Factors affecting pine pitch canker modelled on Michaelis–Menten kinetics[1]

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Abstract: *Fusarium circinatum* Nirenberg and O'Donnell is an important pathogen of pine seedlings and cuttings in South Africa. The fungus causes plant death in nurseries, as well as during establishment of pine plantations. The aim of this study was to consider the effects of wound type, spore concentration, and environmental stress on infection incidence. Pine seedlings were inoculated using three wounding methods and five spore concentrations. Inoculated seedlings were incubated under optimal environmental conditions, suboptimal conditions, and suboptimal conditions combined with a fungicide treatment. Results showed that the mean percentage infection caused by increasing spore concentrations can be described by the Michaelis–Menten function. The gradient of the function, as well as the asymptotic maximum level of infection, was dependant on environmental stress and the physiological state of the host, as well as the wounding method. Spore concentration had the highest influence on infection incidence in physiologically stressed seedlings. Fungicide treatment did not influence the rate of infection incidence in comparison with the treatments conducted under optimal environmental conditions, but significantly lowered the asymptotic maximum level of infection incidence. Seedlings wounded on the stems had the highest infection incidence, when compared with other wounding methods.

Key words: Fusarium circinatum, wounds, environmental stress, spore concentrations.

Résumé : Le *Fusarium circinatum* Nirenberg and O'Donnell constitue un important champignon pathogène des plantules et des boutures de pins en Afrique du Sud. Ce champignon cause la mort des plants en pépinières ainsi qu'au cours de l'installation des plantations de pins. Les chercheurs ont examiné les effets du type de blessure, de la concentration des spores et de stress environnementaux sur l'incidence de l'infection. Ils ont inoculé des plantules de pins en utilisant trois types de blessures et cinq concentrations de spores. Ils ont ensuite incubé les plantules inoculées sous des conditions environnementales optimales, sub-optimales et sub-optimales avec un traitement fongicide. Les résultats montrent que le pourcentage moyen d'infection causé par une augmentation des concentrations de spores peut être décrit par la fonction de Michaelis–Menten. Le gradient de la fonction ainsi que le degré maximum asymptotique d'infection dépendent de l'environnement et de l'état physiologique de l'hôte aussi bien que du type de blessure. La concentration des spores montre l'influence la plus forte sur l'incidence de l'infection chez les plantules physiologiquement stressées. Le traitement fongicide n'a pas influencé l'incidence de l'infection comparativement aux traitements conduits sous des conditions environnementales optimales, mais a diminué significativement le degré asymptotique maximum de l'incidence d'infection. Les plantules blessées sur la tige montrent une plus forte incidence d'infection que les autres types de blessures.

Mots-clés : Fusarium circinatum, blessures, stress environnemental, concentration de spores.

[Traduit par la Rédaction]

Introduction

Pitch canker, caused by *Fusarium circinatum* Nirenberg and O'Donnell (teleomorph *Gibberella circinata* Nirenberg and O'Donnell) (Nirenberg and O'Donnell 1998), is a serious disease of pine trees. The disease has been reported from the southeastern United States (Hepting and Roth 1946), Haiti (Hepting and Roth 1953), California (McCain et al. 1987), Mexico (Santos and Towar 1991), Japan (Muramoto and Dwinell 1990), Chile (Wingfield et al. 2002), Spain (Landeras et al. 2005), and Italy (Carlucci et al. 2007). Damage caused by *F. circinatum* in these regions includes resin-soaked cankers on the trunks and lateral branches of diseased trees (Dwinell et al. 1985). Shoot dieback (Correll et al. 1991) has also been reported. Die-back of female flowers and mature cones (Barrows-Broaddus 1990), reduced germination of seeds (Huang and Kuhlman 1990), as well as pine seedling mortality (Huang and Kuhl-

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man 1990; Carey and Kelley 1994) is commonly caused by this pathogen. Severe outbreaks of *F. circinatum*-infection of nursery plants have been reported in South Africa and Chile (Viljoen et al. 1994; Viljoen et al. 1995; Wingfield et al. 2002). Typical pitch-canker symptoms on older trees have not been observed in Chile (Wingfield et al. 2002). Only recently has the disease appeared, for the first time, on mature *Pinus radiata* D. Don trees in South Africa (Coutinho et al. 2007).

Fusarium circinatum causes disease in seedlings resulting in both pre- and post-emergence damping off (Huang and Kuhlman 1990; Viljoen et al. 1994), as well as mortality of established seedlings (Carey and Kelley 1994; Viljoen et al. 1994). Where pre-emergence damping off occurs, the seed coats and coleoptiles of germinated seeds are heavily colonized by the fungus. In the case of post-emergence damping off, root collars are girdled. Infection also results in needle chlorosis and wilting of seedling tips (Viljoen et al. 1994). Pitch-soaked lesions usually occur at or near the soil surface, but are occasionally found in the region of the cotyledonary node (Barnard and Blakeslee 1980) or in the region of the bud (Carey and Kelley 1994). The pitch-canker pathogen has also been reported to cause severe root rot in established seedlings. Viljoen et al. (1995) noted that established seedlings infected with the pitch-canker pathogen had underdeveloped roots with multiple pitch-soaked lesions. In South Africa, root rot caused by *F. circinatum* is most common on *Pinus patula* seedlings (Viljoen et al. 1994).

The pitch-canker fungus infects pines through wounds (Barrows-Broaddus et al. 1985; Kuhlman 1987). Wounds can be of abiotic origin (Kuhlman et al. 1982) or as the result of insect feeding (Blakeslee et al. 1978). Abiotic wounds, such as those caused by wind damage or silvicultural practices, are the primary points of infection in the southeastern United States (Dwinell et al. 1985). In California, insects are considered to be the main wounding agents (Correll et al. 1991). In South African pine seedling nurseries, a number of horticultural practices, such as transplanting, weeding, and rough handling of seedlings during plantation planting, may provide wounds, which can serve as infection sites.

Conidia of *Fusarium circinatum* are abundant in areas where the pitch-canker disease occurs (Dwinell et al. 1985). Inoculum can be present throughout all seasons (Correll et al. 1991; Fraedrich and Dwinell 1997). Furthermore, Blakeslee et al. (1978) showed that sporodochia containing microconidia occur abundantly on infected branches and dead needles attached to infected shoots. Infected and dying plant organs are shed into the litter where they serve as a source of inoculum (Barrows-Broaddus and Dwinell 1984). A bark-wash survey conducted in California showed that both symptomatic and asympomatic trees could harbour inoculum (Adams 1989). There is some debate as to the minimum number of spores needed for infection to occur, but studies report that tree resistance is not dependant on spore dosage (Gordon et al. 1998*a*; Hodge and Dvorak 2000).

Various stress factors may predispose trees to infection by *F. circinatum*. Moisture deficiency and early frost have been associated with pitch-canker epidemics (Dwinell et al. 1985). Barrows-Broaddus and Dwinell (1983) observed that infections are often latent, and that infected pines harbour the pathogen until environmental conditions permit a disease outbreak. The extent to which environmental stress is involved in pitch canker epidemics is not clear, since epidemics have also occurred in the absence of stress (Dwinell et al. 1985).

Little research has been conducted on the effects of environment on the epidemiology of the pitch-canker fungus. The objective of this study was, therefore, to determine the effect of environmental stress conditions such as heat and water stress on infection incidence in pine seedlings. We also considered the role played by various wound types and different spore concentrations.

Materials and methods

Preparation of inoculum and plants

An isolate of *F. circinatum* (MRC 6213) previously shown to be highly pathogenic (Viljoen et al. 1995) was grown on potato dextrose agar (PDA, Merck, Biolab Diagnostics (Pty) Ltd., Johannesburg, South Africa) in the dark for 2 weeks. Five healthy 16-month-old *Pinus taeda* L. saplings were inoculated with agar discs colonized by the fungal isolate. *Fusarium circinatum* was reisolated from trees 12 weeks after inoculation on *Fusarium*-selective medium (FSM) (Nash and Snyder 1962) and identified from cultures grown on synthetic nutrient agar (SNA) (Nirenberg and O'Donnell 1998). Single conidial cultures were made on PDA from isolates of *F. circinatum* reisolated from inoculated trees, lypophilized in a 15% glycerol solution, and maintained at –80 °C. These cultures were used in later inoculations.

Pinus patula Schiede & Deppe ex Schltdl. seeds were surface disinfected in a 1% solution of NaHClO and sown into a peat seedbed. Six weeks after sowing, seedlings were transplanted into steam sterilized composted bark medium in 26 cm^3 removable plugs in planting trays. Within the trays, seedlings were arranged in blocks of six, with at least one space (25 mm) between the blocks. Eight blocks of six seedlings were fitted into a seedling tray. Seedlings were grown in a greenhouse at an average temperature of 24 °C for 4 months prior to conducting inoculation experiments.

Experimental design of pathogenicity trial

Pinus patula seedlings were arranged in three greenhouse units. In each greenhouse unit, seedlings were arranged in a randomized block design of four blocks. Each block was made up of 24 groups of six seedlings. Each group of six seedlings was considered as one experimental entity and received the same treatment. Four wounding treatments were tested in combination with six spore concentrations. Treatments were replicated in each block and in each greenhouse unit. In each greenhouse unit, different incubation conditions were tested and are, therefore, subtrials of each other.

Wound types

Three different wounding techniques were used prior to inoculations. These included (*i*) root wound (severing a few roots with a sharp scalpel), (*ii*) stem wound (shallow 1 mm cut into the phloem on the lower part of the seedling stem), and (*iii*) a wound simulating rough handling (ripping out a few needles). Within one experimental block, six experimental entities of six seedlings each were treated with each wounding method, and six experimental entities per block were not wounded to serve as controls.

Conidial concentrations

Conidial suspensions of 5, 50, 500, 5000, and 50000 spores/mL were prepared by placing 15 mL of sterile water on cultures of *F. circinatum* and rotating the Petri dishes to dislodge spores. The spore-bearing liquid was removed from the Petri dishes and placed in Erlenmeyer flasks. Conidial concentrations in these flasks were adjusted with sterile water and the number of conidia were counted using a haemocytometer.

Within each block of seedlings, each experimental entity, which received a different wounding treatment, was inoculated with a different spore concentration. For inoculations, 1 mL spore suspensions were applied to wounds with a pipette. Within each block, one experimental entity of each wounding treatment was inoculated with sterile water. Nonwounded seedlings were also inoculated with the five spore concentrations and sterile water. The nonwounded treatment inoculated with sterile water served as the negative control.

Incubation conditions

Inoculation trials were incubated under three different sets of treatment conditions. These included optimal conditions for the pine-seedling growth, suboptimal conditions for the pine seedlings, as well as suboptimal conditions in combination with a fungicide treatment. In the subtrial conducted under optimal conditions, seedlings were maintained at an average temperature of 22 °C. The seedlings were watered three times daily at 8 h intervals for 4 min. In the subtrial conducted under suboptimal conditions, seedlings were placed under water and heat stress. In this subtrial, seedlings were maintained after inoculation at an average temperature of 32 °C, and were watered three times daily at 8 h intervals for 1 min. In the subtrial conducted under suboptimal conditions for pine seedlings in combination with a fungicide treatment, seedlings were treated with a 24 g/L benomyl drench (Runion and Bruck 1988; Runion et al. 1993) 10 d prior to inoculation. The benomyl-treated plants were maintained under the same conditions as the subtrial conducted under suboptimal conditions. Plants were acclimatized to the three different treatment conditions 2 weeks prior to inoculation.

Pathogen reisolation

Three months after inoculation, isolations were made from small pieces of wood from the area immediately surrounding the inoculation sites, with care taken not to isolate from the inoculation site itself. Pieces of wood were removed under near-sterile conditions (bark was treated with sodium hypochloride prior to removal) and plated out onto FSM. Cultures were incubated for 5 d at 25 °C in white light (Osram 58W/77). Isolates with a white or pink mycelium were subsequently plated out on SNA and identified after 7 d using a Zeiss Axioscope at 400× magnification.

Data analysis

The percentage of infected plants was calculated for each unit of six seedlings receiving the various treatments. Mean-to-variance plots for the data demonstrated some heterogeneity of variance, and thus, data were normalized by arcsine transformation. A general linear model (GLM) analysis was conducted using SAS software (version 8.0; SAS Institute, Cary, N.C.). The analysis was done with three fixed factors (spore concentration, wounding, and environmental conditions tested) and one dependent variable (mean percentage infection incidence). Significance was tested using Tukey's *t* test at the 1% significance level. The least significant difference (LSD) was calculated at a 1% significance level. Regression analysis was done on putative interactions using the Pearson's correlation coefficient, and correlations were tested with the r (n) test for significance.

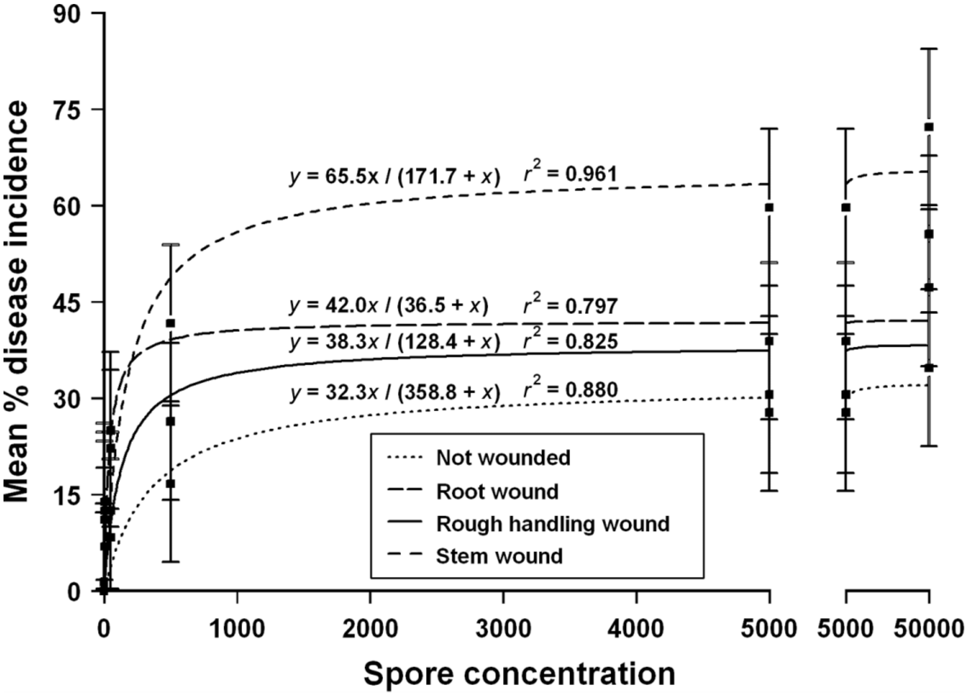
Data analysis was done by averaging the data across factors. For example, when comparing the effect of spore concentration on infection incidence for different wounding methods, the data obtained for the different environmental conditions were pooled for each wounding type. Although significance between the different factors were observed (results not shown), this approach should give a more representative analysis that should be more applicable for field conditions. Furthermore, individual analysis on the separate factors showed similar trends compared with the combined datasets. The results obtained by averaging the data across factors had no significant influence on the observed interactions between factors when compared with the individual datasets.

The mean percentage infection incidence (MPII) was plotted as the observed variable against spore concentration as the controlled variable using the Michaelis–Menten equation. The Michaelis–Menten equation is the basic equation of enzyme kinetics and describes the relationship between an enzyme catalysed reaction velocity and substrate concentration (Cornish-Bowden 2004). In this study the Michaelis–Menten equation is given as $y = \mu_{\max} /(K_s + x)$, where y is the MPII, $\mu_{\max}$ is the maximum value of MPII, x is the spore concentration, and K_s is a parameter that is constant for the specified experimental conditions. The $\mu_{\max}$-value gives the limiting value of MPII when the maximum amount of infection incidence is achieved, in other words all the possible sites for infection are saturated by spores. K_s relates the MPII value to the spore concentration, where K_s is the spore concentration at which the MPII is half-maximal. K_s is also a measure of the suseptability of the plant for infection, or the ease with which the inoculum can cause infection. The lower the K_s value, the lower the spore concentration needed to achieve half-maximum MPII, and the more suseptible the plant to infection by *F. circinatum*.

Results

Considering the effect of spore concentrations, the MPII of the negative control (0 spores/inoculation) differed significantly from all other treatments ($P < 0.01$). The MPII in plants treated with 5 spores/inoculation differed significantly from that of plants treated with 500 or more spores/inoculation, while the MPII of plants treated with 500 spores/inoculation differed significantly from that of plants treated with 5000 or more spores/inoculation ($P < 0.01$). The MPII of plants inoculated with 5000 and 50 000, however, did not differ from each other. Similarly, the mean

Fig. 1. Graph showing the correlations of mean percentage disease incidence with spore concentrations for different wounding methods. Data from the different subtrials were combined. Error bars represent the LSD.



percentage infection values obtained for 500 and 50 spores/inoculation, as well as 50 and 5 spores per inoculation, did not differ significantly from each other. (LSD = 13.4%) (Fig. 1).

Statistical analysis showed that the MPII of seedlings grown at optimal conditions, suboptimal conditions, and suboptimal conditions in combination with a fungicide treatment, differed significantly from each other ($P < 0.01$). Plants placed under water and heat stress after inoculation had the highest MPII (39.1%). Plants incubated at optimal environmental conditions after inoculation had a MPII of 27.3%. Seedlings treated with benomyl had the lowest (8.0%) MPII (Fig. 2) (LSD = 6.1%).

There were significant differences in the mean percentage infection incidence (MPII) between the different wounding treatments ($P < 0.01$). The MPII of plants wounded at the stem was significantly higher than that of plants with no wounds or with wounds simulating rough handling. The MPII of stem wounded plants did not differ significantly from treatments with root wounds. The MPII of root wounded plants did not differ significantly from all the other treatments (LSD = 11.0%).

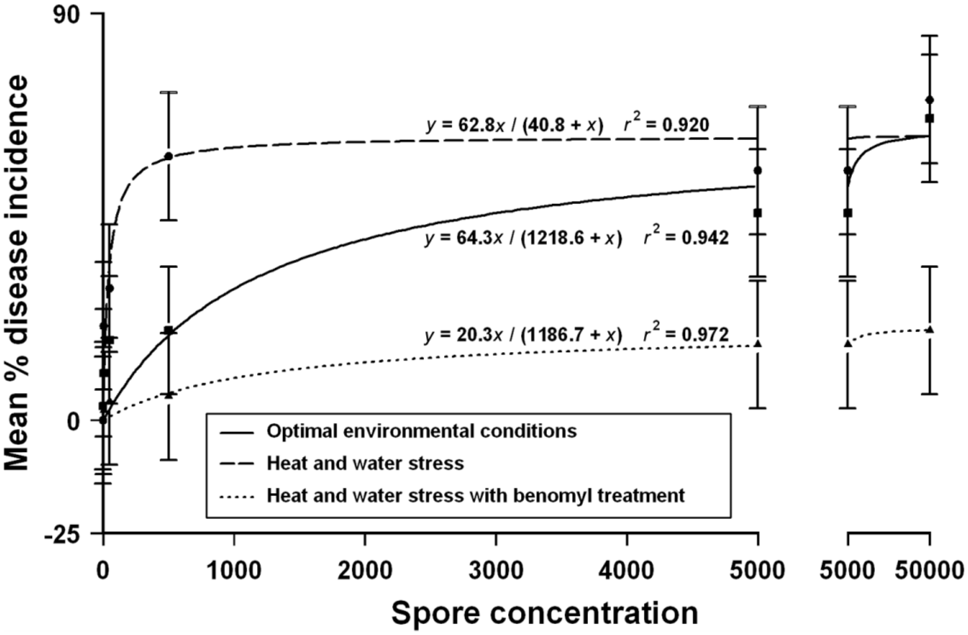
Highly significant interactions between spore concentration and wounding method were observed. The interaction between spore concentration and MPII for the different wounding methods could be plotted on a Michaelis–Menten function $f(x) = \mu_{\max} /(K_s + x)$. Correlations between the MPII for the individual wounding methods and spore concentrations (Fig. 1) were significant at $P < 0.01$, with correlation coefficients ranging from 0.8 to 0.96. The largest $\mu_{\max}$ -value was observed in the Michaelis–Menten function describing stem wounds (65.5%), followed by root wounds (42.0%), rough handling wounds (38.3%), and nonwounded seedlings (32.3). The gradients of the curves describing the different wounding methods as indicated by the K_s-value, did not differ significantly from each other ($P < 0.01$). This indicates that wounding method had little effect on the rate of increase of the MPII at increasing spore concentrations.

Highly significant interactions between spore concentration and environmental conditions were observed. These interactions become evident when plotting the Michaelis–Menten function of MPII and spore concentration for the different environmental conditions. The regression for the combined datapoints of the three environmental conditions yielded the function $f(x) = 44.5x/(134 + x)$, which had a correlation coefficient of 0.894 that was significant at a 1% significance level. Therefore, 89% of the variation in the MPII for the data from the three environmental conditions tested can thus be explained by the spore concentration. Correlations between spore concentrations and MPII for the individual conditions with spore concentrations (Fig. 2) were significant at $P < 0.01$, with correlation coefficients ranging from 0.92 to 0.97. The $\mu_{\max}$-value of the trial incubated under optimal conditions (64.3%) did not differ significantly from that observed in the trial conducted under water and heat stress (62.8%). The $\mu_{\max}$ -value of the trial incubated under water and heat stress in combination with a fungicide treatment was significantly lower (20.3%) than that of the other two trials. The K_s-value of the experiment conducted under optimal conditions (1218.6) and that of the experiment conducted under water and heat stress in combination with a fungicide treatment (1186.7) did not differ significantly from each other ($P > 0.01$), but differed significantly ($P < 0.01$) from that of the experiment conducted under water and heat stress without the fungicide treatment (40.8).

Discussion

Results of this study provide strong evidence that the en-

Fig. 2. Graph showing the correlations of the mean percentage disease incidence with spore concentration for different environmental conditions and plant vigour states. Data from the different subtrials were combined. Error bars represent the LSD.



vironmental conditions of the plant in a genetically nonuniform heterogenic pine seedling population have a major effect on the infection of pine seedlings by *F. circinatum*. It was also evident that the type of wounds affect infection by the pathogen. Our findings also suggest that spore concentration is an important factor in the *F. circinatum* infection process. There were also strong interactions between spore concentration and environmental conditions, as well as interactions between wounding method and spore concentration. These interactions could be described using the Michaelis–Menten function.

Fusarium circinatum is a wound-infecting pathogen. Kuhlman et al. (1982) demonstrated natural infection by placing wounded seedlings under infected mature trees. These researchers showed that no disease was observed in the absence of wounds. Our studies show that *F. circinatum* can infect seedlings and cause infection in the absence of a wounding agent. We consistently reisolated *F. circinatum* from inoculated, nonwounded seedlings in tests conducted in a greenhouse in the absence of insects and nematodes. Infections arising on nonwounded plants originated in all instances from the root collar. Upon closer inspection of the root collar, we found microscopic growth cracks in the periderm, which might have served as points of infection for the fungus. A possible explanation for the occurrence of these cracks might lie in the fertilization program used in our experiments. We applied relatively high rates of potassium, phosphorous, and nitrogen. Growth cracks might thus have originated in the seedlings owing to rapid cell elongation that took place in response to high mineral nutrition in the absence of cell division, cell wall thickening, and lignification. Other researchers have also found that application of high levels of mineral nutrition to pines increases pitch canker induced mortality (Fisher et al. 1981), but these studies were conducted on older pine trees.

There were highly significant interactions between wounding method and environmental conditions. Wounding had little effect under optimal conditions except for increased infection of root wounds, but had considerable effects in the experiment conducted under water and heat stress, where stem wounds were significantly more susceptible. These results are consistent with different epidemiological patterns observed in different climatic regions in the United States. Dwinell et al. (1985) suggested that mechanical damage of abiotic origin was the main driving force of pitch canker epidemics in the southeastern United States. In contrast, no association with abiotic damage was observed in California, where insect inflicted damage plays a major role in the infection cycle of the pitch-canker disease (Correll et al. 1991). Climatic conditions thus appear to be an important factor relating to the susceptibility of wounds to infection by *F. circinatum*.

In our study, spore concentrations had a significant effect on the success of inoculations. In inoculations conducted under optimal environmental conditions, there were no significant differences in infection incidence in plants inoculated with low spore loads (0–500). This result is consistent with those of Gordon et al. (1998*b*), who found no significant differences in lesion length between *P. radiata* clones inoculated with spore concentrations in the range of 50–140 spores. Our results showed that there are no significant differences in infection incidence in plants inoculated with high spore loads (5000–50 000). This supports findings by Hodge and Dvorak (2000), who failed to find significant differences in disease severity for spore concentrations between 50 000 and 100 000. We have, however, shown that there are significant differences between the MPIIs caused by high and low spore loads. Storer et al. (1999), tested a range of spore concentrations between 25 and 1000 spores, and found significant differences between disease severity, which is consistent with the results of the present study.

Interactions between spore concentration and the MPII of the individual environmental states tested were highly significant. Correlation coefficients of the Michaelis–Menten function plotted for the interactions were high, ranging from 0.92 to 0.97, showing that the Michaelis–Menten function can effectively model the effect of spore concentration on the MPII in a genetically diverse population of seedlings inoculated with *F. circinatum*.

The Michaelis–Menten function of the experiments under optimal conditions and under water and heat stress did not differ significantly in their $\mu_{\max}$-values, but the K_s-value was considerably smaller for the stressed plants. Water and heat stress thus act as a competitive activator for infection. The stress conditions lowered the resistance of plants to infection. This can be seen in the higher percentage infection at lower spore concentrations as compared to infection under optimal environmental conditions. However, the maximum percentage infection occurring at higher spore concentrations still remained the same for both stressed and nonstressed plants. Gordon et al. (1998*a*, 1998*b*) suggested that pitch canker of pine is primarily controlled by the interaction between the host and the pathogen, and less so by the environmental conditions. The fact that 89% of the variation in our experiments can be accounted for by spore concentation supports this view. Nevertheless, the significant differences between the gradients of the Michaelis–Menten function plotted for the interaction between spore concentration and environment shows that the interaction between the pathogen and the host is also strongly influenced by the environment. This finding is supported by observations made by Blakeslee and Rockwood (1999), who showed that various stress factors such as moisture deficiency may predispose pine trees to infection by the pitch-canker fungus.

Our results showed that fungicide treatment can act as an uncompetitive inhibitor to infection, since the benomyl treatment lowered the $\mu_{\max}$-value of the Michaelis–Menten curve considerably when compared with the other two subtrials, while the K_s-value is similar to that of the experiment conducted under optimal conditions. Benomyl is a systemic residual fungicide and its application effectively lowered the possible sites for infection. This resulted in a lowering of the percentage of infection resulting from inoculations. The fungicide also appeared to counteract the decreased seedling resistance under stress conditions. The low K_s-value shows that at lower spore concentrations, the stressed plants have a similar resistance to fungal attack to that of plants grown under optimal environmental conditions. Although reduction in infection was significant, owing to fungicide application, infection incidence was still sufficiently high to have an economic impact in a commercial situation.

Conclusion

The Michaelis–Menten function has not been used previously to model the effect of fungal spore concentration on infection incidence in plant pathology. Nonetheless, in our study, the function was useful in modelling various responses of a genetically nonuniform seedling population confronted with a pathogen. We believe that modelling plant infection with the Michaelis–Menten function might be useful in determining the optimal spore concentration in pathogenicity trials conducted under different abiotic conditions. The function might also be useful in determining the importance of different environmental conditions on pathogen attack and might, therefore, aid in devising disease management programmes.

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