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Note

Peroxidase activity of perennial ryegrass and tall fescue seedlings artificially infected with endophytes

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Abstract – An increase in peroxidase activity is a common response of plants to various stresses, especially to infection by pathogens. It seemed interesting to study the effects of symbiotic fungi of fodder grasses on the peroxidase activity of their hosts. The peroxidase activity of tall fescue cv. Clarine artificially infected with *Neotyphodium coenophialum* or with e-endophytes from other hosts (*N. lolii* and LpTG-2 from *Lolium perenne*, *Epichloë festucae* from *Festuca gigantea* or *Koeleria cristata*, *Epichloë bromicola* from *Bromus erectus*) was generally either lower or not significantly different from that of the non-infected control. Similar results were obtained with perennial ryegrass cv. Vigor artificially infected with different e-endophytes. In contrast, artificial infection of both grasses with *Gliocladium*-like fungi belonging to the group of p-endophytes provoked an increase in peroxidase activity. These results suggest that p-endophytes can be considered as parasites while e-endophytes, which are not able to trigger a non-specific host defence response, are really mutualistic. (© 1999 Inra/Éditions scientifiques et médicales Elsevier SAS.)

clavicipitaceous endophytes / *Gliocladium*-like / *Festuca arundinacea* / *Lolium perenne* / peroxidases

Résumé – Activité peroxydasique de plantules de ray-grass anglais et de fétuque élevée inoculées avec des endophytes. Les plantes stressées (ou attaquées par un pathogène) montrent généralement une augmentation de leur activité peroxydasique. Il a semblé intéressant d'observer l'effet de différents champignons endophytes des graminées sur l'activité peroxydasique de la fétuque élevée (var. Clarine) et du ray-grass anglais (var. Vigor). Clarine, artificiellement inoculée par *Neotyphodium coenophialum* ou par des e-endophytes isolés d'autres graminées (*N. lolii* et LpTG-2 de *Lolium perenne*, *Epichloë festucae* de *Festuca gigantea* et de *Koeleria cristata*, *Epichloë bromicola* de *Bromus erectus*) montre généralement une activité peroxydasique inférieure ou comparable à celle du témoin. Des résultats similaires ont été obtenus pour Vigor. En revanche, l'inoculation par un champignon *Gliocladium-like*, qui appartient au groupe des p-endophytes, entraîne une augmentation significative de l'activité peroxydasique sur les deux espèces-hôtes. Ces résul-

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tats suggèrent le caractère quasi-parasitaire des p-endophytes, tandis que les e-endophytes se comporteraient plutôt comme des symbiotes dont la présence ne déclenche pas l'augmentation de l'activité peroxydasique. (© 1999 Inra/Éditions scientifiques et médicales Elsevier SAS.)

e-endophytes / *Gliocladium*-like / *Festuca arundinacea* / *Lolium perenne* / peroxydases

1. Introduction

In recent years, the importance of symbiotic interactions between grasses and clavicipitaceous fungal endophytes has been more fully recognised. Knowledge of their taxonomy and relationships with their hosts has considerably progressed. It has been shown that these endophytes, called e-endophytes because they are related to species of *Epichloë*, form a continuum from antagonism to mutualism according to their mode of transmission [5]. Mutualism concerns symbionts only able to propagate clonally via the seeds of their hosts. These symbionts are classified in the genus *Neotyphodium*, formerly *Acremonium* section *Albo-lanosa*. The main mutualistic effects of clavicipitaceous endophytes are the production of protective anti-herbivore metabolites and their tendency to enhance host growth and stress tolerance [27, 29].

In addition, non-clavicipitaceous endophytes from some species of the genera *Festuca* and *Lolium*, referred to as p-endophytes [10] or a-endophytes [22], have also been described. P-endophytes include *Phialophora*-like and *Gliocladium*-like Deuteromycetes, which often occur co-symbiotically with e-endophytes on *Festuca* spp. and *Lolium perenne*, respectively. Although some results suggest that p-endophytes are commensal with, or antagonistic towards, their hosts [26, 28], the ecological importance of p- and a-endophyte–grass interactions has not yet been determined.

It has been demonstrated that e- and p-endophytes can be introduced artificially into different host grasses [11]. Such experimental inoculations can help to elucidate compatibility patterns and the range of endophyte hosts. Many successful cross-infections of *Epichloë* or *Neotyphodium* endophytes have been achieved within or between the genera *Festuca* and *Lolium* [3, 9, 13, 21]. However, some of these novel associations showed incompatibility reactions such as the presence of stunted tillers on the new host [3] or the degeneration of endophyte hyphae [9].

Little is known on the physiological processes which could play a role in grass–endophyte relationships. No specific pathogenicity or resistance mechanisms have been reported [12]. However, non-specific reactions from the fungus or the plant have been described; these reactions could explain some cases of incompatibility: i) Clavicipitaceous endophytes can express proteases homologous to other proteases involved in fungal pathogenicity of insects or nematodes [16, 23]. Such proteases may facilitate colonisation of the host by degrading the plant cell wall and/or the apoplastic proteins providing a nutritional source for the fungus; ii) Endophyte infection can increase the expression of chitinase activity [24]. Chitinase is one of the pathogenesis-related (PR) proteins involved in non-specific plant defence responses.

The peroxidases constitute a group of enzymes which play the role of oxidoreductases and catalyse the cleavage of the O-O link. In higher plants, the peroxidases are involved in several physiological processes such as the oxidation of indole-3-acetic acid (IAA), the oxidation of phenolic compounds and the assembly of the cell wall. Peroxidases that play a major role in the synthesis of lignin [17] are involved in the response of the plant to pathogenic organisms [18, 19, 31] by strengthening the cell wall. This phenomenon can delay the penetration of pathogens into the plant cells. Therefore, these peroxidases are considered as PR proteins [30].

Since Roberts et al. [24] showed that chitinase synthesis, a non-specific response to fungal infection, can be modified by the presence of an endo-

phyte, it has been assumed that peroxidase activity could also be influenced by endophytes. The objective of this study was to examine the influence of several endophytes on the peroxidase activity of tall fescue and perennial ryegrass.

2. Materials and methods

2.1. Materials

The artificial host–endophyte associations used in this study mostly corresponded to those created by Naffaa et al. [21]. They concerned tall fescue (cultivar Clarine from which the natural endophyte was removed) and perennial ryegrass (cultivar Vigor, naturally endophyte-free) inoculated with various endophytes belonging to different groups: e-, p- and a-endophytes (*table I*).

2.2. Inoculation

Endophyte-free seeds were surface sterilised. Seeds were then placed on water agar in Petri dishes and incubated in darkness at 23 °C ± 1 for 7 days. After germination of the seeds, a longitudinal slit was cut through the tissue of the young seedlings at the junction of the mesocotyl and coleoptile and a drop of solution of ground mycelium in sterile water was placed in the slit. The mycelium used for inoculation was taken at the periphery of the colonies.

For both cultivars, controls consisted of artificially infected plants where a drop of sterile water was introduced into a wound instead of mycelium.

After inoculation, the seedlings were incubated for 4 days in darkness, then 3 days in light prior to planting in pots. Then, the plants were, after adaptation, grown in the greenhouse.

The plants used for this assay were aged 3 months and had two to six young tillers. The assay was carried out on tillers with three totally developed leaves and in addition, several internal young leaves without flag constituting a pseudo-stem. The fungus was present at least in the sheath of the three oldest leaves.

For most associations, compatibility or incompatibility reactions as well as the level of seed transmission, are reported in *table II* (from Naffaa [20]).

The second leaf sheath of each studied tiller was used for peroxidase extraction. The first basal 5 cm of the pseudo-stem of these plants was also used for other extractions.

2.2. Biochemical methods

2.2.1. Extraction of peroxidases

Leaf sheaths and pseudo-stems (600 mg fresh weight) were ground at 4 °C in 0.1 M phosphate buffer

Table I. List of the different novel associations used in this study.

Associations	New host species	Endophytes	Natural host species
C / Lp-1	*F. arundinacea* (cv. Clarine)	LpTG-2	*Lolium perenne*
C / Lp-80	*F. arundinacea* (cv. Clarine)	*Gliocladium*-like	*Lolium perenne*
C / Fa-3	*F. arundinacea* (cv. Clarine)	*N. coenophialum*	*Festuca arundinacea*
C / Fg-a1	*F. arundinacea* (cv. Clarine)	*Epichloë festucae*	*Festuca gigantea*
C / Be-1	*F. arundinacea* (cv. Clarine)	*Epichloë bromicola*	*Bromus erectus*
C / Kc-1	*F. arundinacea* (cv. Clarine)	*Epichloë festucae*	*Koeleria cristata*
V / Lp-1	*L. perenne* (cv. Vigor)	LpTG-2	*Lolium perenne*
V / Lp-13	*L. perenne* (cv. Vigor)	*Neotyphodium lolii*	*Lolium perenne*
V / Lp-80	*L. perenne* (cv. Vigor)	*Gliocladium*-like	*Lolium perenne*
V / Lpe-1	*L. perenne* (cv. Vigor)	*Acremonium sp.*	*Lolium persicum*
V / Fa-3	*L. perenne* (cv. Vigor)	*N. coenophialum*	*Festuca arundinacea*
V / Fg-a1	*L. perenne* (cv. Vigor)	*Epichloë festucae*	*Festuca gigantea*
V / Be-1	*L. perenne* (cv. Vigor)	*Epichloë bromicola*	*Bromus erectus*
V / Kc-1	*L. perenne* (cv. Vigor)	*Epichloë festucae*	*Koeleria cristata*

Table II. Compatibility of the different associations (according to Naffaa [20]).

Associations	Host reactions			Endophyte	Seed
	Growth	Stunted	Production of endophyte-free tillers or plants	Aspect of hyphae	transmission (%)
C / Lp-1	**delayed**	**frequent**	**frequent**	healthy	93
C / Lp-80	**delayed**	rare	**frequent**	**vacuolated**	37
C / Fa-3	normal	rare	rare	healthy	89
C / Fg-a1	normal	rare	rare	healthy	78
C / Be-1	normal	rare	rare	healthy	ND
C / Kc-1	normal	rare	rare	healthy	ND
V / Lp-1	normal	rare	rare	healthy	100
V / Lp-13	normal	rare	rare	healthy	100
V / Lp-80	**delayed**	rare	rare	healthy	67
V / Lpe-1	normal	rare	rare	healthy	0
V / Fa-3	**delayed**	rare	**frequent**	**vacuolated**	25
V / Fg-a1	normal	rare	rare	healthy	ND
V / Be-1	normal	rare	rare	healthy	ND
V / Kc-1	normal	rare	rare	healthy	ND

ND: No Data

pH 7, at a buffer to tissue ratio of 0.5 mL per g of fresh weight. The homogenate was centrifuged at 10 000 *g* for 15 min at 4 °C. The supernatants were used as enzyme extracts. Each extraction was duplicated.

This procedure allows extraction of soluble enzymes, but not of those enzymes linked to the cell wall.

2.2.2. Proteins quantification

The proteins were quantified according to the method of Bradford [2]. Raw extract (5 mL) was added to 1 mL of Bradford reactant, then the whole was homogenised mildly. The optical density was read after 5 min of reaction at 595 nm per min with a spectrophotometre (Philips Unicam).

2.2.3. Determination of peroxidase activity

Peroxidase activity was determined according to the method reported by Boyer et al. [1] which is based on the increase in absorption of a substrate after its oxidation by peroxidases. Raw extract (5 mL) was added to 1 000 µL of reaction medium containing 900 µL of Na-K phosphate buffer (0.15 M, pH = 6.1) with 0.1 % guaiacol and 100 µL of 0.5 % H_2O_2. Absorbance was read at 470 nm after 1 min of reaction. Specific peroxidase activity was expressed as the increase in absorbance at 470 nm per min and per mg of proteins [6].

2.3. Statistical analysis

Specific peroxidase activity was measured twice (from two distinct extractions) leading to two replicates. As Clarine and Vigor were infected by different sets of endophytes, the data sets from the two hosts were analysed separately.

The influence of each endophyte on specific peroxidasic activity in the leaf sheaths and pseudo-stems of each host was analysed by a variance analysis with one main effect (endophyte isolate). In the case of a significant main effect, means were separated by the two-tailed *t*-test of Dunnett which detects whether any treatments are significantly different from the control.

These analyses were performed by means of the procedures GLM of SAS [25].

3. Results

Regardless of the host plant, the results showed that specific peroxidase activity was remarkably higher in leaf sheaths than in pseudo-stems (*tables III* and *IV*) and the main 'endophyte isolate' effect was highly significant ($P < 0.0001$) for each variable (specific peroxidase activity in leaf sheaths

Table III. Effects of different endophytes on peroxidase activity expressed as the increase of absorbance at 470 $nm \cdot min^{-1} \cdot mg^{-1}$ of proteins of *Festuca arundinacea* (cv. Clarine).

a) In the leaf sheath of three-month-old plants

Endophyte isolate	Peroxidase activity	Difference between endophyte isolate and the control means	Lower confidence limit at the 0.05 level	Upper confidence limit at the 0.05 level
Lp-80	589	222 ***	147.1	296.9
Lp-1	401	34	–40.9	108.9
Control	367			
Be-1	347	–20	–94.9	54.9
Kc-1	295	–72	–146.9	2.9
Fa-3	267	–100 ***	–174.8	–25.1
Fg-1a	226	–141 ***	–215.9	–66.1

b) In the pseudo-stem of the three-month-old plants

Endophyte isolate	Peroxidase activity	Difference between endophyte isolate and the control means	Lower confidence limit at the 0.05 level	Upper confidence limit at the 0.05 level
Lp-80	536	438 ***	380.5	495.5
Be-1	159	61 ***	3.5	118.5
Lp-1	122	24	–33.5	81.5
Kc-1	102	4	–53.5	61.5
Control	98			
Fa-3	98	0	–57.5	57.5
Fg-1a	52	–46	–103.5	11.5

*** Significant comparison at the 0.05 level.

and pseudo-stems). The comparison of means gave different results according to the associations considered.

3.1. *Festuca arundinacea*

In the association between Clarine and the *Gliocladium*-like (Lp-80), specific peroxidase activity in the second leaf sheath was slightly higher than that measured in the pseudo-stem (*table III*). In the other associations, the peroxidase activity in the leaf sheath was considerably higher than that measured in the pseudo-stem.

The Dunnett test shows that in associations with the *Gliocladium*-like, the peroxidase activity in sheaths and pseudo-stems was significantly higher than that observed in the controls, while these variables were not influenced by LpTG-2 and one strain of *Epichloë festucae* (Kc-1). Associations with *N. coenophialum* (Fa-3) and *E. festucae* from *Festuca gigantea* (Fg-1a) exhibited a significantly weaker reaction in the leaf sheath than the controls, whereas there was no difference in the pseudo-stem. *E. bromicola* significantly increased the peroxidase activity in the pseudo-stem of tall fescue while it did not influence that observed in the leaf sheath.

3.1 *Lolium perenne*

In most associations, peroxidase activity in the leaf sheath was significantly lower than that of the control (*table IVa*), with the exception of *E. festucae* (Fg-1a), which did not alter the reaction observed in the leaf, and *Gliocladium*-like which significantly increased peroxidase activity. Such a

Table IV. Effects of different endophytes on peroxidase activity expressed as the increase of absorbance at 470 $nm \cdot min^{-1} \cdot mg^{-1}$ of proteins of *Lolium perenne* (cultivar Vigor).

a) In the leaf sheath of three-month-old plants

Endophyte isolate	Peroxidase activity	Difference between endophyte isolate and the control means	Lower confidence limit at the 0.05 level	Upper confidence limit at the 0.05 level
Lp-80	453	198 ***	129.6	266.4
Control	255			
Fg-1a	198	–57	–125.4	11.4
Lp-13	147	–108 ***	–176.4	–39.6
Be-1	141	–114 ***	–182.4	–45.6
Fa-3	122	–133 ***	–201.4	–64.6
Lpe-1	108	–147 ***	–215.4	–78.6
Kc-1	96	–159 ***	–227.4	–90.6
Lp-1	94	–161 ***	–229.4	–92.6

b) In the pseudo-stem of the three-month-old plants

Endophyte isolate	Peroxidase activity	Difference between endophyte isolate and the control means	Lower confidence limit at the 0.05 level	Upper confidence limit at the 0.05 level
Lp-80	202	149 ***	120.1	177.9
Control	53			
Lp-13	52	–1	–29.9	27.9
Lpe-1	50	–3	–31.9	25.9
Fg-1a	45	–8	–36.9	20.9
Fa-3	42	–11	–39.9	17.09
Be-1	41	–12	–40.9	16.9
Lp-1	30	–22	–51.4	6.4
Kc-1	23	–30 ***	–58.9	–1.1

*** Significant comparison at the 0.05 level.

result was not observed in pseudo-stems; only two associations (with *Gliocladium*-like and *E. festucae* from *Koeleria cristata*) showed a peroxidase activity significantly different from that of the control (*table IVb*). In the pseudo-stem, following the general trend, the *Gliocladium*-like increased the level of peroxidase activity; in contrast, the strain of *E. festucae* (Kc-1) decreased this activity.

4. Discussion

The infection of grasses by endophytic fungi is significant from both an ecological and an agronomical point of view, yet very little is known about interactions between the two organisms. A better knowledge of these interactions could be useful for generating novel associations of agricultural interest. Plants exposed to various environmental stresses respond by synthesising a set of proteins, including peroxidases. Moreover, specific lignin-forming peroxidases are considered as PR proteins [30] and classified as PR-6. Therefore, peroxidase activity could be one out of many markers indicating how the host plant responds to endophyte infection.

Our results show that soluble peroxidase activity is influenced by the age of the plant organs and by the presence of the endophyte.

Peroxidase activity was always higher in the leaf sheath than in the pseudo-stem. Such a result is not surprising because peroxidase activity generally increases with age [1]. As the second leaf sheath was older than the leaves forming the pseudo-stem, the age of each organ accounts for the results obtained.

As regards the endophytes, three different reactions were observed: the peroxydase activity of each association was lower, not significantly different, or higher than that of the control (*table III* and *IV*).

An enhanced peroxidase activity was expected because it is a common response of plants to stresses, such as pathogen infection. Moerschbacher et al. [18] observed such a response for resistant near-isogenic wheat lines infected by stem rust. Ye et al. [31] also reported increased peroxidase activity in tobacco infected by the mosaic virus. This activity was linked with biochemical changes in the cell wall and could be due to specific peroxidases (PR-6). Mouzeyar [19] showed that the global peroxidase activity of sunflower was increased by infection by the downy mildew (*Plasmopara halstedii*). In our study, infection by the *Gliocladium*-like significantly increased the peroxidase activity of the host. In the association involving this endophyte, peroxidase activity was high even in young organs. This may indicate that the *Gliocladium*-like triggers a systemic defence mechanism thus confirming that this endophyte is parasitic rather than mutualistic, as postulated by Siegel et al. [28].

According to Naffaa et al. [21], despite the delayed growth of seedlings, the association between perennial ryegrass and *Gliocladium*-like is stable (*table II*). Though *Gliocladium*-like was not in co-symbiosis with an e-endophyte, the host plants looked healthy. Such observations seem inconsistent with the enhanced peroxidase activity. However, the results were quite different in *F. arundinacea*. For tall fescue infected by *Gliocladium*-like, peroxidase activity in leaf sheaths was almost equal to that observed in pseudo-stems. In addition, the plants with this endophyte showed delayed growth and were able to discard their novel endophyte, resulting in a low rate of seed transmission (*table II*). In this case, the enhanced peroxidase activity was associated with morphological responses. This confirms that *Gliocladium*-like is recognised as pathogenic by tall fescue. As this endophyte is not natural for this species, it cannot overcome the defence responses of tall fescue.

Epichloë bromicola (Be-1) induced a significantly enhanced peroxidase activity in the pseudo-stem of tall fescue (*table IIIb*). As this isolate from *Bromus erectus* can form stromata on its natural host, it is antagonistic rather than mutualistic and it is not surprising that it triggers a defence response from tall fescue. On the contrary, it is more surprising to observe that the peroxidase activity of perennial ryegrass in the leaf sheath can be significantly decreased by this fungus. Leuchtmann and Schardl [14] reported that *E. bromicola* contains both stroma-forming and non-stromal strains. Our results suggest that the same isolate of *E. bromicola* might have an antagonist or mutualistic effect, depending on the species into which it is introduced. Unfortunately, plants harbouring *E. bromicola* died as a result of technical problems, and it was not possible to observe whether they formed stromata on the two hosts.

On the other hand, most e-endophytes decreased or did not significantly alter peroxidase activity of their novel hosts. In leaf sheaths of perennial ryegrass, all the e-endophytes used (with the exception of the strain Fg-1a of *E. festucae*), as well as the isolate of a-endophyte, Lpe-1, significantly decreased peroxidase activity (*table IIIa*). The same behaviour was also observed in tall fescue inoculated with *N. coenophialum* (Fa-3) and *E. festucae* (Fg-1a) (*table IVa*). In the pseudo-stem of perennial ryegrass and tall fescue, most associations responded as the control (*tables IIIb* and *IVb*). The different reactions of young and adult leaves can be explained by the process of plant colonisation by e-endophytes; endophytes are more abundant in the first leaf sheaths than in the younger tissues, colonised later [8], and their metabolic activity is higher in mature tissues [7].

It is also worth noting that different strains of a same species can trigger different reactions, as was the case of Fg-1a and Kc-1, both belonging to *E. festucae*. This can be explained by the considerable genetic differentiation of *E. festucae* which can be harboured by several *Festuca* species [15]. Moreover, Christensen et al. [4] noted that different isolates of *E. festucae* provoked different morphological reactions (isolates referred as stunting or non-stunting) when they were introduced into novel hosts. Neither of the two isolates of *E. festucae* used in this study provoked any morphological reaction in their novel host, nor did they enhance its peroxidase activity.

However, there is no direct relationship between peroxidase activity and morphological reactions. Despite the delayed growth of tall fescue seedlings inoculated by Lp-1 (*table II*), their peroxidase activity was not significantly higher than that of the control. This might indicate that tall fescue accepted this novel endophyte, an hypothesis confirmed by the high rate of seed transmission of the endophyte in its novel host (*table II*). Conversely, the association between Vigor and *N. coenophialum* did not appear to be compatible (*table II*) though the peroxidase activity of the host was not altered by the presence of the fungus.

Our results show that e-endophytes, even introduced into novel hosts, do not generally enhance peroxidase activity. Reddy et al. [23] reported that mutualistic fungal endophytes and some *Epichloë* species express a protease close to proteases suspected to be virulent factors in pathogen systems. Moreover, Roberts et al. [24] showed an increased level of chitinase in endophyte-infected tall fescue. Therefore, in our experiment, the host was expected to exhibit defence reactions. But this was not the case. The lack of enhanced peroxidase activity by e-endophytes may facilitate the colonisation of their host. This phenomenon may indicate that many e-endophytes are not recognised by tall fescue and perennial ryegrass. These fungi may have developed similar mechanisms to escape recognition by grasses. However, the peroxidases are only one group of enzymes involved in the defence reaction of higher plants against micro-organisms. Study of additional systems (PAL, chitinases, glucanases, etc.) might lead to slightly different conclusions. In addition, the procedure used for extraction yields only a fraction of the peroxisases (soluble peroxidases) leaving in the pellet the enzymes linked to the cell wall.

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