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Endophyte-Host Interactions III. Local vs. Systemic Colonization

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Abstract

Growth of endophytic fungi within their plant hosts can either be systemic or localized and may have varied influences on the fungal-host interaction. It may also vary depending on the organ that is being colonized. In these studies, the histology and physiology of non-clavicipitaceous endophytic growth in the shoots of bean and barley were investigated in growth chamber and field experiments. The three endophytic isolates of *Fusarium* that were chosen for the experiments grew asymptomatically and intercellularly within the above-ground organs of the hosts. Endophytic colonization of the shoots had no significant effects on yield, carbohydrate metabolism, stress tolerance or induced resistance of the host. Both histological examination and ELISA demonstrated that endophytic colonization of the shoots was localized and limited, presumably explaining the lack of significant effects on the measured physiological parameters. Both our previous results and those of others reported in the literature demonstrate that colonization of the roots is usually systemic. It is discussed that mutualistic infections seem to involve systemic colonization and more often involve systemic colonization of the roots than of the shoots, presumably due to the nature of the shoot infections. An hypothesis that deals with the evolutionary development of these fungal interactions with their hosts is presented.

Keywords: Endophyte, mutualism, *Fusarium*, apoplast, interaction

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1. Introduction

The interaction of a fungus with its plant host varies, depending on the mode in which the fungus infects its host and to what extent there is a plant defense reaction. This colonization may be local or systemic, inter- or intracellular. The effects on the host also vary, ranging from asymptomatic to disease, mutualistic to pathogenic symbioses.

Endophytic fungi colonize their hosts, by definition, asymptomatically. Some endophytic interactions are mutualistic, others quiescent, in others the endophyte is a pathogen in a latent stage of development (Petrini, 1991; Schulz et al., 1998). Some endophytes are host-specific, others colonize only certain organs of the plant host (Chapella et al., 1993; Petrini, 1996). Thus, the range of endophytic interactions is very broad.

Most of the investigations dealing with the non-clavicipitaceous endophytic fungi have concentrated on investigating the species composition of fungi that can be isolated from different plant hosts (Bills, 1996). There have been some reports of induced resistance (Bultman and Murphy, 2000), stress tolerance (Römmert et al., 1998) and improved growth (Varma et al., 2000). Secondary metabolites of endophytic fungi may be involved in these processes and are regarded as sources for novel metabolic structures (e.g. Dreyfuss and Chapela, 1994; Calhoun et al., 1992; Schulz et al., 1995; Krohn et al., 1996; Krohn et al., 1997).

There have been only few investigations dealing with the physiology and histology of plant hosts colonized by non-clavicipitaceous endophytic fungi. Studies on endophytic colonizations of the above-ground organs of plants have shown that these are usually localized (Cabral et al., 1993; Carroll, 1995; Stone et al., 1994). The few histological studies have shown that endophytic growth may be inter- or intracellular. For example, *Rhabdocline parkeri* grew intracellularly in *Pseudotsuga menziesii*, the infections of non-senescent tissue being confined to single cells (Stone, 1988). However, in the culms and leaves of *Juncus* spp., Cabral et al. (1993) found that whereas most of the infections were localized, an unidentified endophyte colonized the air channels of the host mesophyll extensively.

In contrast, most of the limited reports on endophytic colonization of the roots have found fungal growth to be systemic. For example, colonization of the roots of various hosts by the dark septate fungus *Phialocephala* spp. (Jumpponen and Trappe, 1998; Stone et al., 2000) and of *Larix decidua* by *Cryptosporiopsis* sp. and *Phialophora* sp. (Schulz et al., 1999) is extensive and both inter- and intracellular. Colonization of the roots of maize by an endophytic isolate of *Fusarium moniliforme* was systemic, but only intercellular (Bacon and Hinton, 1996).

Investigations on the physiology of the endophyte-host interaction of

non-clavicipitaceous fungi concentrated on studying colonization of the roots of the plant hosts (Schulz et al., 1999) and on studying the interaction in simplified systems (Peters et al., 1998a; 1998b). As yet there have been no reports on the physiological effects of endophytic colonization of the shoot and the influence of these infections on the constituents of the apoplast. Our present investigations deal with the physiology and histology of endophytic colonizations of the shoots of the monocot barley and the dicot bean in order to compare these with our previous results and with reported data on the physiology and histology of root infections.

2. Material and Methods

Hosts

Summer barley, *Hordeum vulgare* cv. Salome, was chosen as the monocotyledonous host; bean, *Phaseolus vulgaris* cv. Saxa, was the dicotyledonous host.

Fungal strains

Endophytes and pathogens were isolated following surface sterilization with 70% ethanol and sodium hypochloride (1, 2 or 3% active chloride) from leaves, shoots and roots of bean and barley plants growing as crops in Lower Saxony, Germany. The length of sterilization and the concentration of sodium hypochloride were optimized for the respective plant tissue (Schulz et al., 1993). The resulting isolates, as well as endophytes from other hosts with known secondary metabolites, were tested for their potential virulence or pathogenity on intact plants. Those from other hosts belonged to the following genera: *Crandallia, Cryptosporiopsis, Gymnoascus, Fusidium, Monodictys, Physalospora, Plectophomella, Pleospora, Sirodothis* and *Torula*. Following tests for potential virulence, three isolates of the genus *Fusarium*, one of the most common endophytic genera isolated from all organs of barley (Dammann, 1997) and bean (Götz, unpublished), were chosen as endophytes and a *Drechslera* isolate as pathogen for experiments using both plant hosts.

Growth chamber experiments

Bean seeds (3 per 22 cm pot) were sowed into a mixture of Composana® and sand (3:1) and cultured under standard conditions (70% relative humidity, 18°C, 16 h light with PAR 210 µmol m^{-2} s^{-1}). After 3 weeks, the plants were decapitated regularly, inoculated by spraying both sides of the leaves with a

spore suspension (~10^7 ml^{-1}) in water + 0.01% Tween 80 and cultured for 3 days at 100% relative humidity, subsequently at 70% rh. Culture of barley differed in that seeds were sowed in 3 rows of 25 each in planters, 1 m in length, and inoculated when the primary leaves had fully developed, but not decapitated; culture was at 18° and 70% rh during the day and 16° and 90% rh at night. The success of the infection was ascertained for each experiment on the 7th day after inoculation and at the end of the experiment by reisolation from inoculated, surface sterilized tissue.

UV-stress was applied with a UV lamp (UVASPOT 400 / T; Dr. Hönle, UV Technik, Medizinische Geräte) 2 × 5 min daily for 5 days, starting 7 days after the inoculation of the respective fungus on young barley and bean plants.

High nitrogen concentrations (N-stress) were applied to three week old decapitated bean cultures inoculated with a spore suspension of *Fusarium* sp. 2 (3.5×10^8 ml^{-1}) using tap water as a control, while being cultered as above. The pots were either additionally fertilized with 50 ml of a 50 mmol l^{-1} Ca $(NO_3)_2$ solution or watered with 50 ml tap water in 5 day intervals. Evaluation of leaf symptoms and plant growth was done weekly, dry weight was determined at the end of the experiments (49th day after inoculation).

To test for potential induced resistance after sowing barley was inoculated with an endophyte and subsequently with the pathogen. Two experiments were conducted with three repetitions each. The endophyte, *Fusarium* sp. 2 (2×10^7 ml^{-1} spores), was inoculated on the 6th day after sowing and the pathogen, *Drechslera* sp. (2×10^5 ml^{-1} spores), after (1) 4 days, (2) 6 days and (3) 10 days after the inoculation of the endophyte. Each inoculation was followed by a 3-day period of high humidity. As controls, spore suspensions of the individual fungi were inoculated suspended in tap water with 0.01% Tween 80.

Field experiments

The field experiments with both bean and barley were conducted during late spring – summer in Lower Saxony, Germany. The garden plots for beans were approximately 5 × 5 m and the two plots for barley were 2.7 × 4 m each.

The bean plants were inoculated twice with one of three endophytes or with the pathogen. (1) After development of the primary leaf, both sides of the leaves were sprayed with a spore suspension of the respective fungi in sterile tap water (10^5–10^7 spores ml^{-1}). Each plant was then covered for three days with a premoisturized plastic bag. (2) Two weeks later all the plants were again inoculated as above and the field was covered with a plastic sheet for three days. Seven days later, the success of the infection was checked by surface sterilizing segments of some of the inoculated leaves. Half of each plot was

shaded with a cotton sheet 1 m above the plants in order to induce stress to the bean plants which normally require good lighting.

The bean plants were evaluated weekly for disease symptoms. The plants in one plot were harvested after 69 days, the plants in the other plot after 105 days. After harvesting, the plants were evaluated for the presence of nodules; yield was determined as number of leaves, pods, and shoot length, as well as the respective wet weights.

The barley plants were inoculated four times by spraying the plants with a spore suspension in sterile tap water with 0.05% Tween with one of three endophytes or with the pathogen. The plots were subsequently covered for 24 hours with a plastic sheet to ensure adequate moisture. The spore concentrations of the *Fusarium* endophytes were approximately 10^7 ml^{-1} and that of the pathogen *Drechslera* sp. was approximately 10^5 ml^{-1}. Inoculation in plot 1 was at 12, 18, 54 and 67 days after sowing, in plot 2 at 12, 19, 42 and 56 days after sowing. Success of infection was checked by surface sterilizing sections of the primary and secondary leaves of 5 plants from each plot one week after the first inoculation. Disease symptoms were evaluated on a weekly basis. After harvesting, plot 1 at 105 days and plot 2 at 93 days, yield was determined on the basis of the wet and dry weights of shoot and ear, and the length of the main shoot.

Tests for fungal virulence

Both sides of primary leaves of 3–4 week-old decapitated bean plants were sprayed with a spore suspension (2×10^6 ml^{-1}) of the respective fungi and subsequently incubated in growth chambers with 100% relative humidity at 18°C and 16 h light (PAR 210 $\mu mol\ m^{-2}\ s^{-1}$). Surface sterilization of inoculated leaves assured the success of the infection. Disease symptoms were evaluated after 7, 14 and 21 days. Intact axenically cultured barley plants were inoculated and disease symptoms evaluated as reported previously (Schulz et al., 1998).

Histological examination

Plant tissue was embedded in Technovit 7100, sections cut with a microtome. The best differential staining was achieved with 0.1% thionine in 5% glycerin (30 sec).

Since the fungal infections often could not be detected directly microscopically, we used a technique which artificially improved endophytic growth enabling analysis of the fungus' mode of growth within the plant tissue. Inoculated leaf segments were incubated for 3–4 days on a 5% (w/v) biomalt (Villa Natura, Kirn, Germany) medium before embedding and staining.

Exoenzymes

Lipase was determined according to the method of Carroll and Petrini (1983), pectin lyase according to Dingle et al. (1953) and Obi (1981). The Bavendamm test (Bavendamm, 1928) was used to determine phenoloxidase activity. Proteases were detected using a lactmus-milk agar. Amylase activity was detected by incubating the fungi on a 2% malt extract agar medium to which 0.2% starch had been added. After 14 days the cultures were flooded with Lugol's solution staining non-degraded starch blue.

Biological activity

The biological activity of the isolates against fungal, bacterial and algal test organisms was according to Schulz et al. (1995). Suspensions of the test organisms were either sprayed onto colonies of the isolates or onto antibiotic assay discs soaked with 0.05 ml culture extract, evaluation was of the radius of the zone of inhibition.

Apoplastic washing fluid

The apoplastic washing fluid (AWF) was extracted according to a modified version of the method employed by Mühling and Sattelmacher (1995) from plants grown in growth chambers. For each sample the wet weight of primary leaves (8 from bean, 140–150 from barley) was determined. Three parallel samples were taken at each sampling time. The samples were then incubated in iced distilled water for 30 min, infiltrated twice at 50 mbar below atmospheric pressure, dried, weighed, the degree of infiltration calculated and subsequently centrifuged for 30 min at 4°C and 300 g (1200 rpm). The pH value and the conductance of the AWF were measured and the dry and wet weights of the remainders of the leaves determined, before both the AWF and the remainders of the leaves were lyophilized and frozen at –70°C pending further investigations.

Constituents of the AWF

To check for possible contamination of the AWF with symplastic fluids, the activity of malate dehydrogenase was determined (Smith, 1983). The activities of invertase and peroxidase were measured using the modified method of Wagner and Boyle (1995b).

The pellet residues were subsequently used for analysis of protein and ash

content following concentration via lyophilization, and filtration following resuspension in distilled water, and analysis of mono- and disaccharides. Sucrose was additionally analyzed according to van Handel (1968), glucose and fructose with a combi-set (Test - Kit Nr. 139 106 + 131 229; Boehringer, Mannheim) or measured via HPLC-analysis directly from the crude extract.

Fungal growth in AWF and synthetic AWF

The growth and biomass production of endophytic and pathogenic fungi were compared in different liquid media with that in sterile filtered AWF: (1) tap water, (2) biomalt (5 g l^{-1} biomalt, 0.13 g l^{-1} sucrose, 0.18 g l^{-1} glucose, 0.01 g l^{-1} fructose), (3) SNA (1 g l^{-1} KH_2PO_4, 1 g l^{-1} KNO_3, 0.5 g l^{-1} $MgSO_4 \times 7\ H_2O$, 0.5 g l^{-1} KCl), (4) AWF concentrated to compensate for the dilution which resulted from the infiltration (2.5 g l^{-1} sucrose, 1.4 g l^{-1} glucose, 0.3 g l^{-1} fructose), (5) SNA with the same concentrations of carbohydrates as found in concentrated AWF (4), (6) SNA5 contained 5 × the carbohydrate concentrations of SNA. The fungi were cultured in the liquid media (110 rpm) at 20°C for 14 days, harvested using a cellulose filter, and carefully dried (n = 6).

3. Results

Endophytic isolates

All of the 120 endophytic fungi of different genera isolated from bean and barley plants were screened for symptomless growth in the two host plants. The isolates that were only obtained from barley belonged to the genera *Ascochyta, Microdochium, Periconia, Rhizoctonia, Verticillium,* those only from bean to *Botrytis, Coniothyrium* and *Harzia*. The following genera were isolated from both host plants: *Acremonium, Alternaria, Coniothyrium, Drechslera, Epicoccum, Fusarium, Geniculosporium, Phialophora, Ramichloridium, Stachybotrys, Mycelia sterila.*

Further experiments were conducted with those isolates whose infection did not cause symptoms within the young host plants under growth chamber conditions and that could be reisolated at a high rate. Microscopic examination assured that the isolates only grew intercellulary in order to decrease the possibility of symplastic contamination of the AWF, even under application of non-damaging stress. Neither a deposition of cell wall material (papillae) nor that of phenolic metabolites was detected with light and fluorescent microscopy as a defense reaction to infection with any of the endophytic isolates.

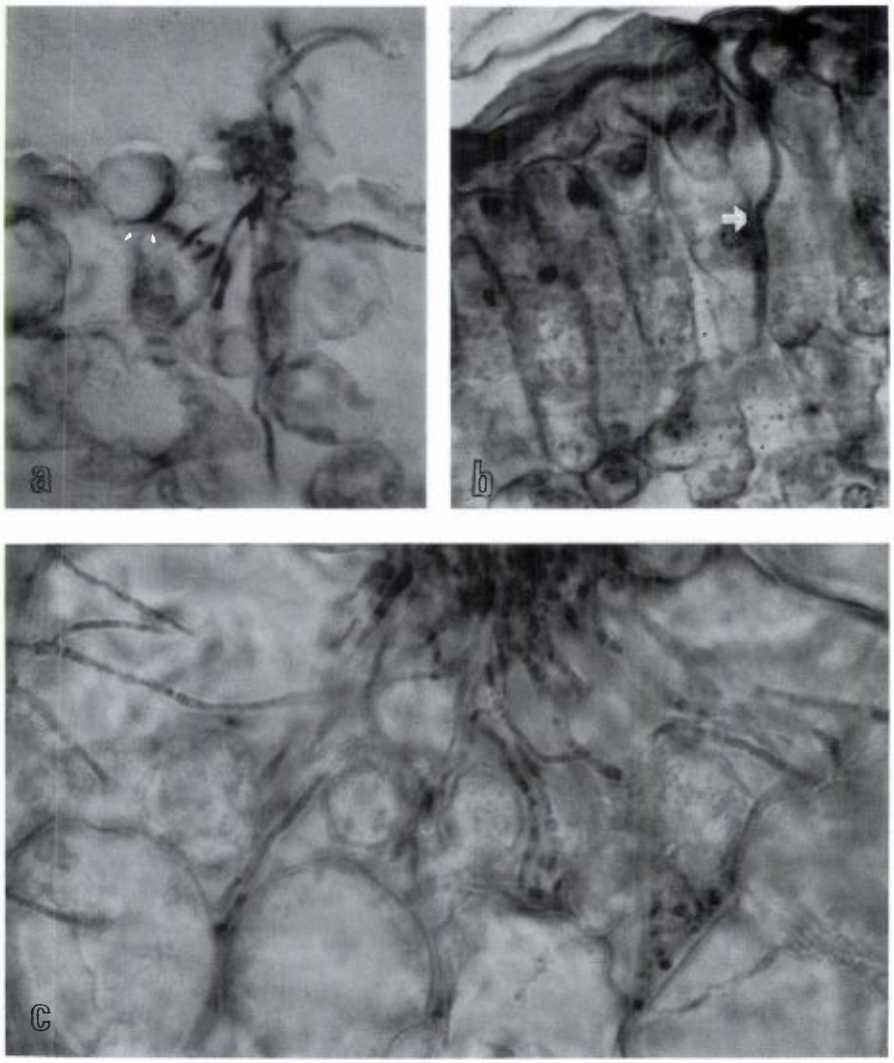


Figure 1. Intercellular growth of *Fusarium* endophytes in bean leaf segments following artificial inoculation and incubation in Petri dishes on biomalt agar: (a) *Fusarium* sp. 2 – invasion of host via stomata, (b) *Fusarium* sp. 2 – growth along the inner cell walls, (c) *Fusarium* sp. 1 – massive intercellular growth along the cell walls of the mesophyll.

Colonization

The success of infection could be verified via reisolation of the fungi from

bean and barley in both field and growth chamber experiments. The endophytic fungi were reisolated from approximately 50% of the leaf segments from each plant, demonstrating that each host plant had been successfully infected with the respective isolate.

The three *Fusarium* endophytes that were selected for further studies invaded both hosts via the stomata and most of them were also able to grow along the anticlinal epidermal cell walls into the mesophyll tissue without penetration of epidermal cell walls. After passing the external barriers, the endophytes grew intercellularly along the inner epidermal cell walls (Fig. 1), and subsequently vertically into the intercellular space along the mesophyll cells (Table 1). Even under weak stress due to wounding, intracellular growth was usually not observed. In contrast to infection with the pathogen, only localized colonization of the endophytes was observed. *Drechslera*, the pathogenic fungus used for both hosts, not only was able to penetrate through the stomata and grow along the anticlinal walls, but also directly through the epidermal cells (Table 1). It grew both inter- and intracellularly without application of stress and *in vitro* produced all the exoenzymes for which it had been tested. Infection with *Drechslera* sp. led to disease symptoms on young plants of both hosts. Although the exoenzymes protease or pectin lyase were not produced by the *Fusarium* isolates sp. 2 and 3, and phenoloxidase was missing in *Fusarium* sp. 1, the enzymes the endophytes could produce were theoretically adequate to enable them to penetrate the host cell wall (Table 2). Nevertheless, penetration of the cell wall by the endophytes was rarely observed.

Biological activity

The pathogenic fungus was able to inhibit the growth of all the test organisms: *Bacillus megaterium* > *Eurotium repens* > *Microbotryum violaceum* with inhibition zones between 15–25 mm, whereas the endophytic isolates *Fusarium* sp. 2 and 3 only inhibited *Chlorella fusca* (20–22 mm). *Fusarium* sp. 1 did not inhibit the coccal green alga, however it did weakly inhibit *Escherichia coli* (10 mm) as well as *E. repens* and *M. violaceum* (3–4 mm).

Apoplastic washing fluid

In order to analyze the effects of intercellular endophytic growth on the constituents of the apoplast of the host, we first checked to see whether or not the selected endophytes were able to grow using the carbohydrates available in the apoplast. After determination of the concentrations of carbohydrates, the main energy sources of the AWF (see also Fig. 3), the growth of *Fusarium* sp.

Table 1. Growth modi of *Fusarium* spp. (endophytes) and *Drechslera* sp. (pathogen) in bean and barley leaves following inoculation of leaf segments

Isolate	Host	Penetration	Colonization		Growth in tissue	
	Bean/barley	Stomata, anticlinal epidermal cell walls, direct	Along the inner epidermal cell walls	Vertically into the intercellular space	Intercellular	Intracellular
Drechslera sp.	be/ba	st, aw	++	++	+	+
Fusarium sp. 1	be/ba	st, aw	+	+	+	–
Fusarium sp. 2	be/ba	st, aw	++	+	+	–
Fusarium sp. 3	be	st	+	+	+	–

Table 2. *In vitro* exoenzyme synthesis by *Fusarium* spp. (endophytes) and *Drechslera* sp. (pathogen)

Isolate	Protease	Amylase	Phenoloxidase	Lipase	Cellulase	Xylanase	Pectin lyase
Drechslera sp.	+	+	+	+	+	+	+
Fusarium sp. 1	+	+	–	+	+	+	+
Fusarium sp. 2	–	+	+	+	+	+	–
Fusarium sp. 3	–	+	+	+	+	+	–

2 and the pathogen in the AWF of young bean plants was compared with that in various liquid media with low or high nutrient content. Both endophyte and pathogen grew best in AWF (Fig. 2), the latter attaining a significantly higher dry weight. Better growth in AWF cannot be a result of its sugar content alone, as demonstrated by a comparison of growth of the isolates in SNA with that in SNA5 medium, since in the latter the sugar concentration was five times higher than in AWF (Fig. 2). Biomalt contains additional nutrient factors and more protein (3.8 g per 100 g), different sugars (50.3 g per 100 g) and fat (0.8 g per 100 g) than AWF. Growth in biomalt resulted, with the exception of tap water, in the lowest biomass. Additional non-microbial growth factors/inducers in sterile filtered AWF with its low carbohydrate content apparently enhanced fungal growth. Further analyses should clarify whether these are e.g. plant hormones, metabolites and/or enzymes.

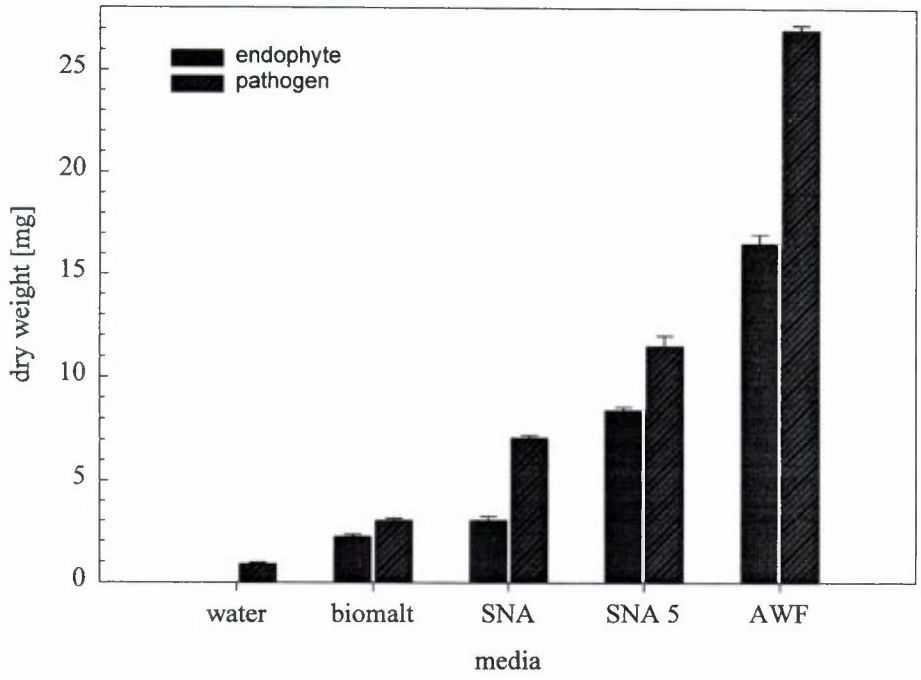
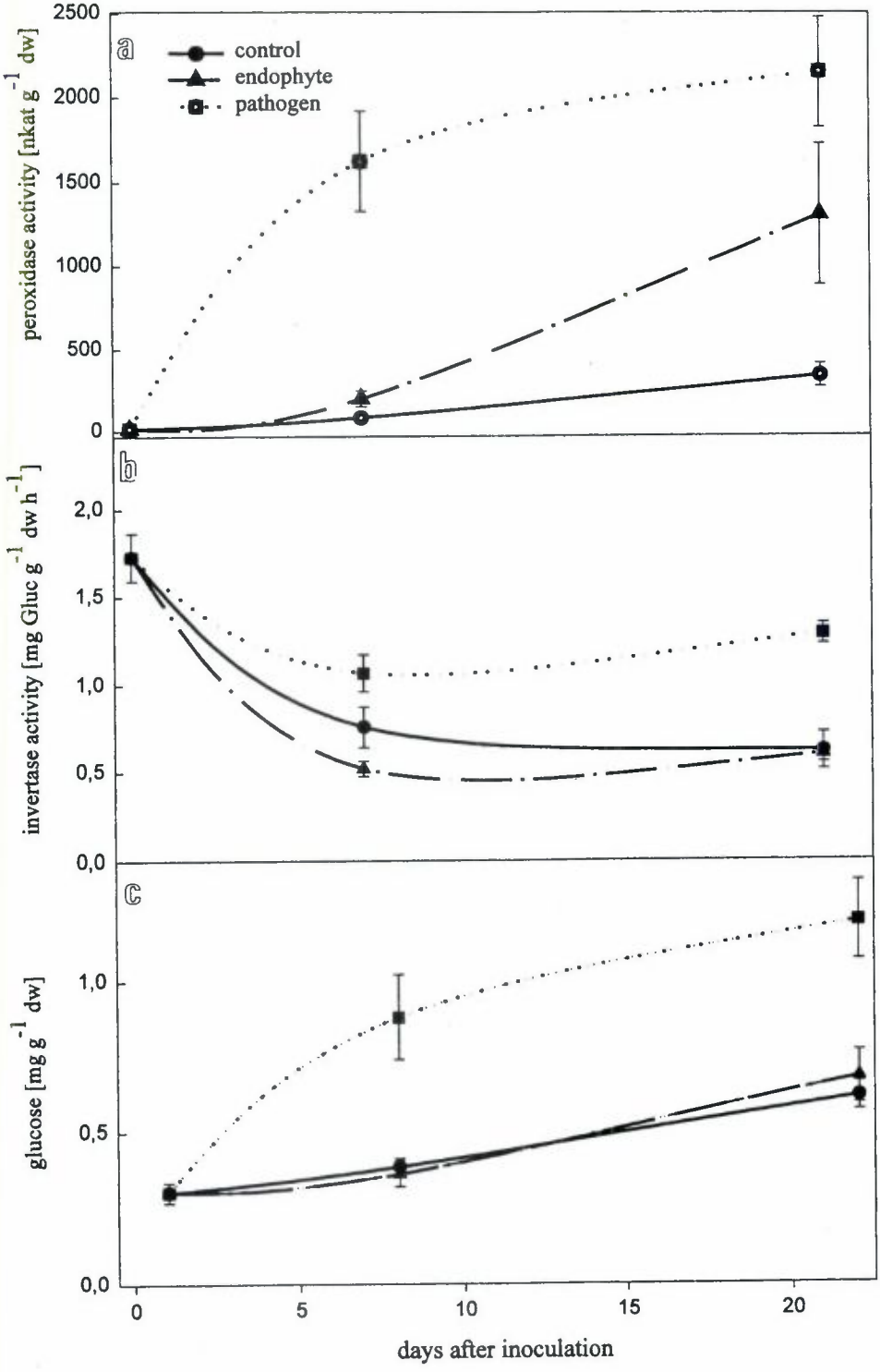


Figure 2. Dry weight of *Fusarium* sp. 2 two weeks after cultivation in different liquid media (110 rpm) at 20°C (n = 6, mean ± standard error). Media: tap water, biomalt (0.5% biomalt 0.13 g l^{-1} succrose, 0.18 g l^{-1} glucose, 0.01 g l^{-1} fructose), SNA mineral medium (1 g l^{-1} KH_2PO_4, 1 g l^{-1} KNO_3, 0.5 g l^{-1} $MgSO_4 \times 7\ H_2O$, 0.5 g l^{-1} KCl, and carbohydrates added as found in AWF), SNA5 (= 5 × carbohydrates of the AWF), AWF (2.5 g l^{-1} sucrose, 1.4 g l^{-1} glucose, 0.3 g l^{-1} fructose).

Significant changes in the concentrations of the constituents of the AWF only occurred when the hosts were infected with the pathogen, but not with any of the three *Fusarium* endophytes. Exemplary for these results were the increase in glucose content of the AWF of barley during the course of infection with the pathogen, but not with the endophyte (Fig. 3c). The data for invertase activity were similar, in that the activity was low in the plants colonized by the endophyte and in the non-infected control, but increased significantly only following infection with the pathogen (Fig. 3b). In the interactions of bean with the endophytes and the pathogen, the results were also similar, although up to the 7th day after inoculation with the endophytes, a non-significant increase was found in the concentrations of all sugars tested (data not shown).

Peroxidase activity was determined as a marker for defense and senescence of the infected plant tissue. While this parameter only increased slightly in the


a
control
endophyte
pathogen
peroxidase activity [nkat g -1 dw]
2500
2000
1500
1000
500
0
b
invertase activity [mg Gluc g -1 dw h -1]
2,0
1,5
1,0
0,5
0,0
c
glucose [mg g -1 dw]
1,0
0,5
0,0
0
5
10
15
20
days after inoculation

AWF of non-infected plants during the 21 days of investigation, the increase was somewhat higher, especially on the 21st day after inoculation for the endophyte-host interactions with barley (Fig. 3a). However, when infected with the pathogen *Drechslera*, the increase in activity of the enzyme commenced on the 7th day after inoculation and reached a higher level than in the plants colonized by the endophyte or non-infected controls. Similar results were obtained with bean as host when infected with *Drechslera*. Peroxidase activity during endophytic infection of bean remained at the level of the control.

Stress

When stress is administered to plants infected by endophytes, the endophytic colonization might either lead to stress tolerance (Römmert, 1998) or alternatively when the host is weakened the endophytes might become weak pathogens (Schulz et al., 1998; Schulz et al., 1999). In a field experiment, shading did not influence the biomass of endophytically infected bean plants in comparison to the controls, similar results were attained following excess N-fertilization in the field as well as under growth chamber conditions. Here, not even an additionally applied UV-A-stress resulted in the development of disease symptoms in either of the hosts when infected with the *Fusarium* endophytes. However, under this stress the biomass of the young bean plants colonized by the endophytes was slightly, though not significantly, higher than that of the non-infected controls. No effect could be measured on barley (UV-A-stress), either on shoot length nor biomass.

Since endophytic colonization might convey the host with induced resistance to subsequent pathogenic infection, barley plants were first inoculated with one of the endophytes and subsequently with the pathogen. Development of disease symptoms was evaluated over a period of 18 days. Independent of the time at which the pathogen was inoculated, the reduction of disease symptoms was not significant during the 18 days of evaluation (Fig. 4). Necrotic disease symptoms developed neither in the control plants nor in the plants infected with the endophyte *Fusarium* sp. 2. Evaluation at the end of the experiment showed that infection with the pathogen, either alone or after preinoculation

See opposite page.

Figure 3. Activity of (a) peroxidase (nkat g^{-1} dw), (b) invertase (mg glucose g^{-1} dw h^{-1}) and (c) concentration of glucose (mg g^{-1} dw) in the AWF of barley leaves inoculated with *Fusarium* sp. 2 (endophyte) or *Drechslera* sp. (pathogen); control sprayed with water. Culture conditions: 18°C, 70% relative humidity, PAR: 210 µmol m^{-2} s^{-1}, growth chamber (n = 5, mean ± standard error).

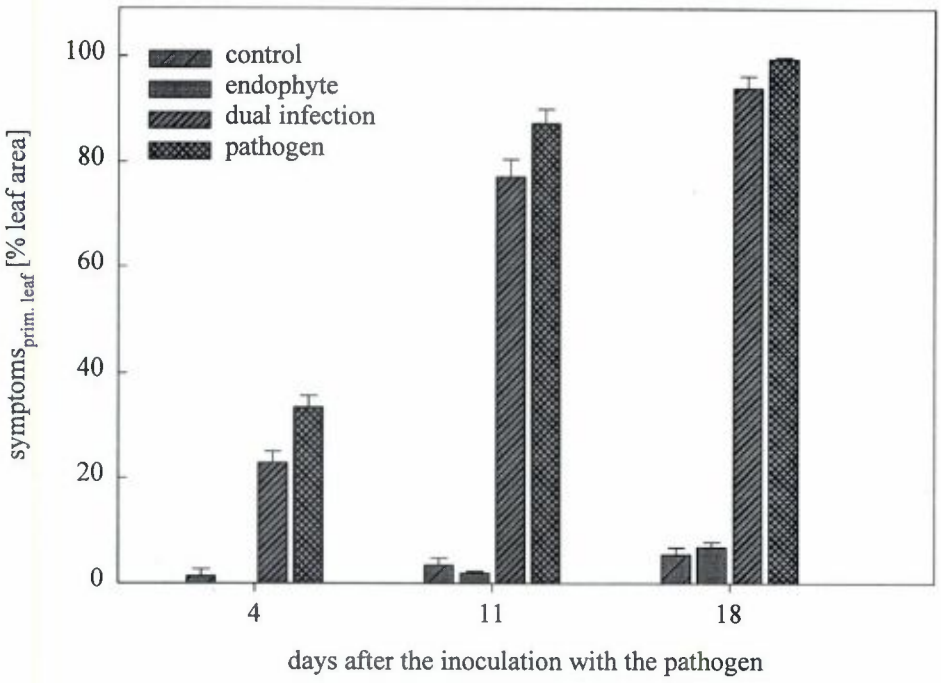


Figure 4. Disease symptoms of pathogen infection on barley previously inoculated with an endophyte. Endophyte = *Fusarium* sp. 2 was inoculated 6 days after sowing, pathogen = *Drechslera* sp. was inoculated 4 days later, control = water. Culture conditions in the growth chambers: 18°C, 70% relative humidity, PAR: 210 µmol m^{-2} s^{-1} (n = 30, mean ± standard error).

with the endophyte, resulted in symptoms covering between 95 to 100% of the area of the inoculated primary area and a reduction of shoot length.

Multiple stress, resulting from variable extremes of precipitation and temperature, short but intensive UV irradiation, as well as coinfection with naturally occurring pathogens was tested in a field experiment on host plants inoculated with endophytes. Even under the multiple stress of field experiments, colonization with none of the inoculated endophytic fungi led to the development of disease visuable symptoms in either of the hosts. Although the success of the infection could be verified by reisolation of the inoculated isolates, microscopic visualization of the infection in the plant tissue was not possible, indicating that growth of the endophytes had remained localized, as had also been observed following inoculation in the greenhouse and growth chamber experiments.

Pathogen infection with *Drechslera* resulted in clear and extensive

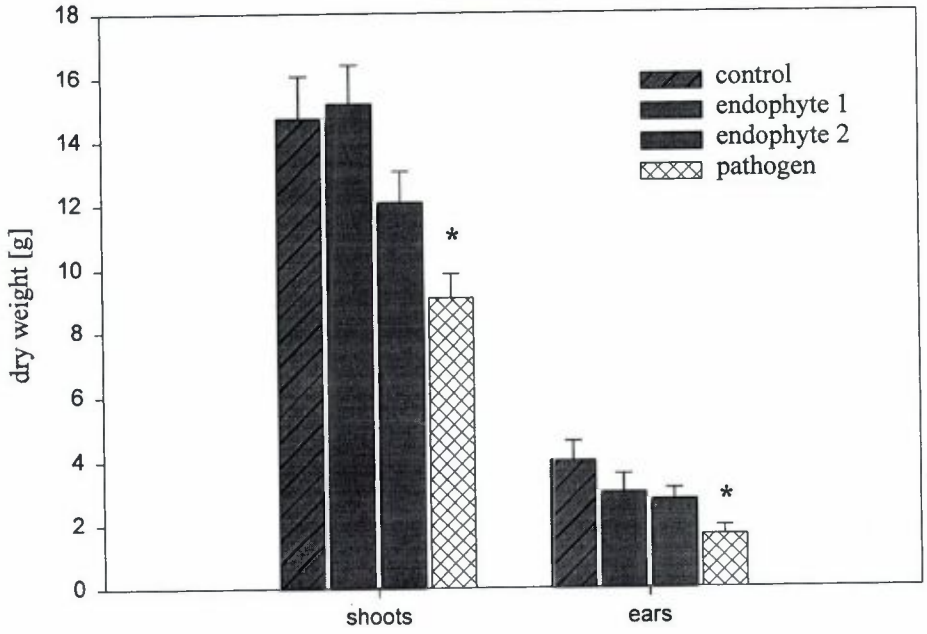


Figure 5. Influence of artificial endophyte or pathogen infection of barley on the dry weights of shoot and ear under field conditions. Endophytes = *Fusarium* sp. 2 (1), *Fusarium* sp. 3 (2); pathogen = *Drechslera* sp. (n = 50, mean ± standard error), control sprayed with water.

development of disease symptoms that were not found in the non-infected control plants. Plant development was defined as number of tillers and leaves per shoot of barley, and number of leaves, blossoms and pods of bean. No significant differences were found in any of these parameters when comparing plants infected with the endophytes with each other or with the control plants. Significant differences could only be demonstrated following infection of barley with *Drechslera*, measured as reduced dry weight of the shoot and ears (Fig. 5), reduced shoot length and fewer ear producing tillers. The effects of endophytic infections with *Fusarium* sp. 2 on these parameters in barley were slightly negative, but not significant, whereas *Fusarium* sp. 1 had nearly no effect on them.

Isolation of fungi following surface sterilization at set intervals showed that the incidence of naturally occurring endophytes in field experiments in the plants inoculated with an endophyte or a pathogen was reduced in comparison to the controls. This evaluation does not include natural infections of obligate biotrophic pathogens such as rust. Infections with this fungus developed in all plots of barley, with and without endophytic inoculation.

Endophytic fungal colonization of the roots in growth chamber experiments could not, even after massive inoculation of the shoots, be directly observed in the tissues of either host, emphasizing the localization of the infections. Quantitative ELISA, kindly done by Prof. Wolf (Göttingen), verified our findings that the shoot infections of barley and bean remained localized: the concentration of *Fusarium* in bean was always low, although a slight increase could be observed up until the 7th day after inoculation, corresponding to the results obtained for peroxidase activity (Fig. 3a). The fungal biomass inside the plant tissue did not increase after this date.

4. Discussion

The three *Fusarium* endophytes did not cause disease symptoms in either host even under conditions of stress. Whereas many species of *Fusarium* are pathogens of shoots and roots of numerous plants, many species and strains of the genus are endophytes within their hosts (Kuldau and Yates, 2000). *Fusarium* was not only found as a common endophyte in all organs of bean and barley (Dammann, 1997), but also in most plants investigated (Guske, 1995; Schulz et al., 1993; 1998; Draeger and Schulz, unpublished). Bacon and Hinton (1996) found that whereas one strain of the same species, *Fusarium moniliforme*, did not cause disease symptoms within maize, another strain of this species did. The non-pathogenic strain they isolated only grew intercellularly in all organs of the host and even in senescent tissue, whereas the pathogenic strain grew both inter- and intracellularly. This is in agreement with the results obtained with the three *Fusarium* endophytes studied here. They only grew intercellularly, even when the host tissue was weakened, senescent or stessed. In order to avoid contamination of the AWF with symplastic fluids, intercellular growth was a prerequisite in our experiments. Intercellular growth is, however, not a prerequisite for asymptomatic growth of an endophyte. There are numerous examples of fungal endophytes that grow intracellularly in the above-ground organs of the host. However, in the reported cases intracellular colonization is confined to single cells, e.g. *Rhabdocline parkeri* in Douglas fir (Stone, 1988) and *Stagonospora innumerosa* and *Drechslera* sp. in *Juncus* spp. (Cabral et al., 1993).

Both the microscopic examinations and the results of the quantitative ELISA of artificially infected leaves suggest that in both hosts endophytic colonization remained localized. This was not a contradiction to the high reisolation frequencies, since the high inoculum density resulted in numerous localized infections. Limitation of these infections was not due to mechanical defense reactions as have been found for some localized infections of endophytes, e.g. *Stagonospora innumerosa* in *Juncus effusus* (Cabral et al., 1993).

Other examples of endophytic colonization in which no visible mechanical defense was observed were *Rhabdocline parkeri* in Douglas fir (Stone et al., 1994), *Fusarium moniliforme* in maize (Bacon and Hinton, 1996) or of an unidentified endophytic fungus in *Juncus bufonius* (Cabral et al., 1993).

Fungal growth was also not limited due to the inability of the *Fusarium* isolates to produce the enzymes required for further growth, since they all had this capacity. Nor was the limited growth of the endophytic *Fusarium* isolates due to their inability to grow in the apoplast, since they grew better in the apoplastic fluid than they did in either complex or synthetic media. However, growth of the endophytes may well have been limited by induced plant defense reactions (Peters et al., 1998).

The limited and localized growth of the *Fusarium* endophytes in bean and barley is in accord with most reports on colonization of the above-ground host organs by non-clavicipitaceous endophytes (Carroll, 1995; Stone et al., 1996). This is in contrast to the extensive hyphal colonization of grasses by clavicipitaceous endophytes (Leuchtmann and Clay, 1995). Only few examples of systemic colonization by non-clavicipitaceous endophytes have been reported, e.g *Pseudocercosporella trichachnicola* in the warm-season grass *Trichachne insularis* (White et al., 1990), an unidentified endophyte in *Juncus bufonius* (Cabral et al., 1993), and *Fusarium moniliforme* in maize (Bacon and Hinton, 1996).

The limited growth observed following infection of the above-ground organs of both hosts with the three *Fusarium* isolates may be due to a balanced antagonism between host and fungus (Schulz et al., 1999). It may also be the reason why no significant changes were measured in the constituents of the apoplastic washing fluid, e.g. in the concentrations of glucose, fructose, sucrose and invertase. Localized and limited growth could also be the reason why endophytic infection in the field experiments with bean and barley did not have any significant effects on yield of the respective crops.

When the localized endophytic infections of the above-ground organs of the host are compared with endophytic colonization of the roots, there are some remarkable differences (Table 3), for example, endophytic colonization of the roots frequently involves extensive systemic infections (Stone et al., 2000; Jumpponen and Trappe, 1998). Fungal-root associations that fit the definition of endophytes and are systemic infections may involve endo- and ectomycorrhizas, ericoid mycorrhizas, the orchid endophytes of the genus *Rhizoctonia* (Stone et al., 2000), the basidiomycete *Piriformospora indica* (Varma et al., 1999), the dark septate endophytes (Jumpponen and Trappe, 1998) or endophytic fungi of other genera, e.g. *Fusarium* (Kuldau and Yates, 2000; Schulz et al., 1998; Schulz et al., 1999), *Cryptosporiopsis* (Kowalski and Bartnik, 1995; Kowalski et al., 1998; Schulz et al., 1999), *Phialophora* (Jumpponen and Trappe, 1998; Schulz et al., 1999) and *Penicillium* sp.

Table 3. A generalized comparison of colonization by non-clavicipitaceous endophytic fungi in the shoots and roots of host plants

	Shoots	Roots
Colonization	Local	Systemic
Degree of	Low	High
Microscopic detection	(+)	++
Immunological detection	(+)	+
Effects on host (compared to control)		
Stress tolerance	0	+
Growth enhancement	0	+
Induced resistance	(+/0)	+
Plant defense reaction	(0/+)	+
Carbohydrate pools/AWF	0	+
Mutualistic fungi	*Neotyphodium*	Endo- and ectomycorrhizal fungi, dark-septate fungi, endophytes, *P. indica*
Antagonistic fungi	Many	Many

(Capellano et al., 1987). Some of these fungi colonize the host roots intercellularly, others both inter- and intracellularly.

Roots may harbor a broad spectrum of endophytic microorganisms, many of these entering into mutualistic interactions with their hosts. These may involve not only eukaryotes, but also prokaryotes, such as Rhizobiaceae, and may involve more than one microorganism. One mutualistic effect of endophytic infection on the host is improved growth (Römmet et al., 1998). Growth of the host is also improved when the roots are systemically colonized by other fungi by improving nutrient supply, e.g. the AM-fungi, ectomycorrhizal fungi (Allen, 1992), *P. indica* (Varma et al., 2000), the orchid endophytes of the genus *Rhizoctonia* (Petrini and Dreyfuss, 1981) and *Phialocephala fortinii* in *Pinus contorta in vitro* (Read, 1982).

Systemic fungal infections of the roots, but not local endophytic infections of the shoots (Table 3), may also counteract the negative effects of stress. Endophytes may alleviate N-stress (Römmert et al., 1998; Römmert et al., unpublished) and that of drought (Varma et al., 2000). Mycorrhizal fungi counteract the stress of drought (Davies et al., 1996), heavy metals (Kaldorf et al., 1999), salt and acid stress (Gupta and Krishnamurthy, 1996).

Systemic colonization of the roots of barley and larch with endophytes led to higher concentrations of plant defense metabolites in the roots infected with

the endophytes as compared to those infected with the pathogens (Schulz et al., 1999). These metabolites might convey the host plants with induced resistance to pathogens. Systemic fungal root infections have been reported to result in induced systemic resistance of the host (Schönbeck and Dehne, 1979; Hallmann and Sikora, 1994; 1996; Bargmann and Schönbeck, 1992; Table 3). Whereas there have also been reports of induced resistance following leaf application e.g. of *Colletotrichum lagenarium* on cucumber (Dean and Kuc, 1987), the localized leaf infections of barley with the endophytic *Fusarium* isolates reported above only tendencially led to induced resistance against further endophytic infection, but not against obligate biotrophs. Analogously, the resistance induced by a localized infection with a tobacco necrosis virus yielded only elevated resistance to anthracnose fungus, but was not adequate to protect against insect or mite attack (Apriyanto and Potter, 1990).

Stress tolerance, induced systemic resistance, improved growth, and increased synthesis of plant defense metabolites resulting from systemic root colonization demonstrate the mutualistic nature of the described root infections. At this stage, it is tempting to hypothesize that mutualistic associations of fungi with plant hosts generally involve systemic root infections. However, in contrast the mutualistic systemic colonization of *Neotyphodium* is confined to the above-ground plant organs. *Neotyphodium* also confers its grass hosts with stress tolerance, improved growth, induced systemic resistance and an increased synthesis of plant defense metabolites (Cheplick and Clay, 1988; Leuchtmann and Clay, 1995; Belesky and Malinowski, 2000; Bultman and Murphy, 2000). Thus, we hypothesize that systemic, but not local, fungal infections may be beneficial for the host and thus may be mutualistic interactions.

The fact that roots in contrast to the above-ground organs of the plants are more frequently colonized systemically by microorganisms may be due to the fact that roots are in close contact with an environment harboring many different mainly degradatively active microorganisms that can potentially provide the plants with water and essential minerals. In the course of time, mutualistic interactions have developed between these microorganisms and the roots as a natural sink of the plants to dual and multiorganismic symbiotic systems. In contrast, due to its photosynthetic capacity, the shoot synthesizes its own metabolites and would not equally benefit from a mutualistic interaction, since the microorganisms could not enhance photosynthesis. In comparison to the roots, the shoot does not have the necessity for development of a mutualistic interaction, nor does it have an equivalent "supply" of potential fungal partners. In addition, the physical contact of the roots with microorganisms is not limited by seasonal fluctuations in the occurrence of spores or in the xeromorphic tissue structures (epidermal wall, wax, etc.) as it is in the shoot. Thus, the evolution of a mutualistic systemic interaction with the roots of the host is more probable than with the above-ground organs.

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