection success. The use of potentially infected nursery grown tree seedlings in forest regeneration creates a new hazard from the disease. Whereas western gall rust infections of older trees can affect form and act as a source of inoculum, infections on nursery seedlings often result in the death of trees before they reach a harvestable age.

Efforts to reduce the impact of the disease have included studies on chemical and biological control methods (Merrill and Kistler, 1976; Kistler and Merrill, 1978; Tsuneda and Hiratsuka, 1981b; Cunningham and Pickard, 1985), the biology and genetics of the host-parasite relationship (Hildreth, 1969; Nelson, 1970; Allen and Hiratsuka, 1985; Hoff, 1986b; Old et al., 1986; Vogler, pers. comm.; Hopkin et al., 1988 in press), and epidemiology of the rust (Peterson, 1973; Chang et al., 1988-in press; Chang and Blenis, in press). A lodgepole pine genetic improvement program now being initiated in western North America will

include the selection of resistance to E. harknessii.

Range and Hosts

The occurrence of western gall rust is as yet restricted to North America (Ziller, 1974). Although found mainly in western Canada and the U.S. (Hiratsuka and Powell, 1976), the fungus has also been reported in eastern Canada (Hiratsuka and Maruyama, 1968; Forbes et al., 1972), the Lake States (Anderson, 1965), and New York State (York, 1926). The disease as found in the northeastern U.S., earlier named the "Woodgate Rust" by York (1926), is now thought to be synonymous with western gall rust (Boyce, 1957; Krebill, 1968). Western gall rust is found on hard pines including Pinus banksiana Lamb., P. contorta Dougl., P. mugo Turra., P. muricata D. Don, P. nigra Arnold, P. pinaster Ait., P. ponderosa Laws., P. radiata D. Don, and P. sylvestris L. (Ziller, 1974). Many other pine hosts have been described, occurring naturally or identified by artificial inoculation (Nelson, 1970).

Although P. radiata is a susceptible host for E. harknessii, there have been no reports of its incidence in areas such as Australia and New Zealand where this species is planted as an exotic forest tree (Parmeter and Newhook, 1967). The disease poses a major threat in these areas where P. radiata seedlings have been planted over extensive areas. Extensive plantations of Canadian provenances of P. contorta are also being developed in Scandinavia and are subject to the same threat of infection by E. harknessii (Martinsson, 1980).

Taxonomic History

Efforts to describe the life history of E. harknessii have been complicated by confusing nomenclature with questionable synonymy and several common names applied to the fungus.

The fungus causing western gall rust was originally named Peridermium harknessii Moore by H.W. Harkness in 1876 (Harkness, 1884; Peterson, 1967). He found the fungus growing on Pinus ponderosa near Colfax, California. As more hosts were listed, and further observations of the fungus were made, there was question as to whether Harkness's description represented a single species (Boyce, 1957). Meinecke (1916, 1920, 1929) identified two forms of P. harknessii suggesting that the coastal form on Pinus radiata was identical to Peridermium cerebrum Peck (later changed to Peridermium ceribroides (?)). The other form, found in mountainous regions, was named Cronartium harknessii Meinecke. Artificial inoculation experiments were conducted using pine and known alternate hosts of Cronartium species in an attempt to identify the life cycle of the fungus. He was able to infect both pine and Castilleja Mutis ex L.f. species with the aeciospores of P. harknessii. From these results he concluded that P. harknessii was

> "...a heteroecious rust possessing, in addition to the usual mode of spreading through basidiospores, faculty of directly infecting pines through aeciospores..." (Meinecke, 1916).

He termed this phenomenon "facultative heteroecism" and used the name C. harknessii to describe the fungus as it existed on both the pine and Castilleja hosts (Meinecke, 1920). Further reports of Castilleja

infection by western gall rust aeciospores were made by Rhoades et al. (1918), Hedgecock and Hunt (1920), and Anderson and French (1965). Attempts by other researchers to duplicate these results have been negative (Wagener, 1958; Zalasky and Riley, 1963; Nelson, 1970; and Ziller, 1970). Moreover, in an analysis of Meinecke's early studies, Wagener (1964) suggested that the observed uredinia formation was the result of contamination from other rusts. The inclusion of P. harknessii in the Cronartium coleosporioides Arth. complex (Arthur, 1922; Anderson and French, 1965; Peterson, 1967) could have resulted in a misrepresentation of the rust's capacity to infect an alternate host. That is, the inoculation experiments demonstrating alternate host infection may not have been conducted with what we now recognize as Endocronartium harknessii.

Another possible explanation of the conflicting evidence is that although western gall rust is usually autoecious, heteroecious races may occur rarely in some locations (Nelson, 1970). Peterson (1967) suggested that the fungus is an extremely variable species and that physiological adaptations could account for the differences in life cycle, host preference, and growth form.

Hiratsuka et al. (1966) compared the nuclear behavior and germ tube morphology of autoecious (Peridermium pini Pers. and P. harknessii) and heteroecious (C. coleosporioides) rust species. In autoecious forms, the aeciospores functioned as teliospores which were termed "peridermioid teliospores" (Hiratsuka et al., 1966). Young aeciospores were usually binucleate, but later became uninucleate at maturity, suggesting nuclear fusion. Upon germination, septation occurred

producing a monokaryotic germ tube. The heteroecious rust had binucleate aeciospores and the dikaryotic condition was maintained through germ tube production, nuclear migration, and appressorium formation. Similar observations were made regarding the nuclear characteristics of P. pini and Cronartium flaccidum (Alb. & Schw.) Wint. (Hiratsuka, 1968). As a result of this work, Hiratsuka placed P. harknessii and P. pini into a new genus, Endocronartium, recognizing their endocyclic life cycles (Hiratsuka, 1969). Recent studies have questioned Hiratsuka's observations and interpretation of the nuclear behavior in germinating spores of these rusts (Epstein and Buurlage, 1988; Gibbs et al., 1988). Epstein and Buurlage recommend the reversion of Endocronartium harknessii to the genus Peridermium. Further studies must be conducted to elucidate the nuclear condition of the fungus throughout its life cycle, with particular attention paid to spore production and germination.

Disease Development

Because E. harknessii is autoecious, its life cycle is less complex than that of most forest tree rusts. Spores are produced by mature galls on branches and/or stems of the pine host from mid-May until early July. Site elevation, aspect, and prevailing climatic conditions determine the time of sporulation for a given year. Chang et al. (in press) showed that spore production was favored by periods of high humidity, at night when the ambient relative humidity was high or on rainy days. Spore release occurs when the relative humidity decreases and the warmer, drier conditions result in the drying of spores (Chang

et al., in press; Peterson, 1973). Spores land on susceptible pine hosts and germinate, given proper environmental conditions of temperature and moisture. York (1929) observed that spores infected the pines "directly through the epidermis of the current season's growth". True (1938) reported on the infection process of the Woodgate rust on Pinus sylvestris. All of the penetrations that he observed were through the epidermis, either inter- or intra-cellularily. No appressoria were noted. Hopkin et al. (1988) observed appressoria formation and direct penetration at the junctions of epidermal cells of young lodgepole pine hypocotyls.

Some reports have been made of successful inoculations through wounds or bark slits (Boyce, 1957; Anderson and French, 1965), but the current season's new growth is generally recognized as the primary site of infection (Meinecke, 1916; York, 1929; Hutchinson, 1935; True, 1938; Boyce, 1957; Nelson, 1970; Hiratsuka and Powell, 1976; Allen and Hiratsuka, 1985).

The position of an infection on a stem seems to be determined by the maturity of the stem tissue. Most of the successful infections observed by True (1938) were of tissues that had not yet developed a periderm. He felt that the presence of a normal periderm played a passive role in host resistance. Nelson (1970), studying Pinus radiata and P. ponderosa found that the elongating stem became nonsusceptible at the base first, and that the nonsusceptible zone "moved up" the stem as it matured. He felt that

> "...the nature of nonsusceptibility with stem maturation...appeared to be correlated with periderm formation as observed by True (1938)

for the Woodgate rust on Scotch pine...".

Other factors contribute to the success of infection. Adequate inoculum levels and suitable temperature and moisture conditions must coincide with the presence of susceptible tissue for infection to occur. In some years, termed "wave-years" by Peterson and Jewell (1968), infection levels were very high in localized areas, suggesting that conditions were optimal for infection. Attempts have been made to describe the environmental conditions prevalent in "wave-years" to aid in the prediction and possible control of the disease (Meyer et al., 1982).

Once the fungus has entered the host in compatible E. harknessii infections, it grows intercellularly through the cortex, forming haustoria in cortical cells. The fungal mycelium proliferates in the cortex, and appears to grow toward starch containing cells (True, 1938). When the fungus reaches the cambium, abnormal growth of the xylem, and to a much lesser extent, phloem, occurs. The most significant abnormality is hyperplasia of xylem cells. An 80 to 100 % increase in the number of these cells has been reported (True, 1938). Growth-ring width is therefore affected; Peterson (1960) observed that rings in infected wood were up to 2-3 times as wide as those in uninfected wood. Another major change that results from infection is an increase in the proportion and number of parenchyma cells. Peterson's studies showed that infected gall tissue consisted of 32% parenchyma and 68% tracheids whereas normal wood was comprised of 6% parenchyma and 94% tracheids. Xylem ray structure is also changed; most rays in infected wood are bi-seriate or multi-seriate whereas very few rays are more than one cell wide in uninfected wood (True, 1938; Peterson, 1960; Hiratsuka, 1975).

Zalasky (1976) described changes in xylem cell structure resulting from infection by E. harknessii. He found that tracheids in galls "...follow a radial order, but with changes in polarity, curvations, intricate branching, and nodulation...".

Such changes begin to occur during the same season as infection, and a swelling of the stem is often noticeable within four months (True, 1938; Boyce, 1957) The galls grow each year, adding growth rings similar to, but wider than those in a normal stem Spores are produced each spring until the branch or stem that the gall occupies is killed or the gall is attacked and inactivated by hyperparasitic fungi (Tsuneda et al., 1980; Tsuneda and Hiratsuka, 1981a). Rodents and insects can also damage or kill western gall rust galls by feeding on the spores and the bark of the galls (Powell, 1971; 1982; Powell et al., 1972).

Disease Impact

Western gall rust is widespread on the hard pines of western North America. Mortality is not normally high in the natural forest, but localized epidemics do occur. These have resulted in high mortality in young stands in the northwestern U.S. (Peterson, 1960). Generally, infections that occur in the natural forest result in the development of branch galls that affect form, but have little effect on growth rate (Gross, 1983). Main stem galls occur less frequently in older stands, as trees with stem galls usually die as they mature (Gross, 1983).

The greatest threat from western gall rust lies in intensively managed stands, plantations, and forest tree nurseries. Christmas tree plantations in New Brunswick have been abandoned because of damage by

western gall rust to tops and branches (Forbes et al., 1972). In Alberta, 63% of the 6 to 12-year-old lodgepole pine stock in a Christmas tree plantation were infected (Powell and Hiratsuka, 1973). Mortality in this case was not high but most of the trees were deformed and had no commercial value.

Western gall rust poses a major threat to plantations of Pinus radiata planted as an exotic species in Africa, South America, Spain, Australia, and New Zealand (Scott, 1960). The pathogen is not yet present in these areas, but seedlings from these plantations have been shown to be susceptible (Parmeter and Newhook, 1967). The relatively limited genetic base from which these trees originated further increases the potential danger.

Another danger from western gall rust is in forest nurseries that produce seedlings for the regeneration of logged areas. Infections in the nursery almost always result in the formation of basal stem galls. These may go unnoticed in the nursery and thus the infected seedlings may be outplanted. In plantations, these infected seedlings occupy valuable space and may receive special silvicultural attention such as pruning, fertilization, etc. Mature galls produce spores that can cause further infection of natural or planted stock. Whereas older trees can sustain branch infections with little detrimental effect, infection of nursery seedlings is ultimately lethal. Controls, therefore, must focus on the prevention of infection in young seedlings or the development of resistant seedling stock.

Disease Management and Control

Various control measures to reduce the incidence of western gall rust

have been considered, including silvicultural methods, chemical and biological control, and the genetic improvement of seedling stock. The choice of control methods and their effectiveness depends on the particular nature of the disease problem.

The primary means of silvicultural control involves the mechanical removal of galls from infected trees. This removes the source of inoculum preventing further spread of the pathogen. This type of control is obviously not feasible in mature natural stands, but can effectively reduce the incidence of the disease in localized areas. Infected trees in and around the Pine Ridge Forest Nursery in Smoky Lake, Alberta have been treated in this way. Further reduction or elimination of western gall rust problems in nurseries can be achieved by locating them in areas that are free of the disease or where pine does not naturally occur. Pruning galls or removing whole trees can help to reduce inoculum levels in intensively managed stands.The removal of galled trees can be implemented during thinning operations, but it is important that thinning crews can recognize the presence of the disease and make appropriate selections. Bella (1985) showed that larger trees in young lodgepole pine stands were more prone than smaller trees to western gall rust damage, so thinning operations that retained larger trees could result in an increase in damage from the disease.

Preliminary tests of chemical control of western gall rust have been conducted, (Merrill and Kistler, 1976) but as yet, field trials have shown limited success. Such controls, if possible, could be used in tree nurseries to prevent infection during that time of the year when the risk of infection is high.

A number of organisms have been observed to parasitize western gall rust galls (Byler, 1970; Byler and Cobb, 1969; Byler et al., 1972; Hiratsuka et al., 1979; Tsuneda and Hiratsuka, 1979; 1980; 1981a; 1981b; Tsuneda et al. 1980). Some of these parasites are abundant in certain localities and have been reported to cause a significant reduction in the activity and number of sporulating galls. Byler and Cobb (1969) reported that 44% of 2-year-old galls, and 97% of the 3 to 6-year-old galls in selected pine stands in California were inactivated by hyperparasitic fungi. Byler (1970) found that

"Nectria fuckeliana Booth was the most common and efficient gall killer in the plots where it was present."

In western Canada, Scytalidium uredinicola Kuhlman et al. and Cladosporium gallicola Sutton were both found to be strongly parasitic on E. harknessii (Tsuneda and Hiratsuka, 1981a). Cunningham and Pickard (1985) showed that maltol, a metabolite of S. uredinicola, caused a 50% decrease in E. harknessii spore germination in vitro. Preliminary greenhouse tests using maltol as a germination suppressant to reduce seedling infection gave promising results (Blenis et al., in press). A third mycoparasite, Monocillium nordinii (Bourchier) W. Gams. was reported by Tsuneda and Hiratsuka (1981a) to be a less effective biocontrol agent in the field. However, its ability to produce antibiotic metabolites (Ayer et al., 1980) raises the possibility of its use in the production of antifungal compounds. Ayer's study showed that the compounds monorden and monocillin I showed pronounced activity against a wide variety of fungi.

These and other mycoparasites are thought to be contributing factors

to the natural control of western gall rust (Byler et al., 1972) Their use as biological control agents has been suggested (Byler, 1970; Tsuneda and Hiratsuka, 1981a), but they remain untested under field conditions.

Genetic tree improvement programs are well established for breeding white pine resistant to Cronartium ribicola J.C. Fisch. ex Rabh. and resistant seedling stock has been produced (Bingham, 1983). Similar efforts to develop hard pines that show resistance to western gall rust are now being initiated as part of a lodgepole pine improvement program that is being started in western Canada and the U.S.. Included in this program are studies to determine the genetics of the host-parasite relationship, to examine the details of the infection process, and to further describe host-parasite interactions.

Symptom Development in Pine Stem Rusts

External symptoms that form soon after infection have been studied in some detail. True (1938) reported the appearance of infection spots on candle tissue of Pinus sylvestris infected with the "Woodgate rust". He observed lesions ranging in color from orange to dark brown and proposed a possible relationship between symptom characteristics and ultimate gall formation. A preliminary study of infection symptoms on young lodgepole pine seedlings infected with E. harknessii has been reported (Allen and Hiratsuka, 1985) as have responses on two exotic pine species, Pinus densiflora Sieb. and Zucc. and P. thunbergii Parl. (Hiratsuka and Maruyama, 1983; Hopkin et al., 1988). Similar studies have been carried out on a number of pine species infected with Cronartium quercuum Miyabe ex Shirai f. sp. fusiforme. Jewell (1960)

observed red spots on the cotyledons and primary needles of slash pine seedlings 4-7 weeks after inoculation. Miller et al. (1976) reported the formation of pigmented lesions as early as 2 weeks after inoculation and found that some symptom responses were correlated with gall formation. Lundquist and Luttrell (1982) and Lundquist and Miller (1984) studied early symptomology on slash pine and loblolly pine families of known resistance. They reported that the amount and rate of pigmentation varied with inoculum load and host family. Lesion types, which changed with time after inoculation, could be related to histopathological reactions of the underlying tissues.

A clear understanding of the significance of the different types of early symptom responses to infection by pine stem rusts could be of potential use in the development of methods for more rapid screening for resistance.

Resistance Reactions in Pine Stem Rusts

A variety of resistance reactions to pine stem rust fungi have been reported (Kinloch, 1980). Hutchinson (1935) studied the resistance of Pinus sylvestris to the Woodgate rust. He described three major reaction types. Type A, occurring in what he classed as susceptible trees, showed little external evidence of infection. Minimal host tissue response was observed, although some tannin[1] formation by cortical cells was evident. Reaction type B showed definite stem discoloration following infection, with bark splitting and resinosis occurring during the following growing season. In these plants, tannin

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[1]The term tannin or tannin-like substance refers to a large group of poly-phenolic secondary plant metabolites that are often involved in plant defence (Swain, 1979).

production by the cortical cells was much faster than in type A plants and the initial cells attacked by the fungus were killed. A barrier of cork cells produced by the host delineated these dead cells, excluding the fungus from the healthy part of the stem. The fungus sometimes broke through this layer, resulting in the reinfection of cortical cells and the development of a second cork barrier. This continued until the fungus was either successfully excluded or was able to reach the cambium and initiate gall development. The third reaction type (type C) developed numerous small discolored spots on the stem a few weeks after inoculation. This was sometimes followed by swellings which did not increase in size, but were gradually sloughed off. In type C trees, the infected host cells were usually killed immediately after invasion. A periderm formed around these dead cells and the necrotic area was sloughed off. Hutchinson felt that the production of tannin by the affected cells represented a local immunity and that the production of cork cells was a normal reaction to wounding and played a less important role in resistance.

Miller et al. (1976) reported three "hypersensitive" resistance reactions to Cronartium quercuum f.sp. fusiforme in slash pine which they designated types 3a, 3b, and 3c. Type 3a reactions involved cell death immediately around the point of infection and were confined to superficial cortical cells. In stems exhibiting type 3b reactions, infection zones were limited to cortical cells and appeared to be separated from healthy cortical tissue by necrophylactic periderm. In type 3c reactions successful infection of the cambium occurred but about 3 months after inoculation, the galls "stabilized" and the seedlings

"recovered".

Recovery from fusiform rust infection was observed by Snow et al.(1963) and later examined in greater detail by Jewell and his co-workers (Jewell and Snow, 1972; Jewell and Speirs, 1976; Jewell, 1979; Jewell et al., 1980; Jewell, 1985; 1988). These studies described the development of "resistance zones"; pathological tissue, often tanninized, which extended from the pith to the epidermis and within which hyphae and haustoria were confined. Abnormal secondary xylem formation was observed as a result of cambial interruption, but in some seedlings, cambial function was restored and the necrotic tissue was overgrown (Jewell and Speirs, 1976). Reactivation of rust infections in apparently-recovered seedlings ultimately resulted in typical gall formation (Jewell, 1979).

The practical value of the observed resistance reactions was evaluated through crosses of highly resistant shortleaf pine (Pinus echinata Mill.) with slash pine seedlings normally susceptible to the rust. Progeny from such crosses were found to be susceptible (Jewell, 1961a; 1961b; 1985). Backcrosses of these progeny with shortleaf parents produced reactions ranging from resistant to susceptible (Jewell, 1988). These results suggest that although hybridization does not ensure resistance, backcrossing can produce resistant F_2 progeny.

Studies on resistance in the white pine blister rust system have described responses similar to those outlined above. Struckmeyer and Riker (1951) observed wound-periderm formation around infected tissue and concluded that this was an important resistance response. Other mechanisms have been observed such as the prevention of needle lesions,

reduced frequency of needle lesion formation, premature shedding of infected needles, short-shoot fungicidal activity, and host tolerance (Hoff et al., 1980).

The genetics of resistance to Cronartium ribicola have also been examined (Kinloch, 1980). For example, the inheritance of bark reactions has been studied (Hoff, 1986b) and in sugar pine (Pinus lambertiana) a hypersensitive reaction to needle infection has been demonstrated to be conditioned by a single dominant gene (Kinloch and Littlefield, 1977).

Problems of a reliance on monogenetic resistance mechanisms that could lead to early breakdown of field resistance have been recognized. Long-term stability in field resistance might be achieved by maintaining diversity of resistance genes in populations of pine bred for resistance (Dinus, 1974; Browning et al., 1985). In this regard, it is important to recognize such variation in resistance mechanisms to endemic pathogen populations and care must be taken not to lose this diversity through selection for easily recognized resistance characters that may be controlled by major genes. This concern emphasizes the need for a thorough understanding of the nature and genetics of resistance mechanisms.

Tissue culture technology has recently been used in an effort to develop more rapid and controlled methods of screening for resistance to pine stem rusts. Amerson and Mott (1978) developed a technique of in vitro inoculation of loblolly pine seedlings with contaminant-free C. fusiforme basidiospores. The histological responses of loblolly pine embryos inoculated in vitro were reported by Gray and Amerson (1983).

Three seed lines of varying field resistance were tested and showed some positive correlations with resistance responses. The formation of necrotic tissue and host wall appositions were thought to be effective host resistance responses. Good correlations between in vitro responses and field resistance ratings have also been obtained with loblolly pine embryos (Frampton et al., 1985).

The successful infection of white pine callus tissue by basidiospores of Cronartium ribicola was described by Harvey and Grasham (1970). However, attempts to infect loblolly pine callus with C. fusiforme were unsuccessful (Jacobi et al., 1982) Inhibition of mycelial growth was attributed to the presence of an inhibitory factor produced by the pine callus tissue (Jacobi, 1982).

Axenic Culture of Pine Stem Rusts

Axenic culture is the growth of a single species in the absence of other living organisms or cells of other species (Dougherty, 1953). Most fungi are relatively easy to grow in this "pure culture" condition. Rust fungi, however, are biotrophic organisms with specialized nutritional needs. As a result, they are difficult to grow in the absence of their hosts and until recently all attempts to culture them axenically were unsuccessful.

The first successful axenic culture of a rust fungus (Gymnosporangium juniperi-virginianae Schw.) was reported by Hotson and Cutter (1951). Subsequently, various levels of success have been achieved in culturing over 25 species of rusts (Maclean, 1982; Yamaoka and Katsuya, 1985). Axenic culture has been reported for two species of pine stem rust fungi, Cronartium quercuum Miyabe ex Shirai f. sp. fusiforme (=

Cronartium fusiforme Hedgc. and Hunt ex Cumm.) (Hollis et al., 1972), and Cronartium ribicola J.C. Fisch. ex Rabenh. (Harvey and Grasham, 1974).

Axenic cultures are initiated by plating contaminant-free rust cells on a suitable growth medium. Different rust fungi have different nutritional needs so the composition of the growth medium is a major factor in culture success. Two basic approaches have been used to initiate cultures. In the first method, infected host cells are plated onto growth medium, establishing a dual culture. Once established, rust cells are removed from the dual culture and grown axenically. Both C. ribicola (Harvey and Grasham, 1974) and C. fusiforme (Hollis et al., 1972) have been cultured in this way.

The alternative method is to apply contaminant-free rust spores directly onto the growth medium. Hare (1978) cultured C. fusiforme from three spore forms, basidiospores, urediniospores, and aeciospores. Urediniospores and basidiospores are most commonly used for the initiation of axenic rust cultures because of the relative ease of producing contaminant-free spores (MacLean, 1982).

Axenic cultures of pine stem rusts will be a useful resource in the study of host-parasite interactions. Basic information may be obtained regarding life history and physiological characteristics of the rusts. Axenic rust cultures have been used in the in vitro study of pine stem rust infections. Diner and Mott (1982a) reported techniques for the inoculation of tissue-cultured sugar pine embryos with vegetative hyphae of axenically cultured C. ribicola. They also used in vitro methods to demonstrate a rapid assay procedure for hypersensitive resistance in

embryos (Diner and Mott, 1982b) and subcultured pine callus tissue (Dinus et al., 1984). Similarly, axenic cultures of C. quercuum f. sp. fusiforme have been used to inoculate and infect loblolly pine embryos (Frampton, 1984).

STUDY OBJECTIVES

The studies in this thesis were conducted in order to gain a greater understanding of the interactions between Endocronartium harknessii and lodgepole pine. The specific objectives of the project were to:

a) document the development of early symptoms in infected pine seedlings and attempt to relate externally visible symptoms with histopathological responses,

b) identify and characterize susceptible and resistant reactions to E. harknessii operating in lodgepole pine, and

c) establish axenic cultures of E. harknessii.

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CHAPTER II

EARLY SYMPTOM DEVELOPMENT IN LODGEPOLE PINE INFECTED WITH *Endocronartium harknessii*

Introduction

Endocronartium harknessii (J.P. Moore) Y. Hiratsuka is the causal agent of western gall rust, an important fungal disease of hard pines in North America. In Alberta, it is of major concern on lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.), particularly in forest nurseries and plantations. Infection by the fungus occurs on the current season's growth; on elongating candle tissue in trees (True, 1938; Hiratsuka and Powell, 1976; Nelson, 1970), and on epicotyl tissue in juvenile seedlings (4- to 6-week old) (Allen and Hiratsuka, 1985) resulting in the formation of galls on branches or stems.

Infection responses have been reported in studies observing the new growth of older trees (Hutchinson, 1935; True, 1938; McKenzie, 1942; Hoff, 1986), and in a preliminary study which examined infection symptoms on juvenile lodgepole pine ilings (Allen and Hiratsuka, 1985). However, no work has been done to follow symptom development and histopathological responses in juvenile seedlings infected with *E. harknessii*. Early symptom development has been reported for pines infected with *Cronartium quercuum* Miyabe *ex* Shirai f. sp. *fusiforme* (=*Cronartium fusiforme* Hedg. & Hunt *ex* Cumm.) (Lundquist and Luttrell, 1982; Lundquist and Miller, 1984).

A greater understanding of early symptom expression and its relationship with resistance and susceptibility may facilitate more rapid screening of resistant host material for use in lodgepole pine

improvement/breeding programs.

This paper documents the development of symptoms in lodgepole pine seedlings infected with E. harknessii, and attempts to relate the externally visible symptoms with histopathological reactions at the light microscope level.

Materials and Methods

Test Materials

Seeds were obtained from six Alberta provenances, ranging from 49 N lat. to 56 N latitude, and were planted in limed peat (2.6 g $CaCO_3$/l peat) in 60 cm^3 Spencer-Lemaire Rootrainers™ (Spencer-Lemaire Ind. Ltd, Edmonton, AB). Seedlings were grown at 22 C under an 18h photoperiod, and fertilized weekly with NPK (229-29-154 ppm) (Carlson, 1979).

E. harknessii spores were collected from various locations in Alberta, including Jasper, Hinton, and St. Albert, and used either fresh or after storage with silica gel in glass vials at -70 C. Trays holding 60 6- to 8-week-old seedlings were inoculated using dry spores. Each tray was sprayed with a mixture of 60 mg of spores and 240 mg of talc, then misted with approximately 40 ml distilled water (Blenis and Pinnell, 1988). Following inoculation, a wire frame was placed on each tray which was then covered with wet paper towels and wrapped in plastic to maintain high humidity. After incubation in darkness at 16 C for 48 hr in a growth chamber, inoculated seedlings were returned to the greenhouse.

Of 660 seedlings inoculated, 486 showed no symptoms within 6-weeks of inoculation. These were retained for assement of gall formation at 4-6 months. The remaining 174 seedlings showed symptoms of infection within

six weeks of inoculation. As symptoms appeared during this period, seedlings were examined weekly with a dissecting microscope and photographed to establish a permanent record of symptom development. At each examination, five to seven seedlings representing a range of external symptoms were sectioned. Paraffin-embedded stems were serially sectioned and observations were recorded in sequence allowing the construction of a three-dimensional "map" of infections within the stem.

Both symptomatic and non-symptomatic seedlings were grown for 4-6 months for assessment of gall formation.

Histological Methods

Stems were sectioned on a cryostat at 16 μm or were fixed in formalin-acetic acid-alcohol (FAA) and processed for paraffin sectioning (Jensen, 1962). Fresh cryostat sections were mounted in water or staining reagents. Paraffin blocks were sectioned at 12 μm and the sections mounted on glass slides using Haupt's adhesive. Epidermal peels were taken from infected epicotyls and mounted in 0.01% aqueous Tween 20.

Specimens were observed on a Leitz Orthoplan microscope using bright-field (BF) or transmitted fluorescence illumination. Fluorescence microscopy employed the use of ultra-violet (UV) (Leitz filters UG1, 300-400 nm + BG38, 300-700 nm excitation, barrier 430) or blue-green (BG) (Leitz filters BG12, 330-500 nm excitation, barrier 530) illumination.

Histochemical Methods

The sections and epidermal peels were stained with safranin-fast green (SFG) (Jensen, 1962) or were examined using the following

methods:

Phenol localization: Cryostat sections were treated with Hoepfner-Vorsatz (HV) reagents (Reeve, 1951). Sections were placed in a mixture of 10% sodium nitrite and 10% acetic acid. After 3 min, sections were flooded with 2N sodium hydroxide. Under BF illumintation, phenolic compounds appeared varying shades of red-brown, and under BG fluorescent illumination they appeared orange-red against a green background.

Lignin localization: Under fluorescent illumination, lignin produced a yellowish autofluorescence in fresh and SFG stained sections. When stained with a saturated aqueous solution of phloroglucinol in 20% hydrochloric acid, the fluorescence was quenched. Lignin appeared bright pink in phloroglucinol-stained sections under BF illumination.

Suberin localization: Suberin produced a bluish autofluorescence under UV illumination. When sections were stained with Sudan IV (saturated in 95% ethanol; glycerine 1:1, v/v) and viewed with BF illumination suberin stained red. Ultra-violet autofluorescence was quenched in Sudan IV-stained sections.

Haustoria localization: Localization of haustoria in thick (20-30 µm) hand-cut fresh sections or cryostat sections was enhanced by staining with 0.01% aqueous aniline blue and viewing under UV fluorescence.

Results

External symptoms.

The first symptoms were observed as early as three days after inoculation. In 83% of the 174 infected seedlings, however, the first symptoms appeared 14-28 days after inoculation. Red pigmentation developed in epidermal cells giving the appearance of red streaks on

the stem (Plate II-1). Considerable variation was observed in the number of and distribution of pigmented cells produced in response to infection. Some seedlings had as few as one affected cell whereas in rare cases, many cells developed pigment giving the infected area a reddish cast (Plate II-2). The most severe responses were obtained in an earlier unpublished experiment when an application of 400 mg fresh spores was used for each tray in the inoculation.

Pigmented cells were most commonly located on the epicotyl, in the axils of primary needles, and on the abaxial bases of primary needles. Pigmentation occasionally occurred on needle bases of control seedlings, but was distinguishable from that on infected seedlings by its appearance and distribution.

The pigment observed in epidermal cells was alcohol-soluble, but insoluble in water.

Epidermal peels were made from epicotyls of pigmented stems 14- to 21-days after inoculation. Haustoria were almost always present in pigmented cells, and often in nearby unpigmented cells. Haustoria were rarely seen in unpigmented tissue more than 10 cells away from the nearest pigmented cells. When epidermal cells were stained with HV, it was clear that the phenolic compounds were associated with infected cells, regardless of whether they were pigmented. Uninfected cells showed little or no evidence of phenolic activity. The dark stained zones in Plate II-3 closely corresponded with the location of infected cells; no fungal elements were observed in unstained tissue.

The color intensity of the pigmentation was affected by cuticular wax present on the stem surface; in stems with a thick layer of wax the

Plates II-1 - II-11: Infection responses in lodgepole pine seedlings infected with Endocronartium harknessii.

Plates II-1, II-2: Variation in the extent of epidermal pigmentation (arrow) on seedling epicotyls 7 days after inoculation. (bar = 1mm)

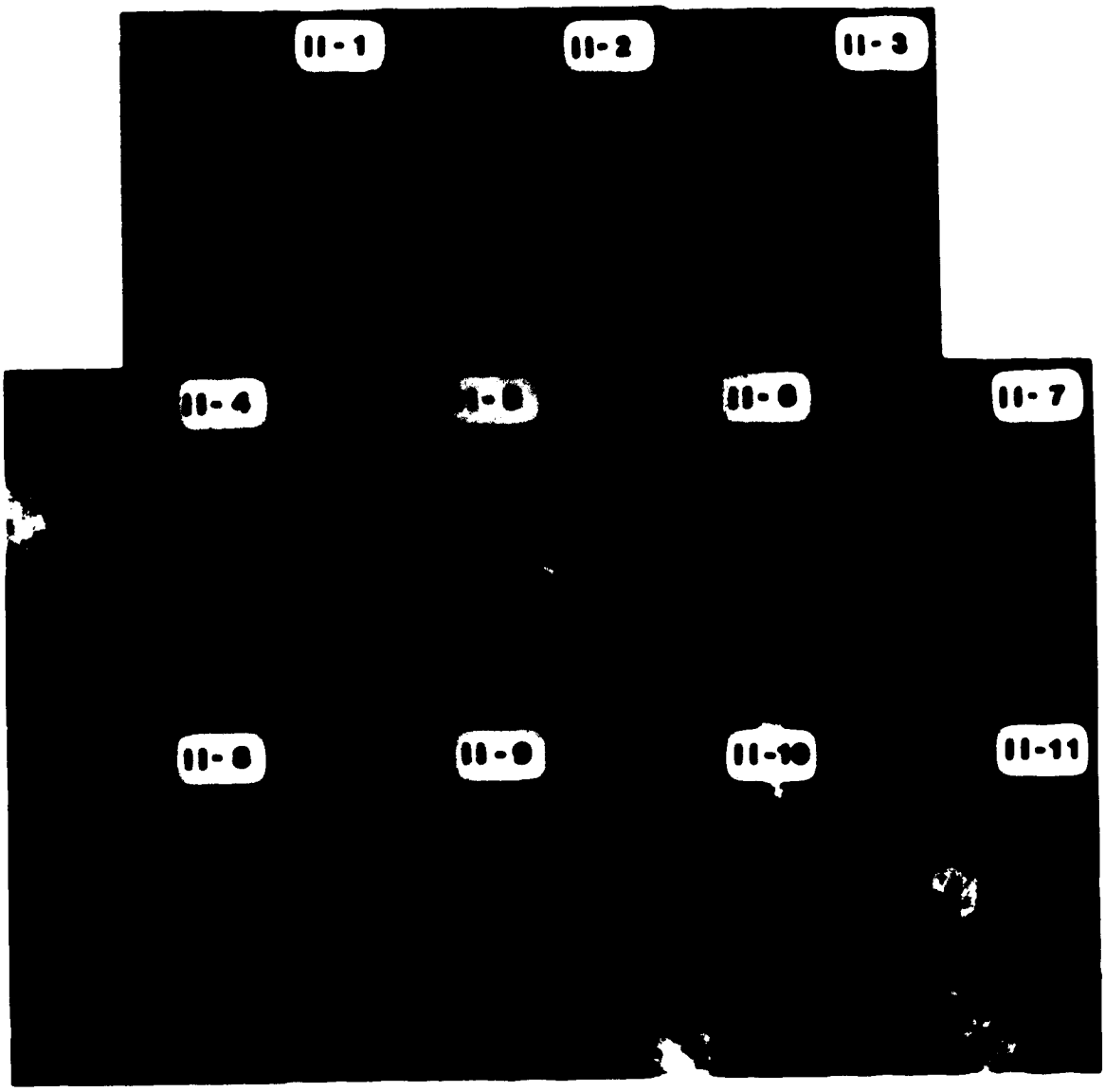
Plate II-3: Epidermal peel 10 days after inoculation showing the location of phenolic compounds in infected tissue stained with HV. (bar = 2mm)

Plates II-4 - II-7: External symptoms visible 22, 28, 35, and 42 days after inoculation, respectively. (bars = 1mm)

Plate II-8: Epidermal pigmentation (arrow) first visible 42 days after inoculation. (bar = 1mm)

Plate II-9: Diffuse lesion, first visible 35 days after inoculation. (bar = 1mm)

Plates II-10, II-11: Variation in the appearance of lesions and stem maturity 28 days after inoculation. (bars = 1mm)


11-1
11-2
11-3
11-4
11-7
11-8
11-9
11-10
11-11

color appeared lighter and more diffuse.

In the weeks following inoculation, early pigmentation responses developed further in extent and intensity of color. Plates II-4 - II-7 illustrate the same stem observed over a 4 wk perod, beginning 22 days after inoculation. After 6-7 weeks of development, lesions were generally necrotic, often showing resinosis and splitting of the outer stem tissues. Little further change was observed in the external appearance of the lesions after this time, and the maturation of the stem resulted in the drying and ultimate exfoliation of the epidermis.

In many cases, symptoms did not appear until as long as 35 days after inoculation. Such late-appearing symptoms were first observed as either red streaks (Plate II-8) as previously described, or as diffuse lesions (Plate II-9). Late, diffuse lesions were generally less intense in color than lesions which appeared soon after inoculation.

Variation was observed in the appearance of lesions of similar age. Plates II-5, II-10, and II-11 show symptoms observed 28 days after inoculation, in all cases, 14 days after their initial appearance. Plate II-5 represents a common 28-day response. Individual pigmented cells were discernable but appeared to be underlain by discolored tissue within the stem. Borders of the lesion were not well-defined. The lesion shown in Plate II-10 was orange-red in color, more discrete, and was bordered by an unpigmented zone of cells with a water-soaked appearance. Plate II-11 also shows a well-defined lesion differing in that the color was dark violet-red and the epidermal tissues surrounding the lesion were cracked and dried. Lesions of this type (Plates II-10, II-11), with discrete borders, changed little in size and appearance in

subsequent examinations, whereas the less defined type (Plate II-5) generally increased in size and color intensity. No differences in symptom development or expression were observed among pine provenances.

In 18 of the 660 inoculated seedlings, pigmentation of epidermal cells was observed 7 and 14 days after inoculation, but thereafter, coincident with normal maturational exfoliation of the epidermis, no symptoms were visible. Such seedlings continued normal development with no further indication of infection.

Many infected seedlings did not show early symptoms. Of the 486 seedlings which did not show infection symptoms, 230 developed galls. Asymptomatic gall production was also observed in an earlier inoculation of 550 seedlings, but only 6 asymptomatic seedlings produced galls (unpublished).

In contrast, sixteen of the 174 symptomatic seedlings did not produce galls.

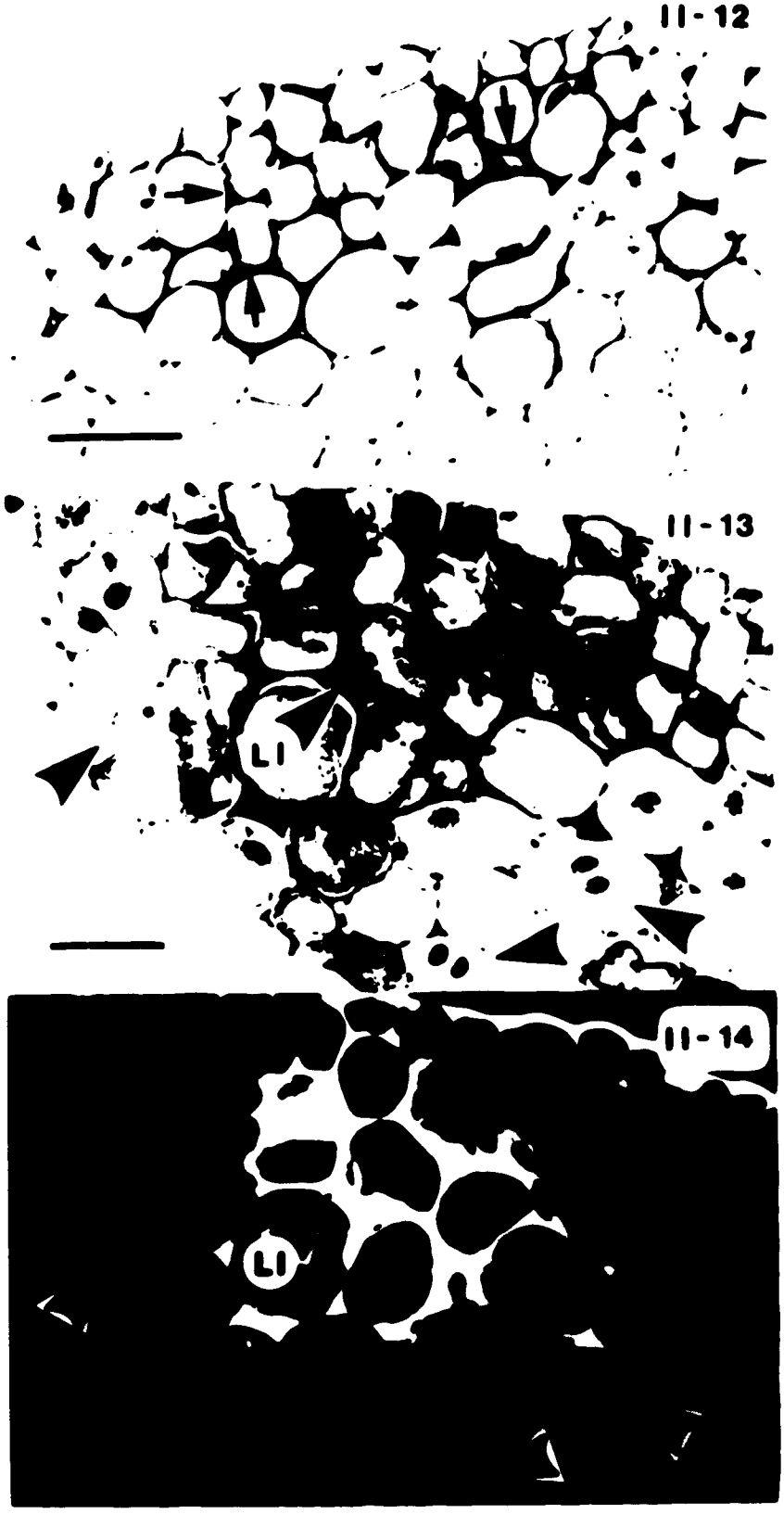
Internal Symptoms

Histological evidence of infection was difficult to locate in pigmented seedlings sectioned 7 days after infection. In most stems examined, haustoria were rare and hyphae were observed to be subcuticular or were restricted to the intercellular spaces around epidermal cells. Generally, seedlings exhibiting more severe external symptoms had a correspondingly greater amount of internal fungal development. Even in such heavily infected seedlings, however, fungal growth did not appear to penetrate deeper than the outer 2-3 cells of the cortex. Plate II-12, a cross-section of the seedling illustrated in Plate II-2, shows intercellular hyphae between epidermal and cortical

Plates II-12 - II-14: Infection responses in lodgepole pine seedlings infected with Endocronartium harknessii.

Plate II-12: Transverse section of the stem illustrated in Plate II-2 showing intercellular hyphae (arrows) in outer cortical cells 7 days after inoculation. Paraffin section stained with SFG. (bar = 5 μm)

Plates II-13, II-14: Necrophylactic phellogen initiation (arrows) is evident by recent periclinal cell divisions around infected tissue 14 days after inoculation. Lignin deposition (LI) is visible in the middle lammella and cellular junctions of infected cells. Transverse paraffin section stained with SFG. Plate II-13, brightfield illumination, Plate II-14, BG fluorescent illumination of the same section. Lignin autofluorescence is bright white. (bar = 5 μm)


II-12
II-13
LI
II-14
LI

cells. Haustoria were present in both epidermal and cortical cells. Both hyphae and haustoria were observed in the outer tissues of approximately one half of the circumference of the stem.

Scattered cells in the infected area stained positive for the presence of phenolic compounds and under UV fluorescence, lignin deposition was faintly visible in the cellular junctions of infected cells in some stems.

No other changes were observed in response to infection 7 days after inoculation although some sections originating from lower, more mature regions of the stem showed rudimentary exophylactic periderm formation associated with normal stem development.

Infections observed 14 days after inoculation showed greater fungal development and more intense histochemical responses than at 7 days. Cross-sections of 14-day stems showed hyphae and haustoria well established in localized regions of the cortex, and in one case, as deep as the cortical cells adjoining the phloem. Most of the hyphal growth observed deep in the cortex was concentrated around the longitudinal parenchyma cells surrounding resin canals.

Cortical cells in infected areas observed at 14 days showed brownish discoloration in unstained sections and stained positive with HV. Lignin was observed in the cellular junctions of infected cells near the outside of the stem.

The first evidence of necrophylactic periderm formation was seen 14 days after inoculation. Periclinal divisions were observed in cells bordering a zone of infected cortical cells suggesting the initiation of

necrophylactic phellogen (Plate II-13). The infected cells stained positive with HV, and examination of the same zone under UV illumination showed that intercellular lignin was restricted to the tissue being isolated by the developing phellogen (Plate II-14).

Exophylactic phellogen developed normally in subepidermal cells in healthy regions of the epicotyl. In infected zones however, differentiation of these cells into phellogen did not occur. Rather, subepidermal cells in infected tissue remained in an immature state (Plates II-13, II-15). Such suppression of exophylactic periderm formation occurred in virtually all infected tissue observed.

Sequential examination of serial sections presented clear evidence of discrete multiple infections on stems observed 14 days after inoculation. For example, one 2.4 mm stem segment had four separate infections at different locations on the stem. In another stem, five discrete infections were observed in a 3.1 mm segment.

A variety of interactions were observed both within and among seedlings within 21 days of inoculation. In one stem, for example, three separate infections were present. Two of the infections were restricted to the outer cortex and were bordered on the inside by a well defined, necrophylactic periderm. In the third infection, less than 500 µm higher on the same stem, hyphae and haustoria were present in abundance throughout the radius of the cortex, infiltrating as deep as the vascular cambium and no periderm was observed.

In eight of the ten seedlings examined in detail 21 days after inoculation, hyphae were observed in the cambial zone. In some cases, hyphae had grown through rays and interfascicular parenchyma tissue, and

Plates II-15 - II-20: Infection responses in lodgepole pine seedlings infected with Endocronartium harknessii. Transverse paraffin sections stained with SFG.

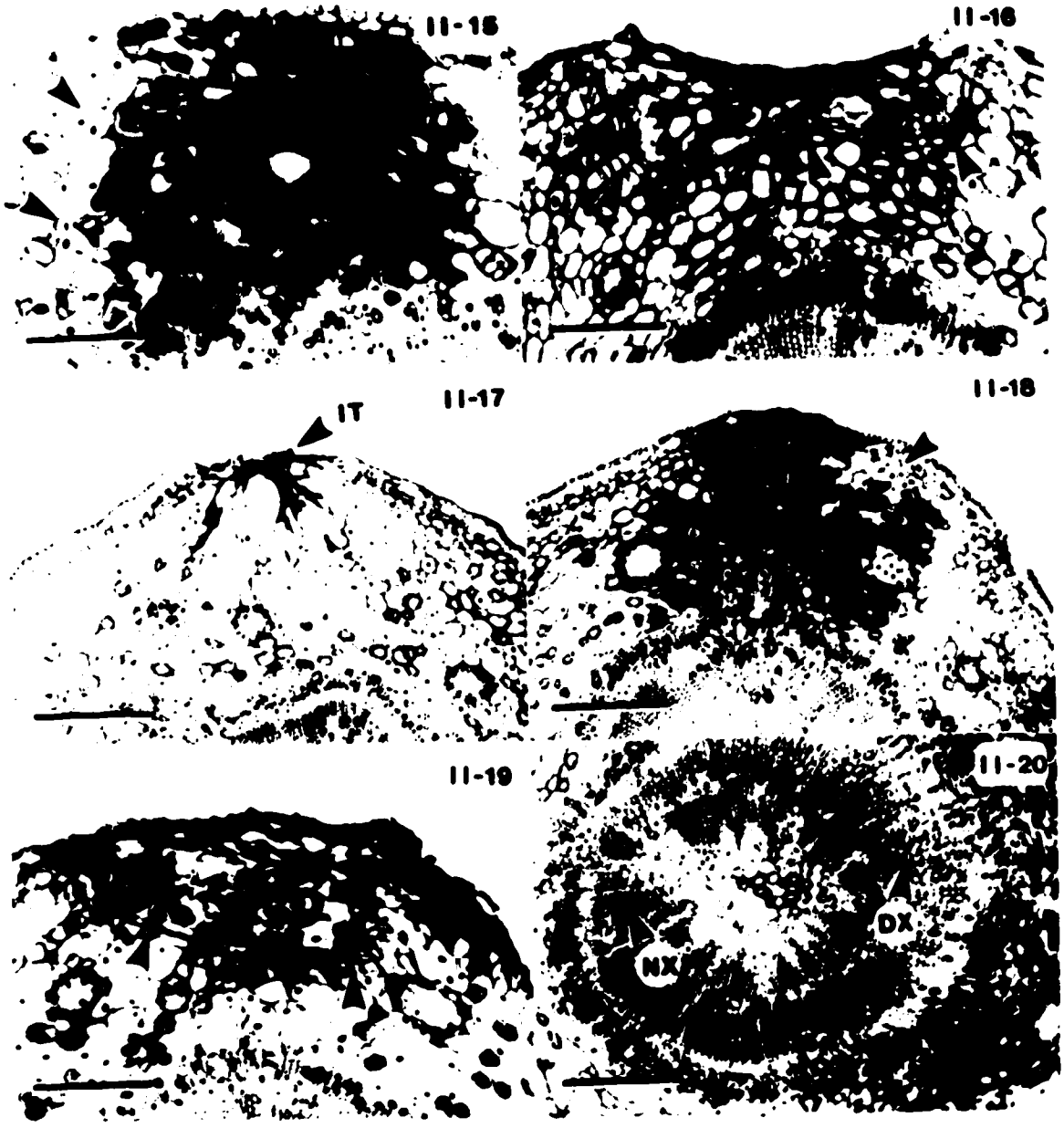
Plate II-15: Phenolic compounds in infected cells 21 days after inoculation. Recent cell divisions (arrows) periclinal to lesion indicate the initiation of necrophylactic phellogen. (bar = 200 μm)

Plate II-16: Necrophylactic periderm (arrows) around infected tissue 21 days after inoculation. (bar = 200 μm)

Plate II-17: Hypertrophic cells bordering necrotic infected tissue (IT) 21 days after inoculation. (bar = 250 μm)

Plates II-18, II-19: Sections of stem illustrated in Plate II-10, 28 day after inoculation. Plate II-18. Phenolic compounds in infected cells showing the typical extent of infection. Arrow indicates necrophylactic phellogen initiation. (bar = 250 μm) Plate II-19. Cross-section of lesion illustrated in Plate II-10. Crushed, necrotic tissue isolated by necrophylactic periderm (arrows). (bar = 200 μm)

Plate II-20: Modified, hyperplastic xylem development 35 days after inoculation. Note distorted (DX) and normal (NX) tracheid alignment in infected and uninfected tissue, respectively. (bar = 250 μm)


II-15
II-16
II-17
IT
II-18
II-19
II-20
DX
NX

had colonized the pith.

At 21 days, hyphae were often closely associated with longitudinal parenchyma cells around resin canals. Fungal growth followed the resin canals forming isolated columns of infected tissue. Hyphae were also concentrated in the phloem and xylem ray parenchyma. Mycelium was present in needle traces from the vascular cambial zone to the bases of primary needles, but was never observed to have developed more than 400 µm beyond the needle bases.

At 21 days, a significant increase in the accumulation of phenolic compounds was observed in cells of infected tissues. Phenolic-filled cells appeared amber to brown in unstained sections, and when treated with HV, the color intensified. Granular materials stained red-brown by HV in cells in infected areas and to a lesser extent in cells scattered throughout the cortex and phloem. Autofluorescence of phenolic compounds was observed under BG fluorescence illumination, and in SFG stained sections, phenolic-filled cells ranged in color from grey-purple to bright red. In the cortex, hyphae and haustoria were generally limited to stained infection zones but were occasionally observed 1-2 cells beyond the edge of stained areas.

An increase in the extent of lignin deposition was also observed at 21 days. Deposits of lignin were limited to infected cortical tissue and, as at 14 days, the initiation of phellogen was observed in association with lignin deposits (Plate II-15).

Well developed necrophylactic periderm was present around infected tissue in some stems 21 days after inoculation (Plate II-16). Outer

cortical and epidermal cells in tissue isolated by this periderm were often crushed and heavily lignified.

A striking anatomical feature first observed in one stem at 21 days was a zone of hypertrophic cells in an infected area. Plate II-17 shows enlarged, radially oriented cortical cells produced by periclinal divisions. The enlarged cells were up to 200 x 50 μm in size compared to 30-50 μm, the normal diameter of cortical cells. Infected outer cortical cells, often lignified, were crushed between the hypertrophic cells and the epidermis. The enlarged cells developed such that their longitudinal axes were perpendicular to the crushed outer cortical cells. In some sections, crushed cells also were observed within the hypertrophic zone. There was little or no accumulation of phenolic compounds, lignin, or suberin in the hypertrophic cells. Resin canals in the zone were modified; longitudinal parenchyma and epithelial cells were distorted, following the orientation of the surrounding cells. Hyphae were observed in crushed cortical tissue, throughout hypertrophic tissue, and in the phloem and vascular cambium zone interior to the affected area.

Little change was observed in infection development between 21 and 28 days after inoculation. In all stems examined, fungal colonies were well developed. Hyphae and haustoria were observed throughout the cortex, in the cambial zone, in unlignified newly formed secondary xylem cells, and through interfascicular parenchyma to the pith. In some stems, hyphae were abundant and packed tightly between cortical cells. In general, the area occupied by infected tissue had increased by 28 days. In most stems, up to one quarter of the cross-sectional

area was infected (Plate II-18). In severe infections, hyphae were present throughout the entire section.

Histochemical responses in 28 day infected tissue ranged from little visible reaction to heavy accumulation of phenolic compounds and significant lignin deposition.

Anatomical changes were similar to those observed at 21 days. Variation was present in the levels of periderm development that occurred (Plates II-18, II-19) and in one stem a hypertrophic response similar to that shown in Plate II-17 was observed. Plate II-19 is a cross-section of the lesion illustrated in Plate II-10. Serial sections showed that the entire lesion was bordered by a well-developed necrophylactic periderm. Complete containment was not achieved however, as hyphae were observed in adjoining cortical tissue. A separate infection in the same stem showed partial periderm development (Plate II-18).

At 35 days after inoculation, all stems examined showed fungal development through to the pith. Longitudinal fungal growth was associated with resin canals and vascular tissue.

Sections of some stems, particularly those exhibiting external symptoms of discrete, dark colored lesions like that in Plate II-6, stained intensely with HV and had extensive deposits of intercellular lignin. Lesions of this type were bordered with a well-developed periderm. Other stems, with diffuse, lightly-colored lesions, as illustrated in Plate II-9, showed similar accumulations of lignin and phenolic compounds, but little or no necrophylactic periderm was present.

The first evidence of hyperplastic xylem growth indicating the initiation of gall development was observed 35 days after inoculation. In Plate II-20, the infected portion of the vascular cylinder shows evidence of accelerated xylem production with distorted alignment of tracheids typical of galled tissue.

Examinations made 42 days after inoculation and later showed more seedlings initiating gall formation, but little other change in infection development was observed. In sections of the stem with late appearing symptoms (42 d) illustrated in Plate II-8, hyphae and haustoria were observed in the cortex and cambial zone. Hyperplastic xylem growth was observed, and although phenolic compounds were present in cortical parenchyma cells, the prevalence and staining intensity of these compounds was less than that of other reactions.

Discussion

Symptom development in E. harknessii infected lodgepole pine seedlings did not always occur in a definite progression in time. Although many seedlings developed symptoms in a similar pattern to those illustrated in Plates II-5 to II-7, late appearing symptoms or the absence of symptoms was also common.

The first externally visible response to infection was the production of red pigment in epidermal cells. Epidermal pigmentation is not a response specific to rust infection. It is observed on maturing conifer hypocotyls (Smith, 1968) and was seen on the abaxial bases of the primary needles of healthy controls. When pigmentation occurred on uninoculated seedlings, it appeared on virtually all needle bases on the

stem and could therefore be distinguished from the more localized responses on infected seedlings. Lundquist and Luttrell (1982) reported (as unpublished results) the induction of pigment formation on epicotyls of slash pine (Pinus elliottii var elliottii Engelm.) seedlings through mechanical wounding, or the application of dry ice, toxic organic solvents, or UV radiation. Although epidermal pigmentation did not occur in response to all infections in the present study, it remains a good indicator of successful penetration in inoculated seedlings. The factors controlling the pattern of pigment formation are not clear. Both pigmented and unpigmented cells contained haustoria and stained positive with HV. The development of red-colored phenolic substances may simply be less advanced in unpigmented cells. More discriminating tests for different types of phenolic compounds may lead to a greater understanding of the pigmentation response.

Subsequent development in the appearance of external symptoms was due to changes in infected cortical cells. The production of phenolic compounds and the death of infected cortical cells contributed to changes in external appearance. The variation in external symptoms observed among seedlings and among infections on the same seedling can be attributed to the characteristics of individual infections. For example, well-defined dark-colored lesions (Plates II-7, II-10, II-11) were typically comprised of necrotic cells underlain by a well-developed periderm (Plate II-16, II-19). Stems exhibiting less severe external symptoms had as much or more infected tissue, but often less intense cellular reactions and more importantly, little or no periderm. The death of cells in lesions isolated by periderm seems to be a key factor

in determining the appearance of some external symptoms.

Other studies have assessed the function of necrophylactic periderm in resistance to pine stem rust fungi, and there is good evidence to suggest that periderm plays an important role (Hutchinson, 1934; True, 1938; Struckmeyer and Riker, 1951; Miller et al., 1976; Walkinshaw, 1978). However, there is clear that the fungus is often not completely isolated by the periderm. Therefore, although some external symptoms may be indicative of a necrophylactic periderm response, such symptoms are not a reliable indicator of resistance.

In the present study, phenolic compounds were observed in association with infected tissue as early as 3 days after inoculation and in all subsequent examinations. The presence of these compounds had no apparent effect on the growth of the fungus nor were they directly related to the induction of periderm. The production of phenolic compounds in infected tissue is a common response observed in many pathosystems (Misaghi, 1982). "Tannins" have been observed in pine stem-rust infections (Hutchinson, 1934, Jewell, et al., 1962) but, as they are formed in both resistant and susceptible stems, their role in resistance is not clear (Walkinshaw, 1978).

Cell wall lignification and suberization have been implicated in the formation of impervious tissue in fungal infected and wounded plants (Biggs et al., 1984; Biggs, 1985; Rittinger et al., 1987) and is considered of importance in the process of phellogen regeneration (Mullick, 1977; Biggs et al., 1984). These reports support the observations of phellogen formation around infected tissue with lignified cell walls (Plates II-13, II-14, II-15). The deposition of

lignin within lesions, and the subsequent development of a suberized periderm, may have resulted in the isolation of the affected tissue from the apoplast and the ultimate death of the cells. These observations are consistent with Mullick's concept of periderm formation (Mullick, 1977; Puritch and Jensen, 1980).

The normal formation of exophylactic periderm in young pine epicotyls occurs in subepidermal cells beginning when the seedlings are about 6 weeks of age (Kozlowski, 1971, Waisel and Liphschitz, 1975; Allen, unpublished results). Normally the phellogen forms a complete cylinder of suberized cells around the stem. However, in all diseased stems observed in this study, no exophylactic phellogen was produced in infected tissue. The inhibition of exophylactic phellogen formation through the application of the growth regulators IAA, GA, NAA, and 2,4-D has been reported in woody tree species (Borger and Kozlowski, 1972; Arzee, et al., 1968). Borger and Kozlowski (1972) demonstrated further that under some treatments, the development of exophylactic phellogen was retarded while necrophylactic phellogen formation remained unaffected. Hormonal changes may be involved in the inhibition of exophylactic phellogen development in E. harknessii infections. Alterations in hormone metabolism have been observed in diseased plants (Sequeira, 1973), and research is currently being conducted on the production of hormones in the E. harknessii pathosystem (R.A. Savidge, pers. comm.).

A much less common response was the development of hypertrophic cells in association with infections in a number of the stems observed. The orientation of periclinal divisions around infected tissue suggested

the production of a modified phellogen although suberization did not occur and there was no apparent restriction of fungal growth. Cellular hypertrophy in the extremes observed here has not been reported as a response to this or other pine stem rusts.

This study provides clear evidence of multiple infections of E. harknessii on young pine seedlings. Multiple gall formation is a common occurrence, and is an obvious consequence of spatially separate infections on the same stem. However, the multiple infections observed herein were generally less than 1 mm apart, often vertically overlapping, and might have been incorporated into the same gall. The resultant gall could therefore contain genetically different fungal material, and spores produced by the gall would not be genetically identical. This could have serious implications in research which assumes genetic homogeneity of spores from single galls.

Lodgepole pine seedlings infected with Endocronartium harknessii may or may not produce externally visible symptoms prior to gall formation. When symptoms do occur, they are variable and often do not clearly indicate the nature and extent of the underlying infection. With our present understanding of the factors controlling symptom expression, the identification of resistant and susceptible trees using the characteristics of external symptoms is not recommended. However, the recognition of external symptoms can be a useful tool to verify infection, and when resistant lines of lodgepole pine become available, further study of symptom types may prove valuable.

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CHAPTER III

HISTOLOGICAL EVIDENCE OF RESISTANCE TO *Endocronartium harknessii* IN *Pinus contorta*

Introduction

Endocronartium harknessii (J.P. Moore) Y. Hiratsuka is the causal agent of western gall rust, an important fungal disease of hard pines in North America. The fungus is an endocyclic, autoecious rust, possessing only one spore state and no alternate host, and therefore efforts to control the disease must focus on the pine host. The impact of western gall rust is not great in natural forests in Canada, but as forest management becomes more intensive, damage by the disease will likely increase.

Trees potentially resistant to the rust have been observed under field conditions (Merrill, 1986). Hoff (1986) observed external evidence of resistance reactions in *E. harknessii*-infected *Pinus ponderosa* Laws. seedlings but no studies have examined specific resistance mechanisms in lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.). Hutchinson (1935) studied resistance to the "Woodgate Rust", thought to be synonymous with *E. harknessii* (Boyce, 1957; Krebill, 1968), on *Pinus sylvestris* L., and described resistant reactions involving tannin deposition and periderm formation. Evidence of resistance has been reported in other pine stem rust systems: in slash pine (*Pinus elliottii* var. *elliottii* Engelm.) infected with the southern fusiform rust fungus, *Cronartium quercuum* Miyabe *ex* Shirai f. sp. *fusiforme* (= *Cronartium fusiforme* Hedgc. & Hunt *ex* Cumm.) (Jewell and Snow, 1972; Jewell and Speirs, 1976; Jewell, 1988; Miller *et al.*, 1976), and in western white pine (*Pinus monticola* Dougl.) infected with

the white pine blister rust fungus (Cronartium ribicola J.C Fisch ex Rabenh.)(Struckmeyer and Riker, 1951; Hoff et al., 1980).

A greater understanding of resistance mechanisms may contribute to the development of rust-resistant lines of lodgepole pine. This paper presents the results of a histological study of host:parasite interactions in trees suspected of showing resistance to E. harknessii.

Materials and Methods

Six-week-old lodgepole pine seedlings were inoculated with E. harknessii spores and observed weekly for infection symptoms during the 5 weeks following inoculation. Symptom characteristics were recorded and the seedlings were photographed. The seedlings were assessed for gall formation 4-5 months after inoculation. Those seedlings which had shown evidence of infection during the first month but which did not have galls 4-5 months after inoculation, were considered potentially resistant and kept for further observation.

Four inoculations were conducted; in March 1985, June 1986, April 1987, and September 1987. Final observations of all inoculations were made in the spring of 1988, providing sampling of trees approximately 3, 10, 20, and 33 months after inoculation. A total of 1960 seedlings were inoculated, and of these, 83 were gall-free 4-5 months after inoculation. Potentially resistant trees as well as trees which exhibited normal gall development were sectioned and examined histologically.

Test materials

Seeds used in the 1985 planting were obtained from Grand Prairie, Alberta. Seeds for subsequent plantings were obtained from six Alberta

provenances ranging from 49 N lat to 56 N lat. These were planted in limed peat (2.6 g $CaCO_3$/l peat) in 60 cm^3 Spencer-Lemaire Rootrainers™ (Spence-Lemaire Ind. Ltd, Edmonton, AB). Seedlings were grown at 22 C under an 18h photoperiod, and fertilized weekly with NPK (229-29-154 ppm) (Carlson, 1979). At 4 months and 2 years, seedlings were transplanted to larger pots (0.5 L and 10 L, respectively) to maximize growth potential.

E. harknessii spores were collected from various locations in Alberta including Jasper, Hinton, and St. Albert, mixed, and used either fresh or after storage with silica gel in glass vials at -70 C. Trays holding 60 6-week-old seedlings were inoculated using dry spores. Each tray was sprayed with a mixture of 60 mg of spores and 240 mg of talc, then misted with approximately 40 mL distilled water (Blenis and Pinnell, 1988). Following inoculation, a wire frame was placed over each tray which was then covered with wet paper towels and wrapped in plastic to maintain high humidity. After incubation in darkness at 16 C for 48 hr in a growth chamber, inoculated seedlings were returned to the greenhouse.

Histochemical Methods

Stems were sectioned on a cryostat at 16 µm or were fixed in a formalin-acetic acid-alcohol (FAA) solution, processed for paraffin microtomy and sectioned at 12 µm. The sections were stained with safranin-fast green (SFG) (Jensen, 1962) or were examined using the following methods:

Autofluorescence: Sections were observed by fluorescence microscopy with a Leitz Orthoplan microscope using transmitted ultra-violet (UV)

illumination (UG1, 300-400nm + BG38, 300-700nm excitation filters, barrier filter 430).

Phenolic localization: Cryostat sections were treated with Hoepfner-Vorsatz (HV) reagents (Reeve, 1951). Sections were placed in a mixture of 10% sodium nitrite and 10% acetic acid. After 3 min, sections were flooded with 2N sodium hydroxide.

Lignin localization: Under UV illumination, lignin produced a yellowish autofluorescence. When stained with a saturated aqueous solution of phloroglucinol in 20% hydrochloric acid the fluorescence was quenched. Lignin appeared bright pink in phloroglucinol-stained sections under bright-field illumination.

Suberin localization: Suberin produced a bluish autofluorescence under UV illumination. When sections were stained with Sudan IV (saturated in 95% ethanol; glycerine 1:1, v/v) suberin stained red under bright-field illumination. Autofluorescence was quenched in stained sections under UV, but enhanced when the filter combination of BG12, 340-500 nm excitation, barrier filter 530 was used (Biggs, 1985).

Peroxidase activity: Peroxidase activity was used to determine the viability of rust haustoria (Walkinshaw, 1978). Sections were placed in a fresh mixture of : 1 ml saturated ammonium chloride, 1 ml 5% EDTA (ethylenediaminetetraacetic acid), 9 ml saturated benzidine, and one drop sodium hydroxide. Haustoria which stained blue were considered peroxidase positive and viable.

Calcium Oxalate localization: Stem sections were stained with silver nitrate-dithiooxamide for identification of calcium oxalate (Yasue, 1969). Sections were flooded with 50% acetic acid (three changes),

rinsed in double-distilled water, and stained in a 5% aqueous silver nitrate solution for 15 min. After rinsing with water, the sections were flooded with a saturated aqueous dithiooxamide solution for 2 min, dehydrated in an ascending alcohol series, and mounted in glycerol.

Crystals were also examined using energy-dispersive x-ray microanalysis (EDX). Paraffin sections were mounted on SEM (scanning electron microscope) stubs using Haupt's adhesive, dewaxed, and coated with gold in a sputter-coater. Microanalysis was conducted on a Cambridge Steroscan 250 SEM equipped with a Kevex Micro-X 7000 analyzer.

Resin: Pine resin autofluoresced bright blue-white under UV light. When stained for 1-3 weeks in saturated aqueous cupric acetate, resin stained green (Catcheside, 1950).

Haustoria localization: Rust haustoria were often visually obscured by the presence of phenolic compounds in lesions and older bark tissues. Oxidation of sections in 6% sodium hypochlorite allowed visualization of haustoria. Localization of haustoria in thick (20-30 μm) fresh sections was often enhanced by staining with 0.01% aqueous aniline blue and viewing under UV fluorescence.

Results

Virtually all seedlings examined that exhibited red epidermal pigmentation were found to be infected by E. harknessii. Pigmentation occasionally occurred on needle bases on uninfected stems but could be distinguished from the infection response by its appearance and distribution.

No differences were observed among provenances regarding symptom development or expression of resistance responses. Within 7-14 days of

inoculation, infection symptoms were visible as red streaks in the epidermal layer of the epicotyl (Plate III-1).

Normal gall formation

Histological examination revealed the presence of intercellular hyphae in the epidermis and outer cortex. In some cases the fungal growth was subcuticular. Within 28 days of inoculation, the fungus proliferated in the cortex, growing radially toward the vascular cambium and circumferentially around the stem. The presence of the fungus in the cortex appeared to stimulate the production of phenolic compounds by these cells. The initiation of periderm development at the borders of phenolic reaction zones was observed in some stems. The fungus reached the vascular cambium as early as 21 days after inoculation, and evidence of gall initiation was visible by 35 days. Gall initiation was characterized by an increase in the number of xylem elements, disorientation of tracheids, and an increase in the ratio of xylem ray cells : tracheids as previously described by Peterson (1960).

Resistance prior to 3 months

In some seedlings epidermal pigmentation indicating successful infection was visible 7 and 14 days after inoculation, but in the following weeks, these symptoms disappeared and no further progress of infections was observed. Normal maturational splitting and exfoliation of the epidermis occurred in the stems, and brownish-red streaks were visible in the moribund epidermal tissues near the original site of the symptoms.

Resistance reaction at 3 months

In one of twelve stems examined three months after inoculation,

Plates III-1 - III-6: Infection reactions in lodgepole pine seedlings inoculated with Endocronartium harknessii.

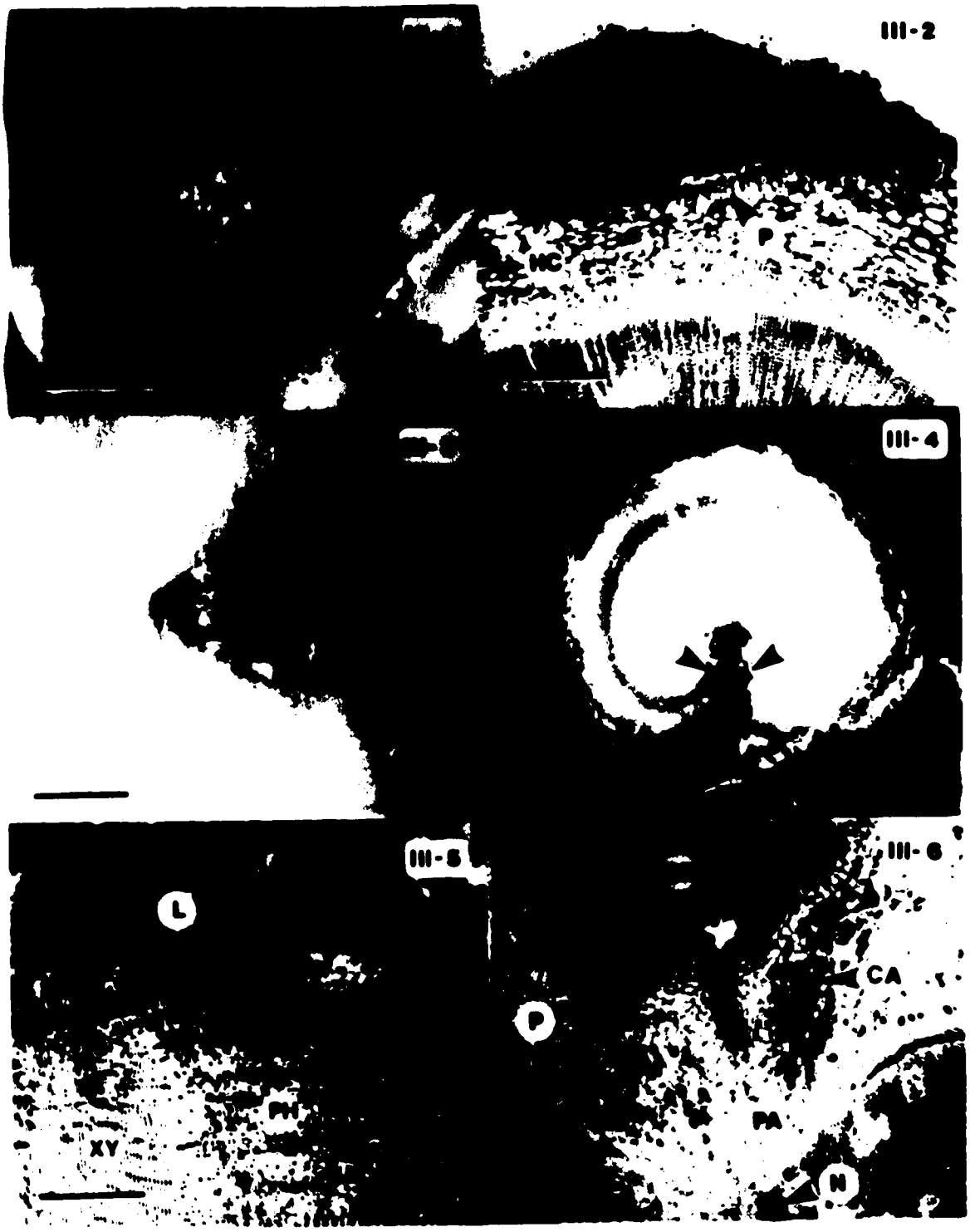
Plate III-1: Red epidermal cells (arrows) on epicotyl of 62-day-old seedling, 21 days after inoculation. (bar = 1 mm)

Plate III-2: Three-month-old cortical lesion (L) separated from healthy cortex (HC) by necrophylactic periderm (P). Transverse paraffin section stained with SFG. (bar = 500 µm)

Plate III-3, III-4: Ten-month-old reaction showing the result of cambial inactivation and a lesion continuous from pith to epidermis. Unstained longitudinal (Plate III-3) and transverse (Plate III-4) faces of fresh stems. Arrows in Plate III-4 indicate the inner extent of vascular cambium into reaction zone. (bars = 1 mm)

Plate III-5: Ten-month-old reaction showing disruption of the axial continuity of xylem (XY) and phloem (PH) at edge of lesion (L). Longitudinal paraffin section stained with SFG. (bar = 500 µm)

Plate III-6: Ten-month-old reaction with cortical necrosis (CN) separated from inner necrotic tissue (N) by suberized periderm (P) and non-necrotic parenchyma (PA). Calcium oxalate crystals (CA) are present in suberized parenchyma cells. Transverse cryostat section stained with silver nitrate-dithiooxamide. (bar = 500 µm)


III-2
NC
P
III-4
III-5
III-6
L
D
CA
PA
N
PH
XY

resistance to infection was observed in cortical tissues. Necrotic cells containing nonfunctional haustoria were isolated from healthy cortical cells by a well-developed, necrophylactic periderm (Plate III-2). No hyphae or haustoria were present in healthy tissue adjoining the lesion.

Resistance reactions at 10 months

Thirteen stems considered potentially resistant were examined 10 months after inoculation. Of these, 10 stems exhibited what were considered to be successful resistant reactions. The remaining 3 appeared to have resisted the fungus for some time, after which gall development began. While the resistant stems had many anatomical and histochemical characteristics in common, there was some variability, and individual stems possessed unique traits. The most common feature observed in resistant stems was disruption of the normal development of secondary xylem, and the replacement of this tissue with a cone-shaped zone of pathological tissue (Plate III-3, III-4). The cells filling the disrupted area were largely thin-walled, parenchyma which stained positive for peroxidase activity and for the presence of phenolic compounds. In three dimensions, the disrupted area appeared as a cone-shaped indentation in the column of secondary xylem. Serial cross-sections and longitudinal sections showed that at the widest point of the disrupted area, the zone of pathological tissue extended radially from the pith to the cortex, disrupting the axial continuity of the xylem and phloem (Plate III-5). In cross-sections above and below this point, the innermost point of the zone was located further out in the secondary xylem. The vascular cambium was also interrupted by the

pathological zone. The cambium extended inward along the periphery of the pathological tissue, but did not extend to the center of the zone (Plate III-4).

In all resistant stems, a zone of necrotic, phenolic-filled cells was present in the cortex, radially opposite to the indentation (Plate III-4). In some cases, the necrosis extended inward from the cortex to the center of the stem (Plate III-4). In other stems, necrotic cells in the center of the stem were separated from the cortical necrosis zone by non-necrotic parenchyma, or rudimentary vascular tissue (Plate III-6). One stem contained a small necrotic lesion near the pith and necrotic tissue in much of the rhytidome, but showed little disruption of the secondary xylem.

In all of the resistant stems observed, necrotic zones were found to contain fungal haustoria. When stained for peroxidase activity, the haustoria were determined to be non-functional. Often non-functional haustoria were observed in cells immediately adjacent to the necrotic tissue. These cells appeared healthy in unstained cryostat sections, however, they always showed some degree of phenolic activity when stained with HV. Functional (peroxidase positive) haustoria were also present in parenchymatous cells of the pathological tissue outside of the necrotic zones (Plate III-7).

Zones of necrotic tissue were usually bordered by suberized periderm cells (Plate III-2). These often formed in successive layers (sequent periderm) in the necrotic cortical tissue (Plate III-8), and frequently the innermost layer was continuous with the normal exophylactic periderm. The layers of periderm appeared to form around cortical

Plates III-7 - III-12: Infection responses in lodgepole pine seedlings inoculated with Endocronartium harknessii.

Plate III-7: Haustoria in necrotic (N) and healthy (H) cells in a 10-month-old lesion. Haustorium marked with arrow is peroxidase positive. Transverse cryostat section stained with HV. (bar = 80 μm)

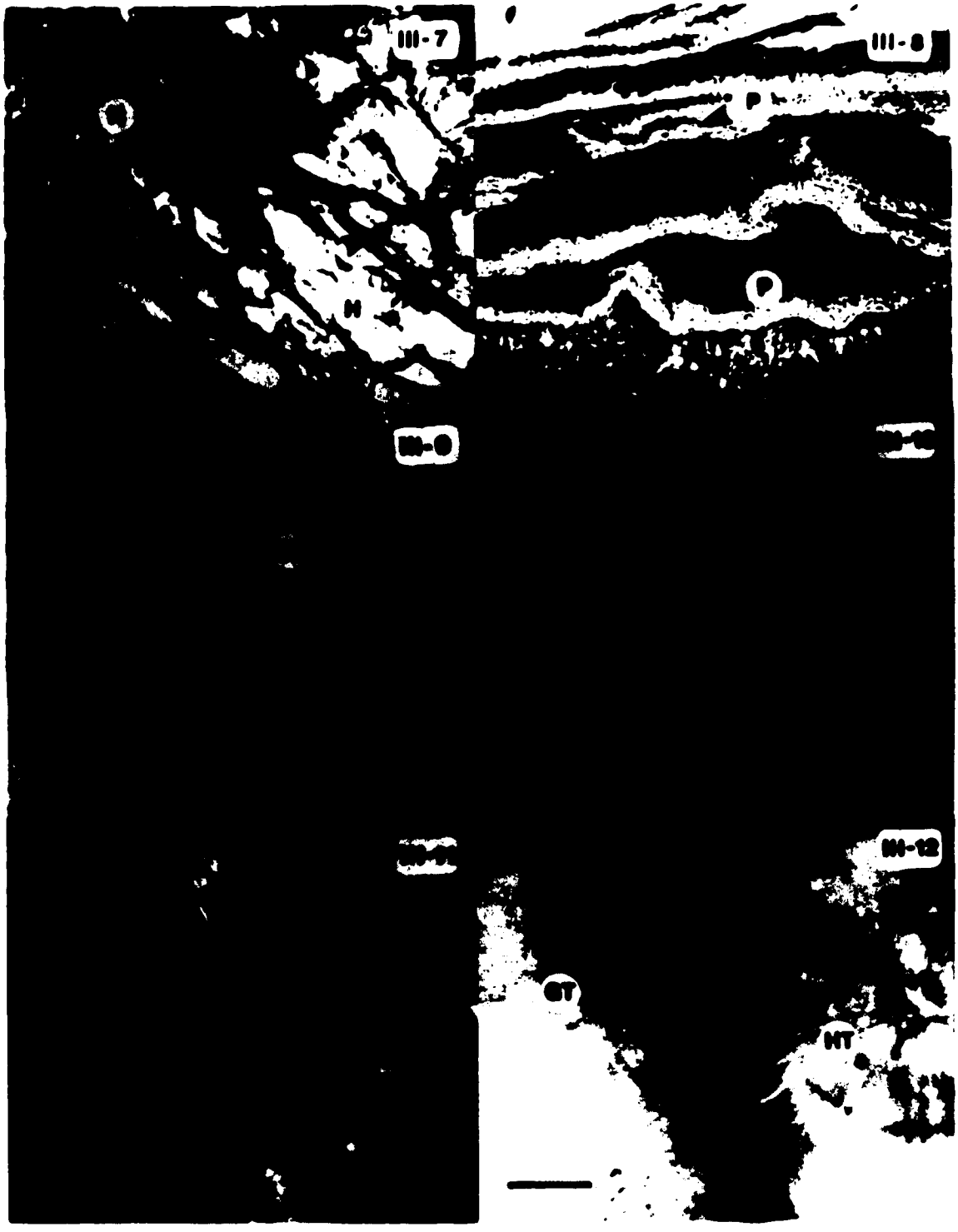
Plate III-8: Sequent periderm (P) in necrotic bark of a 10-month-old infected stem. Cryostat section stained with SFG. (bar = 500 μm)

Plate III-9: Calcium oxalate crystals (Ca) in suberized parenchyma cells surrounding lesion in 10-month-old stem. Transverse cryostat section stained with silver nitrate-dithiooxamide. (bar = 80 μm)

Plate III-10: External symptoms of necrosis and resinosis on a 10-month-old seedling. (bar = 1 cm)

Plate III-11: Lateral shoot development near the infection site on a 10-month-old resistant seedling. (bar = 2 cm)

Plate III-12: Latent gall formation in a 10-month-old seedling. Gall tissue (GT) is separated from the original infection site near the pith by healthy xylem tissue (HT). Phenolic compounds (arrow) present between healthy and galled tissue. Transverse face of fresh stem stained with HV. (bar = 2 mm)


III-7
III-8
III-12
GT
HT

cells infected with mycelium infiltrating from the adjacent lesions.

High concentrations of crystals were observed in parenchyma cells surrounding necrotic zones in four of the stems (Plate III-6). Crystal containing cells were always suberized and easily located with Sudan IV staining or under UV illumination. The crystals varied in length from 15 to 20 µm and appeared in the form of elongate prisms (Plate III-9). They stained dark brown with silver nitrate-dithiooxamide and EDX analysis identified calcium as the predominant element.

External symptoms of resistant stems were also variable, including necrosis and resinosis (Plate III-10), and some swelling of the stem resulting in noticeable longitudinal cracks in the bark. Cross-sections of swollen stems indicated hyperplastic increases in xylem diameter of up to 30% compared to diameters above and below the affected areas. In spite of diameter increases, cell arrangement and composition were not typical of gall tissue and fungal elements were not present. Another external symptom commonly observed was a profusion of lateral shoots near the infection site (Plate III-11). Serial cross-sections through the site showed that the shoots extended from vascular traces originating in the pith. No fungal elements were observed in wood or bark tissues.

Three of the trees that appeared to be resistant after 4 months showed signs of gall development by 10 months. Serial cross-sections of these stems showed that the initiation of gall development began some time after the initial infection event. This was evident by the amount of normal secondary xylem development between the innermost extent of the gall tissue and the center of the stem where infection occurred at

the time of inoculation (Plate III-12). Infected necrotic cells were observed in the rhytidome and functional haustoria were present in galled tissue and adjoining cortical cells.

Resistance at 20 months

A total of 18 trees selected for resistance were observed 20 months after inoculation. Of these, 14 showed no visible internal signs or symptoms, and external symptoms were limited to profuse growth of lateral shoots in four of the stems. In the remaining four stems, necrotic lesions were present in both cortical and internal tissues or in cortical tissues only. Plate III-13 shows a cross-section of a 20 month stem with necrosis extending from the pith to the outside of the stem. Nonfunctional haustoria were observed in phenolic-filled cells, and the entire lesion was separated from healthy tissue by a well-developed, suberized periderm that was continuous with the exophylactic periderm surrounding the healthy part of the stem. Plate III-14 illustrates a stem with a necrotic lesion extending from the pith outward into the secondary xylem, but separated from the cortical lesion through the development of tracheids. As in the previous stem, a suberized necrophylactic periderm separated necrotic from healthy tissue.

Tracheids outside of the lesion near the center of the stem were often resin soaked as indicated by staining with cupric acetate. Under UV illumination, tracheids surrounding internal lesion tissue showed strong autofluorescence (Plate III-15). In subsequent staining with phloroglucinol, lignin autofluorescence was quenched and a substance with weaker luminescence remained lining the tracheid walls.

Plates III-13 - III-18: Infection responses in lodgepole pine seedlings inoculated with Endocronartium harknessii.

Plate III-13: 20-month-old reaction showing a necrotic lesion continuous from pith to epidermis. Lesion is bordered by suberized periderm (P). Transverse cryostat section stained with SFG. (bar = 1 mm)

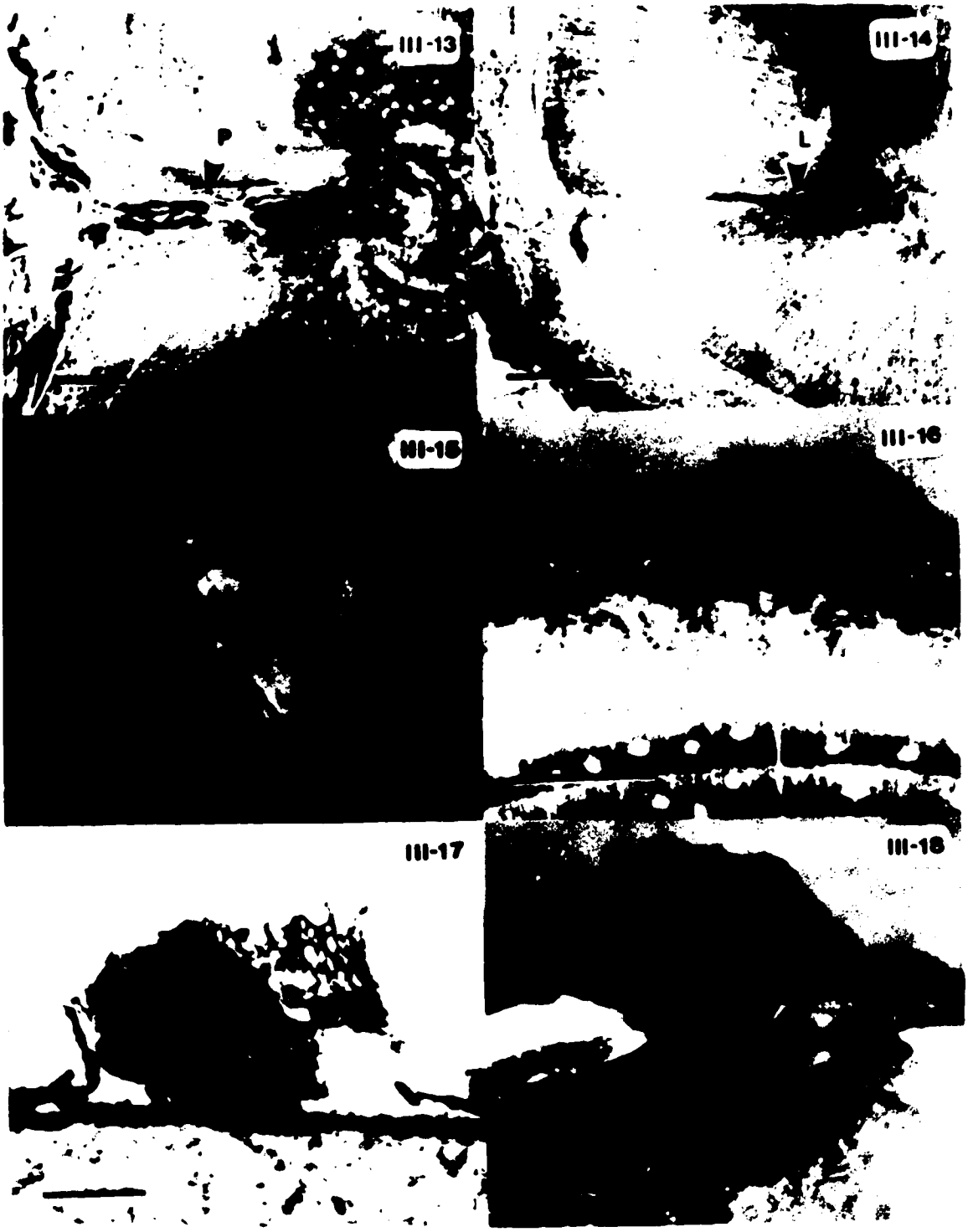
Plate III-14: 20-month-old reaction. Restoration of cambial activity has resulted in the lesion (L) being overgrown by secondary xylem. Unstained cryostat section. (bar = 2 mm)

Plate III-15: Resin-soaked lesion and secondary xylem tracheids in 20-month-old reaction. Unstained cryostat section under UV illumination. (bar = 400 µm)

Plate III-16: Infected necrotic tissue in rhytidome of 20-month-old reaction. Transverse face of fresh stem stained with phloroglucinol. (bar = 1 mm)

Plate III-17: Exfoliated necrotic lesion in rhytidome of 20-month-old reaction. Transverse cryostat section stained with phloroglucinol. (bar = 200 µm)

Plate III-18: Necrotic lesion and cambial disruption in 33-month-old resistant stem. Arrow indicates inner extent of lesion. Transverse face of fresh stem stained with phloroglucinol. (bar = 4 mm)


III-13
P
III-14
L
III-15
III-16
III-17
III-18

In the remaining two stems, lesions were observed that were restricted to the outer cortex and rhytidome (Plate III-16). Within these, non-functional haustoria were observed in phenolic-filled cells of cortical origin. The entire lesions were separated from the healthy tissue by a well developed periderm and a layer of lignified cells. Lesions were almost entirely exfoliated at the time of observation (Plate III-17) and serial sectioning indicated that no fungal elements were present in the healthy stem tissue bordering the lesion.

Resistant reactions at 33 months

Of the 40 seedlings identified as resistant in this inoculation, 26 were available for observation at 33 months. Eight of these were field planted for future testing, and the remaining 18 were sectioned. The predominant external symptom visible on 12 of the 18 trees was the profuse development of lateral shoots on the stem near the infection site (Plate III-11). Only two of the sectioned trees showed signs of fungal presence at 33 months. External symptoms of one of these stems included a necrotic lesion 3-4 cm in length accompanied by resinosis and slight stem swelling. Cross-sections of this stem showed the lesion consisting of necrotic cells in the rhytidome underlain by non-necrotic parenchymatous tissue in the cortex (Plate III-18). Discoloration was observed in xylem ray parenchyma extending from the pith to the outer extent of the secondary xylem. Staining with HV indicated the presence of phenolic compounds in xylem ray parenchyma as well as in necrotic and non-necrotic parenchyma within the lesion. Xylem tracheids in and around the discolored area exhibited autofluorescence characteristics

similar to those observed in 20-month resistant reactions.

Non-functional haustoria were present in necrotic cells within the lesion, but no fungal elements were observed in discolored cells in the secondary xylem. Sequent periderm formation was evident within the necrotic tissue of the lesion, and calcium oxalate crystals were observed in suberized cells bordering the lesion.

A gall was produced in the other 33-month stem that showed signs of fungal infection. In Plate III-19, showing a cross-section of this stem, the gall tissue formed a zone of affected cells radiating out from the point of initiation, 4 mm from the center of the stem. Serial sections through the affected area showed this point to be the inner most extent of gall tissue. A zone of phenolic-filled parenchyma cells was present at the border between normal and affected xylem. Functional haustoria were observed in abundance in these cells and throughout the ray parenchyma of the gall tissue, but were not present in adjacent normal tissue. In the rhytidome, at the exterior of the gall tissue, a necrotic zone of phenolic-filled cells was present. These cells contained abundant non-functional haustoria and layers of sequent periderm.

Near the center of the stem, radially opposite to the point of the galled tissue, was a group of necrotic cells (Plate III-20). These cells contained non-functional haustoria and were surrounded by a layer of suberized cells. No other fungal elements were present in the tissue between this lesion and the point of gall initiation. Rather, normal secondary xylem development, representing approximately one year of growth, separated the internal lesion from the gall tissue.

Plates III-19, III-20: Latent gall forma[illegible] 3[illegible] onth-old lodgepole pine stem infected with Endocronartium harkn[illegible]

Plate III-19: Gall [illegible] (GT) separated from healthy tissue by necrotic parenchyma c[illegible]. Transverse face of unstained fresh stem. (bar = 1 mm)

Plate III-20: Point of gall initiation (arrow) is separated from necrotic lesion (L) at original infection site by normal secondary xylem. Transverse cryostat section stained with SFG. (bar = 1 mm)


III-19
III-20

Discussion

All of the 83 trees examined in detail presented clear symptoms of infection within four weeks of inoculation. The symptoms appeared as red-pigmented epidermal cells which generally developed into reddish lesions. Subsequent examination at 3-4 months identified these trees as having recovered from infection and therefore potentially resistant, showing no further development of infection symptoms at that time. Resistance to E. harknessii appears to operate at various stages in the host-parasite interaction. Three sites of resistance have been tentatively identified: epidermal, cortical , and cambial.

The earliest indication of resistance, but that for which the least evidence is available, is thought to have occurred in the epidermis in the first few weeks following inoculation. The development of red pigmentation in epidermal cells of pine seedlings is a well documented response to infection by rust fungi (Lundquist and Luttrell, 1982; Lundquist and Miller, 1984; Allen and Hiratsuka, 1985). The lack of infection development following the production of these symptoms is suggestive of a hypersensitive response.

Reactions in which fungal development was arrested in the cortex were observed in seedlings 3, 10, and 20 months after inoculation. In all cases, lesions were characterized by haustoria in phenolic-filled cells, all separated from healthy cortical tissue by a suberized necrophylactic periderm. The production of phenolic compounds has been described as a non-specific host response to wounding (Mullick, 1977; Aist, 1983). However, as similar responses are observed in successful

gall-forming infections, the precise role that these compounds may play in resistance to pine stem rusts is not clear (Miller et al., 1976; Jewell and Spiers, 1976; Walkinshaw, 1978). The development of a periderm around infected, "tanninized" cells has been reported as a resistance mechanism (Hutchinson, 1935; True, 1938). True (1938) suggested that such a structural barrier is only successful when infected cells die before the mycelium has time to spread. The results of the current study clearly show that infected cells can be isolated from healthy tissue and sloughed off with the bark. This response is similar to that described by Miller et al. (1976) as type-3b, corticular hypersensitivity, in their evaluation of resistance to C. quercuum f. fusiforme in slash pine. Hiratsuka and Maruyama (1983) also reported similar cortical resistance reactions in Pinus thunbergii Parl. and P. densiflora Sieb. and Zucc. infected with E. harknessii.

Most of the stems examined at 20 and 33 months showed no signs of infection at the time of observation although they had clearly been infected at earlier stages. A possible explanation is that the fungus had been blocked in the cortex and that this tissue was exfoliated by the time of examination.

A third type of response involving cambial disruption occurred, in stems observed 10, 20, and 33 months after inoculation. In all cases, fungal haustoria associated with necrotic lesions were observed near the center of the stem where the vascular cambium had been located when the fungus reached it. Fungal interaction with the cambium at this time resulted in a cessation of normal cambial function rather than the stimulation of cambial activity associated with typical infection and

gall development. As a consequence, normal tracheid development was interrupted in the necrotic zone while adjoining cambial cells continued the production of derivative tissues. This resulted in the abnormal stem anatomy shown in Plates III-3 and III-4. The parenchymatous cells that filled the disrupted zone were continuous with similar cortical cells and likely originated from divisions within the cortex. Meristematic activity of ray parenchyma may also have contributed to the parenchyma in the pathological zone. This is suggested by the high proportion of ray parenchyma observed at the border between pathological and healthy tissue in longitudinal sections and the continuity of ray parenchyma with parenchyma in pathological tissue observed in transverse sections.

In some stems, the restoration of cambial activity resulted in lesion tissue being buried in secondary xylem (Plates III-14, III-20). This was particularly true in older stems or where pathological tissue was limited to a small number of cells. In cases where tracheids had been laid down external to pathological tissue, they were often obliquely or horizontally oriented. However, as growth continued, normal alignment of these cells was restored. Jewell and Speirs (1976) made similar observations in 2-year-old infections of resistant slash pine inoculated with C. quercuum f. sp. fusiforme. In young resistant reactions or those with large amounts of pathological tissue, lesions were continuous from the pith to the cortex (Plates III-3, III-4, III-13).

Sequent periderms were produced in cortical lesions in stems of all ages (Plate III-7). Their production and the presence of viable haustoria in cells interior to them indicated that these structural

barriers were often not effective at blocking the inward spread of mycelium. The maintenance of infective mycelium in the cortex and subsequent reinfection of the vascular cambium likely explains the late initiation of galls in 10 and 33 month stems (Plates III-12, III-19). Latent-type infections have been reported in the fusiform rust disease complex (Jewell, 1978; 1984). The infection dynamics involved in ultimate gall production in these latent-type infections are poorly understood and the possibility of their occurrence must be considered when assessing trees for resistance.

Resinosis was an external symptom commonly observed on resistant stems, and internal lesions and surrounding xylem tracheids were often resin-soaked. Peterson (1960) described resin deposition in the normal development of pine galls. As well, increases in the number of vertical resin ducts and the development of radial resin ducts have been reported for this (True, 1938) and other phloem rusts (Jewell et al., 1962). However, resinosis has not been described in association with resistant reactions. Excessive resin production in these stems is likely a result of tissue damage and not an active resistance response.

The presence of calcium oxalate crystals has not been reported in association with disease in pine although they are a normal component in phloem and bark tissues of healthy stems of many pine species (Srivastava, 1963). In other disease systems, it is hypothesized that pathogen-produced oxalic acid sequesters calcium from the middle lamella and lowers pH, thus activating tissue degrading enzymes (Rao and Tewari, 1987; Marciano et al., 1983). In the present study, the accumulation of calcium oxalate in parenchyma cells surrounding lesions

in some stems did not appear to be associated with tissue degradation nor is it likely that oxalic acid was produced by the rust fungus. Rao and Tewari (1987) showed calcium oxalate crystal production in agar and broth cultures of Mycena citricolor (Berk. & Curt.) Sacc.. However, the examination of axenic cultures of E. harknessii (Allen et al., 1988) grown on media containing calcium gave no indication of calcium oxalate crystal production by the rust. It is not clear whether the observed high concentration of calcium oxalate crystals plays any part in resistance.

Many of the trees identified as being potentially resistant produced a large number of lateral shoots near the infection site. Lateral shoot development is a common symptom on galled pine seedlings, where shoots typically form on the upper hemispheres of stem galls (Boyce, 1957). The occurrence of similar shoot development on infected, non-galled stems poses interesting questions regarding this symptom. It was clear that the shoots developed from pre-formed latent buds originating near the center of the stem. Therefore, the stimulation for additional bud development occurred when the seedlings were young, at about the time of infection. It is possible that the presence of the fungus stimulated the development of lateral buds early in the course of infection. Subsequent resistance reactions that excluded the fungus and prevented gall production had no apparent effect on the further development of lateral shoots. Further study of this phenomenon may add to our understanding of the mechanisms controlling symptom expression in rust infected trees.

Based on the observations of resistant reactions made in this study,

at least three types of resistance to E. harknessii in lodgepole pine appear to be present. In the first type, epidermal resistance, the fungus penetrates the stem and early infection symptoms are produced. However, the infection does not develop further and infected tissue is sloughed off with the epidermis and the seedling develops normally. The second type is characterized by a cortical resistance response. Here the infection is successfully stopped through cell death and periderm formation, and the resulting necrotic lesion is exfoliated with the rhytidome. In the third type, cambial resistance, the fungus penetrates to the cambium where cell necrosis results in the localized interruption of normal cambial function. In time, cambial function is restored and the lesion is overgrown with secondary xylem. Necrotic tissue in the cortex is eventually sloughed off and the stem develops normally. The formation of latent galls is possible in trees which initially exhibit cortical or cambial resistance characteristics. Slow-growing infective mycelium escapes cortical lesions and reinfects the cambium resulting in gall development.

There are close similarities between apparent resistance to E. harknessii in P. contorta and that observed in other stem rust pathosystems (Kinloch, 1982). Cortical or bark-type reactions have been reported in trees infected with white pine blister rust (Struckmeyer and Riker, 1951), fusiform rust (Miller et al., 1976) and gall rust (Hutchinson, 1935; True, 1938; Hoff, 1986). Cambial-type reactions have been observed in fusiform rust-resistant trees (Jewell and Speirs, 1976). These results suggest that mechanisms of resistance to stem rusts in conifers operate in a similar, non-specific manner.

This study provides the first histological evidence of resistance to E. harknessii in lodgepole pine. All examples of resistance observed in various aged stems were similar in the basic manner in which the fungus was stopped or delayed. This involved the death of infected cells, likely associated with the production of phenolic compounds, lignin, and necrophylactic periderm. Further research is required to assess the long-term stability of the resistance observed here, to determine whether similar resistance reactions are expressed in inoculations of other trees, and to ascertain whether other resistance mechanisms are in operation.

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CHAPTER IV

AXENIC CULTURE OF *Endocronartium harknessii*[2]

Introduction

Endocronartium harknessii (J.P. Moore) Y. Hiratsuka is the causal agent of western gall rust, an important fungal disease of hard pines in North America. It is an endocyclic, autoecious rust, producing peridermioid teliospores (Hiratsuka, 1969). Morphologically similar to aeciospores in *Cronartium* spp., these spores are produced on globose galls found on branches and stems of trees 1-2 years after infection. The impact of western gall rust is not high in natural forests in Canada, but as forest management becomes more intensive, the damage caused by the disease will likely increase. Seedlings in nurseries and plantations are more likely to develop main stem galls resulting in unmerchantable trees. This disease is of sufficient importance that resistance to it is being considered as a criterion for selecting superior trees in genetic improvement programs for hard pines.

The first successful axenic culture of a rust fungus (*Gymnosporangium juniperi-virginianae* Schw.) was reported by Hotson and Cutter (1951). Since then, various levels of success have been achieved in culturing over 25 species of rusts (Maclean, 1982; Yamaoka and Katsuya, 1985). Axenic culture has been reported for two species of pine stem rust fungi, *Cronartium quercuum* Miyabe *ex* Shirai f. sp. *fusiforme* (= *Cronartium fusiforme* Hedgc. and Hunt *ex* Cumm.) (Hollis *et al.*,

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[2]A version of this chapter has been published. Allen, E.A., Blenis, P.V., and Hiratsuka, Y. 1988. Mycologia 80(1):120-123.

1972), and Cronartium ribicola J.C. Fisch. ex Rabenh. (Harvey and Grasham, 1974). In both cases, infected tissue and basidiospores have been used to initiate fungal cultures.

This paper reports the successful axenic culture of Endocronartium harknessii from spores and infected host-tissue.

Materials and Methods

Explants were obtained from Endocronartium harknessii galls on vigorously growing 1-year-old lodgepole pine seedlings (Pinus contorta var. latifolia Engelm.). The galls had been initiated by the artificial inoculation of 6-week-old greenhouse-grown pine seedlings and had not sporulated at the time of dissection. Galls were cut from the seedlings, surface sterilized for 10 min in 5% sodium hypochlorite and for 1 min in 70% ethanol before being rinsed three times in sterile distilled water. The outer bark of the gall was removed with a sterile scalpel, and 4 mm^2 sections of rust infected tissue were isolated by making perpendicular cuts through to the cambium. The segments were undercut with a scalpel and placed cambium-side down on the surface of the medium. Explants (approximately 200) were incubated in 100 x 15 mm Petri plates at 15 C in darkness.

The growth medium contained modified Murashigie and Skoog ingredients (Yamaoka and Katsuya, 1984). The growth hormone napthaleneacetic acid (NAA) was used in place of 2,4-dichlorophenoxyacetic acid (2,4-D) based on recommendations for optimal tissue growth of P. contorta (Harvey and Grasham, 1969) and kinetin (6-furfurylaminopurine) was not found to be necessary. The composition (per liter of double distilled water) of the medium was as follows: KNO_3, 475 mg; NH_4NO_3, 412.5 mg; $CaCl_2 \cdot 2H_2O$, 110

mg; $MgSO_4 \cdot 7H_2O$, 92.5 mg; KH_2PO_4, 42.5 mg; Na_2-EDTA, 9.3 mg; $FeSO_4 \cdot 7H_2O$, 6.95 mg; $MnSO_4 \cdot 4H_2O$, 5.8 mg; $ZnSO_4 \cdot 7H_2O$, 2.15 mg; H_3BO_4, 1.55 mg; KI, 0.208 mg; Na_2MoO_4, 0.063 mg; $CuSO_4 \cdot 5H_2O$, 0.006 mg; $CoCl_2 \cdot 6H_2O$, 0.006 mg; myo-inositol, 25.0 mg; nicotinic acid, 0.125 mg, pyridoxine-HCl, 0.125 mg; thiamine-HCl, 0.025 mg; glycine, 0.5 mg; Evans peptone, 2 g; Difco Bacto-soytone, 2 g; glucose, 40 g; NAA, 0.5 mg; Purified Difco-agar, 8 g.

The pH of the medium was adjusted to 5.8 before autoclaving for 20 min an 121 C.

Sample preparations for light microscopy were viewed unstained using interference contrast, or were fixed in formalin-acetic acid-alcohol (FAA), sectioned in paraffin (12 μm sections), and stained with safranin-fast green. To determine the nuclear condition of the cells, unfixed cells were stained for 30 min in a 1 μg/ml aqueous solution of the DNA-specific fluorochrome DAPI (4,6-diamidino-2-phenylindole). Fluorescence was viewed under a Zeiss-Universal epifluorescence microscope, with ultraviolet (UV) illumination using a UG-5 excitation filter, barrier filter 50, and a Neofluar 100/1.30 objective. Lipids were identified by staining cells with 0.001% Nile Blue and observing them under blue-green fluorescence (BG 12 excitation filter, barrier filter 53). Scanning electron microscope (SEM) specimens were fixed in 3% buffered glutaraldehyde, dehydrated through an ascending alcohol series, critical point dried, gold-coated, and examined with a Hitachi S-510 SEM.

Results

Mycelium and haustoria were abundant in the explant tissue (Plate IV-1, arrows). Callus tissue was produced on the lower side of the explant, and as growth continued, the cells grew up and around the explant, sometimes enveloping it completely. After 6 weeks of culture, fungal mycelium had grown from the explant into the loosely packed cells of the callus tissue. Haustorial production in the callus tissue was rare and intercellular hyphae showed unusual form, such as spiral growth. Aerial mycelium was visible on about 70% of the explants (Plate IV-2, arrows). The continuity of aerial mycelium with that growing in the explant was shown by histological examination (Plate IV-3). Like the mycelium in the callus tissue, the structure of the aerial mycelium was unusual. Variation in cell size and shape, anastomoses, and irregular branching were observed (Plate IV-4). In some instances, aerial hyphae grew together forming erect bundles of mycelium up to 3 mm in height (Plate IV-3, arrow). Aerial mycelium was visible on the surface of the callus tissue after about 12 weeks of culture.

After 33 weeks, colonies of fungal cells were visible on approximately 2% of the explants (Plate IV-5). The masses of fungus were a light orange color and covered with hyaline aerial mycelium. Microscopic examination showed septate hyphae, sometimes long and branched, arising from a mass of vesicular cells. The vesicular cells varied in size from 5-10 µm but were as large as 30 µm in diameter (Plate IV-6). Yellow-orange lipid bodies were observed within the cells, similar in appearance to those found in spores of this rust and

ıtes IV-1 - IV-9: Endocronartium harknessii

Plate IV-1: Cross section of [illegible]t tissue explant with abundant rust mycelium (MY) and haust[illegible] (bar = 20 µm)

Plate IV-2: Aerial mycelium (arrows) on explant surface [illegible] weeks of culture. (bar = 1.5 mm)

Plate IV-3: Cross section on explant after six weeks of culture showing internal and aerial mycelium (arrow). (bar = 20 µm)

Plate IV-4: Aerial mycelium on explant surface. (bar = 30 µm)

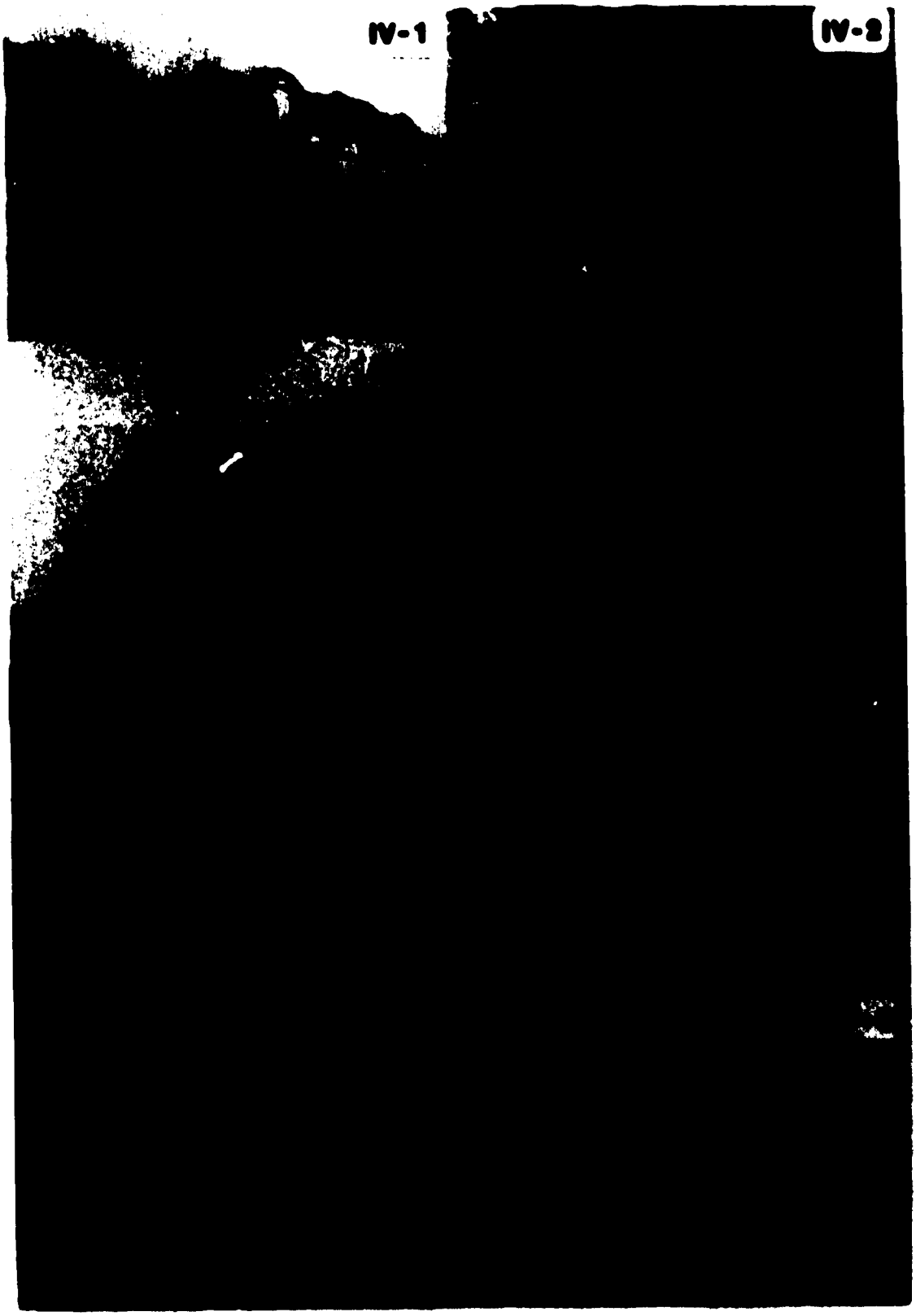
Plate IV-5: Dual culture colony growing on the surface of pine callus tissue. (bar = 3 mm)

Plate IV-6: Vesicular and hyphal cells of fungal colony, 33 weeks viewed under interference contrast illumination. (bar = 20 µm)

Plate IV-7: Monokaryotic cells stained with DAPI and viewed under combined brightfield and UV fluorescent illumination. (bar = 20 µm)

Plate IV-8: Dikaryotic cells stained with DAPI viwed under UV fluorescent illumination. (bar = 30 µm)

Plate IV-9: Axenic colonies grown from dilution subculture. (bar = 1 mm)


IV-1
IV-2

cultured cells of other rust fungi (Harvey and Grasham, 1974). Both monokaryotic and dikaryotic cells occurred in approximately equal numbers (Plates IV-7, IV-8); occasionally cells were multinucleate or akaryotic.

Portions of the colonies (approximately 4 mm^2 in diameter) were separated from the host tissue and plated onto growth medium after 35 weeks of culture. Subcultured colonies increased in size through division of vesicular cells, but showed limited mycelial growth onto the medium. As the colonies aged, some browning was observed, while new growth was light-orange in color. Other observed changes included increased vacuolation, reduction in the size of lipid bodies, and increased hyphal growth on the colony surface. The nuclear condition of the new growth on the sub cultured colonies was predominantly monokaryotic.

Subcultured colonies were diluted in agar-free medium and placed onto fresh agar growth medium. Scattered groups of cells on the surface of the medium produced aerial hyphae and mycelium growing into the medium. Within 4 weeks, discrete colonies were produced from these groups of cells (Plate IV-9).

Discussion

Axenic cultures of pine stem rusts have been initiated using infected host tissue (Hollis et al., 1972; Harvey and Grasham, 1974) as well as by using basidiospores, urediniospores, and aeciospores (Hare, 1978; Diner et al., 1981). In preliminary tests, spores were tried as a starting material, but difficulties were encountered in preventing contamination. Attempts are currently being made to use spores from

field collected galls, sporulating for the first time. Spores excised from beneath an unruptured peridium seem to be free from contaminants and show promising signs of axenic growth. Problems with contamination were also encountered in early attempts at using field-collected gall tissue as the source of explant material; however, the use of greenhouse-grown galls that had not previously sporulated provided virtually contaminant-free explants.

The success of fungal colony production was low, but the numbers are similar to those in other rust culture studies (MacLean, 1982). Modifications of media composition and environmental growth conditions are currently being tested in an effort to improve colony production, hasten growth, and to stimulate sporulation in culture. Inoculation techniques using axenically grown fungal cells on pine seedlings, embryos, and callus tissue are being investigated.

This is the first successful axenic culture of an endocyclic rust fungus, and will provide interesting morphological and cytological information. As well, it will be a useful tool for in vitro investigation of host-parasite relationships, and it may offer a source of clonal inoculum for resistance testing of hard pine material against this fungus in tree improvement programs.

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CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

The development of external symptoms of infection was followed for a period of seven weeks following inoculation. Symptom expression was extremely variable, but was shown to be linked to physiological and anatomical events occurring in underlying tissues. Processes in these tissues such as the production of phenolic compounds, the death of cells, and the development of necrophylactic periderm contributed to the external appearance of infected stems. Similar observations were made by Lundquist and Miller (1984) on slash pine (Pinus elliottii Engelm. var. elliottii) seedlings infected with Cronartium quercuum (Berk.) Miyabe ex Shirai f.sp. fusiforme. An earlier study (Lundquist and Luttrell, 1982) reported differences between known resistant and susceptible slash pine families regarding numbers of pigmented seedlings and the rate of pigmentation. With the establishment of resistant lines of lodgepole pine, and further study of the factors contributing to variation in symptom expression, it may be possible to discriminate more clearly between resistant and susceptible lodgepole pine seedlings on the basis of early symptoms. If this is possible, then screening for resistance could be expedited.

Evidence of three potential resistant responses were identified in lodgepole pine seedlings. Epidermal resistance, characterized by a hypersensitive-type response is thought to have occurred within two weeks of inoculation. The mechanism of this apparent resistance is not clear and further study must be directed at this potentially important

reaction. Cortical and cambial resistance responses were more common, and were very similar to responses described in other pine stem rust systems (Hutchinson, 1935; True, 1938, Struckmeyer and Riker, 1951; Miller et al., 1976; Jewell and Spiers, 1976). Hoff (1986) presented evidence of externally visible resistance reactions on E. harknessii-infected ponderosa pine up to 28 months after inoculation. In the present study, trees which produced resistant responses following infection generally remained gall-free for up to 3 years, although gall development did occur in some cases. Preliminary observations of lodgepole pine trees growing in the field that appear to be resistant to E. harknessii suggest that cambial responses similar to those observed in seedlings may be operating in older trees as well. Forty-year old trees with no E. harknessii galls, growing in stands of heavily infected trees have shown anatomical responses similar to the resistance responses illustrated in Chapter II (Y. Hiratsuka, pers. comm.). These include localized inactivity of the vascular cambium and the resultant modifications in stem anatomy, as well as the maintenance of slow-growing fungal colonies in cortical tissues. These observations support the results reported here but further research must be carried out to establish the relationship between laboratory and field results and to determine the heritability and long-term stability of the observed resistance traits in specific pine families. Provenance trials of western gall rust resistance have demonstrated variation in resistance, but the relative importance of genetic factors and inoculation characteristics are not clear (Hoff, 1986).

The identification of resistance mechanisms in lodgepole pine to

infection by E. harknessii will contribute to the development of techniques of resistance screening. The methods of inoculation and assessment outlined in Chapter II suggest such a technique. Test seedlings could be inoculated at 6-7 weeks of age and examined 4 weeks later for the presence of external symptoms. The seedlings would be reassessed 4 mo later for evidence of gall formation. Seedlings developing early infection symptoms, but subsequently not forming galls, would be selected as potentially resistant. A shortcoming of this proposed method is that selections would not include infected seedlings which do not develop external symptoms but are eventually resistant to the fungus. As well, seedlings exhibiting epidermal resistance will not be identified through this process. Further research must be directed at understanding non-symptom infections and hypersensitive responses.

Resistant lines of lodgepole pine may also be used in the development of clonal pine tissue cultures for possible use in resistance screening. Both axenic rust cultures and pine tissue cultures (Appendix 1) were successfully established in the course of this project. Axenic fungal cultures were produced from both infected pine tissue and spores. Various treatments to induce sporulation were attempted, but none were successful. Likewise, no success was achieved in inoculations of pine callus tissue, clonal pine plantlets, in vitro embryos, or in vivo pine stems of various ages with axenically cultured E. harknessii. The reasons for this failure are not clear. Studies of other pine stem rusts have reported the successful inoculation of pine tissue with axenic cultures. Diner and Mott (1982) demonstrated the use of axenic cultures of Cronartium ribicola J.C. Fisch. ex Rabenh. as inocula for

aseptically grown embryos of Pinus lambertiana Dougl.. A subsequent study reports the successful infection of subcultured colonies of P. lambertiana with axenically cultured C. ribicola (Diner, et al., 1984). Amerson et al. (1984) describe the use of axenic cultures of C. quercuum f. sp. fusiforme as inocula for Pinus taeda L..

The use of tissue culture has great potentia in the study of disease resistance and may prove to be a valuable tool in the development of resistance screening systems. However, major obstacles in ease of handling and manipulation of host and parasite cultures must be overcome before such systems will be operational.

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APPENDIX

PINE TISSUE CULTURE

Clonal plantlet cultures of lodgepole pine were established on a series of defined growth media (Von Arnold and Eriksson, 1981). Seeds were surface sterilized for 8 min in 7% sodium hypochlorite and for 1 min in 70% ethanol before being rinsed three times in sterile distilled water. Embryos were aseptically removed from their seed coats and placed on the surface of a cytokinin-supplemented, bud-induction medium. Cultures were incubated under an 8 hr photoperiod at 20 C. After 4 weeks, adventitious buds had formed and the embryos were transferred to a 1/2 strength, hormone-free medium to stimulate the multiplication and elongation of buds. After a further 3 mo of culture, shoots were dissected from the tissue and plated onto a 1/8 strength medium supplemented with activated charcoal. Spontaneous rooting occurred on some plantlets; the remaining were treated with indolbutyric acid (IBA) to stimulate root formation. Plantlets were also produced from surface sterilized needle fascicles from seedlings up to 3 years old.

Pine callus cultures were produced from embryos, hypocotyls and cotyledons of 1 month-old seedlings, and from surface-sterilized candle tissue from field collected lodgepole pine trees up to about 40 years of age. Callus cultures were grown on a Murashige and Skoog medium (Yamaoka and Katsuya, 1984), modified through the replacement of 2,4-dichlorophenoxyacetic acid (2,4-D) with napthaleneacetic acid (NAA) (based on recommendations for optimal growth by Harvey and Grasham, 1969), and the deletion of kinetin (6-furfurylaminopurine). Pine callus

grew equally well on this medium with or without the addition of Bacto-soytone and Evan's peptone (included in axenic rust culture media). Pine callus was subcultured at regular bi-monthly intervals for up to 1 yr.

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