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Epichloë grass endophytes increase herbivore resistance in the woodland grass *Brachypodium sylvaticum*

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Abstract Endophytic fungi of the genus *Epichloë* and their asexual *Neotyphodium* forms are thought to interact mutualistically with their host grasses, providing protection for the host against herbivores and pathogens mediated by fungal alkaloids. Most previous research has concentrated on agronomically important grasses, such as tall fescue, and its interactions with livestock grazers or invertebrate herbivores. In this study we focus on the woodland grass *Brachypodium sylvaticum* which is infected by the strictly host-specific endophyte *Epichloë sylvatica*. This fungus has two alternative modes of reproduction: the predominant asexual strains are seed-transmitted, whereas the rare sexual strains are capable of contagious spread by ascospores produced on stromata. To assess potential host protection from herbivory, we tested to what extent development of *Spodoptera frugiperda*, a noctuid generalist herbivore, was affected when fed on different genotypes of naturally infected (E^+), artificially infected (F^+), or uninfected (E^-) leaf material of *B. sylvaticum*. In a feeding assay, insect larvae performed significantly better on a diet of uninfected leaves, even though previous studies have not detected alkaloid production by *E. sylvatica*. A possible explanation for this result may be the presence of an unknown compound in infected plants, acting as metabolic toxin against *S. frugiperda* larvae. The negative effect on insect larvae was increased when they were fed on a diet artificially infected by a particular genotype (F), suggesting that content of allelochemicals may depend on the fungal genotype. In a dual-choice test, neonate *S. frugiperda* larvae initially preferred uninfected seedlings over naturally infected seedlings, but only during the first 6 h of the experiment. This suggests that the unknown compounds can act as weak insect-feeding deterrents. To assess herbivory in natural stands of the host grass, nine infected populations of *B. sylvaticum* were examined for feeding damage. Damage due to micro herbivores and macroherbivores was equal in most populations. However, microherbivores (mostly insects) showed a clear preference for tillers bearing fungal stromata, whereas asymptomatically infected tillers were less damaged. Thus, herbivore resistance of infected plants appears to be correlated with the mode of reproduction of fungal genotypes. This result is of evolutionary significance, since asexual, seed-transmitted endophytes depend on host fitness and seed production for their dispersal.

Keywords Anti-herbivore hypothesis · Clavicipitaceous fungi · Plant-insect interaction · *Spodoptera frugiperda* · Symbiosis

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Introduction

Microorganisms are important mediators of plant-herbivore interactions (Berenbaum 1988; Hammon and Faeth 1992), often contributing symbiotically to the chemical defence system of their hosts. A well-known group of such microorganisms are the fungal endophytes of the genus *Epichloë* (Ascomycota, Clavicipitaceae) and their asexual derivatives of the genus *Neotyphodium* (Glenn et al. 1996). These species form systemic and perennial associations with many C_3 grasses (subfamily Pooideae), and are mostly considered to be beneficial for their hosts (Schardl 1996). The fitness of the host can be positively affected by enhanced growth (Clay 1990) and seed yield (Rice et al. 1990), by increased resistance against drought (Malinowski et al. 1997), and also by improved protection against plant pathogens (Schuster et al. 1995) and herbivores. Clavicipitaceous fungal endophytes can have wide-ranging and often dramatic biological effects on growth and reproduction of grass herbivores (Clay 1988, 1990; Breen 1994; Rowan and Latch 1994; Saikkonen et al. 1998).

The increased herbivore resistance of endophyte-infected plants has mostly been studied in cultivated turf

and pasture grasses, particularly species of *Festuca*, *Lolium* and *Poa* (Hardy et al. 1985; Prestidge and Gallagher 1988; Bultman and Ganey 1995; Schardl and Philips 1997; Siegel and Bush 1997; but see also Schmidt 1986; Cheplick and Clay 1988). By contrast, little is known about the role of *Epichloë/Neotyphodium* endophytes in wild grasses of natural plant communities, such as prairies or woodlands (Faeth et al. 1997; Saikkonen et al. 1999). One such association is formed by *Brachypodium sylvaticum* (Huds.) P.B. which is always infected with its specific endophyte *Epichloë sylvatica* Leuchtmann & Schardl (Leuchtmann and Schardl 1998).

The anti-herbivore properties of *Neotyphodium*-infected host grasses are largely attributable to the production of various biologically active alkaloids, which accumulate within infected host tissues (Porter 1994). Several factors can affect the concentration of allelochemicals and thereby the expression of insect resistance in endophyte-infected plants: the taxonomic identity of the endophyte (Siegel et al. 1990; Bush et al. 1993; Christensen et al. 1993), the endophyte concentration within plant tissue (Breen 1992), soil fertility (Bacon et al. 1977), and also the genotype of the endophyte (Breen 1992; Jones et al. 1997). Characteristic compounds associated with infected host grasses include four classes of alkaloids: saturated aminopyrrolizidines (lolines), pyrrolopyrazines (peramines), ergot alkaloids and indolditerpenes (lolitrems) (Bush et al. 1997; Siegel and Bush 1997). Lolines have a broad spectrum of activity against insects, and to lesser extent also against small mammals (Coley et al. 1995; Bultman et al. 1997). Peramine is known to act as feeding deterrent; mainly against the Argentine stem weevil (*Listronotus bonariensis*); most other insects so far tested have been insensitive to this alkaloid (Prestidge and Gallagher 1988; Rowan and Latch 1994). Ergot alkaloids are primarily active against vertebrates, causing the well known toxicosis syndrome of livestock, whereas lolitrems are responsible for neurotoxic disorders of mammals (Prestidge 1993).

The objective of this study was to examine whether infected plants of *B. sylvaticum* are more resistant to herbivory compared to uninfected plants. We used larvae of the generalist herbivore *Spodoptera frugiperda* (J.E. Smith), the fall armyworm, as a model herbivore, to represent other, natural herbivores of *B. sylvaticum*. *S. frugiperda* is an important insect pest of grasses in warmer areas. The ease of rearing, knowledge of its development and its wide graminoid host range make it an ideal model insect for herbivore assays. Earlier experiments have found reduced larval weights and delayed development of *S. frugiperda* larvae reared on endophyte-infected versus uninfected perennial ryegrass, tall fescue, and other grasses or sedges (Hardy et al. 1985, 1986; Cheplick and Clay 1988). In this study we investigated to what extent the development and reproductive success of *S. frugiperda* were affected when reared on different genotypes of naturally infected, artificially infected and uninfected leaf material of *B. sylvaticum*.

Increased herbivore resistance may provide a fitness advantage for infected host grasses in nature and could explain the observed high level of asymptomatic infection with *E. sylvatica*. In a second part of the study, feeding damage by macro- and microherbivores was assessed in nine populations of *B. sylvaticum* at natural sites where all plants are infected. We scored herbivore damage for stroma-forming and infected flowering plants (or tillers) separately, because different levels of herbivore damage in the two types of tillers could indicate different degrees of endophyte protection.

Materials and methods

The study system

The clump-forming grass *B. sylvaticum* is a common element of beech forests in temperate Eurasia. Surveys in Switzerland and other European countries suggest that probably almost all plants of *B. sylvaticum* are infected with the strictly host-specific fungus *E. sylvatica* (Bucheli and Leuchtmann 1996; Leuchtmann and Schardl 1998). The association is characterised by a high rate of asymptomatic, seed-transmitted infections, and only rare sexual reproduction of the fungus (Bucheli and Leuchtmann 1996; Meijer and Leuchtmann 1999). The wide distribution of *E. sylvatica* and the consistent infection of host plants suggest that infected plants have a substantial fitness advantage. Infected plants of *B. sylvaticum* grown both at natural sites and under well-fertilised green house conditions have been analysed for the presence of lolines, ergovalines, and ergot alkaloids but none of these chemicals were detected (Leuchtmann et al. 2000). The predominant mode of reproduction of *E. sylvatica* is asexual. The hyphae grow into the seeds and derived offspring, which are infected with the same fungal clone. This vertical mode of transmission is very efficient, with all seeds of a tiller becoming infected. The sexual stage, also known as "choke-disease" involves the production of ascospores after fly-mediated cross-fertilisation (Bultman et al. 1998). However, stromata are rarely seen at natural sites and are restricted to clusters within populations of the grass. Horizontal transmission by ascospores has been assumed to be rare, but a recent study has shown that it can be quite frequent (Brem and Leuchtmann 1999).

Plants and herbivores

Seeds of *B. sylvaticum* were collected in bulk from a natural stand of the grass at Risleten, in the Sihlwald area, near Zürich (Switzerland). Because all seeds are naturally infected by the endophyte, half of the seeds were heat-treated for 3 weeks at 37°C and 100% humidity to kill viable fungal mycelia and to obtain endophyte-free seedlings and plants (after Nott and Latch 1993). Treated (E^-) and untreated seeds (E^+) were surface-sterilised and placed on water-agar in sterile petri dishes sealed with parafilm. To break dormancy, seeds were incubated for 3 weeks at 3°C, then transferred to an incubator with continuous light at 22°C. At the time of first leaf emergence, all seedlings were checked under the microscope at a magnification of 400× for outgrowing hyphae to insure that they were endophyte-free.

Because naturally infected seeds were collected from several different plants and therefore most likely harboured genetically different endophytes, we experimentally inoculated some of the endophyte-free seedlings with one selected, previously characterised fungal genotype (F) of *E. sylvatica*. This genotype has distinct isozyme patterns and is predominantly found in flowering tillers of *B. sylvaticum* (Meijer and Leuchtmann 1999). After 2 weeks on agar, the seedlings were transferred to trays with standard potting substrate for another 6 weeks. Afterwards all plantlets were transplanted into small plastic pots (diameter 10 cm) in a

commercial substrate (UFA) with equal parts of peat, compost and natural fibres (68%) mixed with perlite (16%) and sand (16%). Pots in the greenhouse were watered daily and fertilised with liquid fertiliser (20:20:15, N:P:K) every other week from the 3rd week on. After 4 weeks, the plants were again checked for endophyte infection. For feeding trials, single leaves (blade and leaf sheath) were clipped from newly formed vegetative shoots and harvested no more than once from any one plant. For the whole experiment, we used 36 naturally infected, 36 uninfected, and 18 plants infected with genotype F.

Larvae of *S. frugiperda* were obtained from the United States Department of Agriculture (Tifton, Ga., USA) and were reared in the laboratory on an artificial diet. The diet consisted of media prepared from 120 g pinto beans, 55 g wheat germ, 35 g torula yeast, 825 ml water and 15 g water-agar. Additional components were 3.5 g ascorbic acid, 1.1 g sorbic acid and 2.2 g methyl 4-hydroxybenzoate. For the experiments, only individuals of one hatching clutch were used.

No-choice feeding assay

Within 6 h after hatching, the 130 larvae were placed in 5 cm petri dishes (one per dish) on moist filter paper and feeding material added. Petri dishes were stacked inside a transparent plastic bag with a bowl of water to ensure high humidity and kept in an environmental chamber under a 14:10 h (L:D) cycle at 28±1°C. Endophyte-free or naturally infected leaf material were fed to 50 larvae each, while 30 larvae were fed on plant material infected with fungal genotype F. Each day, freshly clipped grass leaves from one plant (towards the end of the experiment from two plants) per treatment (E^-, E^+ and F^+) were equally distributed among all petri dishes of a treatment and supplied in abundance daily to avoid any food shortage. After each feeding, petri dishes were rearranged randomly within the growth chamber. Old leaves and faeces were not removed until the end of the experiment when their dry weight was measured. Each larva from each treatment was weighed 8 days and 14 days after the beginning of the experiment. In addition, pupal weight and the time from the start of the experiment to pupation and to emergence of the moth were recorded. Only larval weight after 8 days fitted a normal distribution. To fit the other variables (larval weight after 14 days, pupal weight and weight of faeces) to a normal distribution, data were x^3-transformed. Results for the four variables were analysed in a one-way analysis of variance (ANOVA). Time to pupation and eclosion were analysed by the Kruskal-Wallis non-linear test. A *G*-test (with Yates's correction) was used to compare differences in survival of *S. frugiperda* larvae among treatment diets at the end of the experiment.

Dual-choice feeding assay

In this assay a total of 122 young seedlings were used as living feeding material. Shortly after germination of the *B. sylvaticum* seeds on water agar, the young seedlings were transferred individually to new 9-cm petri dishes with water agar. Each of the 61 replicate petri dishes contained one infected and one endophyte-free seedling completely separated at opposite sides of the dish. After 2 weeks of growth in an incubator, 10–20 neonate *S. frugiperda* larvae were added to each dish. To keep humidity high, and to prevent larvae from escaping, the petri dishes were sealed with parafilm and kept in an incubator in total darkness for the next 48 h of observation. After 6, 12, 24 and 48 h each dish was examined carefully for the number of surviving larvae, and their location was recorded to assess feeding preference. Additionally, after 12, 24 and 48 h, percentage damage to seedlings by feeding activity of the *S. frugiperda* larvae was estimated according to a rating scale with 12 classes (*0* no damaged area, *1* 0–1% damaged area, *2* 2–5%, *3* 6–10%, *4* 11–20%, *5* 21–30%, *6* 31–40%, *7* 41–50%, *8* 51–60%, *9* 61–70%, *10* 71–80%, *11* 81–90%, *12* 91–100% damaged area).

Herbivory in natural populations of *B. sylvaticum*

Nine different populations of *B. sylvaticum* were examined for herbivore damage in late summer 1999. Four populations selected for this part of the study exhibited a high incidence of stroma-forming plants; two were in the Silhwald area (Risleten and Eichbach) and two near Zürich (Albisgüetli and Denzlerweg). In the other five populations, stromata-forming plants were not observed. The Galgenbüel, Waldburg, Hohfurren and Isleren populations were located in the Pfannenstil area (Kt. Zürich), and the Sihlwald population was located in the Sihlwald area. Populations were at least 0.5 km apart from each other. Within each population surveys were made along transects. A 20-m-long string with a knot every 1 m was used to place transects at randomly selected positions in each population of *B. sylvaticum*. The 20 plants nearest to each of the 20 knots were examined carefully. All leaves (of all tillers) of a plant were counted and scored individually on a rating scale with ten classes, to quantify herbivore damage (Table 1). Percent damage was estimated by comparing damage on leaves to dried and pressed template leaves. Classes 1–7 describe damage due to microherbivores including insect larvae, bugs and small snails. The pattern of damage here is mostly characterised by small holes or by lesions of different lengths along the axis of the leaf. Macroherbivores such as rabbits or roe deer bite off a bit of the leaf, mostly perpendicular to the leaf axis. Such damage was referred to classes 8–10. Densely rolled and totally necrotic leaves were excluded from scoring and do not appear in the statistical evaluation. Mean leaf damage per tiller and population was calculated by multiplying the number of leaves within a given damage category by the mid-point of that category (Table 1), adding the total across all damage classes, and dividing by the number of leaves.

Table 1 Rating scale used to measure the extent of leaf feeding resistance of *Brachypodium sylvaticum* against microherbivores (classes 1–7) and macroherbivores (classes 8–10)

Score	Description	Range of damage (%)	Mean injured leaf area (%)
0	No visible damage	0	0
1	One or a few pinholes (0–2 mm)	>0–1	0.5
2	Several pinholes or a few short holes (2–5 mm)	>1–2.5	1.25
3	A few short holes (5–15 mm)	>2.5–3	2.75
4	Several short lesions (5–15 mm)	>3–5	4
5	A few midsize lesions (16–50 mm)	>5–10	7.5
6	Several midsize lesions (16–50 mm)	>10–20	15
7	A few elongated longer lesions >50 mm	>20–30	25
8	A small part of the leaf eaten	10–30	20
9	A medium part of the leaf eaten	>30–60	45
10	A large part of the leaf eaten	>60–100	80

Results

No-choice feeding assay

In the no-choice feeding assay, mean larval weights of *S. frugiperda* on day 8 and day 14 were significantly higher for larvae feeding on uninfected leaf material of *B. sylvatica*, while larvae feeding on leaf material infected with fungal genotype F performed worst (Table 2). Likewise, pupal weight and the amount of dried larval faeces were significantly lower for larvae feeding on naturally infected leaf material. Further, larvae maintained on infected leaves required nearly 3 days more to pupation and 7 days more to eclosion than larvae feeding on endophyte-free grass-material. Survival at the end of the experiment varied among treatments (Fig. 1). All larvae feeding on *B. sylvaticum* leaves infected with the fungal genotype F died by the 2nd week of the experiment. Survival of *S. frugiperda* larvae feeding on E^+ diet was lower than for the uninfected diet (G=14.34, P<0.001), while survival on E^+ and on F^+ leaf material did not differ significantly (G=1.72, P>0.10). Overall mortality was highest during week 1 on all three diets, then the number of larvae was more or less stable until pupation. At the end of the experiment, after 6 weeks, survival dropped further due to uneclosed pupae (Fig. 1). In addition, feeding on an infected diet negatively affected production of eggs by *S. frugiperda*. The five survivors on the naturally infected diet were able to produce eggs but no progeny, while those on an uninfected diet produced 17 clutches of eggs (510 eggs) resulting in 420 hatched neonate larvae (Table 2).

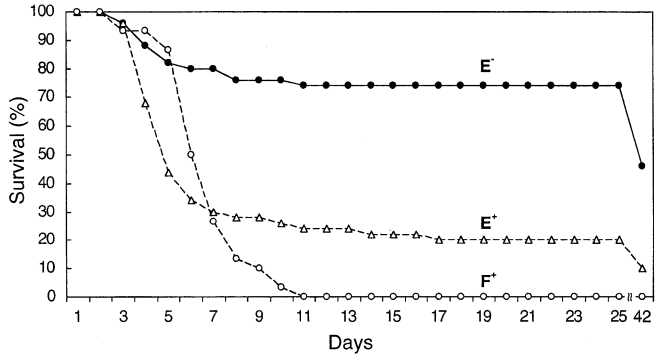

Fig. 1 Survival of *Spodoptera frugiperda* larvae reared on naturally infected (E^+, *open triangles*), infected with genotype F (F^+, *open circles*) or uninfected (E^-, *filled circles*) leaves of *Brachypodium sylvaticum*

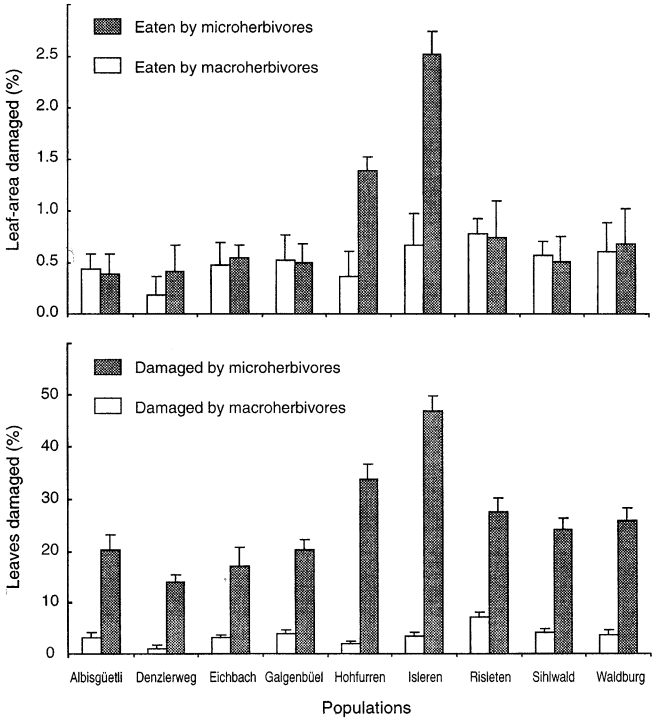

Fig. 2 Proportion of leaves (percentage of total leaf number per population) and leaf area damaged (percentage of total leaf area per population) by natural macro- and microherbivores, for nine different populations of *B. sylvaticum* in summer 1999. *Error bars* represent SEs

Table 2 Effects of endophyte-infected and uninfected leaves of *B. sylvaticum* used as the food source on the development of *Spodoptera frugiperda*, as shown by larval and pupal weights, amount of faeces, days to pupation, days to adult eclosion, and number of eggs and progeny. Weights and days are given ±1 SE with number of replicates in *parentheses*

Leaf tissue of *B. sylvaticum*	Larval weight (mg) at 8 days[a]	Larval weight (mg) at 14 days	Pupae (mg)[a]	Faeces (mg)[a]	Days to pupation[b]	Days to adult eclosion[b]	Eggs	Progeny
Uninfected	519.3±35.6 *a* (38)	3299.5±116.4 *a* (37)	2047.9±30.9 *a* (37)	3883.5±67.4 *a* (37)	17.8±0.2 *a* (37)	26.9±0.5 *a* (23)	510 (23)	420 (23)
Infected with natural genotype	243.0±47.1 *b* (14)	1917.2±352.5 *b* (11)	1583.6±122.9 *b* (10)	2646.0±215.7 *b* (10)	20.5±0.8 *b* (10)	34.0±1.2 *b* (5)	40 (5)	0 (5)
Infected with genotype F	158.8±57.3 *b*	–[c]	–	–	–	–	–	–

[a] Means followed by different *italic letters* within a column are significantly different at $P \leq 0.001$

[b] Means followed by different *italic letters* within a column are significantly different at $P \leq 0.01$

[c] Not further examined as a result of larval death before day 14

Table 3 Dual-choice test assessing larval preference and feeding damage of neonate *S. frugiperda* larvae in the presence of endophyte-infected and uninfected seedlings of *B. sylvaticum* at 6 (not assessed for feeding damage), 12, 24 and 48 h. Means are presented ±1 SE with number of replicates in *parentheses*

	Larval preference (%) after indicated time (h)[a]				Feeding damage after indicated time (h)[a,b]		
Type of seedling preferred	6	12	24	48	12	24	48
Infected	43.1±2.0 *a* (61)	46.5±1.9 *a* (61)	35.6±1.8 *a* (61)	22.1±1.6 *a* (61)	2.54±0.09 *a* (61)	6.22±0.18 *a* (61)	8.65±0.11 *a* (61)
Uninfected	48.8±2.0 *b* (61)	45.5±1.8 *a* (61)	34.4±1.9 *a* (61)	19.5±1.9 *a* (61)	2.72±0.10 *a* (61)	6.38±0.20 *a* (61)	8.80±0.12 *a* (61)
Neither	8.1±0.9	8.0±0.7	30.0±1.8	58.4±2.4			

[a] Means followed by different *italic letters* within a column are significantly different at $P \leq 0.05$

[b] Extent of feeding damage as described in Methods

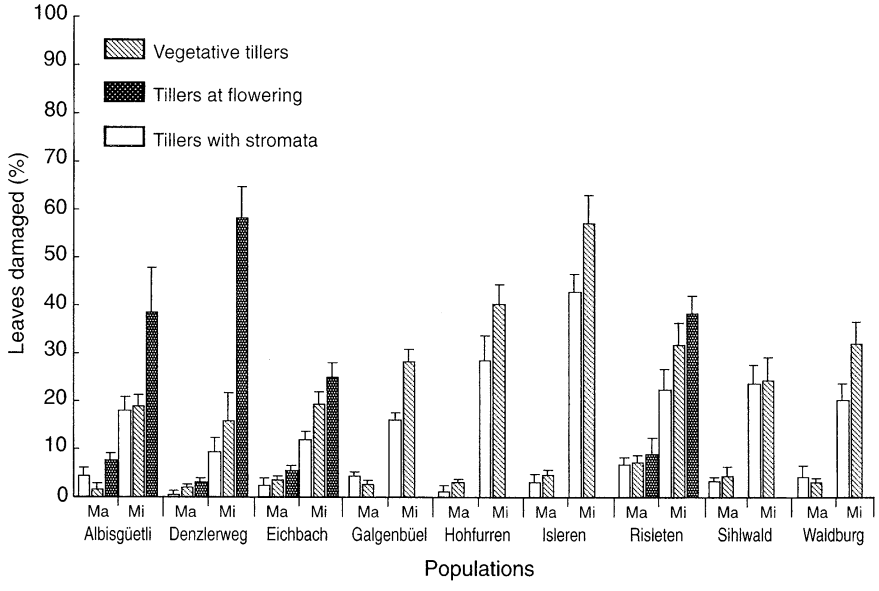

Fig. 3 Herbivore damage by macro- (*Ma*) and microherbivores (*Mi*) in nine populations of *B. sylvaticum*. *Bars* indicate percentages of damaged leaves for tillers with different symptom types. *Error bars* represent SEs

Dual-choice test

S. frugiperda larvae showed some preference ($P<0.05$) for uninfected seedlings over infected seedlings 6 h after the beginning of the experiment (Table 3). At later times, the differences were not significant. Likewise, feeding damage assessed on the rating scale was not significantly different between infected and uninfected plants. However, *S. frugiperda* larvae seemed to weakly favour infected over uninfected seedlings as their diet. The proportion of larvae not feeding, but wandering on the agar increased during the experiment from 8.1% to 58.4%. At the end of the experiment, 624 (of the initial 729) neonate *S. frugiperda* larvae used in the assay survived (85.6%).

Herbivory in nature

Herbivore damage was observed in all populations of *B. sylvaticum*. The total number of leaves examined varied from population to population in a range of 651 (Albisgüetli population) to 1494 controlled leaves (Eichbach population). Leaves of almost all tillers showed at least some traces of herbivore damage. Most of the observed feeding damage could be attributed to microherbivores (Fig. 2). Roe deer were almost exclusively responsible for macroherbivore damage. Because no obvious microherbivores were observed, we suppose that a combination of different insect herbivores (mainly larvae) were responsible for feeding damage. Feeding traces of bugs and snails were only very rarely observed. The proportion of leaves damaged by microherbivores ranged from 17.0% in the Eichbach population to a maximum of 38.2% in the Albisgüetli population. Damage by macroherbivores ranged from 3.1% in the Eichbach population up to a maximum of 6.9% in the Albisgüetli population. Losses of leaf area due to feeding by microherbivores were almost equal to leaf area eaten by macroherbivores in most populations, except for the Hohfurren and Isleren populations, where damage by microherbivores considerably exceeded that by macroherbivores (Fig. 2). Total damaged leaf area due to herbivory varied among populations, ranging from 0.6% in the

Denzlerweg population to 3.2% in the Isleren population (data not shown).

Herbivore damage also varied among tillers of different symptom type or developmental stage within populations (Fig. 3). Tillers with stromata consistently had more leaves damaged by microherbivores than flowering tillers and vegetative tillers, with vegetative tillers usually showing the least damage.

Discussion

Herbivory under laboratory conditions

In the no-choice feeding assay, we found that *S. frugiperda* larvae had significantly lower weights, delayed pupation and eclosion, and a much higher mortality when fed infected leaves of *B. sylvaticum* than when they were fed uninfected leaves. The effects were even more pronounced when *S. frugiperda* larvae were reared on leaf material artificially infected with the distinct fungal genotype F. These results are unexpected because none of the insecticidal alkaloid compounds, commonly found in endophyte infected host grasses (N-acetylloline, N-formylloline, peramine and ergovaline), were detected in samples of the grass material used in this assay nor in any other infected *B. sylvaticum* plant previously analysed (Leuchtmann et al. 2000; C. Schardl, unpublished work). Moreover, the few *S. frugiperda* larvae that survived to mature imagos (10%), produced no viable progeny when reared on infected grass tissues, whereas those reared on uninfected diet produced 420 neonate larvae (Table 2). This indicates that feeding on infected plants could have a significant long-term effect on insect populations. In the dual-choice test using infected and uninfected seedlings as food choice, no clear feeding preference was observed, although initially (after 6 h) more larvae were present on uninfected seedlings. Therefore, infected plants or unknown compounds accumulated in the tissues may not act as feeding deterrents. However, such an effect would be difficult to detect in the closed environment of a petri dish, if feeding deterrence were based on volatile substances. The significant adverse effect on larval development found in the no-choice experiment clearly suggests the presence of metabolic toxins in infected plant material. These toxins could be related forms of known fungal toxins not detectable with the routine methods applied so far (Leuchtmann et al. 2000), or compounds from other chemical classes.

Feeding trials using *S. frugiperda* as a generalist herbivore have been performed with many other endophyte-infected grasses, and have mainly found higher resistance in infected grasses than uninfected controls (Hardy et al. 1985; Ahmad et al. 1987; Cheplick and Clay 1988; Bultman and Ganey 1995). Similar results have been obtained with other noctuids (Schmidt 1986), the hairy chinch bug (Hemiptera: Lygaeidae, Carrière et al. 1998) and with aphids (Homoptera: Aphididae, Siegel et al. 1990), indicating the potential of endophytes to protect grasses from a wide range of insect herbivores. Such protection has also been documented in experimental field situations and in natural stands, particularly of *Lolium perenne* (Prestidge and Gallagher 1988; Johnson-Cicalese and White 1990; Clement et al. 1992). A recent study shows that even populations of leaf-cutting ants (Hymenoptera: Formicidae) are negatively influenced when reared on infected *Festuca arundinacea* (Tibbets and Faeth 1999). Overall, these findings suggest that herbivore resistance found under laboratory conditions with a generalist herbivore should also protect the plant from native herbivores.

Levels of antiherbivore alkaloids are often very high in plants infected with asexual genotypes of the endophyte (Siegel et al. 1990; Leuchtmann et al. 2000). Although the production of secondary compounds must be a considerable metabolic cost for these endophytes, the large investments make sense. Secondary compounds with antiherbivore properties provide increased protection from insects and perhaps other herbivores and thus can enhance host survival. This could be relevant from an evolutionary point of view, because both symbionts are highly dependent on increased fitness and seed-production of the host since they share the same diaspores for dispersal. Therefore, natural selection will favour endophytes (and their hosts), which invest resources into products that enhance host fitness. This could explain in part the wide distribution of this association and the high infection rate with asexual genotypes of the endophyte in populations of *B. sylvaticum* (Bucheli and Leuchtmann 1996; Meijer and Leuchtmann 1999). Alternatively, endophyte infection could also affect seedling establishment and competitive abilities of plants at natural sites and thus may have altered host plant demography as found for other grass/endophyte associations (e.g. Clay and Holah 1999).

Results of feeding assays do not always show that endophyte-infected grasses are at an advantage, particularly in wild grass species (Lopez et al. 1995; Saikkonen et al. 1999). In contrast to cultivars, such as *L. perenne* and *F. arundinacea* which normally contain high levels of alkaloids due to intense selection and breeding, constraints for alkaloid production in wild grasses may be quite different (Saikkonen et al. 1998). Levels of secondary metabolites depend on many different factors, including host plant and endophyte genotype, abiotic factors and even transmission. Therefore, effects on herbivores are expected to be similarly variable, ranging from negative to positive (Clay and Brown 1997; Faeth et al. 1997; Saikkonen et al. 1999; Tibbets and Faeth 1999). We found variation in toxicity levels in our no-choice feeding test, where plant material infected by two fungal genotypes caused different rates of mortality in *S. frugiperda*.

Herbivory in natural populations

Field observations showed that herbivore damage was considerable in natural populations of infected *B. sylva-*

ticum, independent of the location and possible regional differences of the herbivore fauna. Far more leaves were damaged by microherbivores, mostly insect larvae, than by macroherbivores, but the total leaf area damaged was mostly similar for both herbivore groups because macroherbivores ate larger chunks of leaves (Fig. 2). In all nine populations overall leaf damage never exceeded 4%. These values are comparable to insect herbivore damage typically found in herbs of an early succession plant community (Carson and Root 1999).

Because all plants of *B. sylvaticum* are infected in nature, our data on herbivore damage could not be compared to endophyte-free populations of *B. sylvatica*. However, we found that microherbivores have a clear preference for stromata-bearing tillers over flowering or vegetative tillers in populations where stromata occurred (Fig. 3). In symptomless populations, herbivore damage to flowering tillers reached similar levels, which could be due to lack of a choice or to regional differences in the herbivore fauna. Since all tillers appear more or less synchronously and mature at the same time of the year (Brem and Leuchtmann 1999), the feeding preferences of microherbivores were presumably not influenced by age or physiological stage of the tillers, but only by the symptom type. In contrast, herbivory by macroherbivores appeared to be unaffected by symptom type (Fig. 3). It is possible that flowering tillers contain higher levels of an unknown fungal compound (probably an alkaloid) which may have deterred or been toxic to insects but not to mammals. Specific fungal toxins that act on either organismal group are well known from other endophyte associations (Porter 1994; Bush et al. 1997). Further, many asexually reproducing endophytes produce more alkaloids and in higher quantity than their sexual relatives and are therefore better protected from herbivory (Leuchtmann et al. 2000). To explain this finding, it has been suggested that seed-transmitted, asexual endophytes should invest more in host protection than sexual endophytes, because asexual endophytes depend on host fitness and seed production for dispersal, while horizontally transmitted, sexual endophytes do not (Bush et al. 1997). Our observations on *B. sylvaticum* would be consistent with such a hypothesis, assuming that flowering and stromata-forming tillers harbour genetically distinct endophyte genotypes, for which there is evidence (Meijer and Leuchtmann 1999). Of course, alkaloid content may vary in endophyte-infected wild grasses, depending on genetic, geographic or environmental factors (Agee and Hill 1994; Saikkonen et al. 1998) and further studies are needed to test this hypothesis in other natural grass-endophyte associations. A feasible approach would be a manipulative, reciprocal transplant experiment, firstly to assess the effects of environmental conditions on the content of antiherbivore compounds, and secondly to estimate the effect on herbivory of herbivore fauna heterogeneity between populations.

Alternatively, modifications of host tissues caused by the endophyte or high levels of accumulated compounds could be responsible for enhanced protection of flowering tillers against insect herbivory (White et al. 1993). Anatomical alterations such as thickening of epidermal cell walls could act as mechanical barriers against insect herbivores, but not against macroherbivores. The observation by White et al. (1993) of better protection of uppermost stromal leaf blades from insect herbivory is not supported by our data.

Differential protection from insect herbivory of plants or tillers with different symptoms could influence population dynamics of infected *B. sylvaticum*. A possible consequence of higher damage of stromata-forming tillers would be that fewer stromata would reach maturation, resulting in reduced production of inoculum (ascospores) for contagious spread. The number of stromata-forming plants or tillers infected by sexual strains should decrease over time, while the number of asexual associations should increase. In fact, individual plants can be infected by different endophyte genotypes, and plants showing mixed disease expression are most likely infected by two or more strains of *E. sylvatica* (Meijer and Leuchtmann 1999). Because most of the wind-dispersed inoculum will probably land on neighbouring plants, sexual fungal genotypes accumulated in small clusters within the whole population. In fact, local clustering of stromata-forming plants within populations of *B. sylvaticum* is often observed, in agreement with distribution of distinct isozyme genotypes (Bucheli and Leuchtmann 1996).

Conclusions

Results from the two different parts of the study (feeding trials with *S. frugiperda* and observations in natural populations of *B. sylvaticum*) both suggest that asexual strains of *E. sylvatica* may be better able to protect their hosts against insect herbivory than sexual strains. Although other studies of natural or semi-natural grass populations have previously found adverse effects of endophyte infection on herbivores, it has never been shown for a woodland grass. Moreover, our study showed that insect herbivore protection in wild grasses may be variable and dependent on the particular fungal genotype associated with the host-grass. However, given the artificial nature of the model system used here, further experiments with native herbivores are needed to substantiate these conclusions. As in other grass-endophyte associations, we predict that an unknown secondary compound (probably an alkaloid) will be found to be responsible for increased herbivore resistance.

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