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Original article

'Fungicide application method' and the interpretation of mycorrhizal fungus–insect indirect effects

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ABSTRACT

Mycorrhizal fungi, by altering their host plant's physiology, can have indirect effects on insect herbivores. The 'fungicide application method' is a common approach used to investigate the indirect effects of mycorrhizal fungi on insects. This approach works by using initially mycorrhizal plants, and then generating a subset of these plants that are free of mycorrhizal fungi by applying fungicide to their roots. When insect feeding-bioassays are conducted using the resulting mycorrhizal and non-mycorrhizal plants, differences in insect performance are typically attributed to differences in mycorrhizal colonization per se, rather than the application of the fungicide. Thus, the fungicide application method relies on the assumption that there is no direct toxicity of the fungicide on the focal insect species, and no indirect effects on the focal insect resulting from effects of the fungicide on the host plant or on non-target soil micro-organisms. We tested this critical assumption by feeding *Zygogramma exclamationis* (Chrysomelidae) larvae on *non*-mycorrhizal *Helianthus annuus* (Asteraceae) plants whose roots were treated with a solution of the fungicide benomyl or with a distilled water control. Larvae fed on benomyl-treated plants had reduced survival, lower relative growth rate, and lower food conversion efficiency, compared to larvae fed on control plants. Hence, fungicides applied to roots can affect herbivorous insect performance even in the absence of the possibility of mycorrhizal fungi-mediated effects. We recommend caution when using fungicide application and suggest that selective inoculation is a preferable method of generating mycorrhizal and non-mycorrhizal plants when studying mycorrhizal fungi–insect indirect effects.

1. Introduction

Two of the most common and important species interactions involving plants are the plant–mycorrhizal fungus symbiosis and insect herbivory. Because mycorrhizal fungi can alter physiological and morphological traits of their host plant, they are likely to indirectly affect insect herbivores. This observation has resulted in a recent wave of studies examining plant-mediated indirect effects between ecto- or arbuscular mycorrhizal fungi and herbivorous insects (e.g. Rabin and Pacovsky, 1985; Gange and West, 1994; Gange et al., 1994, 1999, 2002, 2005; Borowicz, 1997; Gange and Nice, 1997; Gehring et al., 1997; Manninen et al., 1999; Goverde et al., 2000; Gange, 2001; Wamberg et al., 2003; Koschier et al., 2007;

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reviewed in Gehring and Whitham, 2002; Strauss and Irwin, 2004). One common experimental approach for investigating these indirect effects is the 'fungicide application method', wherein the presence/absence of mycorrhizal fungi is manipulated using fungicides applied to the roots of previously colonized plants (approximately one-quarter of published mycorrhizal fungi–insect studies used this approach; e.g. Gange and West, 1994; Gange and Nice, 1997; and the 'field experiment' of Gange et al., 2005). This method relies on the assumption that any effects of fungicides on herbivores are solely due to changes in the host plant, mediated by reductions to that host plant's mycorrhizal fungi. In other words, the fungicide application method assumes that the fungicide itself does not affect herbivores – either directly, or indirectly through changes in the host plant or the soil microbial community that are not related to mycorrhizal colonization (*cf*. Gange and Nice, 1997, p. 340).

In some cases, this assumption may be warranted. For example, in one of the first studies on the topic of mycorrhizal fungi–insect indirect effects, Gange and West (1994) advanced the argument, based mainly on unpublished data, that the fungicide iprodione was suitable for their study system (*Arctia caja* and *Myzus persicae* feeding on *Plantago lanceolata*). Their argument was founded on the basis that iprodione ''is non-toxic to insects'', that it ''appears to have no toxic effect on growing plants'', and that effects on non-target soil micro-organisms were ''unlikely to be a problem, as these were rarely seen during the mycorrhizal assessments of control plants'' (Gange and West, 1994, pp. 84–86). In another study, Gange and Nice (1997) reported unpublished data that showed no effects of the same fungicide on the gall fly *Urophora cardui* when the fungicide was applied to the roots of *Circium arvense* plants that were grown in sterile soil, implying an absence of direct toxicity. Thus, unintended consequences of fungicide application were probably not an issue in these fungicide–plant–insect systems.

Notwithstanding these arguments, there is a paucity of published data testing the validity of the fungicide application method. Moreover, it is important to consider the 'overall' (direct *plus* indirect) effects of root-applied fungicides on insect herbivores, since it is these overall effects that the fungicide application method assumes to be negligible. Here, we report on an experiment in which we test the main assumption of the fungicide application method – that there are no direct or indirect effects of the fungicide on the insects – by comparing the survival, relative growth rate, relative consumption rate, and feeding efficiency of sunflower beetle larvae (*Zygogramma exclamationis* Fabricius) on fungicide-treated and non-treated sunflower plants (*Helianthus annuus* L.). The fungicide we used was benomyl. Benomyl is effective in reducing arbuscular mycorrhizal fungi (less so with respect to ectomycorrhizal fungi; Unestam et al., 1989), but with few phytotoxic effects (Paul et al., 1989; Sukarno et al., 1993; Merryweather and Fitter, 1996; but see van Iersel and Bugbee, 1997). For example, Paul et al. (1989) found benomyl to be the least phytotoxic fungicide of the four that they tested: none of 19 plant species they examined showed negative effects of benomyl. Even though benomyl can be toxic to insects when it is sprayed on the leaves they consume (e.g. Vickerman and Sotherton, 1983), its generally low uptake by roots (Hershberger and Arce, 1993) suggests that there may be little opportunity for leaf-feeders to come into direct contact with root-applied benomyl. Thus, at least at the outset, benomyl has several properties that might appear to make it a good fungicide for detecting mycorrhizal fungus–insect indirect interactions. In contrast to previous experiments, the plants in our experiment were grown from surface-sterilized seeds in a soil-free growth medium, and therefore were all non-mycorrhizal. However, we purposely did not control for non-target soil micro-organisms. Hence, any costs or benefits to beetle larvae would be manifested as overall effects, and would result from a combination of direct toxic or anti-feedant effects of benomyl, and indirect effects mediated via non-target soil micro-organisms. Not controlling for non-target soil micro-organisms was an important feature of our experimental design, because this matches the situation in field experiments, the most common scenario in which the fungicide application method is employed. We demonstrate that benomyl, when applied to sunflower roots, can have strongly negative effects on leaf-feeding sunflower beetle larvae, even in the absence of mycorrhizal fungi.

2. Materials and methods

2.1. *Study species*

Our focal plant species was the common annual sunflower, *H. annuus* L. ssp. *lenticularis* (Lindl.) Cockerell (Asteraceae) (Moss, 1983), an annual plant native to western North America (Rogers et al., 1982). Although *H. annuus* can form associations with arbuscular mycorrhizal fungi (Chandrashekara et al., 1995), all of the plants used in this experiment were non-mycorrhizal (see '*Root staining and microscopy*'). In addition, many species of insect herbivores feed on *H. annuus* (e.g. Rogers, 1979). Our focal insect herbivore was the sunflower beetle, *Z. exclamationis* Fabricius (Chrysomelidae) (Westdal, 1975; Rogers, 1977). *Z. exclamationis* is a specialist of several species of the genus *Helianthus*, including *H. annuus*, and both larvae and adults consume *Helianthus* leaves (Westdal, 1975; Rogers, 1977). Individuals are in the larval stage for approximately 15 days (Rogers, 1977).

2.2. *Preparation of sunflower plants*

In August 2004, we collected dry *H. annuus* capitula from several hundred plants in a population located near Gem, Alberta, Canada. From these we obtained several thousand achenes ('seeds'). The seeds were refrigerated over the winter. On May 4, 2005, we surface-sterilized the seeds by washing them in a 2% sodium hypochlorite solution for 20 min, and then rinsing them five times in sterile (autoclaved) water, each rinse lasting 5 min. We then scarified the seeds using sterilized sandpaper, and excised the narrow end of each seed coat using a razor blade. We placed the seeds in Petri dishes on filter paper that was moistened with sterile water. The following day, we removed the seed coats of each seed using sterilized forceps. The seeds were then left in the dark to germinate. It was necessary to start with many more seeds than there were plants in the experiment, because the germination success was low.

Following germination, on May 9, 2005, we planted each seed in a 100 mL pot. The pots contained a soil-free growth medium that was free of mycorrhizal fungi (see '*Root staining and microscopy*'). The medium was composed of a 4:3:3 mixture (by volume) of peat moss, perlite and crushed clay. We placed the seedlings in a greenhouse and watered them daily until saturation with distilled water. On May 26, 2005, we transplanted each seedling into 7.6 L pots in the same growth medium, and randomly assigned them to one of two treatments, 'benomyl' (B+) or 'no benomyl' (B−). We treated the plants with either 1 L of 1.25 g L^{-1} benomyl solution ('Benlate' 50% wettable powder; Wilson Laboratories, Dundas, ON) or 1 L of distilled water, applied to the growth medium. There were 28 B+ plants and 28 B− plants. After applying the benomyl treatment, we moved the plants outside, where they stayed for the duration of the experiment on the roof adjacent to the greenhouse. Thereafter, we continued to water them daily until saturation with distilled water.

2.3. Beetle feeding experiment

On June 29, 2005, we collected *Z. exclamationis* larvae from *H. annuus* plants in Dinosaur Provincial Park, Alberta, Canada. We immediately returned the larvae to the greenhouse, where we weighed them and randomly assigned them to a B+ or a B− plant. We placed one larva on each plant's uppermost fully expanded leaf. At that time, the plants had between six and 12 leaves (mean ± SEM: 8.7 ± 0.2 leaves) and none had any open inflorescences. On July 7, 2005, 8 days later, we removed surviving larvae from the plants and re-weighed them. We chose 8 days to ensure that the larvae did not pupate before the end of the experiment while still allowing the plants in our experiment to be an important source of larval biomass (which they were: over the 8 days, the average surviving larva gained >550% of its initial mass).

2.4. Leaf area analysis

Immediately after the termination of the beetle feeding experiment, we trimmed all the leaves off every plant, and scanned them at 200 dpi. We estimated the leaf area eaten by each larva using SigmaScan Pro 2.0 software (Jandel Scientific, San Rafael, CA). First, we measured each plant's leaf area remaining. Next, we digitally filled-in the parts of the leaves that were eaten to determine each plant's total leaf area. Leaf area eaten was estimated as the difference between the two.

2.5. Root staining and microscopy

After the experiment was completed, we collected the roots from five B+ and five B− plants to check whether any evidence of colonization by mycorrhizal fungi could be detected. All the plants were randomly chosen. We used the staining and microscopy (line intersection) techniques of Brundrett (1994) and Brundrett and McGonigle (1994). As expected, starting with surface-sterilized seeds and using a non-soil growth medium meant that we detected no evidence of mycorrhizal colonization in either the B+ or the B− plants (i.e. no arbuscules or vesicles).

2.6. Analysis

All analyses were performed using SAS or JMP software (SAS Institute, Cary, NC). We used a generalized linear model assuming a binomial distribution (=logistic regression) to determine whether there was any effect of initial larval mass and benomyl treatment (and their interaction) on larval survival. We used the final and initial mass to estimate relative growth rate (RGR) of each surviving larva (Waldbauer, 1968; Scriber and Slansky, 1981). We then broke this down into its two component rates, relative consumption rate (RCR) and efficiency of conversion of ingested food (ECI). We assumed RGR, RCR, and ECI were constant over the course of the experiment. Therefore,

$$\mathrm{RGR} = \frac{\ln M_A - \ln M_B}{t}, \tag{1}$$

$$\mathrm{RCR} = \frac{\ln M_A - \ln M_B}{t(M_A - M_B)}L, \tag{2}$$

$$\mathrm{ECI} = \frac{M_A - M_B}{L}, \tag{3}$$

where M_A and M_B are the masses of a larva after and before the feeding experiment, respectively, t is the time the larva fed, and L is the leaf area eaten by the larva. Note that RGR is equal to the product of RCR and ECI. We used *t*-tests or their non-parametric equivalent, Wilcoxon rank sum tests, to test for differences in average RGR, RCR, and ECI between the surviving larvae fed on B+ and B− plants. We also used *t*-tests or Wilcoxon rank sum tests to test for differences in the total leaf area, the leaf area eaten, and the percentage of leaf area eaten for B+ and B− plants. Wilcoxon rank sum tests were used if assumptions of normality were violated as assessed with Shapiro–Wilk's tests or assumptions of homoscedasticity were violated as assessed with Levine's tests. We tested for violation of these assumptions for both untransformed and log-transformed data. The analysis of total leaf area was conducted for all plants in the two treatment groups and then separately for only those plants on which larvae survived for the duration of the feeding experiment.

3. Results

Fungicide application had a significantly negative effect on the survival of sunflower beetle larvae (Fig. 1; logistic regression: $\chi^2 = 26.4$, $p < 0.0001$, $\mathrm{df} = 1$, $n_{B+} = n_{B-} = 28$), with probabilities of survival of larvae in the B−, and B+ treatments being 0.86 and 0.18, respectively. There was also a significant interaction between fungicide treatment and initial larval mass ($\chi^2 = 6.72$, $p = 0.0095$, $\mathrm{df} = 1$, $n_{B+} = n_{B-} = 28$), with survival of larvae decreasing with initial larval mass for B− plants, but increasing for B+ plants. Because of the interaction between benomyl treatment and initial larval mass there was no significant effect of initial larval mass on the probability of larval survival ($\chi^2 = 0.03$, $p = 0.87$, $\mathrm{df} = 1$, $n_{B+} = n_{B-} = 28$).

Of the larvae that survived until the end of the feeding experiment, larvae fed on B− plants had significantly greater relative growth rates than larvae fed on B+ plants (means ± SEMs):

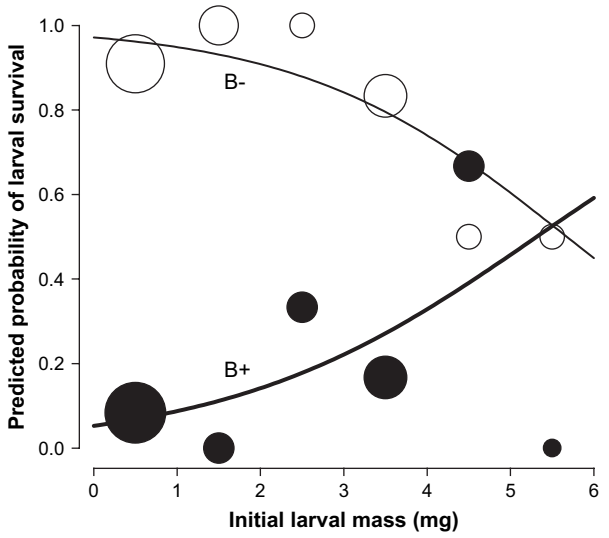

Fig. 1 – Predicted probability of larval survival (lines) over the course of the feeding experiment, as a function of fungicide treatment and initial larval mass. Symbols represent the proportion of larvae that survived in each 1 mg initial mass class; open symbols and the thin line represent larvae fed on B− plants ($n_{B-} = 28$), and closed symbols and the thick line represent larvae fed on B+ plants ($n_{B+} = 28$). Symbol areas are proportional to the sample size of each initial mass class with the closed symbol in the lower-right corner representing a single larva.

0.25 ± 0.0057 mg mg^{-1} d^{-1} and 0.056 ± 0.012 mg mg^{-1} d^{-1}, respectively (Fig. 2a; *t*-test: $t = 14.24$, $p < 0.0001$, df = 27, $n_{B-} = 24$, $n_{B+} = 5$). The effect of benomyl on RGR of surviving larvae occurred primarily because of its effect on ECI and not RCR: Larvae on B− and B+ plants had similar relative consumption rates of 3.38 ± 0.28 mm^2 mg^{-1} d^{-1} and 4.52 ± 0.61 mm^2 mg^{-1} d^{-1}, respectively (Fig. 2b; Wilcoxon rank sum test: $Z = 0.606$, $p = 0.53$, $n_{B-} = 24$, $n_{B+} = 5$). However, the efficiency of conversion of ingested food was significantly greater for larvae on B− than B+ plants: 0.082 ± 0.0059 mg mm^{-2} and 0.018 ± 0.013 mg mm^{-2}, respectively (Fig. 2c; *t*-test on log-transformed data: $t = 7.34$, $p < 0.0001$, df = 27, $n_{B-} = 24$, $n_{B+} = 5$).

The absence of differences in RCR for larvae fed on B+ versus B− plants is explained by there being no differences in either the absolute leaf area eaten (Fig. 3c; *t*-test on log-transformed data: $t = 0.24$, $p = 0.81$, df = 27, $n_{B-} = 24$, $n_{B+} = 5$) or the percentage of leaf area eaten (Fig. 3d; *t*-test on log-transformed data: $t = 1.15$, $p = 0.26$, df = 27, $n_{B-} = 24$, $n_{B+} = 5$). Total leaf area did not differ between treatments whether all plants were included in the analysis (Fig. 3a; *t*-test: $t = -0.66$, $p = 0.51$, df = 54, $n_{B-} = n_{B+} = 28$) or just the plants with surviving larvae were included in the analysis (Fig. 3b; *t*-test: $t = -1.74$, $p = 0.093$, df = 27, $n_{B-} = 24$, $n_{B+} = 5$).

4. Discussion

A single application of the fungicide benomyl resulted in strongly significant decreases in the survival and relative growth rate of *Z. exclamationis* larvae feeding on *H. annuus*

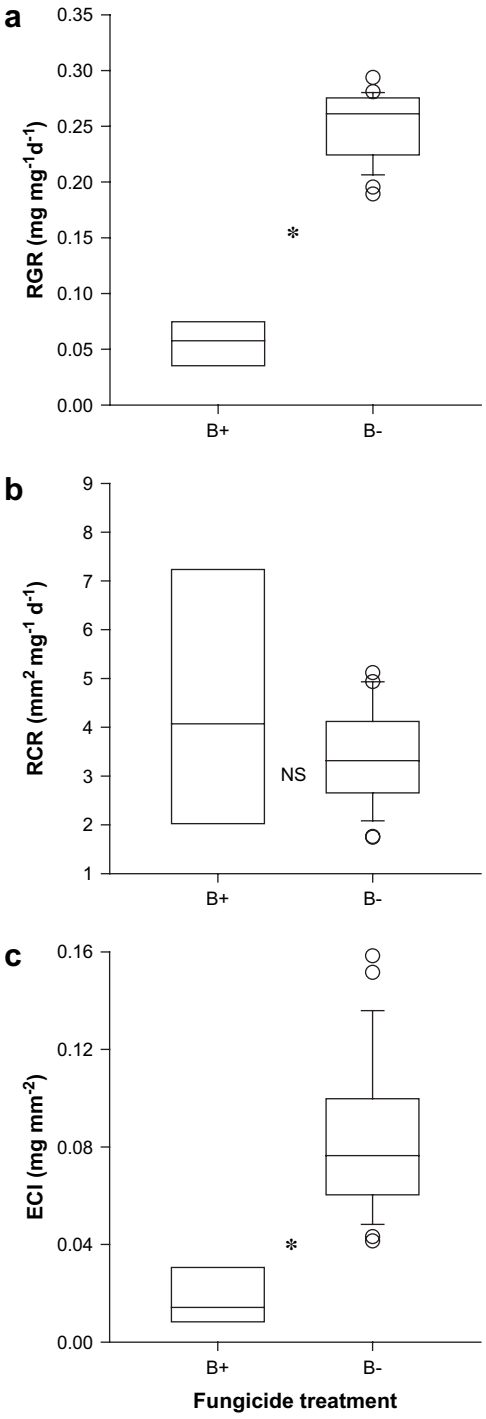

Fig. 2 – (a) Relative growth rate, (b) relative consumption rate, and (c) efficiency of conversion of ingested food as a function of fungicide treatment for larvae that survived the feeding experiment ($n_{B+} = 5$, $n_{B-} = 24$). In the box plots, the whiskers are the 10th and 90th percentiles, the bottom and top edges of the boxes are the 25th and 75th percentiles, the lines inside the boxes are the medians, and the points are data that fell outside the 10th or 90th percentiles. Asterisks (*) between boxes represent a significant difference in the means ($p \leq 0.05$); NS = not significant.

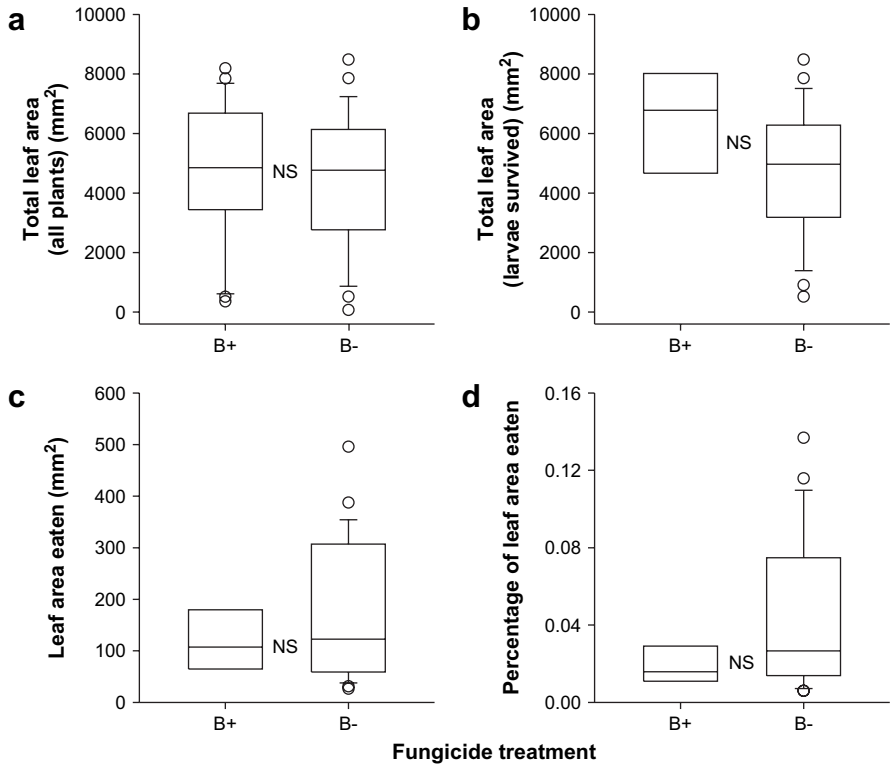

Fig. 3 – (a) Total leaf area on all plants in the study, (b) total leaf area for those plants on which larvae survived, (c) leaf area eaten, and (d) percentage of leaf area eaten as a function of fungicide treatment (for (a) $n_{B+} = n_{B-} = 28$; for (b)–(d) $n_{B+} = 5$, $n_{B-} = 24$). See Fig. 2 for explanation of the symbols in the box plots.

(Figs. 1 and 2a). The negative effect of benomyl on the relative growth rate of larvae was not due to changes in the relative consumption rate (Fig. 2b). In other words, larvae of a given size fed at the same rate, and consumed the same amounts of leaf tissue (Fig. 3), whether they were fed on B+ or B− plants. The negative effect of benomyl on the relative growth rate of larvae was due instead to a reduction in the efficiency of conversion of ingested food for larvae fed on B+ plants compared to those fed on B− plants (Fig. 2c). Therefore, either the leaf tissue of B+ plants was less digestible than that of B− plants, or treatment by benomyl inhibited larvae's abilities to convert plant biomass into larval biomass. In order to provide a strong test of the critical assumption of the fungicide application method, our experiment focused on *overall* effects of benomyl application on *Z. exclamationis* larvae, and therefore did not separate direct causes (e.g. toxicity) from indirect causes (e.g. trophic or phytotoxic effects) of the observed negative effects of benomyl.

A number of other studies have reported lethal and sub-lethal effects of fungicides on invertebrates, although most of these involved foliar fungicides rather than root-applied fungicides (but see Wright (1977) and references therein for negative effects of fungicides on earthworms). These studies examined the effects of an array of fungicides on individuals and populations of species in a number of different insect and mite orders. The results were similarly diverse, with lethal and sub-lethal effects ranging from neutral (e.g. Irving and Wyatt, 1973; Cherry et al., 1992; Moreby et al., 1997; Raudonis et al., 2004) to negative (e.g. Irving and Wyatt, 1973; Nakashima and Croft, 1974; Bailiss et al., 1978; John et al., 1982; Vickerman and Sotherton, 1983; Cherry et al., 1992; Colignon et al., 2003; Lo, 2004).

The results of our study indicate that the fungicide benomyl may not be appropriate for studies investigating the indirect effects of mycorrhizal fungi on insect herbivores. More generally, we suggest that the burden-of-proof rests with researchers using the fungicide application method to demonstrate that their chosen fungicide does not affect their focal insect herbivore, unless this can be justified a priori (e.g. as in Gange and West, 1994; Gange and Nice, 1997). An alternative to the fungicide application approach for investigating mycorrhizal fungi–insect indirect effects is the 'selective inoculation approach'. In this method, plants are grown from seed in either the absence or the presence of propagules (typically spores) of mycorrhizal fungi. Although appropriate mycorrhizal controls are still challenging to achieve with this approach (see Koide and Li, 1989; Gange and Nice, 1997), it avoids the potential problems of fungicide toxicity and anti-feedant properties, as well as indirect effects due to changes in the community of non-target soil micro-organisms. The selective inoculation approach has been used successfully in a number of studies on the indirect effects of mycorrhizal fungi on insects (e.g. Rabin and Pacovsky, 1985; Gange et al., 1994, 1999, 2002; Borowicz, 1997; Goverde et al., 2000; Gange, 2001; Wamberg et al., 2003; Koschier et al., 2007; and the 'phytometer plant experiment' of Gange et al., 2005). The results of our study strongly suggest that the selective inoculation may be a promising approach for future investigations on this topic.

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