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Erik T. Aschehoug

Kerry L. Metlen

Ragan M. Callaway

University of Montana - Missoula, Ray.Callaway@mso.umt.edu

George Newcombe

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Fungal endophytes directly increase the competitive effects of an invasive forb

ERIK T. ASCHEHOUG,^{1,4} KERRY L. METLEN,² RAGAN M. CALLAWAY,¹ AND GEORGE NEWCOMBE³

¹*Department of Biology HS104, University of Montana, 32 Campus Drive, Missoula, Montana 59812 USA*

²*The Nature Conservancy, 33 North Central Avenue, Suite 405, Medford, Oregon 97501-5939 USA*

³*Forest Resources Program, University of Idaho, P.O. Box 441133, Moscow, Idaho 83844-1133 USA*

Abstract. Competitive outcomes among plants can vary in different abiotic and biotic conditions. Here we tested the effects of two phylotypes of *Alternaria* endophytes on the growth, competitive effects, and competitive responses of the exotic invasive forb *Centaurea stoebe*. *Centaurea stoebe* was a better competitor against North American grass species than grasses from its European home range in the absence of endophytes. However, one endophyte both increased the biomass of *C. stoebe* and reduced the competitive effect of North American grasses on *C. stoebe*. The competitive effects of *C. stoebe* on grass species native to North America were enhanced by both fungal endophytes, but not for native European grasses. We do not know the mechanism by which endophytes increased *C. stoebe*'s competitive ability, and particularly against biogeographically new neighbors, but one endophyte increased the competitive ability of *C. stoebe* without increasing its size, suggesting mechanisms unrelated to increased growth. We tested only a fraction of the different endophytic fungi that have been found in *C. stoebe*, only scratching the surface of understanding their indirect effects. However, our results are the first to demonstrate such effects of a fungal endophyte infecting an invasive forb, and one of the few to show that endophyte effects on competition do not have to be mediated through herbivory.

Key words: *Alternaria*; *biogeography*; *Centaurea*; *community*; *competition*; *conditionality*; *endophyte*; *fungus*; *invasion*; *mutualism*.

INTRODUCTION

Competition is a strong organizing force in plant communities (Connell 1983, Grace and Tilman 1990). However, competitive outcomes are highly conditional, varying with abiotic conditions (Callaway et al. 1996), herbivore attack (Louda et al. 1990), and pathogens (Van der Putten and Peters 1997). At the scale of continents, exotic invasions also suggest strong conditionality in competition because some species become much more dominant in their nonnative ranges than in their native ranges (Hierro et al. 2005). This conditionality in dominance would seem to be related, at least in part, to unusually strong competitive suppression of resident species in the newly invaded range (Maron and

Marler 2008). This superior competitive ability of “invaders” in their new ranges has been primarily attributed to release from host-specific enemies (Keane and Crawley 2002), such that reduced herbivore and pathogen attack may give invaders a disproportional competitive edge in their new ranges where native species remain suppressed by their host-specific enemies. Invaders may also directly exert greater competitive effects in their nonnative ranges through their ability to attain higher biomass (Maron and Marler 2008), or through novel traits that confer greater competitive ability beyond that of size (Callaway and Aschehoug 2000, Kim and Lee 2010, Inderjit et al. 2011). However, we know much less about the conditionality of competition between invaders and natives than we do about the conditionality of competitive interactions among native species.

Mutualisms play powerful roles in some successful invasions (Richardson et al. 2000, Rout and Chrzanowski 2009, Callaway et al. 2011) and there is evidence that the competitive ability of some invaders can be

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⁴ Present Address: Department of Biology, North Carolina State University, Campus Box 7617, Raleigh, North Carolina 27695-7617 USA. E-mail: erik_aschehoug@ncsu.edu

improved by associations with mutualists (Marler et al. 1999, Reinhart and Callaway 2004, 2006). Fungal endophytes are mutualists that can provide indirect benefits via herbivore defense (Clay et al. 2005, Koh and Hik 2007; but see Faeth 2002, Faeth and Fagan 2002). Fungal endophytes can also improve plant performance by altering rhizosphere microbial communities (Rudgers and Orr 2009) and helping plants cope with water stress (Elmi and West 1995). Fungal endophytes also appear to directly increase the competitive effects of infected plants on other species (Marks et al. 1991, Rudgers and Orr 2009), but these effects are much less understood than herbivore-mediated effects. Importantly, examples of endophyte-increased competitive effects are limited so far to a relatively small group of endophytes in grasses: those belonging to the family Clavicipitaceae (e.g., *Neotyphodium*; Clay et al. 1993, Clay and Holah 1999).

There has been little investigation into the role of endophytic mutualists in the success of invasive species, with the exception of what has been learned from nonnative, agriculturally important, grass species (Saikkonen et al. 2006). *Centaurea stoebe*, an aggressive invader of western North American grasslands, provides an opportunity to substantially broaden our understanding of how non-clavicipitaceous endophytes affect interactions among plants (Newcombe et al. 2009) and their roles in invasion because *Centaurea stoebe* harbors many fungal endophytes (Shipunov et al. 2008). Some of these endophytes have the potential to enhance the competitive and allelopathic effects of *C. stoebe*, while others may act as pathogens (Newcombe et al. 2009).

We explored the role of two fungal endophytes on the growth of *C. stoebe* and on the competitive effects and responses of the invader when interacting with North American and European grass species. Both endophytes are phylotypes of *Alternaria* (Shipunov et al. 2008).

Some species in the genus *Alternaria* are pathogens of crops and trees, and are found in soils where they act as decomposers (Kwasna 1992). However, species of the polyphyletic genus *Alternaria* also act as mutualistic endophytes with some plant species. For instance, Musetti et al. (2007) found that *Alternaria alternata* is a defense mutualist against the downy mildew *Plasmopara viticola* in grapevines (*Vitis*). Other very closely related pathogens of the order Pleosporales also appear to switch between pathogen and mutualist roles and aid plants in extreme environmental conditions (Márquez et al. 2007, McLellan et al. 2007) and can be common as endophytes (Porrás-Alfaro et al. 2008).

We focused on three primary questions: (1) Do fungal endophytes directly affect the growth and competitive ability of *C. stoebe*? (2) Does *C. stoebe* have stronger competitive effects on and weaker competitive responses to native North American species than European species? and (3) Do fungal endophytes affect competitive interactions between *C. stoebe* and North American natives more than competition with European species?

METHODS

We grew *Centaurea stoebe* in three treatments: (1) endophyte-free; (2) infected with *Alternaria* phylotype alt2b (isolate CID120); (3) infected with *Alternaria* phylotype alt2f (isolate CID73). The alt2b phylotype is closely related to *Alternaria alternata* whereas the alt2f phylotype is closer to *Alternaria longipes*. The CID73 isolate, or fungal individual, that we used was from seed of a *C. stoebe* plant collected along the Clearwater River, Idaho (46.4474333° N, 116.861917° W; elevation 233 m), whereas the CID120 isolate was from Heviz, Hungary (46.8046667° N, 17.2556667° E; elevation 454 m). These endophytes have been found in *C. stoebe* over wider ranges, but were chosen because of differences in their relative abundances in the native and nonnative ranges of *C. stoebe* (Shipunov et al. 2008). The alt2f phylotype (CID73) is much less common in both the native and invaded ranges of its host than alt2b (CID120), the most abundant phylotype of the native range and quite common in the invaded range as well. Endophyte infection rates of sampled populations of *C. stoebe* vary between 0–100%; however, less than 30% of all seeds contain endophytes of any kind (Shipunov et al. 2008).

Centaurea stoebe plants were grown either alone ($n = 10$ per endophyte treatment) or in pairwise competition ($n = 10$ per endophyte treatment per competitor) with each of four North American and four European grass species in a greenhouse at the University of Montana, Missoula, Montana, USA. We selected grass species that are either dominant or common species in their respective native ranges. North American grasses were *Festuca idahoensis*, *Koeleria macranthus*, *Pseudoroegneria spicata*, and *Stipa comata*. European grasses were *Agropyron repens*, *Lolium rigidum*, *Melica ciliata*, and *Poa annua*. All grass seed was wild collected from native prairie surrounding Missoula, Montana and in grasslands containing *C. stoebe* near Iasi, Romania. Endophyte-free seeds of *Centaurea stoebe* were raised from a parent stock of wild collected endophyte-free seed in a greenhouse at the University of Idaho. All species were germinated in Petri dishes over a two-week span prior to transplanting into 2.4-L (18 cm diameter, 22 cm depth) pots to insure germination success and reduce priority effects among competing species that germinate at different times. All pots were randomized after planting to avoid the possible effects of greenhouse microsite variability. Soil in pots was composed of a 1:1 homogenous mix of autoclave-sterilized field-collected soil (from Missoula, Montana, USA) and 20/30 grit sand (632–1000 μm grain size). All soil, sand, and pots were autoclaved prior to planting to remove any confounding soil microbial effects. Fungal endophytes were cultured on potato dextrose agar and applied exogenously to *C. stoebe* roots in the seedling stage prior to planting. To ensure adequate inoculation, seedlings were placed in Petri dishes of the cultured fungal endophytes and allowed to remain in contact with

fungal hyphae for 12 hours. Like other Class 2 endophytes (Rodriguez et al. 2009), the *Alternaria* phylotypes studied here can colonize roots and leaves as well as the seeds from which they were originally isolated making our inoculation procedure an appropriate experimental manipulation that results in reliable colonization (Newcombe et al. 2009).

We tested the direct effects of endophytes on all grass species by directly applying fungal endophytes to the roots of grass seedlings using the same procedure as for *C. stoebe* seedlings. These seedlings and controls without fungal endophytes were planted alone ($n = 10$ seedlings per species per endophyte treatment) in 500-mL cone-shaped pots. Pots were filled with a 1:1 homogenous mixture of autoclave sterilized local native soil and 20/30 grit sand.

All plants were grown for 70 days prior to harvest. Greenhouse temperatures were kept between 15° and 30° C. and natural light was supplemented by metal halide bulbs to maintain PAR above 1200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Plants were watered two to three times per week. Entire individual plants were harvested by washing and manually disentangling roots of competing species. We subsampled the live roots of 36 individual grasses to test for horizontal transfer of endophytes from *C. stoebe* plants to grasses. Subsampled roots were surface sterilized and cultured to determine infection rates. We did live mass to dry mass conversions of all subsampled tissues using a conversion factor from the remaining root mass of subsampled plants.

Harvested plants were dried at 60°C for 72 hours and weighed. We used ANOVA (univariate GLM in Predictive Analytics Software [PASW] v.18.0; IBM SPSS, Chicago, Illinois, USA) where competitor species, region, and endophyte treatment were fixed factors. We also calculated relative interaction intensity indices (RII; Armas et al. 2004) using endophyte-free *C. stoebe* grown alone as the control and competitor \times endophyte interactions as the treatment. RII is a measure of the strength of interaction between species centered on zero with negative interactions (competition) indicated by values between 0 and -1 , and positive interactions (facilitation) indicated by values between 0 and $+1$. RII allows for simple comparisons of interaction strength across taxa and treatments. Statistical analyses and the results presented in the figures for RII were calculated using t test comparisons (Excel 2007; Microsoft, Redmond, Washington, USA) and one-way ANOVA with Tukey's post hoc analysis (Sigmaplot 11.2; Systat Software, San Jose, California, USA) of RII values and SE (Appendix A in Armas et al. 2004) both among and between region \times endophyte treatments to determine whether RII values significantly differ from zero.

RESULTS

When *C. stoebe* was experimentally infected with the CID120 isolate from Hungary, plants were 46% larger (post-ANOVA Tukey test, $P = 0.001$; Appendix: Table

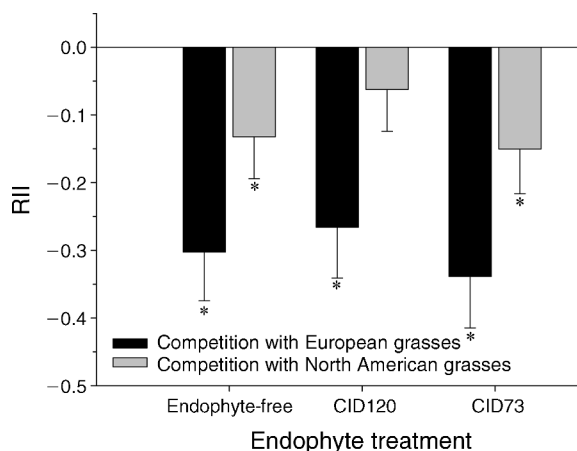


FIG. 1. Response (relative interaction intensity index, RII) of *Centaurea stoebe* to competition with European and North American grass species when either endophyte-free, infected with the CID120 endophyte, or infected with the CID73 endophyte. Asterisks (*) indicate a significant competitive interaction ($P < 0.05$). Error bars indicate SE.

A1) than the uninfected controls and 36% larger (post-ANOVA Tukey's test, $P = 0.003$) than plants infected with the CID73 isolate from the Clearwater River of Idaho.

Endophyte-free *C. stoebe* were more than twice as suppressed by European grass species as by North American grass species (Fig. 1; Appendix: Table A1); however, both European and North American species suppressed endophyte-free *C. stoebe* ($P < 0.001$ and $P = 0.038$, respectively). When *C. stoebe* was infected by CID120, the competitive suppression by European grasses was as strong ($P < 0.001$) as when *C. stoebe* was endophyte-free. In contrast, *C. stoebe* infected by CID120 was unaffected by North American grasses ($P = 0.317$). When *C. stoebe* was infected by CID73, the competitive effect of the four European grass species analyzed as a group significantly suppressed *C. stoebe* ($P < 0.001$) and this effect did not differ from that of either endophyte-free or CID120-infected *C. stoebe*. Unlike the effects of the CID120 endophyte, when *C. stoebe* was infected by CID73, North American plants significantly suppressed the invader ($P = 0.028$).

Endophyte-free *C. stoebe* marginally suppressed European grass species analyzed as a group ($P = 0.058$; Fig. 2; Appendix: Table A2). Endophyte-free *C. stoebe* tended to suppress North American grass species as a group but this effect was not statistically significant ($P = 0.072$). *Centaurea stoebe* infected by CID120 did not change in its effect on European grass species ($P = 0.074$), but strongly suppressed North American grass species ($P = 0.005$). When *C. stoebe* was infected with CID73, there was still a trend toward suppression of European grass species, but this effect was not significantly different than the effects of endophyte-free or CID120-infected *C. stoebe* ($P = 0.100$). In contrast, North American grass species were strongly suppressed

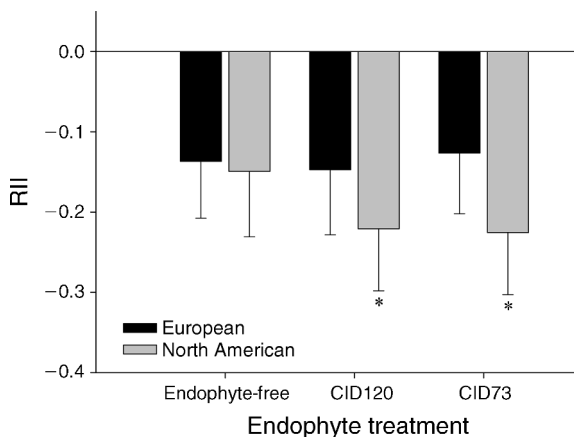


FIG. 2. Competitive effect of *Centaurea stoebe* on European and North American grass species when either endophyte-free, infected with the CID120 endophyte, or infected with the CID73 endophyte. Asterisks (*) indicate a significant competitive interaction ($P < 0.05$). Error bars indicate SE.

by *C. stoebe* infected with the CID73 endophyte ($P = 0.005$).

We found limited evidence for direct horizontal transfer of endophytes from *C. stoebe* to grass species. Of the 20 subsampled European grass species, two were infected by CID120 and two were infected by CID73. For North American grass species, we subsampled 16 individual plants and found three infected by CID120 and none infected by CID73. In our tests of the direct effect of endophytes on grass species, we found no effects on European grass species (Appendix, Fig. 1). However, North American grasses analyzed as a group were significantly inhibited by CID120. This result was highly skewed by the sensitivity of *Stipa comata* to direct infection; *S. comata* was the only species of the eight tested to show significant effects to the direct application of CID120 and CID73 (Appendix: Table A3). But when *S. comata* was grown in competition with *C. stoebe*, we found no significant differences between endophyte-free and endophyte-infected treatments ($P = 1.00$ and $P = 0.760$, respectively; Appendix: Table A2).

DISCUSSION

The most novel contribution of our results is that the direct competitive effects of *C. stoebe* on native species in the invaded range were strongly enhanced by fungal endophytes. Importantly, the enhanced effects of endophytic fungi only occurred against North American natives and not against European natives. The effects of endophytic fungi on competing grass species may have been direct, as horizontal transfer from *C. stoebe* to grasses did occur. However, only 7 of 36 grasses surveyed from the competition experiment showed any evidence of fungal endophyte infection, and only *S. comata* showed negative effects of direct application of either fungal endophyte. If direct effects of the fungal endophyte via horizontal transfer are responsible for the

suppression of grass species, we would have expected the highly sensitive *S. comata* to be suppressed more when in competition with endophyte-infected *C. stoebe* than when in competition with endophyte-free *C. stoebe*. Instead, when *S. comata* was grown in competition with endophyte-free *C. stoebe* and endophyte-infected *C. stoebe*, there was no difference in the amount of suppression of *S. comata* by *C. stoebe*. Therefore, the enhanced competitive effect of *C. stoebe* by fungal endophytes appears to be driven by changes in *C. stoebe*, or synergistic processes involving *C. stoebe* and endophytes, rather than the infection of the grass species by the endophytes themselves.

A second key finding of our study was that *C. stoebe*, with or without endophytes, was far more suppressed by European grass species than by North American grass species (Fig. 1), a general result that is consistent with other studies (Callaway and Aschehoug 2000, He et al. 2009, Thorpe et al. 2009). However, infection of *C. stoebe* by CID120 eliminated even the weak competitive effect of North American grass species on the invader that was manifest in endophyte-free and CID73-infection treatments, suggesting that CID120 improved the competitive response of *C. stoebe* as well as its competitive effect.

Infection of *C. stoebe* by CID120 also resulted in increased size when grown alone, which may explain the increased competitive effects of CID120-infected *C. stoebe* on North American grass species. However, CID73 did not increase the size of *C. stoebe* when grown alone yet CID73-infected *C. stoebe* had much stronger competitive effects on North American grass species than endophyte-free *C. stoebe*. In addition, *C. stoebe* did not significantly differ in size when grown in competition with North American grass species regardless of endophyte treatment (Appendix: Table A1), further suggesting that the effects of fungal endophytes on competition are derived from something other than increasing the size of *C. stoebe*.

There is little information in the literature for how fungal endophytes might increase plant size or influence plant competitive ability in the absence of herbivory, but Rodriguez et al. (2009) reports a number of Class 2 endophytes that increase the root or shoot biomass of their hosts. Endophytes can increase plant defenses against herbivores through the production of alkaloids, which can indirectly increase competitive outcomes through herbivore preference for the less defended competitor (Clay et al. 1993). Fungal endophytes can also alter soil microbial communities (Rudgers and Orr 2009), and this might provide indirect competitive advantages. However, we sterilized all substrates, thus the only biota that were in the pots were the experimentally added endophytes and any organisms that colonized the pots during the experiment. To our knowledge, because we eliminated soil biota and herbivores, our results for *C. stoebe* are the first to demonstrate that endophytes can be a *direct* cause of

increased competitive ability, rather than indirect. Because we worked with an invasive species and fungal mutualists for which biogeographical information is scarce, we limited our study to greenhouse experiments. However, for a better understanding of the ecology of this invader–fungus mutualism, field studies should be conducted in the nonnative and native ranges of *C. stoebe*.

Centaurea stoebe appears to be allelopathic (Ridenour and Callaway 2001, He et al. 2009); however, the allelopathic effects of *C. stoebe* have been highly variable. The fungal endophyte community infecting *C. stoebe* throughout its native and nonnative range is very diverse taxonomically, and the proportion of individual plants infected by endophytes varies dramatically among populations (Shipunov et al. 2008). Thus variation in endophytic infection has a great deal of potential to cause variation in competitive outcomes, and perhaps explain differences among experiments and variation within experiments. Similarly, different endophytic fungi might produce different allelopathic chemicals (Newcombe et al. 2009, Rudgers and Orr 2009) or stimulate different levels of allelochemical production.

The biogeographic native ranges of the fungal isolates (Shipunov et al. 2008) is not clear, but the idea that *C. stoebe* may have picked up novel endophytic “weapons” in North America or imported an important novel weapon when it was introduced suggests important future questions. However, even endophyte-free *C. stoebe* were far more competitively superior against North American than European species, indicating that fungal endophytes enhanced an extant competitive mechanism or provided another mechanism that operated in an additive fashion.

The mechanism by which fungal endophytes increased *C. stoebe* competitive ability is unknown, but because of the strong biogeographic pattern in competitive outcomes, it would appear that the long term evolutionary histories among the interacting species is important (Callaway and Aschehoug 2000). Global *C. stoebe* populations appear to be “mosaics of uninfected and infected plants” (e.g., Faeth 2002) and vary dramatically in the genetic identity of the endophytic fungi they host. This diverse mixture of endophyte and host genotypic combinations may be maintained by different selective pressures including herbivory, abiotic factors and competition, which in turn can affect the growth, survival or reproductive costs of hosting endophytes such that net interactions can range from mutualism to parasitism (Faeth 2002). We tested only two of the more than 90 endophytes known to be found in the seeds of *C. stoebe*, but our results suggest that endophytes can change the outcomes of competitive interactions in newly invaded ranges.

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SUPPLEMENTAL MATERIAL

Appendix

Detailed species results from pairwise competition experiments and direct endophyte infection (*Ecological Archives* E093-001-A1).