Tissue Specific Localization of Root Infection by Fungal Pathogens: Role of Root Border Cells

Uvini Gunawardena^{1,2} and Martha C. Hawes¹

¹Department of Plant Pathology, 204 Forbes Building, University of Arizona, Tucson 85721, U.S.A.; ²Torrey Mesa Research Institute, 3115 Merryfield Row, San Diego, CA 92121 U.S.A.

Submitted 22 April 2002. Accepted 8 July 2002.

When roots of pea seedlings were inoculated uniformly with spores of *Nectria haematococca* or other pea pathogenic fungi, more than 90% developed lesions in the region of elongation within 3 days. More mature regions of most roots as well as the tip showed no visible signs of infection. Yet, microscopic observation revealed that ‘mantles,’ comprised of fungal hyphae intermeshed with populations of border cells, covered the tips of most roots. After physical detachment of the mantle, the underlying tip of most roots was found to be free of infection. Mantle-covered root tips did not respond to invasion of their border cells by activation of known defense genes unless there was invasion of the tip itself, as revealed by the presence of a lesion. Concomitant with the activation of defense genes was the induction of a cell-wall degrading enzyme whose expression is a marker for renewed production of border cells. Mantle formation did not occur in response to nonpathogens. The data are consistent with the hypothesis that border cells serve as a host-specific ‘decoy’ that protects root meristems by inhibiting fungal infection of the root tip.

Additional keyword: pectinmethylesterase.

Plant roots are not uniformly susceptible to infection by soilborne pathogens and symbionts (Bruehl 1986, Curl and Truelove 1986). Except at wounds and points of lateral root emergence, the older, upper parts of roots often are resistant to invasion, presumably as a result of secondary cell wall thickening during tissue maturation (Bruehl 1986). Older tissues also may become invaded when cortical sloughing is initiated as plants age. In healthy young roots, bacteria and fungi as well as nematodes initiate invasion within the zone of elongation, a distinct tissue several mm in length that begins 1 to 2 mm behind the root apex (Baluska et al. 2001; Barlow and Baluska 2000). The root tip itself, a 1- to 2-mm apical region comprising the root meristem and the root cap, usually remains devoid of infection or colonization (Bauer 1981; Cunningham and Hagedorn 1962; Foster et al. 1983; Parkinson et al. 1963; Rovira and Campbell 1974; Royle and Hickman 1963; Zentmyer 1961).

One explanation to account for tissue-specific localization of root infection and colonization is that root border cells (previously known as ‘sloughed root cap’ cells) that surround the root tip of most plants but not the zone of elongation play a role in protecting the tip (Foster et al. 1983; Hawes and Brigham 1992; Hawes et al. 1998; Kroes et al. 1998). Border cells are

programmed to separate from the cap periphery to form a boundary between the root and the environment, and can play a role in mechanical penetration of root tips through soil (Bengough and McKenzie 1997). At the time of their release from the root tip, border cells are viable and can survive indefinitely in culture if supplied with appropriate nutrient media (Hawes and Pueppke 1986; Knudson 1919). How border cells function under natural conditions is unknown but, in the laboratory, they specifically influence gene expression, growth, and behavior in populations of nematodes as well as pathogenic and symbiotic bacteria and fungi (Hawes et al. 2000).

Production and release of border cells is developmentally programmed and occurs in a very consistent pattern among primary and secondary roots of diverse plant species (Hawes et al. 1997). In pea, when seedlings are germinated in aeroponics or on damp filter paper, their accumulation at the cap periphery begins when the root has emerged to a length of 5 mm, and a full set of approximately 4,000 cells is present by the time the root reaches a length of 25 mm. At this point, cell accumulation ceases and this original set of border cells can remain appressed to the cap for days as the root continues to lengthen (Hawes and Lin 1990). If the root tip is immersed in water, however, the population of border cells instantly begins to disperse away from the root into suspension. Within five minutes, synthesis of a new set is initiated by the induction of mitosis in the root cap meristem (Brigham et al. 1998). Several hundred newly synthesized border cells can be collected within an hour, and a full set of approximately 4,000 new cells is present at the cap periphery within 24 h.

The model system of pea (*Pisum sativum* L.) and the fungal pathogen *Nectria haematococca* (anamorph: *Fusarium solani* f. sp. *pisi*) was used to investigate mechanisms underlying tissue-specific localization of root infection. Experiments confirmed that, under most experimental conditions, *N. haematococca* infection is predominantly localized in the region of elongation, while most root tips remain infection free. The current study examined predictions of the hypothesis that the presence of border cells at the root periphery and their ability to separate from the tip play a role in protecting the root tip from infection.

RESULTS

Tissue specific localization of root infection by *N. haematococca*.

To characterize the infection pattern of *N. haematococca* on pea roots, whole roots of pea were inoculated uniformly with 10^5 spores per radicle and were incubated at 24°C. Tan to light brown lesions were visible within 3 to 5 days on $93 \pm 3\%$ of the inoculated seedlings. In $90 \pm 7\%$ of those seedlings with visible infections, the lesions were localized at site 1 (Fig. 1).

Corresponding author: M. C. Hawes; Telephone: +1.520.621.5490; Fax: +1.520.621.9290; E-mail: mhawes@u.arizona.edu.

Site 1 corresponded to the region of elongation, a tissue approximately 1 cm in length (Fig. 1A, double arrow) beginning several mm behind the point that was the root apex at the time of inoculation (Fig. 1A, arrow). Visible infection at site 1 was not correlated with significant reduction in root growth or inhibition of normal development. Thus, despite the presence of an obvious lesion, morphology and growth of roots and above-ground parts were indistinguishable from uninoculated controls (Fig. 1B) during a six-day period of observation.

In most seedlings, there were no visible infections at site 2, a 1- to 2-mm region at the root apex comprising the root cap and apical meristem (Fig. 2A). Thus, 86% of inoculated seedlings had tips that were uniformly white (score = 0) and were indistinguishable in appearance from that of uninoculated control seedlings. In 14% of inoculated seedlings, visible infections were present at site 2; infections ranged from yellow or tan (score = 1) to light brown (score = 2) to dark brown or black (score = 3) (Fig. 2A, left to right).

Even when the temperature was increased from 24°C to 30°C, which stimulates fungal growth and inhibits pea root growth (Hawes and Lin 1990), $73 \pm 5\%$ of root tips escaped the development of visible infection at site 2; and when inoculum concentration was increased from 10^5 to 10^7 spores per radicle, nearly 50% ($49 \pm 9\%$) of seedlings still escaped infection at site 2 (score = 0).

In contrast to infection at site 1, which caused no obvious effects on growth and development during a six-day period of observation (Fig. 1), development of visible site 2 infections correlated strongly with cessation of further growth and development. Thus, in one experiment with 97 inoculated seedlings, 100% of roots (13 of 13) that developed brown or black lesions at the root tip ceased growth within 24 h; in an independent experiment with 115 inoculated seedlings, 17 of 19 seedlings (89%) that developed tan to brown lesions ceased growth within 24 h, and the remaining two seedlings ceased growth within 48 h.

Tissue-specific defense gene expression in roots inoculated with *N. haematococca*.

Three genes known to be induced in pea during infection, glucanase, chitinase, and HMM1 (Chang et al. 1992, 1995; Wu et al. 1997), were used as molecular markers to examine recognition responses at site 1, where infection is evident within 3 days on most seedlings, and to compare the responses with those at site 2, where visible infection seldom occurs within 3 days. In pooled samples of inoculated seedlings, defense gene expression was induced at site 1 within 24 h after inoculation with *N. haematococca* (Fig. 3A, left), whereas it was undetectable at site 2 after 24 h. Only after 60 h postinoculation was there a detectable response (Fig. 3A, right).

To assess the relationship between the presence of visible lesions and defense gene expression, the test was repeated by comparing defense gene expression of seedlings with visible infections at site 2 with that of seedlings without visible infections. Defense gene expression was detected only in the seedlings whose tips had visible lesions (Fig. 3B through D, left). In seedlings without visible lesions at site 2, defense gene expression was indistinguishable from tips of uninoculated control seedlings, even at 72 h postinoculation (Fig. 3B through D, right).

Host-specific colonization of border cells surrounding infection-free root tips: site 3.

Experiments were carried out to examine the developmental biology of root-tip responses to fungal inoculation. Root tips of inoculated seedlings that were white, grew normally and, to the naked eye, appeared to be infection-free (Fig. 2A, score = 0) were immersed in a droplet of water and examined with a dissecting microscope. Surprisingly, the root tips were found to be

ensheathed within a conspicuous network of hyphae intermeshed within and growing among detached border cells (Fig. 2B). When the border cell-hyphal colonies were plated onto culture media, identity of the fungus was confirmed to be *N. haematococca*. These root-tip-associated aggregates of border cells and hyphae, referred to as "mantles" due to their resemblance to the mantles formed by ectomycorrhizae (Fusconi 1983, Hawes et al. 1998, Horan et al. 1988), were designated as site 3 and were examined in detail.

Frequency of mantle formation. Many inoculated seedlings that lacked site 2 infection and failed to express defense gene expression nevertheless did have infection at site 3. Thus, when seedlings were inoculated with 10^5 spores per radicle and incubated at 24°C for 3 days, $77 \pm 4\%$ of the seedlings were found to have site 3 infections, even though most of the seedlings showed no visible signs of site 2 infection (score = 0). Despite the presence of the mantles, the fungus-ensheathed root tips remained white and continued to grow at the same rate as the uninoculated roots (Fig. 1B).

Lack of infection of mantle-ensheathed root tips. When root tips with site 3 infection were immersed in water and gently agitated, the mantles separated from the root tip spontaneously, as described previously (Hawes et al. 1998) or in response to wiping the tip gently with a tissue (Fig. 2C and D). The detached mantles, when placed onto culture medium and incubated overnight, yielded prolific fungal growth (data not shown). In contrast, when uninoculated control root tips (Fig. 2E) or root tips whose mantles had been removed (Fig. 2F) were placed onto culture medium and incubated overnight, no

Fig. 1. Primary site of root infection by pathogenic fungi (site 1). Roots of seedlings were inoculated uniformly with a suspension of spores (left) or sham-inoculated with water (right), then incubated in cellophane growth pouches. The site of the root apex (arrow) was marked on the pouch at the time of inoculation. Within 3 days, most seedlings exhibited pale yellow to tan or brown lesions (double arrow) 5 to 10 mm long, beginning 2 to 3 mm behind the original site of the root apex.

hyphae emerged from the tips. Thus, despite having been ensheathed in viable pathogenic fungus, the root tip had escaped infection. In contrast, roots with a visible lesion at site 2 (score = 2, 3, or 4) or with a mantle that was not removed yielded abundant hyphae after an overnight incubation (Fig. 2G).

The percentage of inoculated root tips that escaped infection was quantified and compared to the number that developed border cell-infected mantles. Seedlings inoculated with 10^5 spores were incubated at 24°C in the dark for 3 days, and site 3 infection was recorded by the presence of mantles using microscopic observation. The mantles were removed by gently wiping with a damp tissue, and then, root tips were surface-sterilized, excised, and placed onto culture medium. Among the inoculated seedlings, $77 \pm 4\%$ exhibited mantles around the root tip 3 days after inoculation. Among those seedlings with mantles, $70 \pm 6\%$ of the root tips yielded no fungal growth.

Mantle formation among different environmental conditions. Mantle formation was not specific to *N. haematococca* infection of pea roots maintained in growth pouches but also occurred on

seedlings grown in sand and vermiculite and on cultured hairy roots (data not shown).

Host specificity of mantle formation. Mantle formation was dependent on the genotype of the plant and the fungus. Other pea pathogenic fungi (including *N. haematococca* MPIV, four different field isolates of *N. haematococca* MPI, as well as *Phoma pinodella*, *Mycosphaerella pinoides*, and *Ascochyta pisi*) formed mantles around pea roots (as in Fig. 2B). In contrast, pea pathogenic isolates of *N. haematococca* did not form mantles on nonhost species including wheat, corn, and cotton (data not shown). An isolate of *Thielaviopsis basicola*, which infects tobacco but not pea, did not form mantles around pea root tips.

Removing border cells prior to inoculation with 10^7 spores is correlated with increased root tip infection.

The impact of border cells on tip infection was examined by washing border cells from the tip prior to adding spore suspensions. Removing border cells from root tips induces renewed

Fig. 2. Other sites of root infection by pathogenic fungi. **A**, In approximately 10% of the seedlings at site 2, a visible lesion was present at the root tip within 1 mm of the root apex. Based on comparison to a white, uninfected tip (score = 0), infections were scored as 1 (yellow), 2 (tan to light brown), or 3 (brown to black). **B**, At site 3, some root tips scored as 0 were found, upon microscopic inspection, to be covered in a mantle of fungus. **C** and **D**, In some cases, mantles detached spontaneously upon immersion in water. When excised, surface sterilized, and plated onto culture medium after mantle detachment, the tip yielded **E**, no microbial colonies as did **F**, surface-sterilized, uninoculated control root tips. In seedlings in which the fungus had penetrated to cause a visible lesion, or when mantles were left on the excised tips, prolific hyphal growth emerged from **G**, surface-sterilized tips within 24 h.

production, so that several hundred cells are present by the time spores germinate after more than 1 h. When roots were inoculated with 10^5 spores and incubated at 24°C for 3 days, the difference in root-tip infection of seedlings with border cells ($14 \pm 7\%$) and seedlings without border cells ($19 \pm 7\%$) was not significantly different. However, when challenged with high inoculum concentrations, removing border cells prior to inoculation was correlated with increased infection of root tips. At 24°C, $54 \pm 6\%$ of seedlings inoculated with 10^7 spores escaped site 2 infection, but when border cells were removed prior to inoculation only $33 \pm 7\%$ escaped tip infection. The values were statistically distinct at $p > 0.001$.

Retardation of border cell separation is correlated with increased root tip infection.

Experiments were carried out to test predictions of the hypothesis that the ability of border cells to separate physically

from the tip plays a role in allowing the root tip to escape infection. Dispersal of border cell populations occurs when roots are immersed in free water, which hydrates the surrounding mucilage, but otherwise, the cells remain tightly appressed to the root-tip surface (Hawes and Brigham 1992). If border cell separation is important in protecting the root tip from infection, then conditions that retard separation would be predicted to increase root-tip infection. When the water in growth pouches was reduced from 15 ml to 7 ml, mean root lengths after 3 days were 50 ± 7 and 55 ± 4 cm, respectively, and were not significantly different. Accumulation of the phytoalexin pisatin in response to inoculation with *N. haematococca* occurred at the same rate in site 1 when seedlings were grown in 7 or 15 ml of water per pouch (data not shown). However, separation of border cells was significantly reduced. After 3 days in pouches, seedlings grown in 7 ml of water retained more than 6,000 border cells at the cap periphery (Table 1). In contrast, in 15 ml of

Fig. 3. Differential activation of defense gene expression at localized sites of infection. **A**, Pooled samples of mRNA from inoculated seedlings were extracted from site 1 (the region that was behind the root tip at the time of inoculation) (left) and site 2 (the root tip) (right) at 2 to 72 h postinoculation. **B**, Seedlings with visible lesions (left) were sorted from seedlings without visible lesions (right) at site 2. mRNA was extracted at 48, 60, and 72 h postinoculation and compared with expression at time 0 and in uninoculated control seedlings after 72 h (72C). **C**, Density quantification of signal was done using the NIH image software program, which was adjusted in relation to the positive control actin gene.

water, border cells detached and were left behind as the roots grew, so that the number present on tips when seedlings were removed from pouches after 3 days was reduced several thousand cells (Table 1).

When seedlings in pouches were inoculated with *N. haematococca* (10^5 spores per radicle) under conditions that inhibited border cell separation, a 2.5-fold increase in the number of site 2 infections and a 1.9- to 2.3-fold increase in the severity of infections was observed. There was no change in the incidence of infection at site 1 in low water conditions ($89 \pm 5\%$) vs. high water conditions ($91 \pm 4\%$).

A gene needed for border cell separation is induced in response to fungal invasion of the root tip.

A gene whose expression is tightly correlated with and whose product is required for border cell separation, *rcpme1*, was used as a molecular marker to determine whether border cell separation is induced in response to fungal inoculation (Wen et al. 1999). High induction of this gene was evident 60 and 72 h after treatment in pooled samples of root tips from inoculated seedlings (Fig. 4A, left) but was absent in water-inoculated control seedlings (Fig. 4A, right). When root tips with visible lesions were separated from tips without visible lesions, the differences were even more striking (Fig. 4B). Induction of *rcpme1* expression was evident within 48 h in root tips with visible lesions (Fig. 4B, left), whereas expression in seedlings without visible lesions was indistinguishable from that of uninoculated control roots, even after 72 h (Fig. 4B, right). The expression of *rcpme1* also was induced in response to treatment with chitosan, a fungal elicitor (data not shown).

DISCUSSION

The results revealed that a uniform exposure of young roots to inoculum under conditions highly conducive to infection, including direct exposure to concentrated inoculum in moist pouches at high temperatures, resulted in infection of more than 90% of the roots within 1 to 2 days. However, infection largely was confined to the region of elongation, with no evidence of lesion formation in older tissues of the root. Most root tips also escaped infection. These results are consistent with previous studies demonstrating a localized pattern of root susceptibility to infection (Bruehl 1986; Curl and Truelove 1986).

One hypothesis to account for such observations is that the region of elongation is preferentially susceptible because it is a primary site for release of root exudates thought to induce germination of resting spores, to attract motile propagules of pathogens, or both (Curl and Truelove 1986). This hypothesis is supported by assays in which ninhydrin positive substances are detected in the region of elongation after incubation of roots on filter paper and by bioassays showing that, in some cases, fungal zoospores are preferentially attracted to the region of elongation (Cunningham and Hagedorn 1962; Hickman and Ho 1966; Ho 1969; Pearson and Parkinson 1961; Royle and Hickman 1963; Zentmyer 1961). However, these same assays, as well as carbon labeling experiments, have revealed

that the root tip per se also is a major, if not the primary, source of exudates (McDougall and Rovira 1970; Schroth and Snyder 1960; Griffin et al. 1976) and that not all pathogens exhibit specific attraction to the zone of elongation. Zoospores of *Phytophthora megasperma* and *Pythium dissotocum*, for example, are specifically attracted to the root apex of certain plants rather than the zone of elongation (Goldberg et al. 1989; Zentmyer 1961).

A second model to explain tissue-specific patterns of root infection is that growth generated by the root tip allows the tip to grow past germinating spores, thus evading infection (Huisman 1982). This hypothesis has been formulated by considering the growth rate of the root tip and the time required for the germination of resting spores by exposure to root exudates and, to date, is not supported by experimental data (Huisman 1982; Nelson 1991; Stanghellini and Hancock 1971).

A third hypothesis that could account for the observed tissue localization is that a fundamental difference exists between the inherent susceptibility of the two tissues to infection. Thus, root-tip tissue may have properties that render it impervious to invasion by fungi and other soilborne organisms. Though the tissues in the root tip as well as in the zone of elongation both are comprised of newly generated cells, those within the zone of elongation are markedly distinct from those within the root tip with respect to cell wall composition, structure, function, and gene expression (Baluska et al. 1996a, 1996b, 2001; Barlow and Baluska 2000; Feldman 1984; Freshour et al. 1996; Knox et al. 1990). The possibility that these variations could influence cellular responses to infection has not been examined previously, but our results were not consistent with the hypothesis that the two regions differ in their responses to infection. As in the region of elongation, there is little or no constitutive expression of defense genes in the root tip, but those that do develop a visible lesion respond with defense gene activation as quickly as when infection occurs in the region of elongation. Unlike infection in the zone of elongation, however, which has little or no impact on growth and development, the development of visible lesions in the root tip was correlated with cessation of root growth.

A fourth hypothesis to explain the results is that the region of elongation, the preferential site of infection, is the first available site of newly generated tissue that is not ensheathed in populations of specialized border cells whose function is to protect the root tip from infection (Hawes et al. 1998, 2000). Fungi ramify quickly throughout border cell populations, forming a sheath that detaches as the root elongates, leaving the tip uninfected. The detachment of a microscopic lesion from peripheral cells of the cap actually has been seen to occur along with the detaching mantle (Hawes et al. 1998). If border cells do function to protect the tip from infection, then inhibiting either production or detachment of the cells would be predicted to result in increased tip infection.

Circumstantial evidence in support of the hypothesis that border cells function to protect the tip from infection includes the observation that removing border cells prior to inoculation, or inhibiting border cell detachment by reducing water, both

Table 1. Increased infection of root tips when border cell separation is inhibited by reduced water levels^z

Amount of water added to pouch	Number of border cells per root tip	Seedlings (%) with tip infection (score > 1)		Severity of infection (average score)	
		10 ⁵ spores	10 ⁶ spores	10 ⁵ spores	10 ⁶ spores
7 ml	5970 ± 480	55 ± 8 ^a	79 ± 7 ^a	1.14 ^a	1.82 ^a
15 ml	2489 ± 143	22 ± 5 ^b	30 ± 5 ^b	0.61 ^b	0.78 ^b

^z Seedlings were inoculated, placed into growth pouches (3 pouches per treatment, 5 seedlings per pouch), and evaluated after 3 days at 24°C. Values represent means and standard errors from at least five independent experiments. Superscripts reflect values that are significantly different (7 ml vs. 15 ml) at $p > 0.05$.

were correlated with increased infection of the tip. The most direct observation consistent with the possibility that border cells play a role in protecting the tip is that, despite their close proximity, the tip often does, in fact, remain free of infection for days, while border cells rapidly become infected. A similar pattern of border cell infection has been reported to occur in early stages of ectomycorrhizal infection of *Cistus incanus* and *Fusarium oxysporum* infection of flax (Fusconi 1983, Kroes et al. 1998; Rodriguez-Galvez and Mengden 1995).

Interestingly, invasion of border cells does not elicit defense gene expression in the root tip of pea, emphasizing the physiological independence of the cap from border cells. Only when there was a visible lesion present on the root tip was there a measurable increase in expression of genes encoding production of antimicrobial activities, including glucanase, chitinase, and the phytoalexin pisatin. At the same time, there was induction of renewed border cell production, as measured by induction of *rcpme1*, a gene known to be required for border cell separation (Wen et al. 1999). The defense gene elicitor chitosan applied to root tips also induced expression of *rcpme1*. The data are consistent with the possibility that invasion of peripheral cells of the cap elicits renewed production and separation of border cells, which would have the effect of jettisoning the infected cells as the root elongates. If this model is correct, then use of transgenic

plants with altered production and separation of border cells in future studies would be predicted to exhibit altered patterns of root infection. Understanding mechanisms that allow some tissues to avoid infection might lead to new methods to prevent infection at sites that normally are infected.

MATERIALS AND METHODS

Growth conditions for pea seedlings, fungal cultures, and enumeration of spores.

Pea (*Pisum sativum*) seeds of cultivar Little Marvel were surface sterilized with 95% ethanol (10 min) and with 1.0% sodium hypochlorite (30 min), then rinsed thoroughly and soaked in sterilized water for 6 h. The imbibed seeds were spread on germination paper overlaid on 1% water agar and were incubated at 24°C for 2 days in the dark.

The primary test organism was *Nectria haematococca* (Berkeley and Broome) MPI isolate 3418. Other strains and species included four field isolates of *N. haematococca* MPI (T8, T23, T403, and T547); *N. haematococca* MPVI, isolate SUF1111; *Mycosphaerella pinodes* (Berkeley and Bloxam); *Phoma pinodella* (L. K. Jones); *Thielaviopsis basicola*; and *Ascochyta pisi* Lib., T422 (Delserone et al. 1999). Cultures were obtained from H. D. VanEtten (Department of Plant Pathology, University of Arizona,

Fig. 4. Activation of expression of a cell wall degrading enzyme in response to root-tip infection. **A**, In pooled samples of root tips from seedlings excised 2, 24, 36, 48, 60, or 72 h after inoculation, *rcpme1* expression was increased within 48 h after inoculation (left) compared with uninoculated controls (right). **B**, mRNA was extracted from root tips with visible lesions (left) or without visible lesions (right) at indicated intervals. In seedlings with visible lesions at the tip that were examined after 48, 60, and 72 h (left), *rcpme1* induction was evident within 48 h when compared with tissue excised at time 0 or from uninoculated control tissue after 72 h. No induction of *rcpme1* expression occurred in root tips without visible lesions (right). Density quantification of signal was done using the NIH image software program, which was adjusted in relation to the positive control actin gene.

Tucson, AZ, U.S.A.) and were maintained on V8 juice agar (Stevens 1974) and incubated at room temperature. For *T. basicola*, an additional 0.1% glucose was added to the medium (H. D. VanEtten, *personal communication*). Conidia were rubbed from the agar surface and were washed in water (3500 g for 1 min). The spores were quantitated using a haemocytometer, and the spore concentrations were adjusted such that 50 μ l of suspension contained the desired number of spores.

Inoculation of seedlings.

Infection assays were carried out in cellophane growth pouches (Mega International, St. Paul, MN, U.S.A.). Five seedlings with full sets of border cells (i.e., with a radicle length of 25 mm) were placed in bags containing 15 ml of water, unless otherwise indicated. Known amounts of spores were applied directly to the radicle of each seedling ensuring that the entire radicle was evenly inoculated. The location of the root tip at the time of inoculation was marked on the bag to monitor growth over 3 days after inoculation. Uninoculated seedlings were set up as controls exactly as described above, except that 50 μ l of water instead of the spore suspension was applied to the radicle.

The pouches were placed in plastic containers and were covered with plastic wrap to prevent excessive drying. The containers were placed in the dark in a 24°C incubator unless noted otherwise. Every 24 h, the pouches were taken out, and the location of the root tip was marked on the bag. Three days after inoculation, the seedlings were visually scored for infection development at the distinct sites.

To inoculate seedlings without border cells, the cells were washed off prior to being placed in the bags by immersing the root tip in water and gently pipetting water onto the root tip to remove the cells. The seedlings were then placed in growth pouches and inoculated as described above.

Observation of mantle formation and quantification of root tip infection.

Three days after inoculation, mantle formation was observed using a dissecting microscope at $\times 10$ to 60 (SV8; Zeiss-Nederland B. V., Weesp, The Netherlands).

After scoring for mantle formation, the root tip was wiped gently with a damp tissue to remove the mantle to evaluate for root-tip infection. The apical regions (2 to 3 mm from the tip) of these roots were surface sterilized by immersing in 2% sodium hypochlorite for approximately 2 min. These were rinsed twice in sterilized water and were placed on plates containing 1% water agar or V8 juice. After 3 days, fungal growth from the root tip was observed visually and microscopically.

Determination of mantle formation on pea seedlings grown in sand or vermiculite.

Plastic cone shaped vials were filled with sand or vermiculite and 2-day-old seedlings with a full set of border cells were inserted into a 2.5-cm deep indentation made in the sand. A spore suspension containing 10^6 spores was directly added to the indentation; controls included plants treated with water only. The seedlings were covered with sand or vermiculite and were covered lightly with aluminum paper to prevent exposure to light. The vials were incubated at room temperature for 4 days and were maintained by constant immersion in water to a depth of approximately 2 in. At the end of the test period, the sand containing the seedling was gently pushed from the vial and separated away from the root system by hand. The roots were placed in water and were observed under a dissecting microscope at $\times 60$ magnification (Zeiss, SV8). All experiments were repeated at least in triplicate, with at least 10 test plants for each treatment.

Determination of retardation of border cell separation.

Two-day-old seedlings with a root length of 25 mm were selected and were inserted into growth pouches containing 7 and 15 ml of water and then incubated at 24°C in the dark for 3 days. The seedlings were removed and border cells of the five seedlings in each pouch were washed into 1 ml of water. Border cells in 10 μ l of this suspension were counted. Four trials, each with two replicate pouches including five seedlings per pouch, were conducted, and the data are presented as the mean number of border cells per seedling.

To determine effects of altered water level, seedlings were inserted into growth pouches containing 7 ml water, and each day's growth was measured by marking the position of the root tip on the cellophane pouch. Values represent means from three independent trials with two replicate pouches containing five seedlings per pouch for each treatment.

Effect of retarded border cell separation on root-tip infection development.

Two-day-old seedlings with a full set of border cells were inserted into growth pouches containing 7 or 15 ml of water. Inoculations were then carried out as described. Incubation, infection scoring (discussed below), and root-length measurements were done as with other experiments. Values represent means from three independent trials with at least two replicate pouches containing five seedlings per pouch for each treatment.

Scoring system for severity of root-tip infection.

At the desired time period after initiation of an infection assay, scores of 0, 1, 2, and 3 were assigned to each of the inoculated and control seedlings after evaluating the condition of the root tip at the appropriate times. No visible tip infection was scored 0, a slight browning at the root tip was scored 1, a light brown lesion at the root tip was scored 2, and a dark brown/black lesion at the root tip was scored 3 (Fig. 2A).

RNA gel blot analysis.

Quantification of total RNA and RNA gel blot analysis were done essentially as described by Sambrook and associates (1989) using Magnacharge (MSI) membrane. Appropriate probes (listed below) were labeled with 32 P by the random primer method (Feinberg and Vogelstein 1983) using the Prime It II kit from Stratagene (La Jolla, CA, U.S.A.). Hybridization was carried out at 42°C for heterologous probes and at 50°C for homologous probes using the Ultra Hyb hybridization buffer obtained from Ambion (Austin, TX, U.S.A.).

Density quantification of the signals obtained from RNA gel blot analysis was done using the NIH image software program (Bethesda, MD, U.S.A.). Relative level means the ratio of the density values obtained for the signal of interest and those of the actin signal for the same evaluated RNA.

Preparation of RNA.

Radicles of 2-day-old seedlings with full sets of border cells were inserted into growth pouches and were uniformly inoculated with the desired spore concentration of *N. haematococca*, as described above. Control seedlings were set up as noted above. At specific time periods after inoculation, the root tips of inoculated and control seedlings were cut, immediately frozen, and stored at -80°C. Isolation of total RNA was carried out using the Trizol method (Gibco BRL, Bethesda, MD, U.S.A.) with slight modification by including an overnight LiCl precipitation to remove contaminating DNA from the RNA samples (Sambrook et al. 1989). Northern blot analysis for all treatments was replicated at least twice.

Elicitor preparation.

Elicitor was prepared according to the method of Hadwiger and Beckman (1980) with slight modifications (L. Hadwiger, *personal communication*). Chitosan (Sigma, St. Louis) was dissolved in water and cleaved by exposure to glacial acetic acid for more than 12 h. Then, the fragments were neutralized by adjusting the pH to 6.0. The resulting mixture (15 ml per pouch; 2.5 mg of chitosan per ml) was added to growth pouches containing five seedlings each. Control seedlings were inserted into growth pouches containing water that was treated similarly to the chitosan solution but without the addition of the chitosan. At appropriate times after exposure, root tips of the chitosan-treated and control seedlings were isolated and immediately frozen and stored at -80°C until ready for use.

Probes used for this study.

A 300-bp *EcoRI/SpyI* fragment of the cDNA of a pea root cap pectinmethylesterase (*RcPME1*) gene was used as the probe (Wen et al. 1999). Bean phenylalanine ammonia lyase (PAL1), alfalfa chalcone synthase (CHS1), and glucanase cDNA clones were obtained from the Samuel Roberts Noble Foundation, Inc. (Ardmore, OK, U.S.A.). A genomic clone of the pea chitinase gene was obtained from L. Hadwiger at Washington State University, Pullman, WA, U.S.A. A cDNA clone of HMM1(hydroxymethyltransferase) was supplied by H. VanEtten at the University of Arizona (Wu et al. 1997). A 600-bp cDNA fragment of *Arabidopsis thaliana* actin gene was obtained from H. Shi, Department of Plant Sciences, University of Arizona.

LITERATURE CITED

- Bauer, W. D. 1981. Infection of legumes by rhizobia. *Annu. Rev. Plant Physiol.* 32:407-449.
- Baluska, F., Volkmann, D., and Barlow, P. W. 1996a. Specialized zones of development in roots: View from the cellular level. *Plant Physiol.* 112:3-4.
- Baluska, F., Volkmann, D., Hauskrecht, M., and Barlow, P. W. 1996b. Root cap mucilage and extracellular calcium as modulators of cellular growth in postmitotic growth zones of the maize root apex. *Bot. Acta* 109:25-34.
- Baluska, F., Volkmann, D., and Barlow, P. W. 2001. A polarity crossroad in the transition growth zone of maize root apices: Cytoskeletal and developmental implications. *J. Plant Growth Regul.* 20:170-181.
- Barlow, P. W., and Baluska, F. 2000. Cytoskeletal perspectives on root growth and morphogenesis. *Annu. Rev. Plant Physiol.* 51:289-322.
- Bengough, A. G., and McKenzie, B. M. 1997. Sloughing of root cap cells decreases the frictional resistance to maize (*Zea mays* L.) root growth. *J. Exp. Bot.* 48:885-893.
- Brigham, L. A., Woo, H. H., Wen, F., and Hawes, M. C. 1998. Meristem-specific suppression of mitosis and a global switch in gene expression in the root cap of pea (*Pisum sativum* L.) by endogenous signals. *Plant Physiol.* 118:1223-1231.
- Bruehl, G. W. 1986. *Soilborne Plant Pathogens*. Macmillan Publishing, New York.
- Chang, M., Hadwiger, L. A., Horowitz, D. 1992. Molecular characterization of a pea beta-1,3-glucanase induced by *Fusarium solani* and chitosan challenge. *Plant Mol. Biol.* 20:609-618.
- Chang, M., Horowitz, D., Culley, D., and Hadwiger, L. 1995. Molecular cloning and characterization of a pea chitinase gene expression in response to wounding, fungal infection and the elicitor chitosan. *Plant Mol. Biol.* 28:105-111.
- Cunningham, J. L., and Hagedorn, D. J. 1962. Attraction of *Aphanomyces euteiches* zoospores to pea and other plant roots. *Phytopathology* 52:616-618.
- Curl, E. A., and Truelove, B. 1986. *The Rhizosphere*. Springer-Verlag, Berlin.
- Delserone, L. M., McCluskey, K., Matthews, D. E., and VanEtten, H. D. 1999. Pisatin demethylation by fungal pathogens and nonpathogens of pea: Association with pisatin tolerance and virulence. *Physiol. Mol. Plant Pathol.* 55:317-326.
- Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
- Feldman, L. J. 1984. Regulation of root development. *Ann. Rev. Plant Physiol.* 35:223-42.
- Foster, R. C., Rovira, A. D., and Cock, T. W. 1983. Ultrastructure of the root-soil interface. American Phytopathological Society, St. Paul, MN, U.S.A.
- Freshour, G., Clay, R. P., Fuller, M. S., Albersheim, P., Darvill, A. G., Hahn, M. G. 1996. Developmental and tissue-specific structural alterations of the cell wall polysaccharides of *Arabidopsis thaliana* roots. *Plant Physiol.* 110:1413-1429.
- Fusconi, A. 1983. The development of the fungal sheath on *Cistus incanus* short roots. *Can. J. Bot.* 61:2546-2553.
- Goldberg, N. P., Hawes, M. C., and Stanghellini, M. E. 1989. Specific attraction to and infection of cotton root cap cells by zoospores of *Pythium dissotocum*. *Can. J. Bot.* 67:1760-1767.
- Griffin, G. J., Hale, M. G., and Shay, F. J. 1976. Nature and quantity of sloughed organic matter produced by roots of axenic peanut plants. *Soil Biol. Biochem.* 8:29-32.
- Hadwiger, L. A., and Beckman, L. 1980. Chitosan as a component of pea-*Fusarium solani* interactions. *Plant Physiol.* 66:205-211.
- Hawes, M. C., and Brigham, L. A. 1992. Impact of root border cells on microbial populations in the rhizosphere. *Adv. Plant Pathol.* 8:119-148.
- Hawes, M. C., Brigham, L. A., Woo, H. H., Zhu, Y., and Wen, F. 1997. Root border cells: Phenomenology of signal exchange. Pages 210-218 in: *Advances and Perspectives on the Function of Plant Roots*. H. E. Flores, ed. American Society of Plant Physiology, Rockville, MD, U.S.A.
- Hawes, M. C., Brigham, L. A., Wen, F., Woo, H. H., and Zhu, Y. 1998. Function of root border cells in plant health: Pioneers in the rhizosphere. *Ann. Rev. Phytopathol.* 36:311-327.
- Hawes, M. C., Gunawardena, U., Miyasaka, S., and Zhao, X. 2000. Role of root border cells in plant defense. *Trends Plant Sci.* 5:128-133.
- Hawes, M. C., and Lin, H.-J. 1990. Correlation of pectolytic enzyme activity with the programmed release of cells from root caps of pea (*Pisum sativum*). *Plant Physiol.* 94:1855-1859.
- Hawes, M. C., and Pueppke, S. G. 1986. Sloughed peripheral root cap cells: Yield from different species and callus formation from single cells. *Am. J. Bot.* 73:1466-1473.
- Hickman, C. J., and Ho, H. H. 1966. Behavior of zoospores in plant-pathogenic Phycomycetes. *Ann. Rev. Phytopathol.* 4:195-220.
- Ho, H. H. 1969. Notes on the behavior of *Phytophthora megasperma* var *sojae* in soil. *Mycologia* 61:835-838.
- Horan, D. P., Chilvers, G. A., and Lapeyrie, F. F. 1988. Time sequence of the infection process in eucalypt ectomycorrhizas. *New Phytol.* 109:451-458.
- Huisman, O. C. 1982. Interrelations of root growth dynamics to epidemiology of root-invading fungi. *Ann. Rev. Phytopathol.* 20:303-327.
- Knox, J. P., Linstead, P. J., King, J., Cooper, C., and Roberts, K. 1990. Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. *Planta* 181:512-521.
- Knudson, L. 1919. Viability of detached root cap cells. *Am. J. Bot.* 6:309-310.
- Kroes, G. M. L. W., Baayen, R. P., and Lange, W. 1998. Histology of root rot of flax seedlings infected by *Fusarium oxysporum* f. sp. *lini*. *Eur. J. Plant Pathol.* 104:725-736.
- McDougall, B. M., and Rovira, A. D. 1970. Sites of exudation of ^{14}C -labeled compounds from wheat roots. *New Phytol.* 69:999-1003.
- Nelson, E. B. 1991. Exudate molecules initiating fungal responses to seeds and roots. Pages 197-209 in *The Rhizosphere and Plant Growth*. D. L. Keister and P. B. Cregan, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Parkinson, D., Taylor, G. S., and Pearson, R. 1963. Studies on fungi in the root region. I. The development of fungi on young roots. *Plant Soil* 19:332-349.
- Pearson, R., and Parkinson, D. 1961. The sites of excretion of ninhydrin-positive substances by broad bean seedlings. *Plant Soil* 13:391-396.
- Rodriguez-Galvez, E., and Mengden, K. 1995. The infection process of *Fusarium oxysporum* in cotton root tips. *Protoplasma* 189:61-72.
- Rovira, A. D., and Campbell, R. 1974. Scanning electron microscopy of microorganisms on roots of wheat. *Microbial Ecol* 1:15-23.
- Royle, D. J., and Hickman, C. J. 1963. Analysis of factors governing in vitro accumulation of zoospores of *Pythium aphanidermatum* on roots. *Can. J. Bot.* 10:151-162.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY, U.S.A.
- Schroth, M. N., and Snyder, W. C. 1960. Effect of host exudates on chlamydospore germination of bean root rot fungus, *Fusarium solani* f. *phaseoli*. *Phytopathology* 51:389.

- Stanghellini, M. E., and Hancock, J. G. 1971. Radial extent of the bean spermosphere and its relation to the behavior of *Pythium ultimum*. *Phytopathology*. 61:165-168.
- Stevens, R. B. 1974. Page 703 in: *Mycology Guidebook*. University of Washington Press, Seattle.
- Wen, F., Zhu, Y., Hawes, M. C. 1999. Expression of a pectinmethylesterase gene in pea root tips influences extracellular pH, cell morphology and border cell separation. *Plant Cell* 11:1129-1140.
- Wu, Q., Preisig, C. L., and VanEtten, H. D. 1997. Isolation of the cDNA encoding (+) 6a-hydroxymaackiain 3-O-methyltransferase, the terminal step for the synthesis of the phytoalexin pisatin in *Pisum sativum*. *Plant Mol. Biol.* 35:551-560.
- Zentmyer, G. A. 1961. Chemotaxis of zoospores for root exudates. *Science* 133:1595-1596.