PRIMING BY ARBUSCULAR MYCORRHIZAL FUNGI OF PLANT ANTIOXIDANT ENZYME PRODUCTION: A META-ANALYSIS

Ami Lokhandwala and Jason D. Hoeksema

Department of Biology, University of Mississippi, University, MS, USA

Abstract: Priming of antioxidant enzyme production of plants, which can act as a defence against pathogens and other stresses, is an under-appreciated benefit often provided by arbuscular mycorrhizal (AM) fungi. Although numerous experiments have demonstrated this phenomenon, this literature has not been synthesised, and we understand little about the factors that may drive variation in the magnitude of this priming phenomenon. We gathered data from 81 original publications in which antioxidant enzyme production of plants was measured in the presence and absence of AM fungi, including factors that varied among studies, such as the identity of plants and fungi, number of species and genera of fungi, the presence and type of plant stress, the particular enzyme measured, and the plant tissues sampled. We used these data to estimate the average magnitude of the priming effect, and to ask whether these study-level variables explained variation in the magnitude of priming. We found that AM fungi increased plant production of antioxidant enzymes by approximately 16%, regardless of whether the plants were under stress. The identity of plants and fungi, number of species and genera of fungi, and the type of stress did not explain variability in the degree of priming. In the absence of stress, priming was higher for peroxidase enzymes than other enzyme classes, and plant root tissues exhibited greater priming responses than other plant tissues. Our best meta-analysis models had substantial unexplained heterogeneity in effect size among studies, suggesting that additional factors not included in our analyses could help explain variation in priming. Overall, the degree of priming observed here suggests the potential for...
mycorrhiza-induced resistance to diseases to have broader application, including for the improvement of sustainable agriculture.

**Keywords:** mycorrhiza-induced resistance, priming of antioxidant enzymes, arbuscular mycorrhizal fungi, meta-analysis

### 1 Introduction

Stress tolerance may be one of the most important benefits provided to plants by arbuscular mycorrhizal (AM) fungi (Jung et al., 2012; Cameron et al., 2013; Berruti et al., 2016; Yang et al., 2016), and yet it is under-appreciated compared to other benefits such as improved growth from alleviation of nutrient limitation. Plants respond to fungal colonisation (both pathogens and mycorrhizal fungi) by undergoing various physiological changes, including alteration of primary and secondary metabolites that may contribute to resistance (Harrison, 1999; Corradi and Bonfante, 2012). For successful mycorrhiza formation, AM fungi must be able to cope with these changes, but the process of AM fungal colonisation may still result in elevation, or ‘priming’, of plant defences, also known as mycorrhiza-induced resistance (Pozo and Azcón-Aguilar, 2007). Priming enhances the outcomes of plant defence strategies such as production of reactive oxygen species, hormonal changes, elicitor production, nutritional improvement, metabolite production, and alteration of signalling pathways (Jung et al., 2012; Bora and Lokhandwala, 2016). Such priming of plants by AM fungi to improve plant responses to stress is in fact an extensively studied mechanism, but this literature has seen little synthesis, and thus there is no consensus on the typical magnitude of priming effects, nor on factors that affect its magnitude. To fully understand the importance of this phenomenon for plants, and to maximise the utility of mycorrhizal inoculations in agriculture and other applied contexts, we sought to estimate the magnitude of priming across different systems and to identify the parameters that affect this magnitude.

Oxidative burst is a sign of stress recognition and activation of plant defence responses (Mendoza, 2011). In order to scavenge the oxygen radicals produced thereafter, plants produce various antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidases, and reductases. Priming of plant responses to stress occurs when plants change their production of these antioxidant enzymes upon AM fungus colonisation (Younesi et al., 2013; Chen et al., 2014; Bora and Lokhandwala, 2016). Therefore, antioxidant enzymes are important indicators of efficiency of mycorrhiza-induced priming (Pozo et al., 2009; Jung et al., 2012; Hashem et al., 2018). However, little is known about which of the antioxidant enzymes are most important for these priming effects.

Moreover, production of antioxidant enzymes due to priming may be influenced by other variables, for example the type of stress applied,
mycorrhizal genus/species colonised, host plant species, location in the plant (e.g. root versus shoot), and when colonisation of AM fungi took place relative to the stress event. Plants encounter a variety of abiotic and biotic stresses, and their tolerance and resistance against these stresses is achieved by a variety of physiological, hormonal, and enzymatic changes and regulation (Mittler, 2006; Rejeb et al., 2014); thus, priming of plant antioxidant enzymes by AM fungi may depend on the type of stress encountered. The degree of priming may also vary among different plant species, fungal species, and their combinations and diversity, given the extent of genetic variation within and among those species for their symbiotic compatibility with each other (Hoeksema et al., 2009, 2018), and because previous meta-analyses have found that the diversity of AM fungi influences plant growth responses to AM fungi (Yang et al., 2017). Detection of priming may also depend on the part of the plant sampled if, for example, oxidative enzyme production is localised to a tissue on which the particular stress is focused. Finally, priority effects, i.e. prior presence of AM fungi, can potentially affect the efficacy of plant defence responses against stresses. If antioxidant enzyme production is constitutively enhanced in response to AM fungi, regardless of the presence of stress, this could increase both the costs and efficacy of these defences, and it is not clear how often this would result in a net benefit.

We performed a meta-analysis of data from 81 previous studies to answer the following questions:

1. Do AM fungi prime plants against stress by changing antioxidant enzyme production, and what is the average magnitude of this priming?
2. Does the magnitude of priming vary among different oxidative enzymes?
3. If priming occurs, is it stress dependent?
   a. Do arbuscular mycorrhiza show equal priming against biotic and abiotic stress?
   b. During abiotic stress, does the degree of priming change with the type of stress?
4. Does species or genus richness of AM fungi affect the magnitude of priming?
5. Is priming more of a local or systemic response in plants, i.e. does it depend on the plant tissues measured?
6. Does the timing of mycorrhizal inoculation (before or after stress) make a difference in the magnitude of priming?

2 Materials and Methods

2.1 Data Collection

We used PubMed Central and Google Scholar to search for published studies on interactions between AM fungi and stressors of plants, in which
production of antioxidant enzymes was measured via enzymatic activity assays, with and without colonisation by AM fungi. Searching terms were (AM fungi * antioxidant enzymes) or (AM fungi * priming) or (AM fungi * stress) or (AM fungi * biotic stress) or (AM fungi * abiotic stress). From the papers found in these searches, we selected those that met the following criteria: (i) inclusion of plant antioxidant enzyme measurements in pairwise control and experimental (AM fungi inoculation) treatments and (ii) clear description of host plant species and stressors (AM fungi could have specific identity or a mixture of unknown genus/species). Ultimately, 81 papers with 1110 observations matched these criteria.

The mean values of antioxidant enzyme production, standard error/standard deviation (SE/SD) values, and sample size (N) were extracted for each observation. Tabular data were extracted directly, while data from graphs were digitised using GetData software (http://getdata-graph-digitizer.com/). Wherever SE was reported, it was transformed to SD by SE * sqrt (N). When SD was missing and could not be calculated from other reported metrics, we imputed the missing SD value using the median from all other studies in which it was reported.

We calculated the log response ratio (LRR) to estimate the effect size of priming, calculated as:

\[
LRR = \ln \left( \frac{\bar{x}_{\text{inoc}}}{\bar{x}_{\text{ctrl}}} \right),
\]

where \(\bar{x}_{\text{inoc}}\) and \(\bar{x}_{\text{ctrl}}\) are the mean enzyme production in an AM fungi treatment and a non-inoculated control, respectively. This value is positive for priming and negative for the opposite of priming (i.e. lower antioxidant enzyme production in the presence of AM fungi). LRR is a standardised, unit-less measurement of overall performance with statistical advantages for meta-analysis (Hedges et al., 1999). The sampling variance of LRR was estimated with the following equation:

\[
\hat{\sigma}^2 = \frac{SD_{\text{inoc}}^2}{n_{\text{inoc}} * \bar{x}_{\text{inoc}}^2} + \frac{SD_{\text{ctrl}}^2}{n_{\text{ctrl}} * \bar{x}_{\text{ctrl}}^2},
\]

where SD_inoc and SD_ctrl are the standard deviation, and n_inoc and n_ctrl are the number of replicates in the inoculated treatment and non-inoculated control groups, respectively (Hedges et al., 1999).

2.2 Overview of Data Analysis

We performed a mixed-effect multi-factor meta-analysis using likelihood estimation of parameters in the metafor package (Viechtbauer, 2010) of the software R (R Core Team, 2018), as described previously by Hoeksema et al. (2018). All models contained the random effects of plant species,
AM fungi species, and experiment. The latter was included to account for non-independence of multiple observations from the same experiment. All the models also included the random effect of study ID, which was a unique identifier for each observation (i.e. effect size); its inclusion specifies the conventional mixed-effect meta-analytic model with random intercepts at the observation level, and its variance component corresponds to the residual between-studies variance (as modelled in more conventional random-effects meta-analyses and typically referred to as the between-studies variance).

The data were divided into two subsets: Stress data and No Stress data. Stress data is data wherein plants were subjected to biotic/abiotic stress whereas No Stress data is wherein plants where not subjected to any kind of stress. These two subsets were analysed separately. Saturated mixed models, i.e. models containing all possible factors, for analyses of both the Stress and No Stress subsets, all contained the main effects of the following four fixed-effect predictors: location of tissue sampling in the plant (whole plant/root/shoot/leaves), number of AM fungal species (1, 2, 3+, or unknown), number of AM fungal genera (1, 2, 3+, or unknown), and enzyme class (catalase, dehydroascorbate reductase, glutathione reductase, S-nitrosoglutathione reductase, lipoxygenase, peroxidase, polyphenol oxidase, superoxide dismutase). In addition, saturated models for the Stress data included the fixed effects of abiotic versus biotic stress and specific stress type (drought, salinity, heavy metal, organic compounds, nutrient level, temperature, and pathogen).

2.3 Estimating the Importance of Fixed-effect Predictors and Magnitudes of Random Effects

Because meta-analysis data sets are observational with respect to differences in study-level fixed-effect predictors, null hypothesis tests of particular fixed-effect predictors can be influenced by correlations among predictors and can vary among models containing different combinations of predictors. Thus, rather than rely on null hypothesis testing for stepwise determination of a single reduced model of fixed effects, we used likelihood model fitting and conducted model selection guided by information criteria (specifically, Akaike’s Information Criterion corrected for small sample sizes or AICc; Sugiura, 1978) to explore the relative importance of fixed-effect predictors among all possible reduced models varying in their fixed effects, all of which contained all of the possible random effects (as in Hoeksema et al., 2018) and which were fit with maximum likelihood (ML). Results from these model selection analyses were summarised by examining the relative variable importance (RVI) for each fixed-effect predictor, calculated for each predictor as the sum of Akaike weights for models containing that predictor. Predictors with RVI near or below 0.5 were considered unimportant in explaining variation in effect size.
Random effects and important fixed effects were characterised by using REML to fit models that were determined to be the best (with respect to which fixed effects were included) according to AICc-based model selection. Fixed effects in these best models were further explored by calculating marginal means using the `predict` function of `metafor`, and these means were compared using pairwise tests with the Holm adjustment of the family-wise error rate for multiple comparisons. The influences of random effects were ascertained by examining the estimated magnitudes of associated variance components. In those best models we also estimated Q as a metric of residual between-studies heterogeneity in effect size, i.e. heterogeneity in effect size not explained by the included factors. To obtain an overall estimate of the weighted mean effect size (LRR), we fit a pure random-effects model with all random effects for each data set separately, using REML estimation. The potential for publication bias in our meta-analysis results was assessed by examination of funnel plots of residuals versus their standard errors from the best likelihood models of both the Stress and No Stress data sets, using the `funnel` function of the R package `metafor`. The dataset and R code used in our analyses are freely available on the University of Mississippi’s eGrove online repository (see https://egrove.olemiss.edu/biology_facpubs/2/).

3 Results

3.1 Overall Degree of Priming

A large proportion of the plants included in our dataset were important agricultural crops, such as pigeon pea (Cajanus cajan, 10.8% of studies), corn (Zea mays, 10.3% of studies), wheat (Triticum aestivum, 8.8% of studies), and tomato (Lycopersicon esculentum, 6.3% of studies). Overall, positive priming did occur on average across all studies, and was apparently not dependent on stress. Priming for the Stress data was 0.171 (±0.040 SE) and for the No Stress data it was 0.153 (±0.047 SE), meaning that AM fungi colonisation tended to increase production of antioxidant enzymes by about 16%. Funnel plots did not show evidence of publication bias.

3.2 AICc Model Selection to Determine the Importance of Fixed-effect of Predictors

Model selection analysis for the Stress data compared 64 candidate models. The best model contained none of the fixed factors, and none of the fixed factors had RVI value above 0.5 (Figure 1). This result indicates that none of the included fixed-factor predictors explained the degree of priming in plants under stress conditions.
Priming of Antioxidant Enzyme Production of Plants

Figure 1  AICc model selection results from meta-analysis of plant antioxidant enzyme response to arbuscular mycorrhizal colonisation, i.e. priming, for plants subjected to stress. None of the predictors had an important association with priming, as their relative variable importance (RVI) scores were all relatively low and none of the fixed effects appeared in the best model.

Model selection analysis for the No Stress data compared 16 candidate models. The best model included both Enzyme and Plant Part, both of which had RVI values above 0.5 (Figure 2). Among the nine antioxidant enzymes included, peroxidase (POD) was most consistently associated with positive priming by AM fungi, significantly more positive than glutathione reductase (GR) and superoxide dismutase (SOD) (Figure 3). Among different plant organs, roots showed highest priming while leaves showed significantly less priming than other plant organs (Figure 4). However, the Q metric of heterogeneity was large and highly significant in the best models for both data sets, indicating unexplained heterogeneity in the effect size and suggesting that there are unknown factors associated with priming that were not included in our analyses (No Stress data: $Q (df = 836) = 120 423.762, P < 0.001$; Stress data: $Q (df = 1056) = 161 267.551, P < 0.001$).

In both data sets, random effects for plant species and AM fungi species were generally small (Stress data: plant species = 0.032, AM fungal species = 0.000; No Stress data: plant species = 0.000, AM fungal species = 0.004), suggesting little or no heterogeneity in priming among different plant species and AM fungal species.
Figure 2  AICc model selection from meta-analysis of plant antioxidant enzyme response to arbuscular mycorrhizal colonisation, i.e. priming, for plants not subjected to stress. Plant type and enzyme had an important association with priming, as their relative variable importance (RVI) scores were substantial. The other two fixed factors were unimportant, with very low RVI scores.

4 Discussion

The present study was an effort to quantify the average magnitude of priming of plant defences by AM fungi, and to elucidate factors affecting the magnitude of priming, focusing on plant production of antioxidant enzymes. We found 81 original studies that had quantified plant antioxidant enzyme production in response to AM fungi, but this literature has not been previously synthesised. We found that AM fungal inoculation increased plant antioxidant enzyme production by 16%, on average, across all studies, regardless of whether plants were subjected to stress or not. Thus, enhancement of plant defence apparently occurs due to AM fungal colonisation (Pozo and Azcón-Aguilar, 2007; Jung et al., 2012; Cameron et al., 2013) irrespective of stress. The magnitude of priming also did not vary substantially among different plant or AM fungal species, nor did it depend on the number of AM fungal species or genera used in inoculation. This observation is notable because previous meta-analyses have found that plant growth benefits from AM fungi vary among plant species and are enhanced in the presence of multiple AM fungal genera (Yang et al., 2017; Hoeksema et al., 2018). Our results suggest that priming of plant antioxidant enzymes
Priming of Antioxidant Enzyme Production of Plants

- CAT DHAR Enzyme GR GSNOR LOX MDHAR POD PPO SOD

LRR of priming (mean ± SE)

Figure 3: Priming by arbuscular mycorrhizal (AM) fungi and production of antioxidant enzymes. LRR is the log response ratio of priming, i.e., the log ratio of antioxidant enzyme production with AM fungi versus without. Enzymes are abbreviated as follows: catalase = CAT, dehydroascorbate reductase = DHAR, glutathione reductase = GR, S-nitrosoglutathione reductase = GSNOR, lipoxygenase = LO, peroxidase = POD, polyphenol oxidase = PPO, superoxide dismutase = SOD.

by AM fungi may be more universal across AM plants and fungi than are plant growth benefits from AM fungi.

This priming of plant antioxidant enzyme production, or mycorrhiza-induced resistance, may allow AM plants to more quickly combat stresses such as pathogens, as compared to non-colonised plants, because the initial steps of activating plant defence systems have already been completed as a part of the AM fungal colonisation process.

AICc model selection results showed that in the presence of stress, none of the fixed factors included to explain variation in degree of priming were important (Figure 1), while in the absence of stress, the magnitude of priming varied depending on the plant tissue analysed and the particular antioxidant enzyme measured (Figures 2–4). Attenuation of disease symptoms in AM-fungi-colonised plants has been shown to be systemic (Pozo and Azcón-Aguilar, 2007; Jung et al., 2012; Pineda et al., 2013; Jacott et al., 2017); however, our results showed that priming of antioxidant enzymes by AM fungi is biased towards the roots (Figure 4). It has been

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suggested that effective plant immunity resulting from below-ground interactions of plants with AM fungi and pathogens results from an array of mechanisms, including competition for colonisation sites, competition for photosynthates, and altered root exudation (Morgan et al., 2005; Siddiqui et al., 2008; Bongard, 2012; Pineda et al., 2013). Moreover, production of root exudates like strigolactones and isoflavones attracts other beneficial microbes, such as plant growth-promoting bacteria, which is known as the mycorrhizosphere effect (García-Garrido and Ocampo, 2002; Besserer et al., 2006; Maillet et al., 2011; Cameron et al., 2013). Roots are the hub of all these aforementioned mechanisms, which may explain why priming is more substantial in roots, even if mycorrhiza-induced resistance is inherently systemic.

Among the antioxidant enzymes in this study, results showed that peroxidase enzymes were most consistently stimulated by AM fungal colonisation. Singlet oxygen (\(1^1O_2\)), superoxide (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)), and hydroxyl radical (\(HO^*\)) are the reactive oxygen species (ROS) produced by plants (Tripathy and Oelmüller, 2012). Ground state oxygen is converted to singlet oxygen by energy transfer and to superoxide (\(O_2^-\)), hydrogen

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**Figure 4** Variation among different plant parts in priming by arbuscular mycorrhizal (AM) fungi, for plants growing in the absence of stress, from meta-analysis of plant antioxidant enzyme response to AM fungal colonisation. LRR is the log response ratio of priming, i.e. the log ratio of antioxidant enzyme production with AM fungi versus without.
peroxide (H$_2$O$_2$), or hydroxyl radical (HO$^\bullet$) via electron transfer reactions (Foyer and Noctor, 2000). Rapid generation of ROS is a common plant response towards abiotic and biotic stress. There are several enzymatic and non-enzymatic mechanisms by which plants can remove ROS, peroxidase being one of them for the removal of hydrogen peroxide (Sewelam et al., 2016). Previous studies have shown that AM colonisation increases peroxidase production (Carole et al., 2018; Liu et al., 2018; Subramanian et al., 2011). Consistent production of peroxidase in response to AM fungi in our analysis builds on those prior results and suggests the potential role of AM fungi in priming plants for the removal of particular ROS species enzymatically.

An important caveat for our conclusions regarding factors affecting priming is that we were only able to consider a limited number of factors that varied among studies. Additional factors such as the age of host plant, the time of sample collection, the type of mycorrhizal inoculation (propagules/spores), and the type of soil may also be important and should be examined in future studies. For example, Afek et al. (1990) mentioned that 10- and 17-day-old onion seedlings were less responsive to AM fungal colonisation than 3-day-old seedlings. Moreover, priming is a cumulative response of all the defence strategies of plants. As such, considering additional secondary metabolite pathways in plants besides antioxidant enzymes, such as activation of jasmonic acid and salicylic acid, could lend further insights. For example, plants with an activated jasmonic acid pathway show higher production of defence-related genes (Wasternack and Hause, 2013). Finally, it is important to consider that the rhizosphere contains more than one symbiotic organism. Along with AM fungi, plant growth promoting bacteria, rhizobia, and endophytic fungi may also be present. It is possible these symbionts have positive or negative effects on mycorrhiza-induced resistance (Jia et al., 2004; Ballesteros-Almanza et al., 2010; Kariman et al., 2014; Pérez-De-Luque et al., 2017), and we were not able to consider these other organisms in our meta-analysis. A better picture of factors affecting priming mechanisms will be revealed with expanded data sets including factors not considered in our study.

Increased understanding of soil biology, especially plant-symbiotic associations such as arbuscular mycorrhiza, has the potential to increase the efficiency and sustainability of agriculture (Barea et al., 2014; Hodson and Lewis, 2016; Thirkell et al., 2017; Sepp et al., 2018). In particular, mycorrhizal symbioses may contribute to sustainable agriculture because of their multifunctional roles in increasing plant performance. Chen et al. (2018) conducted a survey on firms selling AMF products in Europe and worldwide. Results of this survey showed that North America, Europe, Asia, and Latin America are the major producers. Fields of application include gardening, horticulture, landscaping, forestry, golf courses, land reclamation, and soil bioremediation (Chen et al., 2018). An experiment conducted by Hirji (2016) showed that marketable yield of potato when inoculated with commercially produced
Rhizophagus irregularis DAOM 197198 increased by an average of 3.9 tons ha$^{-1}$ which was 79% of the total yield. However, out of all the species of AM fungi identified, very few have been screened for commercial purposes, especially for their ability to mediate mycorrhiza-induced disease resistance and priming in plants. It is possible that this benefit to plants could be increasingly utilised to protect plants from pests and pathogens, and to increase plant growth, in locations with extreme climates or under future scenarios of extreme climatic conditions.

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