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Research

Temporal dynamic of parasite-mediated linkages between the forest canopy and soil processes and the microbial community

Ana Mellado[1], Lourdes Morillas[2,3], Antonio Gallardo[2] and Regino Zamora[1]

[1]Department of Ecology, Terrestrial Ecology Research Group, University of Granada, Av. Fuentenueva s/n, E-18071, Granada, Spain; [2]Departamento de Sistemas Físicos, Químicos y Naturales, Universidad Pablo de Olavide, Crta. Utrera Km. 1, 41013, Sevilla, Spain; [3]Department of Sciences for Nature and Environmental Resources, University of Sassari, Sassari, Italy

Author for correspondence:
Ana Mellado
Tel: +34 676 428 301
Email: anamegar@ugr.es

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Summary

• Parasitic plants are important drivers of community and ecosystem properties. In this study, we identify different mechanisms by which mistletoe (*Viscum album* subsp. *austriacum*) can affect soil chemical and biological properties at different temporal stages of parasitism.

• We quantified the effect of parasitism on host growth and the number of frugivorous mutualists visiting the host canopy. Then we collected, identified, and weighed the organic matter input underneath tree canopies and analyzed its nutrient content. Simultaneously, we analyzed soil samples under tree canopies and examined the chemical properties, microbial abundance, and functional evenness of heterotrophic microbial communities.

• Mistletoe increased the amount, quality, and diversity of organic matter input beneath the host canopy, directly through its nutrient-rich litter and indirectly through a reduction in host litterfall and an increase in bird-derived debris. All these effects gave rise to enriched hotspots able to support larger and more functionally even soil microbial communities beneath parasitized hosts, the effects of which were accentuated after host death.

• We conclude that mistletoe, together with the biotic interactions it mediates, plays a key role in intensifying soil resource availability, regulating the functional evenness, abundance, and spatial distribution of soil microbial communities.

Introduction

There is an increasing awareness that plants and aboveground–belowground subsystems are tightly connected, and thus the need for integrated approaches to understand more clearly how terrestrial ecosystems function is being recognized (Wardle, 2002). Recent studies with parasitic plants have shown their important role in regulating belowground processes by enhancing soil nutrient availability and increasing soil spatial heterogeneity (Bardgett *et al.*, 2006; Quested *et al.*, 2008; Fisher *et al.*, 2013), which may in turn affect the growth of neighboring plants (Quested *et al.*, 2003; March & Watson, 2010). In addition, many parasitic plants are known to be highly connected with organisms of different trophic levels, being considered major resources within communities (Watson, 2001; Press & Phoenix, 2005). However, we still have a limited understanding of the way in which complex interactions involving parasitic plants might change linkages between the forest canopy and the soil. Moreover, it remains unknown how such linkages between the host–parasite system and soils change through time and how they contribute to temporal ecosystem dynamics. This is especially critical for long-lived plants, which substantially change their physical and biotic environment at different stages of their life and may leave behind a spatial footprint that persists long after death (Rodríguez *et al.*, 2011) as a consequence of the continuous enhancement of nutrient inputs, with important implications for local biogeochemical cycling (e.g. Dean *et al.*, 1999; Bardgett *et al.*, 2005; Stahlheber *et al.*, 2015).

Mistletoes constitute appropriate study systems with which to explore this subject, as they are long-lived organisms that maintain multiple long-term biotic interactions. Comprising a diverse group of aerial hemiparasitic plants that obtain water and mineral resources from host plants, mistletoes are considered keystone species in forest ecosystems around the world (Watson, 2001). These plants, in addition to being parasitic, establish mutualistic interactions with pollinators and seed dispersers, which move pollen and seeds in exchange for nutritive nectar and fleshy-fruit rewards (e.g. Aizen, 2003). Herbivorous insects and mammals consume mistletoe foliage (e.g. Umucalilar *et al.*, 2007; Burns, 2009) while many other animals use the bulky structure of this plant as a site for refuge, or to rest or nest (e.g. Cooney *et al.*, 2006). As a result of this variety and bounty of interactions, mistletoes can, both directly and indirectly, modulate soil organic matter and nutrient inputs underneath the canopy of parasitized hosts. Direct effects can be highly noticeable, as mistletoes often contribute large amounts of biomass to the forest floor – including leaves, fruits, and flowers (March & Watson, 2007), which are rich in nutrients and have a high decomposition rate,

enhancing soil nutrient cycling (March & Watson, 2010; Ndagurwa *et al.*, 2014a,b; Muvengwi *et al.*, 2015). Indirect effects of mistletoe through its parasitic and mutualistic interactions may be equally remarkable, but, although sometimes mentioned, these relationships still remain unexplored. On the one hand, the acquisition of host resources reduces host productivity and reproductive fitness (Reid *et al.*, 1994; Silva & Martínez del Río, 1996; Howell & Mathiasen, 2004), which might be reflected in lower input of host biomass into the soil. On the other hand, animal visitors may provide substantial nutrient sources, dropping excrements, feathers, wings, hair, or food leftovers underneath the host (Dean *et al.*, 1999; Van der Wal *et al.*, 2004; Watson, 2009). Given time, high parasitic loads may culminate in host death (Dobbertin & Rigling, 2006; Sanguësa-Barreda *et al.*, 2012), and subsequently in the death of the parasite. Once this occurs, the host–parasite system may expand its effects on the soil through the deposition of dead organic matter (e.g. Facelli & Faceilli, 1993), through the persistence of past changes in nutrient cycling (e.g. Maron & Jefferies, 1999) or through the establishment of new interactions with animals that make new use of them, for instance as perches for birds (e.g. McClanahan & Wolfe, 1993).

In this study, we sought to identify different mechanisms by which mistletoe, together with the parasitic and mutualistic interactions it mediates, jointly affects soil chemical and biological properties at different temporal stages of the host–parasite system. We focus on the mistletoe *Viscum album* subsp. *austriacum* (hereafter *V. a. austriacum*), a long-lived (over 35 yr) common parasite of European forests that specializes in hosts of *Pinus* spp. (Zuber, 2004; Mellado & Zamora, 2014a) and that maintains a mutualism with generalist frugivorous birds (Mellado & Zamora, 2014b). In a Mediterranean mountain with clear signs of mistletoe expansion (A. Mellado & R. Zamora, unpublished data), we selected: nonparasitized trees, representing the starting point of parasitism, as well as a control for the effect of parasitism (hereafter 'unparasitized trees'); parasitized trees, representing the stage of mistletoe parasitism (hereafter 'parasitized trees'); and dead trees, representing the final stage, after the death of parasitized trees (hereafter 'dead trees'). Our main hypothesis was that unparasitized, parasitized, and dead trees exert different influences on soil properties as a result of changes in biological interactions prevailing in the host canopy at each stage. In the absence of the parasite, pine litter is expected to be the dominant resource reaching the soil. When the pine becomes parasitized, mistletoe may either directly or indirectly alter the overall nutrient inputs beneath the host. After host death, the remaining dead structures from the host and the parasite are expected to maintain litter accumulation. We expected differences in the identity, quantity, and quality of organic compounds reaching the soil at different stages to be reflected in the soil nutrient status and the microbial community. In particular, we identified the main direct and indirect links between the canopies of unparasitized, parasitized, and dead parasitized trees in order to evaluate the implications of such linkages for soil nutrient status, as well as for the abundance and functional evenness of soil microbial communities.

Materials and Methods

Study site

The study was performed from 2010 to 2013 in a Mediterranean pine forest located in the Natural Park of Sierra de Baza (southeastern Spain; 2°51′W, 37°22′N, a jagged calcareous mountain range with elevations between 1200 and 2269 m asl). The site shows the typical Mediterranean climate, characterized by cold winters and hot summers with pronounced summer drought (June–August), while precipitation is concentrated in spring and autumn. Sierra de Baza contains a complex mosaic of plant formations. The dominant tree vegetation is pine forest, mainly Austrian pine (*Pinus nigra* Arn.) and Scots pine (*Pinus sylvestris* L.), but also Aleppo pine (*Pinus halepensis* Mill.) and maritime pine (*Pinus pinaster* Ait.), coexisting with oaks (*Quercus ilex* L.) and maples (*Acer opalus* L. ssp. *granatense* Boiss). Many fleshy-fruited shrubs, lianas, and trees form part of the plant community throughout the mountain, including *Crataegus monogyna* Jacq., *Berberis hispanica* Boiss. & Reut., *Prunus ramburii* Boiss., *Juniperus oxycedrus* L., *Juniperus communis* L., *Juniperus Sabina* L., *Daphne gnidium* L., *Daphne laureola* L., *Hedera helix* L., *Lonicera arborea* Boiss., *Phillyrea angustifolia* L., *Sorbus aria* (L.) Crantz, and *Rosa* spp. At lower altitudes non-fleshy-fruited shrubs such as *Cytisus reverchonii* (L.) Link, *Adenocarpus decorticans* Boiss., *Genista cinerea* DC., *Genista scorpius* (L.) DC., and *Genista umbellata* (L'Her) Poir. are common. Higher altitudes are characterized by open vegetation with a basal layer of hard-leaf grasses (e.g. *Festuca hystrix* Boiss., *Poa ligulata* Boiss., and *Koeleria vallesiana* Honk.); cushion shrubs of juniper species and Genisteae (e.g. *Erinacea anthyllis* Link., *Genista versicolor* Boiss., and *Echinospartum boissieri* (Spach) Rothm.); and other thorny plants (e.g. *Vella spinosa* Boiss., *Ptilotrichum spinosum* (L.) Boiss, *Bupleurum spinosum* Gouan., *Daphne oleoides* Schreb., and *Dianthus subacaulis* Boiss.). Part of the zoochorous plant community is the mistletoe *Viscum album austriacum* (Wiesb.) Vollmann (Viscaceae), a hemiparasitic, dioecious epiphyte widely distributed across European coniferous forests. *Pinus nigra* and *P. sylvestris* constitute the most common host species in southern Spain, *P. nigra* being the most numerous at the study site (Mellado & Zamora, 2014a), where *V. a. austriacum* can live for > 35 yr (Zuber, 2004; A. Mellado & R. Zamora, pers. obs.). The most common species of avian seed dispersers in Sierra de Baza include nonmigrants and seasonal migrants such as *Sylvia*, *Turdus* and *Erithacus*, which feed on various fruit species from autumn to winter (Herrera, 1984). Thrushes are the main seed dispersers of *V. a. austriacum* (Zuber, 2004; Mellado & Zamora, 2014b), as well as legitimate dispersers of other zoochorous species of the plant community. Small passerines, such as the robin (*Erithacus rubecula*) and blackcap (*Sylvia atricapilla*), also contribute to the dispersal of both ground- and canopy-dwelling fleshy-fruited plants.

For this study, we selected 125 *P. nigra* trees (hereafter focal trees), of which 55 were parasitized by mistletoe, 55 were unparasitized and 15 were recently dead parasitized trees. The dead individuals still retained needles and twigs, as well as thin branches of

dead mistletoes that often disappear with the passage of time, so that tree death was estimated at *c.* 4–6 yr before the study. Parasitized trees presented moderate to intense parasitic loads (20–40% and 50–80% of the host canopy covered by mistletoe, respectively), bearing at least one mistletoe > 30 yr old. Mistletoe age can be easily estimated based on the dichotomous growth pattern of *V. album* by counting the number of shoot segments of the plant. Dead trees were less numerous than were the specimens in the other categories, being scarce and difficult to find at the study site. However, they were easy to identify because dead mistletoes remained in the branches. The experimental site covers a large range of the mountain's heterogeneity, as focal trees were equitably distributed at three altitudes (1300, 1650 and 1850 m) within stands of different tree densities. Trees were randomly selected and spatially paired (one parasitized and one unparasitized), except for dead trees, which were scattered throughout the mountain because of their rarity. Paired trees were of similar architecture, size (diameter at breast height (mean ± SE): 25.55 ± 1.31 cm), age (90–110 yr old; see Herrero *et al.*, 2013) and height (mean ± SE: 6.87 ± 0.23 m). These were located 40 to 80 m apart to control the environmental variability (e.g. climatic factors and composition of neighboring vegetation). Shrub coverage beneath the tree canopy was similar between focal pines (Mellado, 2016), with leguminous species accounting for < 10% of the total vegetation coverage (A. Mellado & R. Zamora, unpublished data). Thus, the potential contribution of the understory to belowground linkages is expected to be similar at all sites. To address the above-mentioned questions, in all focal pines we quantified the effect of parasitism on host growth and the capacity of mistletoes to attract frugivorous mutualists to the host canopy. Then we collected, identified, and weighed the organic matter input underneath tree canopies and analyzed the nutrient content. Simultaneously, we analyzed soil samples under focal pines and examined chemical properties, nitrogen content in microbial biomass as a proxy of microbial abundance (Joergensen *et al.*, 1995), and functional evenness of heterotrophic microbial communities.

Mistletoe–host interaction

We measured the effect of parasitism on host growth by estimating annual internode growth in unparasitized and parasitized focal pines. Estimates were performed for three randomly assigned branches per tree by measuring (with a tape measure) bud elongation (cm) of three consecutive years (from 2010 to 2012), this being an easy and precise procedure based on the presence of yearly bud scars (see Herrero & Zamora, 2014). We used average growth values within focal pines for the statistical analyses.

Mistletoe–frugivorous bird interaction

We measured the effects of mistletoe on animal mutualists by making direct observations of frugivorous birds visiting parasitized and unparasitized focal pines for three consecutive years (from 2010 to 2012; data published in Mellado & Zamora, 2015). Each census consisted of 5-min observations per focal pine on different days throughout the dispersal season, from the end of September to the end of February. A trained ornithologist (R.Z.) performed observations between 07:00 and 12:00 h, covering the area around each focal tree and identifying birds to the species level. At the end of each season, 12–14 observations were made per focal pine, for a total of 70 observation min per tree per year. We calculated frugivorous bird abundance per focal pine as the cumulative number of birds watched through the dispersal season.

Deposition of organic compounds

We collected, identified, and weighed all of the types of organic compounds reaching the ground beneath the canopy of each unparasitized, parasitized, and dead focal pine. Litter collectors were used throughout the year for two consecutive years (2012–2013). These were flowerpots (0.125 m^2) covered with an aluminum mesh hanging on the lower branches of the tree canopy. These collectors prevented seed predation by rodents and granivorous birds, as well as the intensive uprooting by wild boar that frequently occurs under these trees. Three collectors were hung from three randomly assigned branches of the lower third of the tree height (*c.* 2 m above ground level). Every 6 months, the collectors were emptied and the samples were transferred to the laboratory, where the litter was identified, separated, dried (at 70°C for 72 h), and weighed (g) on a precision balance. We differentiated three main litter sources: (1) host-delivered compounds, that is, needles, cones, flowers, nuts, and bark; (2) mistletoe-delivered compounds, that is, leaves, stems, fruits, flowers, and seeds; and (3) debris from frugivorous birds, that is, food remains, excrement, and seeds from zoochorous species (excluding mistletoe seeds, which were counted as a mistletoe-derived component. We used average biomass per square meter values (g m^{-2}) within focal pines for statistical analyses.

Chemical composition of the organic compounds collected

Senescing pine needles and senescing mistletoe leaves, stems, flowers, fruits, and seeds were gathered from litter collectors for five parasitized focal pines at the beginning of spring 2012. The material was dried (at 50°C for 96 h), well cleaned, ground, and stored in plastic vials until analysis. Each sample of material was analyzed for total carbon (TC) and nitrogen (TN) by combustion at 850°C (Leco TruSpec autoanalyzer; LECO Corp., St Joseph, MI, USA). Potassium (K^+) in the acid extract of organic compounds was determined by atomic absorption spectrophotometry (David, 1960), while phosphorus (P) was determined by the molybdovanadate method (AOAC, 1975).

Soil chemical properties

Beneath the canopy of focal pines, we analyzed the fraction of soil nutrients available for microbes and root plants in spring 2012 (beginning of April), coinciding with the early spring period where maximum soil biological activity is expected. We estimated

soil nutrients (nitrate (NO_3^--N), ammonium (NH_4^+-N) and phosphate (PO_4^{3-}-P)) and K^+ availability using ion-exchange membranes (IEMs; Subler *et al.*, 1995; Durán *et al.*, 2013) on a subset of 76 focal trees: 38 parasitized, 38 unparasitized, and 12 dead trees. This technique takes into account the soil ion diffusion rates, enabling the detection of nutrient accessibility to root plants and microbes over a certain time period. Resins, after being expanded by submersion in distilled water at 82–90°C for 48 h, were cut into 2.5 × 2.5 cm squares and attached to a plastic rod with acrylic glue. Beneath the canopy of the selected focal trees, three cation and three anion IEMs were randomly buried in the soil at a depth of 0.5–3 cm in the mineral soil and were incubated in the field for 30 d (from April to May 2012). After removal, the IEMs were taken to the laboratory and dried at ambient temperature. They were carefully separated from the plastic rod, brushed to remove soil particles, and placed into 125-ml flasks for extraction with 25 ml of distilled water by orbital spinning (1 h at 200 rpm). The NH_4^+-N concentration was directly estimated with the indophenol blue method using a microplate reader (Sims *et al.*, 1995). The NO_3^--N was first reduced to NH_4^+ with Devarda alloy, and its concentration was determined as described above using the indophenol blue method (Sims *et al.*, 1995). The NO_3^--N concentration in the extracts was calculated as the difference between the Devarda-incubated and unincubated samples. The potassium concentration in the extract was determined by atomic absorption spectrophotometry (David, 1960), while the PO_4^{3-}-P content was determined by the Olsen method (Watanabe & Olsen, 1965).

At the same time as the IEMs were installed, we randomly collected three soil cores of mineral soil underneath the canopy of each selected focal tree using a circular soil corer (5 cm in diameter × 10 cm in height). Samples were taken from the top 10 cm of the soil profile because most soil nutrients in a Mediterranean ecosystem accumulate in the first few cm of the soil profile (Lugo *et al.*, 1990). Soil samples were transported in polyethylene bags to the laboratory and sieved at 2 mm to remove stones, roots, and visible plant debris. For each pine, the three samples were combined into a single composite sample for further analysis. A fraction (*c.* 60 g) of each composite soil sample was air-dried at ambient temperature for 7 d and stored until subsequent analyses, and another soil fraction (*c.* 20 g) was kept at 3°C for 3 d and then processed to determine N in microbial biomass (MB-N; Brookes *et al.*, 1985) as a proxy of microbial abundance and dissolved organic N (DON). To measure MB-N, 20 g of fresh soil was fumigated with chloroform for 5 d. The nonfumigated replicates were used to measure DON. The fumigated and nonfumigated samples were extracted with 100 ml of K_2SO_4 0.5 M by shaking for 1 h at 200 rpm at 20°C and filtered through a 0.45-μm Millipore filter (Jones & Willett, 2006). The extracts were first oxidized to NO_3^--N with potassium persulfate ($K_2S_2O_8$) in an autoclave at 121°C for 55 min and then reduced to NH_4^+-N with Devarda alloy (Sollins *et al.*, 1999). The DON content was calculated as total dissolved N minus inorganic N in the digested extracts (Morillas *et al.*, 2013) and determined by colorimetry (indophenol blue method) with a microplate reader (Sims *et al.*, 1995). The MB-N concentration was estimated as the difference between the total N in fumigated and unfumigated digested extracts divided by a Kn (fraction of MB-N extracted after $CHCl_3$ treatment) of 0.54 (Brookes *et al.*, 1985).

From dried soils, we determined the content of soil organic matter (SOM) by incineration at 550°C with a thermo-balance (Leco TGA 710) to constant weight (Sparks, 1996). Total C (TC) and N (TN) were determined by combustion at 850°C (Leco TruSpec autoanalyzer), while total inorganic C (IC) was measured by the acidification method with $HClO_4$ in a TIC analyzer (UIC CM-5014; UIC Inc., Joliet, IL, USA). Total organic C (TOC) was estimated as the difference between TC and IC. The gravimetric soil water content was calculated in fresh 5-g subsamples after drying in an oven at 80°C for 48 h (DeAngelis, 2007), and the soil pH was measured in 1 : 5 soil : water solutions (Kalra, 1995).

Functional measurements of soil microbial communities

We analyzed soil heterotrophic microbial communities with the MicroResp system (Campbell *et al.*, 2003). This method is based on community-level physiological profiles (CLPPs) obtained by the testing of 15 carbon sources that vary in structural complexity (Oren & Steinberger, 2008). Carbon sources were selected depending on their ecological importance to soil and their solubility in water. We used amino acids (L-alanine, L-lysine, arginine, L-cysteine HCl, and N-acetyl-glucosamine (NAGA)), carbohydrates (D-fructose, D-galactose, D-glucose, L-arabinose, and D-trehalose), and carboxylic acids (citric acid, L-malic acid, oxalic acid, oxoglutaric acid, and amino butyric acid (GABA)). In functional terms, the substrate utilization rates of the carbon sources correspond to the catabolic attributes of different soil microbial functional groups (Zak *et al.*, 1994). Even if we cannot evaluate microbial communities in relation to taxonomic or phylogenetic diversity (Øvreås, 2000), we can still use MicroResp results to explain functional evenness shifts. Before performing the MicroResp procedure, air-dried soils were placed in flasks and pre-incubated for 5 d at 25°C. The moisture within the flasks was corrected to 40% water-holding capacity in order to condition the soils and reestablish active microbial populations. To avoid changes in soil moisture content during incubation, we covered the flasks with parafilm. Each carbon source was dissolved in deionized water and added to soils to deliver 30 mg C g^{-1} soil water. Approximately 0.4 g of soil was placed volumetrically in the 96 deep-well plates. To estimate the evolved CO_2, a colorimetric method was used, relying on the change in the pH of a gel-based solution of bicarbonate. The plates were then incubated for 6 h and read at 570 nm. The results were calculated on the basis of water, which represents the basal respiration.

Statistical analyses

The effect of parasitism (parasitized or not) on tree growth was analyzed with linear mixed models (LMMs), using tree condition and year as fixed factors and replicate as a random factor to account for temporal pseudoreplication. The effect of parasitism

on frugivorous counts was analyzed using generalized linear mixed models (GLMMs) with Poisson error distribution and the log-link. To compare biomass inputs, we applied a negative binomial distribution and the log-link because the equidispersion assumption of the Poisson model was not met (Zuur *et al.*, 2009). The nutrient contents of pine and mistletoe litter were compared with analysis of variance (ANOVA) models. Soil chemical properties (TOC, IC, TN, DON, pH, moisture, IEM NH_4^+-N, IEM NO_3^--N, IEM PO_4^{3-}-P, and IEM K^+), as well as N in microbial biomass (MBN) and microbial functional evenness, were analyzed using LMMs, with variables square-root or log-transformed when required in order to meet assumptions of normality and homoscedasticity, followed by Tukey's pairwise comparisons with 95% confidence level. For all analyses, we included the focal tree condition (unparasitized/parasitized/dead) as a fixed factor and, for LMM and GLMM, paired trees as a random factor. We calculated the Shannon index (H′) to assess microbial functional evenness using the soil respiration response to the different C sources as in Shannon (1948):

$$H' = -\sum_{i=1}^{s} pi.\log_e pi$$

(*pi*, the ratio of the activity of a particular C substrate to the sum of activities of all C substrates (Zak *et al.*, 1994)). All C substrates had some activity, and hence the index is a measure of evenness rather than richness. We used a significance level of 0.05. The statistical analyses were carried out using the open source software R 2.15.1 (R Development Core Team, 2012). GLMMs were run using the LMER and GLMER functions of the package LME4 (Bates *et al.*, 2008), and negative binomial GLMMs with the GLMMADMB function of the GLMMADMD package (Skaug *et al.*, 2012). Tukey's pairwise comparisons with 95% confidence level were conducted with the MULTCOMP package (Hothorn, Bretz & Westfall, 2008). Results are presented as the mean ± 1 SE, unless otherwise specified.

Results

For all years, unparasitized trees grew more than did parasitized ones (LMM: tree type: df = 1; $F = 87.42$; $P < 0.0001$; year: df = 1; $F = 1.78$; $P < 0.18$; interaction: df = 1; $F = 1.04$; $P = 0.37$), with mean growth differences of 37% in 2010, 21.30% in 2011 and 23.82% in 2012. Parasitized trees received significantly more visits from frugivorous birds (GLMM: 2010: $\chi^2 = 35.88$; df = 1; $P < 0.0001$; 2011: $\chi^2 = 21.87$; df = 1; $P < 0.0001$; 2012: $\chi^2 = 12.29$; df = 1; $P = 0.0005$; Fig. 1). In general, the greatest accumulated biomass for the two sampling years was quantified beneath parasitized trees (312.85 ± 20.65 g m^{-2}), followed by unparasitized (269.07 ± 24.16 g m^{-2}) and dead trees (53.71 ± 10.96 g m^{-2}). The abundance of different organic matter sources (from the host, the parasite, and the activity of frugivorous birds) differed between unparasitized, parasitized, and dead trees: (1) host needles ($\chi^2 = 302.32$; df = 2; $P < 0.0001$) and other structures, including cones, bark, and pine flowers ($\chi^2 = 14.91$; df = 2; $P = 0.0006$); (2) mistletoe leaves and stems ($\chi^2 = 220.68$; df = 2; $P < 0.0001$), seeds ($\chi^2 = 117.24$; df = 2; $P < 0.0001$), and fruits and flowers (only present under parasitized trees); (3) seeds of co-fruiting zoochorous plant species ($\chi^2 = 57.72$; df = 2; $P < 0.0001$) and bird excrement ($\chi^2 = 85.172$; df = 2; $P < 0.0001$) (Fig. 2). As expected, the soil beneath the canopy of parasitized trees received greater quantities of mistletoe, leaves, flowers, and fruits than that beneath trees without mistletoe (unparasitized: 0.19 ± 0.09 g m^{-2}; parasitized: 103.21 ± 12.31 g m^{-2}; dead: 10.49 ± 3.51 g m^{-2}; $\chi^2 = 519.07$; df = 2; $P < 0.0001$). In contrast, litter from the host was more abundant under unparasitized trees (unparasitized: 268.68 ± 20.61 g m^{-2}; parasitized: 203.66 ± 16.16 g m^{-2}; dead: 39.98 ± 7.67 g m^{-2}; $\chi^2 = 113.68$; df = 2; $P < 0.0001$). The biomass delivered by seed dispersers was greater under the canopy of live and dead

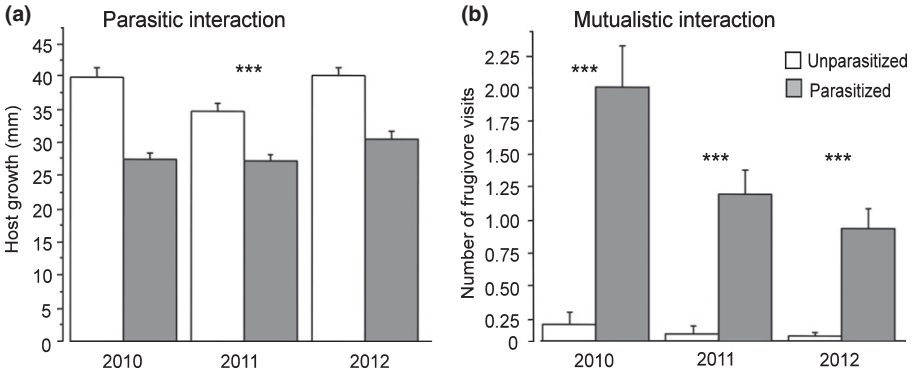

Fig. 1 Tree growth (a) and number of frugivore visits (b) in parasitized and unparasitized *Pinus nigra* trees. Annual internode growth of focal trees was estimated by measuring bud elongations (cm) for three consecutive years (2010–2012) on three randomly assigned branches per tree. The number of frugivore visits was estimated by direct observations in all focal trees during the dispersal season for three consecutive years (2010–2012). We used average growth values within focal pines and the cumulative number of birds watched through the season for statistical analyses. Linear mixed models (using replicate as a random factor to account for temporal pseudoreplication) were used to compare tree growth, while generalized linear mixed models with Poisson error distribution and log-link were used to compare frugivorous bird counts. Statistical differences are indicated: ***, $P < 0.0001$. Results correspond to mean ± 1 SE; $n = 75$ unparasitized trees and $n = 75$ parasitized trees.

New Phytologist

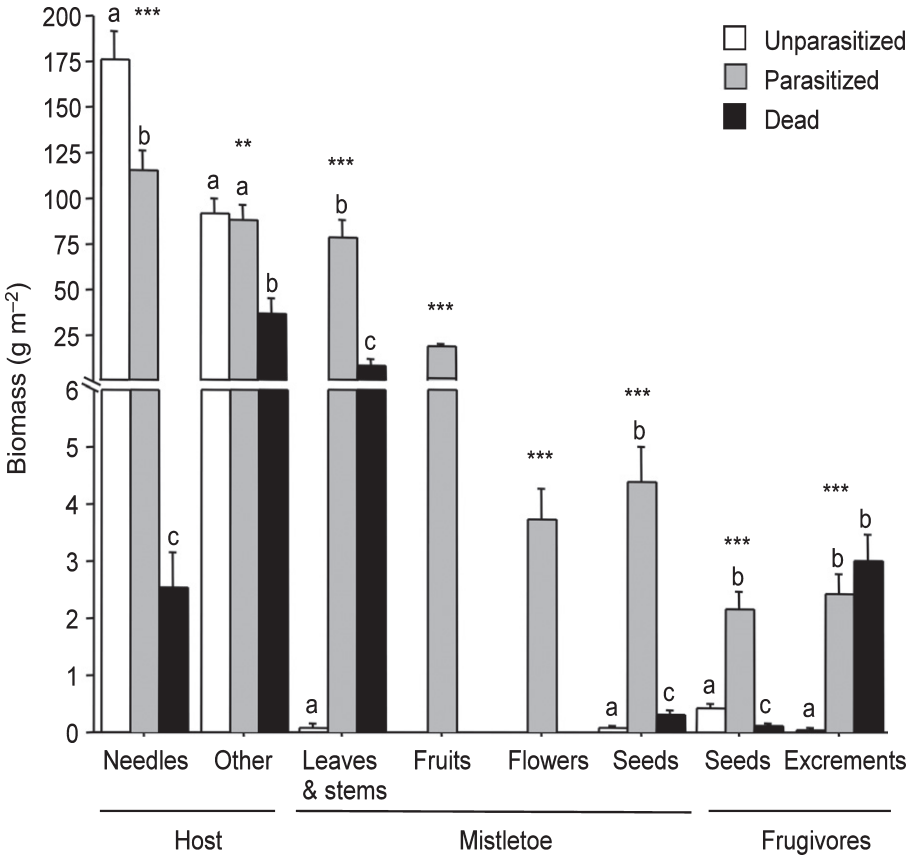

Fig. 2 Comparisons of litter biomass reaching the soil beneath the canopies of unparasitized, parasitized and dead *Pinus nigra* trees. Different organic matter sources come from the host, the parasite (mistletoe) and the activity of frugivorous birds. Analyses were performed using negative binomial generalized linear mixed models (GLMMs). Statistical differences are indicated: **, $P < 0.001$; ***, $P < 0.0001$. Results correspond to mean (± SE) values for two sampling years (2012 and 2013); $n = 75$ unparasitized trees, $n = 75$ parasitized trees and $n = 15$ dead parasitized trees. Tukey's pairwise comparison method was applied to compare tree types. Different letters denote significant differences among tree types (alpha = 0.05).

parasitized trees (unparasitized: 0.42 ± 0.09 g m^{-2}; parasitized: 4.64 ± 0.49 g m^{-2}; dead: 3.23 ± 3.52 g m^{-2}; $\chi^2 = 211.28$; df = 2; $P < 0.0001$), especially in the form of seeds of zoochorous plant species under parasitized trees (Fig. 2), and excrement under dead trees (Fig. 2). Overall, in unparasitized trees, pine needles constituted 99% of total biomass input, while the remaining 1% was from frugivore activity. In parasitized pines, mistletoe litter comprised 33.5% of overall biomass, host litter 65% and frugivore litter 1.5%. In dead parasitized trees, 74% of biomass input pertained to the host, 19% to mistletoe, and 6% to frugivores. Although suspended traps were well prepared to prevent organic matter losses, contributions to nutrient inputs via animal defecation should be considered underestimated, especially for parasitized trees where rates of frugivore visitation were consistently higher, as a fraction of the collected excrement presumably became diluted during rainy days.

N, P, and K concentrations in mistletoe tissues were greater than those of the host (Table 1). Except for seeds, all other mistletoe tissues (leaves, stems, flowers, and fruits) contained 1.5–2.5 times more N, *c.* 1.4 to 4.5 times more P and 4.6 to 9.8 times more K than host needles, and showed lower C : N ratios ($P < 0.001$).

Table 1 The table summarizes the nitrogen (N), carbon (C), phosphorus (P) and potassium (K) contents and the C : N ratio of host senescent needles and mistletoe senescent leaves, stems, flowers, fruits and seeds

	Chemical composition				
Organic matter ($n = 5$)	N %	C %	C : N	P (mg g^{-1})	K (mg g^{-1})
Pine senescent needles	0.71 (0.07)^{a}	49.30 (0.42)^{a}	71.83 (9.22)^{a}	0.65 (0.15)^{a}	2.99 (0.95)^{a}
Mistletoe senescent leaves	1.47 (0.17)^{b}	44.61 (0.56)^{b}	31.92 (3.44)^{b}	2.55 (0.29)^{b}	29.25 (1.66)^{b}
Mistletoe stems	1.76 (0.27)^{b}	47.19 (1.13)^{b}	29.33 (4.24)^{b}	2.02 (0.32)^{b}	14.60 (1.18)^{c}
Mistletoe flowers	1.39 (0.06)^{b}	52.08 (0.34)^{a}	37.65 (1.64)^{b}	2.97 (0.15)^{b}	13.70 (0.33)^{c}
Mistletoe fruits	1.07 (0.15)^{b}	44.45 (0.99)^{b}	45.13 (6.65)^{ab}	2.88 (0.29)^{b}	16.98 (2.09)^{c}
Mistletoe seeds	0.71 (0.06)^{a}	40.86 (0.99)^{c}	59.00 (6.65)^{a}	0.90 (0.29)^{a}	12.58 (2.09)^{c}
	$F_{5,24} = 7.94$; **$P < 0.0001$**	$F_{5,24} = 30.06$; **$P < 0.0001$**	$F_{5,24} = 8.38$; **$P < 0.0001$**	$F_{5,24} = 13.22$; **$P < 0.0001$**	$F_{5,24} = 38.98$; **$P < 0.0001$**

Results are presented of linear models (*F*-values, degrees of freedom, and *P*-value), followed by Tukey's pairwise comparison method with 95% confidence level to compare organic matter types. Significant values appear in bold. Different letters denote significant differences among organic matter types (alpha = 0.05).

According to differences in litter quantity and quality, soils at different stages of the host–parasite system exhibited different chemical and biological properties (Table 2; Fig. 3). In this regard, TOC was highest beneath dead trees, followed by parasitized and unparasitized trees (Table 2), while IC followed the opposite pattern (Table 2). Soil moisture and pH were quite similar ($P = 0.920$ and $P = 0.872$, respectively), with parasitized and dead trees showing slightly more acidic soils. Although the difference was not statistically significant, TN was higher in soils beneath dead trees, followed by parasitized and unparasitized trees (Table 2). The dominant N form was different in each case. Beneath parasitized and unparasitized trees the soils contained a greater abundance of DON and NH_4^+-N, although the difference was not statistically significant, whereas beneath dead pines N prevailed in the form of NH_3^- (Table 2). While IEM PO_4^{3-}-P availability was highest beneath parasitized and dead trees (Table 2), IEM K^+ availability was highest under parasitized trees (Table 2).

Differences in soil chemical properties were similarly reflected in soil microbial features, with soils beneath dead trees hosting more abundant and functionally even microbial communities, followed by parasitized trees and then unparasitized trees (MB-N: LMM: $\chi^2 = 7.36$; df = 2; $P = 0.0025$; H′ based on microbial functional measurements: LMM: $\chi^2 = 4.09$; df = 2; $P = 0.129$). No trend was detected for the mean time course of CO_2 production rate among the three types of focal pines (Supporting Information Fig. S1).

Discussion

Different biotic interactions prevailing in the canopies of unparasitized, parasitized, and dead trees result in different linkages between the forest canopy and the soil, influencing soil chemical and biological properties in different ways.

In unparasitized trees, pine litter was the most important resource reaching the soil (99.9% of total organic compounds) together with small amounts of excrement that birds deposited while perching in the tree canopy (Fig. 2a). Parasitism changed the linkages between the tree and the soil: the parasite increased overall litterfall quantity, including a wide variety of tissue types (i.e. leaves, flowers, trunks, fruits, and seeds; Fig. 2b) of different chemical composition (Table 1), while, as expected, having additional indirect effects. First, the stressful effect of parasitism on the host was reflected by diminished host growth (Fig. 1a), which caused a 1.33-fold reduction in the amount of host litter reaching the soil (Fig. 2a). Second, the increase in frugivorous bird visits (Fig. 1b) raised the input of allochthonous organic compounds (feces and zoochorous plant seeds) by *c.* 15-fold with respect to unparasitized trees (Fig. 2c). Finally, once the host died, senescing litter of both the parasite and the host continued to enrich the soil. Moreover, frugivorous birds used dead standing structures to perch, from where they dropped excrement (Fig. 2c). Overall, parasitized trees, either alive or dead, received organic matter inputs from more diverse sources than did unparasitized ones. Thus, parasitized trees accumulated under their canopy 1.16-fold more litter biomass than did unparasitized trees and 5.8-fold more biomass than did dead trees. These sources, in addition to their abundance, differed in quality (Table 1). Thus, litter from *V. a. austriacum* contained *c.* 2-fold more N, 4-fold more P, and 5.8-fold more K than did that from the host, these nutrients being especially concentrated in leaves, but also in stems, flowers, and fruits (Table 1). Moreover, litter of *V. a. austriacum* contained lower C : N ratios (Table 1), indicating a higher decomposition rate and more rapid nutrient release than in the more recalcitrant litter of the host (Quested *et al.*, 2002, 2005; Ndagurwa *et al.*, 2014b). Furthermore, considerable inputs of bird debris into the soil may provide a large supply of nutrients, mainly in the form of N, P and sodium (Na) (Dean *et al.*, 1999; Van der Wal *et al.*, 2004). In addition to frugivorous birds, other animals, such as deer, squirrels, and cattle, frequently browse on mistletoe shoots (Umucalilar *et al.*, 2007) and numerous insectivorous birds have been reported to feed within mistletoe clumps (Bennetts, 1991). These visitors, although not explicitly considered in this study, may affect nutrient input to

Table 2 Nutrient concentrations and availabilities in the top 10 cm of the soil profile beneath the canopies of unparasitized and living and dead parasitized trees

Variable	Unparasitized ($n = 38$)	Parasitized ($n = 38$)	Dead ($n = 12$)	Model (χ^2, *P*-value)
TOC (mg kg^{-1})	291.93 ± 40.71^a	403.03 ± 40.54^a	536.06 ± 120.41^b	7.017 (**0.029**)
IC (mg kg^{-1})	25.04 ± 6.01^a	16.19 ± 1.80^a	15.03 ± 3.47^a	3.886 (0.143)
TN (mg kg^{-1})	52.58 ± 8.65^a	73.02 ± 11.76^a	117.20 ± 23.42^a	2.116 (0.347)
DON (mg kg^{-1})	53.38 ± 5.91^a	54.34 ± 4.62^a	45.37 ± 4.33^a	1.518 (0.468)
pH	7.29 ± 0.06^a	7.21 ± 0.06^a	7.22 ± 0.15^a	0.273 (0.872)
Moisture (%)	4.50 ± 0.36^a	4.52 ± 0.28^a	4.39 ± 0.71^a	0.165 (0.920)
IEM NO_3 (μg ($cm^2 \times d$)$^{-1}$)	0.249 ± 0.020^a	0.269 ± 0.027^{ab}	0.277 ± 0.048^b	6.132 (**0.046**)
IEM NH_4^+ (μg ($cm^2 \times d$)$^{-1}$)	0.279 ± 0.013^a	0.298 ± 0.016^a	0.247 ± 0.009^a	3.847 (0.172)
IEM PO_4^2 (μg ($cm^2 \times d$)$^{-1}$)	0.043 ± 0.002^a	0.055 ± 0.004^b	0.061 ± 0.007^b	14.932 (**0.0005**)
IEM K^+ (μg ($cm^2 \times d$)$^{-1}$)	0.340 ± 0.026^a	0.465 ± 0.037^b	0.348 ± 0.022^a	28.811 (**0.0001**)

Total organic carbon (TOC), inorganic carbon (IC), total nitrogen (TN), dissolved organic nitrogen (DON), pH, and moisture were analyzed in composite soil samples collected at a depth of 0–10 cm, whereas NO_3^-, NH_4^+, PO_4^{2-} and K^+ availabilities were estimated using ion exchange membranes (IEMs) incubated for 1 month in soils. Results (χ^2 and *P*-values) of a linear mixed model are presented including tree condition (unparasitized/parasitized/dead tree) as a fixed factor and paired trees as a random factor, followed by Tukey's pairwise comparisons with 95% confidence level. Significant values (alpha = 0.05) appear in bold. Values are mean ± SE. Different letters denote significant differences among tree types.

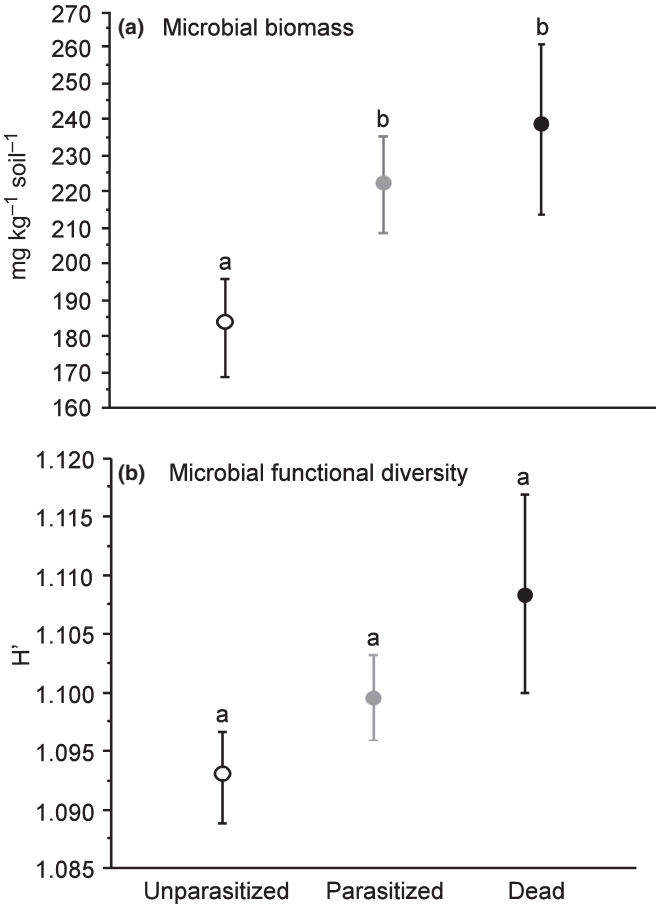

Fig. 3 (a) Nitrogen in microbial biomass and (b) Shannon index (H') based on functional measurements of soil microbial communities beneath unparasitized, parasitized, and dead *Pinus nigra* trees. Nitrogen in microbial biomass was estimated from composite soil samples collected beneath the canopy of focal trees. The Shannon index is based on functional measurements of microbial communities analyzed with the MicroResp system. Results correspond to average values (± SE) of one sampling year (2012); $n = 38$ unparasitized trees, $n = 38$ parasitized trees and $n = 12$ dead trees. Tukey's pairwise comparison method with a 95% confidence level was applied to compare tree types. Different letters denote significant differences among tree types (alpha = 0.05).

the soil by removing mistletoe foliage from the canopy or by boosting organic matter input with their excrement, either offsetting or magnifying the influence of mistletoe infection.

Mistletoe, by augmenting the amount, quality, and diversity of organic matter input, returned a more heterogeneous mixture of resources to the soil that enhanced C accumulation and increased the variety of soil resources that the biota could utilize, giving rise to local 'fertilization islands' under parasitized trees (Table 2). These soil spots were especially enriched in K^+ and PO_4^{3-}-P, reflecting the main elements provided by litter of *V. a. austriacum* (Table 1). This result has been noted in other mistletoe species (March & Watson, 2010; Ndagurwa *et al.*, 2013; Muvengwi *et al.*, 2015). However, soil N content was lower than expected, a result also reported for mistletoes of the African savanna (Ndagurwa *et al.*, 2013; Muvengwi *et al.*, 2015). This could be attributable to a greater mineralization and nitrification of the organic N, which could be stimulated by the lower C : N relationship of the mistletoe or by changes in the abiotic environment produced in the tree canopy as a consequence of parasitism (e.g. greater light infiltration and higher temperature). Alternatively, lower NH_4^+ availability could result from an antagonistic effect of NH_4^+ and K^+ at the soil exchange surface (Ndagurwa *et al.*, 2013; Muvengwi *et al.*, 2015). One notable outcome is that the host–parasite system maintained its effects on the soil after death, despite the fact that organic matter input diminished at late stages. These soils showed greater amounts of organic C and PO_4^{3-}-P than did those under parasitized trees, and the dominant N form switched from NH_4^+-N to $NO3^-$-N, perhaps because, once the tree dies, the sparser crown permits greater light infiltration to the forest floor, thereby raising soil temperature and stimulating nitrification processes. Our findings show that the effects of a parasitized tree after death could be maintained by the additional organic matter input long after the parasitism has ceased, similar to the effects of past agricultural land use (Dupouey *et al.*, 2002; Mattingly & Orrock, 2013) or the effects of dead tree crowns in savannas (Dean *et al.*, 1999; Stahlheber *et al.*, 2015), which may persist for years in the soil (Rodríguez *et al.*, 2011).

Soil heterotrophic microbial communities tended to become increasingly abundant (Fig. 3a) and functionally even (Fig. 3b) as mistletoe parasitism matured, reaching their maximum under dead parasitized trees. These results are consistent with the greater variety and quantity of resource availability found in the soil. The absence of significant differences among the three types of focal pines in the functional evenness of the soil microbial community might be explained by the combination of three mechanisms. First, microbial communities are metabolically extremely flexible and physiologically tolerant to environmental changes, resulting in microbial populations that are surprisingly resistant to soil changes (Meyer *et al.*, 2004; Curiel-Yuste *et al.*, 2014). Second, microbial communities may be able to maintain their functionality despite changes in soil nutritional status as a consequence of functional redundancy (Allison & Martiny, 2008). Third, the compounds used in the MicroResp technique are likely to occur during the degradation of organic matter in soil (Schipper *et al.*, 2001). Although significant differences were not consistently found in soil microbial functional evenness among the three types of focal pines, the tendencies shown by this variable (Fig. 3b) and by soil microbial abundance (Fig. 3a) point in the same direction: these variables were elevated during the process of parasitism, increasing from unparasitized to parasitized and from parasitized to dead trees. This tendency could be one plausible explanation for the different N forms prevailing under the canopy of dead trees (NO^{3-}-N) in contrast to that predominating under unparasitized and parasitized trees (NH_4^+-N). These results support the idea of aboveground diversity boosting belowground communities (Bardgett & Shine, 1999; Zak *et al.*, 2003) at different ecosystem developmental stages (Ohtonen *et al.*, 1999; Schipper *et al.*, 2001), which may potentially affect fundamental ecosystem processes driven by soil microbial organisms (Zak *et al.*, 2003).

In conclusion, mistletoe, together with the biotic interactions that prevail in the forest canopy at different stages of parasitism, can play a central part in increasing soil resource availability

and its spatial variability, in turn regulating the functional evenness, abundance, and spatial distribution of heterotrophic microbial communities inhabiting the soil. These findings strengthen the idea of mistletoes as keystone species and ecosystem engineers (*sensu* Jones *et al.*, 1994), expanding their effects to soil communities and ecosystems, and may have far-reaching implications for soil health and function, as well as for other organisms of the forest community. By increasing soil microbial biomass, mistletoe could indirectly influence a number of key soil functions, including soil carbon sequestration, decomposition, nutrient release, and the suppression of plant pathogens (Pankhurst *et al.*, 1997). In addition, it can contribute to the maintenance of soil structure, which affects the water-holding capacity, infiltration rate and erodibility (Elliot 1996). Also, the increment in soil resource availability could enhance the growth of understory plants (March & Watson, 2007), which could also contribute to altering the nutrient dynamics on the forest floor, and increase the frequency of herbivorous visits. Thus, to further elucidate the fertilizer effect of mistletoes, future studies should quantify the role of interacting animals other than frugivorous birds, as well as the potential effect of mistletoe-induced changes on the understory vegetation. As these parasitic plants are so frequent in forest canopies around the world, there still remains much to be explored regarding their impact on aboveground and belowground communities, as well as general ecosystem functioning.

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Author contributions

R.Z. and A.M. developed the conceptual framework of this study; A.M., L.M., A.G. and R.Z. designed the sampling protocols; A.M., L.M., A.G. and R.Z. participated in data collection, processing and interpretation; and A.M. wrote the paper with contributions from all authors.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 Average production of CO_2 rates responding to the addition of 15 different carbon sources, our measure of soil microbial functional diversity, using the MicroResp method, for soils from parasitized, unparasitized and dead trees.

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