RESEARCH ARTICLE

Exclusionary interactions among diverse fungi infecting developing seeds of *Centaurea stoebe*

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Abstract

Developing seeds are expected to be strongly defended against microbial attack. In keeping with this, only 26% of seeds of *Centaurea stoebe* from its native and invaded ranges in Eurasia and North America were infected with fungi, and 92.2% of those were infected with a single fungus per seed. Even when developing seeds in flower heads were inoculated under conducive conditions for infection with 14 of these seed-infecting fungi, re-isolation of inoculants was only 16% overall, and again limited to the particular inoculant. Environmental fungi (i.e. those not isolated from seed of *C. stoebe*) were present in control flower heads under conditions conducive to infection but they were never re-isolated from fully developed seeds in any experiments. When two or three seed isolates were co-inoculated to compete in flower heads, only one inoculant, and always the same one, was re-isolated from all matured seeds, regardless of maternal plant genotype. PCR-based detection methods confirmed that these fungal interactions were exclusionary rather than suppressive. In these strongly defended, developing seeds, we had expected the plant to control not only the overall level of infection but also the outcome of co-inoculations. Consequences for the next plant generation of this exclusionary competition among seed-infecting fungi included effects on seedling emergence, growth and fecundity.

Introduction

Reproductive tissues (e.g. developing seeds) should be well-defended relative to the vegetative organs of a plant (Zangerl, 1992). The relatively few fungi that are able to infect developing seeds will be detected, or if culturable, isolated, as seed-infecting fungi (Shipunov *et al.*, 2008; Newcombe *et al.*, 2009). Seed-infecting fungi should be ecologically important because, in theory, they should be in prime position to affect seedling emergence and early growth (Newcombe *et al.*, 2009), key events in the plant life cycle.

Previously, we isolated fungi infecting seeds of *Centaurea stoebe* ssp. *micranthos* in both its native range in Eurasia and that part of its invaded range that is in the Pacific Northwest region of North America (Shipunov *et al.*, 2008). Plant-based, fungal operational taxonomic unit (OTU) accumulation curves were not asymptotic, even though a total of 92 sequence-based OTUs from eight classes of Fungi were recovered. Sampling of field leaves and roots indicated that an individual plant hosts a subset of the 92 seed-infecting fungi (Shipunov *et al.*, 2008; Newcombe *et al.*, 2009). Typically however, when a fungus was isolated from a seed, it was the only isolate, and roughly 74% seeds were free of all culturable fungi (Shipunov *et al.*, 2008). We wished to understand whether low incidence of seed-infecting fungi was due to an abiotic environment hostile to infection of developing seeds, or host-genetic resistance, or some combination thereof. The environment and host-genetic resistance might also explain the fact that those seeds that were infected harbored only one fungus. An additional hypothesis aimed specifically at the phenomenon of one fungus per seed: exclusionary interactions among seed-infecting

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fungi. Included here in the abiotic environment are all factors other than host-genetic resistance and exclusionary interactions among fungi (e.g. spore dispersal, and conducive conditions of temperature and wetness for infection).

The environment could easily be limiting to infection of developing seeds of *C. stoebe* because fungal spores in flower heads would require moist conditions to germinate and infect, and flowering of this plant occurs during relatively dry summers, at least in the Pacific Northwest region of North America (Shipunov *et al.*, 2008). Sporulation of the 92 OTUs might also be limited in dry weather such that few or no spores would even be present in a flower head of *C. stoebe* ssp. *micranthos*. Given the way the abiotic environment might limit infection of developing seeds, this was a first hypothesis.

However, host-genetic defense of developing seeds is predicted by the optimal defense hypothesis (Rhoades, 1979), and it could also explain the patterns observed in nature. When conducive conditions for infection did occur plant genetic resistance or defense would be expected to be the remaining, limiting factor for any spores that did germinate (Dangl & Jones, 2001). Host genotype or resistance has been shown to be significant in structuring endophytic fungal communities (Ahlholm *et al.*, 2002), but it is not known whether it can be differential. Here, the expectation for differential host resistance was exclusion from seed of specific fungi by specific host genotypes (Redman *et al.*, 1999; Schulz & Boyle, 2005; Tanaka *et al.*, 2006). Alternatively, genetic defense could limit infection generally in a non-specific manner that allowed observed diversity.

The third hypothesis (i.e. exclusionary interactions among fungi for a limited, strongly defended niche – the developing seed) initially seemed unlikely because it would be at odds with the observed diversity of 92 OTUs in the same manner as that of the famous 'paradox of the plankton' (Hutchinson, 1961) or the paradox of biodiversity generally (Tilman, 1999).

At least two, seed-infecting fungi of *C. stoebe* have been demonstrated to influence early, interspecific competition with other plants in a manner that may enhance invasiveness (Aschehoug *et al.*, 2012). Primary fungi could also have priority effects on subsequent, fungal community assembly (Fukami & Morin, 2003) in ways that would indirectly affect their host plants.

Here, we report our analyses of fungal co-occurrences in seeds from our field study (Shipunov *et al.*, 2008), and new inoculation experiments to test the three hypotheses that might explain, singly or in combination, the limited infection that has been observed: the environmental limitation hypothesis (H_1), the genetic defense hypothesis (H_2), and the exclusionary interactions hypothesis (H_3). We also determined the effects of single, seed- infecting fungi on seedling emergence, growth and fecundity.

Materials and methods

Patterns of single and multiple fungi in field-collected seed

Seedheads of *C. stoebe* ssp. *micranthos* were sampled in 102 sites in its invaded and native ranges, as previously reported (Shipunov *et al.*, 2008). Seed-infecting fungi were isolated onto potato dextrose agar (PDA) from seeds after employing surface-sterilization 'Method I': 30 s in 96% ethanol, 3 min in 6% NaOCl solution, and 30 s in 96% ethanol (Shipunov *et al.*, 2008). Each isolate received its own ID number (CID) and was assigned on the basis of morphology and ITS and *Alt a 1* sequences to an OTU of a fungal genus. Endophytic fungi are infrequently identified to species, in part because they represent an important component of undescribed fungal diversity (Arnold, 2007), so an OTU is a proxy for species. Methods for extraction, amplification and sequencing of the nuclear 5.8S rRNA gene and the two flanking, ITS regions were as previously published (Ganley *et al.*, 2004; Shipunov *et al.*, 2008). For those fungi of *C. stoebe* that could be assigned on the basis of ITS sequences to *Alternaria* and related genera, the *Alt a 1* gene was also sequenced to provide additional discrimination of OTUs (Hong *et al.*, 2005). All sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/). Here, we determined the numbers of isolates and OTUs per seed, and field isolation frequencies.

Plant material and culture

Seedlings selected on PDA as free of culturable fungi were then grown in 6 × 4.5 inch pots containing autoclaved 'Sunshine mix # 1' (Sun Gro Horticulture Inc., Bellevue, WA). It took 4 months for the plants to flower and an additional month for the completion of each experiment. Each plant produces more than 100 composite inflorescences (i.e. flower heads) under greenhouse conditions (see Supporting Information, Fig. S1). Flower heads were pollinated with bee abdomens and pollen from two donor plants; from 22 to 25 seeds typically matured in each flower head. Greenhouse conditions were 16 h of light and 8 h of darkness, with temperatures ranging from 24 to 27 °C, respectively. Plants were watered as required and fertilized at weekly intervals with 200 $\mu g mL^{-1}$ of N [15 : 16 : 17, Peter's Peat Lite Special® (Scotts-Sierra Horticultural Products Co., Marysville, OH)], to the completion of the experiment. Each seedling plant of this obligately outcrossing species is an individual genotype (Harrod & Taylor, 1995).

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Fungal culture, inoculation of flower heads and re-isolations

An OTU could, and often did, comprise more than one isolate. For example, isolates CID63 and CID64 were both assigned to one OTU, '*cla063*', as they shared the same ITS sequence and morphology typical of the asexual form genus *Cladosporium* (Shipunov *et al.*, 2008). Isolates were maintained in petri dishes containing PDA supplemented with streptomycin (0.05 mg mL^{-1}). Fungal inoculum was prepared by homogenizing 10- to 20-day-old cultures in sterile, distilled water (SDW) with a Tissue Tearor. The resulting suspension (0.15 g of wet-weight, fungal tissue mL^{-1} of SDW) was used as inoculum. Concentrations ranged from 2×10^6 to 5×10^6 spores or fragments mL^{-1}, based on preliminary study.

Each flower head received approximately 1 mL of inoculum 24 h after pollination. In the case of 'mixed inoculations' (with two or three inoculants), equal volumes of fungal inocula were combined for a final volume of 1 mL per flower head. Inoculation with SDW served as a wet control providing conditions conducive to infection by airborne, non-OTU fungi; a second control was pollinated only. Inoculations were performed with sterile, 3-mL hypodermic syringes. Inoculum or SDW was carefully spread such that the liquid reached all of the flowers in the flower head. After inoculation, each flower head was enclosed in a polythene bag for 24 h to retain moisture and provide the fungal inoculants with the opportunity to infect developing seeds.

To determine the likelihood of deposition of viable spores of non-inoculant fungi in wet control flower heads, five petri dishes with PDA were exposed to air spora 0.6 m above greenhouse benches for 12 h, then sealed and incubated at 24 °C. The number of non-inoculant, fungal colonies per dish after 7 days was used to estimate viable spore deposition on the disk florets of wet controls.

Re-isolation was carried out by placing surface-sterilized seeds equidistantly from one another in each petri dish of PDA/streptomycin. The efficacy of the surface sterilization was tested by imprinting surface-sterilized seeds in separate dishes. Re-isolation frequency was calculated as follows:

$$\text{Re-isolation frequency (\%)} = \frac{\text{Number of seeds with inoculant}}{\text{Total number of seeds}} \times 100$$

Single inoculations

For a first experiment, 18 isolates representing 14 OTUs were randomly selected from the 92 OTUs (Shipunov *et al.*, 2008) to serve as inoculants. Each fungus was replicated seven times by inoculating one flower head of each of seven plants. Seed totals per fungal treatment varied from 86 to 163. Re-isolation was carried out by following 'Method I' surface sterilization and re-isolation frequency was calculated as described by pooling seeds from all seven flower heads yielding the inoculant.

In a second experiment, the flower heads of six plants were inoculated with each of CID96 (*Botrytis* '*bot017*'), CID63 (*Cladosporium* '*cla063*') and CID 124 (*Fusarium* '*fus124*') that will be hereafter referred to simply as *Botrytis*, *Cladosporium*, and *Fusarium*, respectively. All fungal inoculants and controls were replicated four times by inoculating four flower heads on each of the six plants for a total of 24 replicates per treatment. Re-isolations were carried out after surface-sterilization 'Method I'.

In a third experiment, *Botrytis*, *Cladosporium*, and *Fusarium* inoculations of flower heads of six plants were followed by milder surface sterilization of seeds (Method II: immersion in 70% ethanol, plus Tween, for 5 min, followed by three rinses in SDW).

Mixed or co-inoculations

For these two experiments, *Botrytis*, *Cladosporium*, and *Fusarium* were mixed in all combinations (i.e. three, two-inoculant mixes and one, three-inoculant mix). All mixes were replicated four times by inoculating four flower heads of each of the same six plants (24 replicates per mix). Re-isolations were carried out following surface-sterilization Method I and Method II.

PCR-based detection of fungi in seeds

DNA extraction

Surface-sterilized seeds from co-inoculations with more than one fungus (i.e. three two-inoculant and one three-inoculant combinations) following Method II were placed in petri dishes with PDA. After 5 days, 15 seeds infected with the dominant fungus from each co-inoculation were surface sterilized following Method I (to remove DNA present on the surface) and then used for DNA extractions. Total genomic DNA was extracted using modified 2× CTAB method (Doyle & Doyle, 1987). Silica-dried, surface-sterilized seeds were ground in liquid nitrogen and the resulting powder (16–20 mg) was transferred to a 2 mL microtube and mixed with extraction buffer (1 M Tris-HCl, pH 8, 0.25 M EDTA, 0.87% NaCl, 0.2% β-mercaptoethanol and 10% CTAB). This was followed by extraction with chloroform/ iso-amyl alcohol (24 : 1, v/v). DNA precipitation with carried out in cold isopropanol, the resulting pellet was washed twice with cold 70% and 95% ethanol and resuspended in 150 μL of AE buffer

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after drying. DNA concentration was quantified using the Nanodrop (Thermo Fisher Scientific, Wilmington, MA). Purified DNA samples were stored at −20 °C until further use. Additionally, extractions were also carried out with control seeds to verify their inoculant-free status. Extractions were carried out in three sets with each set consisting of 15 seeds. DNA extractions for the three fungal inoculants in pure culture were carried out as previously published (Shipunov *et al.*, 2008).

Nested-PCRs

To increase the sensitivity of fungus detection, a nested or a two-round, PCR procedure was used. This is an extremely sensitive procedure that is used to detect latent fungal infections in plant tissue (Langrell, 2005; Parfitt *et al.*, 2010). The universal, fungus-specific, ITS1 and ITS 4 (White *et al.*, 1990) primer pair were used in the first round of PCR. PCRs were carried out in a total mixture volume of 25 μL containing template DNA (3 μL 278–315 ng μL^{-1} DNA solution), 2.5 mM $MgCl_2$, 2.5 mM dNTPs (Applied Biosystems), 10 pmol of each primer and 1.5 U Taq DNA Polymerase (New England Biolabs). Samples were incubated in a thermal cycler (MJ Research, Waltham) at 94 °C for 2 min for an initial denaturation. This was followed by 14 cycles of touchdown PCR as follows: 94 °C for 30 s, 58 °C for 30 s with a decrease of 0.8 °C per cycle, 72 °C for 30 s, and then by 25 cycles of the following: 94 °C for 30 s, 45 °C for 30 s and 72 °C for 45 s. Reactions were incubated at 10 °C for 5 min after a final extension at 72 °C for 3 min. One microlitre of the first-round PCR product was used as a template for the second-round PCR using fungal-specific primers. For instance, 1 μL of first-round PCR product from the *Botrytis* and *Cladosporium* co-inoculation was used as template for two separate, second-round PCRs containing *Botrytis*- and *Cladosporium*-specific primer pairs. Similar reactions were carried out for all other co-inoculations. For controls, all three primer pairs were tested in the second-round PCR. Primers specific to *Botrytis*, *Cladosporium* and *Fusarium* (Table 1) were designed based on the ITS sequences from our previous publication (Shipunov *et al.*, 2008). The *Botrytis* primer pair was tested for specificity against template DNA from *Cladosporium* and *Fusarium* pure culture. Similar tests were conducted for *Cladosporium* and *Fusarium* primer pairs. PCR conditions for the second round PCR were as described above except for annealing temperature in touchdown which was 62 °C for 30 s with a decrease of 1.0 °C per cycle for six cycles. PCR product from both first and second rounds were subjected to electrophoresis in 1% agarose gel in 1× TBE running buffer and visualized under UV after staining with SYBR-Safe (Invitrogen).

To determine whether the expected sequence was amplified by fungal-specific primers, three DNA fragments/amplicons obtained from each of the *Cladosporium* and *Fusarium* primers were selected and purified using GelElute extraction kit (5Prime; Gaithersburg, MD) by following the manufacturer's instructions. Sequencing reactions with Big Dye Taq premix were performed using forward primers of *Cladosporium* and *Fusarium* on an ABI 3130*xl* automated sequencer (Applied Biosystems) as described previously in Shipunov *et al.* (2008). The resulting sequences were compared directly to original ITS sequences from *Cladosporium* '*cla063*' and *Fusarium* '*fus124*' isolates deposited in GenBank (Table 2) after aligning them in BIOEDIT, v 7.1.3 (Hall, 2011).

Effects of single, seed-infecting fungi on seedling emergence, growth and fecundity

Seedling emergence was determined by planting seeds infected with either *Botrytis*, or *Cladosporium*, or *Fusarium* in seedling trays. Each tray was filled with one part field soil and one part potting soil (Sunshine Mix #1) and divided into nine blocks representing nine replicates or flower heads. Seeds were planted at 2 × 2 cm intervals and emergent seedlings were counted 7 and 14 days post-planting (DPP). Trays were watered and not fertilized for the duration of the experiment. Fungal inoculant effects on early growth were compared by growing seedlings for 3 weeks then washing them of soil, separating roots from

Table 1. Specific primer pairs used to detect fungi in seeds from co-inoculated flowers

Primer pairs*	Primer sequence (5′- 3′)	Length (bp)	T_m	Target region	Amplicon size (bp)
Botrytis '*bot017*' (F)	CCCACCCTTGTGTATTATTACTTTG	25	61.3	ITS 1	337
Botrytis (R)	ACTGATTTTAGAGCCTGCCATTAC	24	61.2	ITS 2	
Cladosporium '*cla063*'(F)	ATAACCCTTTGTTGTCCGACTCT	23	61.0	ITS 1	406
Cladosporium (R)	TAGCCTCCCGAACACCCTTTAG	23	64.5	ITS 2	
Fusarium '*fus124*' (F)	TTACCGAGTTTACAACTCCCAAAC	24	61.2	ITS 1	417
Fusarium (R)	TTTACTACTACGCAATGGAAGCTG	24	61.2	ITS 2	

*Primers were designed from the original ITS sequences from our previous publication (Shipunov *et al.*, 2008); F, forward primer and R, reverse primer.

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Table 2. Field-isolation and re-isolation frequencies of 18 isolates of 14 OTUs of seed-infecting fungi

OTUs	Genus	Isolate	GenBank Accession No.	Field-isolation frequency (%)	Re-isolation frequency (%)		
				Method I*	Method I	Method I	Method II†
alt002a	*Alternaria*	CID 62	EF589849	2.80	12.24		
cla063	*Cladosporium*	CID 63	EF589865	1.23	2.11	38.6	49.0
cla063	*Cladosporium*	CID 64	EF589865	1.23	3.67		
epi066	*Epicoccum*	CID 66	EF589869	1.42	1.44		
epi066	*Epicoccum*	CID 67	EF589869	1.42	0.00		
alt002b	*Alternaria*	CID 73	EF589849	7.78	42.25		
alt076e	*Alternaria*	CID 76	EF589850	0.37	24.42		
bot079	*Botrytis*	CID 79	EF589856	0.57	1.85		
bot080	*Botrytis*	CID 80	EF589857	0.06	2.11		
pho086	*Phoma*	CID 86	EF589891	0.05	0.00		
bot017	*Botrytis*	CID 96	EF589855	0.74	42.86	7.4	17.1
tri103	*Trichothecium*	CID 103	EF589898	0.06	0.00		
alt002b	*Alternaria*	CID 105	EF589849	7.78	39.13		
ple015	*Pleospora*	CID 107	Ef589895	0.16	1.59		
bot079	*Botrytis*	CID 108	EF589856	0.57	1.72		
bot109	*Botrytis*	CID 109	EF589860	0.03	0.61		
alt002c	*Alternaria*	CID 123	EF589849	1.80	57.81		
fus124	*Fusarium*	CID 124	EF589878	0.10	51.56	56.2	85.3

Re-isolation frequencies were determined in three experiments via inoculation of one isolate per flower head (Fig. S1).
*Method I surface-sterilization of seeds: 30 s in 96% ethanol, 3 min in 6% NaOCl solution and, 30 s in 96% ethanol (Shipunov *et al.*, 2008).
†Method II surface-sterilization: 70% ethanol for 5 min (plus Tween), followed by three rinses in sterilized distilled water.

shoot and drying for 72 h at 60 °C. Then, dry biomass was measured.

An additional experiment evaluated the effects of *Cladosporium* on growth and fecundity. A total of 75 seeds were surface-sterilized (Method II) and transferred onto moistened, sterile blotter paper in petri dishes for 1 week to allow germination. After germination, 30 seedlings were placed in petri dishes with *Cladosporium* culture, such that roots were in direct contact with culture for 24 h. Thirty control seedlings were placed in sterile petri dishes with PDA for the same period. After 24 h, 20 seedlings from each treatment were planted singly into 6 × 4.5 inch pots filled with potting soil (Sunshine Mix #1). Ten seedlings of each treatment were used to confirm infection. Greenhouse plants were never fertilized. Number of flower heads per plant was used as a proxy for fecundity and aboveground biomass was recorded after clipping each plant to the soil surface. This experiment was then repeated.

Data analyses

A paired *t*-test was performed to compare re-isolation frequencies of 18 isolates (14 OTUs) with their respective field-isolation frequencies. Analysis of variance was carried out on the re-isolation frequency data determined on a flower head basis. Maternal plant genotype was treated as a random variable whereas fungal inoculant was fixed as *Botrytis*, *Cladosporium* and *Fusarium* were deliberately chosen for the interaction studies. Differential host resistance to inoculants was expected to yield significant interaction (i.e. fungal inoculant × maternal plant genotype). Differences in means among fungal inoculants were tested against fungal inoculant × maternal plant genotype interaction mean squares (MS), whereas, maternal plant genotype was tested against error MS (Newman *et al.*, 1997). Controls were not included in analyzing re-isolation data as all the seeds were free of culturable fungi.

Re-isolation frequency data following Method I surface sterilization were arcsine-square root transformed with corrections for replications with zero re-isolation frequency by term 1/4n (where, n = total number of seeds for that replication) before analysis to meet the assumptions of normality and equality of variance (Ott & Longnecker, 2001). However, transformation was not required for re-isolation data following Method II surface sterilization as they were normally distributed.

A repeated-measure analysis of variance was performed on seedling emergence data. Maternal plant genotype, fungal inoculant and their interaction were treated as between subject effects, whereas interactions of time with between subject effects were within subject effects. Further, when significant between subjects effects were observed separate univariate analyses of variance were performed for each time period. Although fungal inoculant effects on seedling growth were recorded on a

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seedling basis, for the purpose of analyses all three response variables (shoot, root biomasses and shoot-to-root biomass ratio) were calculated as a mean of total number of seedlings for each replicate or flower head separately. As these values were from a variable number of seedlings from each replicate, the data were analyzed using weighted-analysis of variance, with the number of seedlings in each replicate as a weighted variable. The root biomasses and shoot-to-root biomass ratios were square-root transformed prior to analysis. Maternal plant genotype, fungal inoculant, and maternal plant genotype by fungal inoculant interaction were treated as main effects, all effects were fixed. Fecundity (number of flower heads per plant) and aboveground biomass were analyzed using Student's two-sample t-test with pooled variances. Since quantitatively similar results were observed in the repeat experiment results from the original only are presented. All analyses were performed using SAS 9.1 (SAS Institute Inc., Cary, NC) and SYSTAT version 12.0 (Systat Software Inc., San Jose, CA). Mean comparisons were made using Tukey's HSD ($P = 0.05$). As root, shoot, and shoot-to-root biomasses were not independent variables, mean comparisons were made using the Bonferroni adjustment ($P = 0.05$). Untransformed means and standard errors are presented in the Figures.

Results

Patterns of single and multiple fungi in field-collected seed

It was uncommon to find more than one isolate per seed. Of 1440 seeds with fungi, 1328 (92.2%), 102 (7.1%), and 10 (0.7%) yielded one, two, and three isolates, respectively. Seeds infected with two or three isolates did not simply yield two or three isolates of the same OTU. Of the 102 with two isolates, 91 yielded two OTUs each. All 10 seeds with three isolates produced three OTUs each. The three OTUs employed in the multiple inoculations and most of the single inoculations (*Botrytis*, *Cladosporium*, and *Fusarium*) did not co-occur or coexist with one another at all in the field-collected seeds. They did, however, coexist with a few other OTUs although their three coexistence groups were mutually exclusive or non-overlapping.

Single inoculations

If the abiotic environment even partially limited infection then the re-isolation frequencies of the 18 isolates should have been significantly higher than their field-isolation frequencies (H_1 – Table 3). They were in that they were roughly ten times higher (i.e. 15.9% vs. 1.6% overall–Table 2), demonstrating that the abiotic environment can limit infection of developing seeds (d.f. = 17; t = 3.044; $P = 0.007$). However, if the environment were solely responsible for low levels of infection in the field, we would have expected even higher re-isolation frequencies than 15.9% overall. We would also have expected to re-isolate fungi other than inoculants, especially from controls, but none were obtained. Spore trapping in the greenhouse did demonstrate that spores of non-inoculant fungi must have been deposited on control flower heads prior to overnight moistening. This implies that spores of fungi other than inoculants had the opportunity to infect developing seeds in control flowers.

Having confirmed that the environment is partially responsible for limiting infection of developing seeds, we expected that genetic defense (i.e. H_2 – Table 3) should

Table 3. Predicted outcomes of inoculation experiments according to hypotheses of environmental limitation (H_1), genetic defense (H_2), and exclusionary interactions among fungi (H_3)

Hypothesis	Outcomes with single inoculants per flower head
H_1	If the environment is solely responsible for low levels of infection in the field (i.e. no genetic defense), re-isolation frequencies (after inoculation under favorable conditions for infection) should approach 100% for all inoculants in all plant genotypes. As genetic defense is also the most likely explanation for a selective system, in its absence, non-inoculant fungi should be isolated from seeds from both inoculated and control flower heads
H_2	If the environment plays no role and genetic defense is solely responsible for low levels of infection in the field, re-isolation frequencies should not differ significantly from field-isolation frequencies. Re-isolation frequencies of inoculants should vary with inoculants and host genotypes. Non-inoculant fungi should not infect developing seeds in either inoculated or control flower heads
	Outcomes with multiple co-inoculants per flower head
H_1	All inoculants should be re-isolated from each seed in all plant genotypes. Again, non-inoculant fungi should also be isolated from seeds from both inoculated and control flower heads
H_2	Genetic defense could conceivably limit infection by co-inoculants to a single fungus per seed. The identity of that single re-isolated fungus could vary within and among genotypes
H_3	Exclusionary interactions should result in consistent re-isolation of the same inoculant from mixes both within and among plant genotypes

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also play a partial role. Our results were supportive of this inference. If genetic defense had been solely responsible for low levels of infection in the field, we would have expected those same low levels in inoculation experiments (Table 3), but as already mentioned they were higher. As expected for H_2, re-isolation frequencies did vary among the 18 inoculants representing 14 OTUs: from the *Fusarium* '*fus124*' OTU (57.81%) to the three inoculants (i.e. *epi066*, *pho086* and *tri103*) that were not re-isolated at all (Table 2).

In two subsequent experiments with *Botrytis*, *Cladosporium*, and *Fusarium*, re-isolation frequencies varied significantly with maternal plant genotype, again indicating partial support for H_2 (Table 4), even if genetic defense could not be solely responsible (Table 3). As before, the absence of any non-inoculant fungi in any seeds including those from control flower heads demonstrated that genetic defense is selective. Host defense did not, however, appear to be differential. Of the 12 maternal plant genotypes, only one was completely resistant and only to *Botrytis*; all other maternal plant genotypes were infected to varying extent by each of the three inoculants. The significant plant/fungus interaction expected even for weakly differential defense was not observed (Table 4): first experiment (fungal inoculant × maternal plant genotype: $F_{10,52} = 1.829$, $P = 0.080$) and second experiment (fungal inoculant × maternal plant genotype: $F_{10,52} = 1.22$, $P = 0.301$). Surface-sterilization method did not change the ranking of re-isolation frequencies of the three inoculants in these two experiments: *Fusarium* > *Cladosporium* > *Botrytis* (Table 2).

Mixed, or co-inoculations

These inoculations revealed that inoculants completely excluded one another, as predicted by H_3 (Table 3), in a completely consistent manner. *Cladosporium* excluded *Botrytis*. *Fusarium* excluded *Cladosporium*. *Fusarium* excluded *Botrytis*. Not a single seed was infected with two inoculants, according to re-isolation results. Neither maternal plant genotype nor surface-sterilization method had any effect on this finding of complete exclusionary interactions among the three fungi.

PCR-based detection of fungi in seeds

PCR products from the first-round PCR with the universal, fungus-specific, ITS1/ITS4 primer pair did not produce any amplicons. However, second-round, nested-PCR products with fungal-specific primers from mixed inoculations revealed that inoculants did exclude one another as observed by re-isolation (Table 5). Genomic DNA from seeds that developed in flowers co-inoculated with *Botrytis* and *Cladosporium* gave an amplification product with the *Cladosporium*-specific primer pair, but not with the *Botrytis* primer pair in any of the three sets tested. Similarly, genomic DNA from all co-inoculations involving *Fusarium* (i.e. two two-inoculant and one three-inoculant mixes) did not reveal the presence of either *Botrytis* or *Cladosporium* (Table 5). Comparisons of amplification products sequenced directly from *Cladosporium* and *Fusarium* primer pairs to the original ITS sequences revealed that the sequences were identical to their original, respective ITS sequences (data not shown). DNA from control seeds did not produce amplification products either with the ITS1/ITS4 primer pair or with any of the three specific primer pairs tested. The three primer pairs tested were highly specific, as the *Botrytis* primer pair did not amplify DNA from either *Cladosporium* or *Fusarium* pure culture. Similar specificities were observed for the *Cladosporium* and *Fusarium* primer pairs.

Effects of single, seed-infecting fungi on seedling emergence, growth and fecundity

Repeated-measure analysis of variance showed that seedling emergence in a mix of field soil and potting mix

Table 4. Analysis of variance of re-isolation frequency as affected by fungal inoculants (*Botrytis, Cladosporium* and *Fusarium*), maternal plant genotype, and their interaction following Method I and Method II surface sterilization (see Table 2) of seeds

		Re-isolation frequency (%)					
		Method I*			Method II		
Inoculation	Source of variation	d.f.	*F*	*P*	d.f.	*F*	*P*
Single inoculations	Fungal inoculant	2	13.86	**0.001**	2	175.49	**< 0.001**
	Maternal plant genotype	5	12.06	**< 0.001**	5	6.41	**< 0.001**
	Fungal inoculant × maternal plant genotype	10	1.83	0.080	10	1.22	0.301
	Error	52			54		

Significant effects are in boldface.

*Data were arcsin square root transformed before analysis.

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Table 5. Detection via nested-PCR of fungi in seeds developed from co-inoculated flowers

				Nested-PCR			
				First round amplification†	Second round amplification‡		
Inoculation	Fungal inoculants	Dominant fungus re-isolated from seeds	DNA concentration* (ng μL^{-1})	Fungus-specific primers	*Botrytis* specific primers	*Cladosporium* specific primers	*Fusarium* specific primers
Mixed inoculants	*Botrytis* + *Cladosporium*	*Cladosporium*	311.93	–	–	+	NA
	Botrytis + *Fusarium*	*Fusarium*	278.19	–	–	NA	+
	Cladosporium + *Fusarium*	*Fusarium*	300.12	–	NA	–	+
	Botrytis + *Cladosporium* + *Fusarium*	*Fusarium*	315.05	–	–	–	+
Sterile water	Control	–	295.26	–	–	–	–

+, amplicon observed; –, no amplicon observed; NA, not applicable.

*Each value is a mean of three sets. Fifteen seeds infected with dominant fungus were surface-sterilized following Method I prior to DNA extraction (see Materials and methods).

†3 μL aliquot of total genomic DNA from each mixed combination and control was used as template in the first round of PCR mixture containing universal, fungal-specific primer pair ITS1/ITS 4 (White *et al.*, 1990).

‡A 1 μL aliquot from the first round PCR was used as template in the second round PCR with specific primer pairs. Total genomic DNA from seeds gave an amplification product only with the primer pair for the fungus that was consistently re-isolated.

was significantly affected by fungal inoculant ($F_{3,24}$ = 7.705, $P < 0.001$). *Fusarium* infection of developing seeds significantly reduced subsequent seedling emergence (Fig. 1). Fungal inoculant also significantly affected early growth of seedlings as measured by shoot biomass ($F_{3,24}$ = 6.170, $P = 0.003$) and shoot-to-root biomass ratio ($F_{3,24}$ = 3.02, $P = 0.04$), although differences in root biomasses ($F_{3,24}$ = 1.41, $P = 0.265$) were not significant. Seedlings infected with *Fusarium* had a lower shoot biomass (mean of 0.037 g seedling^{-1}) than controls (0.045), or those infected with *Cladosporium* (0.047) or *Botrytis* (0.044) (Fig. 2). *Cladosporium* had no negative effects on seedling emergence and growth.

In additional experiments, *Cladosporium* increased fecundity (t = 4.235, $P < 0.001$) and even aboveground biomass (t = 3.227, $P = 0.004$) and its infection was confirmed in seedlings. No re-isolates of *Cladosporium* were obtained from controls.

Discussion

As expected, both the abiotic environment (H_1) and genetic defense (H_2) contributed partially to limited infection of developing seeds of *C. stoebe*. If genetic defense had been complete, re-isolation frequencies would not have differed from field-isolation frequencies. Instead, the former were ten times higher than the latter. So, the environment is somewhat limiting. In addition, the wet controls demonstrated a selective system that can be attributed to genetic defense alone: seeds free of any culturable fungi were invariably obtained in the presence of non-inoculant fungi in control flower heads in the greenhouse. Selectivity was

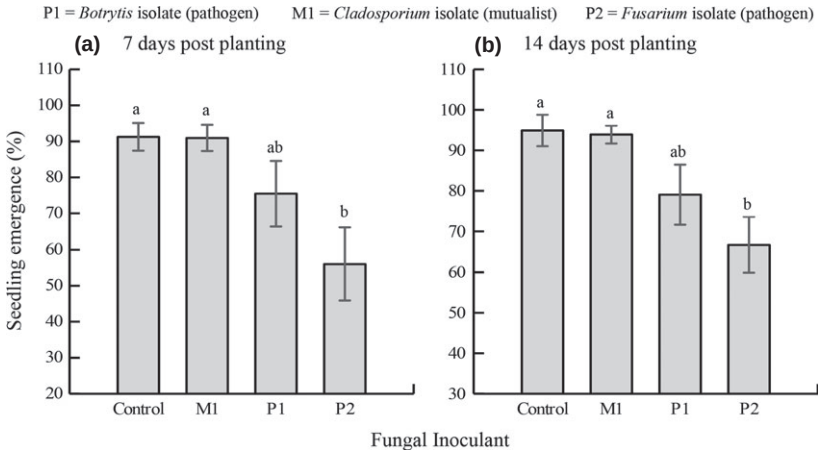

Fig. 1. Effects of seed-infecting fungi on seedling emergence (±SE), (a) 7 days, and (b) 14 DPP. Means with the same letter are not significantly different from one another (Tukey's HSD, $P = 0.05$). $N = 9$. *Fusarium* significantly reduced seedling emergence.

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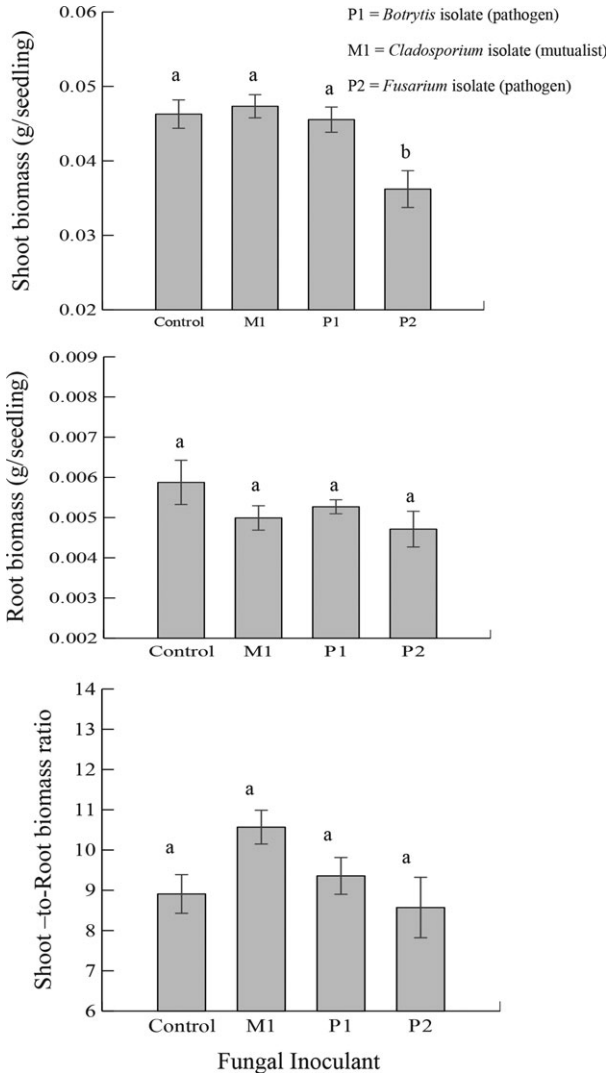

Fig. 2. Effects of seed-infecting fungi on shoot biomass, root biomass, and shoot-to-root biomass ratio. Means with the same letter are not significantly different from one another (Bonferroni, $P = 0.05$). $N = 9$.

also shown by the recovery of inoculants only from inoculated flower heads. Secondly, some fungi that were originally isolated from seeds were not re-isolated at all following the inoculation of 18 isolates (14 OTUs), indicating genetic defense that varies in strength relative to different fungi. Thirdly, plant maternal genotype was a significant factor in analyses of re-isolation frequencies. All of this pointed to host defense as a strong filter of potential, seed-infecting fungi and a partial explanation, along with environment, of low infection or isolation levels with the relatively few fungi able to infect developing seeds of *C. stoebe*.

However, the finding of one fungus per seed following inoculations with multiple fungi is much more simply explained by exclusionary interactions (H_3) than it is by H_1 or H_2. When two or three inoculants were co-inoculated to compete in flower heads, only one, and always the same one, was re-isolated from all seeds, regardless of maternal plant genotype. This was not simply a matter of growth rate on PDA as that of *Cladosporium* is less than that of *Fusarium* which is less than that of *Botrytis*. In other words, the successful inoculant was not simply suppressing the other one or two inoculants by growing out faster on agar. This was also confirmed in the nested-PCR procedure when specific primers were used to detect fungi in seeds from co-inoculations (Table 5). This procedure was highly sensitive, as evidenced by our sensitivity tests which showed that we could have detected both *Botrytis* (in the background of combined host-*Cladosporium* and host-*Fusarium* DNA) and *Cladosporium* DNA (in the background of combined host-*Fusarium* DNA) at levels as low as 24 pg μL^{-1} (Data S1). This level corresponds to 10 000 times less than the combined host-fungus DNA from mixed fungal inoculations observed in this study (Table 5). We are thus left with exclusionary interaction as the simplest explanation for consistent re-isolation of a particular inoculant from seeds of all plants following co-inoculations of flowers.

The complete pattern of interaction for all 92 OTUs would be difficult to determine experimentally, but we expect that it could be non-transitive even though our results to date only demonstrate a transitive pattern. *Fusarium* could, for example, be excluded from seed in the field by fungi other than the co-inoculants employed here. A non-transitive system might explain rather than deepen the paradox of endophyte diversity.

Our finding of strong, exclusionary interactions among microbes might eventually be shown to extend to and include non-culturables. Our experiments emphasized culturable fungi capable of infecting developing seeds. For the time being, we also ignored the potential importance to the next plant generation of microbes on the surface of seeds.

The best communities from which to derive assembly rules may be simple ones (Keddy & Weiher, 1999), especially when the later inhabit 'unproductive, stressful environments' (Booth & Larson, 1999). Fungal communities in developing seeds may provide unique opportunities to determine whether the outcomes of interactions depend on types of interacting symbionts. Mixed inoculations with 'Class 1' endophytes (Rodriguez *et al.*, 2009) in the grass host, *Bromus erectus*, have shown that endophyte-endophyte interactions can be significant (Wille *et al.*, 2002).

Exclusionary interactions might have been favored here by our focus on developing seeds. Vertical transmission

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has been defined as 'the direct transfer of infection from a parent organism to its progeny', and it is thought to 'favor evolution toward mutualism and benign parasitism' (Ewald, 1987). In our system, *Fusarium* tended to be pathogenic with negative effects on seedling emergence and shoot growth (Figs 1 and 2). In contrast, *Cladosporium* tended to be mutualistic as it had no negative effects on seedling emergence, and positive effects on fecundity and growth (the latter in some, but not all, experiments). The success of pathogenic *Fusarium* when competing with mutualistic *Cladosporium* in our system is thus at odds with the vertical transmission model, as presently understood. Finally, and most importantly, the demonstrated, exclusionary interactions in a community with diverse members (Shipunov *et al.*, 2008) provide another example of the paradox of diversity (Hutchinson, 1961). Reconciliation of this paradox may be possible via determination of the extent to which genetic defense and the environment limit opportunities for community members to compete for the single-fungus niche or via evidence that exclusionary interactions are non-transitive overall.

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References

Ahlholm JU, Helander M, Henriksson J, Metzler M & Saikkonen K (2002) Environmental conditions and host genotype direct genetic diversity of *Venturia ditricha*, a fungal endophyte of birch trees. *Evolution* **56**: 1566–1573.

Arnold AE (2007) Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biol Rev* **21**: 51–66.

Aschehoug ET, Metlin KL, Callaway RM & Newcombe G (2012) Fungal endophytes directly increase the competitive effects of an invasive forb. *Ecology* **93**: 3–8.

Booth BD & Larson DW (1999) Impact of language, history and choice of system on the study of assembly rules. *Ecological Assembly Rules: Perspectives, Advances, Retreats* (Weiher E & Keddy P, eds), pp. 206–229. Cambridge University Press, Cambridge, UK.

Dangl JL & Jones JDG (2001) Plant pathogens and integrated defence responses to infection. *Nature* **411**: 826–833.

Doyle JJ & Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* **19**: 11–15.

Ewald PW (1987) Transmission modes and evolution of the parasitism-mutualism continuum. *Ann NY Acad Sci* **503**: 295–306.

Fukami T & Morin PJ (2003) Productivity-biodiversity relationships depend on the history of community assembly. *Nature* **424**: 423–426.

Ganley RJ, Brunsfeld SJ & Newcombe G (2004) A community of unknown, endophytic fungi in western white pine. *P Natl Acad Sci USA* **101**: 10107–10112.

Hall T (2011) Bioedit. Version 7.1.3. http://www.mbio.ncsu.edu/bioedit/bioedit.html.

Harrod RJ & Taylor RJ (1995) Reproduction and pollination biology of *Centaurea* and *Acroptilon* species, with emphasis on *C. diffusa*. *Northwest Sci* **69**: 97–105.

Hong SG, Cramer R, Lawrence CB & Pryor BM (2005) *Alt a 1* allergen homologs from *Alternaria* and related taxa: analysis of phylogenetic content and secondary structure. *Fungal Genet Biol* **42**: 119–129.

Hutchinson GE (1961) The paradox of the plankton. *Am Nat* **95**: 137–145.

Keddy P & Weiher E (1999) The scope and goals of research on assembly rules. *Ecological Assembly Rules: Perspectives, Advances, Retreats* (Weiher E & Keddy P, eds), pp. 1–20. Cambridge University Press, Cambridge, UK.

Langrell SRH (2005) Development of a nested PCR detection procedure for *Nectria fuckeliana* direct from Norway spruce bark extracts. *FEMS Microbiol Lett* **242**: 185–193.

Newcombe G, Shipunov A, Eigenbrode SD, Raghavendra A, Ding H, Anderson CL, Menjivar R, Crawford M & Schwarzländer M (2009) Endophytes influence protection and growth of an invasive plant. *Commun Integr Biol* **2**: 1–3.

Newman JA, Bergelson J & Grafen A (1997) Blocking factors and hypothesis tests in ecology: is your statistics text wrong? *Ecology* **78**: 1312–1320.

Ott RL & Longnecker M (2001) *An Introduction to Statistical Methods and Data Analysis*, 5th edn. Duxbury Publishers, USA.

Parfitt D, Hunt J, Dockrell D, Rogers HJ & Boddy L (2010) Do all trees carry the seeds of their own destruction? PCR reveals numerous wood decay fungi latently present in sapwood of a wide range of angiosperm trees. *Fungal Ecol* **3**: 338–346.

Redman RS, Ranson JC & Rodriguez RJ (1999) Conversion of the pathogenic fungus *Colletotrichum magna* to a nonpathogenic, endophytic mutualist by gene disruption. *Mol Plant Microbe Interact* **12**: 969–975.

Rhoades DF (1979) Evolution of plant chemical defense against herbivores. *Herbivores: Their Interaction with Secondary Plant Metabolites* (Rosenthal GA & Janzen DH, eds), pp. 4–55. Academic Press, FL, USA.

Rodriguez RJ, White JF, Arnold AE & Redman RS (2009) Fungal endophytes: diversity and functional roles. *New Phytol* **182**: 314–330.

Schulz B & Boyle C (2005) The endophytic continuum. *Mycol Res* **109**: 661–686.

Shipunov A, Newcombe G, Raghavendra A & Anderson C (2008) Hidden diversity of endophytic fungi in an invasive plant. *Am J Bot* **95**: 1096–1108.

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Tanaka A, Christensen MJ, Takemoto D, Park P & Scott B (2006) Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. *Plant Cell* **18**: 1052–1066.

Tilman D (1999) Diversity by default. *Science* **283**: 495–496.

White TJ, Bruns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* (Innis MA, Gelfand DH, Sninsky JJ & White TJ, eds), pp. 315–322. Academic Press, New York, USA.

Wille P, Boller T & Kaltz O (2002) Mixed inoculation alters infection success of strains of the endophyte *Epichloë bromicola* on its grass host *Bromus erectus*. *Proc Biol Sci* **269**: 397–402.

(Zangerl AR & Bazzaz FA, eds) (1992) *Theory and Pattern in Plant Defense Allocation*. University of Chicago Press, Chicago, IL, USA.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. (a) Close to 100 flower heads can be produced by a greenhouse plant. (b) Plants were pollinated 24 h prior to inoculation. (c) Inocula were delivered with a 3 mL hypodermic syringe. (d) Flower heads were maintained in a moistened condition within a polythene bag to allow for fungal infection of developing seeds.

Data S1. Sensitivity of nested-PCR procedure in detecting fungi from seeds of mixed or co-inoculated flowers.

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Supporting Information

Method S1. Sensitivity of the nested-PCR procedure

To determine the sensitivity of nested PCR in detecting fungi from co-inoculated seeds, three tests were conducted; one for each of the three two-fungi combinations. First, to simulate detection of *Botrytis* in the background of combined host-*Cladosporium* genomic DNA, seven 1:5 serial dilutions of *Botrytis* genomic DNA from pure culture ranging from 15 ng to 0.19 pg were prepared. One µL from each dilution was added to a PCR reaction mixture containing genomic DNA from *Cladosporium* (single fungus) infected seeds (3 µL, 287 ng genomic DNA μL^{-1}). A second test was conducted to simulate detection of *Botrytis* in the background of combined host-*Fusarium* genomic DNA. For this, 1 µL from each dilution of *Botrytis* was added to a PCR reaction mixture containing genomic DNA from *Fusarium* (single fungus) infected seeds (3 µL, 297 ng μL^{-1}). Finally, to simulate detection of *Cladosporium* in the background of combined host-*Fusarium* genomic DNA, seven 1:5 serial dilutions of *Cladosporium* genomic DNA from pure culture ranging from 15 ng to 0.19 pg were prepared as before. One µL from each dilution was added to a PCR reaction mixture containing genomic DNA from *Fusarium* infected seeds (3 µl, 297 ng μL^{-1}). Reactions were carried out in a total mixture volume of 25 µL and nested PCR's were performed as described in Materials and Methods section. All tests were carried out two twice.

Results

After the second round amplification with specific primer pairs, the detection limit for *Botrytis* in the background of host-*Cladosporium* and host-*Fusarium* DNA was 24 pg μL^{-1}. Similar detection limit was observed for *Cladosporium* in the presence of host-*Fusarium* DNA.

Fig.S1 (a) Close to 100 flower heads can be produced by a greenhouse plant. (b) Plants were pollinated 24h prior to inoculation. (c) Inocula were delivered with a 3 mL hypodermic syringe. (d) Flower heads were maintained in a moistened condition within a polythene bag to allow for fungal infection of developing seeds.