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Hormetic responses in arbuscular mycorrhizal fungi

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ABSTRACT
The concept of hormesis describes that the application of low concentrations of a toxic compound will stimulate growth and activity of an organism. Since it is unknown whether hormesis occurs in arbuscular mycorrhizal fungi (AMF) the present work was designed to reveal whether two fungicides would generate hormetic response curves for AMF performance. The effect of mancozeb and carbendazim on performance of three AMF in symbioses with pea was investigated. The fungicides were mixed uniformly into irradiated soil at three field dose equivalents, which were 1x, 5x and 25x for mancozeb and 0.01x, 0.1x and 1x for carbendazim. A nil fungicide treatment was included for each fungus and a mesh-enclosed, 32P-labelled soil patch enabled the measurement of AMF P uptake. Both fungicides generated biphasic response curves for AMF root colonization, which was largely enhanced by the two lower doses and suppressed by the highest. Besides, the lowest concentration of both fungicides increased the hyphal length-specific 32P uptake by one of the fungi, while 0.1x carbendazim also increased the 32P uptake by another. In contrast, the length of root-external hyphae was either decreased or unaffected by increasing fungicide doses. The biphasic fungicide responses of root colonization and hyphal P uptake were obtained in irradiated soil without AMF antagonists and therefore probably caused by direct effects on the AMF. Such hormetic response patterns may be common in ecosystems where AMF will usually be exposed to a range of abiotic and biotic stressors.

1. Introduction

Low doses of otherwise toxic compounds have been found to stimulate growth and physiological processes in largely all groups of organisms including fungi, a phenomenon known as hormesis; after Greek hormesis - to excite (Calabrese and Baldwin, 2003). Hormesis is understood as an over-compensation to a disruption in homeostasis and leads to biphasic, bell-shaped response curves characterized by low-dose stimulation and high-dose inhibition (Stebbing, 1982).

Hormetic responses in fungi have been documented for more than 100 years (Calabrese and Baldwin, 2000), but have so far not been reported for the ubiquitous arbuscular mycorrhizal fungi (AMF). The AMF engage in mutualistic associations with plants via colonization of roots and ramification of mycelia networks into the soil (Smith and Read, 2008) and the symbiosis appears to play a key role in ecosystem features such as nutrient cycling, plant diversity and plant tolerance to abiotic and biotic stressors (van der Heijden et al., 2015). If AMF show hormetic responses to such stressors, hormesis needs to be considered when analyzing the functional role of AMF in ecosystems. This would seem to be particularly important for natural, unmanaged systems characterized by nutrient scarcity and abundant stressors.

To study whether hormetic responses extend to AMF requires that the selected stressors show AMF toxicity and can be applied in a controlled manner to the experimental system. Specific fungicides such as carbendazim and mancozeb meet these criteria. Carbendazim, a systemic benzimidazole, inhibits polymerization of β-tubulin and hence microtubule formation. It suppresses AMF root colonization (Fitter and Nichols, 1988; Sukarno et al., 1993) as well as AMF phosphorus (P) uptake (Sukarno et al., 1996; Larsen et al., 1996; Schweiger and Jakobsen, 1998). The latter was a conventional threshold model study which however reported that the P uptake by the AMF mycelium tended to be stimulated by the lowest carbendazim concentration used. Mancozeb, a non-systemic dithiocarbamate, inactivates sulfhydryl groups and hence disrupts lipid metabolism, respiration and ATP production. It negatively affects AMF spore germination (Mallmann et al., 2018) and root colonization (Hernandez-Dorrego and Pares, 2010; Channabasava...
The successful use of carbendazim and mancozeb to study whether AMF show hormetic responses to stressors requires that the most relevant regimes of fungus concentrations are selected. The regimes should consider not only the recommended field dose, but also those concentrations that may be expected to reach and infiltrate the soil. Here it is relevant that up to 90% of an applied fungicide is intercepted, depending on crop species and stage of development (Linders et al., 2000) and furthermore that some fungicides such as mancozeb are often applied repeatedly during the growth period (Gullino et al., 2010).

The objective of the present work was to test the hypothesis that hormesis occurs in arbuscular mycorrhizas. Symbioses between pea plants and three different AMF were exposed to a range of mancozeb and carbendazim doses and AMF responses were measured in terms of root colonization, length of hyphae in soil and \( ^{33} \)P uptake by the hyphae.

2. Materials and methods

2.1. Biological materials and fungicide doses

Pea (Pisum sativum L.) cv. Solare plants were grown without mycorrhizas (NM) or in association with each of three AMF originating from an ecologically farmed field in Tåstrup, Denmark: *Claroideoglomus claroideum* (Schenck & Smith) (BEG88), *Funneliformis mosseae* (Nicol. & Gerd.) Gerdemann & Trappe (BEG84) and *F. caledonium* (Nicol. & Gerd.) Trappe & Gerd (H07-1). The fungi were propagated on *Trifolium subterraneum* L. in a soil:sand mixture similar to that used in the experiments and the resulting dry soil inoculum contained spores and colonized root fragments. Mycorrhizal pea plants were grown in soil without fungicides and in soil treated with three concentrations of mancozeb or carbendazim. Mancozeb, is widely used on e.g. potato, tomato and pea crops (Gullino et al., 2010; but no longer approved in the EU) while carbendazim is detrimental to AMF (Fitter and Nichols, 1988). Non-mycorrhizal plants were grown in soil supplied with nil or the highest level of each fungicide. Fungicides were uniformly mixed into the soil as Dithane DG NT (750 g mancozeb kg\(^{-1}\)) and Derosal fl. (516 g carbendazim L\(^{-1}\)) and concentrations were selected to cover the recommended field doses being 2 kg ha\(^{-1}\) and 0.7 L ha\(^{-1}\) respectively. Conversions from recommended field doses to soil concentrations were based on the assumption that field-added fungicide would get evenly distributed in the top 5 cm of soil with a bulk density of 1.2 g cm\(^{-3}\).

Mancozeb was applied at 1, 5 and 25 fold the recommended field dose; levels were chosen to represent scenarios of multiple applications. Carbendazim was applied at 0.01, 0.1 and 1 fold of the recommended field dose; levels were chosen to cover the range used in a previous study indicating positive effects of very low levels (Schweiger and Jakobsen, 1998). The applied doses corresponded to 3.333, 16.665 and 83.325 μg mancozeb g\(^{-1}\) soil and 0.006, 0.060 and 0.602 μg carbendazim g\(^{-1}\) soil.

2.2. Experimental set-up and growth conditions

The growth medium was a 1:1 (w/w) mixture of soil and quartz sand. The soil was a sandy loam (clay 17%, silt 17% and sand 76%; pH (CaCl\(_2\)) 5.6) Luvisol (FAO classification) collected from the long-term nutrient depletion trial at the experimental research farm of the University of Copenhagen in Tåstrup, Denmark. The mixture, hereafter named “soil”, was irradiated (2 \( \times \) 10 KgY, 10 MeV electron beam) and supplied with basal nutrients minus P (Pearson and Jakobsen, 1993) and 30 mg NH\(_4\)NO\(_3\) N kg\(^{-1}\). Its bicarbonate-extractable P content was 9 μg g\(^{-1}\) (Olsen et al., 1954). Fungicides were mixed into the soil as outlined above. Growth containers were non-draining plastic pots which contained a mixture of 950 g soil and 50 g AMF inoculum. Non-mycorrhizal (NM) pots contained 1000 g soil. A 28 g soil patch, labelled with 140 kBq carrier-free \(^{33}\)P\(_\text{O}_4\), was placed in the center of each pot. The soil patch was confined in a small plastic vial that was capped with 25 μm nylon mesh allowing in-growth of hyphae but not roots (Smith et al., 2003). The soil patch also received the relevant fungicide treatment. Treatments had three replicates except for mycorrhizal, nil fungicide treatments which had four replicates.

Two pre-germinated pea seeds were planted per pot and thinned to one after emergence. Pots were watered by weight to 70% of WHC and were maintained in a growth chamber with a 16 : 8 h light:dark cycle with 21 : 16 °C temperatures, respectively. Pots were positioned in a completely randomized design and were repositioned at each watering.

2.3. Harvest and analyses

Shoots were harvested 39 days after planting and dry weights determined after 48h at 70 °C. Soil patch vials were removed and stored frozen. Roots were washed, a subsample was cleared in 10% KOH and stained in trypan blue (Kornamik and McGraw, 1982), and the percentage of root length with AMF colonization was assessed (Giovannetti and Mosse, 1980). Dried shoot samples were digested in a 4:1 mixture (v/v) of nitric and perchloric acids and P concentrations were measured by the molybdate blue method (Murphy and Riley, 1962) using AutoAnalyzer 3 (Seal Analytical, Nordeststedt, Germany). P\(^{32}\) contents were quantified on the same digests in TriCarb (1900) liquid scintillation counter (PerkinElmer, Waltham, MA). Hyphae in aqueous extracts of soil patch samples were collected on Millipore filters and hyphal lengths were measured by microscopy (Jakobsen et al., 1992).

2.4. Data analysis

One of four plants for the treatment *F. mosseae*, nil fungicide died and was assigned as missing in the dataset. Data were transformed if necessary to fulfill the ANOVA assumptions and were analysed using an incomplete factorial design ANOVA in R (R Core Team, 2014). Means were compared by Fisher’s Least Significant Difference (LSD) test using “LSD.test” in the R package ‘agricolae’ (de Mendiburu, 2020).

3. Results

Responses to four levels of mancozeb and carbendazim were obtained for three AMF in associations with pea plants. Shoot dry weights did not respond to AMF inoculation (\( P > 0.59 \)) and were uniform across the three AMF treatments (2.3–2.5 g without any fungicide; Fig. 1a). This facilitated the across-AMF comparison of responses for each of the two fungicides. However, since the 25x mancozeb treatment was phytotoxic and suppressed shoot dry weight by ~30% in both non-mycorrhizal (NM) and AMF plants (Table 1; Fig. 1a), its effect on the measured AMF variables was possibly confounded by this phytotoxicity. In contrast, plant growth was not influenced by carbendazim (Table 1; Fig. 1a) and responses to all carbendazim levels could therefore be ascribed to effects on the AMF. See Table S2 for a summary of ANOVA results for all variables.

3.1. Abundance of AMF in pea roots and soil

While roots of NM plants remained uncolonized, root colonization reached 40, 28 and 43% with *F. caledonium*, *C. claroideum* and *F. mosseae*, respectively, in the absence of fungicides (Table S1). The fungicide treatments resulted in biphasic response curves, largely due to enhancement of colonization by the two lower doses and suppression by the highest dose (Fig. 2). For mancozeb, two-way ANOVA revealed that root colonization was significantly influenced by both fungicide level and fungus and that the two factors interacted (\( P < 0.001 \) in all cases). This interaction reflected that both field dose and 5x field dose markedly enhanced colonization with *F. caledonium* and *C. claroideum* (130–196% increase) whereas *F. mosseae* had its maximum at the field dose (Fig. 2). Root colonization was severely suppressed by mancozeb applied at 25x field dose and was only 1% in the *F. caledonium* symbiosis (Table S1). Carbendazim enhanced root colonization at 0.01 and 0.1x field dose for
all three AMF (19–116%; \( P < 0.001 \)) whereas the effect of the AMF factor was non-significant (Fig. 2). A significant fungicide × AMF interaction (\( P < 0.05 \)) was attributable to the field dose being more suppressive to \( F. \) mosseae than to the other AMF.

Hyphal length in the \(^{32}\)P-labelled soil patch in the absence of fungicides varied greatly among AMF, ranging from 992 m in \( F. \) caledonium, over 274 m in \( F. \) mosseae to 55 m in \( C. \) claroideum (Table S1). The measured hyphal length would have been influenced by the fungicide-induced variation in AMF root colonization and hyphal lengths were therefore normalized on an AMF-colonized root length basis (Table S1).

**Table 1**
Effect of the highest fungicide doses on shoot dry weight and P content for non-mycorrhizal pea plants. Means sharing the same letter are not significantly different (\( P < 0.05 \)).

| Non-mycorrhizal plants | Shoot DW, g | Shoot P content, mg |
|------------------------|-------------|---------------------|
| No fungicide           | 2.6a        | 3.9 ab              |
| 25x mancozeb           | 1.8b        | 3.3b                |
| 1x carbendazim         | 2.8a        | 4.3a                |
These normalized data for hyphal length showed no biphasic response pattern to either fungicide (Fig. 3). Instead, the fungicides had a negative effect on hyphal length of *F. caledonium* and no significant effect on the two other AMF. The increased hyphal length in particular in the 25x mancozeb × *F. caledonium* treatment originated from very small values of colonized root length (Table S1).

### 3.2. Uptake of ³³P and shoot P content

Without fungicides, the AMF-mediated transfer of ³³P from the root-free soil patch and into the plant was significant only in the *F. caledonium* symbiosis (Table S1); levels of ³³P in the two other symbioses were similar to that of the non-mycorrhizal control (0.26 kBq). The fungicide treatments resulted in biphasic responses in total ³³P content of all three symbioses where maximum values were obtained with a field dose of mancozeb and with 0.01x – 0.1x field dose of carbendazim (Table S1). However, since the abundance of hyphae in the ³³P soil patch would vary with fungicide treatment, plant content of ³³P was normalized by the length of hyphae in the ³³P soil patch. The resulting hyphal length specific uptake of ³³P also responded in a biphasic manner to the fungicides (Fig. 4). Mancozeb elicited responses to both fungicide level (P < 0.001) and AMF (P < 0.01) and the two factors interacted (P < 0.05). Similar significant responses were elicited by carbendazim for fungicide level and AMF (P < 0.001) as well as their interaction (P < 0.01). The significant factor interactions were in both cases ascribed to the lack of a biphasic response by *F. mosseae* (Fig. 4, Table S1, Table S2). Also, the length specific ³³P uptake by *F. mosseae* was overall small due to low total ³³P uptakes and relatively abundant hyphae. The contrasting large responses in ³³P uptake by *F. caledonium* were three-fold at 1x field dose mancozeb and six-fold at 0.1x field dose carbendazim. With *C. claroideum* the corresponding maximum responses were two-fold at 1x field dose mancozeb and three-fold at 0.01x field dose carbendazid (Fig. 4, Table S1).

Total P content in shoots was significantly influenced by mancozeb (P < 0.001). As also observed for shoot growth, this effect appeared as a marked suppression at the 25x field dose in both non-mycorrhizal and mycorrhizal plants (Table 1; Fig. 1b). Shoot P content of *F. mosseae*-colonized plants was also significantly suppressed at the 5x field dose. Although plant growth was not suppressed by carbendazim, a field dose of this fungicide still reduced shoot P content in mycorrhizal plants (P < 0.001). This negative response coincided with effects on ³³P uptake; furthermore, the carbendazim × AMF interaction was significant (P < 0.01) since P content in the *F. caledonium* symbiosis was more sensitive to increasing carbendazim doses than the P content in the two other symbioses. Again, such AMF-dependent response pattern to carbendazim was also observed for the ³³P uptake. The field dose of carbendazim had no significant effect on shoot P content of non-mycorrhizal plants (Table 1).

### 4. Discussion

To date, the common biological concept of hormesis has received little attention in AMF – pesticide studies (Hage-Ahmed et al., 2019). Now, this work unequivocally shows that hormesis also applies to AMF since increasing doses of both mancozeb and carbendazim generated biphasic responses in three AMF. These results differ from other AMF studies reporting classical threshold-dose responses to fungicides (e.g. Zocco et al., 2008; Mallmann et al., 2018).

The biphasic response to mancozeb on root colonization in this study extends previous studies reporting neutral (Plenchette and Perrin, 1992) or negative effects on colonization (Hernandez-Dorrego and Pares, 2010; Channabasava et al., 2015; Vuyyuru et al., 2018) as well as negative effects on spore germination (Mallmann et al., 2018). The enhanced root colonization in response to a field dose equivalent of mancozeb could have been caused by direct effects on propagule germination, hyphal extension and branching and the production of AMF myc-factor signals during the presymbiotic phases of AMF. However, since mancozeb is a contact fungicide this should not affect AMF growth inside roots.

The observed suppression of root colonization by a field dose of carbendazim agrees with numerous previous reports (e.g. Fitter and Nichols, 1988; Sukarno et al., 1993). However, the biphasic colonization responses to sub field doses of carbendazim have not been reported before. The stimulation by carbendazim could have worked directly on the presymbiotic phases as for mancozeb, and since carbendazim is systemic, it could also have affected growth of AMF in the root cortex. Although the three AMF showed basic similarities in response patterns to the fungicides, there were also differences such as the nearly complete suppression of root colonization by a field dose of carbendazim in the *F. mosseae* as compared to the two other symbioses. Such variation in fungicide sensitivity across different AMF is in accordance with previous studies (Dodd and Jeffries, 1989; Schreiner and Bethlenfalvay, 1997).

Although the total hyphal length in the ³³p-labelled soil patch also showed biphasic response patterns to increasing fungicide doses, this biphasic response disappeared for hyphal length expressed on a colonized root length unit. Instead, this colonized root length specific hyphal length decreased with increasing fungicide dose, reflecting a typical threshold response pattern. While there are no previous studies on effects of mancozeb on the abundance of AMF hyphae in soil, negative effects of carbendazim have been reported (Sukarno et al., 1996; Kährlund and Vestberg, 2000; de Novais et al., 2019).

The enhanced AMF P uptake in response to a field dose equivalent of

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**Fig. 3.** Effect of mancozeb and carbendazim on hyphal length in root free soil patch in symbioses between pea plants and *Funnelliformis caledonium* (■), *Clafolioedoglomus claroideum* (□), and *F. mosseae* (●). Data for hyphal length were normalized against differences in root colonization and log transformed.
mancozeb and even to 5x the field dose was unexpected and it indicates that single or multiple applications of mancozeb may have a limited toxicity to AMF function. Carbendazim is well known for suppressing AMF P uptake (Sukarno et al., 1996; Kling and Jakobsen, 1997; Larsen et al., 1996); but the observed significant biphasic responses in $^{33}$P uptake by *F. caledonium* and *C. claroideum* extends the results of previous studies (Schweiger and Jakobsen, 1998; Schweiger et al., 2001). In the first study, AMF uptake of P appeared to be stimulated at very low concentrations of carbendazim and in the second, AMF P uptake was enhanced by the recommended field application of Derosal (carbendazim). The results in the present study are further supported by observation of similar biphasic responses to carbendazim in $^{33}$P uptake by *Rhizophagus* sp. BEG87 associated with *Medicago truncatula* (I. Jakobsen and S Rosendahl, unpublished).

The translation of a reduced $^{33}$P uptake into suppressed shoot P contents in treatments with a field dose of carbendazim is similar to effects of silencing a gene encoding an AMF specific Pi transporter in pea (*G. mosseae*; Cruz-Paredes et al., 2011). This demonstrates that hormesis can be induced in natural ecosystems; here, multifactor approaches are highly relevant in agronomic systems. Further, it is evident that hormetic responses in a target pathogen may be a challenge in disease control.

5. Conclusions

This work shows that the hormetic principle extends to AMF and needs to be considered to fully understand the effect of stress factors on AMF function. Although fungicide use is specific to agroecosystems the hormetic response patterns shown in this work may well be the rule for a range of natural AMF stressors such as drought, heat, cold, pH, salinity, metals and antagonists. This may be investigated by including also very mild stress levels in future studies of stress factors towards AMF function in natural ecosystems; here, multifactor approaches are highly relevant (Rillig et al., 2019). In the agroecosystem context it is evident that safe conclusions on fungicide toxicity towards AMF cannot be drawn from experiments with just a single rate of application. The recognition of hormesis in AMF-plant associations combined with future response studies using realistic soil concentrations of agricultural chemicals may help us to understand whether hormesis mitigates the expected negative effects of fungicides on AMF.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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