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Abundance of the soil entomopathogenic fungus *Metarhizium anisopliae* sensu lato in agricultural field and forest soils in Japan

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Abstract

Metarhizium anisopliae sensu lato is a facultative entomopathogenic, soil-inhabiting fungus that has been used for biological control of soil-dwelling arthropod pests. However, little is known about the density of *M. anisopliae* sensu lato in forest soils and the difference in the density among different habitat types and geographical origins. In this study, we determined the density of this fungus in soil samples collected from forest and agricultural fields in Japan by plating method with semi-selective agar medium and analyzed its associations with the habitat types and latitudes of the collecting sites. Mean and mode density of 211 soil samples were 9.4×10^2 CFUs/g soil and 1.0×10^3–1.0×10^4 CFUs/g soil, respectively, which were comparable to the levels previously reported in other countries. The density in forest soil was not significantly different from that of the other habitat types, despite previous studies that concluded *M. anisopliae* sensu lato to be less abundant in forest soil based on occurrence determined by insect bait method. The latitude was also not significantly associated with the density. This study revealed higher abundance of *M. anisopliae* sensu lato in forest soil than the original expectation.

Key words – associations – Clavicipitaceae – habitat – latitude

Introduction

The soil environment is an important reservoir for a variety of entomopathogenic fungi, often contributing to the natural regulation of insect and acari populations, including those of agricultural and forest pests (Foster 1975, Kamata et al. 1997, Jackson et al. 2000). The soil-inhabiting entomopathogenic fungus *M. anisopliae* sensu lato (s.l.) (Ascomycota: Clavicipitaceae) is globally widespread and has been isolated from more than 200 species in 17 families of insects and acari, including soil-dwelling insects (Zimmermann 2007). This species has been frequently isolated from soil samples and shows a close association with plant roots (Hu & Leger 2002, Wyrebek et al. 2011). In a study conducted in Britain, the presence of *M. anisopliae* s.l. was possibly the most important factor affecting the seasonal population fluctuations of a root aphid species (Foster 1975). Because of these ecological traits, this fungus has been studied for its potential application

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as a biological control agent of soil-dwelling insects, such as the larvae of scarabaeid species and root weevils (Ramoutar et al. 2010, Ansari & Butt 2013).

The information pertaining to the background levels of *Metarhizium* spp. play an important role in the evaluation of the persistence of an introduced strain in this genus. According to EU legislation, the amount of a biological control agent applied to soil should be lower than the background level of the same species indigenously occurring at the site (Scheepmaker & Butt 2010). At least 13 published studies have quantified the densities of *M. anisopliae* s.l. in the soil according to a review by Scheepmaker & Butt (2010). The most common methods for the quantification of the CFUs of entomopathogenic fungi in the soil are those with plating on semi-selective agar medium, although a qualitative PCR method was also developed for the quantification of some entomopathogenic fungi in the soil (Schneider et al. 2012). Previous studies have revealed the densities of *M. anisopliae* s.l. in agricultural fields, pastures, and lawns (Scheepmaker & Butt 2010); however, insufficient data have been published for forest soils despite the potential of *Metarhizium* spp. as a forest pests-controlling biological agent, such as emerald ash borers (*Agrilus planipennis*) (Hajek & Bauer 2009). Knowledge of the abundances of *Metarhizium* spp. in semi-natural habitats such as forests is also important in that they may provide a refuge for *Metarhizium* spp. and may allow for recolonization of adjacent arable fields (Schneider et al. 2012). Furthermore, few investigations have been conducted to compare the densities of entomopathogenic fungi in different habitat types and latitudinal origins, although comparisons of the occurrences of *Metarhizium* in Canada indicated its different preference of habitat types (Bidochka et al. 1998).

In this study, we quantified the abundance of *M. anisopliae* s.l. in 211 soil samples collected from various types of environments, including a forest and an agricultural field in Japan, to determine its densities in the soil and the associations among its abundance and the habitat types and latitude.

Materials & Methods

Soil sampling

Overall, 211 soil samples were collected from various environments in Japan from April 2008 to October 2013 (Table 1). After removal of surface litter, soil samples were collected from an approximate depth of 5–10 cm, placed in plastic bags, and stored at 4°C before use.

Quantification of *M. anisopliae* s.l. in soil samples

Each soil sample was suspended in a sterile aqueous solution of 0.05% Tween 80 (0.1 g in 900 µL or 1.0 g in 10 mL), thoroughly shaken, and serially diluted 10 and 100 or 5 and 25 times. Next, two replicates of the original suspension and 2–5 replicates of the two dilutions were spread-plated (100 µL) onto a semi-selective agar medium (6% oatmeal, 1.25% agar, 0.03% chloramphenicol, and 0.1% cycloheximide). The cultures were maintained at 25°C in dark. The colonies that were morphologically identified as *M. anisopliae* s.l. were counted two to three weeks after plating.

The detection limits of the density for each of the soil samples were 45 or 50 CFUs/g soil, which were the values of CFUs/g soil where one CFU was detected on the selective agar plates on which the soil suspension of the original concentration was spread-plated. The mean and standard deviation were calculated by the Kaplan–Meier method using R package NADA. In the calculation, the zero values of CFUs/g soil were regarded as left-censored at their detection limits.

Results

The detection rates and statistical data of CFUs/g soil are shown in Table 1. In addition, the distributions of the density Log_{10} (CFU/g soil + 1) are illustrated in Fig. 1. The modal density of either the total or the individual habitat type soil samples was 1.0×10^3–1.0×10^4. The maximum density was 4.1×10^5 CFUs/g soil, which was determined in a soil sample collected under a shrub.

M. anisopliae s.l. was the dominant species among the fungi that grew on the selective agar plates with a density of more than 1.0×10^4 CFUs/g soil. The differences in the densities of the habitat types were not statistically significant (Forest-Cultural Field: $\chi^2 = 1.7$, d.f. = 1, $p = 0.193$; Forest-Not Forest: $\chi^2 = 3.3$, d.f. = 1, $p = 0.069$).

The relationships between the densities and the latitudes of the sampling sites are presented in Fig. 2. The correlations were not statistically significant (Spearman's rank correlation coefficients were not significantly different from 0) for either the total or the individual habitat type soil samples (Total: r = −0.037, $p = 0.5934$; Cultural field: r = −0.21, $p = 0.15$; Forest: r = 0.11, $p = 0.29$; Others: r = −0.15, $p = 0.23$).

Table 1 Detection rate and density of *M. anisopliae* s.l. in the soil samples.

Habitat types	Detection rate	Density in soil Log_{10}{CFUs/g soil + 1} (CFUs/g soil)		
		Median	Mean	S.D.
Forest	0.75 (70/93)	3.40 (2.5×10^3)	3.13 (1.3×10^3)	1.07
Cultural Field	0.66 (33/50)	2.95 (9.0×10^2)	2.98 (9.6×10^2)	0.91
Others	0.65 (44/68)	2.70 (5.0×10^2)	2.82 (6.7×10^2)	1.15
Total	0.70 (147/211)	3.00 (1.0×10^3)	2.97 (9.4×10^2)	1.09

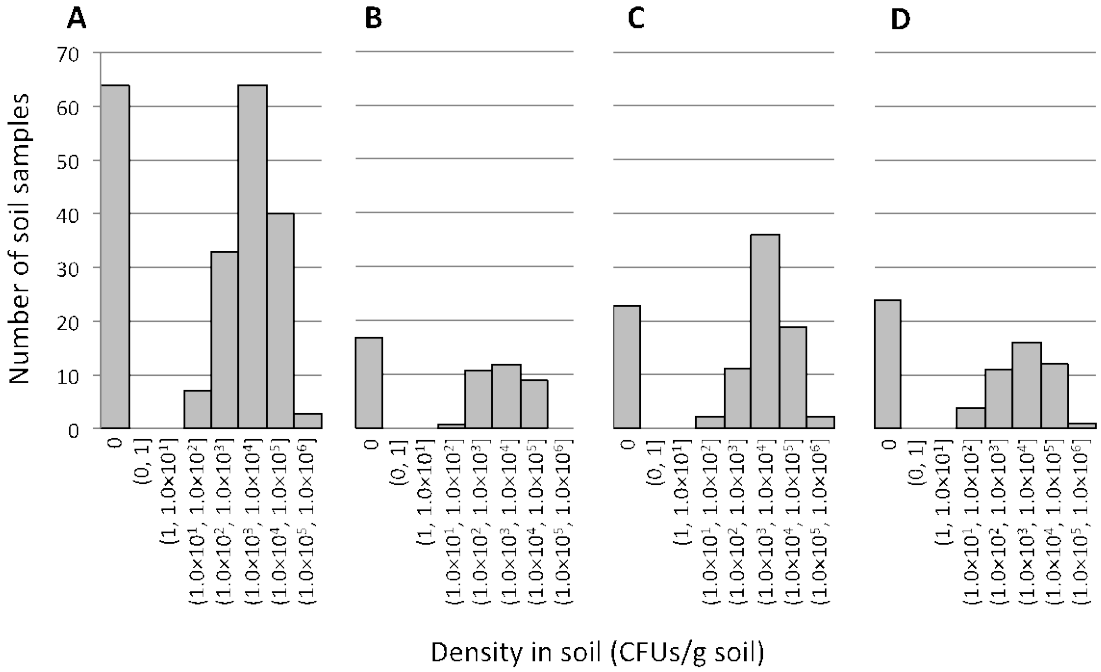

Fig. 1 – Distribution of the densities of *M. anisopliae* s.l. in soils from different habitat types. A Total. B Cultural field. C Forest. D Others.

Discussion

According to the meta-analysis of 13 studies conducted outside Japan by Scheepmaker & Butt (2010), the mean density of *M. anisopliae* s.l. in the soil was 1.04×10^3 CFU/g soil. The modal density in soil from the Macquarie islands was 1.0×10^3–1.0×10^4 CFUs/g soil (Rath et al. 1992). To the best of our knowledge, the maximum value of the density is 1.0×10^6 CFUs/g, which is recorded from a pasture soil in Australia (Milner 1992). In this study, we found that the density of *M. anisopliae* s.l. in soil samples collected in Japan was almost equivalent to those in previous studies conducted outside Japan.

There are little data concerning the density of *M. anisopliae* in forest soils despite its potential as a biological control agent against forest pests (Hajek & Bauer 2009). The present study determined the density of *M. anisopliae* s.l. in forest soil in Japan. The comparison among different

habitat types exhibited no significant difference in the abundance of *M. anisopliae* s.l. between forest and agricultural field soils, although the soils were collected from two habitat types with generally a large number of differences in the environmental factors. This finding is in contrast to those of previous investigations of two entomopathogenic fungal species in soil by using the insect bait method, where *M. anisopliae* s.l. had higher occurrence in agricultural field soils than in forest soils, whereas the other species had comparatively high occurrence in forest soils (Bidochka et al. 1998, Sánchez-Peña et al. 2011). In these studies, the prevalence of *M. anisopliae* s.l. in forest soils may have been underestimated because of the peculiarities of the insect bait method, which is likely to detect only one dominant fungal species, thereby underestimating the occurrence of the subdominant species (Scheepmaker & Butt 2010). Cultivation-independent PCR was used in a study aimed to quantify the abundance of *Metarhizium* clade 1, which almost corresponded to *M. anisopliae* s.l., in which its density was high in semi-natural habitats, such as grassland and field margins (Schneider et al. 2012). However, the exceedingly low detection rate of *Metarhizium* by this method (19.3% of 176 soil samples) hinders the adequate interpretation of the comparisons of the results with those of other studies based on plating method. Forest soils usually have higher organic matter content than agricultural soils (Osman 2013), and higher organic contents in soil was associated with higher occurrences of *M. anisopliae* s.l. in Spain (Quesada-Moraga et al. 2007). Thus *M. anisopliae* s.l. could be more abundant in forest soil, as supported also by our results; both mean and median of the density of *M. anisopliae* s.l. in forest soil was higher than those of in other cultural field and others (Table 1). Among the factors that affect the abundance of entomopathogenic fungi, insect diversities may have a lower impact on the abundance of *M. anisopliae* s.l. in Japan because of its broad host range (Nishi & Sato 2017).

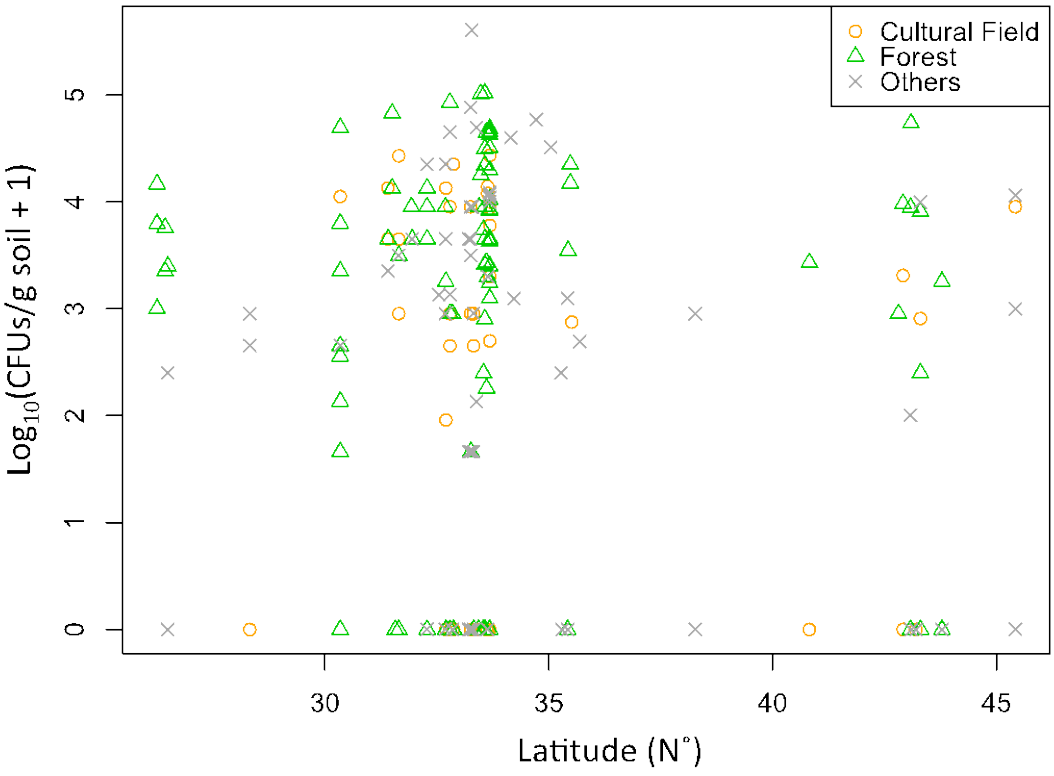

Fig. 2 – Relationship between the densities of *M. anisopliae* s.l. in soils and the latitude of the sampling sites.

The relationships between the latitude and the abundance of soil fungi have rarely been researched, although their species richness is known to be latitudinally gradient (Tedersoo et al. 2014). Wu et al. (2009) reported that the soils in southern China contained relatively more fungi than those in northern China (47.4°N–21.4°N). In previous studies, the occurrence of *M. anisopliae*

s.l. in the soil was associated with the latitudinal origin (Quesada-Moraga et al. 2007, Vänninen 1996). The results of this study showed no significant correlation between the latitude and densities of *M. anisopliae* s.l. in the soil samples collected in Japan (46°N–26°N). In terms of growth temperature, *M. anisopliae* s.l. appeared to have longer optimal growth periods and thus more opportunity to be infected with potential host insects in a year in the lower latitudinal regions in Japan because the optimum growth temperature of *M. anisopliae* s.l. was within the range of 25°C–32°C (Ouedraogo et al. 1997). On the other hand, in another study, the longevity of the conidia of *M. anisopliae* s.l. increased as the temperature declined from 37°C to 4°C (Daoust & Roberts 1983), which suggests that the longevity of conidia in field soils may be longer than that in higher latitudinal regions. The balance between such conflicting factors could result in the lack of significant latitudinal effect.

Our study has determined the density of *M. anisopliae* s.l. in soils in Japan. The information for the background levels provided herein is important for the evaluation of the persistence of any introduced strain of this species to be used as biological control agents in soils in various habitat types. The absence of significant differences in the *M. anisopliae* s.l. densities between forest and agricultural field soils suggests that more subdivided environmental factors, such as temperature, UV radiation, water content, pH, and soil texture, determine their levels. Therefore, to further elucidate the association between *M. anisopliae* s.l. density in the soil and habitat types, multivariate analyzes should be conducted including these environmental soil parameters. Recent studies on *M. anisopliae* s.l. have shown its close association with rhizosphere environments (Hu & Leger 2002, Wyrebek et al. 2011). Quantifications of its densities in the rhizosphere soil of wild plants will also provide further knowledge of ecology and life history of this fungus.

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