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On the use of fungicides in ecological seed burial studies

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Abstract

Evidence for effects of saprophytic fungi on buried seed demography is usually obtained from studies involving the simultaneous burial of fungicide-treated seeds and of untreated seeds. However, any potential influence of fungicide treatment on seed dormancy levels is generally ignored in these studies. Also, some studies assume that a combination of several fungicidal compounds provides better protection against a broader range of fungi, ignoring chemical interactions that may potentially occur between different compounds. To investigate these issues, we carried out a six-month burial experiment using seeds of *Anthriscus sylvestris* (L.) Hoffm., *Centaurea nigra* L., and *Daucus carota* L., and three substrates differing in organic matter content. Three fungicidal compounds, captan, iprodione, and mancozeb, were applied alone and in combination, including an untreated control. All fungicidal compounds and combinations thereof provided protection against fungal-induced seed mortality, and except for a low efficacy of iprodione in protecting seeds of Anthriscus, there were no pronounced differences in seed mortality between different fungicide treatments. Captan temporarily inhibited germination in *Centaurea*, whereas a similar inhibition in *Daucus* seeds caused by mancozeb was more long-lasting, suggesting an induction of secondary dormancy. Organic matter content only had a negligible influence on these results. Our results suggest that the basic conclusions from most seed burial studies are robust with respect to their choice of fungicide. We conclude by discussing further implications of our findings for the design and interpretation of seed burial studies.

Key Words

dormancy, fungal attack, fungicide treatment, mortality, seed burial experiments, seed longevity, soil organic matter content

Introduction

The effects of saprophytic soil fungi on the longevity of buried seeds have been investigated for many different plant species from a wide range of different ecosystems with experiments involving the simultaneous burial of untreated seeds and of seeds treated with fungicides (Fellows and Roeth, 1992; Lonsdale, 1993; Dalling *et al.*, 1998; Leishman *et al.*, 2000; Gallandt *et al.*, 2004) The results underline that fungal-induced seed mortality can greatly affect seed demography.

However, methodological aspects are usually given little consideration in such seed burial studies. One notable exception is the study by Van Mourik *et al.* (2005) which demonstrated that the density of seeds buried in seed bags can markedly affect rates of fungal-induced seed mortality. However, there are at least three other methodological aspects that would in our opinion also merit methodological consideration. First, it is known that fungicides can directly affect live plants even in the absence of the targeted fungi (Paul *et al.*, 1989; Laird and Addicott 2008), and as outlined further below, the same may also apply to the seed stage of plants. Second, fungicide efficacy can crucially depend on soil characteristics such as soil organic matter content (Goring, 1967), although individual fungicides will be affected differently by such characteristics (Lopes *et al.*, 2002; Andrades *et al.*, 2004). Finally, different fungicides, due to their different modes of action, tend to have specific effects on particular fungal taxonomic groups (Paul et al., 1989), and are therefore often combined to protect live plants against a wider range of fungal pathogens (Gisi, 1996). Such a combination of fungicides can sometimes also result in unexpected synergistic or antagonistic effects on fungal pathogens (Scardavi, 1966; Gisi, 1996), and there is also the possibility of unexpected changes in phytotoxicity (Backman, 1978). This third aspect may also deserve more consideration in the context of seed burial studies. Several fungal phyla contain saprophytic genera with the potential to harm seeds (Schafer and Kotanen, 2004), and this provides a motivation for combining several of these fungicides to achieve protection of seeds

against a wider range of fungi (Leishman *et al.*, 2000). This assumption of a combination of fungicides providing a more comprehensive protection against fungal-induced seed decay has however not yet been experimentally verified. On the contrary, it also seems possible that different fungicides used together in a mixture may chemically interact with each other in a way that could negatively affect their efficacy as seed protectants. Combining different fungicides may even result in toxic effects on seeds similar to those observed for particular fungicide-insecticide combinations (Gange *et al.*, 1992).

Similar to the above stated known direct effects on live plants, fungicides can also have direct effects on seeds. It is known from *in vitro* experiments using crop seeds that fungicide treatment can affect germination rates, either by inhibiting or by promoting seed germination (Clark and Scott, 1982; Simmen and Gisi, 1995; Hartz and Caprile, 1995). The same mechanisms can also affect the timing of crop seedling emergence in the field (Smiley *et al.*, 1996).

To explore these various methodological aspects in a full factorial randomized block experiment, we treated the seeds of three grassland plant species with up to three different fungicidal compounds alone and in combination, and buried them in three different substrates representing a gradient in soil organic matter content. The following three main questions were addressed: (1) Do treatments that combine more than one fungicide result in a greater reduction in seed mortality, compared to treatments that use just one fungicidal compound? (2) Do fungicides, alone or in combination with each other, have an influence on the readiness of seeds to germinate when exposed to conditions that are favourable to germination, i.e. are dormancy levels influenced by the fungicide treatments? (3) Do these fungicide effects on readiness to germinate and on seed mortality depend on soil organic matter content?

Material and Methods

105 Field site

106 The burial experiment was carried out in unmanaged ruderal grassland adjacent to the Centre
107 for Environmental Research and Technology (UFT) of University of Bremen, Germany (53°
108 05' N, 8° 48' E). The topsoil at this site consists of almost pure sand, with an average pH of
109 5.2 and an organic matter content of 1.1 % (Mitschunas *et al.*, 2008). Mean annual mean
110 temperature and total precipitation, based on the period 1991-2005, are 9.5°C and 713 mm
111 (Deutscher Wetterdienst, 2008).

112

113 Materials

114 We used seeds of *Anthriscus sylvestris* (L.) Hoffm., *Centaurea nigra* L., and *Daucus carota*
115 L., (nomenclature follows Jäger and Werner, 2002), three grassland species characterised by
116 short-term seed bank persistence between one and five years (Thompson *et al.*, 1997).

117 We used three different fungicidal compounds in our experiment. Two of these
118 compounds, captan and iprodione, have been used previously in ecological seed burial
119 experiments, with captan being very regularly employed in such experiments (Wagner and
120 Mitschunas, 2008). The third compound, mancozeb, has been recommended for seed
121 treatment (Sinha *et al.*, 1988), although it has not been used previously in the context of
122 ecological seed burial studies. Both captan and iprodione are dicarboximide fungicides,
123 whereas mancozeb is a dithiocarbamate fungicide. Captan is considered very effective against
124 seed-rotting fungi (Neergaard 1979), and in an agricultural context it is mainly used against
125 pathogens from the phylum Ascomycota (Whitehead 1998). By contrast, both iprodione and
126 mancozeb are more widely used not only against Ascomycota but also against a wide range of
127 pathogenic Basidiomycota and Oomycetes (Whitehead 1998), the latter group now being
128 recognized as being taxonomically distinct from the true fungi (Deacon, 2006).

129 As in many previous studies (e.g. Blaney and Kotanen, 2001; O'Hanlon-Manners and
130 Kotanen 2004a; Orrock and Damschen, 2005; Van Mourik *et al.*, 2005), seed bags filled with

131 a mixture of soil and seeds were buried. Our seed bags were made of 7 cm × 7 cm pieces cut
132 from nylon stockings. To establish a gradient of soil organic matter content in the seed
133 environment, we used the local topsoil and a green waste compost (pH 5.7) from a local
134 supplier (Kübel-und Pflanzerde; Kompostierung Nord GmbH, Bremen, Germany) as base
135 materials to create three different substrates. These were pure topsoil, pure green waste
136 compost, and a 1:1 volume-ratio mixture of both materials.
137
138 Experimental set-up
139 Prior to the experiment, all three substrates were passed through a sieve of 5.0 mm mesh
140 width. To control for known effects of soil fauna on fungal-induced seed mortality, a sub-
141 sample of each substrate, used for filling the seed bags, was subsequently passed through a
142 1.0 mm sieve and then defaunated by 24 h freezing at -20 °C, followed by 24 h at room
143 temperature and another 48 h at -20 °C (Mitschunas *et al.*, 2006).
144 Each seed bag was filled with 4 ml of respective defaunated substrate and a total of 75
145 seeds (= 25 seeds per species), and then tied up with sewing thread. To ensure recognition of
146 individual treatments at the end of the experiment, each bag was marked using colour-coded
147 pieces of cord. Prior to burial at the field site, the mesh bags from the fungicide treatments
148 were immersed in fungicide solutions prepared from three different fungicidal compounds on
149 their own or in combination.
150 These fungicide solutions were prepared on 20 December 2006 by dissolving specific
151 quantities of each fungicide at 20°C in 1000 cm^3 of distilled water. We used 10g of Merpan
152 80 WDG (active compound: captan 80% w/w; Feinchemie Schwebda GmbH, Eschwege,
153 Germany) for the captan solution, 0.8 g of Rovral 75WG (ac tive compound: iprodione 75%
154 w/w; BASF AG, Ludwigshafen, Germany) for the iprodione solution, and 2 g of Dithane
155 NeoTec (active compound: mancozeb 75% w/w; Spiess-Urania Chemicals GmbH, Hamburg,
156 Germany) for the mancozeb solution. For iprodione and mancozeb, these concentrations

157 corresponded with the recommendations made by the manufacturers for soil application. The
158 concentration of captan was the same as in previous seed burial experiments (Blaney and
159 Kotanen, 2001; O'Hanlon-Manners and Kotanen, 2004a; Kotanen 2007).
160 We did not reduce the concentrations of individual fungicidal compounds when
161 preparing the mixtures, as there was no indication for such a course of action from previous
162 seed burial experiments employing mixtures of different fungicides. Instead, we dissolved the
163 same quantity of each individual fungicide compound when preparing 1000 cm^3 of mixture
164 solutions as was used for preparing single-compound solutions. We also included a control
165 treatment in which mesh bags were immersed in water prior to burial, and thus had all eight
166 possible different fungicide combinations, ranging from no fungicide at all to the combination
167 of all three fungicides. In combination with the three levels of soil organic matter content, this
168 resulted in 24 different treatments. We immersed six replicate seed bags per treatment, i.e. a
169 total of 144 seed bags in the respective solutions on the same day as the fungicide solutions
170 were prepared. Bags were immersed for fifteen minutes to ensure complete saturation. After
171 immersion, the bags were stored over night in plastic trays at 13 °C in the dark, still separated
172 by fungicide treatment. The following day, on 21 December 2006, we established three
173 experimental blocks at our grassland site for seed burial. These blocks were placed in the
174 corners of an equiangular triangle with a side length of ca. 7 m between blocks. Per block, we
175 excavated 48 cylindrical holes of 7 cm diameter and 6 cm depth in a regular grid of 6 × 8
176 across an area of 0.6 m × 0.8 m, allowing for two replicate seed bags of each treatment to be
177 buried in the same block. Individual replicates were assigned at random to positions within
178 the grid. Prior to placing each seed bag in its hole, we filled half of the hole with the same
179 substrate that was used to fill the bag, but passed through a 5.0 mm sieve only and not
180 defaunated. After placement of the seed bags, the holes were filled to surface level with the
181 same substrate, thus ensuring that the substrate around the bags was of the same composition

as the substrate in the bags. After six months, the seed bags were recovered from the field on 20 June 2007.

Seed viability testing

Immediately after recovery, the contents of the seed bags were surface-sterilized by soaking the bags in 70% ethanol for 2 min, followed by soaking in 1.25% sodium hypochlorite solution for 4 min. Finally, each seed bag was rinsed twice for a two-minute period with distilled water. After that, each seed bag was opened and germinated seedlings were counted and removed. Across the whole experiment, a total of four *Centaurea* seeds and five *Daucus* seeds had germinated during burial, and their occurrence was seemingly unrelated to experimental treatments. More regularly, we found germinated *Anthriscus* seeds, but the fraction of germinated seeds of this species never exceeded 12% (= 3 seeds) in a single seed bag, and a three-factorial analysis of variance on arcsine-transformed data, using fungicide combination and substrate as fixed factors and block identity as random factor (results not shown) did not provide any evidence for an influence by the experimental factors. The soil containing the remaining seeds was transferred into 9-cm Petri dishes containing a double layer of filter paper (Whatman No. 1, Whatman International Ltd., Maidstone, England) moistened with 5 cm^3 of distilled water. The Petri dishes were sealed with Parafilm 'M' (Pechiney Plastic Packaging, Chicago, Illinois, USA) and placed in a climate chamber (Sanyo MLR-350H), at constant humidity of 80% and exposed to a diurnal cycle (16 h of light at 25°C, 8 h of darkness at 15 °C) known to promote germination (Thompson and Grime, 1983). Every other day, the Petri dishes were randomized. Seeds showing a visible protrusion of the radicle from the seed coat were considered to have germinated (Kitajima and Fenner, 2000), and counted and removed at weekly intervals. Between counts, the Petri dishes were re-sealed. This germination test was run for a total of three weeks. During this period, only about 2% of *Anthriscus* seeds germinated. By contrast, ca. 98% of the *Centaurea* seeds and ca. 27%

208 of the *Daucus* seeds had germinated by that time, most of them in weeks 1 and 2. To establish
209 the exact status of seeds still ungerminated after three weeks, these were checked for viability
210 under a microscope. Soft seeds were considered dead, as well as seeds containing blackened
211 embryo when dissected. The remaining seeds were stained after dissection with a 0.1%
212 solution of 2,3,5-triphenyl tetrazolium chloride (Cottrell, 1947). After 12 h in an incubator at
213 30 °C, seeds were classified into dead or viable on the basis of embryo and endosperm
214 coloration.
215
216 Data analysis
217 We carried out factorial analyses of variance based on Type III sums of squares using SPSS
218 14.0 (SPSS Inc., Chicago, IL, USA). Prior to statistical analyses, all data were arcsine-
219 transformed to meet distributional requirements (Sokal and Rohlf, 1995).
220 To allow investigation of treatment effects on seed mortality, we calculated for each
221 species in each seed bag the overall proportion of ungerminated dead seeds at the end of the
222 burial period by summarising the proportions of seed determined as dead by visual inspection
223 and of seeds determined dead as a result of tetrazolium testing.
224 To allow testing of treatment effects on the readiness of seeds to germinate, we also
225 calculated for each seed bag the proportion of *Centaurea* and *Daucus* seeds that germinated
226 up to a specific point in time, based on the overall number of seeds that were still viable and
227 ungerminated after the burial period (i.e. seeds germinated in the Petri dishes over the whole
228 three-week period plus seeds that remained ungerminated but viable according to the
229 tetrazolium test). Being interested in both the short-term effects and the longer-term effects on
230 the germinability of viable seeds, we calculated this ratio both based on germination in the
231 first week of the Petri dish trial, and also based on the germination occurring throughout the
232 three-week trial. No similar data analyses were performed for ratios based on an intermediate
233 germination period of two weeks, as these were virtually identical to ratios based on the

whole three-week period. Neither did we analyse *Anthriscus* seed germination, as germination was very low across all treatments, averaging only about 2% of seeds during the whole three-week germination period.

Initial analyses included both substrate type and fungicide combination as fixed factors and block identity as random factor. As the experiment included within-block replication of individual treatments, we were able to follow the recommendation by Quinn and Keough (2002) to also test for treatment x block interactions. Results from these initial analyses indicated that the influence of substrate type on both mortality and readiness of seeds to germinate was negligible and mostly not significant. Therefore, we re-analysed the data for the three soil types combined, dropping the factor substrate type from the analysis. Here, we only report the results of this latter set of analyses.

In the case of significant fungicide treatment effects, we carried out post-hoc comparisons between treatments. For post-hoc comparisons related to seed mortality, we used the Tukey HSD procedure that evaluates any differences in means among all possible pairs of treatments. For post-hoc comparisons related to the readiness of seeds to germinate we used a two-sided Dunnett test procedure instead of the Tukey HSD procedure, as we were only interested in which fungicide combinations significantly affected the readiness of seeds to germinate compared to the untreated control treatment. This procedure is more powerful because pairwise comparisons are restricted to comparing the control treatment with the other treatments, whereas no comparisons are made among the latter (Quinn and Keough, 2002).

Results

Effects on seed mortality

A. sylvestris was characterised by a particularly high proportion of dead seeds at the end of the six-month burial period, with dead seeds making up between 31% and 48% in the

different fungicide treatments, and 66% in the untreated control (Figure 1A). By contrast, in *C. nigra* the proportion of dead seeds never exceeded 20% even in the untreated control (Figure 1B), and *D. carota* was characterised by intermediate proportions of dead seeds (Figure 1C). As indicated by ANOVA, there were highly significant ($P < 0.001$) fungicide effects on seed mortality both in *Anthriscus* and in *Daucus*, whereas there were no such effects in *Centaurea* (Table 1). As indicated by Tukey HSD tests, seed mortality in *Anthriscus* was significantly lowered by all fungicide treatments compared to the untreated control, but was significantly higher in the iprodione only treatment than in the other six fungicide treatments (Figure 1A). Similarly, all fungicide treatments significantly reduced seed mortality in *Daucus*, but in this species there were no significant differences among individual fungicide treatments (Figure 1C).

Effects on dormancy levels

Almost none of the *A. sylvestris* seeds that remained ungerminated viable throughout the 6 month burial period germinated in the Petri dish trial. For this reason, data analyses on the influence of fungicide treatments on readiness of seeds to germinate were carried out only for the other two species, *C. nigra* and *D. carota*. As indicated by ANOVA, the use of different fungicides had a significant impact on the proportion of viable seeds germinating within one week in both species (Table 2A). In each of these two species, particular fungicide combinations were associated with a reduction of the proportion of viable seeds germinating in the first week after seed bag recovery. In *C. nigra*, this effect was highly significant in all treatments involving captan (Dunnett test: $P < 0.001$ in all cases), irrespective of whether this compound was used on its own or in combination with iprodione and / or mancozeb (Figure 2A). A two-way combination of iprodione and mancozeb also resulted in a reduced readiness of *Centaurea* seeds to germinate immediately (Dunnett test: $P = 0.002$). However, at the end of the three-week germination test, germination was close to 100% across treatments (Figure

3B). Although the overall ANOVA test based on this data indicated a significant fungicide effect (Table 2B), according to the Dunnett test procedure, this was not due to any significant pairwise differences between fungicide treatments and the untreated control (Dunnett test $P > 0.44$ for all pairwise comparisons with the untreated control apart from the combination of iprodione and mancozeb, for which $P = 0.054$).

In *Daucus*, a significant reduction of the proportion of viable seeds germinating within one week after seed recovery was found in all treatments combining mancozeb with one or both of the other two fungicides (Table 2, Figure 3A; Dunnett test: $P < 0.02$ in all cases). At the end of the three-week Petri dish trial, only about 27% of all viable *Daucus* seeds, as averaged across treatments, had germinated, and ca. 97% of these had done so in the first two weeks, indicating that in spite of favourable conditions for germination, most of the remaining seeds would likely have not germinated in following weeks, if the Petri dish trial would have been continued for a longer period. Final counts after three weeks indicated that the fungicide effects in *Daucus* observed after one week had persisted throughout the whole three-week period, with final proportions of germinated viable seeds still being significantly lower in two of the treatments involving mancozeb (Table 2B, Figure 3B), both on its own (Dunnett test: $P = 0.042$) and in combination with iprodione (Dunnett test: $P = 0.005$).

Discussion

The combination of several fungicidal compounds generally did not result in any clear further reduction of seed mortality compared to using just one fungicide at its recommended dosage. On the other hand, we could show that individual fungicides can affect the proportion of viable seeds germinating under conditions known to promote germination, and that such effects can be prolonged. However, we found no clear evidence for soil organic matter effects.

Treatment of seeds with fungicides generally increased the survival of buried seeds, although in one of the three test species, *C. nigra*, overall mortality over the six-month burial

312 period was too low to allow any accumulation of significant differences between the untreated
313 control and the fungicide treatments. With the exception of the iprodione only treatment in
314 *Anthriscus* being slightly less effective than the other fungicide combinations, there were no
315 significant differences among individual fungicide treatments. The visual inspection of the
316 results seems to suggest that in the case of *Daucus*, the combination of two fungicidal
317 compounds may tend to provide a slightly better protection against fungal decay than the use
318 of a single compound only, although the observed differences are too small to be significant
319 when comparing individual fungicide treatments pairwise.
320 Individual compounds did in some instances markedly affect the readiness of viable
321 seeds to germinate after retrieval from the field. In *Centaurea*, in the first week of the Petri
322 dish trial an average of 65% of viable seeds germinated from the control treatment. By
323 contrast, this percentage was only 14-23% in the four fungicide treatments containing captan.
324 After three weeks, *Centaurea* seed germination was close to 100% in all treatments,
325 indicating that captan did not induce any longer-lasting dormancy. Similar short-term effects
326 of captan on seed germination were previously observed in wheat seeds (Clark and Scott,
327 1982).
328 Fungicide effects on germination were also found in *Daucus* seeds, where an already
329 low readiness of seeds to germinate after retrieval was particularly low in fungicide
330 combinations involving mancozeb. However, in this case, observed fungicide effects were
331 more persistent: After three weeks, when germination of *Daucus* seeds had largely ceased,
332 significant differences still existed between two of the treatments involving mancozeb and the
333 untreated control. This may be interpreted as a more persistent induction of secondary
334 dormancy in *Daucus* seeds by mancozeb. A similarly persistent but opposite effect on
335 dormancy levels of seeds has been previously documented for wheat seeds stimulated to
336 germinate by the systemic fungicide benomyl (Clark and Scott, 1982). Our results extend the
337 findings of previous studies from crops seeds that tend to germinate readily (Baskin and

338 Baskin, 1998) to non-crop species. Extent and duration of effects on dormancy were both
339 fungicide-specific and species-specific in our study.
340 As outlined in the Materials and Methods section, substrate organic matter content,
341 manipulated by using different substrates based on local topsoil and / or green waste compost,
342 did only marginally influence our results.
343
344 **Conclusions**
345
346 Several conclusions can be drawn from our study with respect to ecological seed burial
347 studies. Our results suggest that, compared to using a single compound at the recommended
348 dosage, a combination of two different fungicidal compounds may often only provide a
349 marginally better protection of seeds from fungal-induced seed mortality, but that such a
350 combination may nevertheless serve as an insurance against unintentionally using a single
351 compound at a dosage too low to provide full protection, as may have been the case in our
352 study for the seeds of *Anthriscus* when treated with iprodione. The benefits of combining a
353 very large number of fungicidal compounds as advocated by Leishman *et al.* (2000) may thus
354 be negligible and may not justify the additional effort involved. While we also did not find
355 any evidence for negative effects resulting from the combination of different fungicidal
356 compounds, our results do not preclude the potential occurrence of such effects when
357 fungicidal compounds other than the ones tested in our study are involved. In the absence of
358 further research on this subject, it may thus be safest to use individual compounds at a
359 sufficiently high dosage or tried and tested combinations of two fungicides that are known to
360 not chemically interact with each other.
361 There were pronounced effects of individual fungicidal compounds on seed dormancy.
362 Mancozeb had a lasting effect on seed dormancy in *Daucus*, and captan initially repressed
363 seed germination in *Centaurea*, although this latter effect was only transient. Captan was the

sole fungicidal compound used in many published studies, but in the absence of proof of more persistent effects, it seems likely that the results of these studies have not been compromised by unexpected side effects on seed dormancy. Nevertheless, given the evidence for a widespread existence of species-specific seasonal germination windows (e.g. Milberg and Andersson, 1997; Vleeshouwers and Bouwmeester, 2001; Schütz, 2002; Baskin and Baskin, 2006), increased levels of seed dormancy as observed in our study for *Daucus* seeds treated with mancozeb have the potential to prevent a sizeable proportion of seeds from germinating in a particular year. This may be an important consideration when planning a study that attempts to assess the effects of fungal exclusion on the *in situ* emergence of seedlings. Such studies are however rare, and in the only study of that kind we know of (Blaney and Kotanen, 2002), captan was used, for which we only found evidence for short-term effects on seed dormancy.

Overall, our results do thus underline the validity of previous seed burial studies using only a single fungicidal compound. Moreover, in our study the differences in mortality between different fungicide treatments were generally only small. It thus seems likely that the basic conclusions from most seed burial studies are unaffected by their choice of fungicide and that reliable conclusions can be drawn from these studies regarding the relative amount of buried seed mortality attributed to fungal attack as opposed to mortality that can be attributed to other causes. However, as the rate of fungal-induced seed mortality in such experiments seems to crucially depend on the density of buried seeds (Van Mourik *et al.*, 2005), we advise for caution when using data generated from such studies in seed demographic models.

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References

Andrades, M.S., Rodríguez-Cruz, M.S., Sánchez-Martín, M.J. and Sánchez-Camazano, M. (2004) Effect of the modification of natural clay minerals with hexadecylpyridinium cation on the adsorption-desorption of fungicides. *International Journal of Environmental Analytical Chemistry* **84**, 133-141.

Backman, P.A. (1978) Fungicide formulation: Relationship to biological activity. *Annual Reviews in Phytopathology* **16**, 211-237.

Baskin, C.C. and Baskin, J.M. (1998) *Seeds: Ecology, biogeography, and evolution of dormancy and germination.* San Diego, Academic Press.

Baskin, C.C. and Baskin, J.M. (2006) The natural history of soil seed banks of arable land. *Weed Science* **54**, 549-557.

Blaney, C.S. and Kotanen, P.M. (2001) Effects of fungal pathogens on seeds of native and exotic plants: a test using congeneric pairs. *Journal of Applied Ecology* **38**, 1104-1113.

Blaney, C.S. and Kotanen, P.M. (2002) Persistence in the seed bank: The effects of fungi and invertebrates on seeds of native and exotic plants. *Ecoscience* **9**, 509-517.

Clark, S.M. and Scott, D.J. (1982) Effects of carboxin, benomyl and captan on the germination of wheat during the post-harvest dormancy period. *Seed Science and Technology* **10**, 87-94.

Cottrell, H.J. (1947) Tetrazolium salt as a seed germination indicator. *Nature* **159**, 748.

Dalling, J.W., Swaine, M.D. and Garwood, N.C. (1998) Dispersal patterns and seed bank dynamics of pioneer trees in moist tropical forest. *Ecology* **79**, 564-578.

Deacon, J. (2006). *Fungal biology* (4th edition). Malden, Blackwell.

Deutscher Wetterdienst (2008) Ausgabe der Klimadaten: Monatswerte. http://www.dwd.de/de/FundE/Klima/KLIS/daten/online/nat/ausgabe_monatswerte.htm. Accessed on 03 March 2008.

Fellows, G.M. and Roeth, F.W. (1992) Factors influencing shattercane (*Sorghum bicolor*) seed survival. *Weed Science* **40**, 434-440.

Gallandt, E.R., Fuerst, E.P. and Kennedy, A.C. (2004) Effect of tillage, fungicide treatment, and soil fumigation on seed bank dynamics of wild oat (*Avena fatua*). *Weed Science* **52**, 597-604.

Gange, A.C., Brown, V.K. and Farmer, L.M. (1992) Effects of pesticides on the germination of weed seeds: implications for manipulative experiments. *Journal of Applied Ecology* **29**, 303-310.

Gisi, U. (1996) Synergistic interaction of fungicides in mixtures. *Phytopathology* **86**, 1273-1279.

Goring, C.A.I. (1967) Physical aspects of soil in relation to the action of soil fungicides. *Annual Review of Phytopathology* **5**, 285-317.

Hartz, T.K. and Caprile, J. (1995) Germination of sh2 sweet corn following seed disinfestation, solid-matrix priming, and microbial seed treatment. *Hortscience* **30**, 1400-1402.

Jäger, E.J. and Werner, K. (2002) *Rothmaler – Exkursionsflora von Deutschland: Gefäßpflanzen, Kritischer Band*. Heidelberg, Spektrum.

Kitajima, K. and Fenner, M. (2000) Ecology of seedling regeneration, pp. 331-359 *in* Fenner, M. (Ed.) *Seeds: The ecology of regeneration in plant communities*. Wallingford, CABI Publishing.

Kotanen, P.M. (2007) Effects of fungal seed pathogens under conspecific and heterospecific trees in a temperate forest. *Canadian Journal of Botany* **85**, 918-925.

Kremer, R.J. (1993) Management of weed seed banks with microorganisms. *Ecological Applications* **3**, 42-52.

Laird, R.A. and Addicott, J.F. (2008) 'Fungicide application method' and the interpretation of mycorrhizal fungus-insect indirect effects. *Acta Oecologica* **34**, 214-220.

Leishman, M.R., Masters, G.J., Clarke, I.P. and Brown,V.K. (2000) Seed bank dynamics: the role of fungal pathogens and climate change. *Functional Ecology* **14**, 293-299.

Lonsdale,W.M. (1993) Losses from the seed bank of *Mimosa pigra*: soil micro-organisms vs. temperature fluctuations. *Journal of Applied Ecology* **30**, 654-660.

Lopes, N.P., de Queiroz, M.E.L.R., Neves, A.A. and Zambolim, L. (2002) Influência da material orgânica na adsorção do fungicida triadimenol pelo solo. *Quimica Nova* **25**, 544-547.

Milberg, P. and Andersson, L. (1997) Seasonal variation in dormancy and light sensitivity in buried seeds of eight annual weed species. *Canadian Journal of Botany* **75**, 1998-2004.

Mitschunas, N., Wagner, M. and Filser, J. (2006) Evidence for a positive influence of fungivorous soil invertebrates on the seed bank persistence of grassland species. *Journal of Ecology* **94**, 791-800.

Mitschunas, N., Wagner, M. and Filser, J. (2008) Increased field emergence of seedlings at high densities of fungivorous soil mesofauna. *Journal of the Torrey Botanical Society* **135**, 272-280.

Neergaard, P. (1979) *Seed pathology*. London, Macmillan Press.

O´Hanlon-Manners, D.L. and Kotanen, P.M. (2004a) Evidence that fungal pathogens inhibit recruitment of a shade-intolerant tree, white birch (Betula papyrifera), in understory habitats. *Oecologia* **140**, 650-653.

O´Hanlon-Manners, D.L. and Kotanen, P.M. (2004b) Logs as refuges from fungal pathogens for seeds of Eastern Hemlock (*Tsuga canadensis*). *Ecology* **85**, 284-289.

Orrock, D.L. and Damschen, E.I. (2005) Fungi-mediated mortality of seeds of two old-field plant species. *Journal of the Torrey Botanical Society* **132**, 613–617.

Paul, N.D., Ayres, P.G. and Wyness, L.E. (1989) On the use of fungicides for experimentation in natural vegetation. *Functional Ecology* **3**, 759-769.

Quinn, G.P. and Keough, M.J. (2002) *Experimental design and data analysis for biologists*. Cambridge, Cambridge University Press.

Scardavi, A. (1966) Synergism among fungicides. *Annual Review of Phytopathology* **4**, 335-346.

Schafer, M. and Kotanen, P.M. (2004) Impacts of naturally-occurring soil fungi on seeds of meadow plants. *Plant Ecology* **175**, 19-35.

Schütz, W. (2002) Dormancy characteristics and germination timing in two alpine *Carex* species. *Basic and Applied Ecology* **3**, 125-134.

Simmen, U. and Gisi, U. (1995) Effects of seed treatment with SAN-789-F, a homopropargylamine fungicide, on germination and contents of squalene and sterols of wheat seedlings. *Pesticide Biochemistry and Physiology* **52**, 25-32.

Sinha, A.P., Singh, K. and Mukhopadhyay, A.N. (1988) *Soil fungicides*. Boca Raton, CRC Press.

Smiley, R.W., Patterson, L.M. and Rhinhart, K.E.L. (1996) Fungicide seed treatment effects on emergence of deeply planted winter wheat. *Journal of Production Agriculture* **9**, 564-570.

Sokal, R.R. and Rohlf, F.J. (1995) *Biometry – the principles and practice of statistics in biology research*. New York, Freeman.

Thompson, K., Bakker, J.P. and Bekker, R.M. (1997) *The soil seed banks of North West Europe: Methodology, density and longevity.* Cambridge, Cambridge University Press.

Thompson, K. and Grime, J.P. (1983) A comparative study of germination responses to diurnally-fluctuating temperatures. *Journal of Applied Ecology* **20**, 141-156.

Van Mourik, T.A., Stomph,T.J. and Murdoch, A.J. (2005) Why high seed densities within buried mesh bags may overestimate depletion rates of soil seed banks. *Journal of Applied Ecology* **42**, 299-305.

Vleeshouwers, L.M. and Bouwmeester, H.J. (2001) A simulation model for seasonal changes in dormancy and germination of weed seeds. *Seed Science Research* **11**, 77-92.

Wagner, M. and Mitschunas, N. (2008) Fungal effects on seed bank persistence and potential applications in weed biocontrol: A review. *Basic and Applied Ecology* **9**, 191-203

Whitehead, R. (1998). *The UK pesticide guide*. Cambridge, Massachusetts, CABI Publishing.

Table captions

Table 1. Effects of fungicide combination (=fixed factor) and of experimental block identity (=random factor) on the proportion of ungerminated dead seeds of *Anthriscus sylvestris*, *Centaurea nigra*, and *Daucus carota* at the end of a 6-month burial period. Analyses are based on arcsine-transformed data. Significant effects ($P < 0.05$) in bold.

Table 2. Effects fungicide combination (=fixed factor) and of experimental block identity (=random factor) on the proportion of viable seeds of *Centaurea nigra* and *Daucus carota* readily germinating within one week (A) and three weeks (B) after retrieval from the field. Analyses are based on arcsine-transformed data. Significant effects ($P < 0.05$) in bold. Due to insufficient germination, *Anthriscus sylvestris* was not analysed.

543 (Table 1)

544

Effect		*A. sylvestris*		*C. nigra*		*D. carota*	
	d.f.	F	*P*	F	*P*	F	*P*
Fungicide combination	7	66.86	**< 0.001**	1.56	0.225	12.70	**<0.001**
Block	2	10.48	**0.002**	0.81	0.465	1.22	0.324
Fungicide combination × Block	14	0.46	0.950	1.24	0.258	1.13	0.339
Error	120						

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(Table 2)

Effect		*C. nigra*		*D. carota*	
	d.f.	F	*P*	F	*P*
A) Germination after one week					
Fungicide combination	7	10.85	**< 0.001**	5.34	**0.004**
Block	2	1.20	0.329	0.50	0.618
Fungicide combination × Block	14	2.18	**0.012**	1.49	0.124
Error	120				
B) Germination after three weeks					
Fungicide combination	7	5.78	**0.003**	4.12	**0.012**
Block	2	2.62	0.108	6.34	**0.011**
Fungicide combination × Block	14	0.34	0.988	1.28	0.231
Error	120				

576 **Figure captions**

577 Figure 1. Percentage of seeds that were dead at the end of a six-month burial period, in
578 relation to particular combinations of captan, mancozeb, and iprodione used alone or
579 in combination: A) *Anthriscus sylvestris*; B) *Centaurea nigra*; C) *Daucus carota*.
580 Fungicide treatments: control = untreated control; C = captan; I = iprodione;
581 M = mancozeb. Bars and error bars indicate back-transformed mean values and 95%
582 confidence intervals. In case of ANOVA significance ($P < 0.05$), differences between
583 different compound combinations are indicated by lower-case characters.

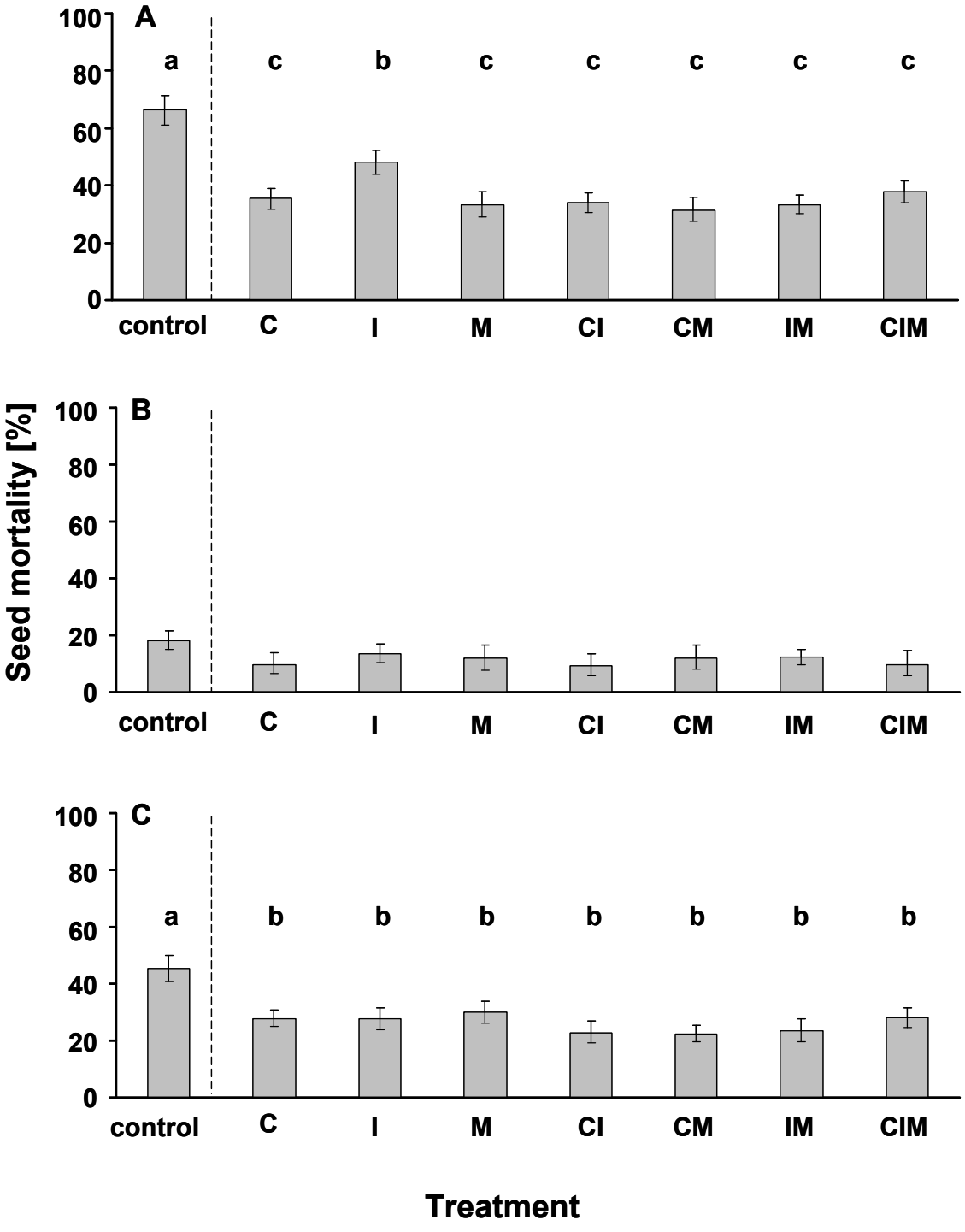
584

585 Figure 2. Percentage of viable *Centaurea nigra* seeds that germinated in the Petri dish test
586 following the six-month burial period, depending on particular combinations of
587 captan, mancozeb, and iprodione alone or in combination: A) after one week; B) after
588 three weeks. Fungicide treatments: control = untreated control; C = captan;
589 I = iprodione; M = mancozeb. Bars and error bars indicate back-transformed mean
590 values and 95% confidence intervals. Particular fungicide combinations that differ
591 significantly from the untreated control as indicated by two-sided Dunnett tests are
592 indicated by asterisks (*** P<0.001; ** P<0.01; * = P<0.05).

593

594 Figure 3. Percentage of viable *Daucus carota* seeds that germinated in the Petri dish test
595 following the six-month burial period, depending on particular combinations of
596 captan, mancozeb, and iprodione alone or in combination: A) after one week; B) after
597 three weeks. Fungicide treatments: control = untreated control; C = captan;
598 I = iprodione; M = mancozeb. Bars and error bars indicate back-transformed mean
599 values and 95% confidence intervals. Particular fungicide combinations that differ
600 significantly from the untreated control as indicated by two-sided Dunnett tests are
601 indicated by asterisks (*** P<0.001; ** P<0.01; * = P<0.05).

(Figure 1)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C
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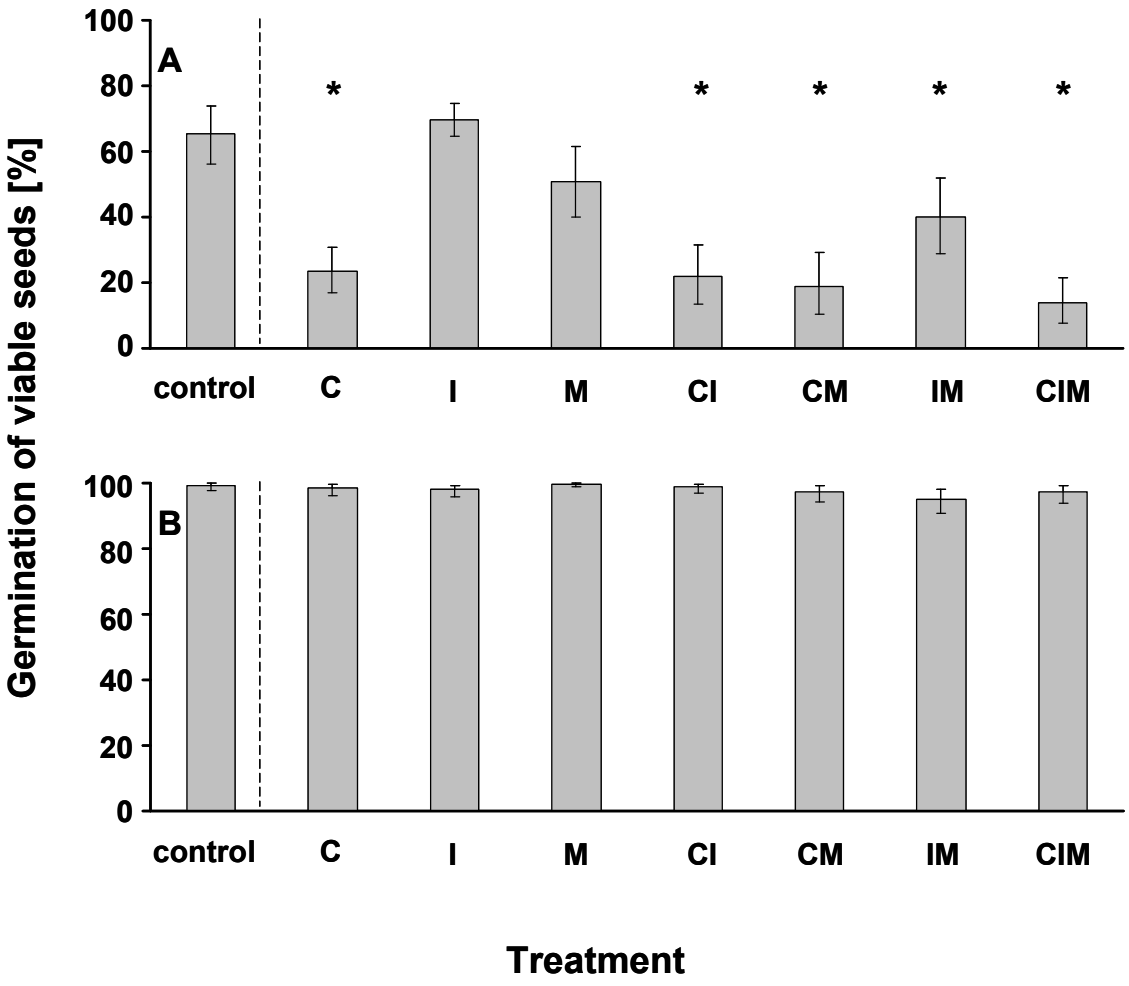
Seed mortality [%]
Treatment

(Figure 2)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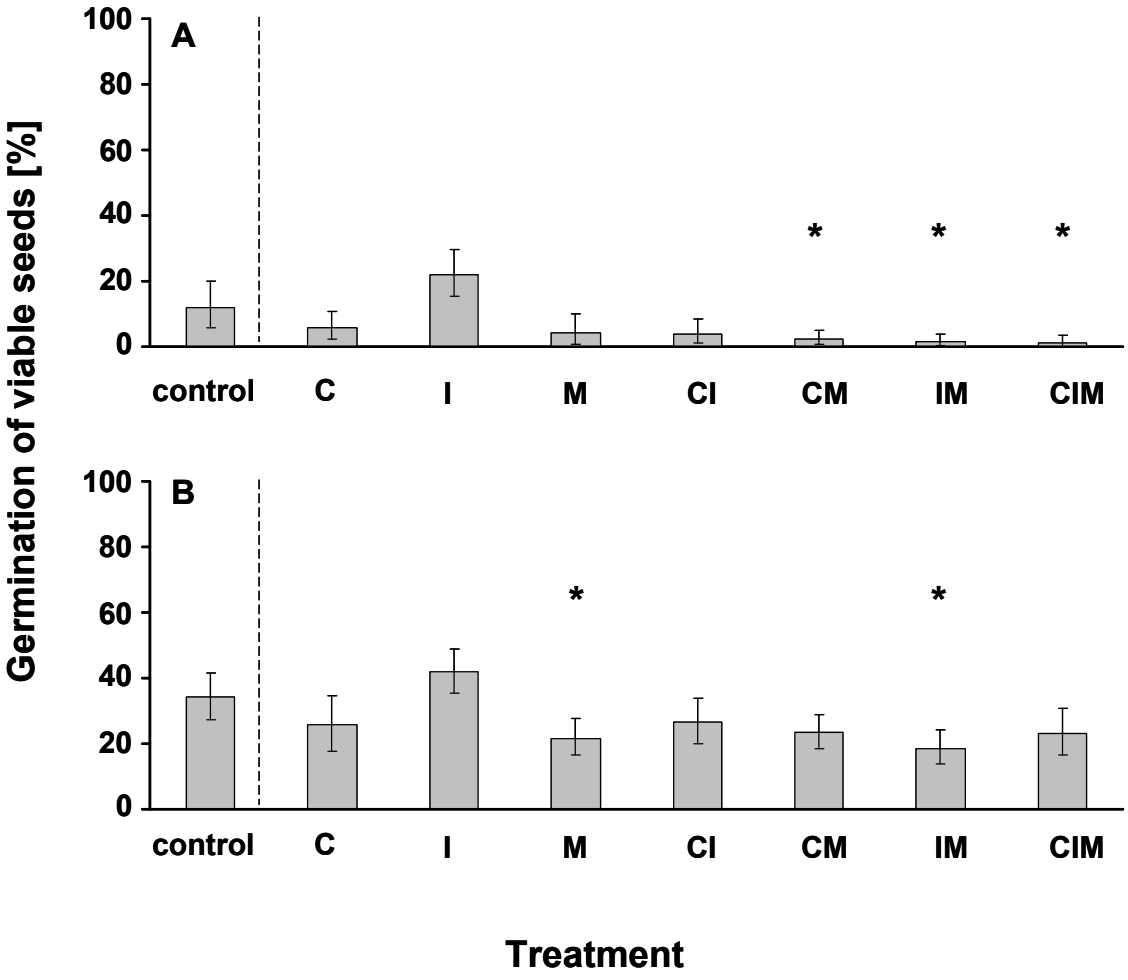
Germination of viable seeds [%]
Treatment

(Figure 3)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