Cutinase of *Nectria haematococca* (*Fusarium solani* f. sp. *pisi*) Is Not Required for Fungal Virulence or Organ Specificity on Pea

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A detailed quantitative and microscopic examination of the infection and colonization process of a wild-type strain of *Nectria haematococca* and cutinase-deficient mutants constructed by transformation-mediated gene disruption was performed. The assays were done under natural conditions. Quantitative analyses were performed by evaluating disease symptoms, measuring plant biomass development and ergosterol content of infected and non-infected plants at different times during the infection process. Qualitative histological observations showed that the wild-type as well as the cutinase-deficient mutants invaded the pea foot by direct penetration of the outer cell layers of epicotyl, hypocotyl, and taproot. After systemic colonization of the root and stem cortex by intra- and intercellular growth wild-type and mutants invaded the vascular system. The infection progressed vertically into the stem by fungal growth into the xylem. The infection of aerial plant parts was restricted to the xylem. This was shown with β-glucuronidase expressing transformants which were stained selectively in the host tissue. Infection of intact or wounded leaves by wild-type or cutinase-deficient mutants was inhibited by a hypersensitive reaction of leaf cells. After having shown recently that cutinase is not required for pathogenicity on pea (Stahl and Schäfer, Plant Cell 4:621-629, 1992), we have now found no evidence that cutinase is a virulence factor of *Nectria haematococca*.

Additional keywords: ergosterol, foot rot, fungus-plant interactions, organ specificity, *Pisum sativum*.

Fungal pathogens can infect their hosts by entering natural openings and wounds or by direct penetration of the outer cell layer. Infecting the outer cell layer of the aerial plant parts, the epidermis, pathogens encounter the cuticle. Two mechanism have been proposed to explain the direct penetration process (Köller 1991). Regarding the mechanical hypothesis, the cuticle can be breached by the physical force of the growing hyphal tip or the cuticle can be weakened or disintegrated enzymatically. The combination of mechanical and enzymatic mechanisms has also been discussed (Kolattukudy 1985).

In the case of the pathogens *Magnaporthe grisea, Colletotrichum lindemuthianum,* and *Colletotrichum lagenarium* the synthesis of 1.8-dihydroxy naphthalin (DHN) melanin in the appressoria is a prerequisite for infection (Valent and Chumley 1990). Melanin is used for the buildup of a hydrostatic pressure in the appressoria, necessary for mechanical penetration (Howard and Ferrari 1989). Genetic analysis of melanin-deficient mutants of *M. grisea* revealed that the mutation of three different genes of the DHN pathway resulted in a loss of pathogenicity (Valent and Chumley 1990). Kubo *et al.* (1991) transformed an apathogenic mutant of *C. lagenarium* lacking a defined gene of the DHN biosynthesis with the library of a wild-type isolate. Transformants with restored DHN biosynthesis regained their pathogenicity.

Cutinolytic activity was first described for the saprophytic fungus *Penicillium spinulosum* (Heinen 1960), and it focused interest on cutinases. Detailed biochemical and molecular biological investigations were performed for the cutinase of the ascomycete *Nectria haematococca* MP VI (*Fusarium solani* f. sp. *pisi),* the causal agent of foot rot of pea. A crucial role of cutinase in infection of plants was indicated by these investigations (Maiti and Kolattukudy 1979; Woloshuk and Kolattukudy 1986; Podila *et al.* 1988; Dickman *et al.* 1989). To evaluate the role of cutinase for the infection process of *N. haematococca* on its natural host, we constructed four cutinase-deficient mutants of a highly virulent strain by transformation-mediated gene disruption (Stahl and Schäfer 1992). The absence of a functional cutinase gene in the mutants was confirmed by gel blot analysis and enzyme assays. Bioassays of the null mutants and the wild-type showed no difference in the pathogenicity of the fungal strains. We have concluded that the cutinase is not essential for the pathogenicity of *N. haematococca.*

To test the possibility that cutinase is involved in virulence of *N. haematococca* to pea we performed a quantitative and microscopical analysis of the infection process. First, we determined macroscopically and biochemically the early infection sites of the foot rot disease. We used the fungal lipid ergosterol to assess the first events of plant-fungus interactions. The determination of ergosterol is thought to give a quantitative measure of pathogen within the plant tissue (Miller *et al.* 1983; Newton 1989) and has been used for disease assessment of pea lines with resistance to foot rot pathogens (Gretenkort and Helsper 1993). Second, virulence of

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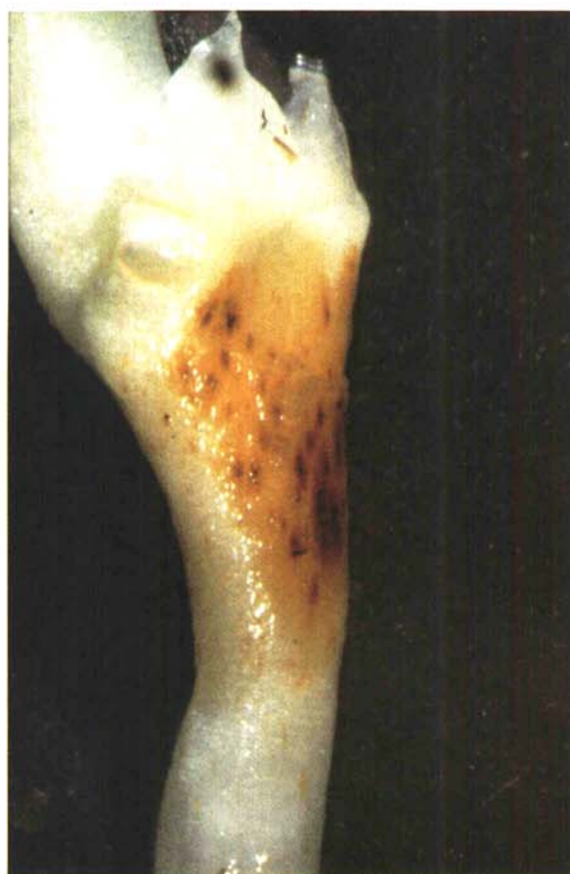
wild-type and cutinase-deficient mutants was assessed semi-quantitatively, by means of disease score, as well as quantitatively by measurement of plant biomass development and ergosterol content of infected plants. Third, the early infection stages were analyzed for microscopical differences and then the total colonization process was investigated by light microscopy.

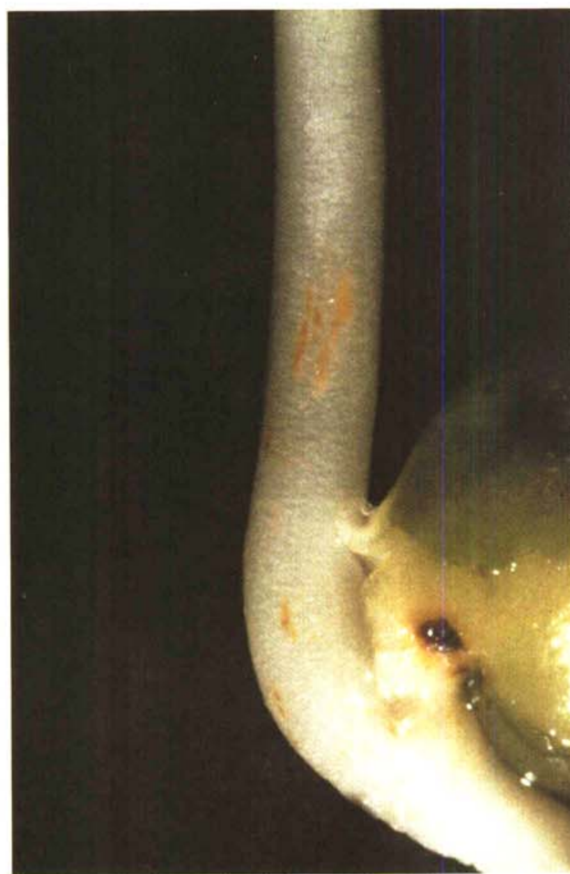
RESULTS

Macroscopic determination of the early infection sites.

Different bioassays were performed to evaluate the pathogenicity of cutinase-deficient mutants (Stahl and Schäfer 1992). The pot assay (VanEtten 1978) was chosen to access virulence. This test system allows the investigation of the infection process under environmental conditions similar to those found in the infested pea field. Surface-sterilized pea seeds were planted in sterile soil and infected with conidia from wild-type and cutinase-deficient mutants. Concentrations of conidia in the soil were adjusted to the level (1,000 conidia per gram of soil) detected in infected pea fields (Kraft and Roberts 1969). To identify the initial centers of fungal attack and the progress of infection, plants were removed 2, 4, 7, 14, and 21 days after infection from the soil, cleaned, and analyzed.

Macroscopic visible symptoms of fungal infection of the seedlings were first observed on the seed coat 2–4 days after infection. *N. haematococca* colonized the seed building a mass of mycelia on the coat. The pea seed is the first point of attack on developing seedlings. From this earliest point of infection, *N. haematococca* colonized the hypocotyl. Four-day-old seedlings showing no obvious symptoms of infection on the taproot or stem, revealed brown lesions on the hypocotyl after removal of the seed (Fig. 1A). After 7 days, brown lesions have always been observed on the underground part of the epicotyl (Fig. 1B), the upper part of the taproot, and the origin of the lateral roots. While the lesions on the epicotyl, the hypocotyl, and the upper taproot quickly increased in size and finally covered the total pea foot, the infection sites around the lateral roots remained small. After 14 days, typical foot rot symptoms appeared for wild-type and cutinase-deficient mutants of *N. haematococca* (Stahl and Schäfer 1992). The underground part of the epicotyl, the hypocotyl, and the upper part of the taproot turned intensively brown because of fungal infection. The lower part of the taproot and the lateral roots showed only minor traces of fungal infection. The observation that the foot region is infected first and most severely is in accordance with earlier reports concerning the infection behaviour of *N. haematococca* (Bywater 1959). During the early infection phase, the cutinase-deficient mu-





A B

Fig. 1. Early infection sites of pea by a cutinase-deficient *Nectria haematococca* strain. Pea seeds had been planted in soil infested with conidia of strain 77-102. **A,** Brown lesions on the hypocotyl of a pea seedling 4 days after planting in the infested soil. The pea seed has been removed for the detection of the infection sites. **B,** Distinct brown lesions of the epicotyl seven days after infection. A part of the seed is visible at right.

tants caused lesions on the seed coat, the taproot, the hypocotyl, and the underground part of the epicotyl. The lesions of the mutants differed neither in size, color, nor in number from the lesions of the wild-type. The appearance of small brown lesions on the underground part of the epicotyl indicated that this cuticle protected plant organ is colonized equally well by wild-type and cutinase-deficient mutants. The brown discoloration of the epicotyl was visible 1–2 cm above the ground where it stopped at a sharp zone, while the rest of the pea stem showed no visible infection symptoms.

Biochemical determination of the early infection sites.

Beside visual observation we measured the ergosterol concentration of different parts of the inoculated plants to determine the early infection sites of *N. haematococca* wild-type and cutinase-deficient mutants 77-102 and 77-75. Gretenkort and Helsper (1993) described a linear correlation of fungal dry mass of *N. haematococca* with amount of ergosterol extracted from the mycelium of a liquid culture during the initial 28 days of incubation. We compared the ergosterol content of wild-type strain and cutinase-deficient mutants 77-75 and 77-102 grown in liquid culture for 1, 2, 4, and 7 days. There was no difference in the specific ergosterol content detectable (data not shown).

To determine which plant organ becomes infected first and most severely, the concentration of the fungal sterol in various plant parts was assessed 4 days after the seeds were planted in infested soil. The ergosterol content of seeds 4 days after infection was in the range of 119–174.6 ng/mg dry weight as shown in Figure 2. A minor, but for all plants very similar, amount of ergosterol (23.9–28.3 ng/mg plant biomass) could be detected in the taproots and hypocotyls infected by the different fungal strains. The ergosterol concentrations of the epicotyls (0.25–2.3 ng/mg plant biomass) were equal or only little above the background level of the uninfected control (0.25 ng/mg plant biomass). However, the slightly higher ergosterol concentrations in the epicotyl could be detected for the cutinase-deficient mutants 77-102 and 77-75 compared to the wild-type.

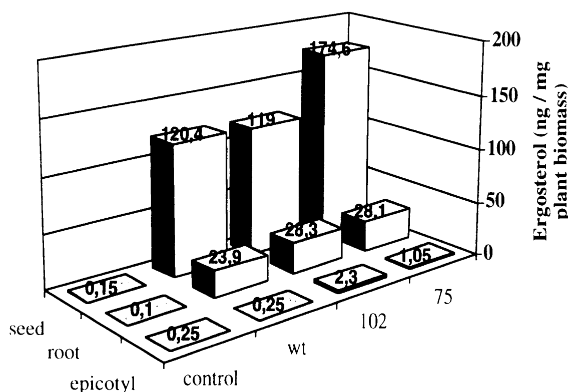


Fig. 2. Biochemical determination of the early infection sites. Ergosterol content of seeds, hypocotyls with upper part of the taproots, and epicotyls of peas was determined. Seeds were sown and grown in with *Nectria haematococca* wild-type and the cutinase-deficient mutants 77-75 and 77-102 infested soil for 4 days. Control plants were grown without fungal inoculum. Ergosterol concentration was calculated per milligram dry plant material. In two independent experiments 10 or 15 plants, respectively, were infected with each of the three fungal strains.

The measurement of ergosterol confirmed the results of the visual analysis. The underground parts of the plants are infected in the following order: seed, hypocotyl, upper part of the taproot, and epicotyl. The epicotyl is the part of the foot region which becomes infected last by *N. haematococca.*

Aboveground symptoms.

After infection of the foot region by wild-type and cutinase-deficient mutant, a wilting of the leaves was observed after 2–3 wk as a first aboveground symptom. Wilting began at the lower leaves and progressed vertically through all leaves to the flowers or pods. Until the wilting appeared, infected plants did not differ in their development from noninfected plants. Wilting was correlated with a reduced growth of the plants and finally with the death of the plants after 5–6 wk. While there was no visual evidence for fungal presence in the aboveground plant parts, cross sections revealed that the vascular system of wilted plants showed the brown discoloration typical for *N. haematococca* infected tissue. Besides these symptoms, damping off was observed in approximately 10% of seedlings 7–9 days after infection. Wilting occurred within the same period, and the numbers of damped off seedlings were comparable for wild-type and cutinase-deficient strains.

Virulence assessment by determination of plant biomass development.

To quantify the virulence of wild-type strain 77-2-3 and cutinase-deficient mutants 77-75 and 77-102 the plant biomass development was analyzed during the infection process. Pot assays were performed as described. Plants were removed after 4, 6, 9, and 13 days after inoculation, cleaned, and weighed. Four-day-old seedlings infected with the different *N. haematococca* strains showed no reduction of weight compared to the uninoculated control plants as shown in Figure 3. After 6

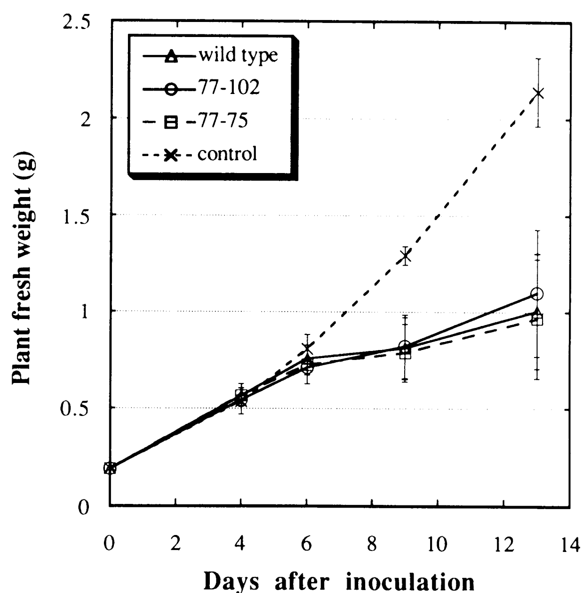


Fig. 3. Virulence assessment by determination of plant biomass development. Fresh weight of plants after growth in soil containing *Nectria haematococca* wild-type, cutinase-deficient mutants 77-75, 77-102 or no fungal inoculum (control) was determined. Bars indicate standard error of the mean.

days a first minor reduction of biomass of infected plants was observed. After 9 and 13 days the biomass of infected plants was dramatically reduced in comparison to the control. However, no difference of plant biomass development among the infected plants could be observed. The three fungal strains caused a similar reduction of plant weight at each stage of the experiment.

Virulence assessment by determination of disease scores and ergosterol content of infected roots and hypocotyls.

The upper part of the taproot and the hypocotyl of plants inoculated with the wild-type and the mutant strains 77-75 and 77-102 were scored for disease and analyzed for fungal biomass by the ergosterol assay 4, 6, 9, and 13 days after

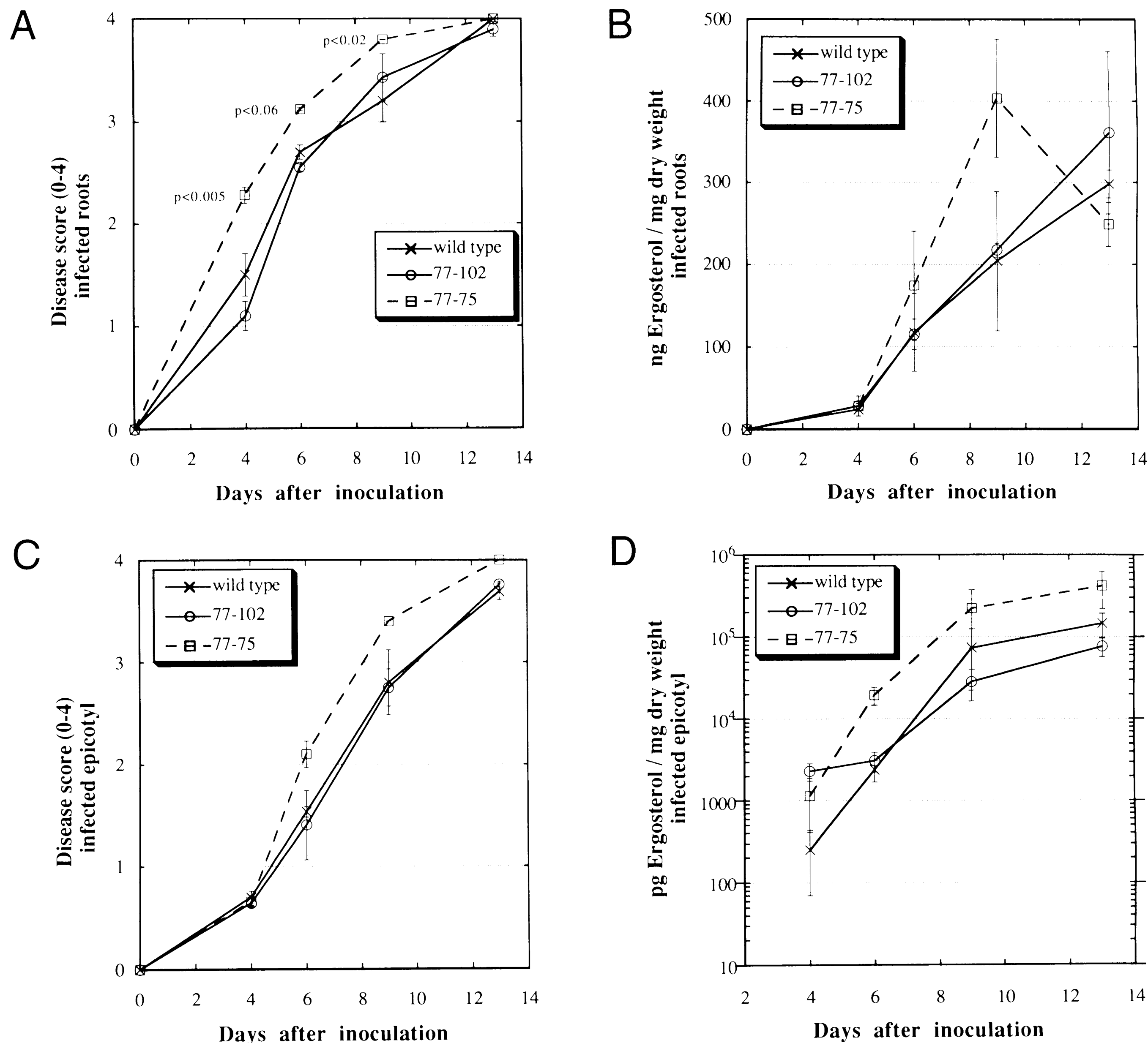


Fig. 4. Virulence assessment by disease scores and ergosterol assays. Disease scores and ergosterol contents of hypocotyls with upper part of the taproots and of epicotyls of peas inoculated with *Nectria haematococca* wild-type and the cutinase-deficent mutants 77-75 and 77-102, assessed at intervals throughout a 13-day period. Plants were first evaluated for disease scores and then the ergosterol assay was performed. **A,** Disease scores of hypocotyls and upper part of taproots. Plants were scored as follows: 0 = healthy plant; 1 = < 25% discoloration of root and hypocotyl; 2 = < 50% discoloration of root and hypocotyl; 3 = < 75% discoloration of root and hypocotyl; 4 = > 75% discoloration of root and hypocotyl. **B,** Ergosterol content of hypocotyl and upper part of the taproot. **C,** Disease score of the epicotyls. Plants were scored as follows: 0 = healthy epicotyl, 1 = < 7 lesions on the epicotyl; 2 = < 25 lesions on the epicotyl; 3 = > 25 lesions on the epicotyl or single lesion width > 1mm; 4 = lesion width > 2 mm. **D,** Ergosterol content of the epicotyls. Data represent two independent experiments with 10 or 15 plants used for each time point and each fungal strain. Bars indicate standard error of mean. Disease scores were analyzed for statistical significance. *P* values are given for the difference between wild-type and cutinase-deficient mutants. Incubation periods without *P* value indicate that there was no significant difference.

inoculation. The amount of ergosterol extracted from infected plant material increased linearly ($r = 0.966$) with the disease scores. It was not posssible to distinguish between the wild-type 77-2-3 and the mutant 77-102 on the basis of the disease scores (Fig. 4A) and ergosterol content (Fig. 4B). However, a difference between wild-type and mutant 77-75 was seen 4, 6, and 9 days after incubation. The cutinase-deficient mutant 77-75 showed higher disease scores (Fig. 4A) and ergosterol

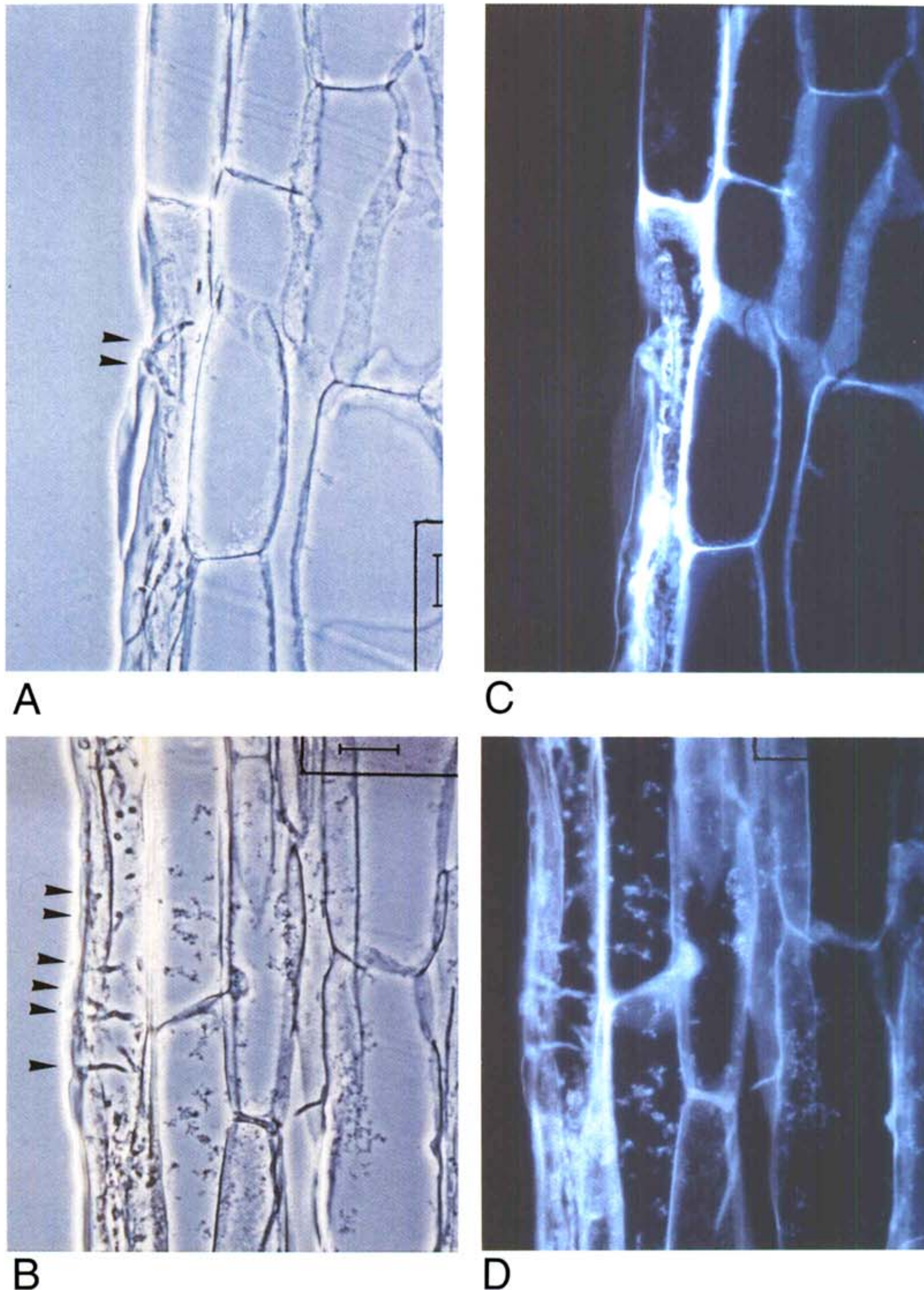


Fig. 5. Direct penetration of the epidermis by the cutinase-deficient mutant *Nectria haematococca* 77-102. Transverse sections of infected epicotyls were analyzed by light and fluorescence microscopy. **A,** Very early direct infection of an epidermal cell by two hyphae of the cutinase-deficient mutant 77-102. The penetrating hyphae are marked by arrowheads. Bar = 10 μm. **B,** Massive infection of several epidermal cells by hyphae of the cutinase-deficient mutant 77-102. The penetrating hyphae are marked by arrowheads. Bar = 25 μm. **C,** Fluorescence micrograph of the section shown in A. The cell wall of the infected epidermal cell shows a distinct autofluorescence. Bar = 10 μm. **D,** Fluorescence micrograph of the section shown in B. The cell walls of the infected epidermal cells shows a distinct autofluorescence. Bar = 25 μm.

contents (Fig. 4B) than the wild-type.

Virulence assessment by determination of disease scores and ergosterol content of infected epicotyls.

The extent of infection of epicotyls was assessed using the disease scores and the ergosterol assay at incubation periods from 4 to 13 days. The disease scoring scheme, again, did not allow differentiation between wild-type and mutant 77-102 (Fig. 4C). A significant difference between the wild-type and the mutant 77-75 was seen after 6, 9, and 13 days of incubation. The cutinase-deficient mutant was more virulent than the wild-type strain (Fig. 4C). The amount of ergosterol extracted from infected epicotyls increased exponentially with disease scores ($r = 0.961$). The dramatic increase of ergosterol in the epicotyls 9 and 13 days after incubation was caused by hyphae advancing vertically from the hypocotyl into the epicotyl. Therefore the direct penetration and colonization of the epicotyl can only be measured at the early stages of infection by the ergosterol assay. At these intervals (4 and 6 days) the ergosterol concentration of the mutants was equal to or higher than the sterol concentration determined for the wild-type (Fig. 4D). After 9 and 13 days of incubation, the mean of wild-type biomass was between the amounts measured for the mutants 77-102 and 77-75.

Microscopic analysis of fungal penetration and plant cellular responses of the foot region.

The mode of penetration of invading hyphae during the infection of the pea epicotyl was examined in thin serial sections of infected plants. The epidermis of the epicotyl was directly penetrated by the hyphae of the wild-type and the null-mutant 77-102. Without producing distinct appressoria but with swollen hyphal tips, the mutant 77-102 entered the epidermal cells (Fig. 5A and B). Single epidermal cells were often infected by several hyphae simultaneously. At this early stage of infection, no macroscopic symptoms were visible. The invasion of the epicotyl through stomata, as described by Bywater (1959), was also observed for wild-type and cutinase-deficient mutants. However, the infection through stomata was observed only occasionally and was of minor importance in comparison to the high number of direct penetration events. It is important to mention that no wounds were required by wild-type or cutinase-deficient mutant to infect the epidermis.

Fluorescence microscopy revealed that the invading hyphae of wild-type and null-mutants induced an autofluorescence of the attacked cell walls. The autofluorescence started at the site of the hyphal penetration and extended over the entire cell wall (Fig. 5C and D). Noninfected neighboring cells did not show this autofluorescence phenomenon (Fig. 5C). The observed autofluorescence and the brown discoloration of infected tissue pointed to a production of phenolic compounds by the infected pea cells.

Colonization of the foot region.

After penetration of the epidermis, wild-type and cutinase-deficient mutants colonized the cortical parenchyma of the epicotyl by inter- and intracellular growth. Hyphae advancing

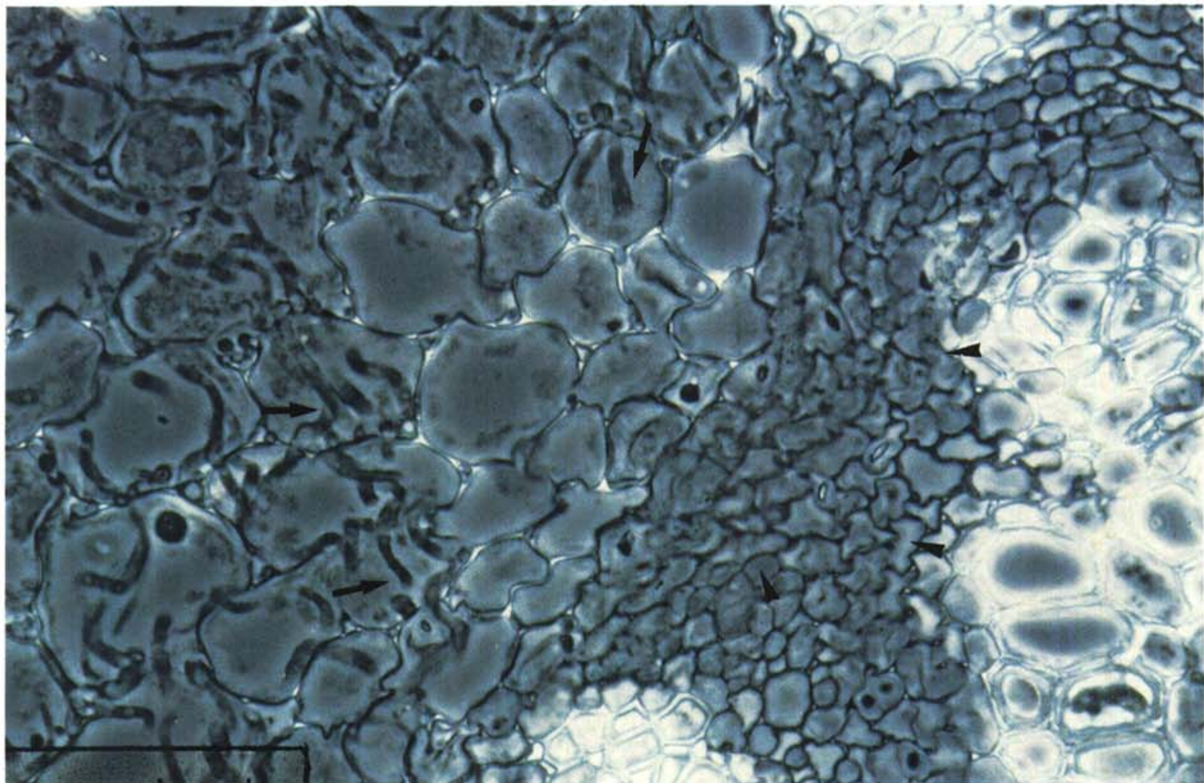


Fig. 6. Colonization of the epicotyl by *Nectria haematococca* cutinase-deficient mutant 77-102. Cross section of the epicotyl after infection by the cutinase-deficient mutant 77-102. Hyphae are growing radially through the cortical tissue to the stele. A disintegration of vascular parenchyma (black arrowheads) is caused in advance of the fungus. Fungal hyphae are still restricted to the cortical tissue (black arrows). Bar = 25 µm.

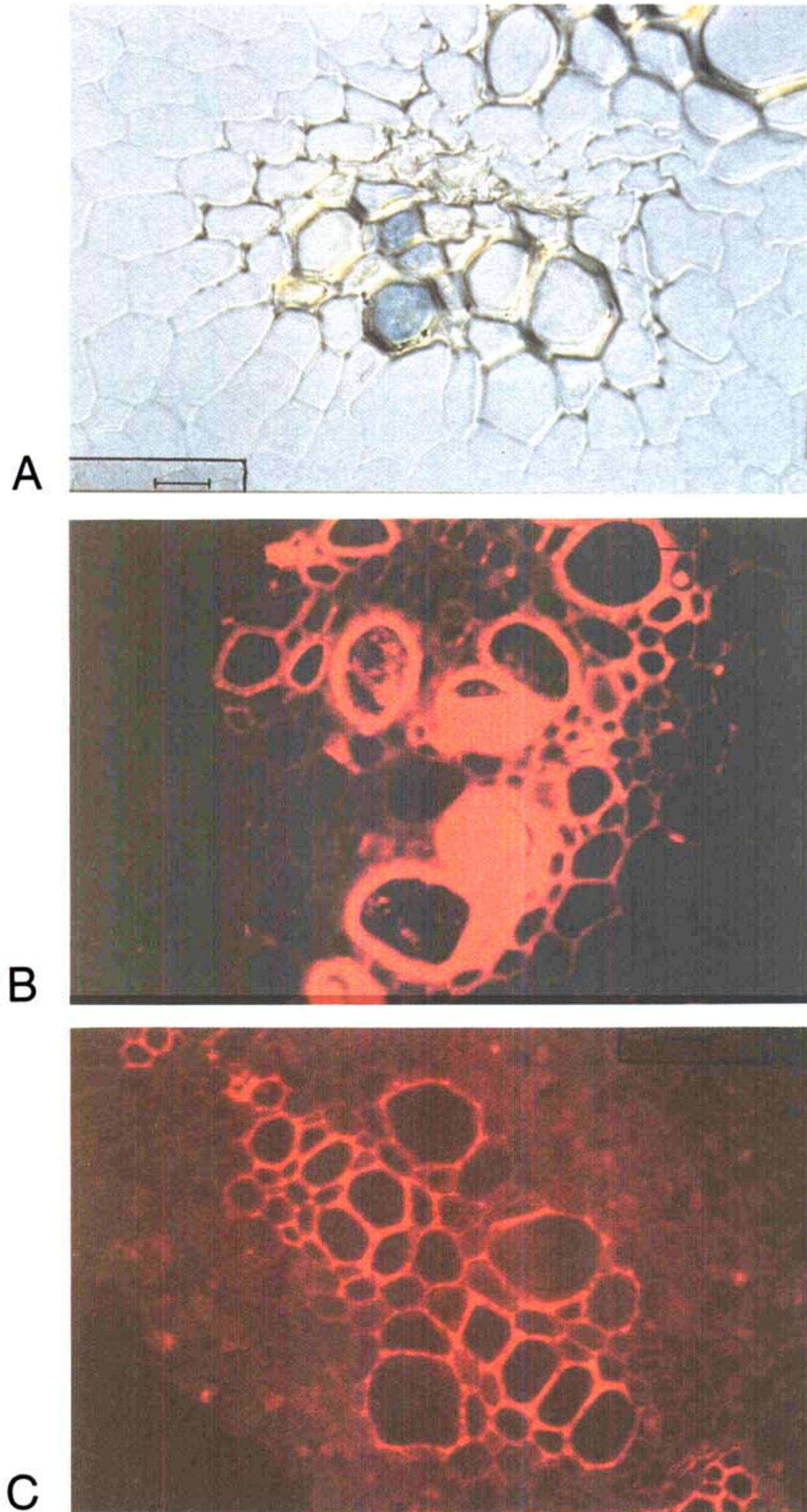


Fig. 7. Detection of a gus⁺ transformant of *Nectria haematococca* in infected pea stem tissue and vascular responses of pea stems infected by *N. haematococca*. **A,** Cross section of the third internode of a plant infected by gus⁺ transformant 77-104 after GUS staining. Fungal hyphae located inside xylem elements turn intensively blue due to β-glucuronidase activity. Bar = 10 μm. **B,** Fluorescence micrograph of a vascular bundle. The plant was infected by a cutinase-deficient mutant. An intensive autofluorescence of the infected xylem elements, the production of vessel coatings and plugs can be observed. **C,** Fluorescence micrograph of an uninfected plant.

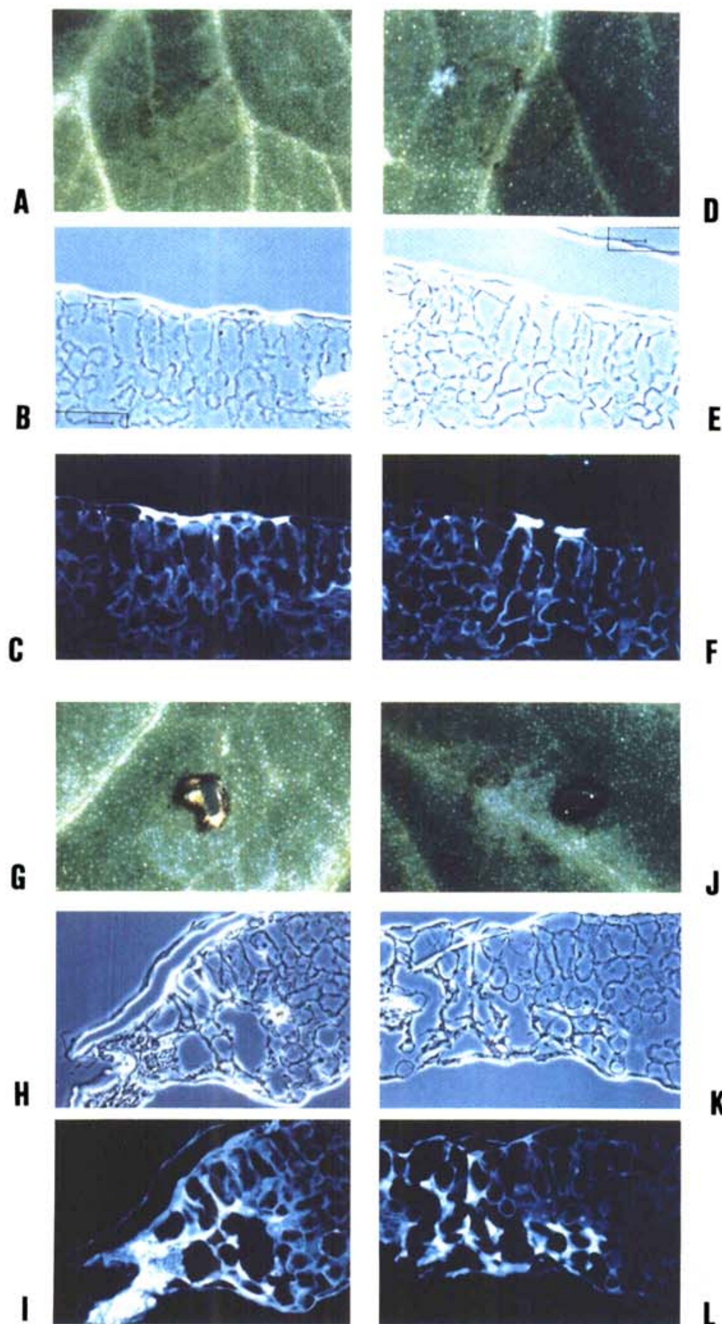


Fig 8. Hypersensitive reaction of intact and wounded pea leaves after infection with *Nectria haematococca* wild-type and cutinase-deficient mutant 77-102. **A,** Unwounded pea leaf 6 days after infection with wild-type isolate 77-2-3. The leaf has been inoculated with a droplet of a conidial suspension (5 $\times 10^4$ spores per 5 µl). A distinct brown spot of the leaf surface has been caused by multiple necrotic lesions. **B,** Transverse section of the unwounded pea leaf infected by wild-type. A group of epidermal cells were collapsed after fungal contact. Bar = 25 µm. **C,** Fluorescence micrograph of the section shown in B. The collapsed cells show a distinct autofluorescence. **D,** Unwounded pea leaf 6 days after infection with the cutinase-deficient mutant 77-102. The leaf has been inoculated with a droplet of a conidial suspension (5 $\times 10^4$ spores per 5 µl). Small necrotic lesions are visible. **E,** Transverse section of the unwounded pea leaf infected by the null mutant. Hypersensitive reaction of the guard cells of a stomata after fungal attack. Bar = 25 µm. **F,** Fluorescence micrograph of the section shown in E. The guard cells are completely filled with fluorescent material. **G,** Symptoms on a wounded pea leaf 6 days after infection by wild-type isolate 77-2-3. The leaf was inoculated with a droplet of a conidial suspension (5 $\times 10^4$ spores per 5 µl). A small necrotic zone has formed around the hole caused by the needle prick. **H,** Transverse section of a wounded pea leaf infected by the wild-type. The transition from infected disintegrated tissue to intact noninfected tissue is shown. **I,** Fluorescence micrograph of the section shown in H. The debris of the disintegrated cells shows a strong autofluorescence. **J,** Symptoms of a wounded pea leaf 6 days after infection by the cutinase-deficient mutant 77-102. The leaf was inoculated with a droplet of a conidial suspension (5 $\times 10^4$ spores per 5 µl). A small necrotic zone has formed around the hole. **K,** Transverse section of the pea leaf infected by the cutinase-deficient mutant. Fungal hyphae are visible between collapsed parenchymatous cells. Vesicles are secreted by a parenchymatous cell in response to progressing hyphae. **L,** Fluorescence micrograph of the section shown in K. Note the strong autofluorescence of the disintegrated cells.

vertically were restricted to the intercellular spaces of the cortex. This observation indicated that the elliptical shape of the lesions was caused primarily by the rapid invasion of the intercellular spaces. After filling the intercellular spaces, the mycelium invaded parenchymatous cells. The enlargement of the lesions by intercellular hyphal growth was correlated with a horizontal extension of the infection sites. Sometimes without apparent reason the mycelium of wild-type or cutinase-deficient mutants grew radially to the stele as shown for the cutinase-deficient mutant 77-102 (Fig. 6). The radial advancing hyphae penetrated the cell walls of parenchymatous cells directly without any morphological change of the hyphal tips. Advancing to the stele, the mycelium reached the endodermis with its well-defined Casparian thickenings. The endodermis stopped slightly the progression of the infection and caused *N. haematococca* to grow into the uninfected cortical tissue. However, the presence of mycelium outside the stele caused a disintegration of the vascular parenchyma (Fig. 6) which resulted finally in a colonization of the vascular system. The vertical progression of the stem infection stopped abruptly in the upper epicotyl as described. Microscopical analysis did not indicate any morphological defense reaction to explain this phenomenon. Hyphae of wild-type and cutinase-deficient mutants accumulated inter- and intracellularly in the cortex of this stem region but did not grow any further.

The colonization of the hypocotyl and the upper part of the taproot by wild-type and cutinase-deficient mutants was similar to the colonization of the epicotyl. The outer cell layer was invaded intra- and intercellularly. After penetration of the epidermal cells these could be filled completely by mycelium. The root cortex was colonized by intercellular vertical growth of the hyphae followed later by an intracellular invasion of the parenchymatous cells. These cortical regions often exhibited a complete disintegration of cell walls. Especially in the hypocotyl, a radial, intracellular growth was observed occasionally as described for the epicotyl. In this case no tissue disintegration was observed. The infection of hypocotyl and taproot progressed until the hyphae reached and entered the stele.

Expression of the *gus*A gene in *N. haematococca*.

The browning of the vascular bundle in stems of infected pea plants suggested that *N. haematococca* wild-type as well as the cutinase-deficient mutants were limited to these cells. To rule out that the fungi were not noticed in the other stem tissues we used the *gus*A gene as a reporter gene system for fungal detection in the infected tissue.

The transformation vector pG1 was constructed for expression of the *gus*A gene in *N. haematococca*. Regulatory elements of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and tryptophan synthase (*trp*C) genes of *Aspergillus nidulans* (Punt *et al.* 1987) have been used successfully for transformation mediated gene-disruption in *N. haematococca* (Stahl and Schäfer 1992). Therefore, we used the same elements for expression of the *gus*A gene. Strain 77-2-3 was transformed with plasmid pG1 and hygromycin B-resistant transformants were isolated. Expression of the β-glucuronidase gene was measured in mycelial extracts of transformants as hydrolysis of 4-methylumbelliferyl-β-D-glucuronide (MUG). Five of eight analyzed transformants showed a 29- to 180-fold increase of GUS activity in comparison to the wild-type (data not shown). For three transformants no significant GUS activity could be observed.

For the histochemical detection of gus$^+$ transformants in infected tissue 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) was used as a substrate. As a prerequisite for this purpose, the ability of intact hyphae of transformants to convert X-Gluc into the blue indigo dye on agar plates was successfully tested.

***N. haematococca* colonizes exclusively the xylem cells of pea stems.**

After development of the *gus*A gene as a reporter gene system for *N. haematococca,* the colonization of the pea stem was investigated. Peas were infected in the pot assay by gus$^+$ transformant 77-104. This transformant had shown the highest GUS activity. The symptoms developed after infection with transformant 77-104 were indistinguishable from symptoms caused by wild-type or cutinase-deficient mutants. Stems of plants wilting 18 days after infection were cut into segments and incubated overnight in the GUS staining solution. After embedding in plastic material, thin cross sections were analyzed by light microscopy. A blue discoloration was observed by hyphaelike structures in the xylem cells of the vessel system (Fig. 7A). No blue could be observed outside the infected xylem cells in the other stem tissues. A control experiment was performed to rule out that hyphae in the xylem cells are easier stainable and therefore detectable as mycelium in the parenchyma. Stems were wounded by a needle and infected with an agar plug (VanEtten *et al.* 1980) bearing mycelium of gus$^+$ transformant 77-104. Four days after infection sections of the infected stems were stained for GUS activity as described. The lesions caused by gus$^+$ transformant 77-104 stained intensively blue. Cross sections of these segments revealed the presence of blue-colored hyphae in the intercellular spaces of the parenchyma (data not shown). These results demonstrated that a gus$^+$ transformant of *N. haematococca* could be histochemically detected in the stem tissue outside the vessel system. Cross sections of stems revealed the presence of fungal hyphae in the xylem of the first five analyzed internodes. Transverse sections confirmed the growth of *N. haematococca* in the vascular system of the stem (data not shown). Outside the xylem, fungal structures were not visible in the internodes above the epicotyl. The fungal presence in the xylem caused morphological changes of the infected cells. Vessel wall coating and plugs could be observed in the infected xylem cells (Fig. 7B). Plugs appeared as amorphous material that filled the vessels. Noninfected control plants showed no vascular symptoms (Fig. 7C). To answer the question whether the vascular coating is caused by the fungal presence or by the wilting process noninfected water stressed plants were analyzed. Despite intensive wilting, no vascular changes could be observed for these plants by light microscopy (data not shown). These results indicated that the vascular changes are induced by the plant-fungus interaction. Transverse sections of infected plants exhibited that the vessel coating started some cells ahead of the growing hyphae (data not shown). Again, no differences between the symptoms caused from *N. haematococca* wild-type or cutinase-deficient mutants were observable.

Organ specificity-infection of pea leaves is inhibited by a hypersensitive response.
The aboveground symptoms of *N. haematococca* infection in the pot assay consisted of wilting and colonization of the vessel system. To assess the virulence of wild-type and null mutants on the cuticle-protected plant organs a new bioassay was performed. Leaves of 13-days-old pea plants were infected with droplets of conidial suspensions from wild-type and the cutinase-deficient mutant 77-102. The concentration of conidia was 5×10^3, 5×10^4, and 5×10^5 per 5 µl water, respectively. The inoculum was placed carefully on the upper surface of intact leaves.

Twelve hours after infection the water of the droplets had evaporated or was absorbed. After 2–3 days, wild-type and the cutinase-deficient mutant had caused many very small brown necrotic lesions at the infection sites on the unwounded leaves (Fig. 8A and D). A positive correlation between the number of lesions and conidia concentration was observed. During the following 4–11 days no further change of the size or color of the lesions appeared. The lesions did not extend beyond the inoculation sites. The infection had been stopped. The microscopic analysis of the infected area revealed that with the exception of some epidermal cells, the leaf tissue was intact and uninfected. Some stomata cells and single or small groups of epidermal cells were filled with refracting material and were collapsed (Fig. 8B, E). Fluorescence microscopy showed a distinct induced fluorescence which filled these cells completely (Fig. 8C, F). Leaves inoculated with water showed neither the appearance of necrotic lesions nor damage to the epidermal cells (data not shown). To explore the role of an intact epidermis for the infection behavior, experiments were repeated with needle pricked leaves. Leaves were infected as described above. A small brown necrotic area arose around the infection site after 2–3 days (Fig. 8G, J). The size of the lesions was independent from the conidia concentration and did not increase during continuous incubation. The microscopic analysis revealed the presence of hyphae in the lesions (Fig. 8H, K). The parenchymatous cells surrounding the hyphae were collapsed and showed a strong induced fluorescence (Fig. 8I, L). A specific morphological change of the parenchymatous cells was observed at the border between infected and uninfected tissue. Cells were reducing the contacts to the neighboring cells and were secreting small fluorescent vesicles (Fig. 8L). The colonization of wounded leaves by wild-type and cutinase-deficient mutant is inhibited like the penetration of intact leaves by a hypersensitive reaction. No difference in the plant response to the wild-type isolate and the cutinase-deficient mutant could be observed.

DISCUSSION

N. haematococca is a soilborne fungal pathogen of pea. In the field it attacks the upper root and lower stem of the plant, where it causes foot rot. Cutinase is an enzyme that can degrade the plant cuticle and was thought to be necessary for pathogenicity of *N. haematococca*.

Several experiments suggested the essential role of cutinase during the penetration step of *N. haematococca*. First, cutinase was shown to be at the site of penetration (Shaykh *et al.* 1977). Second, a mutant with a greatly reduced cutinase activity was less pathogenic to unwounded pea stems. This was partly reversed by the addition of exogenous cutinase to the inoculum (Dantzig *et al.* 1986). Third, antibodies against cutinase in the inoculum impaired the infection of intact but not of wounded sectioned pea stems (Maiti and Kolattukudy 1979). Fourth, chemical inhibition of the enzyme prevented infection of sectioned pea stems, though the growth rate of the fungus was not affected (Köller *et al.* 1982a, 1982b).

As the cutinase gene was cloned and shown to be inducible by cutin monomers, the following model was developed: *N. haematococca* spores secrete low levels of cutinase. This low enzyme activity is sufficient to generate cutin monomers which in turn drastically induce the cutinase gene, thus enabling the fungus to invade the host plant. In contrast to this hypothesis, cutinase-deficient strains of *N. haematococca*, obtained by one-step gene disruption, were as pathogenic as the wild-type using two different bioassays. In experiments under natural conditions disease symptoms were evaluated after several days to weeks (Stahl and Schäfer 1992). So it remained unclear whether cutinase plays a role during the initial penetration event. In this study we used the pea pot assay as a bioassay which allowed us to test fungal virulence under almost natural conditions (VanEtten 1978). Pea seeds were planted in natural soil and incubated with a spore concentration described for infected pea fields (Kraft and Roberts 1969).

The early infection sites of *N. haematococca* wild-type and mutants were macroscopically and biochemically determined. The initial center of fungal attack are first the seed, second the hypocotyl, and then the taproot and the epicotyl. The epicotyl, used in the early cutinase experiments exclusively as a test system, is infected late under natural conditions. Virulence of wild-type and cutinase-deficient mutants were assessed semi-quantitatively, by means of disease score, as well as quantitatively by measurement of plant biomass development and ergosterol content of infected plants. As the specific ergosterol content during *in vitro* culture of the different fungal strains was similar, it is reasonable to compare the fungal strains during plant colonization on the basis of their ergosterol content. It was not possible to distinguish between the wild-type and the mutants based on plant biomass development. Disease scoring and ergosterol assays revealed an identical virulence of wild-type and mutant 77-102. However, reproducible differences between wild-type and mutant 77-75 were found for disease scores and ergosterol contents of seeds, hypocotyls, and epicotyls after prologed periods of infection, i.e., more than 5–6 days. The cutinase-deficient mutant was more virulent than the wild-type. The cause for the increased virulence of one of the two investigated mutants may be an increased parasitic fitness of the transformant. Earlier investigations concerning the parasitic fitness of transformed strains of *Cochliobolus heterostrophus* revealed a decreased fitness of the transformants (Keller *et al.* 1990).

Further, we analyzed the infection process microscopically and, comparing wild-type and a cutinase-deficient mutant, we could not detect any differences. Wild-type and mutant 77-102 were able to colonize epicotyls, hypocotyls, and taproots equally well. The cutinase-deficient strain penetrated the epicotyl directly. Occasionally, it penetrated stomata, as did the wild-type. The plant reacted to the infection by a distinct cell wall autofluorescence of the infected cells. This can be caused by the production of defense-related phenolic compounds as was

shown for other plant fungal interactions. The production of papillae entrapping advancing hyphae was seen sometimes, but there was no obvious general inhibition of the invading hyphae by these possible defense mechanisms. Both fungal strains colonized the cortex of the plant equally well by inter- and intracellular growth. Endodermal cells collapsed in advance of the progressing hyphae and the vascular bundle was colonized. At that phase, the infected cortex cells started to disintegrate. This could be due to extracellular fungal enzymes or toxins, substances which might also be important during the initial penetration event. Fungal growth inside the cortex of the stem stopped abruptly 2–3 cm above the ground where the stem starts to become more intensively green. No other differences in plant morphology could be detected microscopically within this very distinct zone. Nevertheless, infection experiments with plants grown in test tubes which developed totally green stems indicated that the increasing number of chloroplasts do not hinder *N. haematococca* to infect the tissue (data not shown). Above this region the fungus grew exclusively in the xylem vessels. The plant reacted to the growing hyphae by cell wall deposits in the xylem, that lead to clogging of entire xylem cells. Similar plant reactions were observed after the infection of pea by *Fusarium oxysporum* f. sp. *pisi* (Tessier *et al.* 1990), a fungus which causes typical vascular wilt symptoms. The detection of the fungal mycelium was greatly facilitated after expressing the β-glucuronidase gene in *N. haematococca* which allowed us to stain the fungus selectively in the plant tissue and follow the infection microscopically. The *gusA* gene was recently expressed in several phytopathogenic fungi. It was used for transient expression assays (Judelson and Michelmore 1991; Bhairi and Staples 1992; Mönke and Schäfer 1993), constitutively expressed during the pathogenic life cycle of the fungus (Bunkers 1991; Smit and Tudzinsky 1992; Liljeroth *et al.* 1993) and used for promoter analysis of a fungal avirulence gene (Van den Ackerveken *et al.* 1994).

We have demonstrated that wild-type and a cutinase-deficient transformant of *N. haematococca* could equally well invade and colonize the stem under natural conditions, but leaves were only accessible to fungal invasion under conditions of very high humidity (Bywater 1959; Schäfer and Yoder 1994). This organ specificity, i.e., the ability of a pathogenic fungus to infect certain plant organs better than others, is a common feature among fungal pathogens. Nevertheless, the cause of this organ specificity is poorly understood. It has recently been tried to connect it with cutinase activity (Köller and Parker 1989). Following these arguments, fungal pathogens expressing a cutinase with an alkaline pH optimum are root pathogens, while a cutinase with a slightly acidic pH optimum confers foliage specificity. The cutinase of *N. haematococca* has an alkaline pH optimum, and as this fungus is a pathogen of roots and lower stems, it would fit into this scheme (Trail and Köller 1990). We have investigated microscopically the interaction of *N. haematococca* with pea leaves under conditions of rather low humidity. Leaves that were inoculated with conidia showed tiny necrotic spots clearly visible after 2 days. These lesions did not increase in size even after 2 wk. Cross sections revealed that epidermal cells were collapsed and filled with a highly fluorescent material. This is in sharp contrast to root or basal stem cells, where invaded cells did not collapse and showed only a weak fluorescence of the cell wall. Leaf cells reacted with a typical hypersensitive reaction. Even after puncture wounding the leaf, and thus reducing the putative influence of the cuticle in plant defense, the reaction remained basically the same. The fungus could penetrate some cells surrounding the wound site, but was stopped within approximately 1–2 mm of the wound. All types of leaf cells around the infection site showed a strong fluorescence. The described plant reactions to wild-type and cutinase-deficient strains of *N. haematococca* were identical. We conclude therefore that the described organ specificity is determined by totally different reactions of different plant organs towards the fungus and that the resistant plant reaction is not a consequence of the inability of the fungus to degrade the cuticle. In addition, as the cutinase-deficient strain gave the same results, cutinase can not act as an inducer of the resistance reaction. The reasons for these different defense reactions of different organs of the same plant remain unknown. We found no evidence that cutinase is involved in virulence or organ specificity in the interaction of *N. haematococca* with pea.

MATERIALS AND METHODS

Fungal culture.

N. haematococca MP VI isolate 77-2-3 was obtained from H. D. VanEtten (University of Arizona, Tucson). Cutinase-deficient mutants were derived from isolate 77-2-3 as described (Stahl and Schäfer 1992). Fungal isolates were stored as a conidial suspension in 25% (v/v) glycerol at –80° C. For pathogenicity tests fungal isolates taken from the glycerol stocks were maintained on V8 juice agar plates at 23–25° C.

Inoculation procedures.

The pot assay as described by VanEtten (1978) was performed with slight modifications. Pea seeds (cultivar Alaska 2B, Asgrow Seed Co., Kalamazoo, MI) were surface sterilized with 3% sodium hypochlorite and grown in steamed soil contained in plastic pods (11 cm in diameter). Soil was a mixture of Triohum (commercial cutting soil) and sand (3:1). Soil was infested with the conidial suspension at a concentration of 1,000 conidia per gram of soil immediately after the pea seeds had been planted. Control experiments were performed without fungal inoculum. Plants were incubated in an environmental growth chamber with approximately 70% humidity, a 16-hr photoperiod, and 28° C day/23° C night temperatures and investigated at the indicated intervals. Each pot contained five pea seeds. The plants of two or three pots were taken for each interval. Pot assays for histological analysis were performed in Berlin (Germany), virulence assays were performed in Einbeck (Germany).

For the leaf assay, pea plants were cultivated as described above. Leaves of 13-day-old plants were inoculated with 5 μl droplets of a suspension of micro- and macroconidia. The concentration of conidia was 5×10^3, 5×10^4, and 5×10^5 per 5 μl of water, respectively. The inoculum was placed carefully on the upper surface of intact or needle pricked leaves. Approximately 20 leaves of eight plants were inoculated with each conidia concentration in one experiment. Each experiment was repeated twice with the same results. Control experiments were performed with water droplets. Plants were incubated for 2 wk under environmental conditions as de-

scribed for the pot assay. Germination of conidia on V8 juice agar was examined at each inoculation to verify equally high viability.

Virulence assessment.

Plant weight: For virulence assessment plants were carefully removed from soil, soil traces were removed from underground parts, and the fresh weight of the complete plants was determined.

Disease score: The upper part of the taproot and the hypocotyl of plants inoculated with *N. haematococca* wild-type and the mutant strains were scored as follows: 0 = healthy plant; 1 = < 25% discoloration of root and hypocotyl; 2 = < 50% discoloration of root and hypocotyl; 3 = < 75% discoloration of root and hypocotyl; 4 = > 75% discoloration of root and hypocotyl.

The epicotyl of plants inoculated with *N. haematococca* wild-type and the mutant strains were scored as follows: 0 = healthy epicotyl; 1 = < 7 lesions on the epicotyl; 2 = < 25 lesions on the epicotyl, 3 = > 25 lesions on the epicotyl or single lesion width > 1 mm; 4 = lesion width > 2 mm.

Ergosterol content: After disease scoring, foot regions of infected and control plants were dissected for ergosterol measurements. Seeds, epicotyls (1–2 cm), and upper parts of taproots with hypocotyls (5 cm) of corresponding plants were collected, lyophilized, weighed, ground to a fine powder (Retsch Schwingmühle), and assayed for ergosterol. The described method for ergosterol determination by high-performance liquid chromatography (HPLC) analysis was first developed for the host/pathogen-system oilseed rape/*Verticillium dahliae* (Theuerkauf and Heitefuss, in preparation) and could be transferred to the system pea/*N. haematococca* without any modification. Fifty milligrams of the powdered material was used for ergosterol extraction. The samples were saponified under alkaline condition with 10% KOH in ethanol at 85° C for 30 min. Free ergosterol was extracted by using petrolether (60–80° C boiling point), evaporated to dryness and dissolved in mobile phase (acetonitrile/ethylacetate, 9:1). Aliquots were analyzed by reversed-phase HPLC on a C_{18} column (Knauer 250 × 4 mm, nucleosile 100-5 µm) with precolumn. For elution a linear gradient of 95–90% acetonitrile in ethylacetate and a column temperature of 18° C were chosen. Ergosterol was detected at 282 nm and a retention time of 21.5 min. The amount of ergosterol was calculated with reference to an external standard.

Statistical analysis: All experiments were replicated at least twice in a completely randomized design. Statistical analysis of the disease scores was carried out. The significance of differences between the wild-type and the mutant strains was investigated with the U test of Wilcoxon, Mann and Whitney (Sachs 1969).

Microscopic techniques.

Roots, stems, and leaves were cut into short pieces (5–10 mm) and were fixed at 4° C for at least 12 hr in 0.2% glutaraldehyde, 4% formaldehyde, 0.5% Triton X-100, 0.1 M sodium phosphate buffer, pH 7.0. Fixed plant material was dehydrated with ethanol and was embedded in 2-hydroxyethyl-methacrylate resin (Technovit 7100, Kulzer, FRG). Embedded material was sectioned at 5–10 µm using a rotation microtome (Leitz, FRG), collected on glass slides and covered permanently. Light and fluorescence microscopy was performed with a Zeiss Axiophot microscope (Oberkochen, FRG). Induced fluorescence was observed using a HBO UV-lamp (Zeiss) and the excitation filter BP 365, barrier filter LP 397, and beam splitter FT 395.

DNA manipulations and fungal transformation.

The transformation vector pG1 was constructed for expression of the *gus*A gene in *N. haematococca*. The *gus*A gene linked to the *gpd* promoter and the *trp*C terminator was isolated as a 4.5-kb fragment from vector pNOM102 (Roberts *et al.* 1989) and inserted into the vector pDC6 (Stahl and Schäfer 1992). Plasmid construction and DNA gel blot analysis was done as described (Sambrook *et al.* 1989). Transformation and DNA isolation of *N. haematococca* was done as reported recently (Stahl and Schäfer 1992).

GUS assay.

β-Glucuronidase activity of fungi was assayed quantitatively in mycelial extracts according to Jefferson *et al.* (1987) and Mönke and Schäfer (1993) by using 4-methyl-umbelliferyl-β-D-glucuronide (MUG) as substrate.

The detection of gus^+ transformants in infected tissue was performed according Mönke and Schäfer (1993). Infected plants were cut into short segments (0.5–1 cm). Segments were infiltrated with GUS staining solution (2 mM X-Gluc, 0.5%Triton X-100, 50 mM sodium phosphate, pH 7.5) by vacuum infiltration for 30–60 s. Segments were incubated for 6–12 hr at 37° C in the staining solution and then prepared for the microscopic analysis.

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