Endophyte-Host Interactions. I. Plant Defense Reactions to Endophytic and Pathogenic Fungi

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Abstract

To study the nature of the endophyte-host relationship, endophytes and pathogens were isolated from healthy and diseased plant tissue of *Lamium purpureum* and tested for their potential aggressiveness. The plant defense reactions to the endophytes and pathogens which were selected on the basis of this aggressiveness were compared using three test systems: dual cultures of intact plants and fungi, dual cultures of plant calli and fungi, and suspension cultures. In dual cultures in which there was no direct contact between fungus and callus or plant, the activity of phenylalanine ammonia-lyase (PAL) was stimulated more both in dual cultures of intact plants and of plant calli with the pathogen than with the endophytic fungi. In contrast, in suspension cultures, PAL-activity, the accumulation of phenols and the production of H_2O_2 was greater when elicited with endophytic mycelium and elicitor than with those of a pathogen. The different responses in the test systems and the apparently discriminatory reactions of the host plant to pathogenic and endophytic infection are discussed.

Keywords: Dual cultures, endophytes, *Lamium purpureum*, oxidative burst, PAL-activity, plant calli, suspension cultures

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1. Introduction

Research on endophytic fungi during the last fifteen years has mainly focused on documentation and quantification of symptomless infections in healthy plants and on studies of species composition of endophyte assemblages (Petrini, 1991; Stone et al., 1994). In order to better understand the nature of the endophyte-host symbiosis, information about the host response to endophytic infection and its mechanisms of limiting fungal colonization are necessary. A limited amount of research has investigated the mechanisms employed by an endophyte to penetrate its host, showing that these are basically those employed by pathogens (Stone, 1988; Cabral et al., 1993; Viret et al., 1993). Additionally, Viret et al. (1993) and Cabral et al. (1993) demonstrated that a host may respond with the same mechanical defense mechanisms to penetration of endophytes as employed against the penetration of pathogens, e.g. formation of papillae and haloes surrounding infected cells. However, it is an understanding of the physiology of the interactions that will allow us to comprehend what enables one fungus to grow endophytically whereas another fungal infection becomes pathogenic. Much research has been done to investigate the physiology of the pathogen-host interaction, but none as yet to study the physiology of the endophyte-host interaction.

According to Hahlbrock et al. (1995) there are three major types of plant defense responses to fungal infection. The first type of defense occurs very rapidly and includes hypersensitive response, oxidative burst and callose deposition. The second type of defense consists of the induction of the phenylpropanoid pathway and the accumulation of PR-proteins and phyto-alexins. Both types of defense responses are local. In contrast, the third type of defense is induced systemically throughout the entire infected leaf and frequently extends to the whole organism, including the activation of genes encoding several chitinase and glucanase isoforms.

As representative for the first rapid and the second slower types of defense response, the influences of endophytic and pathogenic interactions on H_2O_2-production (oxidative burst) and the induction of PAL-activity and the secretion of total phenolics were compared. To study the interactions of fungi and plants, two dual culture systems were developed, one using intact host plants and the other using host calli. The results of the experiments with these systems were compared to results obtained with elicitation experiments employing suspension cultures of the host *Lamium purpureum* L. and fungal cell wall preparations as elicitors.

2. Material and Methods

Fungal strains

The endophytic isolates *Coniothyrium palmarum* Corda and *Phomopsis* sp. had been isolated from healthy plants of *Lamium purpureum* L. growing in Lower Saxony, Germany and *Geniculosporium* sp. from *Teucrium scorodonia* L. (Schulz et al., 1993). In order to obtain a typical pathogen, fungi were isolated from diseased tissue of *L. purpureum* following surface sterilization (SS) (30 s in 70% ethanol, followed by 3 min in 6% NaOCl with one drop of Tween 80), as described in Schulz et al. (1993). The success of the surface sterilization was checked by imprinting the leaves following SS on MPY-agar medium (20.0 g malt extract, 2.5 g yeast extract, 2.5 g peptone from meat, 12.0 g agar and 1000.0 ml H_2O; pH 6.5).

Leaf infection tests of fungal isolates for potential aggressiveness

L. purpureum plants were cultivated under non-sterile conditions by initially germinating the seeds on moist filter paper in glass petri dishes in growth chambers and incubating for five days in a growth chamber at 20°C with 16 h of light of 270 μmol m^{-2} s^{-1} (PAR). The seedlings were transplanted into auto-claved plastic Phytacon pots (Sigma, Germany) containing 100 ml of Lecaton growth substrate (Leca, Germany) with 30 ml of Knop growth medium (Pierik, 1987). For each isolate to be tested, four leaves, two of which were perforated, were placed onto water agar in petri dishes. These were sprayed with a spore and/or mycelial suspension made by scraping a 2 × 2 cm section of a fungal culture and suspending in 5 ml of sterile tap water, and sealed with Parafilm. The controls were leaves that had been sprayed with sterile water. Incubation was as above. The evaluation was in percent of the leaf surface that had developed necroses or macerations.

Culture of axenic host plants

Seeds of *L. purpureum* were surface sterilized using 70% ethanol (30 sec), followed by 5 min in 30% H_2O_2 and 20 min in 10% NaOCl (Schulz et al., 1993). The effectiveness of each SS was checked as described above. The sterilized seeds were placed for germination into autoclaved glass petri dishes containing sterilized soil (Floragard, Germany) moistened with tap water. Following

germination and an additional 7 days of growth in the soil, the seedlings were transferred to sterile Phytacon pots containing 100 ml Lecaton substrate and 30 ml Knop growth medium and cultivated as above.

Callus cultures

Callus was induced from leaves and shoots of 8-week old sterile plants which had been cultured on SH (Schenk and Hildebrandt, 1962) medium supplemented with furfurylaminopurine (2 mg/l) and 2,4-dichlorophenoxy-acetic acid (2 mg/l). Preliminary experiments had shown that SH-medium was more effective for promoting the callus growth of *L. purpureum* than the methods proposed by Murashige and Skoog (1962), Gamborg et al. (1968) and Lin and Staba (1961). Growing calli were transferred to SH medium supplemented with benzylaminopurine (5 mg/l) and maintained at 25°C in the dark by subculturing in 4-week intervals.

Suspension cultures

For the preparation of suspension cultures approx. 2.0 g fresh weight (fw) of callus were transferred to 100 ml of liquid SH medium supplemented with 5 mg/l naphthalene acetic acid and 2.5 mg/l benzylaminopurine and cultured on a rotary shaker (110 rpm) at 25°C in the dark. Cultures were maintained in a 14 d subculture cycle.

Dual cultures of intact plants and fungi

After 14 d, the roots were cut from the shoot with a sterile scalpel. One shoot was placed in each Phytacon pot containing half-strength SH agar medium, 2 cm from the outer edge of the pot. After one week of monoculture, a 4 × 4 cm segment of a mycelial culture (14 d preculture on MPY agar medium) was inoculated 2.5 cm from the opposite side of the pot. As controls, the fungi and host plants were cultivated in monoculture.

Sampling occurred when growth of the fungal colony had almost reached the plant: *Phomopsis* sp. at 28 dpi, *C. palmarum* at 20 dpi, *Geniculosporium* sp. at 22 dpi and *Alternaria alternata* (Fr.) Keissler at 10 dpi. Following determination of fresh weight of the seedlings, they were immediately frozen

in liquid nitrogen and stored at –70°C. For further processing the samples were suspended in 3 ml of borate buffer (0.5 M $Na_3B_4O_7$, 0.02 M H_3BO_4, pH 8.8) and homogenized for 3×30 s with an homogenizer at 1500 rpm (Potter S, Germany). The samples were then centrifuged for 20 min at 16000 g at 4°C, the supernatant decanted and stored at –70°C.

Dual cultures of calli and fungi

Each petri dish (85 mm), containing 25 ml of SH medium was inoculated 2 cm from opposing sides of the dish with callus (approx. 2.0 g fw of 2-week old cultures) and a 3×3 mm agar plug of a 7 d colony grown on SH medium. For the controls, the callus was cultured alone.

Sampling procedure: all the cultures were inoculated at the same time and sampling of callus material was carried out at 2 d intervals. Four parallels of dual cultures and controls had been inoculated for each sampling time. 1.0 g fw of callus was taken from each culture from the side opposing the fungal colony. Processing was as above.

Preparation of elicitors and mycelial fragments

Cell wall elicitors were prepared according to Schwacke and Hager (1992). To initiate the experiments, 100 µg/ml of the lyophilised material were applied to the suspension cultures. A 3×3 mm plug of mycelium from a 7 d colony grown on SH-medium was used for elicitation with mycelial fragments.

Elicitation of suspension cultures

Aliquots of 50 ml each (approx. 2 g fw) of a 14 d suspension culture were transferred to 500 ml Erlenmeyer flasks containing 150 ml liquid SH medium. Cultures were incubated for 8 d at 25°C in the dark prior to inoculation with elicitor or mycelial preparation. Incubation continued for the indicated time periods. Suspension culture aliquots of 50 ml were sampled and the cells were collected by suction filtration. From this cell material three aliquots of 500 mg fw were used for the determination of PAL-activity. The medium of each sample was used for the detection of secreted total phenolics.

Enzyme assay

Phenylalanine ammonia-lyase (PAL) was extracted and assayed as described by Heinzmann and Seitz (1974). Protein was determined according to Bradford (1976) with bovine serum albumine as a standard.

Determination of phenolics

Phenolics in the culture medium of elicited suspension cultures were determined as described by Seitz et al. (1989).

Determination of hydrogen peroxide

H_2O_2 in the medium of suspension cultured *L. purpureum* cells was measured by a method using oxidation of the pigment phenol red (Grisham et al., 1984). Cells from 8 d cultures were washed twice with SH medium and collected by suction filtration. From the cell material 500 mg fw were suspended in 10 ml sterile SH medium and cultured for 2 h in 50 ml Erlenmeyer flasks on a rotary shaker (110 rpm) at 25°C for equilibration. For the experiments 400 µl phenol red solution (1 mM in SH medium) and 100 µl horse radish peroxidase (Sigma; 500 units/mg; 1 mg/ml) were applied to each culture. The reaction was started by adding 100 µg/ml elicitor or a plug of mycelium. Aliquots of 1 ml were sampled at the indicated times, cells were removed by short centrifugation and the supernatant was mixed with 0.02 ml of 0.5 M NaOH. The extinction of this solution was measured immediately at 558 nm. For quantification of H_2O_2, a calibration curve was constructed using standard H_2O_2 solutions (1–15 µmol/ml).

Tests for statistical significance

T-test statistics (Sigma Stat) were used to test the null hypothesis for no difference between control and treatment.

3. Results

Fungal isolates

The fungi previously isolated from symptomless tissue of *L. purpureum*
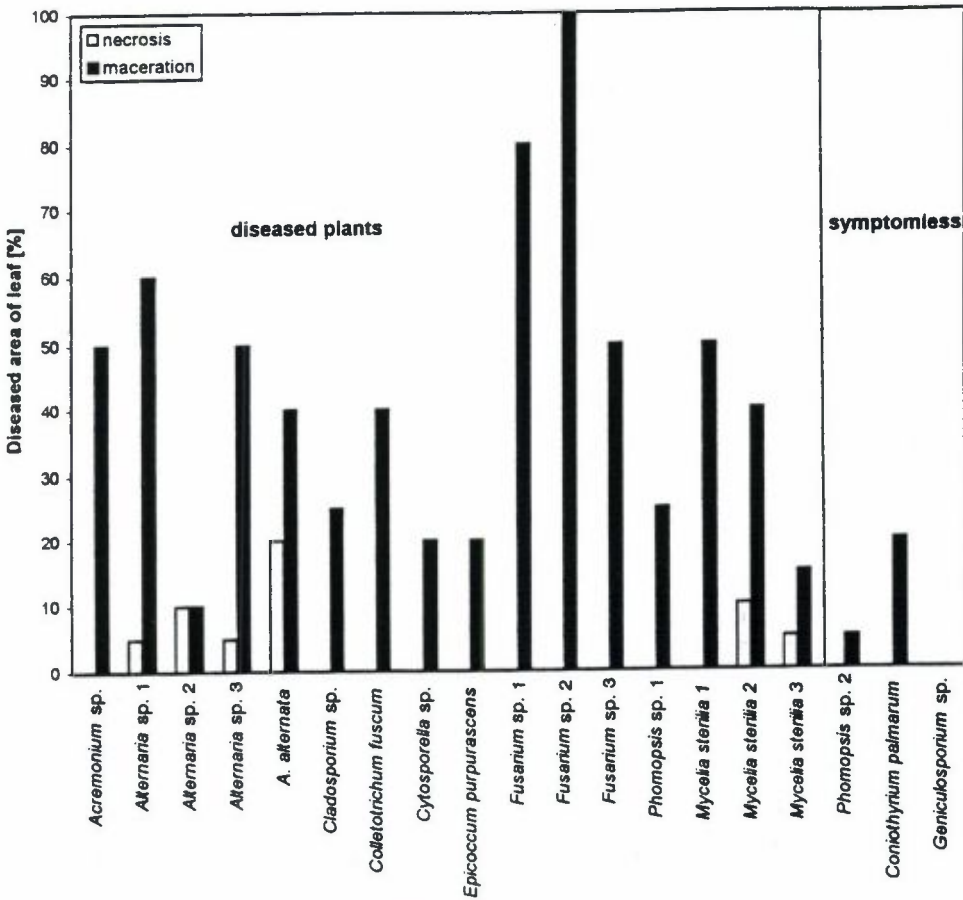
Figure 1.   Leaf infection tests for aggressiveness, showing proportion of the symptomatic leaf with necroses and macerations after inoculation with isolates from diseased and healthy host tissue. Incubation of the leaves was for 5 d in sealed petri dishes at 25°C in the dark.

belonged to the following genera: *Alternaria, Cercospora, Coniothyrium, Crandallia, Fusarium, Gymnoascus, Leptosphaeria, Penicillium, Phialophora, Phomopsis, Podospora, Stemphylium, Tricellula.* The genera of the fungi isolated from diseased tissue were primarily ubiquitous ones and included some

of those that had been found in symptomless tissue: *Acremonium, Alternaria, Cladosporium, Colletotrichum, Cytosporella, Epicoccum, Fusarium, Phoma, Phomopsis, Sporormiella* and *Titaea* as well as a number of *Mycelia sterilia*.

Aggressiveness of the fungal isolates

Of the 16 isolates from diseased tissue and the 3 isolates tested from symptomless tissue, all but one, *Geniculosporium* sp. which was isolated from a different host, caused at least some degree of maceration and/or necrosis in the leaf infection tests for aggressiveness (Fig. 1). The isolates from diseased tissue caused 5–20% necroses and 10–100% maceration, whereas the isolates from symptomless tissue caused no necroses and 0–20% maceration. Although as a group the isolates from diseased tissue caused more disease symptoms in the leaf infection tests than the three isolates from symptomless tissue did, some of the isolates from diseased tissue, i.e. *Alternaria* sp. 2, *Cytosporella* sp., *Epicoccum purpurascens* Ehrenb. and *Mycelia sterilia* 3, were just as unaggressive as those from the symptomless tissue were.

Due to its ability to cause not only macerations but also necroses, *Alternaria alternata* was chosen as a pathogen for further experiments. *Coniothyrium palmarum* was chosen for the experiments in which only one endophyte was tested.

PAL-activity in dual cultures of fungi with intact plants

In dual culture of intact plants with the endophytes and pathogen, the activity of PAL was measured at the time of sampling when growth of the fungal colony had almost reached the plant. With each of the three endophytes as well as with the pathogen, PAL-activity was significantly increased in comparison to the control (Fig. 2). Although the four dual cultures cannot be directly compared due to the varied growth rate of the isolates and thus varied durations of culture, in combination with the fast-growing pathogen, *A. alternata*, the increase of PAL-activity (88%) was greatest in comparison to the monoculture control. The PAL-activity of the intact *L. purpureum* plants in dual culture with the endophytes increased 73% with *C. palmarum*, 68% with *Phomopsis* sp. and 56% with *Geniculosporium* sp., the latter originally having been isolated from *Teucrium scorodonia* L.
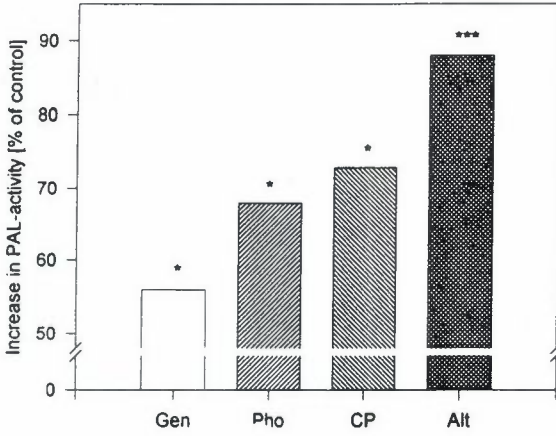
Figure 2. PAL-activity of intact plants following dual culture with endophytes or pathogen, sampled shortly before growth of the fungus had attained the plants. Cultivation was at 20°C, 16h:8h (l/d). Cp = *C. palmarum*; Pho = *Phomopsis* sp.; Gen = *Geniculosporium* sp., Alt = *A. alternata*. Times of incubation and level of statistical significance: Pho – 28 dpi, p<0.05; Cp – 20 dpi, p<0.05; Gen – 22 dpi, p<0.05; Alt – 10 dpi, p<0.001.
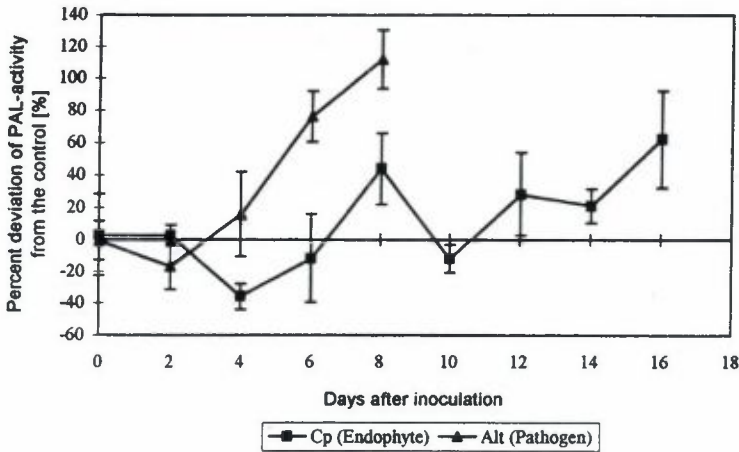

Figure 3. Comparison of the effect of *C. palmarum* (Cp) and *A. alternata* (Alt) on the PAL-activity of *L. purpureum* callus in dual culture, expressed as percent deviation from the control; values are means ±SD; n = 4. Incubation was at 25°C in the dark for 8 d (Alt) and 16 d (Cp).

PAL-activity in dual cultures of fungi with calli

The effects of endophyte and pathogen on the PAL-activity of the host plant *L. purpureum* were also compared in dual culture with calli and are displayed in Fig. 3 as percent deviation from the PAL-activity of the controls. Commencing two days after inoculation of the dual cultures, the fast growing pathogenic fungus induced a rapid and continuous increase of PAL-activity, reaching a level twice as high as in the control cells after 8 d.

Although there were fluctuations in PAL-activity of the calli during the first 10 d of dual culture with the endophytic fungus *C. palmarum*, there was no net change of activity. Not until the 16 d of incubation, at which time the fungal colony had grown to a distance of 2 mm from the callus, was the PAL-activity 60% above that of the controls. This was comparable to the increase in PAL-activity of 72% measured in the intact plants after 16 d of dual culture with *C. palmarum* (Fig. 2).

PAL-activity in elicited suspension cultures

In suspension cultures, the activity of PAL increased significantly after application of the respective cell wall elicitors (Fig. 4). Twenty-four hours after elicitation with *C. palmarum*, PAL-activity reached a level approx. four times that of the corresponding controls and remained at this high level until the end of the experiment at 72 h. After elicitation with *A. alternata*, PAL-activity did not reach its maximum until 48 h, attaining a level three times that of the control at the end of the incubation period. Thus, in contrast to the results obtained using dual cultures in which there was no direct contact between fungus and callus or intact plant, in suspension cultures the elicitor of the endophyte activated PAL of the plant cells more than that of the pathogen did.

Production of hydrogen peroxide in suspension cultures

The application of mycelial segments of *C. palmarum* and *A. alternata* to suspension cultures of *L. purpureum* induced the production of H_2O_2 by the suspension cells as an early defense response (Fig. 5). The mycelium of the endophyte *C. palmarum* stimulated H_2O_2-production more than the mycelium of the pathogen *A. alternata* did. The concentration of H_2O_2 began to increase rapidly 20 minutes after application of mycelium, while in the control cultures the increase of H_2O_2 was not as pronounced.
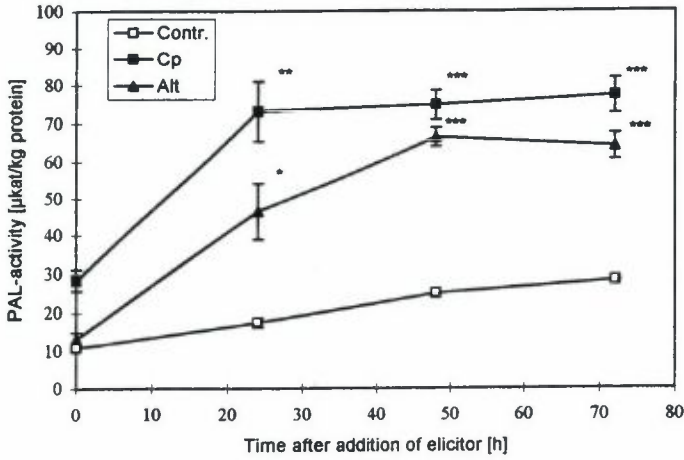
Figure 4.   The effect of cell wall elicitors from a pathogen and an endophyte on PAL-
            activity of *L. purpureum* suspension cultures. Values are means ±SD; n = 3.
            Incubation was in the dark at 25°C. Cp = *C. palmarum*, Alt = *A. alternata*.
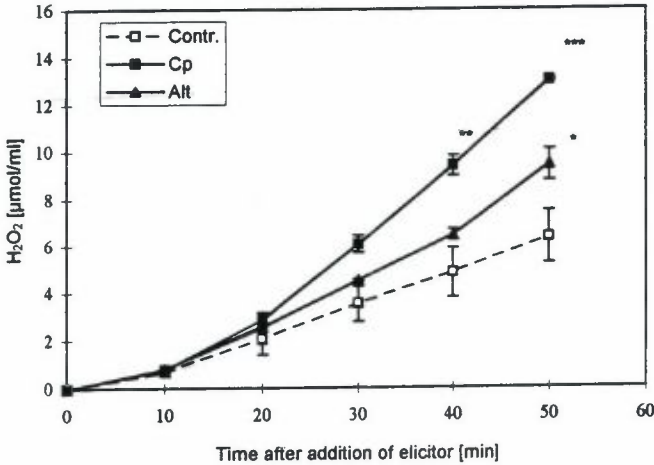   *p<0.05, ***p<0.001.

Figure 5.   Stimulation of H_2O_2-production in *L. purpureum* suspension cultures by
            elicitation with mycelial segments (3 × 3 mm mycelial plug) of *C. palmarum* (Cp)
            and *A. alternata* (Alt). Values are means ±SD; n = 3. Incubation in the dark at
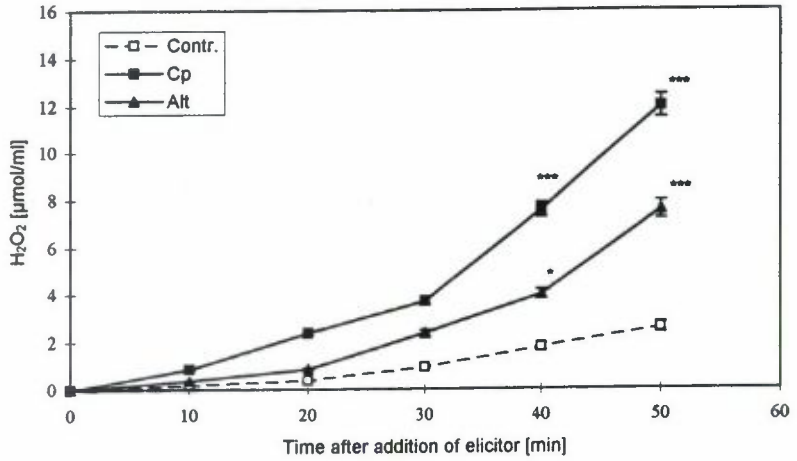   25°C. *p<0.05, **p<0.01, ***p<0.001.

Figure 6.   Stimulation of H₂O₂-production of *L. purpureum* suspension cultures by application of elicitors of *C. palmarum* (Cp) and *A. alternata* (Alt). Values are means ±SD. n = 3. Incubation in the dark at 25°C. *p<0.05, **p<0.01; ***p<0.001.
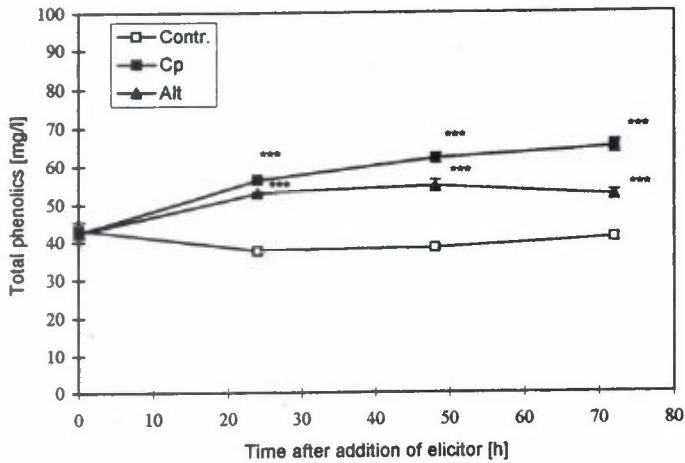

Figure 7.   The effect of elicitation on secretion of total phenolics in *L. purpureum* suspension cultures; values are means ±SD; n = 3. Incubation in the dark at 25°C. ***p<0.001.

The addition of fungal elicitors to suspension cultured cells of *L. purpureum* also significantly increased the production of H_2O_2 (Fig. 6). Again, the elicitor of *C. palmarum* stimulated the cells more to H_2O_2-production than the elicitor of the pathogenic fungus did. In suspension cultures, the elicitation of the production of H_2O_2 correlated positively with the elicitation of PAL-activity, which was significantly and more rapidly stimulated by the endophytic than by the pathogenic elicitor.

Phenolic compounds in elicited suspension cultures

After application of the elicitors of *C. palmarum* and *A. alternata*, the concentration of total secreted phenolic compounds increased only moderately in the culture medium of *L. purpureum* suspension cultures (Fig. 7). The elicitor of the endophyte *C. palmarum* induced an increase in the accumulation of phenolics from 40 mg/l to 65 mg/l after 72 hours of incubation. Following elicitation with *A. alternata* the concentration of phenolics had already reached its maximum of 55 mg/l after 48 hours of incubation. The elicitor of the endophyte *C. palmarum* induced a greater secretion of phenolics than the elicitor of the pathogen did, again correlating positively with the induction of PAL-activity and of H_2O_2-production by elicitors.

4. Discussion

Until now most research on endophytes has concentrated on characterizing the assemblages of fungi that can be isolated from healthy tissues of different hosts. These fungal spectrums have been correlated with various parameters, e.g. environmental, climatic and geographical factors, but also anatomical and host preferences (Bills, 1996). In some cases the ecological role of endophytes within the host plants has been analyzed, a correlation often being assumed between secondary metabolites and ecological niche (e.g. Dreyfuss and Chapela, 1994; Schulz et al., 1995; Bush et al., 1997). Only a limited amount of research has been done on the physiology of the endophyte-host interaction, mainly dealing with methods of penetration, production of enzymes and phytohormones (Petrini et al., 1992; Bacon and Hill, 1996).

It has also become apparent, as discussed by Petrini (1991), Stone et al. (1994), Gange (1996) and Schulz et al. (this volume), that fungi isolated as

endophytes from symptomless tissue comprise a heterogeneous group with different strategies for colonizing their hosts. Some of these infections remain symptomless, the fungi only sporulating saprophytically on dead host tissue. Others are latent or quiescent pathogens. Similarly, as the leaf tests for aggressiveness showed, the fungal isolates from diseased tissue of *L. purpureum* are, at least with respect to their aggressiveness, also a heterogeneous group. Infections with isolates such as *Fusarium* sp. 2 resulted in maceration of 100% of the leaf area, whereas others such as *Alternaria* sp. 2 only caused 20% maceration of the leaf, results similar to those obtained with the endophyte from symptomless tissue, *C. palmarum*. Additionally, the fact that a fungal isolate belongs to a particular genus or species does not mean that it will necessarily be endophytic or pathogenic following reinfection in the host plant (Schulz et al., this volume). For example, in the test for aggressiveness, *Phomopsis* sp. caused 25% maceration of the tested leaves, whereas *Phomopsis* sp. 2, isolated from symptomless tissue, only caused 5% maceration. Bacon and Hinton (1996) also found that not all isolates of a single species, *Fusarium moniliforme* Sheldon, are equally aggressive. Under the experimental conditions, following infection of axenically cultivated plants, some isolates only caused endophytic infections, whereas infection with others led to disease symptoms. The endophytic isolates only colonized the host maize intercellularly and did not induce a plant defense response as measured by the deposition of callose. In contrast, infections with isolates that caused disease symptoms were both inter- and intracellular and induced the deposition of callose.

Our initial investigations on the physiology of the endophyte-host interaction have compared the host defense reactions to endophytic and to pathogenic infection. Three model systems were employed: dual cultures of intact plants and fungi, dual cultures of plant calli and fungi, and suspension cultures with elicitation. In the dual culture experiments, there was never direct contact between fungus and plant or callus because the experiments were terminated before the fungi contacted the plant or the callus. This was done because previous experiments had shown that in dual culture, the calli became necrotic before the fungi had grown up to the calli, presumably due to secreted toxic metabolites (Peters et al., 1998). Another aspect of the dual culture systems is that not all cells are uniformly accessible to changes in the experimental conditions. In contrast, in the simplified system using suspension cultures, all cells are simultaneously and equally effected when substances are

added to the culture medium, in this case elicitors. A disadvantage of the suspension cultures is that these cells do not have all the defense mechanisms of the intact plant.

The plant defense responses to endophytes and pathogen differed in all three test systems, both with respect to the fast and slow defense reactions. In dual cultures of the fungi with the intact plant and with the plant callus, the pathogenic fungus initiated a greater increase in PAL-activity than the endophytes did. In contrast, in suspension cultures induction of the phenylpropanoid pathway, measured as PAL-activity and as total secreted phenolics, was more strongly induced by the endophytic than by the pathogenic elicitor. This may be due to the fact that the reactions in the different test systems were caused by different metabolites. Since in the dual cultures there was no direct contact between fungus and plant or callus, an interaction was only possible via secreted metabolites. As previous experiments had shown, volatile compounds such as ethylene did not play a role in the interaction (unpublished results), hence the active metabolites that induced a plant defense response in dual culture must have been diffusible. In the elicitation experiments using suspension cells the plant cells react to structural compounds on the surface of the fungal cell, for example glycoproteins or glycolipids (Scheel and Parker, 1990; Yoshikava et al., 1993). An "endophytic" mutant of *Colletotrichum magna* also induced an increase in PAL-activity, as well as in the production of peroxidase and lignin deposition (Rodriguez and Freeman, 1993). It is also conceivable that the variation in response with respect to PAL-activity of the plant cells in dual cultures in contrast to suspension cultures is due to the secretion of diffusible suppressors which, as reported by Boller (1995), could delay or reduce elicitor action. Suppressors, which must be actively secreted by intact cells (Shiraishi et al., 1994), cannot play a role in suspension cultures because the response of these cells was a reaction to elicitors or mycelial fragments.

The rapid defense reaction could only be tested in suspension cultures. Both the endophytic mycelial fragments and the elicitor induced a more intense production of H_2O_2 than pathogenic elicitation did. These results correspond to those attained for PAL-activity and the secretion of phenolic compounds in suspension culture following elicitation. The induction of identical reactions by both mycelium and cell wall elicitors in suspension cultures indicates that the same components in mycelium and cell wall elicitors may have been recognized by the plant cells and induced a reaction that differs quantitatively in defense

expression from that induced by the diffusible compound(s) active in the dual cultures.

Since fungi isolated as endophytes and pathogens comprise heterogeneous groups, we cannot be sure that the isolates used in these experiments are representative for all pathogens and endophytes. Thus, although the pathogen was chosen for its aggressiveness and the endophytes for their relative non-aggressiveness in the leaf infection tests, the data must be interpreted cautiously.

5. Conclusion

Our experiments show that there is not only variability in the potential of endophyte and pathogen to cause disease symptoms, but also that in the fungal-host interaction the plant host may be able to differentially recognize endophyte and pathogen, as has been reported for other endophyte-host interactions (Chapela et al., 1991; Petrini, 1996) and thus respond differently to an endophytic and a pathogenic infection. Results we have obtained studying the interactions in other host and endophyte systems in which fungus and host had direct contact yielded comparable results to those obtained here in the suspension cultures in which there was also direct contact between elicitor or mycelial fragment and host cells. Induction of the plant defense response was stronger towards an endophytic than towards a pathogenic infection (Schulz et al., this volume).

In a direct fungal-host interaction in which disease symptoms do not develop, there are several possible explanations of why this does not occur: a) the fungus is not capable of being aggressive enough to cause infection, e.g. the "endophytic" mutant of *Colletotrichum magna* (Freeman and Rodriguez, 1993); b) the host's defense mechanisms are strong enough to prevent the extensive colonization necessary for development of disease, as is the case with incompatible pathogens (Heath, 1981); c) the fungus is recognized as an endophyte and not as a compatible pathogen; d) the fungus' colonization strategy includes a latent or quiescent period (Williamson, 1994; Sinclair and Cerkauskas, 1996). This latter explanation could be a variant of a) and b). Our results suggest that at least some endophytic infections may be equivalent to those of incompatible pathogens (Heath, 1981), in which the defense mechanisms of the plant limit colonization of the fungal endophyte, explaining the fact that endophytic infections frequently remain localized.

Mechanisms that the endophyte might apply to overcome the plant defense reaction could include fungal infection mechanisms, e.g. excretion of enzymes (Reddy et al., 1996), plant growth hormones (Tudzynski, 1997) or of metabolites toxic to the host (Dreyfuss and Chapela, 1994; Peters et al., 1998).

Acknowledgments

REFERENCES

Bacon, C.W. and Hill, N.S. 1996. Symptomless grass endophytes: Products of co-evolutionary symbioses and their role in the ecological adaptations of infected grass. In: *Endophytic Fungi in Grasses and Woody Plants*, S.C. Redlin and L.M. Carris, eds. APS Press, St. Paul, MN.

Bacon, C.W. and Hinton, D.M. 1996. Symptomless endophytic colonization of maize by *Fusarium moniliforme*. *Canadian Journal of Botany* **74**: 1195–1202.

Bills, G. 1996. Isolation and analysis of endophytic fungal communities from woody plants. In: *Endophytic Fungi in Grasses and Woody Plants*, S.C. Redlin and L.M. Carris, eds. APS Press, St. Paul, MN.

Boller, T. 1995. Chemoperception of microbial signals in plant cells. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**: 189–214.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248–254.

Bush, L.P., Wilkinson, H.H., and Schardl, C.L. 1997. Bioprotective alkaloids of grass-fungal endophyte symbiosis. *Plant Physiology* **114**: 1–7.

Cabral, D., Stone, J.K., and Carroll, G.C. 1993. The internal mycobiota of *Juncus* spp.: microscopic and cultural observations of infection patterns. *Mycological Research* **97**: 367–376.

Chapela, I.H., Petrini, O., and Hagmann, L. 1991. Monolignol glucosides as specific recognition messengers in fungus-plant symbioses. *Physiological and Molecular Plant Pathology* **39**: 289–298.

Dreyfuss, M.M. and Chapela, I.H. 1994. Potential of fungi in the discovery of novel, low molecular weight pharmaceuticals. In: *The Discovery of Natural Products with Therapeutical Potential*, V.P. Gullo, ed. Butterworth-Heinemann, Boston, MA, pp. 49–80.

Freeman, S. and Rodriguez, R. 1993. Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. *Science* **260**: 75–78.

Gamborg, O.L., Miller, R.A., and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50: 151–158.

Gange, A.C. 1996. Positive effects of endophyte infection on sycamore aphids. *Oikos* 75: 500–510.

Grisham, M.B., Jefferson, M.M., Melton, D.F., and Thomas, E.L. 1984. Chlorination of endogenous amines by isolated neutrophiles. Ammonia-dependent bactericidal, cytotoxic-induced inactivation. *Journal of Biological Chemistry* 259: 10404–10413.

Hahlbrock, K., Scheel, D., Logemann, E., Nürnberger, T., Parniske, M., Reinold, S., Sacks, W.R., and Schmelzer, E. 1995. Oligopeptide elicitor-mediated defense gene activation in cultured parsley cells. *Proceedings of the National Academy of Science USA* 92: 4150–4157.

Heinzmann, U. and Seitz, U. 1974. Beziehung von Anthocyansynthese und Enzymaktivität der Phenylalanin-Ammonium-Lyase (PAL) bei Kalluskulturen von *Daucus carota*. *Planta* 117: 75–81.

Heath, M.C. 1981. A generalized concept of host-parasite specificity. *Phytopathology* 71: 1121–1123.

Lin, M.L. and Staba, E.J. 1961. Peppermint and spearmint tissue cultures. I. Callus formation and submerged culture. *Lloydia* 24: 139–145.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497.

Peters, S., Draeger, S., Aust, H.J., and Schulz, B. 1998. Interactions in dual cultures of endophytic fungi with host and nonhost plant calli. *Mycologia* 90.

Petrini, O. 1991. Fungal endophytes of tree leaves. In: *Microbial Ecology of Leaves*, J.H. Andrews and S.S. Hirano, eds. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo, Hong Kong, Barcelona, Budapest.

Petrini, O. 1996. Ecological and physiological aspects of host specificity in endophytic fungi. In: *Endophytic Fungi in Grasses and Woody Plants*, S.C. Redlin and L.M. Carris, eds. APS Press, St. Paul, MN.

Petrini, O., Sieber, T.N., Toti, L., and Viret, O. 1992. Ecology, metabolite production, and substrate utilization in endopyhtic fungi. *Natural Toxins* 1: 185–196.

Pierik, R.L.M. 1987. *In vitro Culture of Higher Plants*. Martinius Nijhoff Publishers, 64 pp.

Reddy, P.V., Lam, C.K., and Belanger, F.C. 1996. Mutualistic fungal endophytes express a proteinase that is homologous to proteases suspected to be important in fungal pathogenicity. *Plant Physiology* 111: 1209–1218.

Rodriguez, S. and Freeman, S. 1993. Response from Rodriguez and Freeman. *Trends in Microbiology* 1: 254.

Scheel, D. and Parker, J.E. 1990. Elicitor recognition and signal transduction in plant defense gene activation. *Zeitschrift für Naturforschung* 45c: 569–575.

Schenk, R.U. and Hildebrandt, A.C. 1962. Medium and techniques for induction and growth of monocotyledenous and dicotyledonous plant cell cultures. *Canadian Journal of Botany* 50: 199–204.

Schulz, B., Wanke, U., Draeger, S., and Aust, H.J. 1993. Endophytes of herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycological Research* **97**: 1447–1450.

Schulz, B., Sucker, J., Aust, H.J., Krohn, K., Ludewig, K., Jones, P.G., and Döring, D. 1995. Biologically active secondary metabolites of endophytic *Pezicula* species. *Mycological Research* **99**: 1007–1015.

Schulz, B., Guske, S., Dammann, B., and Boyle, C. 1998. Endophyte-host interactions. II. Defining symbiosis of the endophyte-host interaction. *Symbiosis* **25**: x–x.

Schwacke, R. and Hager, A. 1992. Fungal elicitors induce a transient release of active oxygen species from cultured spruce cells that is dependent on Ca^{2+} and protein-kinase activity. *Planta* **187**: 136–141.

Seitz, H.U., Eilert, U., De Luca, V., and Kurz, W.G.W. 1989. Elicitor-mediated induction of phenylalanine ammonia-lyase and tryptophan decarboxylase: accumulation of phenols and indole alkaloids in cell suspension cultures of *Catharanthus roseus*. *Plant Cell, Tissue and Organ Culture* **18**: 71–78.

Shiraishi, T., Yamada, T., Saitoh, K., Kato, T., Toyoda, K., Yoshioka, H., Kim, H., Ichinose, Y. Tahara, M., and Oku, H. 1994. Suppressors: determinants of specificity produced by plant pathogens. *Plant Cell Physiology* **35**: 1107–1119.

Stone, J.K. 1988. Fine structure of latent interactions by *Rhabdocline parkeri* on Douglas-fir, with observations on uninfected epidermal cells. *Canadian Journal of Botany* **66**: 45–54.

Stone, J.K., Viret, O., Petrini, O., and Chapela, I.H. 1994. Histological studies of host penetration and colonization by endophytic fungi. In: *Host Wall Alterations by Parasitic Fungi*, O. Petrini and G.B. Ouellette, eds. APS Press, St. Paul, MN, pp. 115–126.

Tudzynski, B. 1997. Fungal phytohormones in pathogenic mutualistic associations, In: *The Mycota V, part A*, Carroll, G. and Tudzynski, eds. Springer-Verlag, Berlin and Heidelberg.

Viret, O., Scheidegger, C., and Petrini, O. 1993. Infection of beech leaves (*Fagus sylvatica*) by the endophyte *Discula umbrinella* (teleomorph: *Apiognomonia errabunda*): low temperature scanning electron microscopy studies. *Canadian Journal of Botany* **71**: 1520–1527.

Volpin, H., Phillips, D.A., Okon, Y., and Kapulnik, Y. 1995. Suppression of an isoflavonoid phytoalexin defense response in mycorrhizal alfalfa roots. *Plant Physiology* **108**: 1449–1454.

Williamson, B. 1994. Latency and quiescence in survival and success of fungal plant pathogens In: *Ecology of Plant Pathogens*, J.P. Blakeman and B. Williamson, eds. CAB International, Wallingford, UK, pp. 187–207.

Yoshikawa, M., Yamaoka, N., and Takeuchi, Y. 1993. Elicitors: their significance and primary modes of action in the induction of plant defense reactions. *Plant Cell Physiology* **34**: 1163–1173.