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Fungal diversity increases soil fungistasis and resistance to microbial invasion by a non resident species

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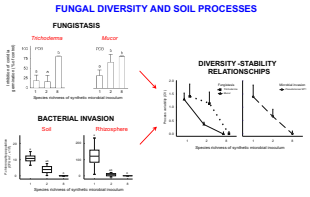
HIGHLIGHTS

- Fungal diversity affects soil fungistasis and bacterial invasion?
- Microcosms were assembled with fungal diversity ranging from 1 to 8 species.
- Soil fungistasis was inversely related to initial microcosm diversity.
- Bacteria capability to invade soil and rhizosphere decreased with microcosm diversity.
- Loss of fungal microbial diversity may adversely affect ecosystem functionality.

GRAPHICAL ABSTRACT



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ABSTRACT

Biodiversity decline is a major concern for ecosystem functioning. Recent research efforts have been mostly focused on terrestrial plants, while, despite their importance in both natural and artificial ecosystems, little is known about soil microbial communities. This work aims at investigating the effects of fungal species richness on soil invasion by non resident microbes. Synthetic fungal communities with a species diversity ranging from 1 to 8 were assembled in laboratory microcosms and used in three factorial experiments to assess the effect of diversity on soil fungistasis, microbial invasion of soil amended with plant litter and of plant rhizosphere. The capability of different microbes to colonize environments characterized by different resident microbial communities was measured. The number of microbial species in the microcosms positively affected soil fungistasis that was also induced more rapidly in presence of synthetic communities with more species. Moreover, the increase of resident fungal diversity dramatically reduced the invasibility of both soil and plant rhizosphere. We found lower variability of soil fungistasis and invasibility in microcosms with higher species richness of microbial communities. Our study pointed out the existence of negative relationships between fungal diversity and soil invasibility by non resident microbes. Therefore, the loss of microbial species may adversely affect ecosystem functionality under specific environmental conditions.

1. Introduction

Traditionally, most studies on biological invasions have focused on invasive plants and animals (Elton, 1958; Mooney and Hobbs, 2000), while only a few considered the effects of invasive microbes. These invisible biota, including causal agents of human, animal or plant diseases, can have major impacts on ecosystems which, in some cases, can change their appearance and functioning (Gerlach, 2001; Waring and O'Hara, 2005).

It is thought that the scarcity of studies on microbial invasion is largely due to a lack of scientific knowledge of the microbe biogeography and distribution (Green et al., 2008), and not to their limited potential impact on ecosystem functions. In fact, the

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limited attention to microbial invasion has been directed to microbial pathogens for their easily noticeable destructive impact. The European epidemics of *Phytophthora infestans*, the causal agent of potato late blight that led to the Great Irish Famine during 1845–1849, are probably the most prominent example. When considering invasive fungi, thoughts turn first toward destructive plant diseases, such as chestnut blight by *Cryphonectria parasitica*, Dutch elm disease by *Ophiostoma ulmi*, and sudden oak death by *Phytophthora ramorum*, which caused a dramatic decline of their respective tree hosts in the native habitat. Invasive plant pathogens may also have dramatic impacts on the structure and functioning of natural ecosystems. The introduction of *Phytophthora cinnamomi* into south-western Australia has substantially altered the native plant communities, by killing not only the dominant Eucalyptus species, but also many understory plant species, thus determining a major shift in the vegetation composition and soil microbial communities (Cahill et al., 2008). Besides emerging threats caused by well known plant and animal pathogens, the impact of non resident microbes on ecosystem structure and functions have not been extensively characterized. In the case of saprophytic soil microbes, the effects of introduced species have not been assessed even because of the limited ecological data available on invaded ecosystems (van der Putten et al., 2007). Moreover, the mechanistic bases of the abundance and disruptiveness of pathogenic or non resident species are not well understood (Parker et al., 2001).

In general terms, the invasion of an environment by new species is influenced by three main factors: (i) the number of propagules entering the new environment (propagule pressure); (ii) the characteristics of the invasive species (invasiveness); and, (iii) the susceptibility of the environment to invasion by new species (invasibility) (Lonsdale, 1999). The success of many invaders has been related to the invasibility of the recipient ecosystem i.e. the vulnerability or susceptibility of a community or ecosystem to invasions, resulting from its intrinsic properties. In this context, Elton (1958) hypothesized that ecosystems with higher diversity are less readily invaded by exotic species. Several mechanisms have been proposed to explain the often observed negative relationship between diversity and invasibility (Tilman, 1999). First, beside the lower occurrence of empty ecological niches and the presence of competitors that may preclude invasion by alien species, communities with higher diversity are assumed to use resources more completely and, therefore, limit the ability of invaders to establish. In addition, more diverse communities are believed to be more stable since they use a broader range of niches (complementary effect) than species-poor communities (Dukes, 2002; Levine and D'Antonio, 1999; Tilman, 1999). Finally, other researchers demonstrated that greater diversity increases the chances that a superior species, in terms of productivity and resource use, be present in the community (sampling effect) driving to more efficient ecosystem functions (Tilman, 1999). In soil ecosystems, competition for resources is crucial for inducing fungistasis and, consequently, for limiting the spread of microbial species (Garbeva et al., 2011). Soil fungistasis causes the inhibition of the germination and growth of soil-borne fungi in presence of optimal soil abiotic conditions (e.g. temperature, moisture, pH, redox potential, etc) (Lockwood, 1977). Fungistasis can be related to the depletion of labile organic carbon compounds and nutrients, as well as to the microbial release of organic compounds with inhibitory effects (Lockwood, 1977). Induction of soil fungistasis is, consequently, a critical mechanisms to limit soil invasion by both pathogenic and saprophytic microbes. Previous studies focused on the sensitivity of different fungi to fungistasis (Lockwood, 1977), how organic amendments affect this phenomenon (Bonanomi et al., 2013), and which role volatile inhibitory compounds play in it (Xu et al., 2004). Only few studies addressed the relationship between fungistasis and microbial diversity (Wu et al., 2008).

Past experimental work, mainly conducted on higher plants, showed that in synthetically assembled ecosystems (i.e. manipulatively assembled communities in which the experimental design determines species richness and composition of the assemblage) invasibility was significantly lower at higher diversity levels (Dukes, 2001; Eisenhauer et al., 2008). However, the diversity-resistance hypothesis, according to which diverse communities being highly competitive readily resist invasion, has been rarely tested with soil microbes. The few experiments carried out with microbial systems used plant-colonizing bacteria (Hodgson et al., 2002), aquatic microbial communities consisting of protists and rotifers (Jiang and Morin, 2005), or hyphomycete fungi (Dang et al., 2005). The use of synthetically assembled communities, originally developed to assess the biodiversity–ecosystem functions in terrestrial ecosystems (e.g. Naeem et al., 1994), is increasingly applied also to microbial systems (Jiang and Morin, 2005; Bell et al., 2005). This approach, by using well-controlled model systems, allows a complete control of environmental conditions and diversity level of microbial communities. However, a claimed drawback is due to the fact that only a minor fraction of soil microbes are culturable in laboratory, thus preventing from using assemblages fully representative of natural communities. Consistently, previous studies used simplified systems with a lower species number (usually less than 10 species) compared to natural systems (e.g. Dilly et al., 2004). In the last years an increasing effort was addressed to study the biodiversity–ecosystem function in the context of soil functions (Griffiths et al., 2000; Tiunov and Scheu, 2005). In details, a large fraction of such studies were done in freshwater systems using hyphomycete fungi (Dang et al., 2005; Costantini and Rossi, 2010), but yet no studies are available for ecosystem functions relevant in agro-ecosystems such as fungistasis and suppression towards soilborne pathogens.

In this study, we use fungal communities synthetically assembled at different species richness levels, ranging from monospecific to 8 species, to test fungistasis and invasibility of two different model systems (i.e. soil amended with leaf litter and plant rhizosphere). Specifically, we aim at testing if (1) soil fungistasis increases with microbial diversity; (2) fungal diversity positively affects resistance to microbial invasion; and (3) the variability in processes rates decreases as microbial diversity increases.

2. Materials and methods

2.1. Experimental approach

To assess the effects of fungal diversity on soil susceptibility to the invasion of non resident species we used laboratory microcosms inoculated with synthetically assembled microbial communities. In such systems, microbial communities were randomly assembled from a known species pool (synthetic assemblage). The use of synthetically assembled communities, originally developed to assess biodiversity–ecosystem function (BEF) in terrestrial ecosystems (e.g. Naeem et al., 1994; Tilman et al., 2001), has being increasingly applied also to microbial systems (Dang et al., 2005; Langenheder et al., 2010). Such an approach allows the control of the diversity levels of microbial communities and the underlying environmental conditions, thus providing an useful setup to investigate the relationships between biodiversity and ecosystem functions.

2.2. Species pool and synthetic microbial communities

Eight fungal strains (Table 1) were obtained from the Department of Agriculture of the University of Naples "Federico II", Italy. The species pool included different functional groups (soilborne and airborne pathogens, saprophytes, antagonistic microbes and

Table 1
List of fungal species used in this study comprising soilborne and airborne pathogens, saprophytes and antagonistic microbes.

Species	Phylum	Functional group
Aspergillus niger	Ascomycota	Saprophyte, plant pathogen
Cladosporium cladosporioides	Ascomycota	Plant pathogen
Fusarium oxysporum	Ascomycota	Saprophyte, plant pathogen
Mucor sp.	Zygomycota	Saprophyte
Penicillium restrictum	Ascomycota	Plant pathogen
Trichoderma harzianum	Ascomycota	Saprophyte, mycoparasite
Trichoderma atroviride	Ascomycota	Saprophyte, mycoparasite
Umbelopsis ramanniana	Zygomycota	Saprophyte

mycoparasites) that represent different ecological functional types of agro-ecosystems.

Concerning microbial inoculum, in all experiments three levels of species richness were used (1, 2 and 8 species). In addition, sterile control was always included. Synthetic microbial communities were randomly assembled, since producing all the possible combinations of 8 species was logistically not feasible (Bell et al., 2005). With the exception of the highest level of species richness that was replicated three times with the same species combination, at each diversity level the microbial species were randomly selected from the species pool for assemblage. Random assemblage is a crucial requisite of the experimental design to effectively test the effect of diversity on ecosystem functions and exclude confounding factors (Huston, 1997). The species combinations always included all monospecific microcosms (8 for each experiments) and four replications at all other diversity levels (Table 2). The use of such biodiversity experimental design enabled the assessment of the "species sampling effect" *vs* "species complementarity effect", because the performance of multiple species mixtures can be compared with that of the most effective monoculture (Huston, 1997; Wardle, 1999).

Microcosms were inoculated keeping constant the amount of fungal conidia at each diversity level. Moreover, all experiments were designated to start with a relatively low inoculum level (1×10^4 conidia) to reproduce a realistic colonization of the microcosms. For microcosms with more than 1 species, the number of conidia inoculated for each species were reduced in proportion to the species number of the consortia (i.e. in case of consortia with four species, each fungal species contributed with 2.5×10^3 conidia for a total amount of 1×10^4 conidia). Fungal inoculum was obtained from ten day old pure cultures grown over Potato Dextrose Agar (PDA, Oxoid). Ten ml of sterile water were added to such cultures and the culture surface was scraped to remove conidia. The suspension was filtered, centrifuged (2395*g* for 10 min), twice washed with sterile water and adjusted to a concentration of 10^5 conidia ml^{-1} by hemocytometer. Inoculum was obtained by the addition of 500 μl of conidia suspension after proper adjustment of the concentration depending on the species richness of the consortia combination.

2.3. Soil fungistasis

The effect of fungal diversity on soil fungistasis was assessed in microcosms designed to simulate soil ecosystems. Experimental units were gnotobiotic systems consisting of Magenta vessels with aerated lid (Sigma–Aldrich, Co. LLC.) filled with 50 g of sterile quartz sand. In preliminary experiments, not amended sterile quartz sand was fungistatic. Sand was amended with two different organic materials: (i) water extracts (0.5%, w/v) of *Medicago sativa* leaf litter, a worldwide occurring forage plant (Bonanomi et al., 2011a) and; (ii) Potato Dextrose Broth (PDB Oxoid) at 5% of full strength, a standard organic substrate for fungal growth. After microcosm amendments, a synthetic microbial community (see Table 2 for species assemblage) was applied at each microcosm. After 3 and 10 days of incubation (24 °C in dark conditions; not limiting water conditions maintained by adding sterile distilled water, after determining gravimetrically the required amount), suspensions were prepared by shaking (200 rpm) 10 g of soil with 50 ml of sterile water for 30 min at room temperature (water:soil ratio 1:1). Electrical conductivity and pH of microcosm water extracts were measured every 2 days. Thereafter, soil suspensions were centrifuged (2395*g* for 10 min), sterilized (microfiltration with 0.22-μm pore filter), and stored at −20 °C until bioassay. The complete experimental design consisted of 80 microcosms with 13 fungal combinations (Table 2).

Table 2
Schematic representation of the synthetically assembled microbial communities used in each experiment. Species combinations were randomly drawn from the pool of 8 fungi (Table 1). The microbial combination with the maximum species richness (eight species) was replicated four times with the same species composition.

Species richness	Number of combinations	Number of identical replicates	Number of microcosms
1	8	1	8
2	4	1	4
8	1	4	4
TOTAL	13	–	16

Fungistasis in sterile soil suspensions was assessed by conidia germination and hyphal growth (Alabouvette et al., 2006) of the two fungi *Trichoderma harzianum* and *Mucor* sp. In preliminary experiments, spores of these two fungi were found to be sensitive to fungistasis because they do not germinate in water and, consequently, can be considered nutrient-dependent spores (Berendsen et al., 2012). Fungal inoculum was obtained as above described and the conidia suspension was adjusted to a concentration of 10^5 conidia ml^{-1}. Spore suspension was prepared in 50 μl of sterile water. Soil suspensions were applied in 96-well plates and incubated at 24 °C. Hyphal growth of germinating spores was measured after 18 h of incubation by using a spectrophotometer (λ 590 nm).

2.4. Invasion by non resident bacteria

Experiments were carried out in two types of microcosms designed to simulate biological invasion of agro-ecosystems: (i) soil amended with litter, and; (ii) plant rhizosphere. The strain M71 of *Pseudomonas chlororaphis* subsp. *aureofaciens* was selected as microbial invader. This strain was selected for two main reasons: (i) it is an effective and well characterized biological control agent (Raio et al., 2011); (ii) being rifampicin resistant, it can be easily detected and its population density rapidly and accurately assessed by a selective substrate.

For the invasion of crop residues, microcosms were assembled as described for soil fungistasis (see previous section). Briefly, Magenta vessels filled with 50 g of sterile sand were amended with water extracts of *M. sativa* leaves. Thereafter, a synthetic microbial community (Table 2) was inoculated into each microcosm and incubated at 24 °C in dark conditions with not limiting water conditions. Electrical conductivity and pH of microcosms water extract were measured every 2 days. Three days after the addition of the synthetic communities, the invader was inoculated into each microcosm. The inoculum of M71 strain was made of 5 mL of physiological solution (NaCl 0.9% w/v) with 1×10^3 mL^{-1} cell concentration. After 7 days of incubation (24 °C in dark conditions), microcosms were destructively sampled to quantify the population level of the invasive strain. The population level of M71 strain was assessed by culturing serial dilutions of microcosms in LB (Lysogeny Broth, Fluka) plates amended with 100 ppm of Rifampicin and incubated for 3 days at +28 °C. In this case, the complete experimental design consisted of 40 microcosms with 13 fungal combinations (Table 2).

For the invasion of plant rhizosphere, we used the model plant *Lepidium sativum* (Bonanomi et al., 2011b). Microcosms consisted

of square Petri dishes (size 12 × 12 × 1.5 cm) with a layer of sterile filter paper. Surface-sterilized (treated with sodium hypochlorite for 5 min) seeds of *L. sativum* (5 for each dish) were transferred onto a filter paper placed in Petri dishes with a 45° slope on a horizontal surface to favor germination and root growth downwards along the plate by positive geotropism. Plates were covered with opaque sheets. Immediately after seed placement into the plates, Petri dishes were inoculated with the microbial synthetic communities described in Section 2.2 (see also Table 2) and incubated in growth chamber (220 μmol photons m^{-2} s^{-1}; photoperiod of 16:8 day–night ratio and not limiting water conditions). The inoculum was uniformly distributed over the root system and the wetted filter paper sheet. Three days after the inoculum of the synthetic communities, the M71 strain was added to each microcosm. The bacterial inoculum was made of 2 mL of physiological solution (NaCl 0.9% w/v) with 1×10^4 mL^{-1} cell concentration. In order to obtain the desiderate bacteria concentrations, for each species, population density of liquid culture was spectrophotometrically evaluated (λ 530 nm) and, thereafter, opportunely diluted. For both experiments, bacterial inoculum was obtained from M71 pure culture refreshed over NA (Nutrient Agar broth, Oxoid) for 3 days at 20 °C. Subsequently, the bacterial culture was centrifuged and refreshed with sterile physiological solution. After 7 days of incubation the population level of the strain M71 was assessed as described above. In this case, the complete experimental design consisted of 40 microcosms with 13 fungal combination (see Table 2).

Overall, 160 microcosms were assembled (80 for fungistasis, 40 for soil invasion and 40 for invasion of plant rhizosphere). Given the large number of microcosms, the assessment of the species richness realized during the experiments was logistically and economically not feasible. We acknowledge that assessing the realized niche at the end of an experiment is relevant for a complete understanding of the role of microbial diversity. Our study, however, allows a direct comparison of microbial inocula with different initial species richness.

2.5. Data analysis

Generalized linear models (GLMs) were used to analyze the results of the soil fungistasis and invasion bioassays. In the case of soil fungistasis, main and second-order interactive effects of fungal species (*T. harzianum* and *Mucor* sp.), type of soil organic amendment (litter water extract, LWE, and PDB), species richness of the synthetic microbial assemblage (1, 2, and 8 species), and incubation time (treated as a continuous variable) were tested on the inhibition of conidia germination. The response, for each treatment combination, was obtained as the complement to 100% of the conidia germination, expressed as percentage of the average value observed in the sterile control microcosms. In the cases of amended soil and plant rhizosphere invasion experiments, mixed GLMs were applied to test the effects of the synthetic microbial assemblages on the population levels of the invader, *P. chlororaphis* subsp. *aureofaciens* strain M71. In particular, both for soil amended with leaf litter of *M. sativa*, and for rhizosphere of *L. sativum*, the species richness of synthetic microbial assemblage was considered as a fixed factor with 3 levels (1, 2, and 8 species), whereas the community identity was treated as a random factor. For all models, significant between-groups differences were statistically assessed by Duncan post-hoc test. Significance was evaluated in all cases at $p < 0.05$.

3. Results

3.1. Effect of fungal diversity on soil fungistasis

The two fungal species showed a similar response to experimental factors: in both cases microbial diversity and incubation time significantly affected conidia germination (Table 3 and Fig. 1). After three days of incubation, the inhibition of fungal growth increased with the number of species inoculated in the microcosms (Fig. 1). In microcosms amended with PDB, *T. harzianum* was strongly inhibited only in presence of the communities with the highest number of species (Fig. 1). After 3 days of incubation, considering all the experimental conditions (two fungi, each with two amendment types, PDB and *M. sativa* extracts) monospecific inocula mostly induced significantly lower fungistatic levels compared to the 8-species assemblages, with comparable values only in 2 cases out of 32.

After 10 days of incubation, fungal growth showed a general inhibition in all microcosms (Fig. 1). However, the inhibition of both *T. harzianum* and *Mucor* sp. was lower, although not statistically significant, in presence of monocultures, compared to microcosms with two- and especially eight-species assemblages (Fig. 1). Fungistatic conditions (e.g. the complete inhibition of fungal growth) was induced only by the synthetic communities with the highest species richness (Fig. 1). These results were generally consistent for both fungi and for microcosms amended with PDB and *M. sativa* extracts (Fig. 1). However, a significant effect of the type of amendment was also found (Table 3): *T. harzianum* growth was inhibited more in soil amended with litter water extracts compared to PDB, at both values of incubation time. Fungistasis variability, expressed as coefficient of variation of the inhibition of conidia germination, was negatively related to fungal species richness in the synthetic assemblages, consistently decreasing with the increase of species richness (Fig. 2A). Amendments with PDB and *M. sativa* induced a slight but significant increase of pH and electrical conductivity. However, the number of species inoculated in the microcosms did not affect such parameters (data not shown).

3.2. Effect of fungal diversity on resistance to invasion by non resident bacteria

Fungal diversity significantly affected the ability of *P. chlororaphis* strain M71 to invade the microcosms, both in soil amended with *M. sativa* litter and in rhizosphere of *L. sativum* (Table 4). The effect of species richness of the synthetic microbial assemblages was not related to the specific composition of the assemblage, which did not affect significantly the invader. The invader populations progressively declined with the increase of diversity in the microcosms, approaching zero values in the presence of eight-species assemblages (Fig. 3). It is noteworthy that, considering the two experiments, the resident monocultures, in all tested cases ($N = 16$), showed a lower resistance to microbial invasion, compared to the microcosms inoculated with the 8-species assemblage. The stability of microcosms invasibility consistently increased with fungal species richness in microcosms, with the CV of the invader population level decreasing accordingly (Fig. 2B). Microcosm amendments with *M. sativa* significantly increased pH and electrical conductivity, but, as observed in the

Table 3
Summary of the generalized linear model (GLM) testing the effects of fungal species (F), type of substrate amendment (S), species richness of synthetic microbial inoculum (R) and incubation time (T) on soil fungistasis.

	SS	d.f.	MS	F	*p*
Fungal species (F)	278.57	1	278.57	0.97	0.3266
Type of amendment (S)	6452.85	1	6452.85	22.51	<0.0001
Species richness (R)	15380.14	2	7690.07	26.82	<0.0001
Days of incubation (T)	17700.57	1	17700.57	61.74	<0.0001
F × T	92.89	1	92.89	0.32	0.5705
S × T	1044.32	1	1044.32	3.64	0.0591
R × T	5613.48	2	2806.74	9.79	0.0001
Residuals	29242.36	102	286.69		

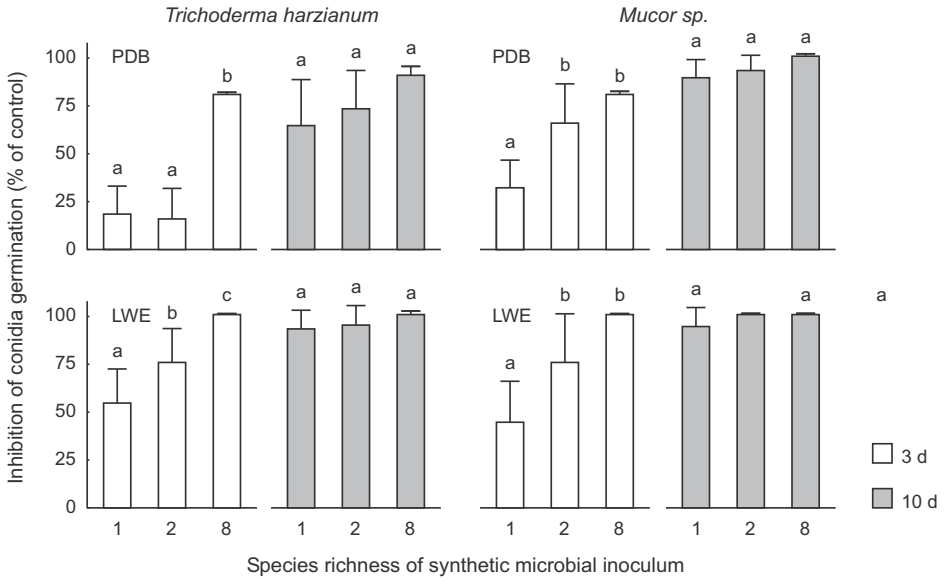

Fig. 1. Inhibition of conidia germination in *Trichoderma harzianum* (left) and *Mucor* sp. (right) incubated for either 3 (open bars) or 10 (gray bars) days in microcosms amended with Potato Dextrose Broth (PDB, above) or water extract of *Medicago sativa* litter (LWE, below) and inoculated with synthetic microbial communities with different levels of species richness. Different small letters indicate significant differences between-groups (Duncan test, $p < 0.05$).

fungistasis experiment, the number of species inoculated in the microcosms did not affect such parameters (data not shown).

4. Discussion

In this study soil fungistasis was positively affected by increasing levels of microbial diversity of synthetic communities. Moreover, higher diverse communities dramatically reduces the capability of *P. chlororaphis* strain M71 to colonize the soil and a plant rhizosphere. We also found a lower variability in fungistasis and soil resistance to invasion when a higher number of fungal species was present. This evidence indicates that the level of species richness of resident microbial communities affects the resistance to biological invasions.

In a recent study, Wu et al. (2008) reported that soil fungistasis declined along a gradient of bacterial diversity, obtained by applying increasing temperatures to soil. However, to our knowledge, this is the first study about the role of diversity on soil fungistasis by using synthetically assembled microbial communities. The effects of species diversity on soil fungistasis and resistance to microbial invasion are likely determined by additive/synergistic or competitive interactions among the different fungi participating to the synthetic consortia. The dramatic fungistasis relief observed

Table 4
Summary of the generalized linear model (GLM) assaying the effects of synthetic microbial inoculum (species richness and community identity) on the invasion by *Pseudomonas chlororaphis* subsp. *aureofaciens* strain M71 of soil amended with *Medicago sativa* leaf litter (a) and rhizosphere of *Lepidium sativum* (b).

	Effect	SS	d.f.	MS	F	*p*
(a) Soil amended with litter						
Species richness	Fixed	431.72	2	215.86	7.63	0.0084
Community identity	Random	166.69	10	16.67	0.59	0.7933
Residuals		311.19	11	28.29		
(b) Plant rhizosphere						
Species richness	Fixed	54908.0	2	27454.0	31.26	<0.0001
Community identity	Random	21712.2	10	2171.2	2.47	0.0767
Residuals		9660.5	11	878.2		

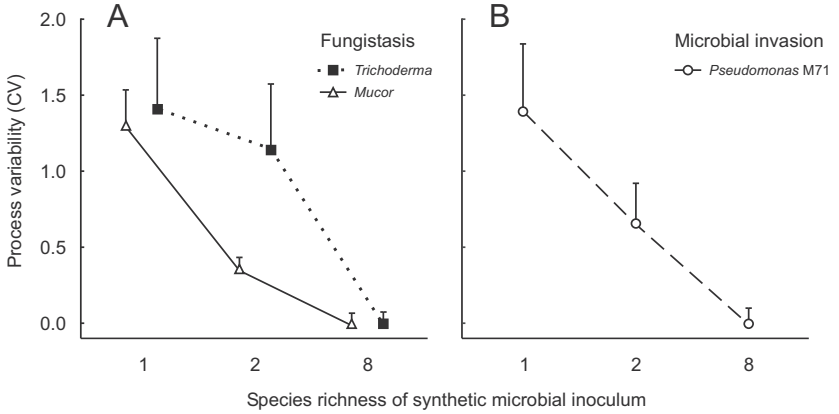

Fig. 2. Diversity–stability relationships of the studied processes. Coefficient of variation (CV) of fungistasis (A) and microbial invasion (B) is shown as a function of species richness of synthetic microbial communities inoculated in experimental microcosms. Values are: (A) CV of inhibition of conidia germination for two fungal species (*Trichoderma harzianum* and *Mucor* sp.), averaged over two incubation periods (3 and 20 days, respectively) and two substrates (PDB, Potato Dextrose Broth; LWE, water extract of *Medicago sativa* litter); and (B) CV of *Pseudomonas chlororaphis* subsp. *aureofaciens* strain M71 abundance (CFUs) averaged over two substrates (*Medicago sativa* leaf litter, and rhizosphere of *Lepidium sativum* plants).

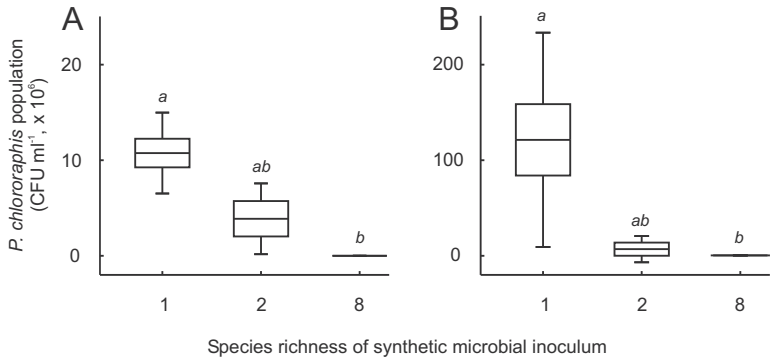

Fig. 3. Population level of *Pseudomonas chlororaphis* subsp. *aureofaciens* strain M71 in soil amended with *Medicago sativa* leaf litter (A) and rhizosphere of *Lepidium sativum* plants (B). The two soils were inoculated with microbial consortia with different species richness levels. Values are average (bars) and superior confidence limit (whisker) of microcosms with the same species richness; different small letters indicate significant differences between-groups (Duncan test, $p < 0.05$).

when microcosms were amended with both PDB and *M. sativa* extracts provides indirect support to the competition based hypothesis (Steiner and Lockwood, 1970). However, the effect of species richness on fungistasis was time-dependent. At short-term (3 days) highly diverse inocula induced fungistasis faster than species-poor inocula, while no diversity effect was recorded at medium-term (10 days). In this respect, we suggest that induction of a faster fungistasis may be ecologically relevant, considering that fungistasis is a sort of "race" to gain soil resources. In other words, the limited effect of diversity observed at medium-term was an expected outcome, given that fungistasis is usually restored 10–20 days after organic amendments (Bonanomi et al., 2013).

In our experiments, the occurrence of increased competition among microbes may explain the rapid induction of fungistasis observed in mixed microbial consortia, compared to monocultures. Ecological niche partitioning among different species may be another possible underlying mechanism. Spatial and temporal niche partitioning may occur when microbes colonize different litter chemical fractions, as frequently occurs during microbial successions over organic substrates (Frankland, 1998). For instance, some saprophytic "sugar" fungi (Garrett, 1963) that thrive when simple sugars are abundant, but are unable to decomposed more complex C compounds (e.g. cellulose and lignin), can be observed over recalcitrant organic substrates because they can metabolize by-products of lignin released by the activity of decomposer fungi (Berg and McClaugherty, 2008). Alternatively, the induction of soil fungistasis could be related to the release of antifungal compounds (Dobbs and Hinson, 1953; Xu et al., 2004). It is possible that strong interspecific interactions within mixed microbial consortia (Boddy, 2000; Romaní et al., 2006) may increase the antagonistic capability of saprophytic microbes. For instance, some bacteria that were non-antagonistic, when tested alone, gave an important contribution to soil fungistasis in mixture with other microbes (De Boer et al., 2007). To better explain the relationships between fungal diversity and soil fungistasis, further works are required to investigate if mixed microorganism communities restore fungistasis faster because of resource competition and/or because of the production of new and more abundant antifungal compounds.

Previous experiments manipulating plant diversity have found consistent positive diversity effects on ecosystem resistance to biological invasion (Knops et al., 1999; Dukes, 2001; Hooper et al., 2005). In contrast, experiments made to test the diversity-invasibility hypothesis reported contrasting results when microbial communities were used (Hodgson et al., 2002; Jiang and Morin, 2005). For instance, Matos et al. (2005), studying the colonization of wheat rhizosphere by the opportunistic pathogen *Pseudomonas aeruginosa*, reported an inverse relationship between rhizosphere community richness and invasibility. This is in agreement with our finding that the colonization capability of *P. chlororaphis* subsp. *aureofaciens* strain M71 dramatically declined when the diversity of fungi resident in the rhizosphere of *L. sativum* and in the soil amended with plant litter increased. Although these studies clearly demonstrate an inverse relationship between microbial diversity and ecosystem invasibility, no insight is provided about the underlying mechanisms. A possible explanation is based on the simple assumption that an invading species must have access to available resources (e.g. nutrients, organic substrate and water) and will be more successful in invading a community if it does not have to compete with resident species. Given this assumption, any factor that increases the availability of limiting resources will increase the vulnerability of a community to invasion (Davis et al., 2000). Resource availability can increase basically in two ways: the use of resources by the resident communities can decline, or resource supplies can increase at a rate faster than the resident communities can sequester it. Since diverse communities are assumed to use resources more completely, they may limit the ability of invaders to establish (Tilman, 1999). As a consequence, invasibility is not an inherent property of communities, but it changes in time as the amount of unused resources fluctuate. If more diverse soil microbial communities are less prone to invasion because of a faster resource sequestration rate compared to monocultures remains to be assessed.

Many experimental studies were addressed to clarify whether positive BEF is due to the statistical mechanism called sampling effect, or to a real ecological effect related to species complementarity (e.g. Huston, 1997; Wardle, 1999; Hooper et al., 2005). In the case of sampling effect, the positive BEF depends on the higher probability that mixed communities include more functioning species (Tilman, 1999). Differently, in the case of niche complementarity, species may use resources more efficiently because of niche partitioning. Loreau and Hector (2001) reported that "sampling effect" and "species complementarity" could be statistically partitioned if the performances of single species in mixed communities are tracked. The application of this approach to microbial communities is very difficult because the role of individual species on BFE cannot be easily measured. However, we found that the communities composed of eight species were always more resistant to the invasion of the *P. chlororaphis* subsp. *aureofaciens* M71 strain compared to the best monoculture. Overall, taking into account all our experiments on fungistasis and resistance to invasion, the best monocultures are equivalent to the 8-species inocula only in 2 cases out of 48 (4.16%). Since the species complementarity hypothesis predicts that polycultures might outperform the best monocultures, our results indicate that the observed positive BEF could be predominantly determined by complementarity, and only in part by the sampling effect.

In the last years, the relationships between diversity and stability of process rates has been matter of an intense debate (e.g.

Tilman, 1999; McCann, 2000), which still remains debated after several studies provided contrasting evidence (Tilman et al., 2006; Gamfeldt et al., 2008). In our work we showed that the variability of both soil fungistasis and resistance to microbial invasion was considerably reduced by increasing fungal species richness of mixed communities. This is consistent with results achieved for other ecosystem processes, including manipulative experiments with synthetically assembled communities composed of microbes (McGrady-Steed et al., 1997; Dang et al., 2005), mosses (Mulder et al., 2001), and vascular plants (Tilman, 1999). Interestingly, the possibility of lowering the rate of the processes analyzed in this study, by increasing community specie richness, can be of practical importance for the stability and predictability of important ecosystem properties, such as soil suppressiveness (Bonanomi et al., 2010).

Finally, it is important to recognize the caveats of our experimental approach. The use of synthetically assembled microbial communities allows to test microbial functionality at different diversity levels, but such an approach can be considered as a "black box" because the mechanisms underlying the observed effects remain unclear. However, our study demonstrates that, at the lower end of a microbial species richness gradient, diversity matters for ecosystem functions. While several studies demonstrated a consistent pattern for basic soil functions, such as respiration, litter decomposition, and nutrient cycling, this work provides a first insight for fungistasis and resistance to invasion in agro-ecosystems. In other words, we showed that species-rich microbial inocula, compared to species-poor assemblages, are able to induce faster fungistasis and higher resistance to bacterial invasion in soil and rhizosphere. Our findings are far from bearing immediate impacts, but provide a baseline for exploring the implications and range of applications of species-rich microbial consortia in agricultural fields. Future studies in this direction are urgently required, considering the recent commercialization of several specie-rich microbial consortia to improve crop yields and control plant pathogens without a robust scientific background.

5. Conclusion

In this study we found that soil fungistasis, as well soil resistance to microbial invasion, were positively correlated with the level of fungal diversity of resident communities. This new evidence suggests that species diversity positively affects the resistance of microbial community to biological invasions. Although our study used oversimplified microbial communities, compared to diversity commonly observed in soil microbial communities (Frankland, 1966; Dilly et al., 2004), it may be a starting point for further research. We are aware that the implications of our findings, as related to the understanding of fungistasis and microbial invasion in real soil systems, are limited by the use of few microbial species interacting with simplified resident communities. For a more comprehensive analysis, synthetic microbial communities should be assembled taking into account their representativeness of natural communities, not only in terms of species richness and composition, but also considering different functional groups (e.g. bacteria, actinobacteria, primary saprotrophic "sugar" fungi, cellulose and lignin decomposer fungi). With such an approach, based on more realistic experimental systems, future studies are likely to provide improved assessments of the relationships between microbial diversity and soil functionality.

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