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Pathogenesis in Pine Wilt Caused by Pinewood Nematode, *Bursaphelenchus xylophilus*[1]

RONALD F. MYERS[2]

Abstract: The progression of events in the development of pine wilt disease following the invasion by *Bursaphelenchus xylophilus* is reviewed from early migration through pine tissues until symptom development on foliage. Disease resistance in pines, especially the hypersensitive reaction that is successful in controlling many potential pests and pathogens, is explored. Pathologies resulting from the activities of pinewood nematode include cortical trails and cavities; formation of cambial gaps and traumatic resin cysts; browning and death of cortex, phloem, cambium, and ray tissues; granulation and shrinkage of cell cytoplasm in rays; and destruction of resin canal epithelial and ray parenchyma cells. Death of parenchyma, production of toxins, and leakage of oleoresins and other material into tracheids are typical of the hypersensitive reaction occurring in pines following migration of small numbers of pinewood nematodes. The hypothesis presented is that a spreading hypersensitive reaction results in some of the observed pathologies and symptoms and eventually causes pine death. The growth-differentiation balance hypothesis is used to help explain predisposition, oleoresin production and toxicity, susceptibility and resistance, and the effects of variation in climate on host pines as related to pinewilt disease.

Key words: Bursaphelenchus xylophilus, pathogenesis, pathology, pine wilt disease, pinewood nematode.

Pathogenesis is defined as the progression of events in the development of pine wilt disease caused by pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970. Susceptibility and resistance and virulence of the pathogen alter the outcome of pathogenesis. Yet little information is currently available on the relative virulence of proposed pathotypes (8), and a practical method of identifying pathotypes needs to be developed. Whereas pathologies relating to susceptibility and resistance in pines infected with PWN have been described, they need to be unified into one hypothesis to explain the nature of pine wilt disease. Data have been merged from several disciplines in an attempt to put into perspective the pathogenesis caused by PWN. In examining pine wilt, consideration is given to primary infection, histopathology, and the responses of susceptible seedlings and mature trees to PWN invasion, especially during the early dispersal of PWN through tissues. No attempt is made to explain the resistance mechanisms that permit the majority of native North American pines to survive infection by PWN. Secondary transmission at vector oviposition followed by pathogenic activities of nematodes is not included because oviposition generally takes place on weakened, dying, or dead trees, or on logs (15,17,53).

The growth-differentiation balance hypothesis was developed to explain bark beetle–pine tree interactions. A study of this hypothesis and associated papers produces many facts concerning the reaction of pines to pathogens. Many of these data seem applicable to pathogenesis of pine wilt disease, since recognition is given to predisposition to pathogens, oleoresin production and toxicity, susceptibility and resistance, and the modifying effects of variations in climate (19,20).

The main thesis and hypothesis presented in this review is that invasion and migration of PWN through tissues invokes an innate hypersensitive defense mechanism, especially in susceptible pine species, and this leads to tree death. The hypersensitive reaction results in the release of phenolics, synthesis of toxins and phyto-

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[2] Department of Plant Pathology, Cook College, Rutgers University, New Brunswick, NJ 08903.

alexins during the catabolism of oleoresins and storage products, and the compartmentalization of xylem and other tissues followed by flooding of tracheids with oleoresin and toxic substances. This pathogenic process is triggered by the mobile parasite, PWN, rapidly migrating in low numbers throughout tree tissues. Tree death results from the pine's excessive response to the invasion of PWN.

Infection of Pines and Nematode Migration

Initial or primary transmission by the phoretic fourth-stage dauer larvae of PWN occurs during maturation feedings by long-horned beetles, *Monochamus* spp., following emergence of the beetles from PWN-infected logs or living trees (1,15,53). Maturation feeding, which normally takes place between bud break and candle maturation, is mainly on primary and secondary cortex, phloem, and cambial tissues produced on elongating candles (apical growth) and 1-year-old twigs of pine trees. Dauer larvae transmigrate from their vectors and enter the wounded tissues (15,26), probably in response to the monoterpenes of *Pinus* spp. (2). When trees are infected using nematodes extracted from fungal cultures, the population consists of a mixture of adults and juveniles and usually does not contain dauer larvae. Activity of these nematodes in pines appears similar to natural infection by dauer larvae which have exited from long-horned beetles.

Most dauer larvae molt within 48 hours after contacting pine tissues (26). Dispersal of PWN from the site of infection throughout *P. thunbergii* Parl. (Japanese black pine) and *P. densiflora* Sieb. and Zucc. (Japanese red pine) takes place in two distinct phases (24). During phase one, most nematodes remain close to the site of inoculation in the surrounding chlorophyllus cortical tissues while low numbers of nematodes wander throughout the tree (46). I have also observed a similar phenomenon in seedlings of several other pine species. Following infection, some PWN straighten out and become moribund, trapped after contact with oleoresin (32,49), while others recovering or avoiding contact gain immediate entry into the cortical tissues. Low numbers of PWN then enter the phloem, cambium, and xylem. These wandering nematodes, traveling with maximum speed of 40–50 cm/day, become randomly distributed throughout susceptible pine trees (13,46). Numbers may increase slowly or the nematodes may disappear from the wood, although it is probable that small numbers of PWN continually disperse from the original site of infection. The lesion increases in size, oleoresin flow ceases, and PWN reproduction commences.

In the second dispersal, there is a widespread movement of nematodes away from the infective lesion and throughout the resin canals of the xylem. The second phase of dispersal does not occur until after a reduction in oleoresin pressure and flow and in the rate of transpiration (46,47). In addition, tracheids are blocked by abnormal leakage of oleoresins from adjacent radial and axial resin canals (35,38,50). Areas of blockage in the tracheids (xylem) gradually enlarge resulting in whitewood formation. This is paralleled by death of parenchyma cells in the rays and resin canals. Tracheid blockage finally restricts water conduction and reduces transpiration. Death of parenchyma cells, which precedes nematode distribution throughout the xylem, has been interpreted as a chemical reaction to nematodes present at the site of the original lesion (11,24,29,46). These phenomena occur with greater rapidity in young trees and seedlings than in older, mature trees.

Migration of PWN is enhanced by the presence of loose chlorophyllus parenchyma and embedded axial resin canals in the cortex, a layer of thin-walled derivatives produced by the cambium, and the interconnecting radial and axial resin canal system. PWN create trails of migration as they move intercellularly and intracellularly through the cortex. PWN aggregate in cortical resin canals and in cavities formed by cell breakage and collapse. Such

trails may close after a few days because of pressure produced on thin-walled cortical cells by secondary phloem growth. Greenish-brown areas usually develop from injured cells and at locations immediately surrounding the PWN-penetrated cortical resin canals. These areas coalesce to girdle the stem (33,34). When enough damage accumulates, trees form cork cambium (phellogen) in the outer cortex and within the phloem tissue. PWN escape across newly developing periderm but not in *P. rigida* Mill. (pitch pine) where moribund nematodes become encapsulated. Cortex enclosed by periderm differentiates into stone cells, desiccates, partially collapses, and is flooded with oleoresin. The formation of wound periderm is a common defense reaction against invading micro-organisms (44).

During the early part of the year when the growth phase predominates, pines may be severely damaged by PWN. Both terminal and diameter growth proceed rapidly as temperatures and day length increase. Oblique cell division followed by sliding intrusive growth produces a layer of thin-walled cells in the cambium. Maturation lags so that a layer of cell derivatives is present in various stages of differentiation to mature phloem, xylem, and ray tissue. PWN migrate through the radial rays or directly through phloem to the cambium before moving both vertically and circumferentially. Migrating and feeding PWN destroy fusiform and ray initials and their derivatives. Circumferential and vertical trails of destruction occur in the cambium, and cambial gaps containing nematodes are present. Cross sections of young living stems show infected and injured cortical tissues and progressively darkening cambial tissues. The phloem in some pines, such as *P. nigra* Arn. (Austrian pine), separates from the xylem. Such activity destroys the future water and food conducting tissues—the xylem, phloem, and rays. Trees infected during late summer and fall, when only scattered cambial mitoses occur after cambial derivatives have differentiated into xylem, phloem, and rays (33,34), become more resistant to PWN injury.

Cambial gaps in pines contain adult males, females with eggs, and juvenile nematodes (24,25,32–34). Such gaps form by destruction of precursor cells of axial resin canals or traumatic resin cysts. Traumatic or wound resin cysts are rapidly generated by the cambium in response to infection by PWN in *P. strobus* L. (eastern white), Japanese red, Japanese black, *Picea pungens* Engelm. (Colorado blue spruce), and *Pseudotsuga menzezii* (Mirb.) Franco (Douglas fir) (32–34,45). After destruction by nematodes, these oleoresin producing, secretory parenchyma cells appear as a row of gaps along the periphery of the xylem. Such gaps may be incorporated into the xylem if the cambium regenerates. Traumatic resin cysts do not interconnect with the normal axial and radial resin ducts. Gaps also exist where needles and branch traces emerge through the phloem and cortex. PWN destroy the developing epidermal parenchyma cells following entry along the cambial layer (33,34). Whereas resin cysts and sometimes canals are present in many of the Abietineae, only *Larix, Pseudotsuga, Picea,* and *Pinus* possess an interconnecting system of axial and radial resin canals. The most highly developed system is found in the genus *Pinus.* Epithelial cells lining axial resin canals have thickened walls in *Pseudotsuga, Larix,* and *Picea,* whereas those of *Pinus* remain thin walled. Epithelial and parenchyma cells lining the resin canals and in the rays synthesize and remetabolize oleoresin and stored nutrients and secrete oleoresin, polyphenols, terpenes, and phytoalexins. These compounds are transported by the canals to help control disease organisms (41). This network of interconnecting resin canals permits axial migration of PWN throughout trees lacking cortical tissues and probably provides a site for PWN to overwinter. PWN destroy the parenchyma and epithelial cells of axial resin canals during migration, apparently by feeding and through their reproductive activities (29,30,34).

In the new primary growth (candles) and 1-year-old stems of pine, multiseriate rays penetrate the phloem and cambium tissues before passing through the xylem to con-

nect with the arms of the pith. PWN follow these natural pathways into the center of young stems. During secondary growth, trees produce additional uniseriate and multiseriate rays. Uniseriate rays consist of thin-walled parenchyma, one cell wide and 2–5 cells deep. Multiseriate rays contain 2–4 rows of parenchyma and surround individual resin cells or a single radial resin canal. Adult and juvenile nematodes do not move through uniseriate rays, although uniseriate rays die inwardly after PWN invade the cambium. Several days after infection, uniseriate rays show granulated and coalescing cytoplasm and collapsing and dead cells near the cambium, but often not deeper within the xylem. PWN enter the multiseriate rays from the cortex, phloem, or cambium through the embedded radial resin canals which generally lead to the axial resin canals.

There is no available comparative study on the density and placement, nor the degree of interconnection between radial and axial resin canals among pine species. These canals are extremely important in the dispersal of PWN. Densities of radial resin canals are, however, quite variable within and between individual trees, species, or genera. In tangential wood sections, radial resin canals number more than $60/cm^2$ in spruce and larch, 60–70 in *P. sylvestris* L. (Scots pine), 50–85 in *P. elliotti* Engelm. (slash pine), 35–50 in *P. ponderosa* Laws. (ponderosa pine), and 35–60 in *P. contorta* Dougl. (lodgepole pine) (43). Radial resin canals in 3-year-old Japanese black and Scots pines are estimated at about 120 and $100/cm^2$, respectively (32). PWN does not penetrate the xylem of Colorado blue spruce, pitch pine, or Douglas fir. Multiseriate rays are absent from young Douglas fir and only low numbers of radial resin canals are embedded in the multiseriate rays of pitch pine and *P. resinosa* Ait. (red pine). Resin canal and cyst densities are affected by fluctuating weather, abundance of food reserves, injuries, temperature, and other factors (43). Internal events in pine trees eventually are expressed as foliar symptoms. The rate at which these symptoms develop is governed by the size and age of the trees. Needle drying, wilting, and discoloration to blond, yellow, grayish-green, or red may be seen. Often older needles are affected first and branch flagging develops in resistant trees. Bleached or blond needles observed in early spring sometimes develop from needles that died the previous year. When only top foliage dies, PWN frequently coexists with fungi. Symptoms are reported in several publications (1,3,6,10,22,23,29,32,54). Since trees do not die instantly, needle symptoms become apparent over a period of time lasting from less than a month in some seedlings to a year or more in mature susceptible trees.

Resistance and Predisposition to Pinewood Nematode

The growth-differentiation balance hypothesis provides a basis for understanding the interactions of host pines and southern pine beetle, *Dendroctonus frontalis* Zimmn. (19,20), but many of these interrelationships could have been written about the interactions among PWN, host trees, longhorned beetles, and pine wilt disease. The main thesis of the growth-differentiation balance hypothesis is that photosynthates are partitioned, when water is not limiting, into new cell production with little cell differentiation. Cell division and enlargement take place first and differentiation occurs later when conditions become less favorable. It is during the differentiation process that pine trees become more resistant to disease. Both processes overlap considerably, but during differentiation metabolism of photosynthates shifts to synthesis of gums, oleoresins, terpenes, essential oils and similar products, as well as production of secondary cell walls and their lignification. Under conditions of mild stress, differentiation is favored, and the biomass has a higher content of lignin, wax, and other materials rather than predominately protein and primary cell walls. Competition between oleoresin formation and wood production is an example of growth-differentiation balance (21).

During late wood formation, when temperatures are higher, oleoresin production

increases and oleoresin flows more readily because of lower viscosity. An abundance of sugars (photosynthate), during periods of moderate water deficit, slows growth and promotes oleoresin production. Factors that retard crown development and reduce radial growth also restrict synthesis of oleoresin and its flow from tree wounds. Dense stands, severe moisture stress, old age, and overtopped trees result in less photosynthate production. The photosynthate is used for restricted growth, so oleoresin production diminishes. This growth-differentiation balance concept and supporting literature provides a basis for partially understanding the phenomena of tree resistance and susceptibility, disease severity, and effects of climatic stress.

A useful classification of resistance in pines to pathogens was developed by Klement and Goodman (14). Preformed or static resistance in host pines is present before invasion by pathogens, while induced or dynamic resistance develops after infection. Induced resistance requires metabolic responses in the host and was subdivided by Klement and Goodman (14) into 1) premunity or resistance acquired after infection by avirulent organisms, or by a pathogenic species that triggers the resistant mechanisms, and 2) hypersensitivity which is considered a universal defense mechanism in plants resulting from an incompatible host–parasite interaction. All three types of resistance are present in pine wilt disease.

Preformed resistance in host pines is present because of the innate toxicity of oleoresins and polyphenols, and probably because of other toxins, nutritional inhibition, antibiotics, or other substances. There has been little research on preformed resistance in relationship to PWN, with the exceptions of toxicity of oleoresin (7,34,36) and the toxicity in aqueous extracts of twigs or bark of *P. taeda* L. (loblolly) and Japanese black pines (2).

Oleoresin, a nonaqueous secretion of resin acids dissolved in a terpene hydrocarbon oil, originates in the living cells of resin canals, ray parenchyma, and other parenchyma cells. Oleoresin is not necessarily a terminal product but may be remetabolized. Its flow is regulated by additional synthesis and by the osmotic pressure of epithelial cells that line the resin canals, until pressure is diminished by oleoresin loss into injured tissues, externally, or into tracheids. Oleoresin pressure and flow is lower in drought and higher following rainfall, fluctuates diurnally, and declines with increasing temperature, light, and decreasing humidity (43). Resistance in pines appears to be associated with the number and interconnections of canals and with oleoresin toxicity. Oleoresin flow ceases in PWN-susceptible pines (15,27), but only reduces before resuming in resistant trees (18,47). Attempts to show that quantity, chemical composition, toxicity, or exudation pressure of oleoresin in relation to resistance to fungi and insects have proven inconclusive (4,43).

One probable example of induced resistance against pine wilt disease, perhaps the result of the synthesis of phytoalexin, is provided by Kiyohara (12). Low numbers of avirulent PWN inoculated into pines induces an acquired resistance to subsequent challenge by virulent PWN. Would pines infected by other aphelenchoid parasites produce a protective barrier against pine wilt disease? The chemical mechanism of this induced resistance remains unexplained. Another example of resistance, although it might be considered part of the hypersensitive reaction, probably results from newly synthesized phenolic compounds in PWN-infected eastern white pines. When PWN isolated from Japanese black pine in New Jersey are used to infect eastern white pine seedlings, the nematodes rapidly migrate through the cortical tissues to the tops and bases of the small trees. Rapidly developing darkened areas of tissue form and PWN emerge from these necrotic tissues (32). In addition, phytotoxins produced following invasion of Scots pines immobilize and kill PWN (7).

A common reaction in plants infected with certain pathogens is rapid tissue necrosis and death of cells. This is generally

designated the hypersensitive reaction (16,31). Hypersensitivity starts with an increase in oxidative enzymes, and is followed by increased synthesis of phenolic compounds and a darkening of tissues. Such a reaction is caused by an incompatible pathogen–hypersensitive host combination (31).

The resistant reaction of a wound or infection in the inner bark or phloem is characterized by rapid desiccation and necrosis of cells and the synthesis of terpenes, polyphenols, and other compounds by these cells. Wound periderm containing compounds toxic to invading pathogens develops around the necrotic area, isolating it from the normal tissues (4). Invaded cambium produces undifferentiated parenchyma cells that die after synthesizing terpenes, or the cambium proliferates callus that differentiates into traumatic resin cysts. Polyphenols synthesized from stored or translocated carbohydrates by dying parenchyma cells mix with oleoresin secreted into canals. These toxins further disorganize ray cells in the xylem as they flow into a pressure deficit created in the affected area, usually in advance of an infection (14). This reaction also is induced in oviposition sites in bark (48). Normally, the necrotic lesion advances with greater speed than the pathogen, as the cells in resistant pines rapidly die, until the pathogen itself is confined and dies. Darkening of tissues, possibly caused by polyphenol accumulation, and cell death in advance of PWN damage were reported in pine wilt disease (28,32–34).

Shain (41) showed that in pines infected with *Heterobasidion annosus* (Fr.) Bref. a reaction zone forms in the xylem with resulting death of ray and resin canal parenchyma, loss of starch, accumulation of free phenols, increase of oleoresin content, and a decrease in moisture. These data suggest that oleoresin diffuses or flows from one tracheid to another. Oleoresin-soluble phenols also diffuse from the radial ray and resin canal parenchyma, effectively blocking water movement. Such areas of tissue or compartments desiccate after nutrient removal. They contain mostly dead cells and flood with oleoresin soluble toxins such as phenolics and phytoalexins (42). This controlled, defensive, pathogen-isolating mechanism is a nonspecific reaction that might be initiated by any agent injurious to pine sapwood. It is somewhat analogous to sapwood transformation to heartwood.

Tracheid blockage (9,38), aspiration of border pits, and altered metabolism exist in pine wilt disease (11). Phytotoxic materials are rapidly produced in pines infected by pathogens (36,40). This is considered part of the hypersensitive response generated by infecting pathogens and wilt has been described as one of the toxic symptoms (7,37,39,51,52). Such toxins may be synthesized from oleoresin (7). Unlike in other infections, toxin production, tracheid blockage, transpiration reduction, and decreased oleoresin result from the wandering of a relatively few PWN and are not directly caused by nematode metabolism, plant tissues response to obvious parasite damage, or to direct physical blockage of tracheids by PWN (39). The ability of an agent to survive and move away from the necrotic area of the hypersensitive reaction determines the pathogenic potential of an organism.

The growth-differentiation balance hypothesis may prove useful in predicting susceptibility to pine wilt disease because physiological conditions, such as adverse environmental conditions that cause stress, increase the risk of disease. Stressed trees produce less oleoresin and photosynthate, and the photosynthate is utilized in maintenance and restricted growth. Pine wilt disease is of major concern in areas where trees are grown outside their natural ranges and where virulent isolates of PWN have been introduced. For example, when red pines are grown outside their normal range, they show severe symptoms of pine wilt disease and have a PWN incidence of up to 77% (10), whereas this species is unaffected in Minnesota, its normal range (54). In Japan, pine wilt disease is frequently more destructive to trees on dry exposed sites than it is to trees on more favorable

sites (47), indicating that high temperatures and low moisture favor the disease (27). In addition, the mortality in Scots pines grown in Illinois appears related to the amount of rainfall (23).

I believe that the hypersensitive reaction in pines, which successfully controls many fungi, bacteria, viruses, and insect pathogens, is responsible for their ultimate death after invasion by PWN. It can be hypothesized that migrating nematodes defeat this innate defense mechanism by moving rapidly through the pine tissues and continually triggering the hypersensitive defense mechanism to extend the boundary of the reacting zone or injury until large areas of the stem are involved. Coalescing zones of dying tissues release oleoresins, polyphenols, and toxins, ultimately creating an area of excessive blockage of water-conducting tracheids. Toxins diffusing from these dying tissues probably enter normal tracheids and water columns and finally affect the foliage. The pine tree, therefore, weakens and ultimately kills itself. This process encourages the build-up of dense populations of long-horned beetles in susceptible, nematode-infected, weakened trees, and the reinforced disease cycle continues.

Whether a virulent pathotype was introduced or evolved (8), or whether the indigenous pines of Japan are more susceptible than those of North America, is not known, but it is clear from my visit to Japan in 1986 that vector pressure is higher in Japan. An epiphytotic situation exists as the result of PWN interacting with Japanese black and red pines and an efficient vector, *M. alternatus* Hops. Resistant genes appear to be either absent or not expressed because of environmental conditions. Japanese black pine grown along the eastern seaboard of the United States, however, do not rapidly succumb to pine wilt even though long-horned beetles and PWN are both present. Weather conditions along the eastern seaboard favor a slow growth rate. Temperatures are cooler and summers are dryer than in the disease endemic areas of Japan where high rainfall continues throughout most of the summer growing season. Warm, wet summers promote growth rather than differentiation, and as predicted in the growth-differentiation balance hypothesis, susceptibility to disease is greatest during rapid growth.

The ultimate purpose for studying pine wilt disease should be to stabilize resistance in susceptible pines (5). The multiplicity of defense mechanisms in pines helps to control pine wilt disease. An understanding of possible resistant mechanisms can be partially achieved by studying why pines are killed by pine wilt disease, but it is equally important to study resistant pines and the virulence of pathotypes of PWN. Such studies will ultimately provide an understanding of the genetic basis of resistance in pine trees and by genetic selection should lead to their coexistence with the nematode pathotypes and cerambycid vectors.

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