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Article Addendum

Endophytes influence protection and growth of an invasive plant

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We investigated the symbiotic activities of fungal endophytes isolated from spotted knapweed, *Centaurea stoebe*. Previously, an analysis of community similarity had demonstrated differences in the endophyte communities of *C. stoebe* in its native and invaded ranges. Here, we found that specific endophytes can exert positive effects on their host, whereas others exert negative effects. Endophytes produced metabolites that inhibited germination of a competitor of *C. stoebe*. Endophytes also repelled a specialist insect herbivore, perhaps by producing biologically active volatiles. Yet other endophytes acted as cryptic pathogens of *C. stoebe*, suppressing its germination, reducing its growth, increasing the abundance of a generalist insect herbivore, and delaying or suppressing its flowering. Since, as reported here, endophytes are not functionally interchangeable, previously reported community differences could be contributing to the invasiveness of *C. stoebe*.

Recently we reported significant diversity in endophytic fungi in an invasive plant, *Centaurea stoebe*, or spotted knapweed.[1] Communities in the invaded and native ranges differed according to an analysis of similarity. Preliminary experiments to investigate functional activities of endophytes suggest that differences in the presence or absence of key endophytes could affect the invasiveness of this plant that is native to Eurasia and invasive in North America and elsewhere.

Positive Effects

Culture filtrates of 12 endophytes (Experiments 1–3, Table 1) suppressed germination of *Festuca idahoensis*, a plant that competes with *C. stoebe* in its invaded range in western North America.[2] This result demonstrated that specific endophytes produce allelopathic effects that might aid *C. stoebe* in competition with other plants.

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Symbionts can also have positive or mutualistic effects on their hosts by protecting them. Two endophytes, *Alternaria* CID62 and *Epicoccum* CID66 (CID = Cultivation Identification Number, or endophyte isolate number. A complete list of our CIDs is here[1]), appeared to protect *C. stoebe* from *Larinus minutus*, a seed-feeding weevil from the native range of *C. stoebe*, that was deliberately released in North America for biological control.[3] In dual-choice laboratory bioassays (Experiments 4–9), mated *Larinus minutus* females spent more time on uninoculated, control flowerheads than on those inoculated with either *Alternaria* CID62 or *Epicoccum* CID66, and preferred flowerheads inoculated with *Epicoccum* CID66 to those inoculated with *Alternaria* CID62 (Fig. 1). A similar pattern occurred when the isolated fungi were applied to cotton-flower mimics, except that the difference in preference for *Epicoccum* CID66 over *Alternaria* CID62 was not significant (Fig. 1).

The effects we have detected thus far are potentially mediated by chemical factors. We sampled each of 16 endophytes for their capacity to release volatile organic compounds (VOC) in pure culture (i.e., Experiment 10), following methods similar to those that have been used to detect biologically active VOC produced by an endophytic fungus.[4] Fourteen of these isolates in pure liquid culture produced at least one volatile sesquiterpene. *Fusarium* CID124 produced 20 distinct sesquiterpenes. Total production of sesquiterpenes ranged from zero to 236.8 ng/0.5 h/20 ml sample of culture. Volatile sesquiterpenes are implicated in many interorganismal interactions.[5]

Negative Effects on Flowering

Although the endophytes reported thus far[1] are not overt pathogens they could be cryptic pathogens.[6] In Experiment 11, knapweed seedlings inoculated with *Alternaria* isolate 'CID62' produced fewer flowering heads than seedlings inoculated with *Epicoccum* CID66, *Fusarium* CID107, and an uninoculated, E^- (i.e., endophyte-free) control (ANOVA $F_{1,38} = 5.276$, $p = 0.03$). In Experiment 12, seedlings inoculated with *Alternaria* CID123 and *Fusarium* CID124 flowered significantly later than E^- controls (ANOVA $F_{2,46} = 17.173$, $p < 0.001$).

Negative Effects on Seed Germination

We also performed knapweed germination assays following inoculation with endophyte cultures (Experiments 13–15), or following treatment with liquid culture filtrates (Experiments 11–13); germination was 100% suppressed by *Botrytis* CID360, *Alternaria* CID120

Table 1 **A summary of experiments**

Experiment(s)	Description
1–3	Effect of liquid culture filtrates of listed CIDs in a *Festuca idahoensis*, seed-germination assay, based on the design of Blair et al.[11] Culture age was varied.
4–9	Choice experiments with adults of *Larinus minutus*, real and artificial flowers. *L. minutus* mated females were exposed to individual, severed flowers of *C. stoebe*. One flower was placed at either end of a 2 cm-diam x 8 cm-long plastic tube. The location of a single weevil was recorded at 1-h intervals over 15 h. Twenty-five insects were tested individually for each of 6 pairwise comparisons.
10	Experiment to determine sesquiterpene production of 16 CIDs, in terms of numbers of detectable compounds and total amounts trapped during 0.5 hours over a 20 ml sample of each culture. VOC were analyzed from headspace of 10 ml sample liquid cultures of selected fungal isolates. A solid phase micro extraction (SPME) fiber was exposed to headspace for 0.5 h. VOC were desorbed, separated, identified and quantified by GC/MS.
11 and 12	Growth and flowering of inoculated *C. stoebe* in the greenhouse (day and night temperatures 27 and 24 C, respectively; photoperiod 16;8, L:D).
13–15	Effect of inoculation of listed CIDs in a *C. stoebe*, seed-germination assay. Three inoculation methods: (a) direct contact with agar-based, endophyte culture for 12 h; (b) continuous contact with agar-based culture for entire observation period of 14 days; (c) continuous immersion in liquid culture for entire observation period.
14–16	Survival, growth and final biomass of inoculated seedlings of *C. stoebe* in the greenhouse (day and night temperatures 27 and 24 C, respectively; photoperiod photoperiod 16;8, L:D).
17	Biomass and aphid population density of inoculated *C. stoebe* plants in the greenhouse (day and night temperatures 27 and 24 C, respectively; photoperiod photoperiod 16;8, L:D)

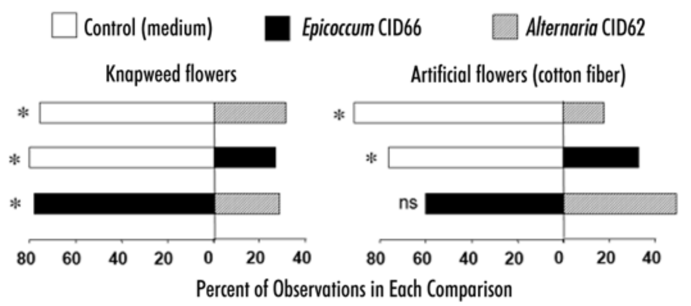

Figure 1. Results of 6 dual-choice experiments to determine the settling behavior of *Larinus minutus* on individual flowerheads or artificial flowers of spotted knapweed with and without inoculation by endophytes. Bars show the relative proportion of observations of weevils on the two treatments being compared over a 2-hour period. Asterisks indicate whether the results depart from equal proportions on each treatment (χ^2, p = 0.05).

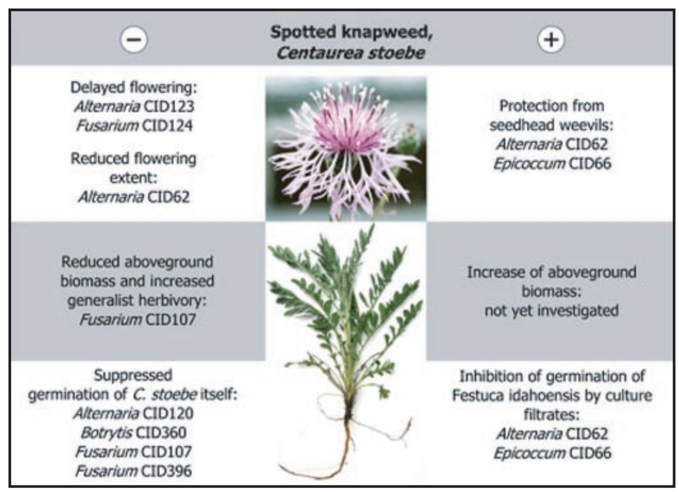

Figure 2. Growth, flowering and biotic interactions of *C. stoebe*, all significantly influenced by specific endophytes. Endophyte genera are followed by CID numbers that are keyed to GenBank accession numbers and to isolation frequencies in the native and invaded ranges of *C. stoebe*.[1]

and *Fusarium* CIDs 107 and 396, and many lesser, but still significant, effects were recorded.

Not only was germination of knapweed seeds entirely suppressed by *Fusarium* CID107, but a viability test with 0.1% unbuffered tetrazolium solution showed that seeds that failed to germinate were actually dead.

Negative Effects on Growth of *C. stoebe*

Some seedlings survived if they were first germinated and then inoculated with *Fusarium* CID107 (Experiments 14–16), but survivors had fewer and shorter leaves (ANOVA $F_{1,52}$ = 8.987, p = 0.004 for number of leaves and ANOVA $F_{1,52}$ = 7.307, p = 0.009 for length of maximal leaves) during a forty-day period of growth, and fewer mature, dissected leaves (χ^2 test for independence, χ^2 = 4.103, p = 0.043) than E^- controls. Final, aboveground biomass was lower for *Fusarium* CID107-inoculated plants (ANOVA $F_{1,50}$ = 11.292, p = 0.001) than E^- controls.

Negative Effects on Protection of *C. stoebe*

Fusarium CID107 also attracted a generalist herbivore, the aphid, *Myzus persicae*, to plants it had infected. In Experiment 17, abundance of aphid infestations differed on E^+ and E^- knapweed seedlings (ANOVA $F_{3,35}$ = 5.023, p = 0.005). *Fusarium* CID107-inoculated seedlings hosted aphid populations 6.3 times higher than plants inoculated with *Alternaria* CID62, *Epicoccum* CID66, or controls, although this difference eventually disapppeared when aphid populations became very large on all treatments (ANOVA $F_{3,36}$ = 0.951, p = 0.426).

Balance of Positive and Negative Effects

With both negative and positive effects on characters associated with fitness (Fig. 2), it seems likely that endophytes strongly influence the ecology and invasiveness of *C. stoebe*. The effects of endophytes were seen in all growth stages of *C. stoebe*, from germination to flowering. Increases in aboveground biomass due to endophytes have been observed in other plants,[7-10] although not yet in *C. stoebe* (Fig. 2). We expect that with further experimentation, we will discover many additional, biotic interactions mediated by endophytes in *C. stoebe*.

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