

Infection by the systemic fungus *Epichloë glyceriae* alters clonal growth of its grass host, *Glyceria striata*

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Parasites and pathogens are hypothesized to change host growth, reproduction and/or behaviour to increase their own transmission. However, studies which clearly demonstrate that parasites or pathogens are directly responsible for changes in hosts are lacking. We previously found that infection by the systemic fungus *Epichloë glyceriae* was associated with greater clonal growth by its host, *Glyceria striata*. Whether greater clonal growth resulted directly from pathogen infection or indirectly from increased likelihood of infection for host genotypes with greater clonal growth could not be determined because only naturally infected and uninfected plants were used. In this study, we decoupled infection and host genotype to evaluate the role of pathogen infection on host development and clonal growth. We found that total biomass production did not differ for clones of the same genotype, but infected clones allocated more biomass to clonal growth. Disinfected clones had more tillers and a greater proportion of their biomass in the mother ramet. Infected clones produced fewer tillers but significantly more and longer stolons than disinfected clones. These results support the hypothesis that pathogen infection directly alters host development. Parasite alteration of clonal growth patterns might be advantageous to the persistence and spread of host plants in some ecological conditions.

Keywords: clonal growth patterns; plant–pathogen interactions; endophyte infection of grasses

1. INTRODUCTION

Parasites may change the morphology, phenology, growth and/or reproductive behaviour of their animal hosts (Dobson 1988; Moore & Gotelli 1996; Webb & Hurd 1999; Gourbal *et al.* 2001). It is often assumed that these changes benefit the parasite by increasing its transmission rate (Bakker *et al.* 1997; Levri 1999; McCurdy *et al.* 1999). For example, increased susceptibility to predation by infected hosts has often been reported for systems where parasites have complex life cycles with alternate hosts (Lafferty 1999; Pulkkinen *et al.* 2000; Knudsen *et al.* 2001). However, questions have been raised about the generality of the host manipulation hypothesis when it has been subject to detailed analyses (Hurd 1998; Levri 1999; Brown *et al.* 2001; Franz & Kurtz 2002). Nevertheless, these systems may provide insights into host–parasite conflict, pathways to parasite fitness and mechanisms by which parasites affect normal host physiology and behaviour.

There are fewer reported cases of host manipulation by plant parasites. The best evidence of host manipulation by plant parasites comes from systemic parasites, primarily fungi (Roy 1993). Plants may be more difficult for parasites to manipulate than animals because of their indeterminate growth form and modularity, but systemic infections provide the opportunity for coordinated alteration of the whole plant. Alteration of particular organs or plant parts that are directly or indirectly involved in parasite reproduction provides the best evidence of host manipulation. Increased attractiveness of infected plants to herbivores such as aphids that act as virus vectors rep-

resents an analogue to increased predation of animal hosts (Orenski 1964). Alternatively, changes in floral morphology or the production of flower mimics can increase parasite transmission when the parasite is dispersed by pollinating insects (Batra & Batra 1985; Roy 1993; Naef *et al.* 2002). *Arabis* sp. infected by the rust *Puccinia monoica* produce pseudoflowers that are actually bright yellow leaves covered with rust reproductive structures (Roy 1993). Pseudoflowers produce nectar to attract pollinators and are often more successful at attracting pollinators than nearby flowers (Roy 1994a). Likewise, systemic *Epichloë* fungal endophytes use *Botanophila* flies to vector gametes (spermatia) (Bultman *et al.* 1998). Spore-bearing stromata are produced that ‘choke’ the host inflorescences (Clay 1998) and emit volatile compounds to attract the flies (A. Leuchtman, personal communication).

Pathogens can influence the growth pattern and spatial spread of clonal plants by altering the number of stolons or rhizomes, the longevity of clonal connections or the placement of daughter ramets. Previous research has generally shown detrimental effects of pathogens on clonal growth (García-Guzmán & Burdon 1987; Nus & Hodges 1990; Wennström 1994; D’Hertefeldt & Van der Putten 1998; Piqueras 1999). For instance, *Poa pratensis* plants infected by *Ustilago striiformis* produced fewer rhizomes than uninfected plants (Nus & Hodges 1990). The fitness of systemic pathogens is particularly tied to their host plants because they are propagated along with the growth of their hosts. Thus, greater growth and survivorship by hosts could lead to greater growth and survivorship for the pathogen.

In a previous study, we found that infection by the systemic fungus *Epichloë glyceriae* was associated with greater clonal growth of its host grass *Glyceria striata*, although total biomass did not differ between infected and uninfected plants (Pan & Clay 2002). Greater clonal growth

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could increase resource acquisition, competitive ability, survival of the host (Pan & Price 2001), as well as survivorship and spore production of the fungus if stromata production increases with host size and spread. However, infection and host genotype were confounded because only naturally infected and uninfected *Glyceria* plants were used. The observed growth patterns may have been due to either infection status or inherent differences in host genotypes (Roy 1994b). Genotypes with greater clonal growth could have a greater chance of becoming infected because they are a bigger 'target'.

The objective of this study was to experimentally separate the effects of infection and host genotype by testing the following hypotheses using infected and disinfected plants of the same host genotypes: (i) infection by *E. glyceriae* directly alters *G. striata*'s biomass allocation towards greater clonal growth; or (ii) *G. striata* genotypes with greater clonal growth are more likely to become infected. If infected and disinfected plants have different growth patterns, then the first hypothesis would be supported. If, however, infected and disinfected plants have similar growth patterns, then the second hypothesis would be supported.

2. MATERIAL AND METHODS

(a) *Study system*

Glyceria striata (tribe Meliceae, Poaceae; hereafter *Glyceria*) is a cool-season, perennial wetland grass found throughout the United States (Hitchcock & Chase 1971). Flowering occurs in the late spring or early summer and is followed by growth through tiller or stolon production, which we refer to as vegetative or clonal growth, respectively (Pan & Clay 2002). Plants used in this study were collected from three locations in southern Indiana, USA: Beaver Bog (BE) (Owen County) Bloomfield Barrens (BL) (Spencer County) and Thomson Park (TH) (Monroe County).

Above-ground parts of *Glyceria* plants can be systemically infected by the host-specific fungus *E. glyceriae* (Clavicipitaceae, Ascomycotina (Schardl & Leuchtman 1999)). Infections are perennial and apparent only when *Glyceria* flowers, at which time *E. glyceriae* (hereafter *Epichloë*) forms stromata that envelop the top of reproductive culms (Hill 1994). Infected *Glyceria* produce only these 'choked' inflorescences and are sterilized as a result. Stromata produce conidia (asexual spores) and/or ascospores (sexual spores) (for details see Schardl (1996)), which may infect uninfected adult plants or florets. We determined *Glyceria* infection status both morphologically, whether plants produced inflorescences (uninfected) or stromata (infected), and microscopically, by examining leaves and culms for the presence of fungal hyphae (Bacon & White 1994).

(b) *Experimental design*

We collected two infected plants from each of three populations (refer to Pan & Clay (2002) for a more detailed description of plant collection and genotype determination). To obtain infected and uninfected *Glyceria* clones of the same genotype, tillers were first separated from each infected plant. Then half of the tillers were potted in a styrofoam cup filled with sand and disinfected by applying 40 ml of the fungicide Benomyl (1 g l^{-1}) every two weeks for up to three months. Remaining tillers were placed into pots filled with a 1 : 1 mixture of soil and peat and were left untreated. After the first month of fungicide appli-

cation, young tillers were separated from the original tiller and planted in a 1 : 1 mixture of soil and peat. To eliminate residual fungicide effects, all tillers were propagated for four or five tiller generations from the initially treated tiller, which spanned six to eight months. During this time most of the disinfected tillers flowered, confirming that they were no longer infected. Plants were also examined microscopically and only infection-free plants were used for the disinfected group. Microscopic examination of a subsample of plants at the end of the experiment indicated that infection status did not change.

Four replicate tillers were used from each of 12 treatment groups (three populations, two genotypes from each population, and two infection levels for each genotype), for a total of 48 experimental plants. Tillers were randomly assigned to and planted into $28 \text{ cm} \times 200 \text{ cm}$ cells in the greenhouse at the Indiana University Botany Experimental Field following the techniques of Pan & Clay (2002). Plants received only water throughout the course of the experiment and were subjected to ambient light and temperature regimes.

Above-ground biomass was harvested after two and a half months, separated into clonal growth (stolons and daughter ramets) and the mother ramet (tillers produced by the original planted tiller), dried and weighed. This separation reflects biomass allocation to lateral spread (clonal growth) and space occupation (vegetative growth) (de Kroon & Schieving 1990). We also counted the number of tillers in the mother ramet, primary stolons, secondary stolons (stolons produced on primary stolons), the number of ramets per stolon, and measured stolon length and spacer lengths (distance between ramets). Below-ground roots were not harvested because it was not possible to separate roots of different ramets.

(c) *Statistical analyses*

All statistical analyses were conducted using PROC GLM in the SAS software package, v. 6.12 (SAS Institute Inc., Cary, NC, USA). First, we determined whether overall growth of plants was different by conducting univariate ANOVAs on two variables that estimated total growth, the number of tillers plus primary stolons produced by the mother ramet and total biomass per plant. We then used a MANOVA to determine whether plant growth patterns and architecture of clonal growth differed (Stevens 1996). The model of infection, population, genotype nested within population, infection by population interaction, and infection by genotype nested within population interaction was tested using the following growth variables: total number of tillers produced by the mother ramet, the total number of stolons per plant, mother ramet biomass and clonal growth biomass. Three stolon characteristics, biomass per stolon (including daughter ramets), mean spacer length of primary stolons and mean stolon length, were used in the analyses for architecture of clonal growth.

To determine which response variables differed, univariate ANOVAs were conducted on all response variables used in the MANOVA. The Bonferroni inequality was used to control for type 1 error rate. Effects were considered significant at $p = 0.025$ for those variables used in the MANOVA for plant growth pattern and $p = 0.033$ for architecture of clonal growth. We used $\alpha = 0.10$ to maintain a reasonable balance between type 1 and type 2 error rates and to increase power (Bray & Maxwell 1985; Stevens 1996).

All analyses were conducted on untransformed data; ANOVA and MANOVA procedures are generally robust to violations of equal variances and normality when sample sizes are equal

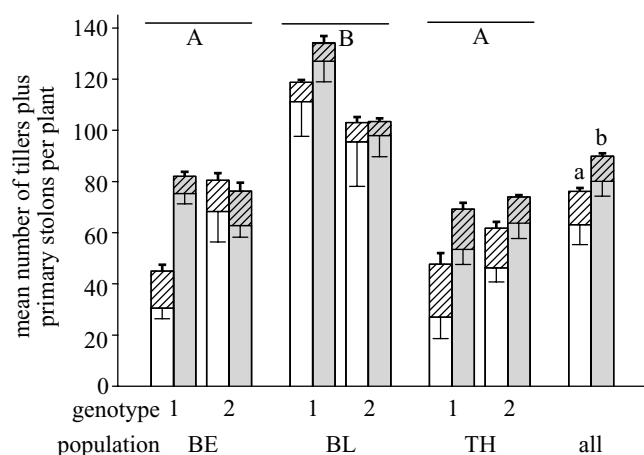


Figure 1. Overall growth of infected (I) and disinfected (D) *Glyceria striata* plants at the end of the study. Means (\pm s.e.) for the number of tillers plus primary stolons per mother ramet by genotype nested within population ($n = 4$), and across all populations (all, $n = 24$). Standard error bars for primary stolons have smaller caps and are in bold. Populations are Beaver Bog (BE), Bloomfield Barrens (BL) and Thomson Park (TH). Individual bars or sets of bars connected by the same line with different letters are significantly different (lower case letters are for infection and capital letters are for population). White bars, I-tillers; white hatched bars, I-stolons; grey bars, D-tillers; grey hatched bars, D-stolons.

(Stevens 1996; Underwood 1997). The infected plant genotypes used in this study were also used in Pan & Clay (2002) and found to have different growth patterns from naturally uninfected plants. Having the same infected genotypes in both of these studies allowed for a qualitative comparison between disinfected and naturally uninfected plants. Therefore, because infected plant genotypes used in this study were not randomly chosen, effects for all analyses were considered fixed.

3. RESULTS

(a) Overall growth

Total biomass did not differ between infected and disinfected plants ($F_{1,47} = 0.03$, $p = 0.86$), indicating that fungal infection had no effect on overall growth of host plants. However, fungal infection affected the number of tillers plus primary stolons produced by the mother ramet ($F_{1,47} = 6.37$, $p = 0.02$). Disinfected plants had more tillers plus primary stolons in the mother ramet than infected plants, primarily reflecting the greater number of tillers (figure 1). The effects of infection did not vary with population or genotype nested within population.

(b) Growth patterns

Infection, population, genotype nested within population and the infection by genotype within population interaction significantly affected *Glyceria* growth patterns (MANOVA, Pillai's trace, $p < 0.05$ for all effects; table 1). Disinfected plants produced more tillers in the mother ramet but had fewer stolons than infected plants (figure 1), although the infection by population interaction approached significance for number of stolons (table 2). Consequently, disinfected plants had greater mother

Table 1. Effects of infection status, host population and genotype nested within population on *Glyceria striata* growth patterns.

(MANOVA using number of tillers produced by the mother ramet, mother ramet biomass, total number of stolons and clonal growth biomass. Significant effects are in bold; see § 2c for criteria. Abbreviations: num. d.f., numerator degrees of freedom; den. d.f., denominator degrees of freedom. F -values are for Pillai's trace. I, infection status; P, host population; G(P), genotype nested within population.)

effect	num. d.f.	den. d.f.	F -value	p -value
I	4	33	23.63	0.0001
P	8	68	13.08	0.0001
G(P)	12	105	4.06	0.0001
I \times P	8	68	1.32	0.25
I \times G(P)	12	105	2.13	0.02

ramet biomass but less clonal growth biomass than infected plants (figure 2), although infection status approached significance only for clonal growth biomass (table 2).

(c) Stolon architecture

Stolon architecture significantly differed by infection, population, genotype within population and the infection by genotype within population interaction (MANOVA, Pillai's trace, $p < 0.05$ for these effects). Stolons from infected plants were slightly longer than stolons from disinfected plants ($F_1 = 8.18$, $p = 0.01$, mean \pm s.e. = 30.14 ± 1.20 and 26.48 ± 0.98 cm, respectively). The effect of infection on spacer lengths depended on population and genotype nested within population. Spacer lengths were longer for infected plants compared with disinfected plants in the BE population (mean \pm s.e. = 1.94 ± 0.24 versus 1.31 ± 0.12 cm, respectively), but were slightly shorter for infected plants in the BL and TH populations (BL: mean \pm s.e. = 1.18 ± 0.04 versus 1.36 ± 0.12 cm; TH: 2.19 ± 0.21 versus 2.30 ± 0.18 cm, infected versus disinfected, respectively). In each population, one genotype had significantly longer spacer lengths when infected, while the opposite was true in the second genotype.

In addition to the three variables used in the MANOVA, we also determined whether numbers of primary and secondary stolons changed with infection status. Infected plants had more primary and secondary stolons than disinfected plants, although the number of secondary stolons varied by infection and population (figure 3). Not only did infected plants have a greater number of primary and secondary stolons ($F_1 = 4.81$, $p = 0.03$ and $F_1 = 11.74$, $p = 0.002$, respectively), secondary stolons made up a larger proportion of their total stolons ($F_1 = 4.47$, $p = 0.04$, mean \pm s.e. = 0.18 ± 0.03 for infected and 0.11 ± 0.03 for disinfected plants). Plants from the BL population produced few secondary stolons, and infected and disinfected plants had similar numbers of them. Infected BE and TH plants generally produced more secondary stolons than their disinfected counterparts. Thus, greater clonal growth by infected plants was composed of greater numbers of both primary and secondary stolons (figure 3).

Table 2. Effects of infection status, host population and genotype nested within population on *Glyceria striata* growth patterns. (Univariate analyses for each of the four response variables used in the MANOVA. Significant effects are in bold; see § 2c for criteria. Degrees of freedom are: I = 1, P = 2, G(P) = 3, I × P = 2, I × G(P) = 3 and error = 36. Abbreviations: I, infection status; P, host population; G(P), genotype nested within population.)

response variable	effect	mean square	F-value	p-value
number of tillers produced by the mother ramets	I	3434.08	10.45	0.003
	P	16 394.27	49.87	0.0001
	G(P)	1170.13	3.56	0.02
	I × P	187.77	0.57	0.57
	I × G(P)	927.21	2.82	0.05
mother ramet biomass	I	1699.24	83.88	0.0001
	P	1699.24	83.88	0.0001
	G(P)	333.08	16.44	0.0001
	I × P	28.24	1.39	0.26
	I × G(P)	60.16	2.97	0.04
total number of stolons	I	432.00	10.43	0.003
	P	702.25	16.96	0.0001
	G(P)	156.75	3.79	0.019
	I × P	142.75	3.45	0.04
	I × G(P)	72.83	1.76	0.17
clonal growth biomass	I	1453.53	4.50	0.04
	P	3643.63	11.28	0.0002
	G(P)	1338.81	4.15	0.01
	I × P	903.52	2.80	0.07
	I × G(P)	168.00	0.52	0.67

4. DISCUSSION

(a) *Pathogen-induced host changes*

The first step to demonstrating parasite manipulation of hosts to increase parasite transmission rate is confirming that the parasite is responsible for changes in host growth or behaviour. In this study, we have shown that the fungal pathogen *E. glyceriae* modifies growth patterns of its host grass *G. striata*. *Glyceria* plants with the same genotype had different growth patterns depending on whether they were infected or disinfected, supporting the hypothesis that *Epichloë* infection induced changes in host growth patterns. Infected plants produced more stolons and had greater clonal growth than their disinfected counterparts, indicating that *Epichloë* infection leads to greater allocation of host resources to clonal growth (i.e. lateral spread). Disinfected *Glyceria* allocated proportionally more resources to the mother ramet (i.e. to space occupation *sensu* de Kroon & Schieving (1990)).

The differences in clonal growth biomass and stolon characteristics between infected and disinfected plants indicate a change in resource distribution and function (lateral spread versus space occupation) when the pathogen was removed. However, there was no difference in total biomass, indicating that infected and disinfected plants had similar resources available for growth, in contrast to other clonal plant-pathogen systems (García-Guzmán & Burdon 1997; D'Hertefeldt & Van der Putten 1998; Wennström 1999). Thus, equivalent resources were allocated differently between lateral spread and space occupation for infected and disinfected *Glyceria*. In other systems, reduced biomass allocation to one or both functions typically results from pathogen infection (De Battista *et al.* 1990; Nus & Hodges 1990).

Results from this study on infected versus disinfected plants differed from those obtained from a previous study

using infected versus uninfected plants (Pan & Clay 2002) for three clonal growth characteristics: clonal growth biomass, spacer lengths and stolon lengths. Genotypic variation is likely to be responsible for the differences observed for clonal growth biomass and spacer lengths between the two studies. For both clonal growth biomass and spacer lengths, the difference between infected and disinfected plants of the same genotypes (this study) was smaller than between infected and uninfected plants of different genotypes (Pan & Clay 2002). However, infected plants had longer stolons than disinfected plants (this study), but had stolons of similar length to those of uninfected plants (Pan & Clay 2002), suggesting that *Epichloë* infection caused an increase in stolon length. The tendency towards longer stolons is opposite to what has been reported in other clonal plants, where infected plants had shorter stolons than uninfected plants (García-Guzmán & Burdon 1997; D'Hertefeldt & Van der Putten 1998; Piqueras 1999). Longer stolons could lead to greater spatial distribution by infected *Glyceria*.

The most dramatic effect of infection was on stolon number. Overall, infected plants produced more primary and secondary stolons than disinfected plants, although this differed by population. These results contrast with findings from other studies, where stolon production was reduced in pathogen-infected plants (Nus & Hodges 1990; D'Hertefeldt & Van der Putten 1998; Piqueras 1999). These differences among plant-pathogen systems could be the result of variation in the specific interaction between plant and pathogen (Ahlholm *et al.* 2002; Wille *et al.* 2002). Our results suggest that *Epichloë* is not as detrimental to *Glyceria* compared with other pathogen-clonal plant systems. Except for sterilization of inflorescences, there is no evidence of any harmful effects of infection in this system.

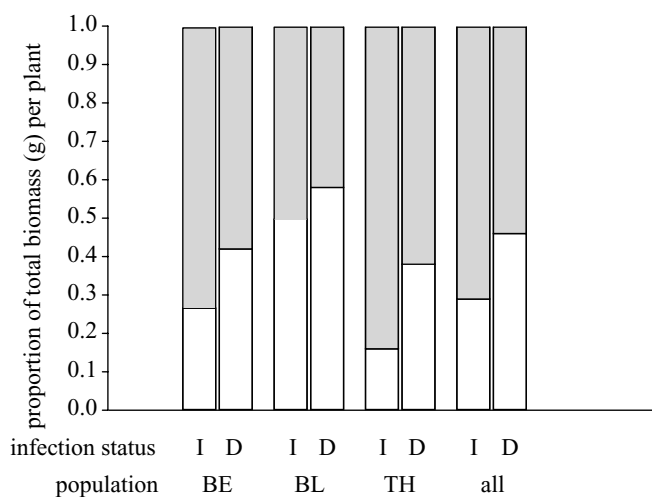


Figure 2. Proportion of total biomass in the mother ramet (white bars) versus clonal growth (grey bars) for infected (I) and disinfected (D) *Glyceria striata* from each population and across populations (all).

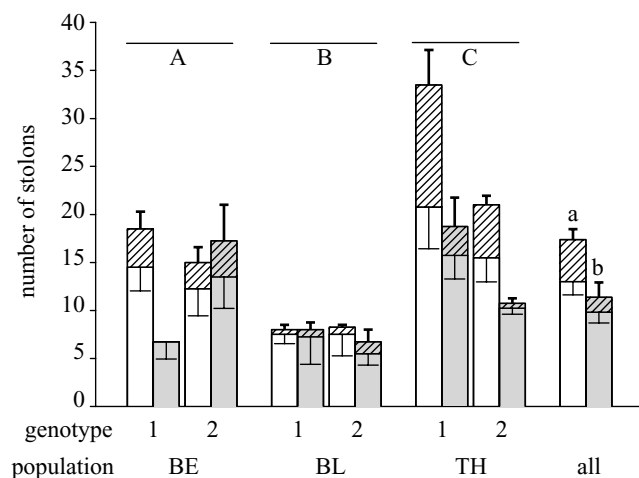


Figure 3. Mean number of primary (–s.e.) and secondary stolons (+s.e. with smaller caps and in bold) for infected (I) and disinfected (D) *Glyceria striata* at the end of the study by genotype nested within population and across populations (all). Disinfected plants of BE genotype 1 did not have any secondary stolons. White bars, I-primary stolons; white hatched bars, I-secondary stolons; grey bars, D-primary stolons; grey hatched bars, D-secondary stolons. Refer to figure 1 for sample sizes and other figure information.

A second necessary condition to support the parasite manipulation hypothesis is that pathogen-induced changes in the host increase parasite transmission. Although we did not directly test for increased parasite transmission, the changes in both stolon number and architecture of *Glyceria* are consistent with increased fitness for *Epichloë*. First, because infection of new host plants appears to be a rare event (Chung & Schardl 1997), a systemic fungus infecting a compatible host can spread and maintain itself in the population through clonal growth of its host. More daughter ramets would lead to more independent units that carry the fungus if clonal connections break down. Second, because *Epichloë* is heterothallic (requiring two mating types for sexual reproduction (Schardl 1996)), clonal spread could also lead to

increased transmission of *Epichloë* through greater intermingling of infected plants with compatible mating types and increased contact with susceptible hosts. Greater densities of infected plants could lead to larger patches of stromata during the reproductive period, which may be more attractive to *Botanophila* flies than smaller patches because they would emit a greater volatile-compound signal. High-density plots including pseudoflowers of *Arabis* sp. have been found to receive more insect visitors than low-density plots (Roy 1996).

(b) Smut infection

During the experiment, infected BE genotypes also exhibited symptoms of infection by the systemic stripe smut, *Ustilago striiformis* (Ustilaginaceae; Basidiomycotina) (Farr *et al.* 1989; R. Wagner, personal communication). Disinfected BE plants did not exhibit symptoms of smut infection, suggesting that they were disinfected of the smut as well as *Epichloë*. Because infected BE plants were doubly infected, we expected that infected and disinfected BE plants would exhibit greater differences in growth pattern than infected and disinfected plants from the other two smut-free populations. However, the population by infection interaction was not significant for the MANOVA or any univariate test. Changes in growth pattern for BE plants were similar to those for TH plants, suggesting that either the smut had very little effect on the host or that the BE *Epichloë* strains were not as effective at altering host growth form as strains from TH. Moreover, total biomass of infected BE plants was not significantly lower than that of infected plants from the BL population, despite their less vigorous appearance. Although we cannot be absolutely certain that BL and TH infected plants were infected solely by *Epichloë*, plants did not exhibit symptoms of other diseases and only *Epichloë* grew out when surface-sterilized tissues of infected *Glyceria* were plated. These findings indicate that smut infection was not a significant resource drain on *Glyceria* and that the effects of *Epichloë* predominated. Other studies have also found minimal effects of *U. striiformis* on hosts under conditions where resources were not limiting (Hodges 1977; Nus & Hodges 1990).

(c) Potential consequences for *Glyceria*

The *Epichloë*–*Glyceria* system is similar to other grass–endophyte systems in that there was a shift in resource allocation with infection. However, in contrast to non-clonal endophyte-infected plants that allocated more resources to space occupation (tiller production) when infected (Bradshaw 1959; Clay 1998), *Glyceria* allocated more resources to spatial spread (clonal growth). Clonal growth can benefit the host by extending longevity of a genotype by spreading the risk of mortality among many ramets, and by potentially increasing the relative fitness of a genotype locally through daughter ramet production (Harper 1977; Cook 1979; Pan & Price 2001). Moreover, daughter ramets have a higher probability of establishment than seeds, particularly in harsh environments, because daughter ramets can be subsidized by mother ramets until they are established (Shumway & Bertness 1992; Shumway 1995; Pennings & Callaway 2000).

For *Glyceria* in particular, clonal growth may enhance persistence in wetland habitats. *Glyceria* occurs in areas

that experience periodic flooding. Seedlings are often washed away during floods because they are not firmly rooted (S. Traub and J. J. Pan, personal observation). By contrast, daughter ramets are unlikely to be washed away if they are attached to a stolon of a well-rooted mother ramet. The benefits of stolons are especially noticeable in *Glyceria* plants that reside along creeks, where all above-ground parts of the plant point downstream (K. McCall and J. J. Pan, personal observation). Thus, *Epichloë* infection may be favoured in habitats that experience frequent flooding because of greater host stolon production and the reduced importance of seedling establishment. Moreover, if greater vegetative growth by the mother ramet increases its survival, then infected *Glyceria* plants differ from many other clonal plants by favouring structures that increase spatial spread (clonal growth) and fitness of the genotype rather than ramet survival (Piqueras 1999).

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