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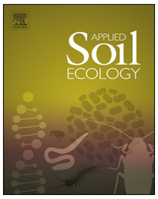

Drivers of entomopathogenic fungi presence in organic and conventional vineyard soils

Deniz Uzman[a,*], Jasmin Pliester[a], Ilona Leyer[b], Martin H. Entling[c], Annette Reineke[a]

[a] Hochschule Geisenheim University, Department of Crop Protection, Von-Lade-Str. 1, 65366 Geisenheim, Germany
[b] Hochschule Geisenheim University, Department of Applied Ecology, Von-Lade-Str. 1, 65366 Geisenheim, Germany
[c] University of Koblenz-Landau, Institute of Environmental Science, Fortstraße 7, 76829 Landau, Germany

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ABSTRACT

Entomopathogenic fungi (EPF) are important antagonists of soil-dwelling insect pests adapted to living in agricultural soils. Little is known about EPF in vineyards, where they could be effective against soil-borne pests such as grapevine phylloxera or larvae of the June beetle. However, the high frequency of fungicide applications might reduce the effectiveness of EPF in vineyard soils. We compared effects of organic and conventional management, fungicide applications and soil parameters on natural occurrence of EPF in vineyards. In 15 pairs of organic and conventional vineyards in Germany, soil was sampled and tested for EPF with a baiting method and subsequent genetic identification. A set of plant protection and soil parameters were measured per site. Three taxa could be verified with *Metarhizium* being the most abundant one. Presence of EPF in general and of *Metarhizium* spp. was enhanced by a high C:N-ratio. Differences between management systems were minor, with *Metarhizium* spp. being more abundant in conventional vineyards. We could not detect a negative effect of fungicides on the presence of EPF. *Metarhizium* spp. was more frequently detected with increasing copper content of vineyard soils. We conclude that EPF are able to persist even in intensively managed vineyard systems. Thus, it would be worthwhile to further explore their potential for the biological control of vineyard pests.

1. Introduction

Alterations of ecosystems for agriculture are obvious in most of the world's land regions with ~40% of the global land surface covered by croplands and pasture (Foley, 2005). Land use intensification is a major threat to biodiversity and frequently reduces bird, mammal, arthropod, and plant diversity (Attwood et al., 2008; Flynn et al., 2009; Storkey et al., 2012). Although less visible, below-ground organisms are equally affected by agricultural practices (see Bardgett and Van Der Putten (2014) for a recent review). Within the diverse soil biota in agricultural landscapes, entomopathogenic fungi (EPF) represent a particularly interesting group. EPF are ubiquitous beneficial soil organisms (Vega et al., 2009), which may act as biological pest control agents or enhance crop performance and health as endophytes (Roy et al., 2010). EPF are available as commercially formulated products for a variety of different crops. The hypocrealean ascomycete *Metarhizium anisopliae* is ideally suited for biological pest control. This cosmopolitan species infests hosts ranging from termites and cockroaches to pests including white flies or grape vine phylloxera or even disease transmitting insects like tse tse flies (Maniania and Ekesi, 2013; Zimmermann, 2007). *M. anisopliae* is an active ingredient of several different agrochemical products. As endophytes, EPF can protect their host plants from pathogens and herbivores (Vega et al., 2009), provide them with insect-derived nitrogen (Behie et al., 2012), and increase plant growth and productivity (Barelli et al., 2016). The various benefits from different EPF species emphasizes the importance of supporting natural and diverse communities of EPF in agricultural cropping systems as an important component of ecosystem functioning.

To support natural communities of EPF, it is important to identify optimal soil and environmental conditions which affect their functioning within agricultural cropping systems. Diversity and abundance of EPF in agricultural soils is influenced by physico-chemical soil parameters, management practices, and climate (Lacey et al., 2015). Within the physico-chemical soil parameters, both soil texture and structure influence the availability of nutrients, oxygen, water, shelter and food for EPF (Eilenberg and Hokkanen, 2006). High percentages of sand facilitate soil water movement and air diffusion as well as the movement of fungal propagules (Jaronski, 2007). Elevated clay fractions can impede movement, but may also increase the abundances of EPF as conidia are adsorbed by clay particles (Eilenberg and Hokkanen,

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[*] Corresponding author.
E-mail address: deniz.uzman@hs-gm.de (D. Uzman).

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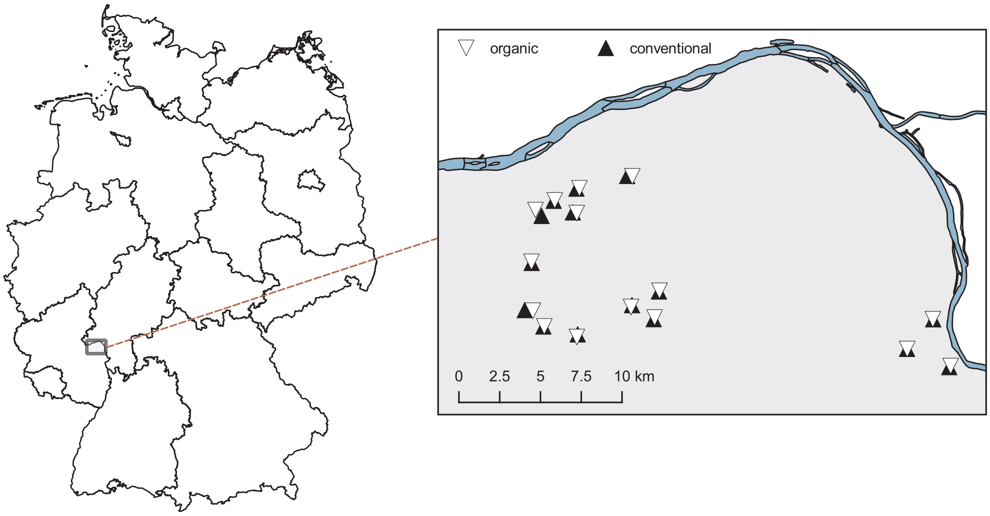

Fig. 1. Location of vineyards used for sampling entomopathogenic fungi (EPF). 15 conventional (filled triangle) and 15 organic (open triangle) vineyards in the German grapevine growing region Rhinehessen were selected (see inlet, Rhine river marked in blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2006). Besides soil texture, quality and extent of soil organic matter is an important driver for the diversity, number, and activity of a variety of soil biota (Thiele-Bruhn et al., 2012). It is not clear if the net effect of organic matter for EPF is positive or negative. On the one hand, a high biological activity and presence of antagonistic organisms can reduce EPF in soils with high organic matter content. On the other hand, higher diversity and density of insects in soils with high organic matter content also implies a higher diversity of hosts for EPF and could thus have a positive effect (Eilenberg and Hokkanen, 2006). Besides the organic matter content, nutritional conditions and the carbon:nitrogen-ratio (C:N-ratio) have been shown to be important for sporulation, growth, propagule yield and virulence of different EPF species (Gao et al., 2007; Jackson, 1997; Shah et al., 2005).

While some of these soil characteristics are site-specific and cannot be influenced by agricultural practices, others can be modified by certain management techniques, including tillage, fertilization, irrigation, and pesticide application. Viticulture, as a perennial cropping system, covers around 7.1 million ha worldwide (FAO, 2018) and has a high potential to serve as a habitat to soil biota like EPF, with grapes cultivated either in conventional or organic production systems. Organic viticulture does not use synthetically produced agrochemicals and fertilizers, but replaces them with natural products where necessary (McGourty et al., 2011). The effects of organic farming on biodiversity and ecosystem services have been intensively investigated. However, these studies have mainly focused on arable production systems (e.g. Hole et al., 2005, Bianchi et al., 2013 or Clark and Tilman, 2017). Organic farming has positive, negative or no effects depending on the examined organism group, ecosystem function or crop system. Organic farming systems have been reported to have higher soil organic matter contents (Coll et al., 2011; Mondelaers et al., 2009) and increased soil microbial diversity and biomass (Mäder et al., 2002), as well as higher richness of soil biota (Hartmann et al., 2015). This suggests that the management system, i.e. organic versus conventional farming, also affects the habitat conditions for EPF. Studies comparing the effects of organic and conventional farming on EPF in different crop systems have reported clear positive (Ramos et al., 2017), partly positive (Clifton et al., 2015; Tkaczuk et al., 2014) or no effects (Goble et al., 2010; Meyling et al., 2011).

While vineyards can potentially foster beneficial soil biota like EPF, as they offer a relatively stable habitat, negative effects on soil organisms can result from the management of fungal grape pathogens. In temperate climates, grapevine typically receives more than ten fungicide applications per year, including the use of copper. Copper is especially, but not exclusively, used in organic viticulture (Komárek et al., 2010). It is known to accumulate in vineyard soils and to affect fungal and microbial soil communities (Giller et al., 2009). In contrast to the generally assumed toxicity of copper, a few studies have shown a tolerance of EPF to this heavy metal (Bååth, 1991). Fungicides have been shown to cause dose-dependent retardation of conidial germination of different EPF species in a laboratory experiment (Shah et al., 2009).

Accordingly, a number of factors potentially influence the presence and diversity of EPF in agricultural soils, yet studies trying to identify the main drivers have been inconclusive, with contrasting results. A mere division into organic and conventional management seems to provide an insufficient explanation of their presence. We suggest that the intensive application of organic and inorganic fungicides in viticulture and the type of management system (organic vs. conventional) may have an effect on EPF. To our knowledge, there are no studies which assess EPF in organic and viticultural soils. In this study, we compared pairs of organic and conventional vineyards for EPF using a baiting method. Habitat conditions were characterized by physicochemical parameters as well as pesticide applications. We hypothesize that (i) organic versus conventional management will result in different soil conditions for EPF, (ii) fungicide and copper applications will reduce EPF and that (iii) a combination of soil parameters and plant protection measurements can explain EPF occurrence better than organic versus conventional management alone.

2. Material and methods

2.1. Study area

The study was conducted in spring and summer 2016 in Rhinehessen (49°51′9.07″N/8°21′0.71″E, 49°58′16.29″N/7°58′4.79″E, Rhineland-Palatinate, Germany, Fig. 1). Due to its fertile soils, the region is characterized by intensive agriculture and viticulture. Rhinehessen has a temperate oceanic climate with annual mean temperatures of 10.8 °C and total annual precipitation of 532.3 mm (Gau-Algesheim, 2001–2017, Agrarmeteorologie Rheinland-Pfalz). Fifteen vineyard pairs were selected, each consisting of one organically and one conventionally managed site. The minimal distance between vineyard pairs was 1000 m. Distances between sampling sites within each pair's organic and the respective conventional vineyard ranged between 16 and 512 m. These 30 vineyards for the study were provided by 17 different farmers (6 organic and 11 conventional). All vineyards had an alternating tillage treatment, i.e. every second inter-row (alley between vine rows) was tilled at least once a year while the other inter-row was not tilled. In 2016, some vineyards were not tilled at all due to heavy rainfalls, but the latest tillage before soil sampling took place in 2015 for all vineyards. Age, size and grape variety were heterogeneous across vineyard sites. Based on the plant protection schemes for the vegetation period 2016 provided by each farmer, two measures of plant protection were used: total number of pesticide treatments (as the number of spraying events applying one or more plant protective substances) and the total number of fungicide treatments (comprising all active ingredients of both organic and inorganic fungicides, see Table S1a/b).

2.2. Soil sampling and soil parameters

Soil sampling was conducted in May and August 2016. Per sampling date, upper-soil of two central inter-rows (till/no-till) was sampled per vineyard (see scheme in Supplementary material S2). One mixed soil sample was taken per inter-row, consisting of 8–12 soil cores from the upper 10 cm soil layer, spread over a distance of 10 m. This resulted in a total of 4 soil samples per vineyard, of which all 4 were used for Galleria baiting and only the samples from May were used for soil parameters (see below). Soil samples were taken with a soil corer (AccuCore TM, diameter = 5 cm, height = 10 cm). In the no-till inter-rows, vegetation cover was removed before sampling. Samples were kept for up to 4 months at 4 °C before further processing.

In order to identify differences in soil properties between management systems and to relate presence of EPF to physico-chemical soil parameters, soil texture, soil organic matter, C:N-ratio, bioavailable copper content as well as plant available phosphor (P), potassium (K) and magnesium (Mg) contents were measured for soil samples collected in May 2016. For those soil parameters, a set of standard procedures listed below were used following Schaller (2000). Subsamples of about 250 g were taken, dried overnight at 50 °C and sieved with a mesh size of 2 mm. Due to the homogeneity of soils in the area, soil texture and bioavailable copper were only measured for one sample per vineyard (no-till inter-rows). For every other parameter, both mixed soil samples taken in May were analysed separately and mean values per vineyard were used for calculations. Bioavailable copper was assessed using the $CaCl_2$–DTPA method (CAT method). Total carbon and total nitrogen contents were determined by the Dumas combustion method using the vario MAX CNS analyser by ELEMENTAR and the C:N-ratio was calculated subsequently. Organic matter contents were extrapolated from the organic carbon contents. Plant available P and K were measured according to the CAL (Calcium Acetate Lactate) method. Plant available Mg was extracted using a $CaCl_2$-solution.

2.3. Galleria bait method

To isolate EPF from the soil samples taken, the Galleria bait method was applied following the protocol of Zimmermann (1986) and Meyling (2007). The baiting was set up separately for both sampling times (May and August). Late instar larvae of the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) were obtained from a commercial provider. Before baiting, the soil samples were sieved through a 4–5 mm mesh and moistened with water to about half field capacity. For every soil sample, three subsamples of 100 g of soil were filled into plastic boxes with lids (diameter = 8 cm, height = 5 cm, see sampling scheme in S2). As a control treatment, autoclaved standard gardening soil was used. Before being baited, all larvae were treated with 56 °C water for 10 s to inhibit silking. Eight *G. mellonella* larvae were put in each box and samples were incubated in a climate chamber at 21 °C ± 1 °C without light. To ensure permanent soil contact, all boxes were inverted every other day. After 7, 10 and 14 days, respectively, the boxes were checked for dead larvae. The dead larvae were isolated and surface sterilized in 1% sodium hypochlorite for 10 s. Subsequently, the dead bodies of larvae were separately incubated in small tubes with a cotton plug moistened with distilled water. These larvae were kept at the same conditions as the soil boxes and were visually checked for mycosis after incubation for at least 14 days. For the Galleria baiting, 2880 larvae were used during both sampling turns. After 14 days of baiting, 2209 larvae were dead. 490 larvae showed possible mycosis and were used for DNA isolation.

2.4. Identification of entomopathogenic fungi

From every larvae showing mycosis, DNA was isolated following a modified protocol by Marzachí et al. (1998). In short, mycosed tissue was cut off and homogenized with a micropestle in 500 µl CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl pH 8.0). Subsequently, the suspension was vortexed and incubated for 30 min at 60 °C before being centrifuged at 13,000 rpm for 10 min. The supernatant was transferred completely to a new tube and 500 µl chloroform:isoamyl alcohol (24:1) was added, then inverted and centrifuged. Again, the supernatant was transferred and cold isopropanol was added to precipitate the DNA. The DNA pellet was washed with 500 µl of 70% ethanol after centrifugation, dried and then dissolved in deionized sterile water (20 µl). For DNA amplification, 1 µl of the DNA solution (in a dilution of 1:1, 1:10 or 1:100) was added to a PCR mixture containing 5 µl 10× Dream Taq PCR buffer, 1 µl of dNTP mixture (10 mM each), 2 µl of each primer (universal fungal primers TW81/AB28, 10 pmol/µl, Curran et al., 1994) and 0.2 µl of Dream Taq-Polymerase (Thermo Fisher) and filled up with sterile distilled water to a final volume of 50 µl. Cycling conditions included an initial denaturation step of 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 55 °C for 1:30 min and 72 °C for 2 min, and finalized with one cycle at 72 °C for 5 min. Sanger sequencing was done by Macrogen Europe, Netherlands. Fungal genera were identified by their sequences using the BLAST data-base.

2.5. Data analysis

For statistical analyses, R 3.4.1 (R Core Team, 2014) and the packages lme4 (Bates et al., 2015), MuMIn (Barton, 2018), plyr (Wickham, 2011), reshape (Wickham, 2007) and ggplot2 (Wickham, 2009) were used. To test physico-chemical soil parameters and plant protection measures for differences between organic and conventional management systems, linear mixed models (LMMs) with vineyard pair as random factor and management system as explanatory variable were built. For the plant protection measures, farmer ID was used as random factor as all vineyards belonging to one farmer were treated identically. Every model was tested with a likelihood ratio test against the respective null-model.

Detections of EPF were coded as a binomial variable with an upper limit of 4 possible detections per sampled vineyard. If one or several larvae within the three soil containers per sampled inter-row were infected by an EPF, the sample was registered as one detection. Accordingly, every sampled vineyard could have a maximum number of four detections (two sampled inter-rows times two sampling turns, see S2 for a graphical scheme). We defined a value of 4 as an occurrence probability of 100%. These occurrence probabilities were calculated for EPF in general as well as separately for the taxa *Metarhizium* spp. and *Clonostachys rosea.* For *C. rosea*, a detection score of 3 was defined as an occurrence probability of 100%, as it was detected maximally 3 times per sampled site. *Beauveria* spp. was omitted from further analyses due to low detection rates. As a first step for data analysis, data exploration was carried out following a protocol by Zuur et al. (2010). Explanatory variables with variance inflation factors > 3 were omitted from analyses to avoid collinearity. Generalised Linear Mixed Models (GLMMs) were fitted on the proportional data for each of the three response variables with a binomial distribution and vineyard pair as a random factor. Subsequently, stepwise best-model selections were conducted with scaled and centered explanatory variables. The maximum number of predictors for model selection was set to 3. Akaike information criterion (AIC) was used for selection of the best model. For model selection, the initial set of explanatory variables comprised C:N-ratio, organic matter content, bioavailable copper content, soil texture (sand, silt and clay contents), numbers of pesticide and fungicide treatments as

Table 1
Differences in physico-chemical soil parameters and plant protection measurements between organic and conventional vineyards (N = 15). LMMs were tested with a likelihood ratio test against the respective nullmodel. Estimates and standard errors for organic management are presented as well as X^2 and p-values for the likelihood ratio test.

	Means ± Standard Error		Organic management		Likelihood ratio test	
	Conventional	Organic	Estimate	Std. Error	X^2	p-Value
Organic matter [%]	3.97 ± 0.21	5.15 ± 0.37	1.18	0.29	11.62	$< 0.001^{***}$
Bioavailable Cu [ppm]	15.96 ± 2.31	22.00 ± 2.95	6.04	3.17	3.46	0.06 .
C:N-ratio	12.83 ± 0.53	12.47 ± 0.40	-0.37	0.34	1.20	0.27
K [mg/100 g]	51.01 ± 3.09	61.14 ± 4.87	10.13	5.48	3.28	0.07 .
P [mg/100 g]	37.43 ± 3.71	38.98 ± 4.83	1.56	5.54	0.09	0.77
Mg [mg/100 g]	12.26 ± 0.74	14.50 ± 0.89	2.24	1.07	4.12	0.04^{*}
Sand [%]	26.54 ± 3.18	25.37 ± 3.70	-1.17	2.70	0.20	0.66
Clay [%]	19.62 ± 1.69	25.19 ± 2.83	5.57	2.32	5.18	0.02^{*}
Silt [%]	53.85 ± 3.94	49.44 ± 3.56	-4.40	2.52	2.95	0.09 .
Pesticide treatments	11.20 ± 0.34	15.27 ± 0.38	3.57	0.75	15.49	$< 0.001^{***}$
Fungicide treatments	23.07 ± 0.78	28.53 ± 1.38	3.40	2.12	2.69	0.10

Significance levels: (.) $p < 0.1$, $(^{*})$ $p < 0.05$, $(^{**})$ $p < 0.01$, $(^{***})$ $p < 0.001$.

continuous variables and management system as a categorical variable (organic/conventional). Due to collinearity, silt content (correlated with sand content) and pesticide treatments (correlated with numbers of fungicide treatments) were omitted from model selection. Two parallel full models were built for model selection because of collinearity between management system and organic matter content, clay content and total number of fungicide applications (Table 1) as following: Both models contained C:N-ratio, bioavailable copper content and sand content. Additionally, one model contained management system (organic/conventional) while the other included organic matter content, clay content and total number of fungicide applications. Model selection was applied to both full models. From both parallel model selections, only results for the model selection resulting in lower AICc values are presented. For final models, model validation was performed as described by Zuur et al. (2009). For all models within ΔAICc < 2 to the best model, marginal and conditional R^2s were calculated (Nakagawa and Schielzeth, 2013).

3. Results

3.1. EPF taxa in vineyards and bait infection rates

Presence of EPF could be verified for 27 out of 30 vineyards over both sampling turns, for 26 vineyards in the first and for 19 vineyards in the second sampling turn, respectively. 247 DNA samples showed visible PCR products and were sequenced. Overall, an infection by EPF could be confirmed for 181 bait larvae by the final BLAST analysis. Among these 181 infections, three taxa of EPF were detected: *Metarhizium* spp. (48.1%), *C. rosea* (32.6%) and *Beauveria* spp. (19.3%). None of the larvae incubated in autoclaved standard gardening soil as a control treatment were infected by EPF. Hence, all EPF detected via baiting were soil-borne in the sampled vineyard soils and were not present due to contamination in the experimental set-up or in the material used.

3.2. Differences between organic and conventional vineyards

Organic vineyards were characterized by higher organic matter, magnesium and clay contents compared to conventional ones (Table 1). C:N-ratios, phosphorous and sand contents did not differ between organic and conventional sites (Table 1). Organic vineyard soils showed a tendency towards elevated bioavailable copper and potassium contents, but differences to conventional soils were not significant (Table 1).

Organic vineyards received more plant protection treatments than their conventionally managed counterparts, meaning four additional application events of plant protective substances in 2016 on average (Table 1). Total numbers of fungicide applications in the vegetation period 2016 did not differ between both management systems (Table 1).

3.3. Drivers of EPF presence

To identify drivers of EPF occurrence in viticultural soils, model selection was conducted as described for EPF in general and separately for *Metarhizium* spp. and *C. rosea*. Final models and the best predictor variables for each group are presented in Table 2a and b. For EPF in general as well as for *Metarhizium* spp. and *C. rosea* alone, a high C:N-ratio enhanced occurrence probabilities significantly. The C:N-ratio was the only predictor contained by the best model for EPF in general and *C. rosea* (Fig. 2a and b). The management system, whether organic or conventional, had no significant effect on either EPF in general or *C. rosea* (Fig. 3a and c). By contrast, *Metarhizium* spp. was detected nearly twice as frequently in conventional than in organic vineyard soils (Table 2, Fig. 3b). Regarding effects of soil copper contents and fungicide applications on EPF, we found an increase of *Metarhizium* spp. with bioavailable copper contents in addition to the positive effect of the C:N-ratio and conventional management (Table 2, Fig. 4). Fungicide applications and plant protection treatments did not explain occurrence probabilities of any of the examined groups.

4. Discussion

4.1. EPF in viticultural soils

Of the three entomopathogenic fungal taxa detected in vineyard soils in the present study, *Metarhizium* spp. was the most frequent, followed by *C. rosea* and *Beauveria* spp. This is in accordance with other studies indicating that *Metarhizium* spp. can be the dominant EPF genus in agricultural soils (Meyling et al., 2011; Rath et al., 1992; Tkaczuk et al., 2014). A study in Slovakia showed that *M. anisopliae* was not negatively affected by agricultural practice while *B. bassiana* was found more frequently in natural habitats (Medo and Cagáň, 2011). While *B. bassiana* was more frequent than *M. anisopliae* in another study conducted in Spain, here the authors concluded that this was due to climatic differences, as all studies conducted in northern countries found *Metarhizium* to be the most common genus (Quesada-Moraga et al., 2007). *C. rosea* is a fungus with a wide range of habitats, from cultivated land to coastal soils (Sutton et al., 1997). A generally low diversity of entomopathogenic fungi in agricultural soils is in accordance with a couple of other studies where no more than 4 species or genera were detected (Goble et al., 2010; Jabbour and Barbercheck, 2009). However, different fungal isolation methods can lead to the detection of

Table 2

Best models explaining the frequency of entomopathogenic fungi (EPF) in vineyard soils. Summary tables for GLMM model selection. (a) Models with ΔAICc < 2 to the best model are presented. (b) Estimates and standard errors for best models. Bold values indicate $p < 0.05$. For (†), full models contained management system instead of total number of fungicide applications and sand and clay contents. For *Clonostachys rosea* (‡), a maximal detection score of 3 was used.

(a) Set of candidate models

Model	df	ΔAICc	Marginal R^2	Conditional R^2
Entomopathogenic fungi				
Y ~ Intercept + C:N-ratio + (1|Pair)	3	0	0.11	0.19
Y ~ Intercept + copper + C:N-ratio + (1|Pair)	4	1.49	0.14	0.19
Y ~ 1 + (1| Pair)	2	1.62	0.00	0.22
Metarhizium spp. †				
Y ~ Intercept + copper + C:N-ratio + organic/conventional + (1| Pair)	5	0	0.26	0.37
Y ~ Intercept + copper + organic/conventional + (1| Pair)	4	1.06	0.18	0.35
Y ~ Intercept + C:N-ratio + organic/conventional + (1| Pair)	4	1.46	0.19	0.35
Clonostachys rosea ‡				
Y ~ Intercept + C:N-ratio + (1| Pair)	3	0	0.13	0.22
Y ~ Intercept + copper + C:N-ratio + (1| Pair)	4	1.77	0.15	0.24
Y ~ 1 + (1| Pair)	2	2.00	0.00	0.25

(b) Variable estimates of selected best model

	Estimate	SE
Entomopathogenic fungi		
Intercept	0.34	0.29
C:N-ratio	**0.63**	**0.29**
Metarhizium spp. †		
Intercept	−0.04	0.38
bioavailable copper [ppm]	**0.61**	**0.29**
C:N-ratio	**0.62**	**0.31**
management system: organic	**−1.51**	**0.53**
Clonostachys rosea ‡		
Intercept	−0.43	0.33
C:N-ratio	**0.74**	**0.34**

different EPF species (Goble et al., 2010; Medo and Cagáň, 2011).

For viticulture, promoting natural EPF populations of the detected groups could contribute to the biocontrol of soil-dwelling pest insects. For example, treatments with *M. anisopliae* led to higher mortality rates of adults of *Hyalesthes obsoletus*, a plant hopper species with a soil-dwelling larval stage transmitting grapevine yellow disease (Bauer, 2008; Langer et al., 2005). For root damaging viticultural pests, efficacy of both *M. anisopliae* and *B. bassiana* has been shown against larvae of the June beetle *Polyphylla fullo,* also known as “pine chafer” (Erler and Ates, 2015). Efficacy of *M. anisopliae* and *B. bassiana* against grape-vine phylloxera *Daktulosphaira vitifoliae* has recently been discussed by Benheim et al. (2012). Our study provides the first record of *Metarhizium, Clonostachys* and *Beauveria* in German vineyard soils. Thus, the potential of these fungi for biological pest control in viticulture should be investigated by further research.

4.2. Fungicides

Fungicide applications could not explain EPF occurrence in our study. Even though dose dependent inhibition of conidial germination of EPF by fungicides was shown in a laboratory experiment where 15 commercially available fungicides were tested on 4 EPF species, no effects on their virulence was found (Shah et al., 2009). Similarly, in a field study by Mietkiwski et al. (1997) where different fungicides were

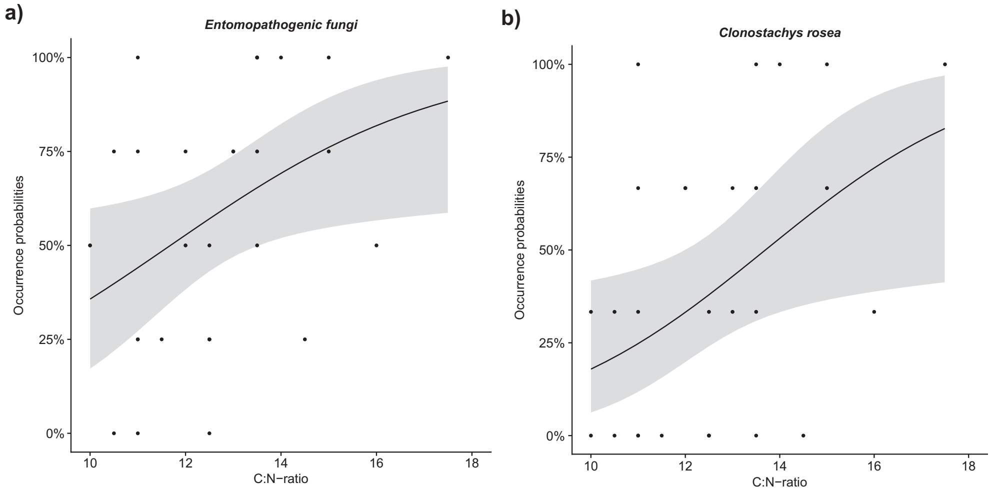

Fig. 2. Predicted and observed occurrence probabilities of (a) entomopathogenic fungi and (b) *Clonostachys rosea* in relation to the C:N-ratio in organic and conventional vineyard soils in Rhinehesse, Germany. Solid lines represent the respective GLMM with binomial distribution obtained after model selection. Grey ribbons represent confidence intervals of 95%.

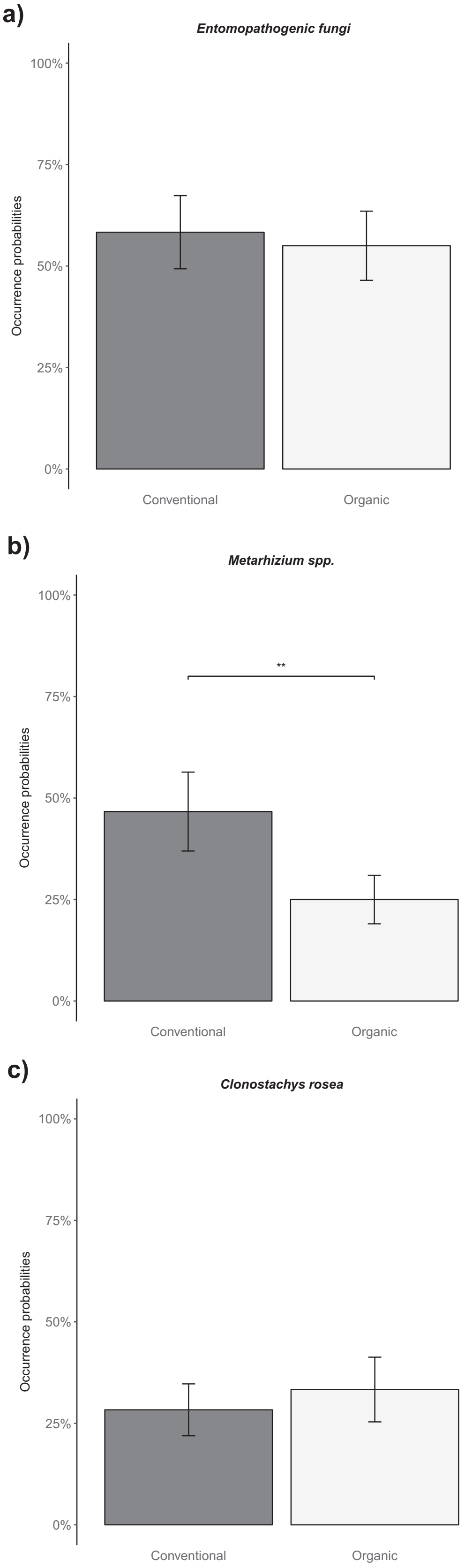

Fig. 3. Occurrence probabilities of (a) entomopathogenic fungi in general, (b) *Metarhizium* spp. and (c) *Clonostachys rosea* in 15 conventional (dark grey) and 15 organic vineyards (light grey) in Rhinehesse, Germany. Means and standard errors are displayed. Significance levels are obtained from model selection (Table 2) with ** for $p < 0.01$.

applied on field plots, only the fungicide benomyl reduced the infection rates of bait larvae by *B. bassiana.* In the same study, application of fungicides in the laboratory inhibited fungal growth of all tested EPF species. This suggests that even though detrimental effects of fungicides on EPF could occur, under field conditions, these effects might not be strong enough to diminish EPF presence, their recolonization abilities or their virulence.

Interestingly, we found a positive relationship between *Metarhizium* spp. and bioavailable copper. Tolerance against copper of *B. bassiana* and *M. anisopliae* was described by Bååth (1991) and may be advantageous for living in copper-loaded habitats like vineyard soils. However, Jabbour and Barbercheck (2009) found *M. anisopliae* to be negatively affected by copper. These contrasting findings may be due to the presence of different fungal strains in the two studies, or due to different copper content analyzing methods. Remarkably, the range of total copper contents in our study was about 10 times higher than in the investigation by Jabbour and Barbercheck (2009), suggesting a high tolerance of *Metarhizium* spp. towards copper in our study region. This emphasizes the suitability of EPF for the use in viticulture.

4.3. *Environmental drivers of EPF*

Our study supports the hypothesis that other factors are more important for EPF in vineyards than conventional versus organic management alone. We found no differences in the occurrence of EPF between both management systems apart from *Metarhizium* spp. being more frequent in conventionally managed vineyards. This observation is in line with other studies concluding that it is not the management system, but rather the environmental factors which are drivers of EPF occurrence. No differences in abundance of soil-born EPF between organic and conventional management systems were detected for a vegetable cropping system in Denmark (Meyling et al., 2011), winter cereals in Poland (Tkaczuk et al., 2014) and citrus orchards in South Africa (Goble et al., 2010). Environmental factors and soil properties were suggested by the authors to have a stronger effect on EPF, but could not be verified as no further parameters were measured. In a 2 year study in corn and bean fields in the USA, higher abundances of EPF in organic systems were only found in the first year (Clifton et al., 2015). In this study, *M. anisopliae* was positively related to the silt content and the use of organic fertilizer, but no further soil properties were assessed. In arable fields in Norway, Klingen et al. (2002) found higher occurrences of EPF in organic sites. The most frequent species in this study was *Tolypocladium cylindrosporum,* which we did not detect in our sites. In the same study, no differences between organic and conventional management were found for *M. anisopliae* and *B. bassiana*. Again, additional environmental variables were not measured, as in a Cuban study in beans (Ramos et al., 2017) that found *B. bassiana* to be more abundant in organic sites. Additionally, an experiment on long term effects of organic viticulture led to a higher abundance of fungal feeding nematodes (Coll et al., 2011), which may explain why we could not confirm organic vineyards to be a more suitable habitat for EPF. The next step would accordingly be to analyze impacts of the management system on fungal antagonists.

We identified a wide C:N-ratio as a predictor of EPF occurrence in vineyard soils. This was true for EPF in general as well as specifically for *Metarhizium* and *C. rosea.* A wide C:N-ratio implies lower biological activity and nitrogen availability (Scheffer et al., 2009). In several laboratory experiments with different media, it has been shown that a wide C:N-ratio can promote growth and sporulation of EPF in biocontrol substances (Gao et al., 2007; Jackson, 1997; Shah et al., 2005). We believe that EPF have an advantage in nutrient-poor environment, because i) it has been shown for *M. anisopliae* that nutrient stress resulted in increased virulence and germination (Rangel et al., 2008; Shah et al., 2005) which could explain higher detection rates in soils with a high C:N-ratio, and ii) EPF are able to mobilize insect-derived nitrogen when plant-derived nitrogen is scarce, and 'trade' it as endophytes for plant

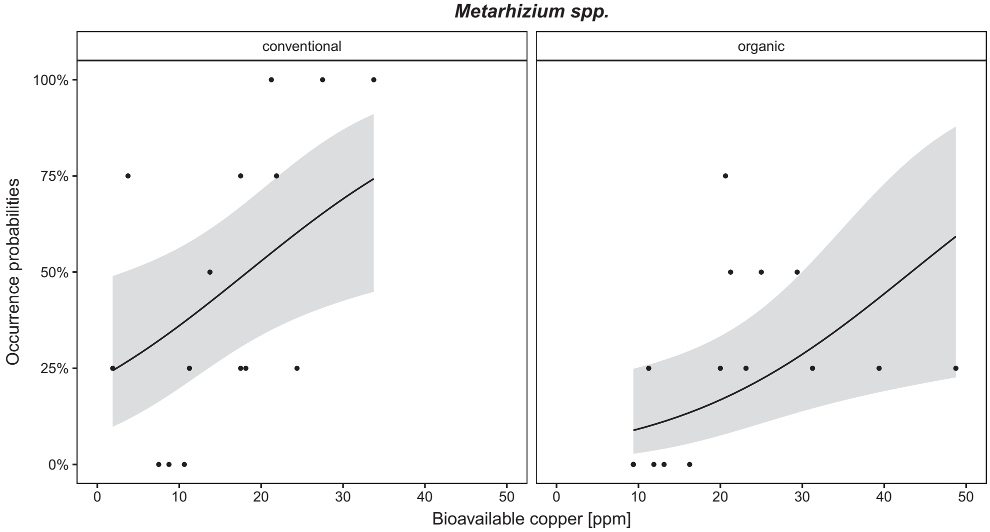

Fig. 4. Observed and predicted occurrence probabilities of *Metarhizium* spp. in relation to the bioavailable copper content in organic and conventional vineyard soils in Rhinehesse, Germany. Solid lines represent the respective GLMM with binomial distribution. Grey ribbons represent confidence intervals of 95%.

carbohydrates (Barelli et al., 2016). This can give EPF an advantage over other soil-microorganisms, allowing survival in difficult environments and profit from reduced competition and antagonist presence. However, low nutrient availability could have adverse effects on other groups of beneficial soil organisms, as well as plant nutrition status and thus grape yield and quality.

Furthermore, Quesada-Moraga et al. (2007) identified additional parameters when comparing different natural and cultivated soils in Spain, including longitude and latitude (for *M. anisopliae* and *B. bassiana),* organic matter (for *M. anisopliae)* and pH and clay content (for *B. bassiana)*. In our study, organic matter did not affect *Metarhizium* spp., but again, the sets of measured environmental variables differed between both studies. Soil temperature and moisture have also been identified as important factors affecting EPF presence (Eilenberg and Hokkanen, 2006; Jaronski, 2007), but this was not measured in our study due to the geographical proximity of the selected study sites. Drawing final conclusions on the most important factors for EPF in agricultural environments remains challenging with different sets of variables measured in different studies.

4.4. Organic and conventional management shape different soil environments

Even though the management system only had minor effects on the presence of EPF in vineyard soils, we found that organic and conventional management resulted in different environmental soil settings, which may affect other biocontrol agents and pest management. In accordance with previous studies, organic vineyard soils were characterized by higher amounts of organic matter and magnesium, as well as higher clay contents when compared to their conventional counterparts. The elevated organic matter content is in line with results from a meta-analysis for general agricultural practices (Mondelaers et al., 2009), as well as from a long-term experiment in viticulture (Coll et al., 2011). This may reflect the use of organic fertilizers, which is standard practice in organic viticulture (McGourty et al., 2011). Surprisingly, organic vineyards showed higher clay contents, which was unexpected due to the paired experimental design with neighbouring organic and conventional sites chosen to minimize geological differences. We suggest that this may be the result of interactions between different soil compartments. As a typical soil formation process, clay particles are leached to lower soil horizons, but soil organic substances can build aggregates with clay particles that are more stable and resistant to leaching processes (Scheffer et al., 2009). As organic vineyards also had higher organic matter content, we suggest this may reflect a possible artefact of the study, as only the upper 10 cm of the soil layer were sampled, which may explain the differences in clay contents with clay particles accumulating close to the surface in organic vineyards. As ionic magnesium attaches to clay particles (Scheffer et al., 2009), this could also explain the elevated magnesium contents in our organic sites. Magnesium is important for vine growth and photosynthesis (Bauer, 2008) and is applied as a mineral fertilizer in conventional viticulture and in form of green manure or mined minerals in organic viticulture (McGourty et al., 2011). In terms of heavy metal contamination, we found high copper contents in soils of both management systems, with a yet not significant trend of elevated contents in organic compared to conventional vineyard soils. Similar results were obtained by Coll et al. (2011) in vineyard soils in Southern France. Both organic and conventional farmers participating in our study used the same fungicide (on a copper hydroxide basis), which may explain similar soil copper contents.

In terms of differences in plant protection strategies, we found more frequent plant protection treatments in organic vineyards. Higher numbers of application events in organic vineyards were caused by more frequent applications of a combination of inorganic fungicides (notably copper hydroxide and wettable sulphur) and plant strengtheners (e.g. potassium bicarbonate against powdery mildew), see Table S1a and b. Total numbers of (organic and inorganic) fungicide applications alone were not statistically different between both management systems. In organic vineyards, only inorganic copper-based agents were applied, while the fungicides used in conventional vineyards contained a wide array of organic substance classes, as well as copper hydroxide and several sulfur-based agents. This is in accordance with other reports, where 13–21 pesticide application events per growing season were reported, depending on management system and year (Fermaud et al., 2016).

In our study, organic viticulture had higher soil organic matter contents which is more favourable for soil biota and plant roots (Thiele-Bruhn et al., 2012). However it was surprising that these systems were characterized by more frequent plant protection applications. This elevated disturbance in organic vineyards is notable, as generally, organic management is associated with the aim to reduce external inputs when compared to conventional systems (Mondelaers et al., 2009), especially in annual crop systems. This implies that in organic viticulture, possible positive effects by renouncing the use of herbicides and insecticides and by higher soil organic matter contents contrast with the negative effect of higher disturbance. In accordance with

Bruggisser et al. (2010), we suggest that those contrasting mechanisms of organic viticulture have to be paid attention to in further research on effects of management system on diversity and biocontrol in vineyards.

5. Conclusions

In spite of the intensive fungicide applications, soil-borne EPF and especially *Metarhizium* have high frequencies in vineyard soils. They are able to persist even in nutrient-poor environments, as a high C:N-ratio was the main predictor for all analyzed EPF groups in this study. Thus, their potential for natural biocontrol in viticulture deserves further investigation. Natural persistence of *Metarhizium* in soil seems compatible with intensive management and use of organic and inorganic fungicides. We found no major differences in EPF occurrence between organic and conventional vineyards despite different soil conditions. With further knowledge about their habitat preferences, we will be able to derive strategies that enhance natural populations and lead to more sustainable agro-ecosystems with reduced agrochemical inputs. Therefore, it is important to close the knowledge gaps on the species specific sets of environmental factors affecting their presence and abundance.

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2018.09.004.

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