

Early-successional vegetation changes after roadside prairie restoration modify processes related with soil functioning by changing microbial functional diversity

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ABSTRACT

Because of their rapidly changing vegetation dynamics and harsh environmental conditions, roadside prairies in semi-arid regions represent an exceptional study system in which to investigate the effects of plant–soil interactions on ecosystem functioning. We conducted a two-year field experiment on two roadside embankments in semi-arid central Spain differing in construction age to answer the following questions: (i) do commonly used restoration treatments (hydroseeding, fertilization and irrigation) affect soil microbial functional diversity and processes related to soil functioning (basal respiration, total N and P and *in situ* N availability rate)? (ii) what portion of plant effects on processes related to soil functioning is mediated indirectly by microbial functional diversity? Except for a small and negative irrigation effect on the microbial functional diversity in the three-year old site, the restoration treatments employed did not affect this variable. Fertilization increased plant diversity, an effect likely mediated by the enhanced soil nutrient availability with this treatment at early stages of secondary succession. In contrast, hydroseeding did not affect processes related to soil functioning. The total effect of the plant community on these processes was higher than that of the microbial functional diversity alone, suggesting that the studied slopes are to the greater extent regulated by plants. However, soil microbes are a key proximate influence in the system, as the indirect effects of plant community on soil functioning processes mediated by soil microbes represented 37–41% of the total plant effects observed. Our results indicate that the restoration of recently built slopes can potentially be improved with treatments that promote plant compositional shifts, such as fertilization, or alter soil function, such as the enhancement of soil microbial functional diversity. They also highlight that plant–soil interactions are an important process that can be manipulated for restoration purposes in early-successional stages, especially in nutrient-poor semi-arid ecosystems.

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1. Introduction

Most of the world's ecosystems are now impacted by humans to a greater or lesser extent (Vitousek et al., 1997), and therefore ecological restoration of degraded systems play a major role in re-establishing ecosystem structure and functioning (Hobbs et al.,

2006). Ecosystem succession is a suitable framework for guiding restoration efforts aiming to recover plant communities and soil processes because it incorporates the temporal dynamics of the ecosystems and the barriers to their development (Walker et al., 2007). Knowledge of classical successional dynamics (Connell and Slatyer, 1977) has guided the development of restoration techniques in several systems (Walker et al., 2007). Nevertheless, ongoing environmental changes and the increasing prevalent anthropogenic disturbance may result in novel ecosystems whose composition and/or function differ from any historical system (see Cramer et al., 2008 for a review). Degraded landscapes such as opencast mines, quarries or roadside slopes represent good examples of novel communities which may or may not function as

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natural assemblages (Hobbs et al., 2006). Thus, ecosystem functioning in this potentially new succession context remains unknown, as well as the suitability of widely used restoration treatments for recovering vegetation and soil processes (Matesanz et al., 2006).

With more than 50% of net primary production being returned to the soil (Wardle et al., 2004), plant-soil interactions are of major importance in understanding the role of biodiversity in controlling ecosystem processes and properties (Van der Putten et al., 2001). However, little is known about these links in the context of ecosystem restoration and succession (Kardol et al., 2006). In order to study the responses of ecosystem functioning to restoration actions, special attention should be paid to the relationships between plants, microorganisms and soil processes (Harris, 2009). Plant community succession can drive the development of ecosystem functioning (Chapin et al., 1994). Since the responses of single plant species fail to match patterns observed in the field where multiple species interact with each other (Blomqvist et al., 2000), a community perspective would be very useful (Ehrenfeld et al., 2005). Given the relevance of temporal variations in plant-soil interactions to control ecosystem development (Kardol et al., 2006), it is worth including soil communities in the study of newly developing soils (Bardgett et al., 2007). In the same way, the effectiveness of restoration treatments must be partially assessed belowground, where the manipulation of the communities enhances the rate of recovery of degraded soils (Harris, 2009). Therefore, in order to provide a more inclusive community viewpoint that improves management practices, it is necessary to perform community level studies that test plant-soil interactions (Ehrenfeld et al., 2005). This implies to achieve a better knowledge of how changes in plant composition, and therefore in resource input quality, affect soil communities (Bardgett et al., 2005). To adequately interpret this interaction, it is particularly useful to assess the ability of soil microbial communities to metabolize a range of substrates that vary in structural complexity (Schipper et al., 2001; Oren and Steinberger, 2008). Relatively few studies have simultaneously considered plant effects on both soil biota and soil processes (Wardle et al., 1999; Porazinska et al., 2003), and many of them have focused on individually grown plants in pot experiments (Besmear et al., 2006) and only a few have attempted to determine these effects in natural ecosystems (e.g. Wardle, 2005; Maestre et al., 2009).

Prairie communities of roadside slopes in semi-arid regions represent an exceptional study system in which to investigate plant-soil diversity effects on ecosystem functioning with a succession perspective. First of all, similarly to opencast sites or strip mines, both plant and soil communities are nearly completely reset due to the use of subsoil and foreign construction materials re-instated after storage (Harris et al., 2005). Therefore, changes in the microbial community follow a similar trajectory to those recorded during primary succession (Harris, 2009). Second, these prairies, dominated by annual plant species, are extraordinarily dynamic, with rapid structural and compositional changes (Wali, 1999). These novel communities are also characterized by a high proportion by exotic species artificially introduced in revegetation practices because they are cheap, readily available and easy to establish on disturbed sites (Matesanz et al., 2006). Thus, since legacy effects of past biota on ecosystem functioning have largely been erased, and because succession occurs more quickly than in most other ecosystems, investigators can easily track its effects on ecosystem functioning. In addition, the soil is nutrient-poor and limited by the short duration of available water (Bochet et al., 2007). Temporal patterns of soil biodiversity are context-dependent, being more evident in managed systems with nutrient-limited soils (Bardgett et al., 1996). Restoration treatments inducing shifts in the composition of soil communities thereby give a sign of

improvement in the efficiency of nutrient cycling and decomposition processes.

Community-level studies focusing on plant-soil interactions in a restoration context have looked at grassland types in different biogeographic areas (Donnison et al., 2000; Grayston et al., 2004). However, not many studies to date have evaluated whether soil microbial communities and soil functioning are driven by changes in plant community composition along a continuum of plant diversity plots with similar climatic conditions and grazing pressures, and whether these interactions change along succession. To overcome this gap in our knowledge, we conducted a field experiment on two different roadside prairie slopes in semi-arid central Spain differing in construction age. A previous study carried out on the same sites indicated that plant community responses to the restoration treatments evaluated (irrigation, fertilization and hydroseeding) were site-specific and responded to the dominance of several fast-growing plant species (García-Palacios et al., 2010). In the present study, we aim to evaluate the effect of these treatments on belowground microbial communities and soil functioning. Microbial functional diversity and surrogates of soil functioning were sampled in parallel with the vegetation to answer the following questions: i) do commonly used restoration treatments (hydroseeding, fertilization and irrigation) affect soil microbial communities and processes related to soil functioning (basal respiration, total N and P and *in situ* N availability rate)? (ii) what portion of plant effects on processes related to soil functioning is mediated indirectly by microbial communities?

2. Materials and methods

2.1. Study area and experimental design

The experiment was developed at two roadside embankments with similar slope and aspect located in the center of the Iberian Peninsula. The climate is semi-arid, with cold winters and a severe summer drought; annual mean temperature and precipitation are 15 °C and 450 mm, respectively (Getafe Air Base climatic station 40°18'N, 3°44'W, 710 m a.s.l., 1971–2000). One of the sites was a recently built embankment, where construction was finished three months prior to the field surveys. Therefore, vegetation succession and soil dynamics were just recovering after perturbation of land movements. The other site was a three-year old embankment. Both sites are nutrient poor, with low levels of soil organic carbon (hereafter SOC), total N and P, scarce soil biological activity and alkaline pH (Table 1). Three restoration treatments (hydroseeding, fertilization and irrigation) were evaluated in this study. Six blocks containing 12 1 m × 1 m plots per block, with at least 1 m buffer between plots, were randomly established at each site. Each block contained a full factorial design with the three treatments employed, which were randomly assigned to the plots

Table 1

Main characteristics and soil properties of the two roadside slopes studied at the beginning of the experiment (December 2006). Numerical values are means ± SE (n = 30).

	Recently built site	Three-year old site
Initial plant cover (%)	12	58
Water holding capacity (ml water g ⁻¹ soil)	0.36 ± 0.03	0.43 ± 0.03
Total N (mg N g ⁻¹ soil)	0.14 ± 0.01	0.34 ± 0.04
Total P (mg P g ⁻¹ soil)	0.16 ± 0.01	0.35 ± 0.01
Basal respiration (mg CO ₂ -C g ⁻¹ soil day ⁻¹)	0.01 ± 0.01	0.04 ± 0.01
SOC (g C kg ⁻¹ soil)	5.30 ± 0.06	9.90 ± 0.09
pH	8.35 ± 0.14	8.06 ± 0.15

within each block. Realistic treatment and seeding rates (García-Palacios et al., 2010) were chosen to achieve marked soil resource availability and plant richness gradients. In December 2006, we added three hydroseeding levels (control, seeding and seeding + mulch). The control and seeding addition levels consisted of no seeding addition and the application of a commercial seed mixture (Zulueta Corp., La Rioja, Spain; dose of 30 g/m²), constituted essentially by Leguminosae, Gramineae and Compositae species (Table ST.1). The ingredients of the seeding + mulch level were stabilizer (Stable, dose of 10 g/m²; Projar, Valencia, Spain), wood fiber mulch (Hortifibre, dose of 100 g/m², Projar, Valencia, Spain), water (dose of 3 l/m²) and the seed mixture. Fertilization was applied twice (December 2006 and January 2008) at two levels (control and fertilized). Fertilized plots received a 20 g/m² dose of a slow release N:P:K (16:11:11) inorganic fertilizer (Scott Corp.), while control plots were not fertilized. Irrigation was conducted from March to June in both 2007 and 2008, coinciding with the peak growing season of annual plant communities characterizing our study sites. This treatment was applied at two levels (0 and 50% of the monthly total precipitation median from the 1971–2000 period).

2.2. Data collection

Plant communities were sampled at the two sites in May 2008, the optimal phenological moment to measure the annual herbaceous communities studied. Plant community composition was established by visually estimating plant species cover in each 1 m square plot. We collected soils in December 2006, prior to treatment addition, and again in June 2008, to see the effects after two plant growing seasons. We removed four soil cores (5 cm diameter, 7.5 cm depth) from random locations in each plot. Soil cores were bulked by plot and homogenized in the field. In the laboratory, the samples were sieved (2 mm mesh) and separated into two fractions. One fraction was immediately frozen at –80 °C for microbial analysis; the other was air-dried for 1 month for biogeochemical analyses.

2.3. Assessing of microbial functional diversity

We analyzed soil heterotrophic microbial communities with the MicroResp™ system (Campbell et al., 2003). This is a whole-soil method based on community level physiological profiles (CLPP) obtained by testing of 15 carbon sources that vary in structural complexity (Oren and Steinberger, 2008); here 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), carboxylic acids (citric acid, L-malic acid, oxalic acid and γ amino butyric acid [GABA]) and a fatty acid ester polymer (Tween 80) (all Sigma Aldrich, UK). 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 assess microbial communities in relation to taxonomic or phylogenetic diversity (Øvreås, 2000), we can use MicroResp data to interpret changes in functional diversity. Prior to carrying out the MicroResp™ method, defrosted soils were introduced into the plates and pre-incubated for five days at 25 °C. The moisture within the plates was corrected to 40% WHC in order to condition the soils and reestablish active microbial populations. Although potential changes in microbial communities may have occurred due to freeze–thaw cycles, samples are still comparable because all the soils had the same treatments. To avoid changes in soil moisture content during incubation, plates were covered with parafilm and introduced in a large sealed box containing a dish of self-indicating soda lime and lined

with wet paper towels. An open air plate substrate-in-soil incubation period of 2 h was set to allow any abiotic CO₂ release from our soils. After that, the plates were incubated for 6 h and read at 570 nm. This protocol was repeated after 24 h to check for longer term catabolism of the substrates and help in the interpretation of CO₂ rates. The results were calculated on the basis of the 16th substrate (water), which represents the basal respiration. Common artifacts in the measurement of respiration in weakly oxygenated and arid alkaline soils include: low responses to amino acid substrates due to ammonia production and high responses to carboxylic acids due to abiotic CO₂ release (Oren and Steinberger, 2008). Therefore, soil aeration was promoted by maintaining soil moisture to 40% WHC prior the addition of the carbon sources. Since we are interested in the relative differences between treatments instead of the absolute CO₂ rates and have compared samples within the same soil context (roadside slopes), we expected any abiotic artifacts to similarly affect all the plots evaluated.

2.4. Surrogates of soil functioning

The following soil variables related to nutrient cycling were measured as surrogates of soil functioning in the samples collected in December 2006 and June 2008: basal respiration, total N and P and *in situ* N availability rate. Basal soil respiration rates were determined by NaOH absorption of the CO₂ evolved during an aerobic five days-incubation in the dark at 40% WHC and 25 °C, followed by titration with HCl (Froment, 1972). This variable is strongly related with biological activity (Kuzakov, 2006). Total N and P were obtained on an SKALAR San⁺⁺ Analyzer (Skalar, Breda, The Netherlands) after digestion with sulphuric acid. Total P has been found to be positively related to fractions of available P (e.g. Pautler and Sims, 2000), and has been employed when assessing soil functioning in semi-arid steppes (Maestre and Puche, 2009) and model grasslands (Orwin et al., 2010). Total soil N has been broadly used in herbaceous ecosystems to study changes in soil functioning during the course of secondary succession (Garnier et al., 2004). To estimate *in situ* soil N availability rate, we used anionic and cationic exchange membranes (types I-100 and I-200, Electropure Excellion, Laguna Hills, California). These ionic exchange membranes (IEMs) are related with N mineralization (Subler et al., 1995) and provide one of the most reliable indices of plant nutrient availability (Ziadi et al., 1999). IEMs were previously conditioned in the lab by immersing them in demineralized water at 82–90 °C for 48 h. Two 2.5 × 2.5 cm IEMs were positioned in each sampling plot 5 cm below of the top of the surface horizon and incubated for 25 days between May and June 2008. Upon retrieval, we extracted the nutrients from the membranes with a 2 M KCl solution and calculated NH₄⁺ and NO₃⁻ availability rates by colorimetry (indophenol blue method) using a microplate reader (Sims et al., 1995). Mineral N was taken as the sum of NH₄⁺-N and NO₃⁻-N. As N availability was measured over a period of 25 days rather than by means of individual observations, we referred to it as inferred availability rate. All these soil variables are critical determinants of the functioning of arid and semi-arid ecosystems (Whitford, 2002). Comparisons of different soil techniques and approaches (*in situ* vs. laboratory one-time measurements) are not straight forward; therefore we only interpret the trends and relative changes between samples and variables. We used the term potential soil functioning to reflect that lab incubations (potential conditions) have been used as surrogates of soil functioning.

2.5. Statistical analysis

We evaluated the effects of restoration treatments on the functional diversity of the soil microbial community and on the

surrogates of potential soil functioning evaluated in June 2008 using a four-way permutational ANCOVA-type test. We used block as random factor and hydroseeding, fertilization and irrigation as fixed factors. Initial (December 2006) microbial functional diversity, basal respiration, total N and P were introduced into their corresponding analyses as covariates. All ANCOVA-type tests were conducted using the semi-parametric PERMANOVA approach (Anderson, 2001; see also McArdle and Anderson, 2001). This approach does not make distributional assumptions and is compatible with any distance measure. We used the Euclidean distance and 9999 permutations (permutation of raw data) in each analysis. The *P*-values used in the analyses were obtained from a random Monte Carlo sample from the asymptotic distribution of the pseudo *F*-statistic under permutation (Anderson, 2001; Anderson and Willis, 2003). All soil microbial community analyses were performed with the first 6 h incubation period data. To aid our interpretation of the PERMANOVA analyses, we also did a canonical analysis of principal coordinates (CAP; Anderson and Willis, 2003).

To assess the extent to which plant and soil microbial functional diversity explained differences in potential soil functioning, we carried out a distance-based linear model called DISTLM (McArdle and Anderson, 2001). This approach is analogous to a traditional regression, but allows the use of matrices of data as either dependent or independent variables. Our three variables were (all spring 2008 data): plant community (a matrix of percent cover data for all plants species), soil microbial functional diversity (a matrix of utilization rates of various C substrates; using the first 6 h incubation period), and potential soil functioning (a matrix of basal respiration, NH_4^+ , NO_3^- , total N and P). We constructed a global model in which plant community was a direct predictor of both soil microbial functional diversity and soil functioning, but also indirectly affected soil functioning through the interactions with soil microbial functional diversity. To reduce the probability of identifying spurious predictors as important (Johnson and Omland, 2004), we removed rare plant species (those having less than three occurrences) from the plant community matrix. Using the results from the DISTLM models, we also conducted a simple path analysis (Shipley, 2000). Path analysis allows us to move beyond bivariate correlation statistics and partition multiple pathways that one variable may have upon another and calculate direct and indirect effects and their total. For example, plant community may affect soil functioning *directly* or *indirectly* via altering microbial functional diversity, and the total effects of plant community on soil functioning is the sum of the two pathways. A path coefficient, ranging from 0–1 and analogous to regression weights or partial correlation coefficients, is estimated and describes the strength of each pathway. We obtained our path coefficients by taking the square root of the R^2 of the following DISTLM models: plant community \rightarrow soil microbial functional diversity, plant community \rightarrow potential soil functioning | soil microbial functional diversity, soil microbial functional diversity \rightarrow potential soil functioning | plant community (note: the latter two are partial tests, and “|” may be understood as “independent of the effects of”). The interpretation is similar, except that a “variable” is actually a distance matrix based upon multiple conceptually related variables using Euclidean distance. R^2 of endogenous variables was calculated using the formula in McCune and Grace (2002). While our model is one of hypothesized causation, path coefficients do not prove a cause. However, we can confirm whether the results are consistent with the cause hypothesis.

We selected parsimonious models using Akaike's information criterion (AIC, Burnham and Anderson, 2002) and step-wise DISTLM procedure to determine which matrix components were most influential in the predictor matrices. The model with the

lowest AIC value was selected as the best model in each case. Before DISTLM analyses, we checked for collinearity between explanatory variables using Spearman correlation coefficients (ρ). The maximum ρ found between variables was 0.58, suggesting that our data does not suffer from strong collinearity problems (Anderson et al., 2008). A separate model was constructed for each site. Multiple regressions were performed between the matrix components identified in our parsimonious models and the predictor matrices. ANCOVAs and distance-based linear models were carried out using the PERMANOVA + module for the PRIMER software (PRIMER-E Ltd., Plymouth Marine Laboratory, UK; Anderson et al., 2008). Multiple regressions were performed with SPSS version 14.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effects of restoration treatments on soil microbial functional diversity and potential soil functioning

Irrigation was the only treatment that significantly changed the physiological profile of microbial communities in the three-year old site (Fig. 1B; see Table ST.2 for PERMANOVA analysis). The majority of the carbon substrate CO_2 rates were $1 \mu\text{g CO}_2\text{-C g}^{-1} \text{h}^{-1}$ lower on

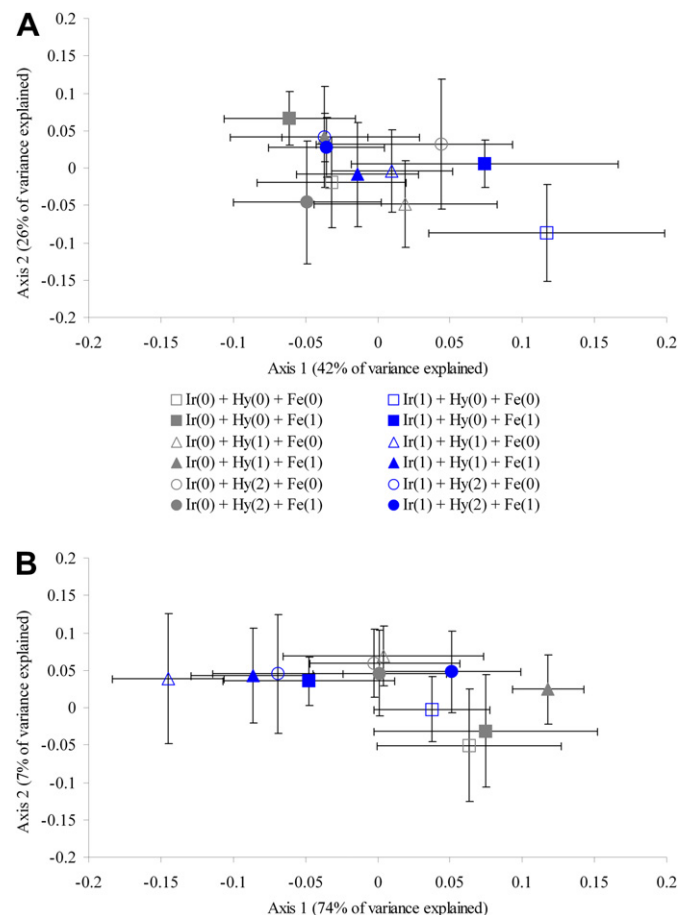


Fig. 1. Results of the canonical analysis of principal coordinates, showing the effects of irrigation (Ir(0) and Ir(1) = 0 and 50% of the monthly precipitation median), hydroseeding (Hy(0), Hy(1) and Hy(2) = control, seeding and seeding + mulch) and fertilization (Fe(0) and Fe(1) = 0 and 20 g/m^2) on the microbial functional diversity in both the recently built (1A) and the three-year old site (1B). The data from the different treatments were pooled; values represent means \pm SE ($n = 60$). An entire block was lost due to lack of enough soil to carry out MicroResp analyses.

average in treatments receiving irrigation (Fig. SF.2). However, this effect was not significant in the recently built site (Fig. 1A; see Table ST.2 for PERMANOVA analysis). All the carbon sources showed lower mean CO₂ evolution rates in the recently built site than in the three-year old site (Figs. SF.1 and SF.2). Results did not change when we introduced the physiological profile of the microbial communities in December 2006 as a covariate

NO₃⁻ availability inferred rate was about three times as high in fertilized plots ($F_{1,4} = 9.89$, $P = 0.043$), although the NH₄⁺ inferred rate was not affected by any effect or interactions in the recently built site (Fig. 2A). In the three-year old site, we found a significant fertilization effect ($F_{1,4} = 19.78$, $P = 0.013$) and an irrigation × fertilization interaction ($F_{1,4} = 18.75$, $P < 0.001$) when analyzing the NO₃⁻ availability inferred rate (Fig. 2B). The same interaction ($F_{1,4} = 15.97$, $P = 0.018$) was also found when analyzing the NH₄⁺ inferred rate. However, this interaction was the opposite between the two components of the mineral N. The NO₃⁻ inferred rate was about two times as high in fertilized plots, but only 10% higher when we also irrigated. However, the NH₄⁺ inferred rate was increased by 36% by the additive effects of both fertilization and irrigation.

A three-way significant interaction (hydroseeding × irrigation × fertilization) was found when analyzing total soil N and P ($F_{2,10} = 4.53$, $P = 0.039$ and $F_{2,10} = 5.97$, $P = 0.018$ for total N and P, respectively); a significant hydroseeding × fertilization interaction ($F_{2,10} = 4.45$, $P = 0.042$) was also found for total P, both in the recently built site. However, the main effects were not significant for either N or P. Post-hoc tests failed to distinguish which groups accounted for the difference, although some non-significant groups contrasted the most. In the three-way interaction, the seeding ($t = 2.25$, $P = 0.071$; $t = 2.58$, $P = 0.052$) and hydroseeding ($t = 1.97$, $P = 0.111$; $t = 2.32$, $P = 0.069$) levels slightly differ from the control when we irrigated and no fertilization was added for both total soil N and P, respectively. In the hydroseeding × fertilization interaction, the seeding ($t = 1.915$, $P = 0.112$) and hydroseeding ($t = 1.78$, $P = 0.136$) levels differ from the control when no fertilization was added. Neither significant main effects nor interactions were found in the three-year old site.

The same was found when analyzing soil basal respiration at both sites. Initial (December 2006) soil N, P and basal respiration were used in the analysis as covariates, but their effects were not significant at any site.

3.2. Links between vegetation, soil microbial functional diversity and potential soil functioning

The overall variation explained by the model was lower at the recently built than at the three-year old site (Fig. 3A and B) for both soil microbial functional diversity ($R^2 = 0.24$ and 0.32 , respectively) and potential soil functioning ($R^2 = 0.47$ and 0.70 , respectively). The total effect of plant community on the latter was higher than that of microbial functional diversity at both sites ($r = 0.72$ and 0.54 at the recently built site; $r = 0.85$ and 0.61 at the three-year old site, for plant and microbial functional diversity, respectively). The parsimonious models constructed (Table 2) showed that some plant species and soil microbial functional groups were especially important in influencing the response matrices but this link was only significant in the three-year old site. In our model for this site, the plant species *Silybum marianum* (L.) Gaertn was responsible for 10% of the soil microbial functional diversity variation ($P = 0.012$). The relative cover of *S. marianum* is positively related with several microbial functional groups belonging to the four types of carbon sources employed: amino acids (NAGA: $R^2 = 0.169$, $P = 0.004$ and alanine: $R^2 = 0.114$, $P = 0.019$), carbohydrates (glucose: $R^2 = 0.189$, $P = 0.002$ and trehalose: $R^2 = 0.106$, $P = 0.024$), carboxylic acids (GABA: $R^2 = 0.127$, $P = 0.013$) and fatty acids (Tween 80: $R^2 = 0.095$, $P = 0.033$). The microbial functional groups responsible of catabolizing the oxalic acid and trehalose, respectively, explained 15% ($P < 0.001$) and 7% ($P = 0.013$) of the soil functioning variation. Their CO₂ rates were positively related with several surrogates of nutrient cycling (NO₃⁻ availability rate: $R^2 = 0.130$, $P = 0.012$ and total N: $R^2 = 0.092$, $P = 0.036$ for oxalic acid; basal respiration: $R^2 = 0.096$, $P = 0.032$; NO₃⁻ availability rate: $R^2 = 0.164$, $P = 0.004$; NH₄⁺ availability rate: $R^2 = 0.185$, $P = 0.002$; total N: $R^2 = 0.098$, $P = 0.030$ and total P: $R^2 = 0.185$, $P = 0.002$ for trehalose).

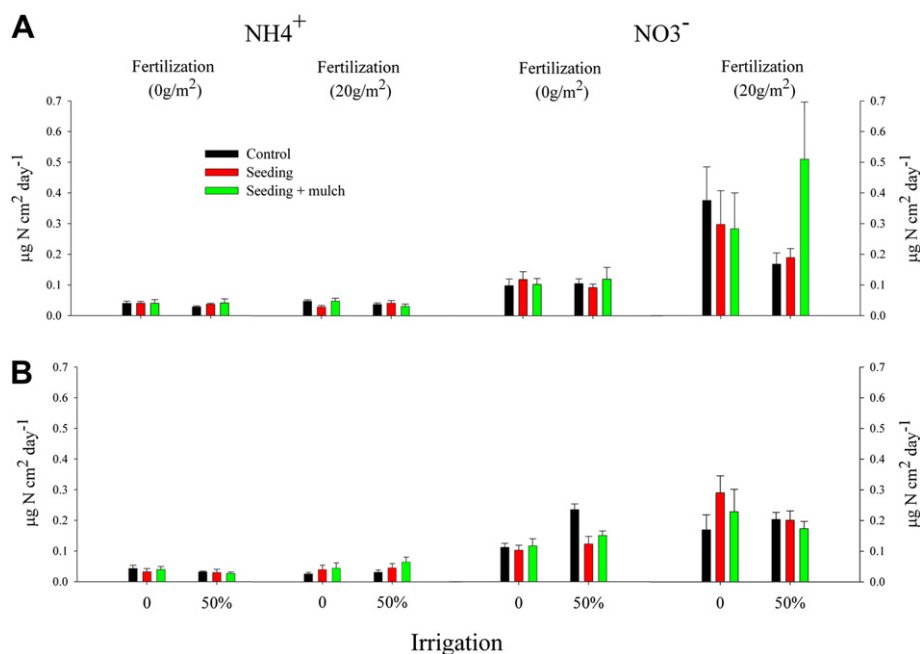


Fig. 2. Treatment effects on inorganic N availability rates (NH₄⁺ and NO₃⁻) in both the recently built (2A) and the three-year old site (2B). Irrigation levels (0 and 50%) correspond to the control and 50% of the monthly precipitation median, respectively, and fertilization levels (0 and 20 g/m²) to the control and the dose applied, respectively. Data represent means ± SE ($n = 60$). Ionic exchange membranes were measured in five blocks only.

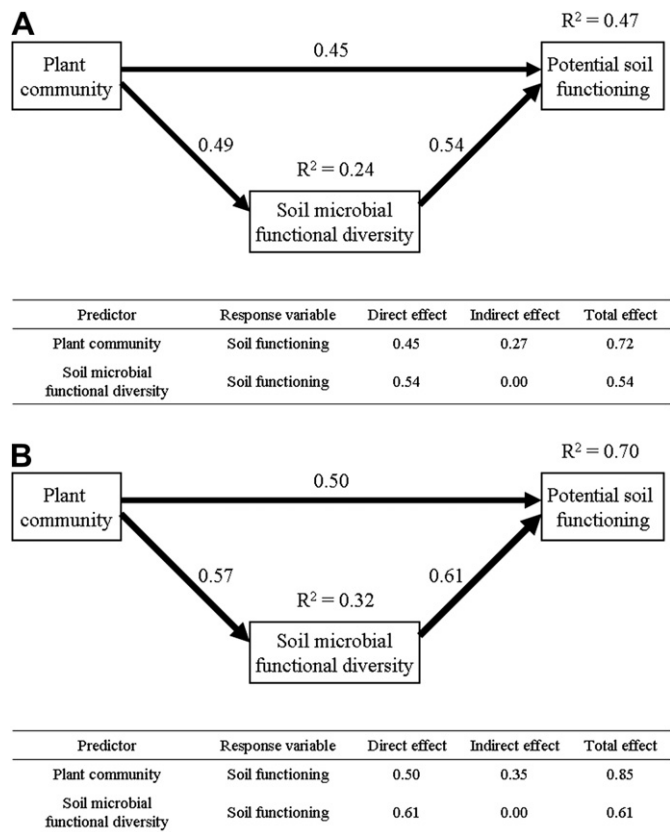


Fig. 3. Results of path analysis, showing the causal relations found between plant community, soil microbial functional diversity and potential soil functioning in both the recently built (3A) and the three-year old site (3B). Numbers adjacent to arrows are partial correlation coefficients, analogous to regression weights among matrices and indicative of the strength of each pathway. Width of arrows is proportional to path coefficients. As in other linear models, R² signifies proportion of variance explained and appears above every response variable in the model. Inset tables reflect the direct, indirect and total effects that one variable may have upon another.

4. Discussion

4.1. Direct effects of roadside slope restoration on soil microbial functional diversity and potential soil functioning

The restoration treatments employed were relatively ineffective in enhancing soil microbial functional diversity in the studied roadside prairies. Irrigation was the only treatment with significant

effects on the microbial functional diversity, with a surprising negative effect on the CO₂ rate of all the carbon sources at the three-year old site. This is an unexpected effect compared to other dry ecosystems of the world, where water availability is one of the major parameters limiting soil microbial activity and function (Wagener and Schimel, 1998; Saul-Tcherkas and Steinberger, 2009). Intense rainfall pulses can cause stress conditions for microbial communities (Fierer et al., 2003) when they come after a dry period (Kieft et al., 1987). However, we do not think that this effect is occurring at our field site because we irrigated in spring, the wettest period in the study area. We do not have a full explanation to this unpredicted result, but we suggest the following hypothetical plant-mediated mechanism. García-Palacios et al. (2010) found a significant increase in plant total cover with irrigation at this site, mainly because of the increase in the cover of the dominant *Carduus tenuiflorus* (32% of total site cover). Wardle et al. (1998) found that *C. tenuiflorus* had one of the lowest leaf N concentrations and specific leaf areas of New Zealand grasslands. These values are related to recalcitrant litter and low decomposition rates (Wardle et al., 1998), which can negatively affect soil biological activity and microbial biomass (Wardle et al., 2004), thereby suppressing the microbial ability in utilizing different carbon sources. Amino acids, such as GABA, arginine, alanine and cysteine, showed the lowest CO₂ rates in the first 6 h incubation period, supporting results found in arid soils from Israel (Berg and Steinberger, 2008). These unutilized substrates within the first incubation period were eventually catabolised in the second incubation after 24 h, probably due to growth of microorganisms in response to the added substrates. The apparent utilization rates of the three carboxylic acids (malic, oxalic and citric) remained equal (Fig. SF.1) or considerably decreased (Fig. SF.2) after 24 h, presumably due to abiotic CO₂ release along the initial 6 h period.

The lack of response of soil functioning surrogates to the restoration treatments applied matched that of microbial functional diversity. Soil N and P increased over time in both sites (data not shown), probably because of the rapid accumulation of dead plant material (Berendse, 1998), but this pattern was not affected by our treatments. Fertilization was able to enhance the availability of *in situ* mineral N, mainly through an increase in the NO₃⁻ availability, in both sites (Fig. 2). This short-term increase in nutrient availability is commonplace on grasslands fertilized during restoration efforts (Baer et al., 2004). However, this soil fertility enhancement only affected plant communities in the recently built site, where fertilization increased the Shannon Index by 67%, an important structural but not functional change (García-Palacios et al., 2010). The NH₄⁺ availability inferred rate was very low at both sites. The volatilization of NH₄⁺ to ammonia, typical of dry

Table 2
Results of the parsimonious models tested in the recently built and the three-year old sites. In each site, the first model represents the causal relation between plant community and soil microbial functional diversity, and the second the causal relation between plant-soil community interactions and potential soil functioning. AIC = Akaike Information Criterion, and % Var = % of variance explained by each predictor (between 0 and 1). Significant R² (P < 0.05) values are shown in boldface.

Site	Response variable	Predictor	AIC	Pseudo-F	P	% Var	R2 (cumulative)
Recently built	Soil microbial functional diversity	<i>Bromus rubens</i> L.	88.66	2.81	0.060	0.06	0.06
		<i>Hirschfeldia incana</i> (L.) Lagr.-Foss.	88.63	1.95	0.105	0.04	0.10
		<i>Melilotus officinalis</i> (L.) Pall.	87.93	2.54	0.054	0.05	0.15
	Soil functioning	<i>Medicago lupulina</i> L.	352.02	2.84	0.071	0.06	0.06
		D-Galactose	351.67	2.26	0.090	0.05	0.10
Three-year old	Soil microbial functional diversity	<i>Silybum marianum</i> (L.) Gaertn.	165.40	5.28	0.012	0.10	0.10
		<i>Lolium rigidum</i> Gaudin	165.27	2.04	0.134	0.04	0.14
		<i>Hirschfeldia incana</i> (L.) Lagr.-Foss.	165.26	1.88	0.149	0.04	0.18
	Soil functioning	Oxalic Acid	362.32	8.37	<0.001	0.15	0.15
		Trehalose	360.16	4.07	0.013	0.07	0.22
		<i>Bromus rubens</i> L.	359.88	2.14	0.106	0.04	0.26
		D-Galactose	359.88	1.83	0.156	0.03	0.29

ecosystems with soil pH higher than 7 (Schlesinger and Peterjohn, 1991), could be an explanation for this pattern. Irrigation modulated the outcome of fertilization in the three-year old site. The application of this treatment may have slightly reduced soil pH, allowing for a certain NH_4^+ accumulation. In contrast, irrigation may reduce fertilization effects on NO_3^- availability through microbial immobilization or nutrient leaching (Gallardo and Schlesinger, 1995). Fertilization is an efficient restoration tool to increase plant diversity because it enhances soil nutrient availability at early stages of secondary succession under poor soil conditions. In contrast, hydroseeding was not able to affect belowground communities and processes, and thus can be considered of little value to improve microsite characteristics for late-successional species, the main goal of this treatment during the restoration of semi-arid roadside slopes (Matesanz et al., 2006).

4.2. Links between aboveground-belowground communities and potential soil functioning, and their application

Because the overall variation in the potential soil functioning data explained by the model was considerably higher in the three-year old site (Fig. 3A and B), we suggest that short-term temporal dynamics of plant-soil interactions play a critical role determining ecosystem development under secondary succession (Kardol et al., 2006). The fact that the total effect of vegetation on potential soil functioning was higher than that of the microbial functional diversity in both sites suggest that the studied slopes are to the greater extent regulated by plants. However, the functional diversity of soil microbes exerted a key influence, as the indirect effects of plant community on potential soil functioning mediated by this variable represented 37 and 41% of the total plant effects in the recently built and three-year old site, respectively. In addition to accounting for a substantial proportion of the total plant effects, soil microbial functional diversity exhibited the greatest direct influence on potential soil functioning at both sites ($r = 0.54$ and 0.61 , respectively). These results point to a key influence of the functional diversity of soil microbes on soil functioning in roadside prairie slopes, and suggest that soil microbes are not completely controlled by the plant community during the earliest stages (0–5 years) of ecosystem development. Therefore, in order to enhance successional rates in newly developing soils such as roadside prairies, management practices should be evaluated in the context of plant-soil interactions.

Our parsimonious models (Table 2) showed that some plant species and soil microbial functional groups had more influence than others in the three-year old site. This influence accounted for an average of 31% of the overall variance explained in the model for both soil microbial functional diversity and soil functioning. The cover of *S. marianum* was positively related with several microbial functional groups belonging to the four types of carbon sources employed. Although this is a causal and not a direct result, it suggests that the seeding of this species could be an appropriate restoration tool to increase the functional diversity of microbial communities. However, *S. marianum* is a common weed in roadside slopes capable of establishing itself in tall dense patches that eliminate other plant species by shading or competition for water and nutrients (Young et al., 1978). Therefore we cannot recommend its application as a restoration tool. The microbial functional groups responsible of catabolizing oxalic acid and trehalose were positively related with several surrogates of nutrient cycling. This result suggests that the processes related with soil functioning evaluated can be altered via the enhancement of soil microbial functional diversity. Although not evaluated in this study, the inoculation of microbial communities from functionally diverse soils could be an option to enhance the restoration success of degraded roadside

slopes (Graham, 2005), and facilitate the establishment of late-successional plant species (Kardol et al., 2006).

During the initial phases of succession on nutrient-poor, mineral substrates, plant diversity alters microbial community composition and functioning, which in turn, increases the supply of soil N to plants (Berendse, 1998). This feedback is likely to be most evident in ecosystems with low organic matter content such as studied, where existing substrate pools in the soil are small relative to amounts entering soil from plant detritus (Zak et al., 2003). The rapidly changing vegetation and soil dynamics occurring in early-successional stages in roadside prairie slopes enhance the importance that plant-soil interactions have to drive soil functioning. However, more time is probably needed to recover rates of soil functioning similar to nearby natural areas. The restoration of recently built slopes can potentially be improved with treatments that promote plant compositional shifts, such as fertilization, or that alter soil function, such as the enhancement of soil microbial functional diversity. The necessity of intensive and costly management interventions should be confronted with the ability of plant-soil interactions to improve the restoration of these harsh environments along succession.

4.3. Conclusions

In conclusion, our results indicate that restoration treatments based on the increase in the availability of resources (seeds, nutrients and water) do not enhance neither soil microbial functional diversity nor key soil processes related to nutrient cycling. They also suggest that the plant community is not strongly controlled by treatments, but rather by the identity of early colonizers which have attained dominance. These plants go on to exert strong influences on belowground processes and organisms. Plant-soil biota interactions are capable of explaining potential soil functioning in roadside prairie slopes after only a few years of secondary succession, and therefore plant-soil interactions are of major importance to determine the restoration of these emerging ecosystems. However, our study is not without limitations. We acknowledge that our results are highly-dependent on the methods employed, and that they are limited by the one-time measure of soil variables and microbial functional diversity; therefore we can only talk about potential soil functioning. The MicroResp™ system does not include information about taxonomic or phylogenetic diversity of soil microbial communities (Øvreås, 2000). Rather, it provides useful information for plant-soil interaction studies aiming to evaluate how changes in plant community composition, and thus in the quality of plant inputs to the soil, affect the functioning of microbial communities (Lagomarsino et al., 2007). The use of higher resolution methods to assess soil microbial communities, such as molecular tools (Fierer et al., 2010), and the direct measurements of soil functions instead of the surrogates employed, such as N mineralization (Allen et al., 1986), could improve our models, increasing their ability to investigate plant-soil interactions along successional gradients.

The effect of plant-soil interactions upon soil functioning was evaluated using casual relationships, which predictive value is always contingent on the causal structure imposed by the modelers (Antoninka et al., 2009). Our model is only valid for the type of relations we are evaluating, but does not deny the possibility of other kind of relations within our study system (e.g. feedbacks). In this regard, our modeling approach should be considered as a guide in identifying essential interactions on which empirical efforts should be targeted to confront the results obtained with purely observational approaches such as that employed in this study (Grace, 2006). Instead of implementing standard treatments, restoration projects should focus on plant-soil interactions as an

important process that can be manipulated for restoration purposes (Harris, 2009), especially in nutrient-poor, semi-arid ecosystems such as studied.

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Appendix. Supplementary material

Supplementary material associated with this paper can be found, in the online version, at doi:10.1016/j.soilbio.2011.02.014.

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