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Impacts of the alien trees *Ailanthus altissima* (Mill.) Swingle and *Robinia pseudoacacia* L. on soil nutrients and microbial communities

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ABSTRACT

Ailanthus altissima (Mill.) Swingle and *Robinia pseudoacacia* L. are two aggressive invasive trees in riparian areas in Central Spain. We aim to test whether soil properties, nutrient mineralization rates and soil bacterial communities of riparian forest dominated by the native *Populus alba* L. can be altered by the presence of *A. altissima* or *R. pseudoacacia*. In autumn 2011 and spring 2012 we conducted a field soil sampling in three sites where invasive and native trees were paired. In addition, in a 6-month greenhouse experiment (GHE), we grew *A. altissima*, *R. pseudoacacia* and *P. alba* from seeds in a soil collected from a native area. We quantified soil organic matter (OM), nitrogen (N), phosphorous (P), nitrate (NO_3^-–N), ammonium (NH_4^+–N), pH, potential net ammonification and nitrification rates, phosphomonoesterase (PME) activity and the composition of soil bacterial community in soils from the field study and from the GHE. Both the field and the GHE results showed the capability of *A. altissima* to decrease soil total N and of *R. pseudoacacia* to increase soil mineral N. In the field, all invaded soils had greater NO_3^-–N than *P. alba* soils. *R. pseudoacacia* field soils had greater PME activity, total N and net ammonification rates while *A. altissima* soils had lower OM, NH_4^+–N, net nitrification and total N mineralization rates than those of *P. alba*. Differences in the composition of soil bacterial communities were only found in the field, being more evident between *A. altissima* and *P. alba* than between *R. pseudoacacia* and *P. alba* field soils. Symbiotic N_2 fixation could explain the capability of *R. pseudoacacia* to increase soil mineral N, while the potential of *A. altissima* to decrease total soil N may be attributed to changes in the balance between N input and losses from the soil. Although the GHE results indicated that the invasive trees can start changing soil conditions during early stages of establishment, more impacts found in the field study suggests that several soil properties, the composition of soil bacteria communities and microbial activities need longer time since invasion to be altered.

1. Introduction

Invasive plants are introduced alien species able to establish viable populations and to expand to areas distant from the sites of introduction (Richardson et al., 2000). Once established, invasive plants may affect native plant communities by reducing their diversity and abundance (Vilà et al., 2011). This effect may be caused directly by allelopathy and competition for resources (Callaway and Ridenour, 2004; Vilà and Weiner, 2004; Maron and Marler, 2008) or indirectly by modifying the environment to the detriment of native species in their own benefit (Haubensak and Parker, 2004; Niu et al., 2007). Recent reviews suggest that the overall effect of invasive plants is an increase of nutrient pools and acceleration of fluxes (Ehrenfeld, 2003; Liao et al., 2008; Vilà et al., 2011; Castro-Díez et al., 2014). The alteration of ecosystem properties by plant invaders may also increase the habitat invasibility for other plants in a process named “the invasion meltdown” (Simberloff and Von Holle, 1999; Von Holle et al., 2006). Moreover, the effects of invasive species may persist during years after the invader removal maintaining the risk of invasion and hampering the recovery of the

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ecosystem or restoration with native plants (Marchante et al., 2009; Von Holle et al., 2013).

Invasive plants may change the composition and activity of microbial communities. For instance, Hawkes et al. (2005) found that invasive grasses increased the abundance and change the composition of ammonium oxidizing bacteria, which caused greater nitrification rates in soil. Likewise, changes in soil pH caused by different plant species can alter the composition of the microbial community (Thoms and Gleixner, 2013). Greater impacts on the composition and activity of soil microbial communities can be produced by invasive species differing from natives in nitrogen use strategies (Boudsocq et al., 2012), allelochemical compounds (Callaway et al., 2008; Lorenzo et al., 2013) or the quantity and chemical composition of plant tissues and root exudates (Wolfe and Klironomos, 2005; Rodgers et al., 2008; Weidenhamer and Callaway, 2010). Moreover, the establishment of positive plant–soil–microbe feedbacks in the invaded range is considered as a cause of the invasion success of some species and a mechanism to alter microbial communities (De la Peña et al., 2010; Rodríguez-Echeverría et al., 2013). For instance, invasive species may accumulate soil pathogens, affecting native plants (e.g. the invasive weed, *Chromolaena odorata*, increased the abundance of the soil pathogenic fungi, *Fusarium semitectum* (Mangla et al., 2008)). Invasive species may also disrupt belowground mutualisms between native plants and arbuscular mycorrhizal fungi (Reinhart and Callaway, 2006) or symbiotic nitrogen-fixing bacteria (Rodríguez-Echeverría, 2010; Rodríguez-Echeverría et al., 2012). Lastly, plant invasion may also lead to the introduction of exotic soil mutualistic microorganisms (Rodríguez-Echeverría et al., 2011; Nuñez and Dickie, 2014).

Riparian forest ecosystems are highly susceptible to plant invasion due to their more buffered temperatures and moister conditions compared with surrounding ecosystems (Hood and Naiman, 2000). In addition, human activities, such as river canalization or flood regulation, cause the decline of native vegetation which forms gaps, giving exotic species a chance to be established (Zedler and Kercher, 2004). In the Iberian Peninsula, the invasive trees *Ailanthus altissima* (Mill.) Swingle (Simaroubaceae) and *Robinia pseudoacacia* L. (Fabaceae) are found colonizing riparian ecosystems (Castro-Díez et al., 2009, 2012). *A. altissima* is native to China and North Vietnam while *R. pseudoacacia* is native to Appalachian Mountains (Southeast of USA) (Kowarik and Säumel, 2007; Cierjacks et al., 2013). They are both included in the Spanish Atlas of Invasive Plants and considered among the 20 most harmful species in Spain and among the 100 worst invasive species in Europe (Sanz Elorza et al., 2004; GEIB, 2006; DAISIE, 2009). Both species have allelopathic compounds in their tissues (Kowarik and Säumel, 2007; Cierjacks et al., 2013) and they both have shown the ability to increase soil nitrate concentration and net nitrification rates in nutrient-poor soils likely due to high quality leaf litter of *A. altissima* and the ability of *R. pseudoacacia* to fix N_2 from the atmosphere (Rice et al., 2004; Gómez-Aparicio and Canham, 2008; Von Holle et al., 2013). However, the effect of both species on the composition of soil microbial communities together with soil properties and nutrient mineralization rates in riparian ecosystems remains unexplored even when soil microorganisms control important ecosystem processes, such as mineralization of soil organic matter or soil nitrate production and assimilation (Booth et al., 2005; Myrold and Posavatz, 2007).

The aim of this study was to assess the effects of the invasive trees, *A. altissima* and *R. pseudoacacia*, on soil properties and on the structure and activity of soil bacterial communities of riparian forest dominated by the native tree *Populus alba* L. (Salicaceae). We used two complementary approaches: 1) a field study comparing soil properties between invaded and paired non-invaded sites (*A. altissima*–*P. alba* and *R. pseudoacacia*–*P. alba*) and 2) a greenhouse experiment (GHE), where the invaders *A. altissima* and *R. pseudoacacia* and the native *P. alba* were grown for six months in a native soil. It is possible that there were pre-existing soil characteristics, which contributed to invasion of the tree species (Dassonville et al., 2008). Therefore, GHE growing exotic invasive trees in non-invaded soils allow to distinguish if differences observed in the field are due to the presence of the plant invader or to the preexisting site conditions (Ehrenfeld et al., 2001).

2. Materials and methods

2.1. Field sampling

The study was conducted in the riparian zone of the Henares River (Tagus Basin, Central Spain), where *P. alba* is the dominant tree, which is accompanied by other native tree species, such as *Tamarix gallica*, *Salix alba*, *Populus nigra*, *Fraxinus angustifolia* and *Ulmus minor* (Martínez, 2000). We selected five sites (Table 1) where invaded patches by *A. altissima* or *R. pseudoacacia* were close to native patches (i.e. vegetation dominated by *P. alba*). Two sites were invaded by the exotic *A. altissima* (Chiloeches and Guadalajara), two by *R. pseudoacacia* (El Encín and Jadraque), and one invaded by both exotic trees (El Val). In this way we had three sites (replicates) per species. Geographical coordinates, a soil taxonomic classification and vegetation cover of each study site are shown in Table 1. In each patch we selected five adult dominant trees (*P. alba*, *A. altissima* or *R. pseudoacacia*), which were considered as pseudoreplicates. Below the canopy of each tree, soil samples were collected in the seasons with greatest microbial activity, i.e. autumn (12–15 December 2011) and spring (17–25 April 2012). Each soil sample consisted of the mixture of four sub-samples taken at 1 m distance around the tree trunk by means of a metallic rectangular core (11 cm depth, 7.5 cm width). Soil samples were kept in polyethylene bags and carried to the lab, spread on trays, air-dried at room temperature, sieved (1 mm mesh) (Hawkes et al., 2005; Niu et al., 2007; Lorenzo et al., 2010) and divided in two parts. One was stored at −32 °C for bacterial DNA analyses and the other part was conserved at 4 °C for the analysis of soil properties (nutrients, percentage of organic matter, pH and mineralization rates). In the autumn, the litter layer (a 21 cm diameter surface) above each of the four soil subsamples was taken and pooled in a single sample. Litter samples were kept in paper bags and brought back to the lab, divided by plant part (leaf and woody), oven dried (60 °C $\geq$ 48 h) and weighed (Balance Sartorius BP211D, 0.0001 g) (Table 1).

In December 2011, we measured the basal perimeter of the selected trees (Table 1). In April 2012, we established a square plot (4 × 4 m) containing each sampling tree in the centre. Within all square plots the canopy cover of the sampling tree species was greater than 75%. In each square plot, we registered the herbaceous and shrubs species cover (%), as well as the cover of the sampling tree seedlings and saplings (Table 1).

2.2. Greenhouse experiment

Soil from a non-invaded area dominated by *P. alba* in the Jadraque site (Table 1) was collected on 27 April 2012 to fill five 0.5 L plastic pots per species (*A. altissima*, *R. pseudoacacia* and *P. alba*). As a control, five additional pots were left without plants during the six-month experiment (named as “control t6”). In addition, an aliquot of the soil sample was taken at the beginning of the experiment (named as “control t0”). Seeds were collected in the field from at least 5 trees per species. Seeds were disinfected with 10% bleach. *R. pseudoacacia* seeds were subsequently scarified mechanically with sand paper and wings of

Table 1
Geographical coordinates, altitude and soil classification of the study sites (CH = Chiloeches, G = Guadalajara, J = Jadraque, E = El Encín, V = El Val) and mean values (±SE, N = 5) of plant variables at understory level in the study patches: invaded (I) by *Ailanthus altissima* or *Robinia pseudoacacia* and dominated by the native *Populus alba* (N).

Site name	Longitude	Latitude	Altitude (m)	Soil classification (dominant soil + associated soils)[a]	Patch[b]	Dominant tree species	Herbaceous cover (%)	Shrubs cover (%)	Cover of tree seedlings/saplings (%) (DBH < 7 cm)[c]	Leaf litter (g)	Woody litter (g)	Total litter (g)
CH	3° 13′ W	40° 34′ N	609	Calcic Cambisol + Calcaric Regosol	I	*A. altissima*	33 ± 11	13 ± 13	70 ± 17	58 ± 6	15 ± 2	73 ± 9
					N	*P. alba*	90 ± 4	13 ± 5	0 ± 0	25 ± 2	16 ± 3	41 ± 4
G	3° 11′ W	40° 37′ N	633	Calcic Cambisol + Calcaric Regosol	I	*A. altissima*	44 ± 11	0 ± 0	66 ± 5	101 ± 8	133 ± 18	235 ± 19
					N	*P. alba*	75 ± 4	0 ± 0	38 ± 5	155 ± 18	61 ± 11	215 ± 21
J	2° 56′ W	40° 56′ N	800	Calcic Cambisol + Euthric Litosol + Rendzina + Chromic Luvisol	I	*R. pseudoacacia*	92 ± 4	10 ± 5	26 ± 5	43 ± 4	30 ± 7	80 ± 10
					N	*P. alba*	98 ± 2	0 ± 0	0.2 ± 0.2	89 ± 23	38 ± 6	127 ± 23
E	3° 17′ W	40° 31′ N	594	Calcaric Fluvisol + Calcic Cambisol + Gleyo-calcaric Fluvisol	I	*R. pseudoacacia*	48 ± 14	0 ± 0	34 ± 9	55 ± 4	31 ± 12	99 ± 11
					N	*P. alba*	3 ± 1	0 ± 0	38 ± 5	72 ± 8	24 ± 7	97 ± 2
V	3° 20′ W	40° 29′ N	587	Calcaric Fluvisol + Calcic Cambisol + Gleyo-calcaric Fluvisol	I	*A. altissima*	86 ± 3	0 ± 0	24 ± 7	49 ± 10	47 ± 12	99 ± 18
					I	*R. pseudoacacia*	92 ± 1	0 ± 0	0 ± 0	44 ± 12	46 ± 12	99 ± 24
					N	*P. alba*	29 ± 6	9 ± 2	25 ± 8	57 ± 5	22 ± 4	79 ± 7

[a] FAO/IIASA/ISRIC/ISSCAS/JRC, 2012. *Harmonized World Soil Database (version 1.2)*. FAO, Rome, Italy and IIASA, Laxenburg, Austria.
[b] I – Invaded, N – Non-invaded.
[c] Only for the dominant tree species.

A. altissima seeds were removed. Seeds from *P. alba* were not manipulated before sowing. On 4 May 2012 we sowed the filled pots with seeds from the three target species to have one plant per pot. Sowed and control t6 pots were kept in an incubation chamber at 20 °C with 12–12 h dark–light photoperiod. After 21 days, pots were moved to an experimental outdoors plot with 65% of full sunlight (González-Muñoz et al., 2011) placed in the Botanical Garden of Alcalá de Henares (Madrid, Spain). Pots were irrigated when necessary and weeds were removed. Periodically the position of the pots was randomly changed to reduce the effect of micro-environment. After 182 days (six months), we harvested the above and belowground part of plants and took four soil subsamples with 1-cm diameter PVC tubes per pot, following Ehrenfeld et al. (2001). Soil samples were air-dried and divided in two parts to perform bacterial DNA and soil properties analyses (see field study in the above section). The remaining soil in the pot was preserved at −32 °C until it was washed in a sieve to separate the roots from the soil. Plants and clean roots were oven-dried at 60 °C for at least 48 h and weighed (plant above and belowground biomass). The above- and belowground biomass ratio was calculated.

2.3. Soil properties

To analyze total nitrogen (N) and phosphorus (P) concentration, 0.5 g of soil were digested with H_2SO_4 and Cu–KSO_4. To analyze nitrate (NO_3^-–N) and ammonium (NH_4^+–N), 5 g of soil were mixed with 100 mL KCl 2 N and shaking the mix for 2 h. The solution was filtered with 0.45 μm Millipore filters and preserved at −20 °C until analysis (Allen et al., 1986). The digested solution (total N and P) and the KCl solution (NH_4^+–N and NO_3^-–N) were analyzed with segmented flux autoanalyzer (Skalar San ++). The ratio between NO_3^-–N and NH_4^+–N concentration (NO_3^-–N:NH_4^+–N) was calculated. Total mineral N was calculated as the sum of NO_3^-–N and NH_4^+–N. The percentage of total N belonging to organic N was calculated as: Organic N(%) = 100 × [(total soil N − total mineral N)/total soil N]. The organic matter (OM) of the soil was determined by weighing the dry soil (at 105 °C, ≥48 h) before and after ignition at 400 °C for 24 h (Nelson and Sommers, 1973). To measure the soil pH, 20 g of soil were mixed with 40 mL of distilled water (slurry texture) (Allen et al., 1974). The mixture was measured for pH using a pH-meter (micropH 2001, Crison Instruments, Barcelona, Spain).

2.4. Mineralization rates

The potential net nitrification, ammonification and mineralization rate of soil N were assessed as the difference in NO_3^-–N, NH_4^+–N, and the sum of both, respectively, in the soil before and after incubation at 30 °C for 14 days. Two aliquots of 5 g per soil sample (both from the field and the experimental pots) were taken and mixed up with 15 g of washed sand (SiO_2, Panreac) in a 200 mL polypropylene bottle. In one of the soil aliquots, mineral N was extracted immediately while in the other aliquot mineral N was extracted with 6 mL of distilled water after incubation, at 30 °C for 14 days. Mineral N was extracted with 100 mL KCl 2 M during 2 h in a shaker. The solution was then filtered through 0.45 μm Millipore filters and preserved at −20 °C until analysis (Allen et al., 1986). NH_4^+–N and NO_3^-–N were analyzed with segmented flux autoanalyzer (Skalar San ++).

The potential mineralization rate of soil P was estimated by the activity of the acid phosphomonoesterase (PME) enzyme, which is the main enzyme responsible for organic P mineralization (Olander and Vitousek, 2000). This activity was determined as the liberation of p-Nitrophenol (*p*-NP) from p-Nitrophenyl phosphate (*p*-NPP), which is an organic phosphate analog. Five grams of each sample (both from the field and the GHE) were mixed with 0.2 mL of 0.1 M of maleato buffer at pH of 6.5 and 0.5 mL substrate (*p*-NPP). The mixture was incubated at 30 °C for 90 min. The reaction was stopped with cold temperature (4 °C, 15 min) and then 2 mL of NaOH and 0.5 mL of $CaCl_2$ 0.5 M were added to get an alkaline pH (pH ≈ 9) where *p*-NP forms a yellow colour. The mixture was centrifuged at 3000 rpm for 15 min. The *p*-NP concentration was measured with a spectrophotometer at 398 nm. The PME activity was expressed as the μmol of *p*-NP produced per g dry soil per hour.

2.5. DNA extraction and amplification and Denaturing Gradient Gel Electrophoresis analysis (DGGE)

DNA was extracted from each soil sample leading to total of 110 extractions (5 trees × 11 patches × 2 seasons) from the field and to 20 extractions ((5 × 3 trees) + 5 controls) from the GHE. Soil DNA was extracted from 0.25 g of soil per sample using a PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., CA). Specific eubacteria primers (primer 1, 5′-C CTACGGGAGGCAGCAG-3′; primer 2, 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGG-GAGGCAGCAG-3′) were used to amplify 16S rRNA genes (Muyzer

et al., 1993) from total DNA extracted. All reactions were carried out in a final volume of 25 μL containing 2.5 μL of buffer (160 mM $(NH_4^+)_2SO_4$, 670 mM Tris–HCl pH 8.8, 0.1% Tween-20, 25 mM $MgCl_2$) (BIORON, Germany), 400 nM of each primer, 200 μM dNTPs, 0.5 U of DFS-Taq polymerase (BIORON, Germany), and 1 mL of template DNA. The PCR conditions were: an initial denaturing step at 94 °C for 5 min followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, followed by a final extension step at 72 °C for 30 min. The size and integrity of PCR fragments were checked in agarose gel electrophoresis (1%, w/v) stained with GelRed™. All PCRs were performed using a GeneAmp 9700 (Applied Biosystems, PerkinElmer, CA, USA). For field-collected samples, equal volumes of each PCR were mixed to get a single sample per season, site and tree species. In this way we mixed variability of pseudo-replicates but maintained three replicated sites per species. Each mixture was analyzed by Denaturing Gradient Gel Electrophoresis (DGGE). For GHE samples, individual PCR products were run using DGGE. We performed DGGE with a DGGE-2001 system from CBS Scientific (CA, USA). 15 μL of each PCR product (or mix of PCR products in the case of the field samples) was used for DGGE analysis. Gels contained 8% (w/v) acrylamide and a linear gradient of 45–68% denaturant were used. The 100% denaturing acrylamide was defined as containing 7 M urea and 40% (v/v) formamide. Gels (22 cm × 17 cm) were run in 21 L 1 × TAE buffer at 20 V for 15 min, followed by 16 h at 70 V and maintained at a constant temperature of 60 °C. Gels were stained for 20 min in 1× GelStarR® and destained for 30 min in distilled water prior to visualization. Gel Compar II (Applied Maths, Belgium) was used to obtain the DGGE gel bands on digitalized images of the gels.

2.6. Statistical analysis

For each invaded-native situation (*A. altissima*–*P. alba*, *R. pseudoacacia*–*P. alba*), soil samples collected in the field below native and invasive trees were ordered according to their values of soil variables (soil properties and mineralization rates) using redundancy analysis (RDA), which is a linear constrained form of the principal component analysis (PCA) (Lepš and Šmilauer, 2003). We used species, season, total litter and tree basal perimeter as potential predictors. Permutations for the Monte Carlo test (9999) were conducted and restricted according to three independent sites replicated across two seasons (spring and autumn). Site was included as a covariate to account for its variability. Temporal dependency in data due to repeated measures was accounted for in the restriction of permutations at the whole-site level. In support to the direct ordination analysis we constructed a *t*-value biplot for species which allowed us to test and plot the relationship between the predictor variable (species) and each soil variable in a multivariate plot. This analysis assumes that the relationship between the predictor variable and each soil variable is significant ($P < 0.05$) if *t* values of respective regression coefficients are >2 units. Significant relationships between soil variables and predictors are indicated with circles to distinguish positive from negative relationships (Van Dobben circles; Lepš and Šmilauer, 2003). Vectors (soil variables) fully falling within a Van Dobben circle indicate that they are significantly different between two levels of the predictor variable (species) (Lepš and Šmilauer, 2003). Similarly, greenhouse soil samples were ordered according to their values of soil variables using RDA, with species as potential predictor. *t*-Value biplots were also constructed to compare soil properties of control t6 samples with soil samples conditioned by the different tree species (*A. altissima*, *R. pseudoacacia* and *P. alba*). In addition, *t*-value biplots were constructed to compare soil properties between samples conditioned by the growth of invasive species and those conditioned by the growth of the native *P. alba*. Plant biomass was compared across species using one-way ANOVA.

Bacterial richness was calculated as the total number of DGGE gel bands per sample. Bacterial diversity was estimated from the total number of bands and their relative intensity following Lorenzo et al. (2010). Gel bands were classified according to their intensity in four categories. Diversity was calculated using a modification of the Shannon index, $H' = -\sum[(n_i/N)\mathrm{Ln}\,(n_i/N)]$ where n_i had one of four possible values (1–4) depending on band intensity (Lorenzo et al., 2010). Statistical differences in soil bacteria richness and diversity of soils from field-collected samples were assessed using two-way ANOVA with species and season as fixed factors. To assess differences in diversity and richness of bacterial communities of soils from the experiment, one way ANOVA with species as fixed factor was used. Differences in soil bacteria community based on the DGGE results were assessed by Non-metric Multidimensional Scaling (NMDS) analysis, one of the most effective ordination methods for ecological community data (McCune and Grace, 2002). The sample positions on the NMDS biplot were calculated using Sorensen distances from the original data. Pair-wise correlations between NMDS axes and soil variables were performed in order to know the likely effect of the different soil variables on the structure of bacterial communities. To perform these pair-wise correlations we used for each soil variable the mean of 5 trees per site and tree species in the field study (N = 3) and individual replicates of each soil variable in the GHE (N = 5). RDA and *t* value biplots were conducted in CANOCO 4.5 (Lepš and Šmilauer, 2003). ANOVA was conducted using R package 3.0.2 (R Development Core Team, 2011). NMDS were performed using Community Analysis Package (CAP) 2004, V. 3.1 (www.pisces-conservation.com). Pair-wise correlations were performed in JMP, Version 7 (SAS Institute Inc., Cary, NC, 1989–2007).

3. Results

3.1. Soil properties and mineralization rates

In the field study, soil from patches invaded by *A. altissima* had higher NO_3^-–N and NO_3^-–N:NH_4^+–N ratio and lower total N, OM, NH_4^+–N, PME activity, potential rates of net nitrification and total N mineralization than soils collected in adjacent native *P. alba* patches (*t*-value biplot, $P < 0.05$) (Fig. 1a). The lower soil total N was attributed to lower organic N in *A. altissima* (98.14 ± 0.17%) than in *P. alba* (98.88 ± 0.09%) field soils (Table S1). Patches invaded by *R. pseudoacacia* had higher soil NO_3^-–N, NO_3^-–N:NH_4^+–N ratio, total N, total mineral N, potential net ammonification rate and PME activity (*t*-value biplot, $P < 0.05$) than adjacent *P. alba* patches (Fig. 1b). In this case, the higher soil total N was attributed to higher mineral N in *R. pseudoacacia* than in *P. alba* field soils (Fig. 1b).

In the GHE, after six months of growth in a native soil, *P. alba* showed the greatest mean plant biomass, although differences were marginally significant (Table 2). The three species differed in the allocation between above and belowground biomass, the ratio being the largest in *R. pseudoacacia* and the smallest in *A. altissima*. *P. alba* showed an intermediate value which did not differ from either of the exotic trees (Table 2). Compared with *P. alba* soil, *A. altissima* soil had lower total N (*t*-value biplot, $P < 0.05$) (Fig. 2a). The lower soil total N was attributed to lower organic N in *A. altissima* (98.17 ± 0.19%) than in *P. alba* (98.24 ± 0.25%) field soils (data not shown). *R. pseudoacacia* soil had greater NH_4^+–N, NO_3^-–N and total mineral N while lower OM and rates of potential net nitrification, ammonification and total N mineralization (*t*-value biplot, $P < 0.05$) (Fig. 2b). Compared with t6 control soils, the growth of the native *P. alba* increased soil total N, potential net nitrification and N mineralization rates, while decreased soil

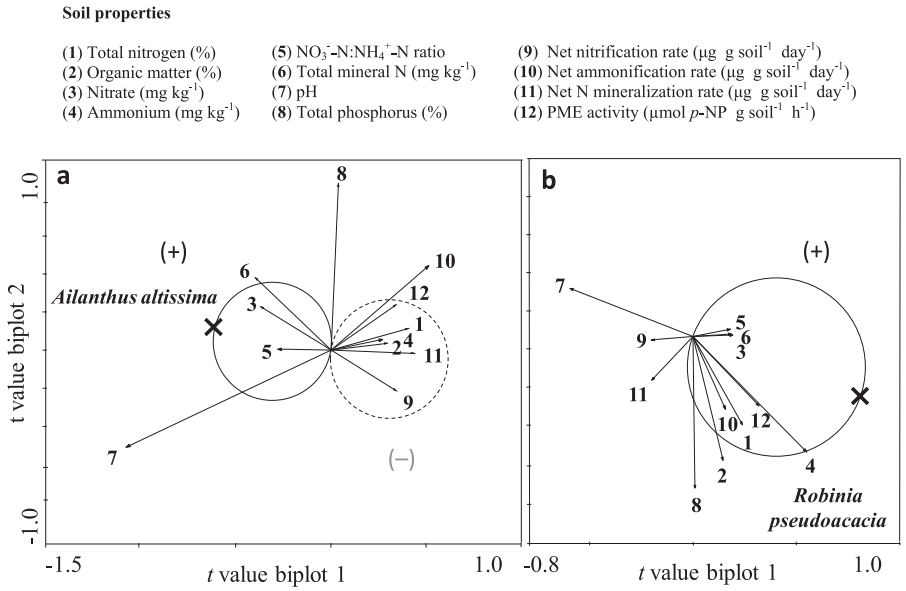

Fig. 1. Field sampling: t-value biplots for the relationship between soil properties (vectors with numbers) and the invasion of *Ailanthus altissima* (a) and *Robinia pseudoacacia* (b) compared with non-invaded patches (dominated by *Populus alba*). Crosses indicate the centroids of the invasive trees, *A. altissima* and *R. pseudoacacia*. Soil property vectors fully falling within a Van-Dobben circle (Lepš and Šmilauer, 2003) indicate a significant relationship between the focal soil property and the presence of the invasive species: *A. altissima* (a) and *R. pseudoacacia* (b). Relationships in t value biplots may be positive (solid line circle) or negative (dashed line circle) and are based on t values of regression coefficients of soil properties expressed as linear combinations of *A. altissima* or *R. pseudoacacia* presence. Mean (±SE) values of soil variables used are available in Supplementary material (Table S1).

NO_3^-–N concentration and total mineral N (*t*-value biplot, $P < 0.05$) (Fig. 2c). The growth of *A. altissima* increased soil net N mineralization rate and decreased soil total mineral N (*t*-value biplot, $P < 0.05$) (Fig. 2d). However, the growth of *R. pseudoacacia* increased soil total N and PME activity while decreased soil OM (*t*-value biplot, $P < 0.05$) (Fig. 2e).

3.2. Soil bacterial community

The NMDSs performed with the data obtained from DGGE of field soil samples had stress values lower than 0.20 and therefore allowed reliable interpretations (McCune and Grace, 2002). The composition of field soil bacterial communities differed between invaded and non-invaded patches (Fig. 3). Both in autumn and spring, *A. altissima* and *P. alba* soil bacterial communities were separated by axis 2, while sites were separated along axis 1 (Fig. 3a and b). Axis 2 of the NMDS for autumn was negatively correlated with soil NO_3^-–N concentration ($r^2 = -0.91$, $P = 0.013$) and NO_3^-:NH_4^+–N ratio ($r^2 = -0.94$, $P = 0.006$), and positively correlated with potential net nitrification rate ($r^2 = 0.93$, $P = 0.008$) (Fig. 3a). These correlations indicated that in autumn, the soil bacterial community in *A. altissima* patches was related with greater NO_3^-–N concentration and NO_3^-:NH_4^+–N ratio and with lower net nitrification rate (i.e. greater NO_3^-–N assimilation). However, in spring we found a different interaction since axis 1 of the NMDS was positively correlated with potential net ammonification rate ($r^2 = 0.95$, $P = 0.004$) and PME activity ($r^2 = 0.87$, $P = 0.023$) (Fig. 3b), indicating that the bacterial community of Guadalajara was related to greater net ammonification rates and PME activity, as compared to Chiloeches and El Val sites. In the case of *R. pseudoacacia*–*P. alba* sites, axis 1 of the NMDS plot mostly separated sites, but differences between invaded and non-invaded plots within sites were more subtle (Fig. 3c and d). The largest difference between invaded and native soils was found in El Encín in spring (Fig. 3c). The axis 2 of the NMDS for spring soil samples was positively correlated with soil NH_4^+–N concentration ($r^2 = 0.88$, $P = 0.020$) and PME activity ($r^2 = 0.93$, $P = 0.008$) (Fig. 3d). In the GHE, NMDS plot did not show clear differences in bacterial community composition among plant species (Fig. 4). Axis 1 was positively correlated with NH_4^+–N (r = 0.55, $P = 0.010$) and total mineral N ($r = 0.45$, $P = 0.039$) concentrations and negatively with net ammonification rate ($r = -0.50$, $P = 0.020$). The remaining soil variables did not significantly correlate with NMDS axes ($P > 0.05$). In the field, species richness and diversity of soil bacteria did not differ between invaded (either by *A. altissima* or *R. pseudoacacia*) and non-invaded patches but both variables were greater in spring than in autumn (Fig. S1). Similarly, in the GHE there were not significant differences in bacterial richness or diversity between soils where the different species grew (Fig. S2).

4. Discussion

Both the field and the GHE results consistently indicated the capability of invasive species to alter some soil properties.

Table 2
Values (mean ± SE, N = 5) of total, aboveground and belowground plant biomass and the ratio between above- and belowground biomass (A:B ratio) for each plant species after six months of growth in the greenhouse experiment.

Variables	*A. altissima*	*R. pseudoacacia*	*P. alba*
Plant aboveground biomass (g)	0.61 ± 0.12 b	0.89 ± 0.15 ab	1.15 ± 0.08 a
Plant belowground biomass (g)	1.26 ± 0.13 ab	0.87 ± 0.13 b	1.34 ± 0.11 a
Total plant biomass (g)	1.87 ± 0.19 a	1.76 ± 0.25 a	2.48 ± 0.16 a
A:B ratio	0.49 ± 0.11 b	1.07 ± 0.19 a	0.87 ± 0.07 ab

Different letters in a row mean significant differences between species (ANOVA, Tukey HSD test, $P < 0.05$).

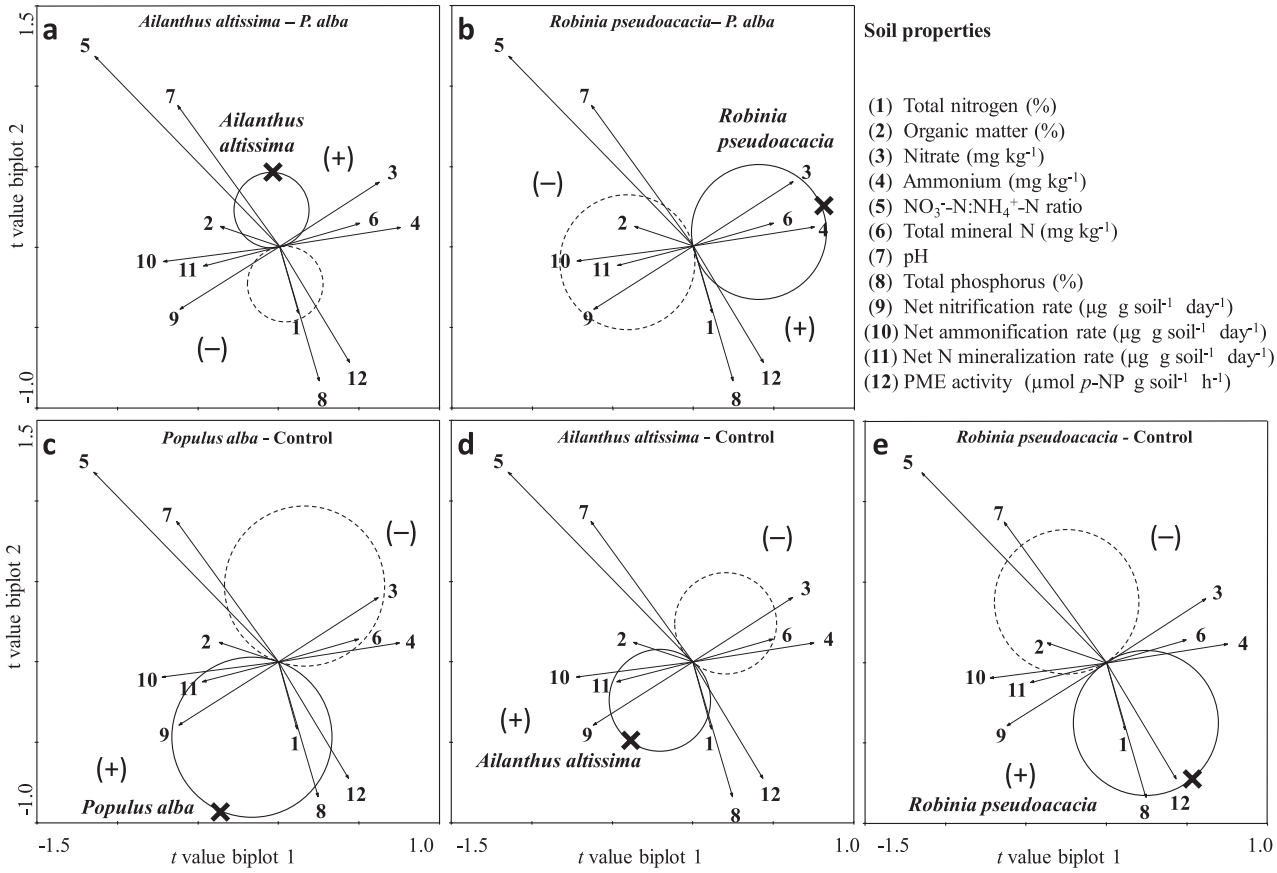

Fig. 2. Greenhouse experiment: t-value biplots comparing soil properties (vectors with numbers) in samples conditioning by the growth during six months of *P. alba* samples with those of *Ailanthus altissima* (a) and those of *Robinia pseudoacacia* (b). T-value biplots comparing soil properties of control samples (soils where no plant grew) with soil samples conditioning by the growth during six months of the tree species: *Populus alba* (c), *Ailanthus altissima* (d), *Robinia pseudoacacia* (e). Crosses indicate the centroids of the tree species (*A. altissima*, *R. pseudoacacia*, or *P. alba*). Vectors fully falling within a Van-Dobben circle (Lepš and Šmilauer, 2003) indicate a significant relationship between the focal soil property and the tree species. Relationships in t-value biplots may be positive (solid line circle) or negative (dashed line circle) and are based on t values of regression coefficients of soil properties expressed as linear combinations of *A. altissima*, *R. pseudoacacia*, *P. alba* or control samples. Mean (±SE) values of soil variables used are available in Supplementary material (Table S2).

R. pseudoacacia increased mineral N (NO_3^-–N and NH_4^+–N) of riparian forest dominated by *P. alba*. This resulted from the N-fixing ability of *R. pseudoacacia*, which can directly release N by means of root exudates and increase soil fertility (Zahran, 1999; Fustec et al., 2010). A similar trend was previously reported by other studies comparing soils under *R. pseudoacacia* and under pine or oak trees (Rice et al., 2004; Von Holle et al., 2013). By contrast, *A. altissima*, decreased total soil N both in the field and in the GHE, mainly due to a decrease of the organic N, which represented more than 95% of total N. In a previous study we found that *A. altissima* leaf litter decomposed faster than *P. alba* leaf litter, probably due to the higher litter quality of the former (Medina-Villar et al., 2015). The low organic N in *A. altissima* patches suggests that the rate of soil organic N loss by mineralization was higher than the rate of organic N gain by litter decomposition. However, in the GHE, six-month plants produced a negligible amount of leaf litter which unlikely could affect soil N. Consequently, belowground mechanisms may also account for the lower total N in *A. altissima* than in *P. alba* soils. These mechanisms may include that *A. altissima* 1) had lower production of N-rich root exudates or root litter, 2) had higher uptake of N-rich organic monomers (e.g. amino acids, nucleic acids), 3) enhanced the activity of N-mineralizing microorganisms by improving the physical connection between organic N and soil decomposers (Schimel and Bennett, 2004). The suggested higher N mineralization rate in *A. altissima* soils contrasts with the lower net N mineralization found in the field experiment. However, this apparent contradiction is compatible with an increase of gross N mineralization in *A. altissima* soils, but a faster loss of mineral N either by a faster increase of the uptake by microorganisms or by a faster increased of denitrification rates, promoted by the higher nitrate availability in *A. altissima* soils (Moreau et al., 2015).

The rest of the results with significant difference between invasive and native trees found in the field were not found in the GHE, and vice versa, possibly because the effects of these species on soil properties may vary with invasion time or plant age (Strayer et al., 2006). The lower soil OM in *R. pseudoacacia* than in *P. alba* soils, which was only found in the GHE, may be due to the lower root biomass found for the invader seedlings, given that dead roots are an important source of OM for the soil (Frank and Groffman, 2009). In addition, N mineralization activity has been found to be affected by plant age (Tolman et al., 1990; Abbès et al., 1995; Côté et al., 2000), which may explain that *R. pseudoacacia* was found to increase net ammonification in the field, but to decrease it in the GHE, along with net nitrification. The higher PME activity in *R. pseudoacacia* field soils as compared with *P. alba* ones, is in accordance with the stimulation of this activity reported for legumes, which demand P for N-fixation (Reinsvold and Pope, 1987; Makoi and Ndakidemi, 2008). However, the fact that this trend was not found in the GHE may be explained because PME activity increases with plant age and size (Makoi and Ndakidemi, 2008;

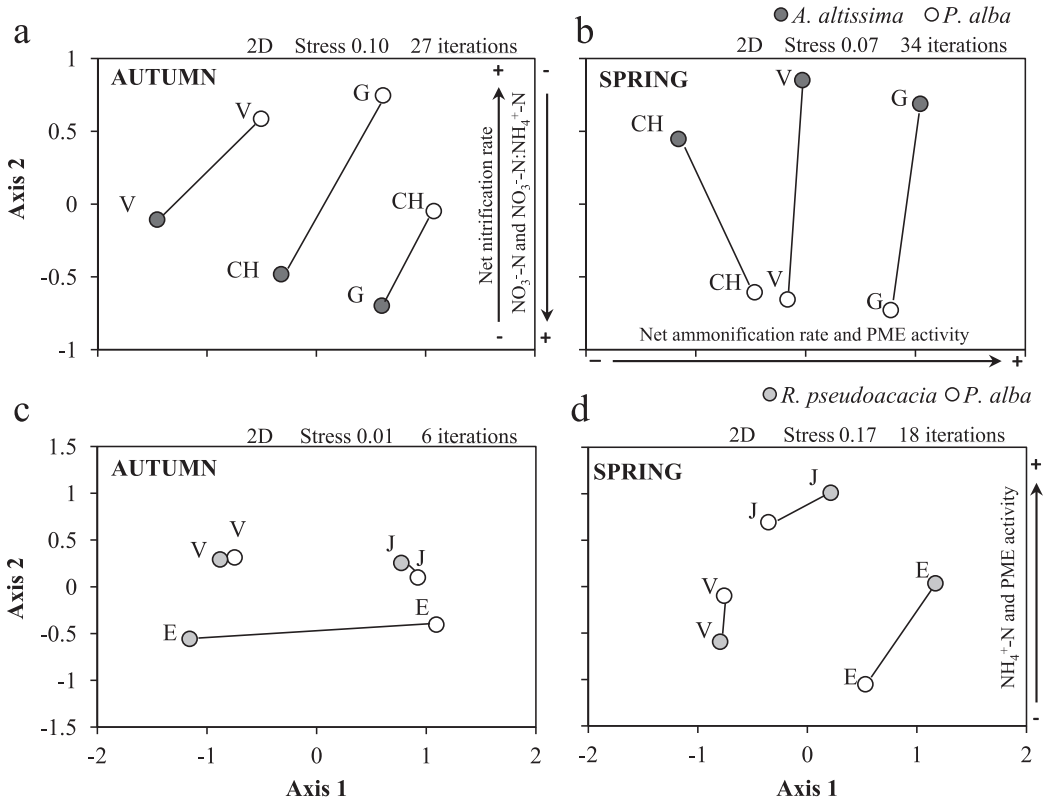

Fig. 3. Field sampling: Two dimensional Non Metric Multidimensional Scaling (NMDS) plots, based on DGGE data, showing soil samples clustered according to the composition of soil bacteria community in *A. altissima* − *P. alba* (a and b) and *R. pseudoacacia* − *P. alba* (c and d) paired sites, in autumn (a and c) and spring (b and d). CH = Chiloeches site; G = Guadalajara site; V = El Val site; J = Jadraque site; E = El Encín site. White points mean native patches and grey points invaded patches. Lines join invaded and non-invaded patches of each site to highlight the potential effect of the invader. Arrows beside the axis indicate significant ($P < 0.05$) pair wise correlations between the axes and the indicated soil variables: ammonium concentration (NH_4^+–N), nitrate concentration (NO_3^-–N), nitrate:ammonium ratio (NO_3^-–N:NH_4^+–N), phosphomonoesterase activity (PME), net ammonification and nitrification rates.

Orczewska et al., 2012). Low root development of *R. pseudoacacia* plants in the GHE also implied low root surface to exude PME enzymes and to support the PME enzymes associated to root cells (Rejsek et al., 2012).

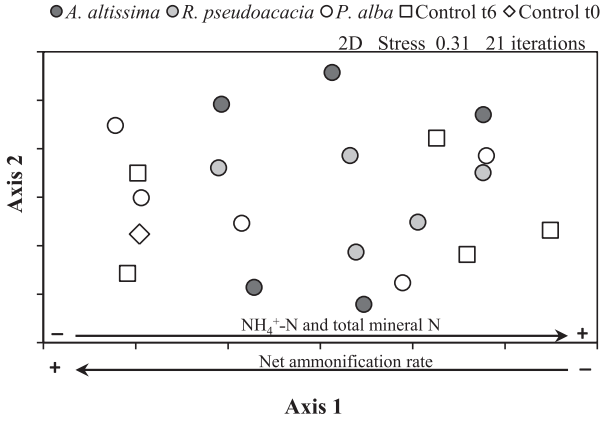

Fig. 4. Greenhouse experiment: Two dimensional Non Metric Multidimensional Scaling (NMDS) plot showing soil samples clustered according to their composition of soil bacteria community under the influence of the growth of *A. altissima*, *R. pseudoacacia* and *P. alba* during 6-months and without the influence of any plant growth at the beginning (Control t0) and the end (Control t6) of the 6-months experiment. Arrows beside the axis indicate significant ($P < 0.05$) pair-wise correlations between the axis and the indicated soil variables: ammonium concentration (NH_4^+–N), total mineral N and net ammonification rate.

Changes in bacteria-related processes, such as nutrient cycling, might be explained by modifications of soil bacterial communities (Hawkes et al., 2005). Accordingly, in our study, the change of soil bacterial community in *A. altissima* patches, compared to that in *P. alba* patches, was related to a lower potential net nitrification rate and with higher NO_3^-–N and nitrate:ammonia ratio. Therefore, *A. altissima* may be affecting soil bacteria related to net nitrification rates (e.g. nitrifiers, nitrate-reducing bacteria or any microorganisms that can take NO_3^-–N). Due to the limitation of the DGGE technique (Fakruddin and Mannan, 2013), we could not determine which organisms changed in the studied bacterial communities. However, our study constitutes a first step to assess differences in bacterial communities between invaded and non-invaded riparian forests. In spite of its limitations, DGGE is still a valid and useful methodology to describe soil communities and explore differences among them (Souza-Alonso et al., 2015). Recent studies have also demonstrated that DGGE and new generation sequencing techniques, such as pyrosequencing, are equally useful to detect differences in soil microbial communities (Buscardo et al., 2015). Differences in soil bacterial communities between *R. pseudoacacia* and *P. alba* patches were only appreciable at one site and season (El Encín in spring) and were related to greater ammonium concentrations and PME activity in *R. pseudoacacia* than in *P. alba* soils. Other studies of soils from maize crops also found that changes in PME activity coupled with changes in soil bacterial community (Kandeler et al., 2002). Besides, dominant tree species affected the microbial communities more in spring than in autumn likely due to the higher tree activity and advanced decomposition of the leaf and

root litter (Thoms and Gleixner, 2013). GHE also showed that the composition of soil bacterial communities was related to N cycle (i.e. ammonium concentration, net ammonification rate and total mineral N concentration). However, the lack of differences among tree species in soil bacterial communities indicated that more than six months were needed for tree species to modify soil bacterial communities.

The increase in overall N availability due to *R. pseudoacacia* invasion may hinder restoration with native species (Haubensak and Parker, 2004; Niu et al., 2007), although some native species could be suitable for the restoration of N-enriched invaded soils (Rodríguez-Echeverría et al., 2015). Greater nitrate in invaded than in non-invaded soils can persist up to 14 years after the removal of the invasive species (Von Holle et al., 2013). Elevated nitrate in invaded patches also increases the probability of nitrate to be leached and reach groundwater and freshwater ecosystems (Cameron et al., 2013). Nitrate accumulation in water stream has also been reported in wetlands adjacent to *R. pseudoacacia* stands (Williard et al., 2005). Future studies on the suitability of soils conditioned by *A. altissima* or *R. pseudoacacia* for the growth of native species, and their usefulness to reduce N leaching, are needed to assess the consequences of soil modification by these invasive species on restoration.

5. Conclusions

Our study showed the capability of *A. altissima* and *R. pseudoacacia* to alter soil properties of riparian forests dominated by *P. alba*. These alterations were species-specific with *A. altissima* decreasing total N and *R. pseudoacacia* increasing mineral N. Soil bacterial communities in the field differed between invaded and non-invaded soils, these differences were greater between *A. altissima* and *P. alba* than between *R. pseudoacacia* and *P. alba*. These differences were related to nitrate concentration and net nitrification rates in *A. altissima* soils and to ammonium concentration and PME activity in *R. pseudoacacia* soils. Our study also showed that the studied invasive species can alter some soil properties, such as N concentrations, in just six months but the composition of soil bacterial community and soil microbial activity needs longer time of invasion to be affected.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.01.015.

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